

PROPERTIES AND ORGANIZATION OF THE PROTEINS
IN THE OUTER MEMBRANE OF ESCHERICHIA COLI

By

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ABSTRACT

Two major proteins of the outer membrane of Escherichia coli, the matrix protein, A (M.W. 36,500) and the heat-modifiable protein, B were purified and partially characterized. Both have a low content of cysteine, an excess of acidic amino acids over basic and a moderate content of hydrophobic amino acids. Protein B (M.W. 28,500) was converted to form B* (M.W. 33,400) upon heating in the presence of sodium dodecyl sulfate at temperatures higher than 50°C. Physical studies showed that protein B unfolds upon heating without a large increase in binding of sodium dodecyl sulfate. It is proposed that protein B as extracted from the membrane contains some native structure which is lost upon heating. The level of protein A₁, a major outer membrane protein in glucose-grown cells, was decreased in cells grown on other carbon sources with a concomitant increase in the amount of protein A₂. Both proteins were tightly associated with the peptidoglycan and had similar amino acid composition, suggesting that they play the same role in the outer membrane. The organization of proteins in the outer membrane of E. coli was studied by proteolytic digestion, covalent labelling and crosslinking. The proteins of the outer membrane were inaccessible to pronase in intact cells and the cells altered in their lipopolysaccharide component. The protein components of isolated outer membrane preparations varied in their rates of digestion and labelling with fluorescamine, suggesting that they are asymmetrically arranged in the

membrane. The proteins most rapidly degraded (proteins B, C, D₁ and E) were judged to be exposed at the surface of the membrane, while those resistant to digestion (proteins A₁, A₂ and D₂) must be protected by their arrangement in the membrane. Digestion of outer membrane preparations with pronase left a fragment derived from protein B (protein Bp) embedded in the membrane. This fragment was not enriched in hydrophobic amino acids relative to protein B. Protein B could be reassociated with itself, without phospholipid or lipopolysaccharide such that pronase digestion of the reassociated material gave protein Bp. These results suggest that protein B may not be held in the membrane primarily by hydrophobic interactions. The resistance of proteins A₁ and A₂ to protease digestion is likely due to protein-protein interactions since oligomers of protein A could be isolated. Treatment of protein A₁- or A₂-peptidoglycan complexes with dithiobis (succinimidyl propionate) or glutaraldehyde produced dimer, trimer and higher oligomers of protein A. No crosslinking of protein A to the peptidoglycan was detected. The proteins of the isolated outer membrane varied in their ease of crosslinking. Protein B, but not the pronase-resistant fragment, protein Bp, was readily crosslinked to give high molecular weight oligomers, while protein A formed dimers and trimers under the same conditions. No crosslinking of protein A to B was detected. Crosslinking of cell wall preparations showed that protein B and the free form of the lipoprotein, F, could be linked to the peptidoglycan. A dimer of protein F, and protein F linked to protein B, were detected.

These results suggest that specific protein-protein interactions occur in the outer membrane. A model for the arrangement of the proteins in the outer membrane of E. coli, summarizing the results of proteolytic digestion, covalent labelling and crosslinking, is presented.

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ABBREVIATIONS

cAMP:	3',5' cyclic adenosine monophosphate
CD:	Circular dichroism
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
DSP:	Dithiobis (succinimidyl propionate)
EDTA:	ethylenediaminetetraacetic acid
Hepes:	N-2-hydroxyethylpiperazine N'-2-ethane-sulfonic acid
mRNA:	Messenger ribonucleic acid
ORD:	optical rotatory dispersion
RNase:	Ribonuclease
SDS:	Sodium dodecyl sulfate
TEMED:	N,N,N',N' tetramethylethylenediamine
Tris:	Tris-(hydroxymethyl)-aminomethane
uv:	ultraviolet

Other abbreviations used in figures are defined in the legends.

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Protein

"He was supposed to be our commencement speaker," said Sandra.

"Who was?" I asked.

"Dr. Hoenikker - the old man."

"What did he say?"

"He didn't show up."

"So you didn't get a commencement address?"

"Oh, we got one. Dr. Breed, the one you're gonna see tomorrow, he showed up, all out of breath, and he gave some kind of talk."

"What did he say?"

"He said he hoped a lot of us would have careers in science," she said. She didn't see anything funny in that. She was remembering a lesson that had impressed her. She was repeating it gropingly, dutifully. "He said, the trouble with the world was . . ."

She had to stop and think.

"The trouble with the world was," she continued hesitatingly, "that people were still superstitious instead of scientific. He said if everybody would study science more, there wouldn't be all the trouble there was."

"He said science was going to discover the basic secret of life someday," the bartender put in. He scratched his head and frowned.

"Didn't I read in the paper the other day where they'd finally found out what it was?"

"I missed that," I murmured.

"I saw that," said Sandra. "About two days ago."

"That's right," said the bartender.

"What is the secret of life?" I asked.

"I forget," said Sandra.

"Protein," the bartender declared. "They found out something about protein."

"Yeah," said Sandra, "that's it."

INTRODUCTION

Cell Envelope of Gram-negative Bacteria

The cell envelope of gram-negative bacteria such as Escherichia coli is composed of three physically and chemically distinct layers (Fig. 1), the inner or cytoplasmic membrane, the murein or peptidoglycan, and the outer membrane (1-8). The peptidoglycan and the outer membrane comprise the cell wall layer. The peptidoglycan, located between the two membranes, varies in thickness in different gram-negative bacteria (5) and can be removed by digestion with lysozyme (10). The inner and outer membranes show a typical unit membrane (20) appearance, 75 Å thick, under the electron microscope (9,10).

The inner and outer membranes can be separated by sucrose density gradient centrifugation of spheroplasts, prepared by lysing cells treated with lysozyme in the presence of EDTA (14,35,71,73-76). Cells can also be disrupted in a French Pressure Cell and the cell wall, consisting of the outer membrane and peptidoglycan, can be readily separated from the inner membrane by sucrose density gradient centrifugation (13) or electrophoresis (70).

Cytoplasmic membrane

The inner membrane of gram-negative bacteria is composed of phospholipid (40%) and proteins (60%) and has a structure similar to that of most biological membranes (11-15). The phospholipids, consisting mainly of phosphatidyl ethanolamine

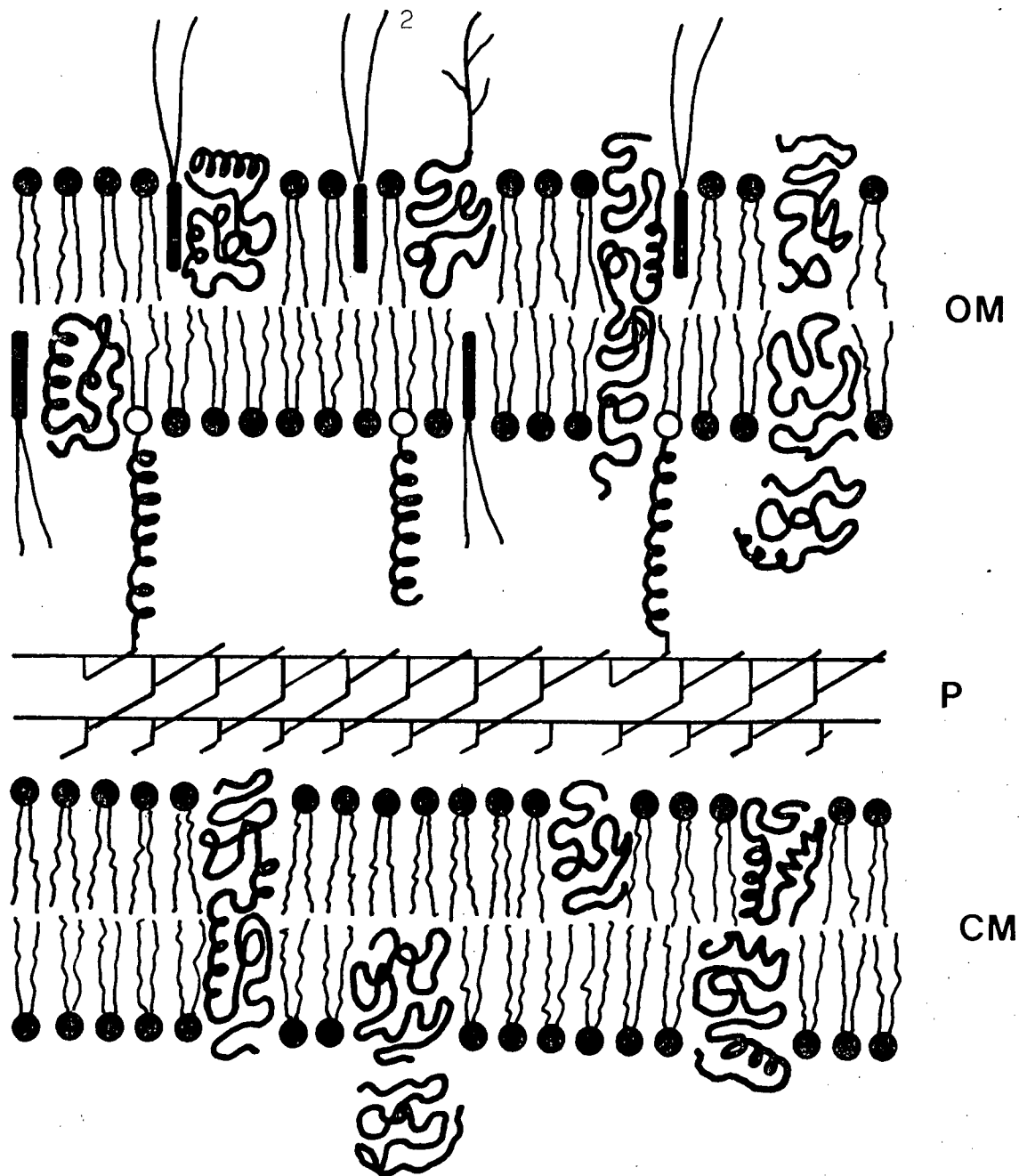


Fig. 1. Model of the cell envelope of *E. coli*. OM, outer membrane; P, peptidoglycan; CM, cytoplasmic membrane; \backslash , phospholipid; — , lipopolysaccharide; — , protein; — , lipoprotein.

(78%) and phosphatidyl glycerol (11%), are arranged as a bilayer, providing a hydrophobic zone which is cleaved during freeze-etching (16,17). The protein components of the membrane are seen as globules in these preparations (18,19). The proteins of the cytoplasmic membrane include those responsible for electron transport and oxidative phosphorylation (21,22), active transport of solutes (23-26), and the biosynthesis of phospholipids (27-30), peptidoglycan (2,31-33) and lipopolysaccharide (2,6,14,34-38).

Peptidoglycan

The peptidoglycan is composed of alternating residues of N-acetylmuramic acid and N-acetylglucosamine joined by β (1,4) linkages (33). Peptide crossbridges, linked to the carboxyl groups of the chains, result in the formation of a huge macromolecular net (40). Fig. 2 presents the structure of the peptidoglycan from E. coli. The peptidoglycan may be involved in maintaining the shape of the organism since the cells bounded only by their peptidoglycan (41) and isolated sacculi (42,43) retain the original shape of the bacterium. The peptidoglycan layer may also play a role in the assembly of the outer membrane components since it is attached to the outer membrane by a lipoprotein (44).

Outer membrane

The outer membrane of gram-negative bacteria is a unique membrane in a number of respects. As already stated, the outer membrane is linked to the peptidoglycan by lipoprotein (44).



Fig. 2. Structure of the peptidoglycan lipoprotein complex of E. coli. The cylindrical section indicates a part of the murein with the shape of the rod-like E. coli cell. It is not known in which direction the glycan chains span the cell, relative to the long axis of the cylinder. In this model they are arbitrarily drawn parallel. The murein is composed of roughly 10^5 repeating units, to which approximately 10^6 lipoprotein molecules are covalently bound. The lipoprotein replaces d-alanine on the diaminopimelate residue. The amino acid sequence is represented in a way that demonstrates the possible evolution of this molecule from a gene that coded originally for 15 amino acids, which was duplicated, and then only the C-terminal half was added four times. The dashes represent hypothetical deletions of amino acids that may have occurred during evolution. The stars indicate the hydrophobic amino acids at every 3.5th position. Taken from Braun (44).

A second unique feature is that the outer membrane contains lipopolysaccharide (20%) in addition to phospholipid (20%) and protein (60%) (14). Thirdly, the outer membrane in the presence of magnesium ions is resistant to disruption by detergents such as Triton X-100 (66,67,72) and sodium lauryl sarcosinate (69). The cytoplasmic membrane is completely solubilized under these conditions. Finally, the protein composition of the outer membrane is much simpler than that of the inner membrane, containing only a few proteins, present in major amounts (12,13).

Components of the Outer Membrane

Lipopolysaccharide

The structure, biosynthesis and assembly of this unique cell wall component has been extensively studied (2,6,14,34-38,52-58). The lipopolysaccharide molecule is composed of three regions (Fig. 3), lipid A, inner and outer core, and O-antigen chains consist of oligosaccharide repeating units which determine the serological grouping of the bacterial strain. The O-antigen region is synthesized separately from the rest of the lipopolysaccharide molecule and is then transferred to a completed core. The core region is made by sequential addition of sugars. A series of mutants of E. coli and Salmonella have been isolated in which the lipopolysaccharide is progressively more defective in the core region (Fig. 3). The series progresses from smooth strains which contain O-antigen side chains to deep-rough mutants which contain only the lipid A portion of the lipopolysaccharide. The lipid A portion of

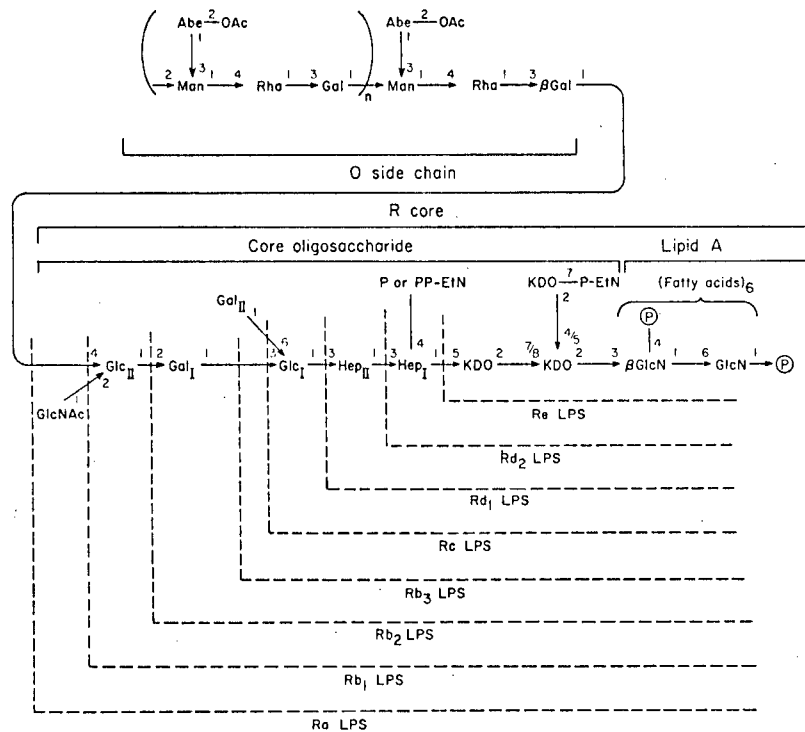


Fig. 3. Structure of *S. typhimurium* LPS. This structure shows a basic "monomer" unit, which is presumably cross-linked with other units. Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-manno-octulosonic acid; EtN, ethanolamine; Ac, acetyl. To some sugar residues in the core oligosaccharide region roman numeral subscripts have been added in order to differentiate one from the other. All sugar residues are α -anomers except where β -conformation is specified. The biosynthesis of LPS starts at the lipid A portion and the core oligosaccharide is elongated toward the "left" (i.e., non-reducing end) in this scheme. Thus, mutants defective at various stages of core oligosaccharide produce incomplete LPS; the structure of these LPS molecules and their "chemotype" are indicated by dotted lines. Taken from Nikaido (38).

the lipopolysaccharide is essential since no mutants lacking this region have been isolated (38). Lipid A may be required for stabilization of the outer membrane since phosphatidyl ethanolamine, the predominant phospholipid of the outer membrane does not form vesicles readily in aqueous solutions (59,60). Mutants defective in the core region are more sensitive to antibiotics, dyes and detergents (5,38,61-65) suggesting that the lipopolysaccharide is involved in the barrier function of the outer membrane.

The hydrophobic lipid A portion is embedded in the membrane contributing, along with the phospholipids, to a central hydrophobic domain (47-49). The lipopolysaccharide is distributed primarily on the outer surface of the membrane with the carbohydrate chains extending into the medium (45,68). The lipopolysaccharide molecules are laterally mobile, although their freedom of translation is lower than that of the phospholipids (50). This lateral mobility could account for the equilibrium between EDTA-extractable and non-extractable lipopolysaccharide (5).

Phospholipids

The phospholipids of the outer membrane consist primarily of phosphatidyl ethanolamine (81%) with small amounts of phosphatidyl glycerol (17%) and cardiolipin (2%). The level of cardiolipin in cells has been shown to increase upon change of growth phase from exponential to stationary (112). The fatty acids of the phospholipids are sensitive to growth temperature,

becoming more saturated with higher growth temperatures (113).

A phospholipid bilayer may not be the basis of the outer membrane since only 25-40% of the phospholipids of the outer membrane take part in phase transitions compared with 60-80% for the cytoplasmic membrane (159). In addition, it has been suggested that the phospholipids are distributed primarily on the inner leaflet of the bilayer, with the outer surface being composed of mainly lipopolysaccharide (160). This is supported by the finding that intact cells are resistant to phospholipases A₂ and C unless the lipopolysaccharide is removed by EDTA extraction (111). Phospholipid has also been shown not to be required for the structural integrity of the outer membrane (131).

Outer membrane proteins

Resolution of the proteins of the outer membrane of E. coli strain NRC 482 by SDS-polyacrylamide gel electrophoresis reveals a limited number of protein bands (Fig. 4). Fig. 4 illustrates the unusual behaviour that these proteins exhibit on SDS-polyacrylamide gel electrophoresis. As seen in gel 2, proteins A₁, A₂, and B are not resolved on SDS-polyacrylamide gels run with phosphate buffer (90) at a neutral pH (System 1) (12, 13, 79, 81, 82, 88). On the basis of this observation it was suggested that the outer membrane of gram-negative bacteria is composed of a single major protein with a structural role (12, 13, 89). SDS-polyacrylamide gels run at alkaline pH (system 2) (79) resolved the major protein of the outer membrane of E. coli NRC 482 into three bands, protein A₁ (MW 44,000), protein A₂ (MW 38,100), and protein B (MW 33,400) as seen in gel 4.

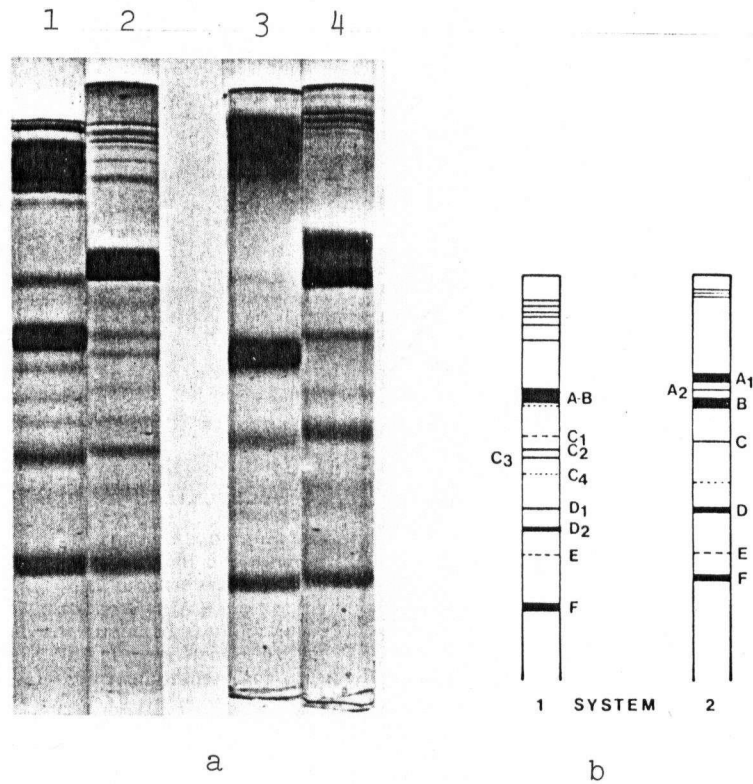


Fig. 4a. Electrophoresis on 10% polyacrylamide gels of proteins of the *E. coli* outer membrane. The gels from left to right are (1) membrane dissolved in dodecyl sulfate-urea-mercaptoethanol at 37°C; (2) solution subsequently heated at 100°C; (3) same as (1); (4) same as (2). Electrophoresis was carried out with System 1 for gels (1) and (2), and with System 2 for gels (3) and (4). In system 1, both buffer compartments contained 0.1% SDS in 0.1M sodium phosphate buffer, pH 7.2. In system 2, the cathodic buffer compartment contained 0.1% SDS in 0.1M Na_2HPO_4 , pH 11.4, while the anodic buffer contained 0.1% SDS in 0.1M NaH_2PO_4 , pH 4.1. Taken from Bragg and Hou (79).

Fig. 4b. Schematic diagram of separation of outer membrane proteins of *E. coli* on 10% polyacrylamide gels using Systems 1 and 2 are shown. The samples in dodecyl sulfate-urea-mercaptoethanol had been heated to 100°C.

Similar results have been obtained with other strains of E. coli (88,91). Further resolving power is obtained through the use of thin SDS-polyacrylamide gel slabs run with a discontinuous buffer system (83,92). Lutenberg et al. (114) have resolved the major outer membrane proteins of E. coli K12 into four closely-spaced bands using a modified Laemmli system. Application of two dimensional gel systems (93-97) to the separation of outer membrane proteins provides a highly-resolving and sensitive method for the detection of all polypeptide components of the outer membrane. The widespread adoption of a two dimensional system such as that devised by O'Farrell (94), and adapted by Ames (97), for use with membrane proteins should greatly facilitate the comparison of results from different laboratories.

Confusion as to the number and molecular weights of the outer membrane proteins had not only arisen by the use of different SDS-polyacrylamide gel systems but it was found that the preparative conditions affected their migration on SDS-gels (79-85). The temperature and time of heating was found to profoundly alter the pattern seen in SDS-gels. If the outer membrane is solubilized at 37°C and the proteins resolved on SDS-polyacrylamide gels the pattern seen in Fig. 4, gels 1 and 3 are found. If the sample is heated at 100°C prior to electrophoresis the patterns in gels 2 and 4 are seen. Protein B changes its apparent molecular weight from 28,500 to 33,400 upon heating. This behaviour has been designated as heat-modifiable (79). Secondly, high molecular weight aggregates of protein A are broken down into lower molecular weight species.

The proteins of the outer membrane also vary greatly in

their ability to be solubilized from the cell wall. It has already been noted that neither Triton X-100 in the presence of magnesium ions nor sodium lauryl sarcosinate could solubilize the proteins of the outer membrane. Extraction of the cell wall with Triton X-100 in the presence of EDTA released about half of the outer membrane protein (67).

Protein B is readily solubilized from the cell wall by SDS at 37°C. The protein migrates as a band with an apparent molecular weight of 28,500 on SDS-polyacrylamide gels until heated at 100°C (79,81,87). Protein A is not extracted by SDS at temperatures less than 60°C (79,80) and remains associated with the peptidoglycan. Extracts made at low temperatures will therefore not contain this protein (79,83,86). The tight binding of protein A to the peptidoglycan has been useful in its purification (80). Extraction of the cell wall at 70°C with SDS for 20 min (82) releases protein A in the form of oligomers (81) that have not bound their full complement of SDS (80). The protein migrates atypically in SDS-polyacrylamide gels as a broad band with a molecular weight of about 60,000 (80-82). Heating the extract to 100°C results in further SDS binding and the protein now migrates as a sharp band with a molecular weight of 36,500 (80,81). All the proteins of the outer membrane except the bound lipoprotein are solubilized by SDS at 100°C. The lipoprotein is covalently linked to the peptidoglycan and is not released until the peptidoglycan is treated with trypsin or lysozyme (44).

Different nomenclatures have been applied to the proteins

of the outer membrane by different research groups on the basis of the migration of the proteins in SDS-polyacrylamide gels. The likely relationships of the proteins studied by other workers to the nomenclature used in this thesis are given in Table I. Comparisons are difficult to make because of the different SDS-polyacrylamide gel systems in use, and the effects of preparative conditions on the migration of the proteins. Also, the corresponding proteins may not be identical due to differences in strain or culture conditions.

Properties of the Outer Membrane Proteins

Bound lipoprotein

The protein responsible for the attachment of the outer membrane to the peptidoglycan is a lipoprotein (Fig. 2) which has been extensively characterized by Braun and his coworkers (4,44). There are 250,000 copies of this protein per cell. It is covalently attached to the peptidoglycan (120). The ϵ -amino group of the C-terminal lysine residue is linked to about every tenth diaminopimelate residue of the peptidoglycan by an amide bond (118,119). The lipoprotein is released from the peptidoglycan by proteolytic digestion (117) or by lysozyme treatment (121).

Lipid is attached to the N-terminal cysteine residue of the protein (117,119,122). Two molecules of fatty acid are esterified to the glyceryl side chain, while the amino group of the cysteine residue is blocked by a fatty acid linked by an amide bond. The ester-linked fatty acids have a composition

Table I

Nomenclatures for the outer membrane proteins of E. coli

	Bragg and Hou (79)	Schnaitman (88)	Henning and Haller (98)	Inouye (120)	Lutenberg et al (114)	Koplow and Goldfine (177)	Chai and Foulds (154)
Matrix protein (80)	A ₁	1	Ia Ib	peak 4	b c	B	
	A ₂	2	-		-	-	
Heat-modifiable protein (79)	B/B*	3a 3b	II*	peaks 6/7	d	C/D	G
Free lipoprotein F (120)			IV	peak 11			

similar to those of the cellular phospholipids (45% palmitic acid, 11% palmitoleic acid, 12% 9,10 methylenehexadecanoic acid, 8% 11,12 methylenooctadecanoic acid and 24% cis-vaccenic acid). The amide-linked fatty acids are mainly palmitic acid (65%), palmitoleic acid (11%) and cis-vaccenic acid (11%).

The lipoprotein has a molecular weight of about 7,000 and its complete structure has been determined (Fig. 2). The amino acid composition is unique in that the protein from E. coli lacks histidine, proline, glycine, phenylalanine and tryptophan. Circular dichroism studies showed that the protein contains about 80% α -helical structure (44,124). The structure was regained after heat denaturation when the protein, as a solution in SDS, was cooled from 100°C as shown by circular dichroism measurements and antibody binding (44,123). The unusual resistance of the lipoprotein to digestion by trypsin can be attributed to its high α -helical content (44).

The amino acid sequence of the protein revealed that when the protein is arranged in an α -helical conformation, all the hydrophobic amino acids are aligned on one side of the helix (44). The aggregation of the lipoprotein in aqueous solutions may be due to the interaction of these hydrophobic regions. An additional feature of the lipoprotein is the repetitive nature of the sequence. This suggests that the gene coding for the lipoprotein has arisen by gene duplication (44).

Free lipoprotein

Inouye has found that about two-thirds of the lipoprotein

exists unattached to the peptidoglycan in the cell envelope of E. coli (125-127). The covalently-bound lipoprotein, released by lysozyme digestion could be distinguished from the free form since it contained two repeating units of peptidoglycan and therefore migrated more slowly in SDS-polyacrylamide gels (120). The free form, which is located in the outer membrane (128), contains a similar composition of fatty acids as the bound form and has a very high α -helical content (127,129,271).

The free form of the lipoprotein has recently been crystallized into several different forms (271,272). The crystals are readily formed by acetone precipitation of the protein from SDS solutions, probably due to the interaction of the hydrophobic regions of the molecule. The protein forms a rigid α -helix, 87 Å in length, with the fatty acids extending a further 21 Å from one end of the molecule.

Other gram-negative bacteria such as Salmonella typhimurium and Serratia marcesens have both free and bound forms of the lipoprotein (44,130,132). Proteus mirabilis lacks both forms of this protein (44,130) during the exponential phase of growth. However, a bound lipoprotein appears in the stationary phase of growth (267).

Matrix protein (protein A)

Several groups have purified and characterized a major protein of the outer membrane of E. coli which is non-covalently bound to the peptidoglycan (80,81,87,88,91,140-142,148,149). There are approximately 1.5×10^5 molecules of protein A in a cell,

arranged hexagonally to cover about half of the outer surface of the peptidoglycan (80). The protein is not extracted by SDS at temperatures up to 60°C, but is readily solubilized at 100°C. This protein does not bind SDS until heated at temperatures above 60°C, accounting in part for its atypical migration on SDS-polyacrylamide gels.

The protein isolated by Rosenbusch (80) from E. coli B was a single polypeptide (probably equivalent to protein A₁) with a molecular weight of 36,500 and an N-terminal sequence:

Ala-X-Tyr-Asx-His-Lys-Glx-

Other E. coli strains may contain multiple forms of this protein (87,91,140-142). Schnaitman has observed differences in the major outer membrane proteins from different strains of E. coli (91). In addition to the matrix protein, 1, some strains contained another protein, 2 (probably A₂), that migrated slightly faster in SDS-polyacrylamide gels run at alkaline pH. In strains of E. coli non-lysogenic for phage PA 2, protein 1 is a predominant protein of the outer membrane (116). In cultures lysogenic for this phage, the amount of protein 1 is reduced and protein 2 is made in its place. Protein 1 is proposed to be the receptor for this phage and protein 2 is made to prevent superinfection (116). The synthesis of protein 2 is also under catabolite repression (91) since cells grown on carbon sources other than glucose, or in the presence of cAMP, contain increased levels of protein 2, with a decrease in protein 1.

Both proteins 1 and 2, before and after heating, were eluted together from a column of Sephadex G-200, equilibrated with 0.1% SDS (81,88,148). Ion exchange chromatography of

Triton X-100 extracts containing protein aggregates resulted in separation of proteins 1 and 2 (88). Analysis of the purified proteins by cyanogen bromide cleavage revealed a spectrum of peptides including partial cleavage products. On the basis of these experiments, Schnaitman concluded that proteins 1 and 2 are different polypeptides (88).

Henning's group has also found that the matrix protein from different E. coli strains is composed of multiple polypeptide species (87,140-142). They found that the matrix protein could be separated by electrophoresis in SDS-polyacrylamide gels into two components, Ia and Ib (142). Both proteins could be further separated into a number of isoelectric species. Cyanogen bromide cleavage patterns revealed that proteins Ia and Ib were essentially the same polypeptide with a modification in one peptide only. The possible correspondence of the matrix proteins from different E. coli strains has been summarized in Table I.

The matrix proteins isolated by Henning's group (87,149) had a similar amino acid composition to the protein isolated by Rosenbusch (80). The protein is only moderately hydrophobic and none of the cyanogen bromide fragments exhibit a low polarity (141). The matrix protein may not be involved in hydrophobic interactions in the membrane in contrast to membrane proteins isolated from other organisms (143-146).

The matrix protein has a high content of β -structure (80) and may be responsible for the β -structure seen in the intact outer membrane (138). The β -structure of the outer membrane

disappears upon heating in SDS (139) due to denaturation of the proteins by the detergent.

Protein A is not digested when cell envelope, cell wall, outer membrane or the protein A - peptidoglycan complex are treated with proteolytic enzymes (79,80,131,135-137). The protein when solubilized is completely degraded to small peptides by trypsin or pronase (79,80) showing that it is not intrinsically resistant to digestion. The insensitivity to proteolytic digestion must therefore be due to the inaccessibility of suitable peptide bonds to the proteases as a result of protein folding and protein-protein interactions.

Mutants missing the matrix protein have been isolated. Schnaitman has reported that a multiple phage-resistant mutant of E. coli (T4,T5,T6,T7, λ) lacked the matrix protein (91). There was an increase in the amount of protein B in this mutant, such that the phospholipid to protein ratio of the outer membrane was the same as in the parent. This compensation of one protein for another is also seen when growth conditions are altered (91). In contrast, Hancock and Reeves (103) found that a lipopolysaccharide-defective mutant of E. coli had a decreased amount of protein A but had a normal amount of protein B. Mutants of E. coli resistant to phage TuI were isolated by Henning's group (142). The mutants either lacked both proteins Ia and Ib or Ib only, suggesting that protein Ib is the receptor for phage TuI. Henning's group has also isolated a mutant missing all the major outer membrane proteins, A₁, A₂ and B (98). Surprisingly, no serious defects affecting the integrity of the

outer membrane were observed. In addition, loss of individual major outer membrane proteins did not change the fracture faces of the outer membrane (104).

The results obtained with these mutants suggest that protein A is not required for the integrity and perhaps normal functioning of the outer membrane. Why E. coli contains such high amounts of this protein in the outer membrane is not clear. However, deep-rough mutants of E. coli and Salmonella, which lack proteins A and B have a seriously disrupted outer membrane, both morphologically and functionally. These mutants will be discussed in the section concerning the barrier function of the outer membrane.

Heat-modifiable protein (protein B)

Protein B is a major outer membrane protein which changes its apparent molecular weight from 28,500 to 33,400 upon heating in SDS-containing solutions (79). The same change was observed by Schnaitman for his protein 3 (88) and Henning's group for their protein II* (87). The cause of this phenomenon has been investigated by Schnaitman (81). He has found that there was an increase in the intrinsic viscosity of the protein on heating in SDS solutions. He suggested that only after heating does the protein unfold to form the rigid rod conformation proposed by Reynolds and Tanford (152) for protein-SDS complexes. It has been shown that the heated form, B* migrates typically on SDS-polyacrylamide gels (131,148) supporting Schnaitman's suggestion. Further physiochemical analyses of protein B and the heat-

modified B*, described in this thesis, also support Schnaitman's view that the protein unfolds upon heating.

Protein B has been purified by a number of workers and some of its properties have been studied (81,87,88,110). Amino acid analysis has revealed that the protein has a molecular weight of 27,000 and is only moderately hydrophobic (87). The purified protein from E. coli K12 contains five methionine residues and the cyanogen bromide cleavage products can be separated into five bands on SDS-polyacrylamide gels. The sum of the molecular weights of these bands is 27,000, the same as determined by amino acid analysis and close to the molecular weight of the unheated form. The true molecular weight of protein B is unknown. Values based on SDS-polyacrylamide gel electrophoresis are subject to error (204). Also, calculation of the minimum molecular weight by amino acid analysis based on a single amino acid is not reliable. This is especially true of cysteine which can only be readily detected in hydrolysates of protein B as cysteic acid and is present in very low amounts (87). Obviously, further studies on this protein, preferably in dissociated form, free of detergent, are required before the true molecular weight of this protein can be established with certainty.

Protein B from E. coli K12 has an N-terminal alanine residue (87). However, three additional Edman cycles did not release any further amino acid derivatives. Henning believes he is dealing with a single polypeptide since mutants resistant to phage TuII* either lack protein II or produce a protein of altered electrophoretic mobility (87,153). In contrast Schnaitman's

work suggests that some strains of E. coli contain two similar heat-modifiable proteins (83). He was able to separate protein 3 prepared from E. coli J-5 grown on succinate-minimal media into two species, 3a and 3b by ion-exchange chromatography in the presence of Triton X-100 and EDTA. The proteins gave different peptides on cyanogen bromide cleavage. The difference between Schnaitman's results and those of Henning may be due to the differences in the strains used or growth conditions as is the case for protein A.

Chai and Foulds (154) have found that E. coli mutants (tol G) tolerant (157) to the bacteriocin JF 246 lack the heat-modifiable protein B. Ames and Nikaido (97) have confirmed this by two-dimensional electrophoresis. These mutants show an increased sensitivity to antibiotics, detergents and dyes, suggesting that the integrity of the cell envelope has been altered. This is in contrast to the results obtained by Henning (98) on a mutant of E. coli lacking both proteins A and B which had normal resistance to these agents. It has been proposed that tol G (tut) is the structural gene for this protein (153).

The heat-modifiable protein is absent in a mutant of E. coli defective in conjugation (105). This mutant is also resistant to phage K3 and therefore protein B may provide the attachment site for this phage, as well as for the pilus. That these are the only roles of protein B is improbable since this protein is present in large amounts in the outer membrane.

Other major outer membrane proteins

Henning has isolated a protein (III) with a molecular

weight of 17,000 which is present in major amounts in the outer membrane (81,149). Only preliminary characterization has been performed. However, its identity with a lipopolysaccharide-protein isolated by Wu and Heath (158) has been ruled out. Moldow et al. (106) have purified some outer membrane proteins but they have not been characterized except by SDS-polyacrylamide gel electrophoresis. The outer membrane of gram-negative bacteria contains many proteins in addition to those present in major amounts (83,97). The only enzyme localized to the outer membrane is phospholipase A (285). Other proteins have been purified and their function elucidated. These will be discussed in the following sections.

Functions of the Outer Membrane Proteins

Role of proteins in the barrier function of the outer membrane.

The cell wall of gram-negative bacteria provides a barrier against the penetration of antibiotics, dyes, detergents and other components (5,161-163). It also prevents the loss of proteins from the periplasmic space, the zone bound by the cytoplasmic membrane and the outer membrane (164). The peptidoglycan alone does not act as a permeability barrier (282) suggesting that the outer membrane is the barrier. The outer membrane must be selective, allowing the passage of nutrients while excluding toxic compounds. Selection is made on the basis of molecular size. Thus, pentalyisine cannot penetrate, in contrast to tetralysine and smaller peptides (170). The outer membrane is impermeable to oligosaccharides with molecular weights

greater than 1,000 (173,282). In contrast to phospholipid bilayers, the outer membrane of wild-type cells is impermeable to hydrophobic compounds (107).

Treatment of cells with EDTA disrupts the barrier function, increasing the permeability of the outer membrane to quaternary ammonium compounds (165), lysozyme (166), actinomycin and other drugs (163,167). EDTA chelates magnesium and calcium ions essential for the integrity of the outer membrane, causing release of from one third to one half of the lipopolysaccharide (167,168). Rough mutants defective in their lipopolysaccharide are more sensitive to dyes, detergents, lysozyme, and antibiotics, and leak periplasmic enzymes (5,174-176,179). Although the change in permeability has been related to the altered LPS structure, recent results have suggested that outer membrane proteins are also involved (86,177-179).

Ames, Spudich and Nikaido (86), and Parton (178) found that heptose-deficient mutants of Salmonella had decreased amounts of the major outer membrane proteins, A₁, A₂ and B. Koplow and Goldfine (177) found a similar effect in heptose-deficient mutants of E. coli where the phospholipid to protein ratio was increased 2.4 fold. These results differ from those obtained by Hancock and Reeves (103) and Wu (108) in which defects in the lipopolysaccharide were accompanied by a decrease in a single major outer membrane protein. Parton (178) correlated the defects in lipopolysaccharide with loss of outer membrane proteins. He found that a decrease in the amount of major outer membrane proteins occurred between Rb and Rc mutants (Fig. 3).

Not only are the major outer membrane proteins greatly decreased in heptose-deficient mutants, but the level of minor proteins such as the receptor for lambda phage was found to be decreased (180). This suggests that the defect is not in the assembly of a complex of lipopolysaccharide with the major outer membrane protein but perhaps reflects the role of lipopolysaccharide in stabilizing the outer membrane (180). The proteins lost were not found in the medium and so were not excreted (86). Hancock and Reeves (103) however concluded from their studies in a heptose-deficient mutant that contained normal amounts of the major outer membrane protein that "lipopolysaccharide core is not required for the presence of major outer membrane proteins in normal amounts." The relationship between the lipopolysaccharide and major outer membrane proteins is therefore still undefined and further work on this aspect of the outer membrane is required.

The effect of the deep-rough mutations on membrane structure has also been studied by electron microscopy and freeze-etching (181-183). A more distinct double-track appearance of the outer membrane was observed and a stronger cleavage occurred in the outer membrane suggesting that deep-rough mutants have a more hydrophobic zone in their membranes than do smooth strains. Nikaido (182) suggests that in wild-type cells most of the phospholipid is on the inner leaflet of the bilayer while the lipopolysaccharide occupies the outer surface (68,160). Loss of proteins results in an increase in the amount of phospholipid which now is distributed on both the inner and outer leaflet of

the bilayer (160,182). This would provide a more hydrophobic domain which would be more readily cleaved during freeze-etching, and would also allow the penetration of hydrophobic compounds such as antibiotics (107).

Reconstitution studies have shown that the permeability properties of the outer membrane are due primarily to the protein components. Vesicles composed of phospholipids and lipopolysaccharide are not permeable to hydrophilic molecules as small as sucrose (173). However, addition of outer membrane proteins (172) or a complex of protein A (134), but not of the lipoprotein, to lipopolysaccharide/phospholipid vesicles restored the permeability properties found in the native membrane. This suggests that protein A forms channels through the outer membrane and is responsible for the penetration of small hydrophilic molecules through the outer membrane.

Role of Outer membrane proteins in maintaining the shape of the organism

Some proteins of the outer membrane are present in major amounts and therefore could play a structural role (12,13,80,131,184,185). Presumably this would involve a repeating or regularly-spaced arrangement of the proteins. Protein A is arranged in an ordered manner on the peptidoglycan (80) and the bound lipoprotein is also attached to the peptidoglycan regularly at about every tenth repeating disaccharide (44).

There is some evidence to suggest that the major outer membrane proteins do help maintain the shape of the bacterium.

Hemming's group has been able to isolate ghosts, free of peptidoglycan and cytoplasmic components, that still retain the shape of the organism (131,136,149,187). The ghosts contain phospholipid (25%), lipopolysaccharide (25-30%) and four proteins (45-50%), proteins I, II, III, and IV (Table I). These proteins could form a repeating oligomeric structure responsible for cell shape, and crosslinking studies have confirmed that extensive protein-protein interactions do occur in the outer membrane (137,186). Moreover, removal of phospholipid from the ghost membrane did not alter its unit membrane appearance which suggests that a phospholipid bilayer may not be the basis for this membrane (131).

There is some evidence against the proposition that the proteins of the outer membrane maintain the shape of the organism. A temperature sensitive shape mutant of E. coli contained a normal complement of outer membrane proteins but was missing a single protein of the cytoplasmic membrane (191). In addition, a mutant of E. coli missing all the major outer membrane proteins had a normal morphology (98). Proteus mirabilis is rod-shaped but lacks both the bound and free forms of the lipoprotein during the exponential phase of growth (132). Obviously, the major outer membrane proteins are not the sole agents responsible for maintaining cell shape.

Role of the outer membrane proteins in DNA replication

The cell needs to coordinate the processes of DNA replication and cell envelope growth in order for cell division to take place

normally. Changes in several outer membrane proteins have been related to alterations in DNA synthesis. Inouye (209,210) has found that a protein found in the outer membrane of E. coli he calls Y protein with molecular weight of 34,000 decreased in amount whenever DNA synthesis was inhibited by nalidixic acid or thymine starvation. Another outer membrane protein with a molecular weight of 15,000 was overproduced under these conditions (211). The rate of appearance of this protein was decreased by inhibiting cell elongation. James has proposed that this protein is a structural protein of cell elongation (211). It is not known if these changes are primary effects of the inhibition of DNA synthesis.

The best evidence for the role of an outer membrane protein in DNA replication comes from the work of Gudas, James and Pardee (212). A protein which the authors call protein D (M.W. 80,000) was incorporated into the outer membrane of synchronous cultures of E. coli towards the end of the cell cycle (212). This protein is similar to one described by Churchward and Holland (213). Inhibition of DNA synthesis delayed the appearance of this protein. Upon removal of the inhibition, protein D was synthesized, followed by a burst of DNA synthesis. Disruption of peptidoglycan synthesis by the amidinopenicillamic acid, FL 1060 also increased the amount of this protein. Protein D bound to DNA in vitro. The authors suggested that this protein acts as an attachment site for DNA to the cell envelope and provides a metabolic link between peptidoglycan synthesis, protein synthesis and DNA initiation.

Involvement of outer membrane proteins in high affinity transport systems

Iron transport: E. coli transports ferric ion as a complex with citrate (214), enterochelin (215) or ferrichrome (216,217). Outer membrane proteins appear to be involved in the last two systems. Hantke and Braun (218) have shown that transport of iron by the ferrichrome system is dependant on the ton A gene product. This gene locus was originally classified as a phage T5 receptor (219). Braun and coworkers have isolated the receptor for phage T5 and have shown that this protein (M.W. 85,000), located in the outer membrane, is also the binding site of colicin M (220-222). Absorption of phage Φ 80 to cells was inhibited by ferrichrome (102,224) suggesting that ferrichrome, phages T5 and Φ 80 and colicin M all bind to the same receptor in the outer membrane.

Bacteria with a reduced ability to utilize iron contained an increased level of colicin Ia receptor in the outer membrane (277) suggesting a role for this receptor in iron uptake. Enterochelin, and colicins B and D likely bind to the colicin Ia receptor (279). Mutants resistant to colicins B and I map at the ton B locus and overproduce enterochelin (225,226). The resistance to colicins is not due to competition of the excreted enterochelin with the colicin for the receptor since ton B mutants unable to synthesize enterochelin (aro E) remain colicin tolerant (218). The ton B mutants are also defective in Φ 80 infection (224) and lack an outer membrane protein necessary for the absorption of Φ 80 and the uptake of enterochelin. The colicin Ia receptor has

been characterized as an outer membrane protein complex with a molecular weight of 307,000 (283). Growth of cells on iron-deprived media results in an increased level of two proteins (M. W. 95,000 and 85,000) of the outer membrane (100,101,268,278). These proteins may be the ton A and ton B gene products.

Vitamin B12: The first step in the transport of vitamin B12 in E. coli is binding to a receptor in the outer membrane (227), which also acts as the binding site for colicins E₂ and E₃ (228) and bacteriophage BF 23 (229). The colicin E₃ receptor from E. coli has been purified and characterized as a protein with a molecular weight of 60,000 (230).

Maltose: The product of the lam B gene in E. coli is the receptor for bacteriophage λ (231). It is an outer membrane protein with a molecular weight of 55,000. The lam B-mutants are phage λ -resistant and are unable to use maltose when this sugar is present in limiting amounts (232). In addition, chemotaxis towards maltose is impaired in these mutants (233) suggesting that the phage λ receptor is involved in maltose transport.

Organization of proteins in the outer membrane of E. coli

Early models describing the organization of proteins in the cell envelope of E. coli were based on observations under the electron microscope (Fig. 5a and b). Protein granules, likely equivalent to the bound lipoprotein, were visible on the peptidoglycan layer (207). In the model presented by Martin (208) in 1963 (Fig. 5a) the outer membrane was visualized as two non-descript layers composed of lipopolysaccharide and

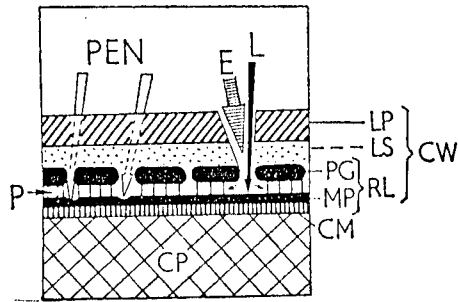


Fig. 5a. Schematic representation of bacterial cell wall structure (1963). Gram-negative bacterium with complex triple-layered cell wall. Separation of the layers can be achieved by solvent extraction and by treatment with proteolytic enzymes (P). Both lysozyme (L), aided by EDTA (E) and penicillin (PEN) induce depolymerization of the rigid mucopolymer (MP), although probably to a different degree and in a different way. LP, lipoprotein layer; LS, lipopolysaccharide layer; RL, rigid layer; PG protein granula; MP, mucopolymer; CM cytoplasmic membrane; CP, cytoplasm. Taken from Martin (209).

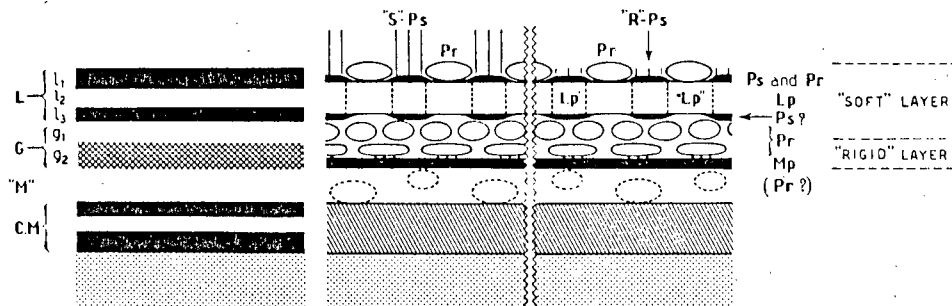


Fig. 5b. Schematic model of the cell wall of *E. coli* (1967). Dimensions, shape, and arrangements of the actual single constituent elements of the cell wall layers are still unknown. The approximate correspondence of the various components with the structure visible in thin section is shown on the left. The left part of the diagram represents the wall of a "smooth" cell, in which the polysaccharides ("S"-Ps) are assumed to be composed of a basal structure and specific side chains on the right wall of a "rough" form, which the polysaccharides ("R"-Ps) lack the specific side chains. Proteins (Pr) and polysaccharides (Ps) are considered to be distributed in a mosaic on the surface of the wall (l_1). The bulk of the lipids (Lp) is situated at the level of the L membrane, associated either with polysaccharide or possibly with proteins. Two rows of protein globular elements are drawn at the level of the G layer to indicate that it probably contains proteins both covalently linked to the mucopeptide layer (murein, Mp) and unlinked to it (see text). CM, cytoplasmic membrane. Taken from DePetrìs (10).

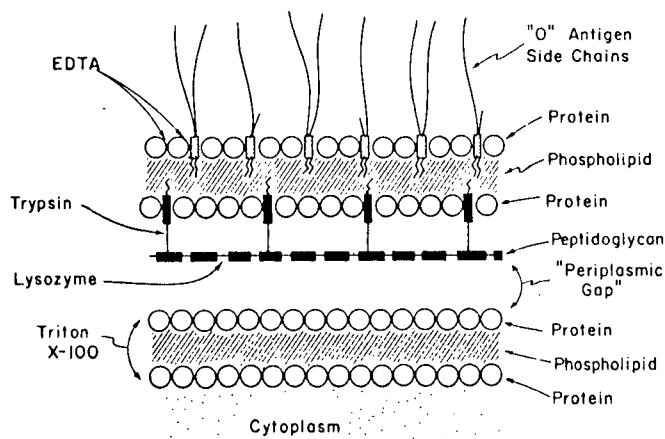


Fig. 5c. Schematic representation of the envelope of *E. coli* (1971). For simplicity the outer membrane are shown as simple bilayers. Arrows indicate the site of action of EDTA in the release of proteins and lipopolysaccharide from the outer membrane, and the action of trypsin in cleavage of the murein lipoprotein. Taken from Schnaitman (67).

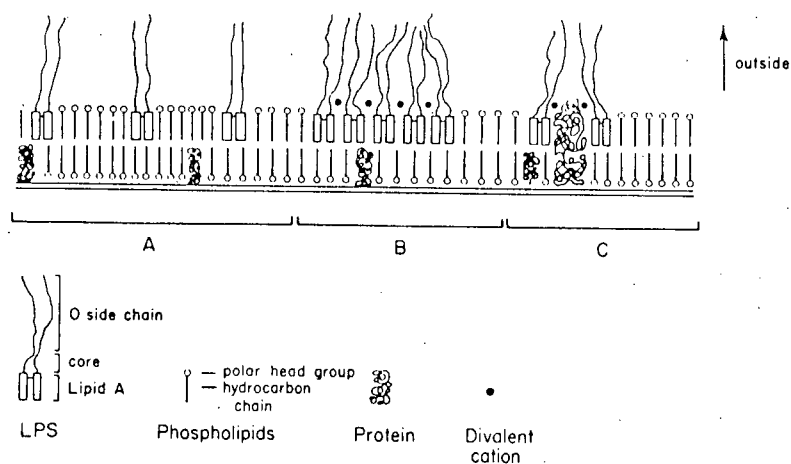


Fig. 5d. A possible arrangement of molecules in the outer membrane layer (1973). In region A, LPS molecules are interspersed among phospholipid molecules reducing the electrostatic repulsion between the KDO-heptose regions of the core oligosaccharides. In region B, divalent cations reduce this electrostatic repulsion, so that at least the outer leaflet of the bilayer is composed predominantly of LPS molecules. In region C, the protein molecules are thought to interact with phospholipids as well as LPS. LPS-protein interaction is here shown as though it involves divalent cations. The outer membrane is assumed to be held in place by the penetration of the small protein molecules, which are placed 100 Å apart and are covalently linked to the peptidoglycan. Taken from Nikaido (33).

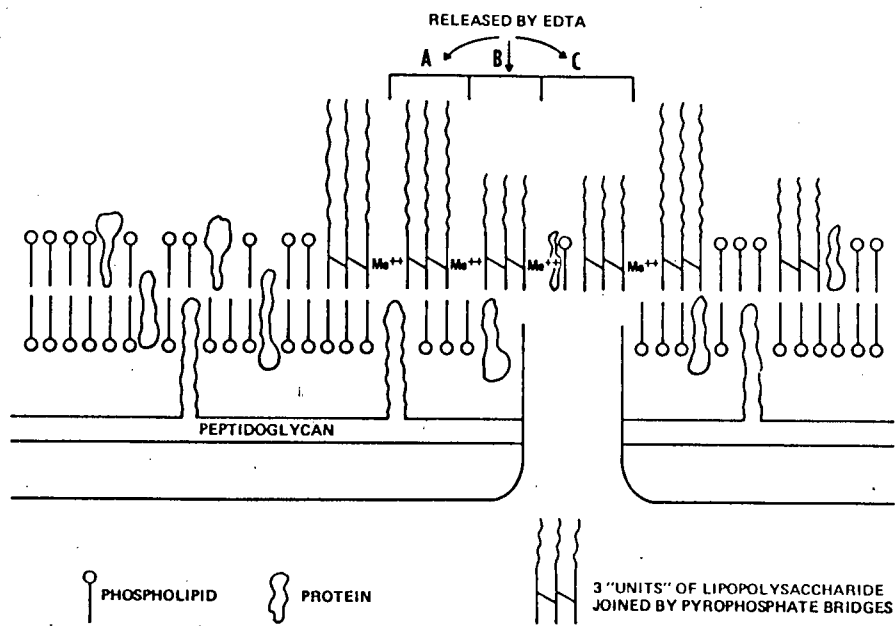


Fig. 5c. Model of outer membrane of gram-negative bacteria (1974). Taken from Leive (61).

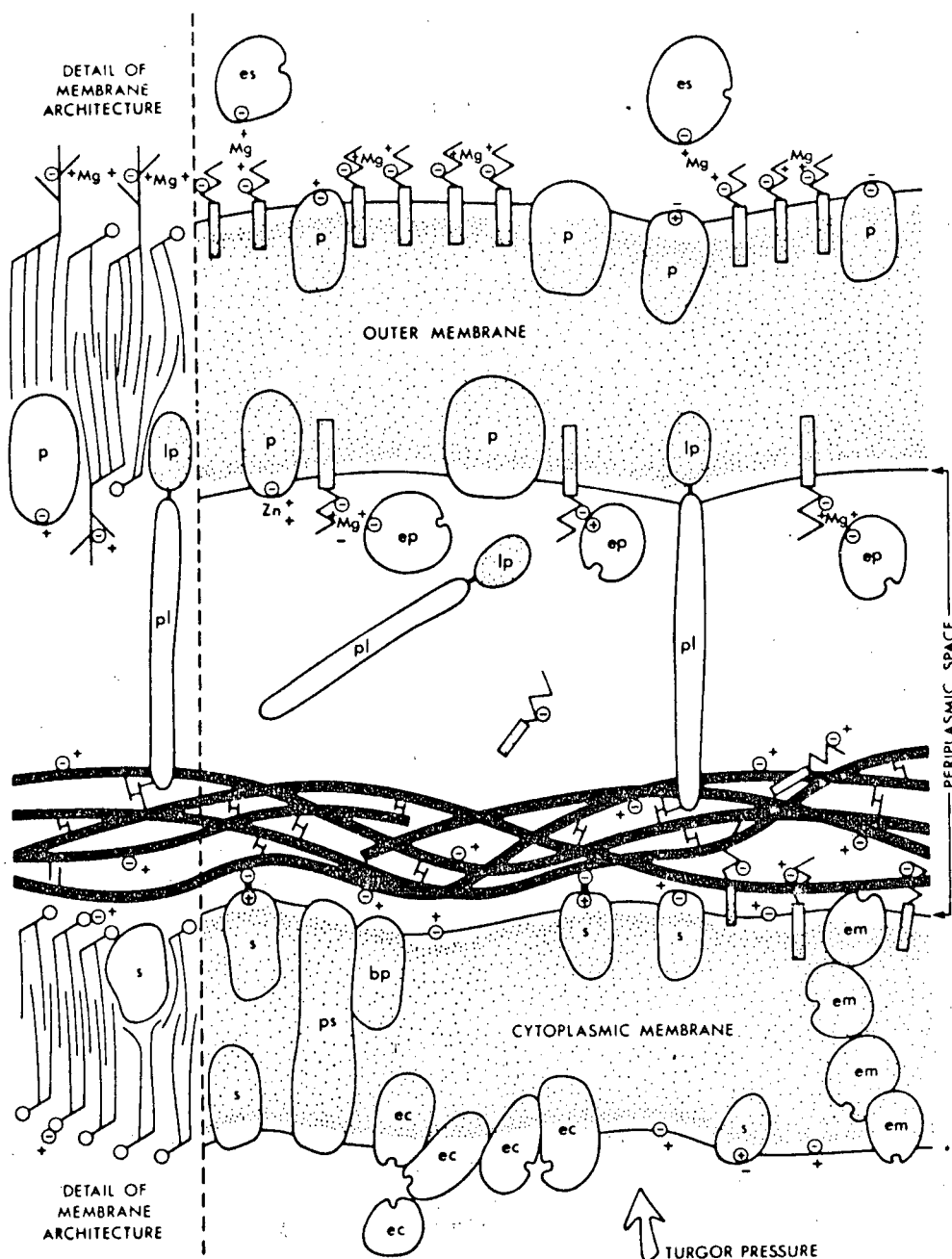


Fig. 5f. Schematic diagram of gram-negative cell envelope (1974). +, Free cation; -, free anion; $\oplus \ominus$ bound cation; bound anion; adhesion point produced by ionic bonding; \sim hydrophobic zone; — covalent bond; --- cross-linking polypeptide in the peptidoglycan; --- polysaccharide portion of peptidoglycan; e enzymatically active protein; p phospholipid; lp lipopolysaccharide; pl lipopolysaccharide (schematic); bp , binding protein; ec , enzymes associated with the cytoplasmic membrane whose function is directed to the cytoplasm; em , enzymes associated with the cytoplasmic membrane which synthesize macromolecular components of the cell wall; ep , enzymes localized in the periplasmic zone; es , enzymes localized at the cell surface; lp , lipid portion of Braun's lipoprotein; p , structural and enzymatic proteins of the outer membrane; pl , protein portion of Braun's lipoprotein; ps , permease; s , structural protein of cytoplasmic membrane. Taken from Costerton, Ingram and Cheng (5).

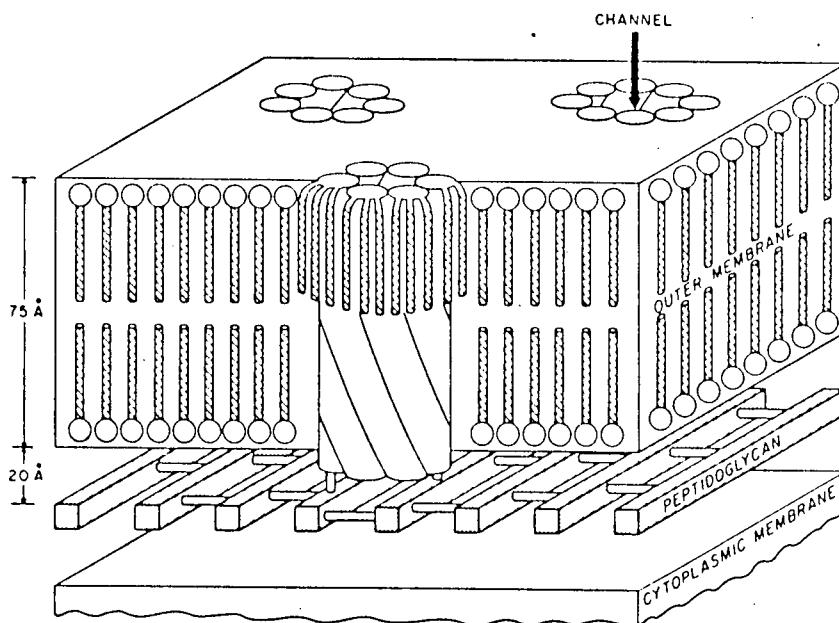


Fig 5g. Schematic illustration of the outer membrane structure (1975). A superhelix made of six α -helices is shown to be inserted into the outer membrane and to span the full 75Å-thick membrane. The three hydrocarbon chains attached at the top of each molecule are flipped over, hanging down from the top, and are anchored in the lipid bilayer of the outer membrane. At the bottom (carboxyl-terminal ends of the lipoproteins) of the assembly, two molecules are linked to the peptidoglycan layer, as shown by small bars. The peptidoglycan layer is illustrated by rectangular blocks (for the glycan chains) and small bars (for the peptide portions) which are cross-linking the glycan chains. Phospholipids forming the lipid bilayer are shown by hydrophilic, open, circular heads and hydrophobic, hatched, long tails. Channel opening of 7- and 8-membered assemblies are also illustrated on the surface of the outer membrane. Taken from Inouye (120).

- | | | | |
|-------|--|----|--|
| + | Free cation | cc | Capsular carbohydrate |
| - | Free anion | cp | Capsular protein |
| • | Bound cation | ec | Enzymes associated with the cytoplasmic membrane whose function is directed to the cytoplasm |
| • | Bound anion | em | Enzymes associated with the cytoplasmic membrane which synthesize macromolecular components of the cell wall |
| •• | Adhesion point produced by ionic bonding | ep | Enzymes localized in the periplasmic zone |
| ••• | Hydrophobic zone | es | Enzymes localized at the cell surface |
| •••• | Cross-linking polypeptide in the peptidoglycan | lp | Braun's lipoprotein |
| ••••• | Polysaccharide portion of peptidoglycan | p | Structural and enzymatic proteins of the outer membrane |
| ••••• | Enzymatically active protein | ps | Permease |
| ••••• | Phospholipid | s | Structural protein of cytoplasmic membrane |
| ••••• | Lipopolysaccharide | | |
| ••••• | Lipopolysaccharide (schematic) | | |
| ••••• | bp | | |
| ••••• | Binding protein | | |

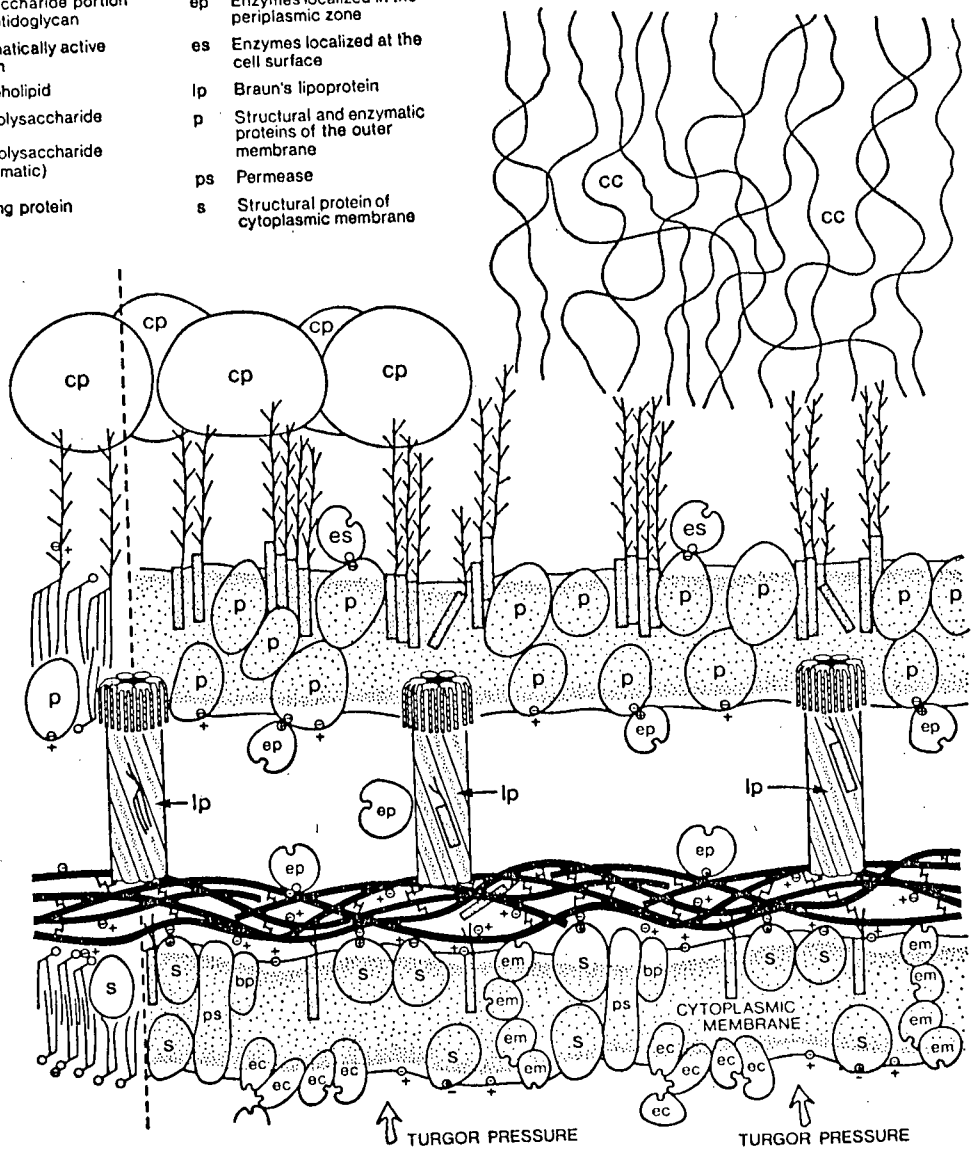


Fig. 5h. Schematic diagram of the gram-negative cell envelope (1975). Taken from Costerton and Cheng (62).

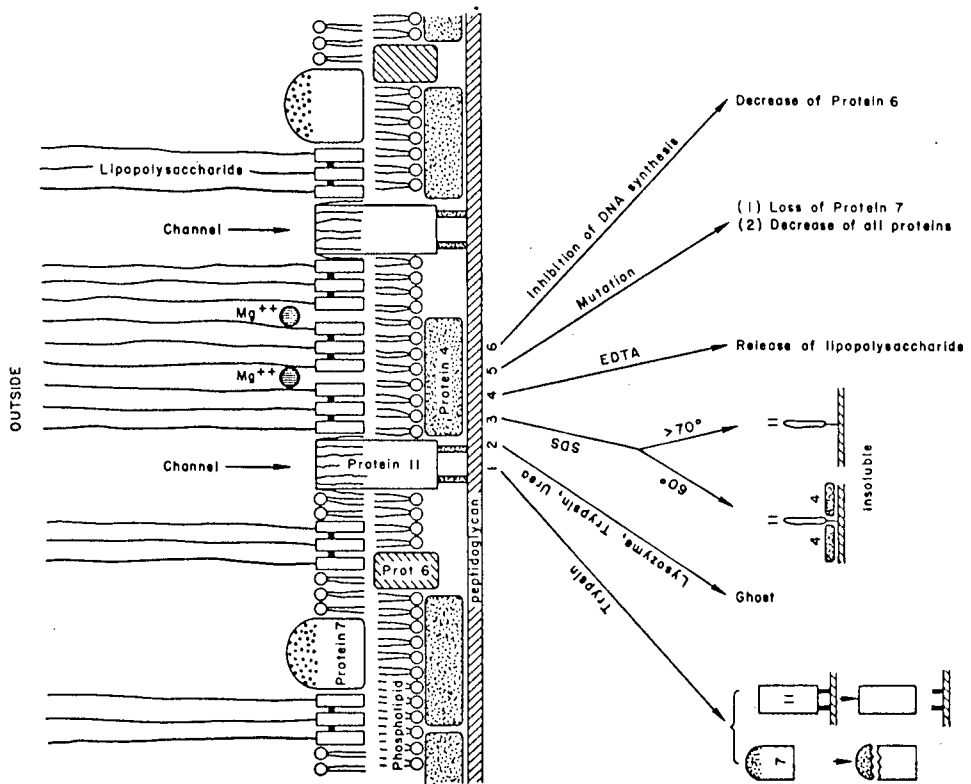


Fig. 5i. Molecular model of the outer membrane (1975).
See table I for protein nomenclature. Taken from Inouye (120).

lipoprotein. De Petris (10) in 1967 suggested that the outer membrane is composed of lipopolysaccharide, lipids and proteins arranged in a mosaic pattern. In this model (Fig. 5b) the carbohydrate chains of the lipopolysaccharide protude from the external surface of the membrane. The protein visualized in the gl area may be the matrix protein A since De Petris found that this protein was non-covalently attached to the peptidoglycan.

The simplified scheme proposed by Schnaitman (67) in 1971 (Fig. 5c) is based on the Davson-Danielli model (205). The proteins are organized on the inner and outer surfaces of a phospholipid bilayer. The hydrophobic lipid A portion of the lipopolysaccharide interacts with the hydrophobic core of the membrane and the hydrophilic carbohydrate chains extend into the medium. The bound lipoprotein links the outer membrane to the peptidoglycan.

Models for the outer membrane which are based on studies of the lipopolysaccharide have been presented (Fig. 5 d and e). These models arrange the lipopolysaccharide and phospholipid molecules into a bilayer and emphasize the role played by divalent cations in stabilizing the outer membrane. The lipopolysaccharide molecules are arranged in regions corresponding to EDTA-releasable and non-releasable fractions. The protein components are presented as globules dissolved in the bilayer as proposed by Singer and Nicholson (240). The bound lipoprotein is visualized as penetrating only part way through the outer membrane. Costerton, Ingram and Cheng (5) have presented a more detailed model (Fig. 5f) illustrating essentially the same features.

Inouye (120,130) has proposed that bound and free lipoproteins form channels through the outer membrane. In this model (Fig. 5g), six lipoprotein molecules, two bound and four free, are arranged in a superhelix with a hydrophilic channel 12.5 Å in diameter in the centre. The assembly is 76 Å in length and could span the 75 Å thick outer membrane.

Braun has proposed (123) that the lipoprotein spans only part of the thickness of the outer membrane in contrast to the proposal by Inouye (133). This conclusion was drawn from extensive immunological studies with antibodies directed against various forms of the lipoprotein. In wild-type cells, the lipoprotein was unable to react with antibodies to it, but became accessible in cells with a defective lipopolysaccharide (123). This does not necessarily contradict Inouye's proposal since the carbohydrate chains of the lipopolysaccharide may restrict access of the antibody to the lipoprotein which extends just through the membrane (Fig. 5g).

A model (Fig. 5h) based on Inouye's superhelix, but not allowing the lipoprotein to penetrate the membrane, was recently proposed by Costerton and Cheung (101). This arrangement of the lipoprotein is very unlikely since all the hydrophobic amino acid residues on the outside of the helix would be in contact with the water of the periplasmic space.

A more detailed model postulated recently by Inouye (120) is illustrated in Fig. 5i. The matrix protein, 4 (equivalent to protein A) is associated with the peptidoglycan (80). Protein 7 (protein 8) is exposed at the external surface of the

outer membrane (137). Proteolytic digestion of the outer membrane will result in the hydrolysis of the exposed part of protein B to leave a resistant fragment embedded in the membrane (79,82,131,135,136,149). Fragments with a molecular weight of 25,000 and 20,000 are produced by the action of trypsin and pronase respectively. Protein 11, the lipoprotein is arranged as a superhelix that penetrates the membrane. Trypsin releases this complex from the peptidoglycan (117,121) while treatment of the outer membrane with pronase results in digestion of the lipoprotein (79).

Results obtained recently suggest that the model proposed by Inouye (Fig. 5i) is no longer adequate. Protein A likely serves as a receptor for bacteriophage (41,103,142) suggesting that it is exposed on the outer surface of the membrane. This is confirmed by the finding that protein A could form channels through lipopolysaccharide/phospholipid vesicles (134) and therefore spans the membrane. In addition, Nakae (134) found that the lipoprotein did not form channels through these vesicles.

Treatment of isolated outer membrane preparations with proteolytic enzymes have suggested that the proteins of the outer membrane are arranged asymmetrically (79). As already discussed, protein A is resistant to digestion while a portion of protein B is exposed. Bragg and Hou also found that proteins D, E and F were readily digested and were therefore exposed at the surface of the membrane. Proteins C_2 , C_3 and D_2 were not digested significantly.

The relationships between results obtained with isolated outer membrane preparations such as outer membrane, cell

wall and cell envelope and intact cells is not clear. Schindler and Teuber (238) found no labelling of cell envelope proteins when intact cells of Salmonella typhimurium were treated with dansyl chloride in phospholipid micelles in order to be nonpenetrating. In contrast, all polypeptides could be labelled by dansylation of isolated cell envelopes. The difference could not be due to interference by the carbohydrate portion of the lipopolysaccharide since a deep-rough mutant of Salmonella which had only KDO left in the lipopolysaccharide also did not react with dansyl chloride. The authors suggest that this was because there were no free amino groups on the surface of the cell.

Lactoperoxidase-catalyzed iodination of exposed tyrosine residues did not result in extensive labelling of outer membrane proteins in intact cells. A single protein band (M.W., 13,000) in Salmonella (236) and a phospholipoprotein (M.W. 16,000) in Pseudomonas (266) were iodinated by this technique. The lack of more general labelling may be accounted for by inhibition of the iodination reaction by the cells. Gow, Parton and Wardlaw (237) have shown that bovine serum albumin, which is readily iodinated by this technique, was not labelled when mixed with intact cells of Bordetella pertussis.

The polypeptides of the outer membrane of intact cells were not readily crosslinked through the formation of disulfide bonds, catalyzed by the CuSO_4 -O-phenanthroline reagent (137). However, in order to be crosslinked, the proteins must be accessible to the reagent and have suitably disposed, free

sulfhydryl groups. Some outer membrane proteins of deep-rough mutants could be crosslinked by this technique, suggesting that the lipopolysaccharide protects the outer membrane proteins from the reagent. The proteins of ghosts and cell envelopes could also be readily crosslinked. The proteins crosslinked by this technique presumably are arranged on the outer surface of the ghost membrane. About thirty different polypeptides were crosslinked, however, none of the major outer membrane proteins (A,B or F) were involved.

Henning (186) has shown that treatment of intact cells with dimethyl imidoesters results in extensive crosslinking of proteins I (A_1), II* (B) III (likely D_2) and IV (F). The ability of the imidoesters to react with the amino groups, which contrasts with results obtained with dansyl chloride is probably due to the ability of the esters to penetrate the membrane. The resulting sacs were the same shape as cells and were resistant to disruption by boiling 1% SDS. Ghosts, containing these four proteins plus some higher molecular weight proteins could be similarly crosslinked. Trypsin treatment of ghosts, which cleaved a portion of protein B, precluded formation of these detergent-resistant sacs. On the basis of these results, Henning has suggested that extensive protein-protein interactions occur in the outer membrane and that the model proposed by Capaldi and Green (239) may more adequately describe this membrane than the fluid mosaic model of Singer and Nicholson (240).

A number of problems are still unresolved. Many outer membrane proteins must be exposed on the outer surface of the cell since, as noted previously, these proteins serve as recep-

tors for phage, colicins, etc. The inability to detect exposed proteins in intact cells may be due to limitations of the technique used or perhaps because of interference by the carbohydrate chains of the lipopolysaccharide. Both aspects of this problem are explored in this thesis. In addition, the spatial relationships of the individual outer membrane proteins to one another will be studied.

Biosynthesis and assembly of outer membrane proteins

Biosynthesis

The proteins of the outer membrane are synthesized on ribosomes located in the cytoplasm or on the inner aspect of the cytoplasmic membrane. However, the synthesis of envelope and cytoplasmic proteins differed in this sensitivity to ribosome-directed antibiotics (120,241). Envelope protein synthesis was more resistant to kasugamycin and puromycin than cytoplasmic protein synthesis, while the reverse is true for tetracycline and sparsomycin. Chloramphenicol inhibited both envelope and cytoplasmic protein synthesis to the same extent (241,242). However, no degradation of incorporated inner and outer membrane proteins occurred under these conditions (276).

Furthermore, inhibition patterns of the synthesis of individual envelope proteins differed. The synthesis of the lipoprotein and a major protein molecular weight of 38,000 were very resistant to the action of certain antibiotics.

The difference between the sensitivity of envelope

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protein and cytoplasmic protein synthesis has been attributed (120) to a number of possible causes. There is evidence for a difference in mRNA molecules. The stability of mRNAs for envelope proteins was determined from the effect of rifampicin on their biosynthesis (241). The mRNAs for the outer membrane proteins are more stable (half-life, 5.5 mins) than those for the synthesis of inner membrane and cytoplasmic proteins (243). Similar results were found in E. coli minicells which lacked DNA but in which protein synthesis was sensitive to chloramphenicol but resistant to rifampicin. In these cells, the mRNAs had half-lives of 40-80 minutes and coded for the outer membrane proteins.

The difference between outer membrane protein synthesis and inner membrane and cytoplasmic protein synthesis may also be due to differentiations of the ribosome population. Purified ribosomes of E. coli were found to be heterogeneous with respect to the content of ribosomal proteins (244,245). However Randall and Hardy (246) examined the polysomes containing the stable mRNAs following rifampicin treatment and concluded that there was no difference between the protein composition of the ribosomes synthesizing outer membrane proteins and those synthesizing total proteins. Another possible explanation for the difference could be the compartmentalization of the ribosomes. For example, ribosomes synthesizing alkaline phosphatase, or periplasmic enzyme are associated with the cytoplasmic membrane (247).

The biosynthesis of the two forms of the lipoprotein has been studied in greatest detail (44,120). The lipoprotein does

not contain histidine and is the only envelope protein synthesized by a histidine auxotroph in the absence of this amino acid (248). Tetracycline and chloramphenicol were highly inhibitory to lipoprotein synthesis under conditions of histidine starvation. Rifampicin, however had little effect, confirming the stable nature of the mRNA.

This stable mRNA has been isolated and translated in both an E. coli cell-free system (249) and a wheat-germ system (250). The mRNA contained two species which were 230 and 250 nucleotides long. Since a sequence of 180 bases are required for translation into a protein of 58 amino acids, 50-70 bases must be untranslated. A single protein characterized as the polypeptide portion of the lipoprotein was formed in the wheat-germ system. A second protein with about twice the molecular weight of the lipoprotein was also synthesized. How this was accomplished with the mRNA molecules previously characterized is not clear, however, the large product may be a precursor normally found in the cell.

The complete biosynthesis of the lipoprotein requires the attachment of the lipid moiety. Little is known about this process except that the cis diol group is derived from glycerol and that attachment of the diglyceride occurs after synthesis of the apoprotein (270). This is consistent with the finding that the ester-linked fatty acids are similar to those found in the cellular phospholipids (122). The availability of the lipid-free protein formed in vitro and E. coli mutants altered in the bound lipoprotein (279) should be of considerable use in esta-

blishing the steps involved in the biosynthesis of the unique lipid portion of this molecule.

The free form of the lipoprotein is the precursor of the bound form and this process is not inhibited by chloramphenicol, amino acid starvation or carbonyl cyanide m-chlorophenylhydrazine, an energy uncoupler (126). No lipoprotein-mucopeptides are found in the cytoplasmic membrane and the lipoprotein is incorporated into newly-made murein at discrete sites (251). The maximum rate of incorporation of the lipoprotein into the outer membrane occurs at the time of septation (252). In addition, a mutant defective in coupling outer membrane invagination with septation contained a decreased amount of bound lipoprotein and a corresponding increased level of the free form (109). The authors suggest "that the morphogenetic defect may result from a defect in formation of covalent bonds between the free lipoprotein of the outer membrane and the murein of the nascent septum".

Assembly of the proteins of the outer membrane

Cell division involves the coordination of DNA replication, septation and cell envelope growth (32,253,255). Cell wall growth requires coordinated synthesis and assembly of peptidoglycan, phospholipid, lipopolysaccharide and protein. A defect in this coordination may result in the production of morphological mutants (192-203).

Coordination of phospholipid and membrane protein synthesis has been studied in a glycerol auxotroph mutant of E. coli (256).

Upon glycerol deprivation, this mutant continued soluble and envelope protein synthesis causing the protein to phospholipid ratio to increase by 60%. The authors concluded that the biosynthesis of inner and outer membrane phospholipids and proteins are not tightly coupled. This is supported by the finding that inhibition of β -ketoacylthioesterase by cerulenin did not prevent the incorporation of the λ phage receptor into the outer membrane (257). In addition, no differences in the polypeptide composition of the outer membrane was observed in fatty acid auxotrophs of E. coli supplemented with various fatty acids (284).

Reconstitution

The reassembly of the isolated components of biological membranes into a form identical to the native membrane will aid in the understanding of the role of the components in the membrane (258). Membranes are self-assembling systems (259) that can be reformed from dissociated components upon removal of the solubilizing agent, which is usually a detergent (260). Reconstitution can be monitored by electron microscopy (258,261). A second measurement of reconstitution is the restoration of function in the reassembled membrane (258) as has been accomplished with the components of oxidative phosphorylation (258,262, 263).

De Pamphilis (72) dissociated purified outer membranes with Triton X-100 in the presence of EDTA and found that the solubilized material reassembled into vesicles upon removal of the

detergent in the presence of magnesium ions. Bragg and Hou (79) have solubilized the outer membrane with SDS and recovered reformed membranes upon removal of the detergent by dialysis. The reconstituted membranes had a similar morphology, density and chemical composition to that of the native membrane. More significantly, the proteins appeared to be arranged in a similar manner in the native and reformed membranes as judged by pronase digestion. A similar study has been reported by Sckizawa and Fukai (264).

Nakamura and Mizushima (265) have separated the protein, lipopolysaccharide and phospholipid components of the outer membrane and have studied the role of the purified components in the reassembly of membrane vesicles. Removal of detergent by dialysis in the presence of magnesium ions was essential for vesicle formation. Although lipopolysaccharide alone reassembled into a trilaminar structure, phospholipid was required for vesicle formation. Protein from the outer membrane but not the cytoplasmic membrane was reincorporated into the membranous vesicles. Recent studies (115) have shown that reassembly of the outer membrane on the peptidoglycan requires the presence of the bound lipoprotein and the matrix protein A.

Nikaido and coworkers have studied the permeability properties of reformed vesicles. Vesicles composed of phospholipid and lipopolysaccharide were not permeable to molecules as small as sucrose (173). Protein was required for penetration of small oligosaccharides (172) with protein A being responsible for the formation of a channel through the

lipopolysaccharide/phospholipid vesicle (134). The reconstitution technique may also help define the nature of the interactions between outer membrane components, and the use of defective components (eg. lipopolysaccharide) in reconstitution systems may lead to an understanding of the function of the altered component.

Thesis Project

The unique features of the outer membrane suggest that it may not be a typical membrane, making it an interesting system for study. Two major proteins of the outer membrane, the matrix protein, A, and the heat-modifiable protein, B, were purified and characterized since little was known about the properties of the outer membrane proteins, except for the bound lipoprotein. Protein B changes its apparent molecular weight upon heating in SDS-containing solutions. The reason for this change was investigated by comparing proteins B and the modified form, B*, by a number of physical techniques. The organization of the proteins in wild-type and nutritionally or genetically-altered membranes was studied by proteolytic digestion, covalent labelling, cross-linking and reconstitution studies. A model for the organization of the proteins in this unique membrane is presented.

METHODS AND MATERIALS

Reagents

All chemicals were reagent grade. The sources of the chemicals were: acrylamide, bis(N,N-methylenebisacrylamide, Bio-Gel P-150 and Dowex AG1-X4 were purchased from BioRad Laboratories; sodium dodecyl sulfate (specially pure), British Drug Houses Ltd.; α -chymotrypsin, α -chymotrypsinogen, bovine serum albumin, egg-white lysozyme, equine hemoglobin, human γ -globulin, phenylmethylsulfonyl Fluoride, pronase, riboflavin, riboflavin-5'-phosphate, Triton X-100, trypsin, Calbiochem; Cheng-Chin polyamide layer sheets were obtained from Gallard-Schlesinger Chemical Corp.; 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine, phenylisothiocyanate, Eastman Kodak Company; D₂O, ICN Pharmaceuticals Inc.; glutaraldehyde (histological grade), Matheson, Coleman and Bell; deoxyribonuclease, Nutritional Biochemicals Corp.; dithiobis (succinimidyl propionate), Pierce Chemical Company; Sephadex G-100 and Sepharose 6-B, Pharmacia; egg-white lysozyme, ovalbumin, Sigma Chemical Company. Fluorescamine (Hoffmann-La Roche) was a gift from Dr. E.P.M. Candido of the Department of Biochemistry, University of British Columbia.

Bacterial strains

E. coli strain 482 of the culture collection of the National Research Council of Canada was used for the majority of the work described in this thesis. E. coli strain JE 1011 (F⁻, Thr⁻, Leu⁻, Trp⁻, His⁻, B₁⁻, Lac⁻, Gal⁻, Xyl⁻, M+1⁻, Str^R) and its heptose-deficient

mutant, NS-1 were used in studies on lipopolysaccharide-defective cells. The bacteria were maintained on agar slants on a medium containing 3 g of Trypticase Soy medium Difco, 0.33 g of yeast extract and 1.5 g of agar per 100 ml.

Growth conditions (143)

E. coli strain 482 was routinely grown on a minimal medium of 0.4% glucose and salts (0.7% K_2HPO_4 , 0.3% $KHPO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% sodium citrate, 0.002% Ferric citrate and 0.1% $(NH_4)_2 SO_4$. In experiments studying the effect of the carbon source, glucose was replaced by either 0.4% glycerol, 1.4% disodium succinate or 0.7% sodium acetate. When 0.5% casein amino acids were used, glucose and $(NH_4)_2 SO_4$ were omitted. Cells were also grown on a complex medium made by dissolving 17.5 g of Penassay medium (Difco) or 30 g of Trypticase Soy medium (Difco) in 1 l of water. E. coli JE 1011 and NS-1 were grown on Penassay medium. Distilled water was used to prepare all media which were sterilized for 15 min in an autoclave before use.

The normal procedure for growing 4 l batches of cells was as follows. A culture tube containing 10 ml of medium was inoculated from a nutrient agar slant and incubated at 37°C for 24 h. This liquid culture was used to inoculate a further 200 ml of medium contained in a 1-l flask. The flask culture was grown at 37°C in either a Reciprocal Water Bath Shaker (New Brunswick Scientific Co. Inc.) at 100 cycles per min or a Controlled Environment Incubator (New Brunswick Scientific Co. Inc.) at 200 revolutions per min overnight. The 200 ml flask culture was used to inoculate 4 l of medium. The bacteria

were grown at 37°C with vigorous aeration provided by forcing water-saturated air through a sintered glass sparger. Cell growth was monitored by measuring the absorbance of the culture at 420 nm and the cells were harvested at the appropriate phase of growth by centrifugation at 4,000 x g for 15 min at 4°C. Cells were either used immediately or stored frozen at -20°C.

Cell Fractionation

Preparation of Triton-extracted cell envelopes (cell wall) (79)

Unless stated otherwise, all operations were performed at 0-4°C. Cells (50 g, wet weight) were suspended to a volume of 160 ml with 10 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂. A few crystals of DNase were added and the cells were broken by passage through a pre-cooled French Pressure Cell at 20,000 lbs/inch². The lysed cell suspension was centrifuged at 4,000 x g for 10 min to remove unbroken cells. The resulting supernatant was then centrifuged at 120,000 x g for 1 h to sediment cell envelopes. The cell envelope fraction was extracted with 1.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.0, containing 35 mM MgCl₂ at 22°C for 1 h by suspending the pellet to a volume of 160 ml and agitating vigorously on a wrist-shaker. This resulted in solubilization of the inner membrane. The cell wall fraction consisting of outer membrane and peptidoglycan, was sedimented by centrifugation at 120,000 x g for 1 h. The resulting pellet was washed with 0.1 M Tris-HCl buffer, pH 8.0 containing 35 mM MgCl₂ and resedimented at 120,000 x g for 1 h.

Sequential extraction of cell wall

The cell wall fraction, derived from 50 g (wet weight) of cells was suspended to 160 ml of 0.5% SDS and incubated at 37°C for 1 h. The SDS-extracted cell wall was sedimented at 120,000 x g for 1 h at 15°C to give "SDS extract 1". The pellet was reextracted with 80 ml of 1% SDS at 100°C for 15 min. Peptidoglycan was removed by centrifugation at 120,000 x g for 1 h at 15°C to yield "SDS extract 2". Extract 1 was used in the purification of protein B.

Preparation of protein A-peptidoglycan complexes (80)

Cell envelopes, prepared from 50 g (wet weight) of cells, were suspended to 160 ml of 2% SDS and incubated at 60°C for 1 h. The insoluble peptidoglycan was sedimented by centrifugation at 120,000 x g for 1 h at 15°C. The extraction step was repeated once and the resulting protein A-peptidoglycan complex was washed four times with an appropriate buffer to remove residual SDS.

Preparation and lysis of spheroplasts (155)

Cells were harvested in the mid-exponential phase of growth ($A_{420,5.0}$). The cells were washed once with 0.18 M NaCl, 40 ml per g (wet weight) of cells. The washed cells were suspended at 22°C to a concentration of 1 g per 40 ml in 30 mM Tris-HCl, pH 8.0 containing 20% (w/v) sucrose and the suspension was stirred gently for 20 min. Lysozyme (5 mg/g of cells) was then added as a solution in distilled H₂O and the suspension was stirred for an additional 30 min at 22°C. The suspension was

diluted rapidly by pouring into 10 volumes of 0.2 mM MgCl_2 , containing a few crystals of DNase. The decrease in turbidity was monitored at 420 nm until a constant absorbance was reached. The suspension of lysed cells was centrifuged at 15°C at 4,000 x g for 5 min to remove intact cells, and then centrifuged at 15,000 x g for 30 min at 4°C to obtain the preparation of spheroplasts used in this thesis. Outer membrane was prepared from the spheroplast membranes by extraction of the inner membrane with 1.5% Triton X-100 in 0.1 M Tris-HCl, containing 35 mM MgCl_2 at 22°C for 1 h. Membrane was recovered by centrifugation at 120,00 x g for 1 h at 4°C.

Release of outer membrane from spheroplasts (77,78)

Cells were grown to mid-exponential phase of growth. The harvested cells (ca 3.5 g, wet weight) from 1 l of culture were suspended to 35.5 ml with distilled water in a 1 l flask cooled in ice-water. The following additions of ice-cold reagents were added in order and allowed to incubate for the indicated times:

- (a) 20 ml of 0.1 M Tris-HCl, pH 8.3 for 5 min,
- (b) 18 ml of 2 M sucrose for 5 min,
- (c) 3.5 ml of 1% EDTA, PH 7.0 for 2 min, and
- (d) 3.5 ml of 0.5% lysozyme for 2 min.

The suspension was warmed to 30°C and kept at that temperature for 1 h. The spheroplasts were removed by centrifugation at 20,000 x g for 30 min at 40°C. The supernatant, containing the released outer membrane, was then centrifuged at 120,000 x

g for 1 h at 4°C to sediment the outer membrane. The membranes were washed with either 0.2 M triethanolamine buffer, pH 8.5 or 0.1 M Tris-HCl, pH 8.0, containing 35 mM MgCl₂ and then centrifuged as before. The membrane pellet was washed four times with one of the above buffers to remove contaminating lysozyme. This preparation of outer membrane was used in cross-linking and proteolytic digestion studies.

Separation of inner and outer membrane by sucrose density centrifugation (14,35,156)

The outer membrane which had been released from the spheroplasts was analysed by sucrose density gradient centrifugation. This technique separates the inner and outer membranes of gram-negative bacteria. The outer membrane derived from 1 l of mid-exponential phase culture was suspended to 1 ml in 0.1 M EDTA, pH 7.5. The samples (1 ml) were applied to the top of 30-55% sucrose gradients in 5 mM EDTA, pH 7.5 and centrifuged at 51,500 x g for 20 h in a SW 25.1 rotor at 4°C. At the end of the run, the bottom of each tube was pierced and fifteen 2 ml fractions were collected. The absorbance at 280 nm, the refractive index, and the protein content of each tube was measured.

Purification of outer membrane proteins

Purification of protein B

Detergent was removed from SDS extract 1 by dialysis against several changes of distilled water at room temperature for 24 h and then at 4°C for 2 to 5 days. The dialyzed solution was

then freeze-dried and stored at -20°C . In a typical experiment, 40 mg of extract 1 was dissolved in 10 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 0.1% 2-mercaptoethanol and incubated at 37°C for 1 h. The clear solution was applied to a column of Sephadex G-100 (5 x 40 cm) connected in series to a column of Sepharose 6B of the same dimensions. The proteins were eluted at room temperature with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. Fractions (10 ml) were collected and the absorbance measured at 280 nm. Fractions were also assayed for their content of protein and lipopolysaccharide. Column fractions (50 and 100 μl) were incubated at 37°C and 100°C with added urea to 4 M concentration and 2-mercaptoethanol and run on 10% polyacrylamide gels containing 0.1% SDS.

The fractions containing protein B were pooled, dialyzed against distilled water and lyophilized. The protein was dissolved in 10 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 0.1% 2-mercaptoethanol, heated at 100°C for 15 min, and reapplied to the double column system. The fractions from the column were examined as before and those containing protein B* were pooled, dialyzed against distilled water and lyophilized. The purified protein was subjected to amino acid analysis, N-terminal analysis and cyanogen bromide cleavage.

Purification of protein B for reconstitution

Protein B isolated as described above is in the heat-modified form, B*. For the preparation of unmodified protein B,

free of phospholipid and lipopolysaccharide, the scheme was modified as follows. The cell envelope fraction was prepared as described. The inner membrane, lipopolysaccharide and phospholipid were extracted by 2% Triton X-100 in 10 mM Hepes buffer, pH 7.4, containing 5 mM EDTA at 0°C for 30 min (67,81). The extracted membrane was recovered by centrifugation at 120,000 x g for 1 h. The extraction was repeated once and the resulting pellet was washed twice with distilled water at 0°C.

Protein B was extracted from this pellet by treatment with 0.5% SDS at 37°C for 1 h and further purified by gel filtration in 1% SDS in the double column system of Sephadex G-100 (5 x 40) and Sepharose 6B (5 x 40). In experiments where proteins B and B* were to be compared, samples were prepared by dividing a solution containing protein B into two equal parts and heating one half at 100°C for 15 min.

Purification of protein A

Protein A was extracted from the protein A-peptidoglycan complexes by treatment with 80 ml of 2% SDS at 100°C for 15 min per original 50 g (wet weight) of cells. The peptidoglycan was removed by centrifugation at 120,00 x g for 1 h at 15°C. The SDS extract was either dialyzed against distilled water to remove the detergent and freeze-dried, or concentrated 5-fold by ultrafiltration through a PM 10 ultrafilter in a Model 420 cell (Amicon Corporation). A solution of protein A (10 ml at 1-5 mg/protein/ml) was applied to the double column gel filtration system described in the purification of protein B and

the protein was eluted with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. In some cases, a single column (1 x 45 cm) of Bio-Gel P-150, equilibrated with the same buffer was used. The fractions containing protein A were pooled, dialyzed against distilled water and freeze-dried. The purified protein was subjected to amino acid analysis and cyanogen cleavage.

Purification of protein Bp, the pronase-resistant fragment derived from protein B

The Triton-extracted cell envelope preparation was digested with pronase at an enzyme: protein ratio of 1:25 for 2 h at 37°C. The digested membrane was recovered by centrifugation for 1 h at 120,00 x g and extracted with 0.5% SDS at 37°C for 1 h. This extraction step removed all the proteins from the outer membrane except proteins A and Bp. The extracted membrane was recovered by centrifugation at 15°C and re-extracted with 1% SDS at 100°C for 15 min. The resulting extract was freed of peptidoglycan by centrifugation at 120,000 x g for 1 h at 15°C and lyophilized after removal of the detergent by dialysis against distilled water at 22°C. Proteins A and Bp, contained in this extract were readily separated by gel filtration in a Bio-Gel P-150 column (1 x 45 cm), equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS, or in the double column system of Sephadex G-100 and Sepharose 6B already described. Fractions containing protein Bp were pooled, dialyzed against distilled water and lyophilized. The amino acid com-

position of the purified protein was determined.

Preparation of lipopolysaccharide (161)

Ten grams (wet weight) of cells was suspended to 20 ml with distilled water and held at 65°C. Twenty ml of 90% phenol preheated to 65°C was added with vigorous mixing and the mixture was kept at this temperature for 15 min. After cooling to about 10°C in an ice bath, the emulsion was centrifuged at 4,000 x g for 10 min, which resulted in the formation of three layers, a water layer, a phenol layer and an insoluble residue at the interphase. The water phase was removed and the phenol layer and the insoluble residue were treated at 65°C with another 20 ml portion of distilled water. The water extracts were combined and washed by shaking with an equal volume of 90% phenol. The water extract was dialyzed for 3-4 days at 4°C against several changes of distilled water (8 l) to remove phenol and low molecular weight material. The extract was then dialyzed against 4 l of 0.01 M Tris-acetate buffer, pH 7.5, containing 0.1 M NaCl for 2 h at room temperature. RNase (500 µg) was added to the extract and the absorbance at 260 nm was measured both inside and outside the dialysis sac. When no further change was observed, a further 500 µg of RNase was added. The extract was then dialysed against 4 l of 0.1 M MgSO₄ for 2 h at room temperature. DNase (100 µg) was added and the absorbance at 260 nm was monitored. An additional 100 µg of DNase was added when no further change in absorbance was observed. Two volumes of ice-cold ethanol were added and the solution was

stored at -20°C overnight. Finally, the solution was centrifuged at $20,000\times g$ for 30 min in sealed stainless steel tubes, the pellet resuspended in distilled water and freeze-dried. The dried lipopolysaccharide was stored at -20°C until used in reconstitution studies.

Small-scale preparation of total outer membrane proteins (110, 179)

The procedure used to prepare the outer membrane proteins from 1 g (wet weight) of cells was similar to that described for the preparation of Triton-extracted cell envelope. The cells were suspended to a volume of 10 ml with 10 mM Tris-HCl, pH 7.5, containing 10 mM MgCl_2 and broken by passage through a French Pressure Cell. The cell envelope fraction was prepared by centrifugation and then extracted with 15 ml of 1.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.0 containing 35 mM MgCl_2 at 22°C . The cell wall was recovered by centrifugation at $120,000\times g$ for 1 h and extracted with 5 ml of 1% SDS at 100°C for 15 min. The insoluble peptidoglycan was removed from the solution of outer membrane proteins by centrifugation at $100,000\times g$ for 1 h at 15°C . The solubilized proteins were subsequently analyzed by SDS-polyacrylamide gel electrophoresis.

Digestion of membrane preparations with proteolytic enzymes

The cells (4 g, wet weight) or cell envelopes derived from this weight of cells, were suspended to 60 ml in 0.1 M Tris-HCl, pH 8.0, containing 35 mM MgCl_2 and warmed to 37°C . The pro-

teolytic enzyme at an enzyme : protein ratio of 1:25 was added and the suspension was incubated at 37°C for 2 h. Samples (15 ml) were removed at timed intervals, 0.75 ml of a phenylmethylsulfonyl fluoride solution (7mg/ml in ethanol) was added immediately, and the solution was cooled in ice. The digested cells were broken in a French Pressure cell at 20,000 lb/inch² and the cell envelope fraction was recovered by centrifugation under the usual conditions. The inner membrane was removed from the cell envelope by Triton extraction at 22°C. The outer membrane proteins were solubilized from the Triton-extracted cell envelope by 1% SDS at 100°C for 15 min and analyzed by SDS-polyacrylamide gel electrophoresis.

Crosslinking of outer membrane proteins.

Membrane samples were suspended at about 1 mg protein/ml of 0.2 M triethanolamine buffer, pH 8.5. Various amounts of DSP, from a freshly prepared solution at 20 mg/ml in dimethyl sulfoxide were added to 100 μ l portions of membrane preparation at 22°C. The reaction was allowed to proceed for 30 sec and then excess 1 M Tris-HCl buffer, pH 8.5, was added in a volume equal to the volume of DSP added. Finally, 2-9 volumes of a solution containing 1% SDS, 10% glycerol and 0.005% Bromophenol Blue in 0.0625 M Tris-HCl, pH 6.8 (SDS-electrophoresis sample buffer without 2-mercaptoethanol) was added and the solution was heated at 100°C for 3 min to solubilize the proteins.

Membrane samples in 0.2 M triethanolamine buffer, pH 8.5 were also crosslinked at 22°C by 0.05-1.0% glutaraldehyde

(v/final volume of solution).

Removal of SDS from protein samples (144-146)

Dialysis: SDS could be removed from protein samples by dialysis against distilled water. The usual procedure was an initial dialysis step against 100 volumes of distilled water at 22°C for 24 h. The distilled water was replaced daily and dialysis was continued at 4°C for 2 to 5 days. Not all of the SDS could be removed from the protein by this method since the presence of detergent could be detected even after extensive dialysis.

Acetone precipitation: Protein was readily precipitated from SDS solutions by addition of 9 volumes of ice-cold acetone followed by storage of the mixture at -20°C for 24 h. Protein was recovered by centrifugation at 10,000 x g for 20 min at 4°C in sealed stainless steel tubes.

Ion exchange: Dowex AG1-X4 was converted to the acetate form by sequential equilibration with 2 N sodium hydroxide, 4 N acetic acid, and 50 mM Tris-acetate buffer, pH 7.5. The resin was washed extensively with distilled water between each equilibration step. The resin was dispensed into disposable pipettes to give 1 ml settled bed volume, and equilibrated with 50 mM Tris-acetate buffer, containing 6 M urea. Protein samples (1 ml) in SDS solutions were made 6 M in urea by addition of solid urea, applied to the column, and eluted at 22°C with 2 ml of the final equilibration buffer. The columns were discarded after each

use.

Cyanogen bromide cleavage of proteins (148,281)

Freeze-dried samples containing ca. 1 mg of protein were dissolved in 1.5 ml of 70% formic acid. Cyanogen bromide (5-100 fold excess over protein by weight) was added as a solid or as a solution (1 g/10 ml) in 70% formic acid. The solution was stored in the dark for 24 h at 22°C. Twenty volumes of distilled water were added and the sample was freeze-dried, and subsequently analyzed by SDS-polyacrylamide gel electrophoresis.

Analytical techniques

SDS-polyacrylamide tube gel electrophoresis - system 1 (79,90)

Reagents: 0.1 M sodium phosphate buffer pH 7.2, containing 0.1% SDS

Cyanogum 41 gelling agent (a mixture of 95% w/w acrylamide and 5% w/w bisacrylamide)

Ammonium persulfate

TEMED

Sample solvent (1% of SDS, 4 M urea, 0.1% 2-mercaptoethanol and 0.01% Bromophenol Blue)

Procedure: A 10% polyacrylamide gel was formed by dissolving 1.0 g of Cyanogum to 10 ml in sodium phosphate buffer. This solution was deaerated by suction with a water pump and then 10 μ l of TEMED and 10 mg of ammonium persulfate were added. After the ammonium persulfate had dissolved, the gel solution was dis-

pensed into eight glass tubes (7.5 x 0.5 cm) which were plugged at the bottom with polythene closures. The gel surface was overlaid with distilled water and the gels were left to polymerize for 1 h at room temperature. After polymerization was complete the water overlay was removed and the tubes were placed in a Shandon gel electrophoresis apparatus. Sodium phosphate buffer was added to the upper and lower reservoirs and the sample (10-100 μ l) was layered on the gel surface with a micropipette. Electrophoresis towards the anode was carried out at 22°C at a constant current of 10 mA per tube until the dye front was about 0.5 cm from the bottom of the gels (2.5 h). The gels were then removed for staining.

SDS-polyacrylamide tube gel electrophoresis at an alkaline pH-System 2 (79)

Polyacrylamide gels were prepared as already described. The upper reservoir buffer was 0.1 M sodium phosphate buffer, pH 11.4, containing 0.1% SDS and the lower reservoir buffer was 0.1 M sodium phosphate buffer, pH 4.2, containing 0.1% SDS. Electrophoresis was carried out as described for System 1, except that a nylon mesh around the bottom of the glass tube was required to prevent the gel from slipping from the tube.

Preparation of samples of SDS-polyacrylamide tube gel electrophoresis

Freeze-dried protein samples were dissolved in the sample solvent (1% SDS 0.1% 2-mercaptoethanol and 4 M urea) at concen-

trations of protein of 0.1 to 2 mg/ml. Solutions containing protein were diluted with 1 volume of a 2X concentrated sample solvent. Unless stated otherwise, all samples for electrophoresis were heated at 100°C for 5 min.

Staining polyacrylamide gels for protein

The polyacrylamide gels were removed from the tubes and the dye front was marked with black India ink applied with a fine needle. The gels were placed in filtered staining solution (0.25% Coomassie Blue in 25% methanol, 10% acetic acid) and heated at 60°C for the following times: 5% gel, 30 min, 7.5% gel, 40 min; 10% gel, 50 min and 12.5% gel, 60 min. The gels were destained at 60°C with 2-3 successive changes of 10% acetic acid for 1 h each change. Finally, the gels were kept in fresh 10% acetic acid overnight. The gels were stored in 10% acetic acid and then scanned at 550 nm in a Gilford Model 240 Spectrophotometer equipped with a linear transporter. The R_f of a stained protein band was calculated as:

$$R_f = \frac{\text{distance migrated by protein band}}{\text{distance migrated by Bromophenol Blue}}$$

Staining polyacrylamide gels for carbohydrate (167)

Carbohydrate was detected in polyacrylamide gels by the periodic acid-Schiff base reaction. The gels were stained by sequential immersion in:

- (a) 12.5% trichloroacetic acid for 30 min,
- (b) distilled water for 1 min,

- (c) 1% periodic acid in 3% acetic acid for 50 min,
- (d) distilled water, overnight,
- (e) Fushsin-sulfite stain for 50 min in the dark,
- (f) 0.5% metabisulfite for 10 min, three times, and
- (g) distilled water, overnight.

The gels were stored in 10% acetic acid. The Fushsin-sulfite stain was prepared by dissolving 160 mg of potassium metabisulfite and 0.21 ml of concentrated HCl in 20 ml of distilled water. Basic Fushsin dye (80 mg) was added and the solution was stirred gently for 2 h. The solution was allowed to stand for a further 2 h, when a small amount of Darco charcoal (grade G-60) was added and the solution was filtered within 15 min. The colourless dye was stored at 4°C and was usable until the solution turned pink. The 0.5% metabisulfite solution was prepared just before use.

Discontinuous SDS-polyacrylamide gel electrophoresis-Laemmli system (83,92)

Reagents: Separating gel buffer, 4 X concentrated (1.5 M Tris-HCl, pH 8.8, containing 0.4% SDS)

Stacking gel buffer, 4 X concentrated (0.5 M Tris-HCl, pH 6.8, containing 0.4% SDS)

Reservoir buffer, 5 X concentrated (0.125 M Tris and 0.960 M glycine, pH 8.3)

Sample buffer (0.0625 M Tris-HCl, pH 6.8, containing 1% SDS, 1% 2-mercaptoethanol and 10% glycerol)

Acrylamide (30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide in distilled water)

TEMED

Ammonium persulfate (10% (w/v) aqueous solution,
prepared fresh daily.

Only high quality SDS was used ("specially pure" grade from British Drug House Ltd.). The pH of the reservoir was not adjusted, but the solution of Tris and glycine (ammonia-free) consistently gave a pH of 8.3 when diluted. The protocol used to prepare the different separating gel concentration is given below:

Volume (ml) per 15 ml solution

% gel	Separating gel buffer	Distilled water	Acrylamide	TEMED	10% persulfate
7.5	3.75	7.50	3.75	15 μ l	75 μ l
9.0	3.75	6.75	4.50	15 μ l	75 μ l
10.0	3.75	6.25	5.00	15 μ l	75 μ l
12.0	3.75	5.25	6.00	15 μ l	75 μ l
15.0	3.75	3.75	7.50	15 μ l	75 μ l

Stacking gel solution (4% acrylamide) was prepared by dissolving 0.2 g of Cyanogum in a mixture of 1.25 ml of stock stacking gel buffer and 3.75 ml of distilled water. TEMED (10 μ l) and 10% ammonium persulfate (15 μ l) were then added to the solution.

Electrophoresis was carried out using a Bio-Rad Model 220

slab gel apparatus. Gels were formed between two glass plates, separated by a 0.75 mm thick spacer, greased with a small amount of Cello Seal (Fisher Chemical Co.). The plates were mounted on the centre core of the apparatus and the bottom was sealed with the sealing bar. A ten or twenty slot former was inserted between the plates and deaerated separating gel solution (15 ml) was added with a Pasteur pipette. The slab "sandwich" was filled to 1 cm below the slot former and was then overlaid with t-butanol. The gel was allowed to polymerize at 22°C for 30 min and then the slot former was removed. The t-butanol was absorbed on a piece of filter paper and the gel surface was washed twice with diluted (4-fold) separating gel buffer. The slot former was reinserted and deaerated stacking gel solution (5 ml) was added to fill the gel "sandwich". No overlay solution was required and polymerization was allowed to proceed at 22°C for 30 min. The slot former was then removed carefully and the sample slots were washed twice with diluted (4-fold) stacking gel buffer. Diluted reservoir buffer was added to the electrophoresis chambers (1.5 l in the bottom and 300 ml in the top). The samples (5-25 µl in a 20 slot gel, 10-100 µl in a 10 slot gel) were applied through a fine piece of polyethylene tubing attached to a micropipette. Cold tap water was circulated through the centre core of the apparatus. Electrophoresis was carried out at a constant current of 30 mA per gel slab until the dye front was 1 cm from the bottom of the gel (1.5 h).

Staining of polyacrylamide slab gels

After electrophoresis, the gel "sandwich" was immersed in a large pan of water. The plates were separated and the gel was transferred to a Pyrex dish (8.5 x 8.5 x 2 inches deep) and 300 ml of staining solution (0.05% Coomassie Blue in 25% isopropanol, 10% acetic acid, filtered before use) was added. The gels were stained at 22°C for 1 h. The staining solution was removed by suction and was replaced by 300 ml of 10% acetic acid. The gel was stored in this solution overnight. Finally, the gel was washed with several changes of fresh 10% acetic acid until the background was clear.

Analysis of DSP-crosslinked proteins by two-dimensional SDS-gel electrophoresis

Crosslinked products were analyzed in a two-dimensional SDS-polyacrylamide gel system using a Bio-Rad Model 220 slab gel apparatus. The gel solutions were prepared as already described. Crosslinked proteins were first resolved on a 7.5% polyacrylamide slab gel, 0.75 mm thick. A 3 mm wide strip from this gel was placed on top of a previously-formed second dimension gel and embedded in warm (45°C) 1% agarose, 2% 2-mercaptoethanol in four-fold diluted stacking gel buffer. The agarose was allowed to cool for 5 min. The time taken from stopping the first dimension electrophoresis to starting the second dimension did not exceed 15 min. The second dimension gel was stored overnight, covered with a layer of four-fold

diluted stacking gel buffer containing 10% 2-mercaptoethanol. The buffer overlay was removed just before insertion of the first dimension strip. With the second dimension gel, a constant current of 40 mA was applied until the dye was 1 cm from the bottom of the gel (2.5 h). The two-dimensional gels were stained with Coomassie Blue as described for 0.75 mm thick slab gels except the staining time was 2 h.

Polyacrylamide gel electrophoresis in the presence of urea

Reagents: Gel buffer (0.375 M Tris-HCl, pH 8.7, containing 6 M urea)

Reservoir buffer (10 mM Tris, 80 mM glycine, pH 8.3)

Sample buffer (50 mM Tris-HCl, pH 6.8, containing 6 M urea, 1% 2-mercaptoethanol and 0.01% Bromophenol Blue)

TEMED

Ammonium persulfate

Procedure: A 5% polyacrylamide gel was formed by dissolving 0.5 g of Cyanogum to 10 ml in the gel buffer. The solution was de-aerated and 10 μ l TEMED and 10 mg of ammonium persulfate were added. The gel solution was dispensed into eight glass tubes and the gel surface was overlaid with distilled water. The gels were left to polymerize for 1 h at 22°C before the water overlay was removed and the gels placed in a Shandon gel electrophoresis apparatus. The reservoir buffer was added to the upper and lower chambers and the samples were applied to the gel surface with a micropipette. Electrophoresis was carried

out at 22°C at a constant current of 3 mA per tube until the dye front was 0.5 cm from the bottom of the gel (2 h).

Determination of molecular weight by gel filtration

Gel filtration was performed at 22°C using a column (1 x 37 cm) of Sepharose 6B equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. Standard proteins were dissolved at 1 mg/ml in the column buffer containing 0.1% 2-mercaptoethanol and heated at 100°C for 5 min before application to the column. The elution positions of the proteins was determined by assaying all fractions (1 ml) for protein by the method of Lowry et al. (171) and by subjecting all fractions to analysis by SDS-polyacrylamide electrophoresis. The void volume (V_o) and total volume (V_t) of the column was determined from the elution positions of Blue Dextran, and sodium chloride (detected by ionic strength) or triethanolamine (detected by the Lowry protein assay) respectively. A calibration curve was obtained by plotting \log_{10} (molecular weight) versus K_{av} , where

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Sucrose density gradient centrifugation of proteins B and B*

Sucrose density gradient centrifugation was performed on 5-20% sucrose gradients prepared in 1 mM Tris-acetate buffer, pH 7.0, containing either 0.1, 0.2, or 0.3% SDS. Samples (0.20 ml), containing ca. 1 mg protein/ml were layered on top of 5.0

ml gradients and centrifuged in a Spinco SW 50-L rotor at 45,000 rpm for 22 h at 20°C. After the run, 20 ten-drop fractions were collected from the top of the gradient by pumping a 60% sucrose solution, containing Blue Dextran, through the bottom of the tube. The refractive index and the protein content of each fraction were measured. Sedimentation coefficients and partial specific volumes were calculated as described in the Appendix.

Measurement of binding of SDS to proteins

The amount of SDS bound to protein B and B* was determined by equilibrium dialysis. Protein samples (1 ml) were dialyzed at 22°C against 1 l of 1 mM Tris-acetate buffer, pH 7.0, containing various concentrations of SDS. The amount of SDS inside and outside the dialysis membrane was determined as described by Hayashi (169).

Measurement of intrinsic viscosity

Intrinsic viscosities were measured with a Cannon-Manning viscometer, immersed in a water bath thermostated to 25 ± 0.01°C. The method used to calculate the intrinsic viscosity in the Appendix.

Spectral measurements

Ultraviolet spectra were taken at 22°C with a Perkin-Elmer model 356 Dual wavelength Double Beam Spectrophotometer. Circular dichroism and optical rotatory dispersion measurements

were made at 25°C with a Jasco J-20 Automatic recording Spectropolarimeter.

Amino acid analysis

The protein sample (0.5-2 mg) was placed in a 13 x 100 mm Pyrex test tube and 0.5-1.0 ml of 6 N HCl was added. The tube was sealed under vacuum and the solution was heated at 110°C for 24, 48 or 72 h. After hydrolysis, the sample was dried under vacuum and then dissolved in 100-500 µl of distilled water. Samples were analyzed on a single column of Aminex A-5 resin using sodium citrate buffers, on a Beckman Model 120C amino acid analyzer or on an instrument constructed by Mr. J. Durgo of the Department of Biochemistry, University of British Columbia. The content of amino acids was determined by calculating the peak areas for each amino acid.

Determination of cysteine (176)

Reagents: Sodium phosphate buffer (0.1 M sodium phosphate, pH 8.0, containing 2% SDS and 0.05% EDTA)
DTNB (40 mg of 5,5'-dithiobis (2-nitrobenzoic acid) in 10 ml of 0.1 M sodium phosphate buffer, pH 8.0)

Procedure: The sample containing 0.01-0.04 µmoles of protein was dissolved in 6 ml of sodium phosphate buffer and 0.1 ml of DTNB solution was added to each of two three ml portions. The colour was allowed to develop for 15 min at 22°C and then the absorbance was measured at 410 nm against a reagent blank. A standard curve was also constructed using cysteine.

Amino-terminal analysis

Protein samples were dissolved to ca. 1 mg/ml in 0.5 M NaHCO_3 , pH 9.8, containing 1% SDS and 0.5 volumes of a dansyl chloride solution (5 mg/ml in acetone) was added. The dansylation reaction was allowed to proceed for 20 min at 37°C , then the protein was precipitated by addition of 2 volumes of ice-cold 20% trichloroacetic acid. The protein was recovered by centrifugation at $3,000 \times g$ for 5 min. The precipitate was washed once with 1 N HCl to remove dansylic acid. The sample was hydrolyzed in 6 N HCl at 105°C for 6 h in an evacuated glass tube and then evaporated to dryness in a heated vacuum desiccator. The residue was dissolved in 50% aqueous pyridine (v/v) and applied to one side of a 5 x 5 cm polyamide plate. A standard mixture of dansyl-amino acids was applied on the reverse side of the plate. The sample was resolved by chromatography in Solvent I (1.5% (v/v) aqueous formic acid), dried and examined under ultraviolet light. The plate was turned 90° and subjected to chromatography in a second dimension with Solvent II (benzene-acetic acid, 9:1), dried and examined under ultraviolet light. Fluorescent spots were identified by comparison to the standard mixture of dansyl-amino acids.

Preparation of dansyl-amino acids

Amino acids (10 mM) in 0.5 M NaHCO_3 buffer, pH 9.8 were mixed with an equal volume of dansyl chloride in acetone such that there was a 5-fold molar excess of dansyl chloride. The reaction was carried out for 30 min at 37°C and then terminated

by addition of 1/30 volume of 88% formic acid. This mixture was used directly for spotting on the chromatogram.

Determination of protein

Protein was determined by the method of Lowry et al. (171), except samples containing membrane preparations were made 1% in SDS and heated at 100°C for 15 min before assaying for protein. Bovine serum albumin was used to construct a standard curve.

Determination of carbohydrate (273)

Samples (1 ml containing 0-1 mg carbohydrate/ml) were mixed with 50 μ l of 80% phenol and then 2.5 ml concentrated H_2SO_4 was added with rapid mixing. The absorbance of the solution was measured at 490 nm after 30 min. Glucose (0.25 μ moles/ml) gave an absorbance of 1.0.

Determination of lipopolysaccharide (274,275)

Reagents: 0.04 N HIO_4 in 0.125 N H_2SO_4
 3% sodium arsenite in 0.5 N HCl
 0.3% thiobarbituric acid

Procedure: Samples (0.2 ml, containing 0-10 nmoles of 3-keto-2-deoxyoctonic acid) were mixed with 0.25 ml of HIO_4 solution and incubated at 22°C for 20 min. Sodium arsenite solution (0.5 ml) was added with shaking and the solution was allowed to stand for 2 min. Thiobarbituric acid solution (2 ml) was then added and the solution was heated at 100°C for 20 min. The

absorbance of the solution was measured at 548 nm. An absorbance of 1.9 was obtained with 100 nmoles of 3-keto-2-deoxyoctonic acid.

Determination of phosphate in protein samples (280)

Reagents: 10% $\text{Mg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ in ethanol

Ascorbic-molybdate reagent (1 part 10% ascorbic acid to 6 parts of 0.42% ammonium molybdate $\cdot \text{H}_2\text{O}$ in 1 N H_2SO_4 , prepared fresh)

1 N HCl

Procedure: Samples (0.01-0.05 ml containing 0-50 nmoles) were placed in 13 x 100 mm Pyrex tubes and mixed with 0.05 ml of the magnesium nitrate reagent. The mixture was evaporated with rapid shaking to a white ash over a strong flame until the brown fumes had disappeared. After the tube had cooled, 0.3 ml of 1 N HCl was added. The tube was capped with a marble and heated in a boiling water bath for 15 min to hydrolyze to inorganic phosphate any pyrophosphate formed in the ashing procedure. Ascorbic-molybdate mixture (0.7 ml) was added to tube and after 20 min at 45°C the absorbance of the solution was read at 660 nm against a blank solution. An absorbance of 0.24 was obtained with 10 nmoles of phosphate as Na_2HPO_4 .

RESULTS

Use of various cell fractions

A number of different cell fractions were used during the course of the research described in this thesis. Figure 6 illustrates diagrammatically the procedures used to prepare these cell fractions and the selective extraction of outer membrane proteins by SDS at different temperatures.

Spheroplasts: Cells could be lysed after lysozyme-EDTA treatment to produce spheroplasts, composed of inner and outer membranes. Outer membrane was prepared from spheroplasts by solubilization of the inner membrane with Triton in the presence of Mg^{2+} . Outer membrane was also released during spheroplast formation and could be prepared by centrifugation without detergent treatment. These preparations served as controls in studies on the organization of proteins in the outer membrane.

Envelope: Spheroplast preparations were not a convenient source of material for the large scale isolation of outer membrane proteins and therefore cell wall preparations (Triton-extracted cell envelope) were used for this purpose. The outer membrane proteins (ca. 5 mg protein/g wet weight of cells) were prepared from the cell wall fraction by solubilization with SDS at $100^{\circ}C$ leaving an insoluble residue of peptidoglycan and covalently-bound lipoprotein. The proteins could also be differentially extracted from the cell wall by SDS at lower temperatures. Thus, extraction at $37^{\circ}C$ to give extract 1

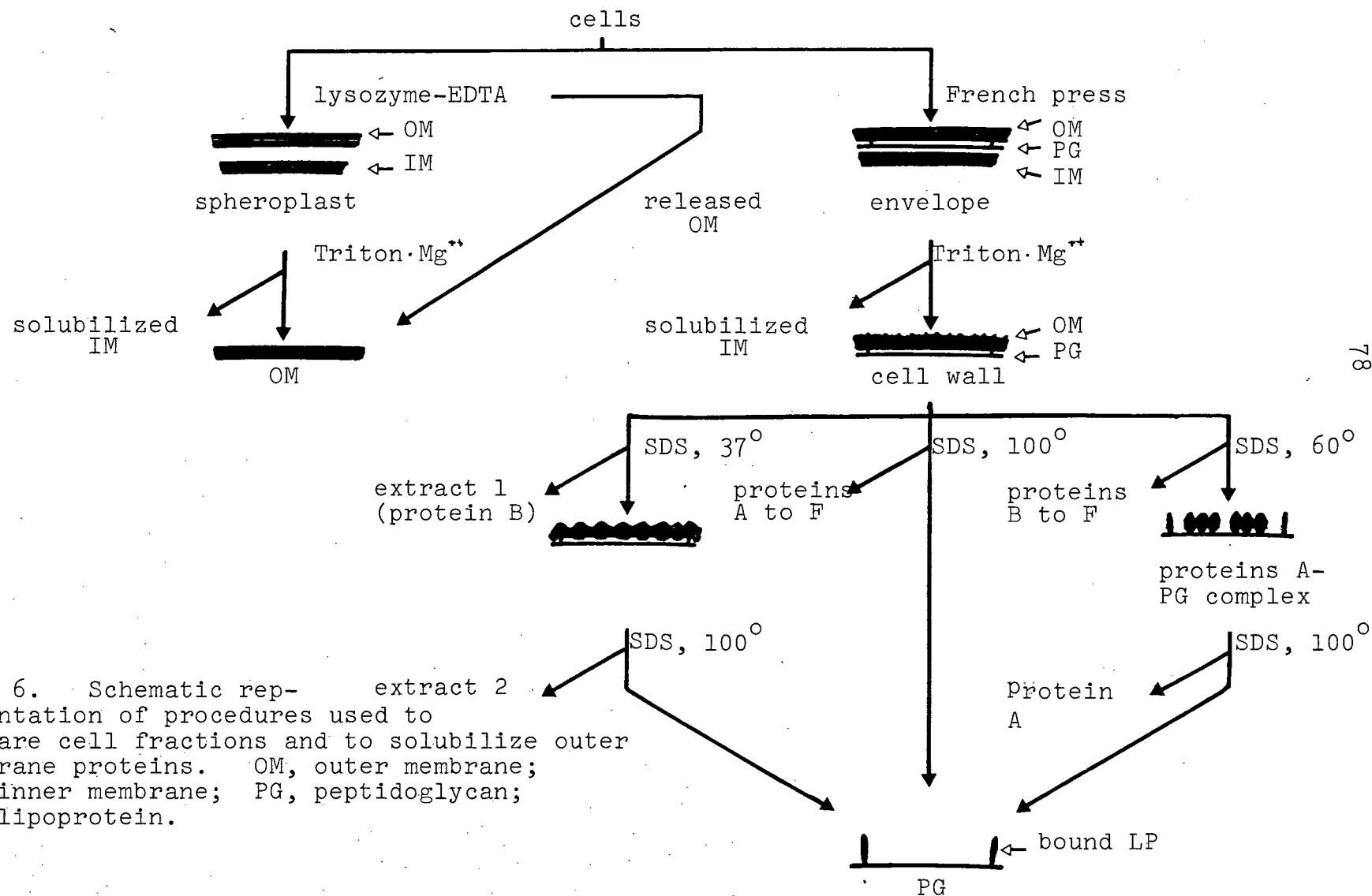


Fig. 6. Schematic representation of procedures used to prepare cell fractions and to solubilize outer membrane proteins. OM, outer membrane; IM, inner membrane; PG, peptidoglycan; LP, lipoprotein.

(2 mg protein/g wet weight of cells) released primarily protein B. This extract was used in the purification of protein B. A further extraction at 100°C to give extract 2 (3 mg protein/g wet weight of cells) solubilized the remaining outer membrane proteins, except for the bound lipoprotein. Extraction of the cell wall with SDS at 60°C removed all proteins except protein A and the bound lipoprotein. Protein A could be subsequently solubilized by SDS at 100°C and this extract was used in the purification of protein A. The purification and properties of proteins A and B are presented in the first part of the results section.

The organization of the proteins in the outer membrane was studied by proteolytic digestion, covalent labelling and cross-linking. The results obtained with the different preparations were compared to rule out gross artifactual rearrangement of the proteins during preparation of the cell fractions. The results of these studies are presented in the second part of this section.

Purification and properties of the outer membrane proteins

Major proteins of the outer membrane

The proteins of the outer membrane were extracted from the cell wall by 1% SDS at 100°C for 15 min and then resolved by SDS-polyacrylamide gel electrophoresis using different buffer systems (Fig. 7). Electrophoresis in phosphate buffer at pH 7.2 did not resolve the major outer membrane proteins A and B (scan 1).

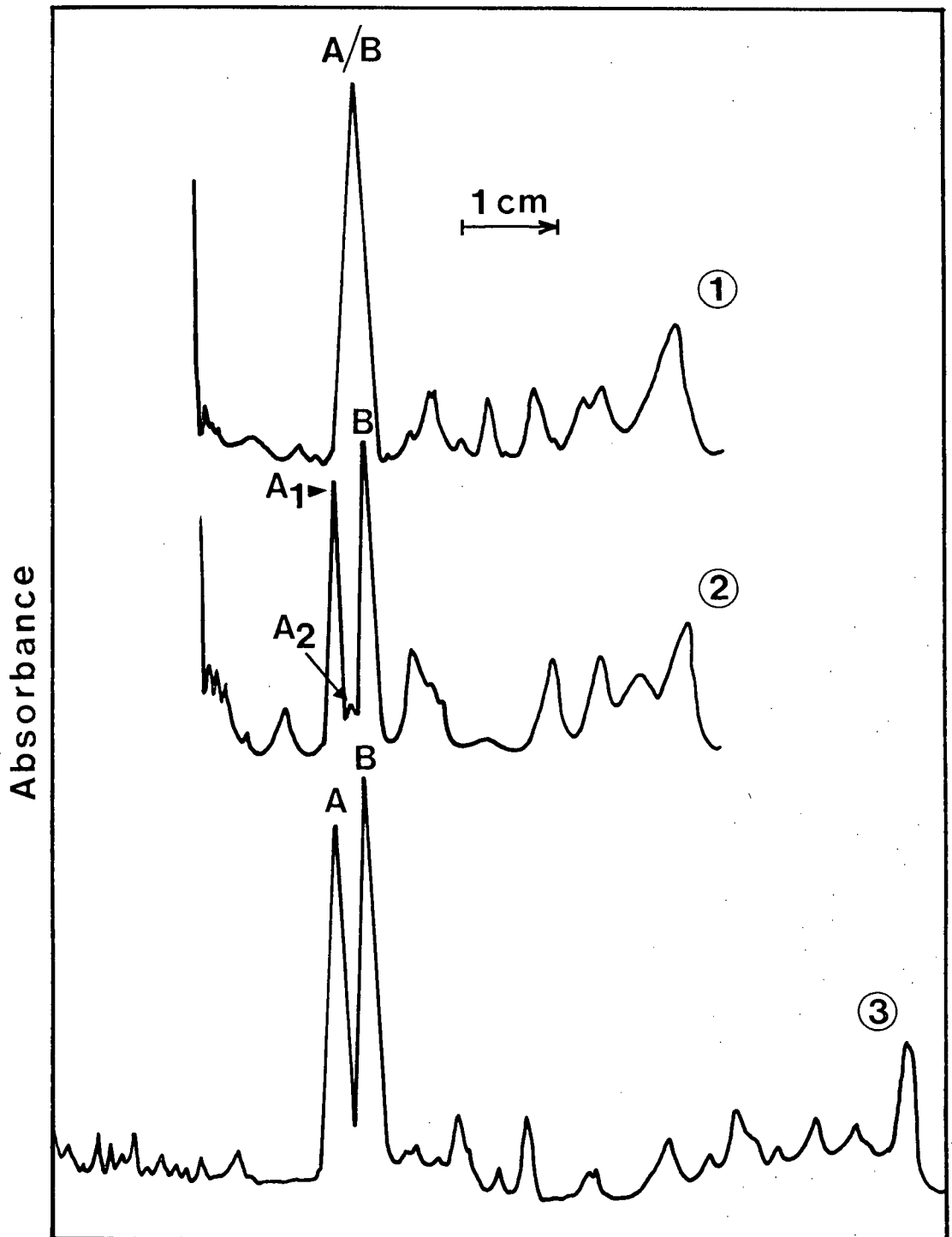
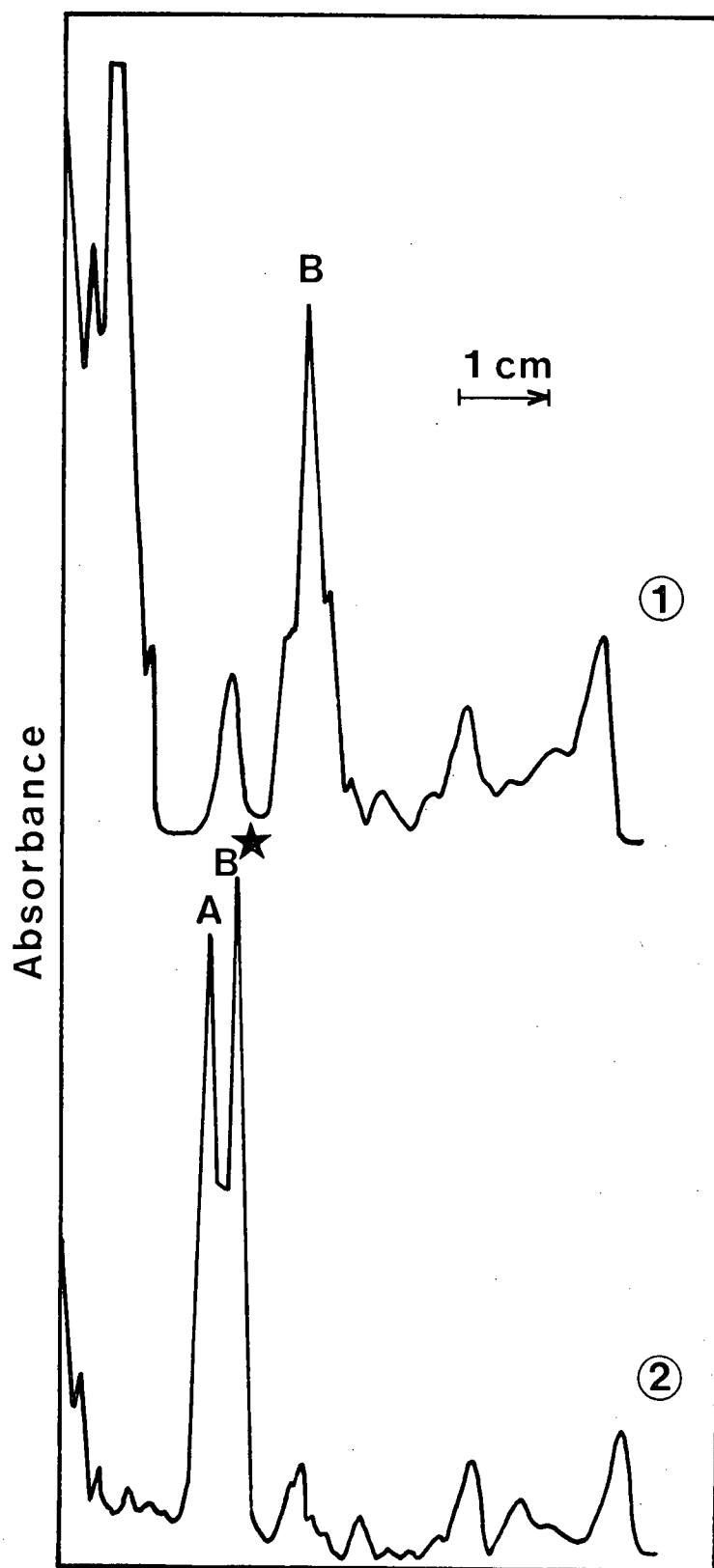


Fig. 7. Resolution of outer membrane proteins by SDS-polyacrylamide gel electrophoresis. Scan 1, 10% polyacrylamide gel run in system 1; scan 2, 10% gel run in system 2; scan 3, 12.5% gel run in the Laemmli system. All samples were heated at 100°C for 5 min. Gels were stained with Coomassie Blue and scanned at 550 nm.

The apparent molecular weight of this band was 40,000. The major outer membrane proteins were readily separated into three closely spaced bands, A_1 (MW 44,000), A_2 (MW 38,000) and B (MW 33,400) by electrophoresis at an alkaline pH. This gel system (System 2 of Bragg and Hou (79)) was used routinely to resolve these proteins. The use of the discontinuous electrophoresis system designed by Laemmli (92) further resolved the other outer membrane proteins. However, proteins A_1 and A_2 migrated as a single band with a molecular weight of 37,000. The fastest migrating band seen in all the gel scans (protein F) is the free form of the lipoprotein (MW 7,200).

The effect of heating the protein samples in SDS on the migration of outer membrane proteins is shown in Fig. 8. Outer membrane prepared from spheroplasts by Triton X-100 extraction in the presence of Mg^{++} was heated in SDS-electrophoresis sample buffer at $37^{\circ}C$ for 1 h (scan 1). The sample in scan 2 was heated at $100^{\circ}C$ for 5 min before application to the gel. As seen, the method of sample preparation profoundly altered the pattern seen in the SDS-polyacrylamide gels. Heating caused the depolymerization of high molecular weight aggregates consisting primarily of protein A and also changed the mobility of protein B. The effect of heating on protein B was studied further since the change in apparent molecular weight might be due to a conformational change that might be functionally significant.

Fig. 8. Effect of heating on the migration of outer membrane proteins in SDS-polyacrylamide gels run in system 2. Scan 1, outer membrane solubilized at 37°C for 1 h in SDS-electrophoresis buffer, scan 2, sample heated at 100°C for 5 min.



Heat-modifiability of protein B

Protein B was readily solubilized from the cell wall by extraction with 0.5% SDS at 37°C for 1 h. Sixty percent of the protein in this extract (extract 1) was protein B, with an apparent molecular weight of 28,500 as determined by SDS-polyacrylamide gel electrophoresis (Fig. 9A). Upon heating a solution containing 1% SDS, 0.1% 2-mercaptoethanol and 4 M urea, protein B was converted to a form B* (MW 33,400). This behaviour is designated as heat-modifiable. The mobility of all other proteins in extract 1 was unaffected by the heat treatment (Fig. 9A). In addition, the presence or absence of 2-mercaptoethanol had no effect on the migration of proteins B or B*. Measurement of peak areas of the gel scans showed a quantitative conversion of protein B to B*, proceeding more rapidly at higher temperatures (Fig. 9B). No change was detected after a 2 h incubation of the sample at 40°C, while heating at 100°C resulted in a rapid conversion.

The rates of conversion (k) are plotted as a function of reciprocal temperature in Fig. 10. The activation energy for this process is 36.5 kcal per mole. Protein B* did not revert back to form B on cooling or after prolonged storage. These results suggest that protein B was irreversibly denatured upon heating.

The behaviour of protein B on gel filtration was changed by heating in the presence of SDS (Fig. 11). The large peak (fraction 71) absorbing at 280 nm was Triton X-100. This was

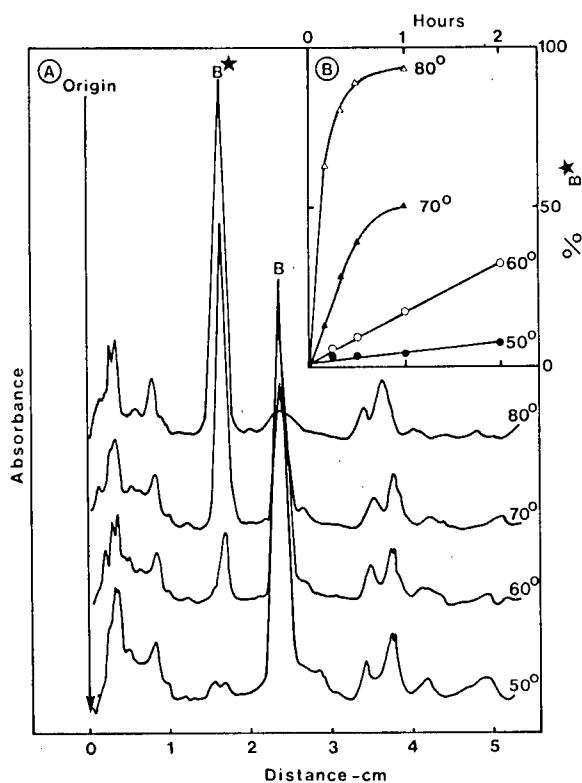


Fig. 9. Effect of heating on migration of protein B in SDS-polyacrylamide gels. A: Densitometer scanning traces of gels. Samples were heated at the indicated temperatures for 20 min. B: Time course of conversion of protein B to B* at various temperatures.

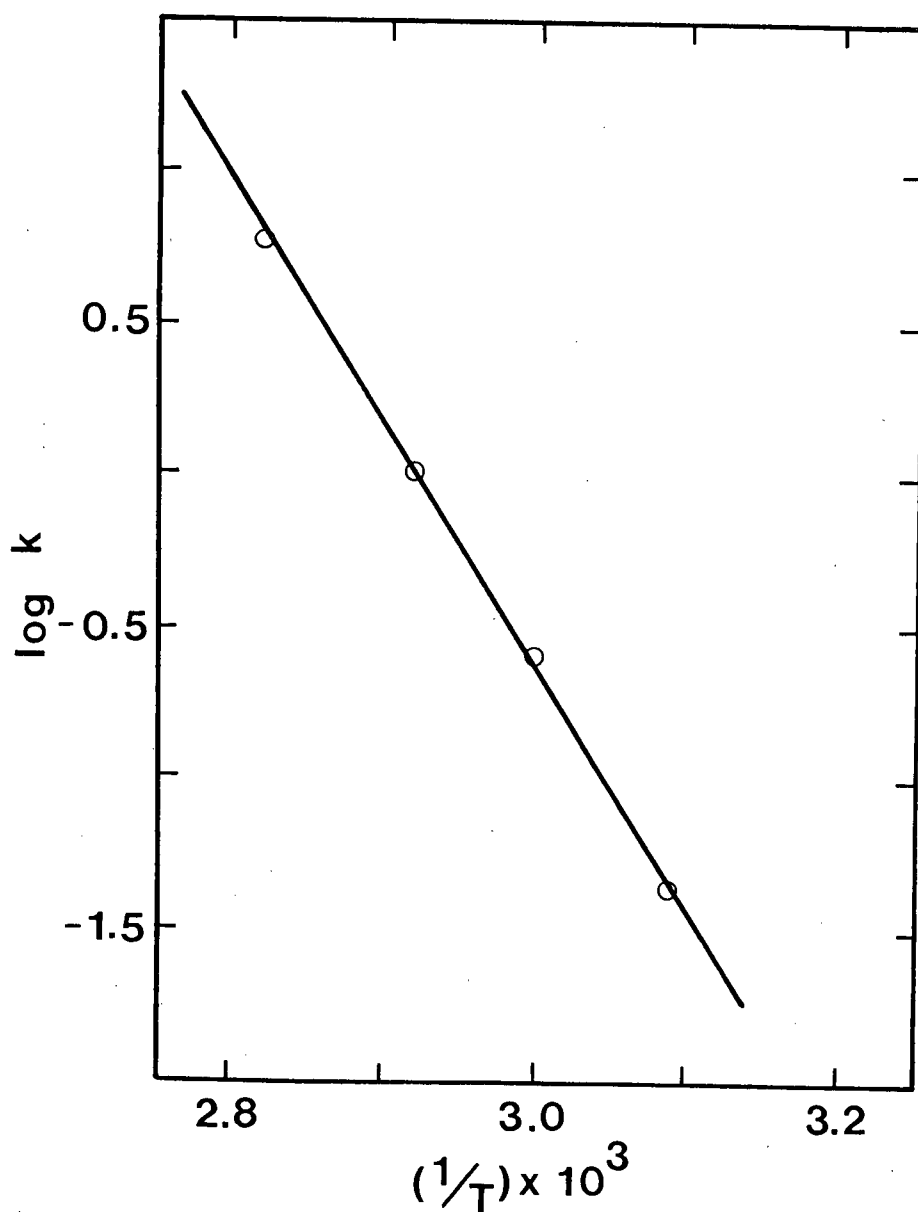
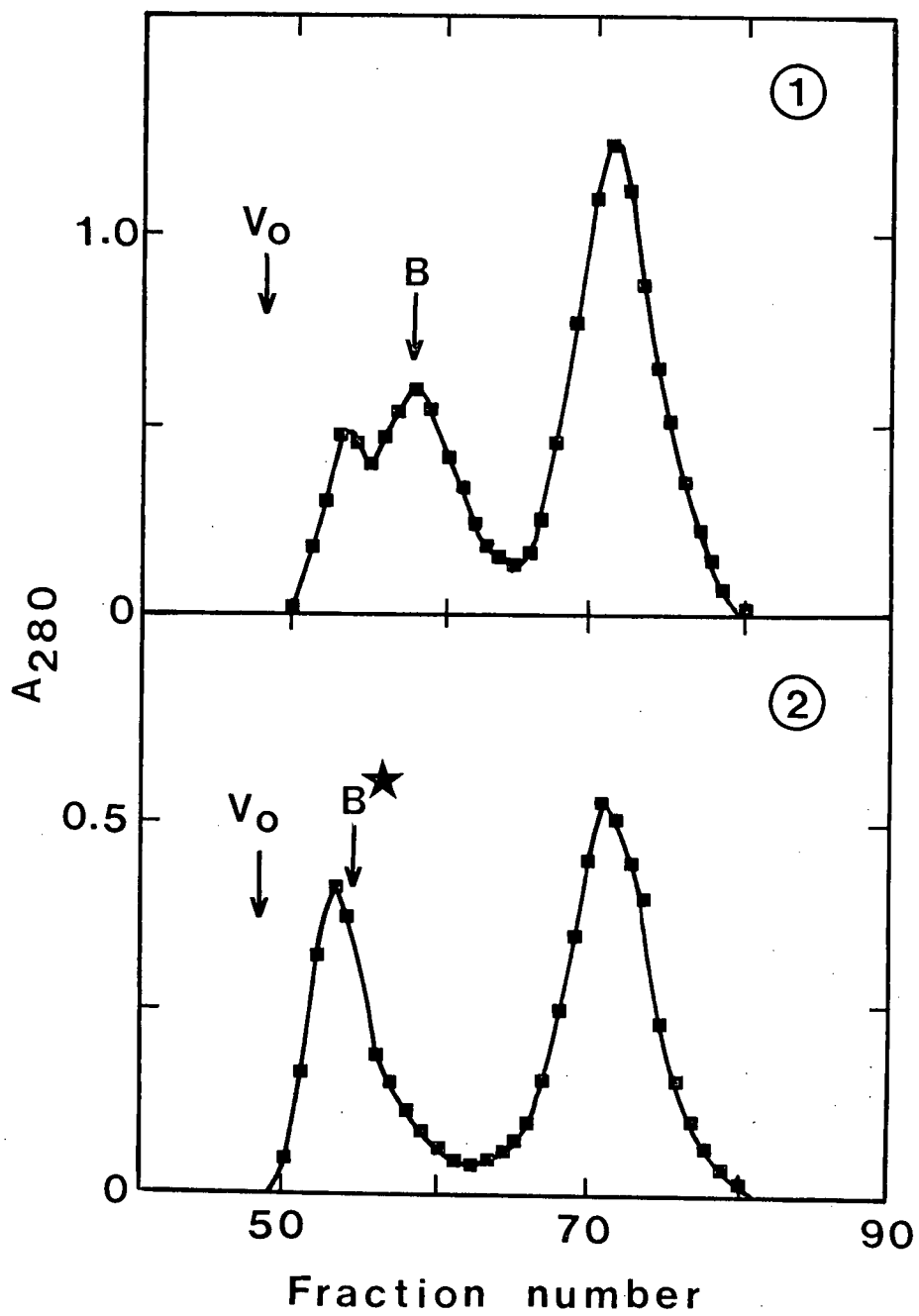


Fig. 10. Arrhenius plot for the conversion of protein B to B*. The rate of conversion, K , is defined as the change in the percentage of protein B* per min.

Fig. 11. Effect of heating on the elution position of protein B from a column (5 x 40 cm) of Sephadex G-100 equilibrated with 0.1M sodium phosphate buffer, pH 7.2, containing 1% SDS. The freeze-dried sample of extract 1 was dissolved in 10 ml of column buffer containing 0.1% 2-mercaptoethanol and heated at 37°C for 1 h (sample 1, 40 mg proteins) or 100°C for 15 min (sample 2, 20 mg protein) and then applied to the column. Fractions (10 ml) were collected and analyzed by SDS-polyacrylamide gel electrophoresis.

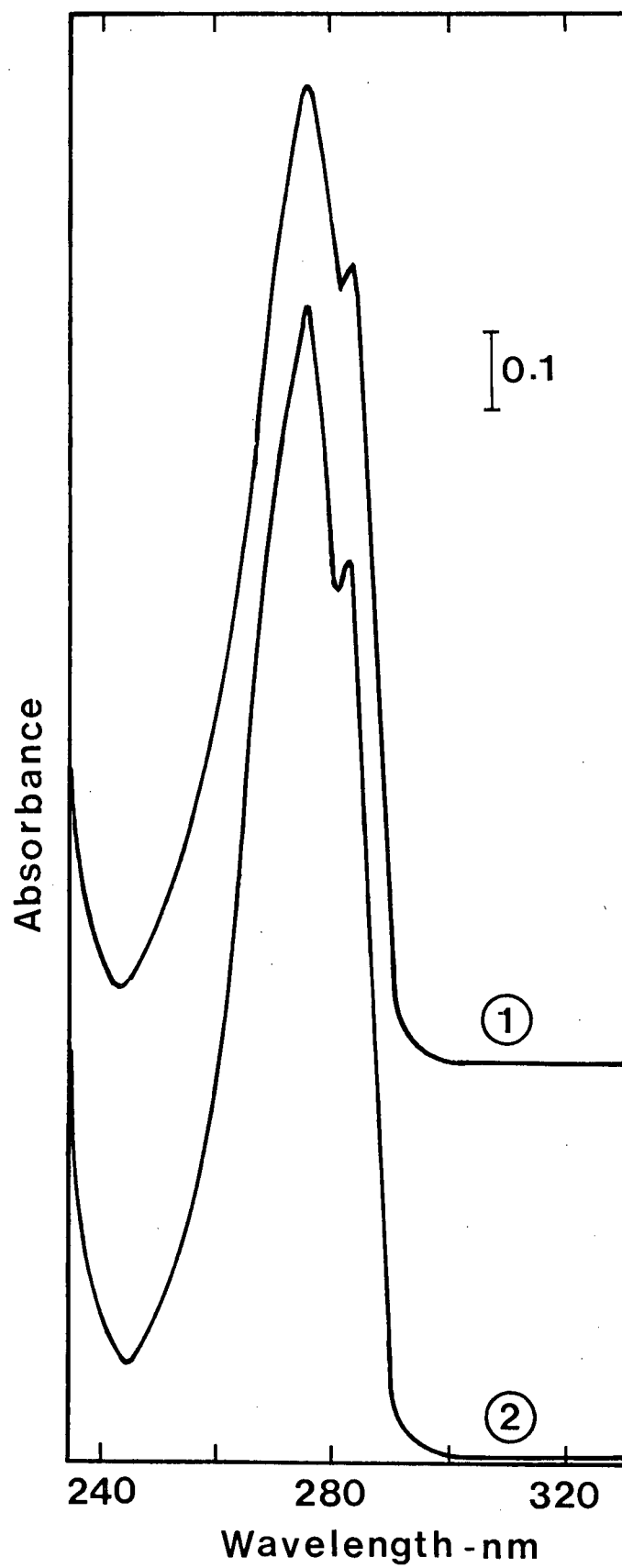


shown by comparing the ultraviolet spectrum of the peak fraction (Fig. 12, scan 1) with Triton X-100 dissolved in the column buffer (Fig. 12, scan 2). The amount of Triton in the peak fraction (Fig. 11, profile 1) was about 0.4 mg/ml (Fig. 13). The elution position of protein B, indicated by the arrows in Fig. 11, changed upon heating extract 1 in the presence of SDS. Incubation of the sample at 37°C for 1 h resulted in the elution of protein B at fraction 58. Heating at 100°C caused protein B to elute at fraction 54. Protein B in fraction 58 of profile 1 migrated as a band with a molecular weight of 28,500 on SDS-polyacrylamide gels, while the protein in fraction 54 of profile 2 migrated as the heat-modified form B*, with a molecular weight of 33,400. The ability to distinguish protein B from the heat-modified form B* by gel filtration suggests that the increase in molecular weight observed in SDS-gels was not due to an artifact of electrophoresis.

Purification of protein B*

The altered elution position of protein B after heating suggested that protein B could be purified by gel filtration in the presence of SDS. Extract 1 was resolved into well-separated protein peaks by gel filtration in a column (5 x 40 cm) of Sephadex G-100 connected in series to a column of Sepharose 6B of the same dimensions, in the presence of SDS (Fig 14A). The largest peak absorbing at 280 nm contained Triton. Protein B could be separated from contaminating Triton, most of the lipopolysaccharide and the other proteins of the outer membrane

Fig. 12. Ultraviolet spectrum of Triton X-100 in 0.1M sodium phosphate buffer, pH 7.2, containing 1% SDS. Scan 1, spectrum of fraction 71 of extract 1 resolved by gel filtration (Fig. 11, profile 1); scan 2, spectrum of 0.05% Triton X-100 in column buffer.



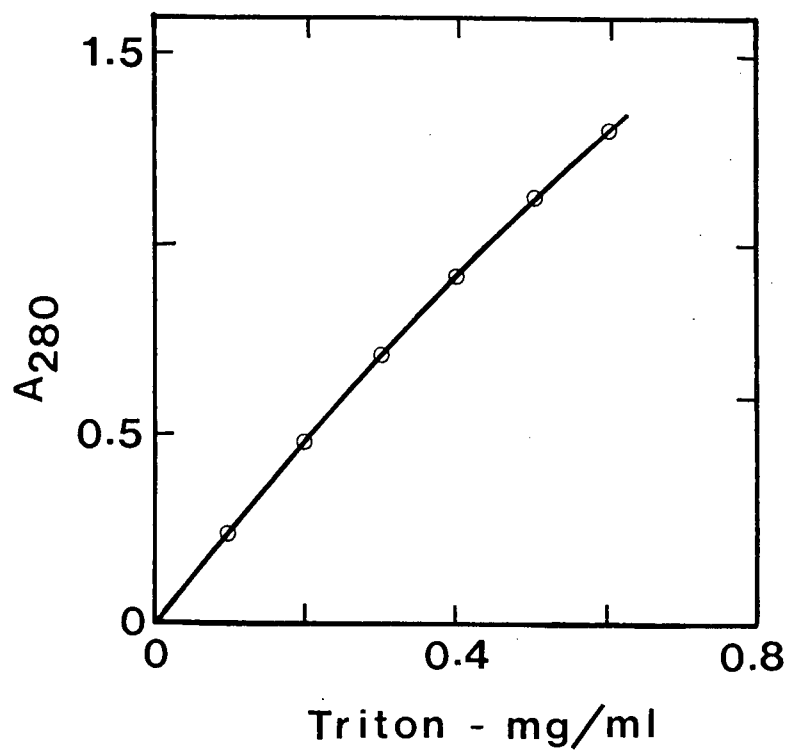


Fig. 13. Standard curve for the determination of Triton X-100 in 0.1M sodium phosphate buffer, pH 7.2, containing 1% SDS by absorbance at 280 nm.

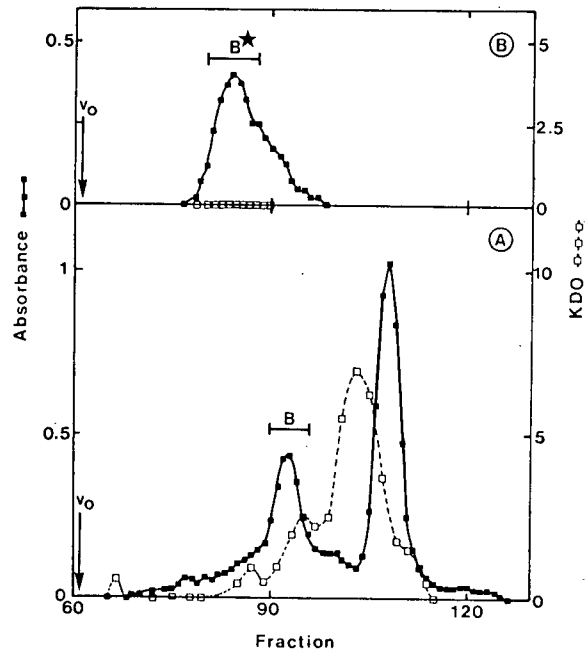


Fig. 14. Separation of proteins of extract 1 by gel filtration in a column (5 x 40 cm) of Sephadex G-100 connecting in series to a column of Sepharose 6B of the same dimensions and in the presence of 1% SDS. A: gel filtration of extract 1 without prior heat-treatment of sample. The fractions under the bar contained only protein B. B: gel filtration of half of protein B from separation A following heating at 100°C for 15 min. The fractions under the bar contained only B*. The concentration of 2-keto-3-deoxyoctonic acid (KDO) is expressed as nmoles/0.2 ml sample. The absorbance of the fractions was measured at 280 nm. Fraction volume, 10 ml. V_o , void volume of double column system.

by this method. Protein B contained in fractions 90 to 96 migrated as a single band of molecular weight 28,500 on SDS-polyacrylamide gels. When protein B was heated at 100°C for 15 min in SDS and rerun in the double column system, it eluted in fractions 80 to 88 (Fig. 14B). The protein in these fractions gave a single band of molecular weight 33,400 on SDS-polyacrylamide gels. The heating procedure enabled resolution of pure protein B* from small amounts of contaminating proteins and lipopolysaccharide which co-eluted with protein B.

Amino acid composition

The amino acid analysis of extracts 1 and 2 are given in Table II. More vigorous conditions were required to obtain extract 2 than extract 1, suggesting that the proteins of extract 2 were more tightly associated in the membrane. This difference was not reflected in the amino acid compositions which were similar. The amino acid composition of protein B is given in Table III. Protein B had a high content of proline and acidic acids. Little cysteine was found and the protein was only moderately hydrophobic.

Cyanogen bromide cleavage

Treatment of either protein B or B* with cyanogen bromide produced the same two fragments which had molecular weights of about 18,000 and 15,000 on SDS-polyacrylamide gels (Fig. 15). The results presented show the most complete cleavage that was obtained. Generally, numerous other bands with molecular

Table II

Amino acid composition of SDS extracts

Amino Acid	Extract	
	1	2
Lys	5.35	5.60
His	1.50	0.90
Arg	4.90	4.30
	11.7	10.8
Asp	11.80	16.70
Glu	9.40	10.70
	21.2	27.4
Val	6.35	6.30
Met	1.90	1.70
Ile	4.15	3.00
Leu	7.30	6.20
Tyr	4.30	5.20
Phe	6.00	3.50
	30.0	25.9
Thr	6.05	6.30
Ser	5.25	6.60
Pro	4.75	1.50
Gly	10.80	9.30
Ala	10.20	12.30
$\frac{1}{2}$ Cys	Trace	Trace
	37.1	36.0

Values are expressed as moles percent. The data are grouped and summed as basic, acidic, hydrophobic and neutral residues. Duplicate analyses were performed after hydrolysis in 6 N HCl at 105°C for 25 h. Cysteine was determined as cysteic acid and tryptophan was not measured.

Table III

Amino acid composition of purified proteins

Amino Acid	Protein B	Protein Bp	Protein A			
			From glucose-grown cells (A ₁)		From CAA-grown cells (A ₂)	
Lys	6.2	5.6	6.4		6.9	
His	2.2	3.2	0.4		0.4	
Arg	4.5	3.8	3.3		3.6	
	12.9			10.1		10.9
Asp	11.1	10.9	15.4		15.7	
Glu	8.7	9.6	8.2		7.8	
	19.8			23.6		23.5
Val	7.6	6.0	6.4		5.7	
Met	2.3	1.7	0.9		0.7	
Ile	5.4	5.2	4.2		4.5	
Leu	7.8	7.8	8.1		7.4	
Tyr	4.6	6.3	6.9		7.3	
Phe	3.1	3.9	6.5		6.9	
Thr	6.0	5.9	5.9	33.0	5.8	32.5
Ser	4.5	5.2	3.6		4.4	
Pro	5.4	3.7	1.4		1.2	
Gly	10.9	11.6	12.6		12.6	
Ala	9.3	9.1	9.5		9.1	
½Cys	0.5	0.6	0.3		0.3	
	36.6			33.3		33.4
		36.1				

Values are expressed as moles percent. The data are grouped and summed as basic, acidic, hydrophobic and neutral residues. Duplicate analyses were performed after hydrolysis in 6N HCl at 105°C for 24 h. Cysteine was determined as cysteic acid.

Abbreviation: CAA, casein amino acids.

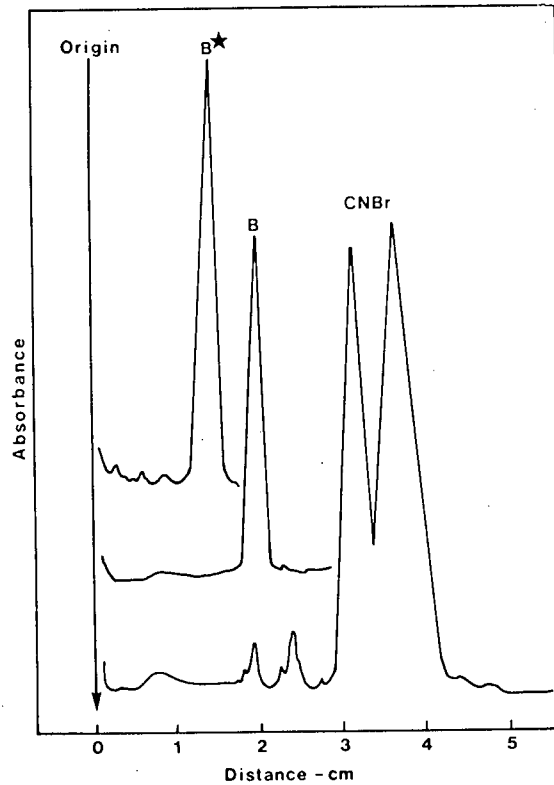


Fig. 15. Cyanogen bromide cleavage of protein B. The lower gel scan shows the separation of the cleavage products of protein B in a 10% SDS-polyacrylamide gel run with system 1. Gels were stained with Coomassie Blue and scanned at 550 nm. Gel scans of proteins B and B* are given for reference.

weights of 30,000 to 18,000 were produced. The protein in the faster migrating peak was probably heterogeneous since the amino acid composition suggests that there are about five methionine residues in protein B.

Amino-terminal analysis

Amino-terminal analysis of protein B and B* failed to produce an amino-terminal dansyl derivative (Fig. 16). However, the amino-terminal lysine of lysozyme could be determined under the same conditions. The nature of the blocking group was not investigated further.

Physical properties of proteins B and B*

The results presented above are consistent with proteins B and B* being the same polypeptide. However, the reason for the change in the apparent molecular weight of protein B upon heating in solutions containing SDS was not clear. Protein B was therefore purified free of phospholipid and lipopolysaccharide and the difference between the unmodified form, B, and the heat-modified form, B*, in SDS was investigated by a number of physical techniques.

Protein B in SDS extract 1 was shown to increase its apparent molecular weight upon heating (Fig. 9). Purified protein B when heated in 1% SDS changes its migration in SDS-polyacrylamide gels run in the Laemmli system (Fig. 17). Protein B was converted to form B* with an increase in the apparent molecular weight from 29,500 to 34,700 (Fig. 18, panel 1).

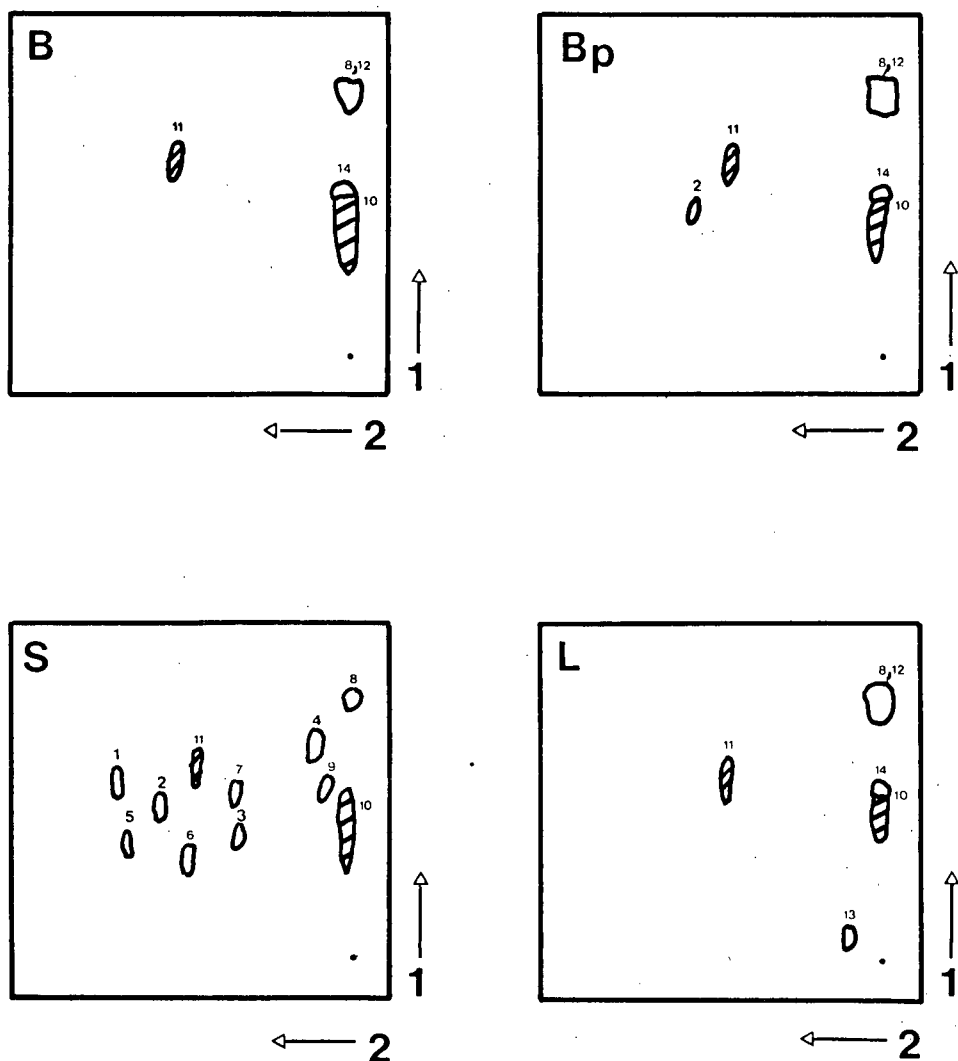
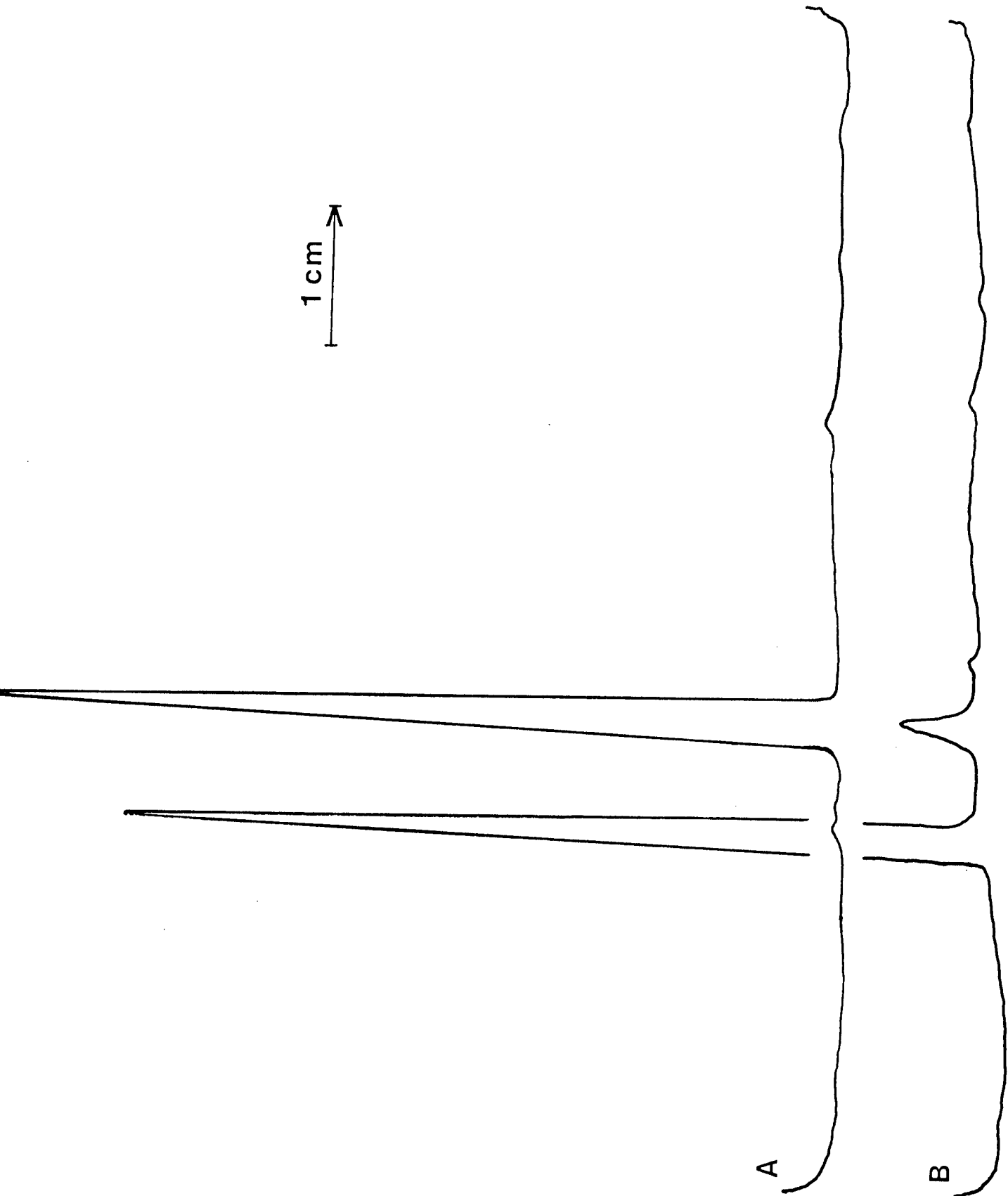


Fig. 16. Amino-terminal analysis of proteins B and Bp, tracings of the fluorescent spots obtained after two-dimensional chromatography of the dansylated derivatives on polyamide sheets (5 x 5 cm) are shown. The direction of chromatography is indicated by the large numbers beside the plates. S, standard dansyl amino acids: 1, proline; 2, valine; 3, methionine; 4, serine; 5, isoleucine; 6, phenylalanine; 7, glycine; 8, arginine; 9, aspartic acid. L, B and Bp, fluorescent derivatives formed by dansylation of lysozyme, protein B and protein Bp respectively: 10, dansylic acid; 11, dansyl amine; 12, E-dansyl lysine; 13, bis (α , ϵ) didansyl lysine; 14, O-dansyl tyrosine.

Fig. 17. SDS-polyacrylamide gel electrophoresis of purified protein B. Scan A; sample heated at 37°C for 20 min. Scan B; identical sample heated at 100°C for 5 min. Gels were 12.5% polyacrylamide, run in the Laemmli system (92). The gels were stained for protein with Coomassie Blue and scanned at 550 nm using a Gilford Model 240 spectrophotometer. The arrow indicates direction of migration toward the anode.



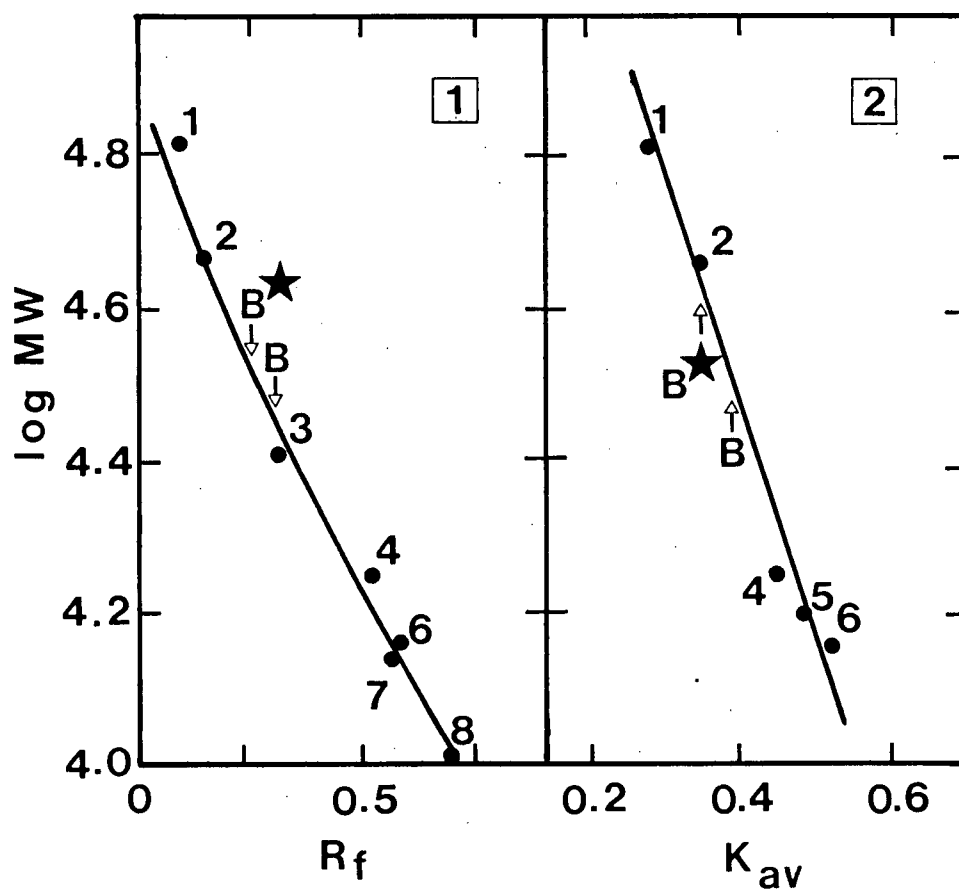


Fig. 18. Determination of the molecular weights of proteins B and B* by SDS-polyacrylamide gel electrophoresis (panel 1) and by gel filtration in the presence of SDS (panel 2). Gels (12.5% acrylamide) were run in a discontinuous buffer system, stained with Coomassie Blue, and scanned at 550 nm. Gel filtration was performed in a column (1 x 45 cm) of Sepharose 6B, equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. Standard proteins, 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen A chain; 4, β -lactoglobulin; 5, haemoglobin; 6, lysozyme; 7, chymotrypsin B chain; 8, chymotrypsin C chain, were heated at 100° for 5 min in sample buffer containing 0.1% 2-mercaptoethanol.

The molecular weights of proteins B and B* were also determined by gel filtration in a column of Sepharose 6B equilibrated with 1% SDS in 0.1 M sodium phosphate buffer, pH 7.2 (Fig. 18, panel 2). When compared to standard proteins run under identical conditions, protein B was eluted in a volume corresponding to a molecular weight of 29,000, while the molecular weight of protein B* was 42,600. Thus, the true molecular weight of protein B is still in doubt.

Schnaitman has found that the intrinsic viscosity of protein B increased on heating from 28 to 35 cc/g, when measured at SDS concentration below the critical micellar concentration (81). He suggested that heating caused unfolding of protein B with an increased binding of SDS to the protein molecule. This experiment was repeated at concentrations of SDS above the critical micellar concentration to ensure complete saturation of the protein with SDS. The results are presented in Fig. 19. The intrinsic viscosity of protein B increased on heating from 28.5 to 34 cc/g in agreement with Schnaitman's data. In order to check the validity of these measurements, the intrinsic viscosities of three proteins, BSA, ovalbumin and lysozyme, previously characterized by Reynolds and Tanford (152) were measured. The values determined for these proteins, 59, 32 and 8 cc/g, respectively, agree with those found previously.

Calculation of axial ratios (a/b) for proteins B and B* using the Simha relationship (see Appendix) and assuming a prolate ellipsoid with a hydration of 0.9 g/g protein at a binding level of 1.4 g of SDS per g of protein (152,286) gave values of

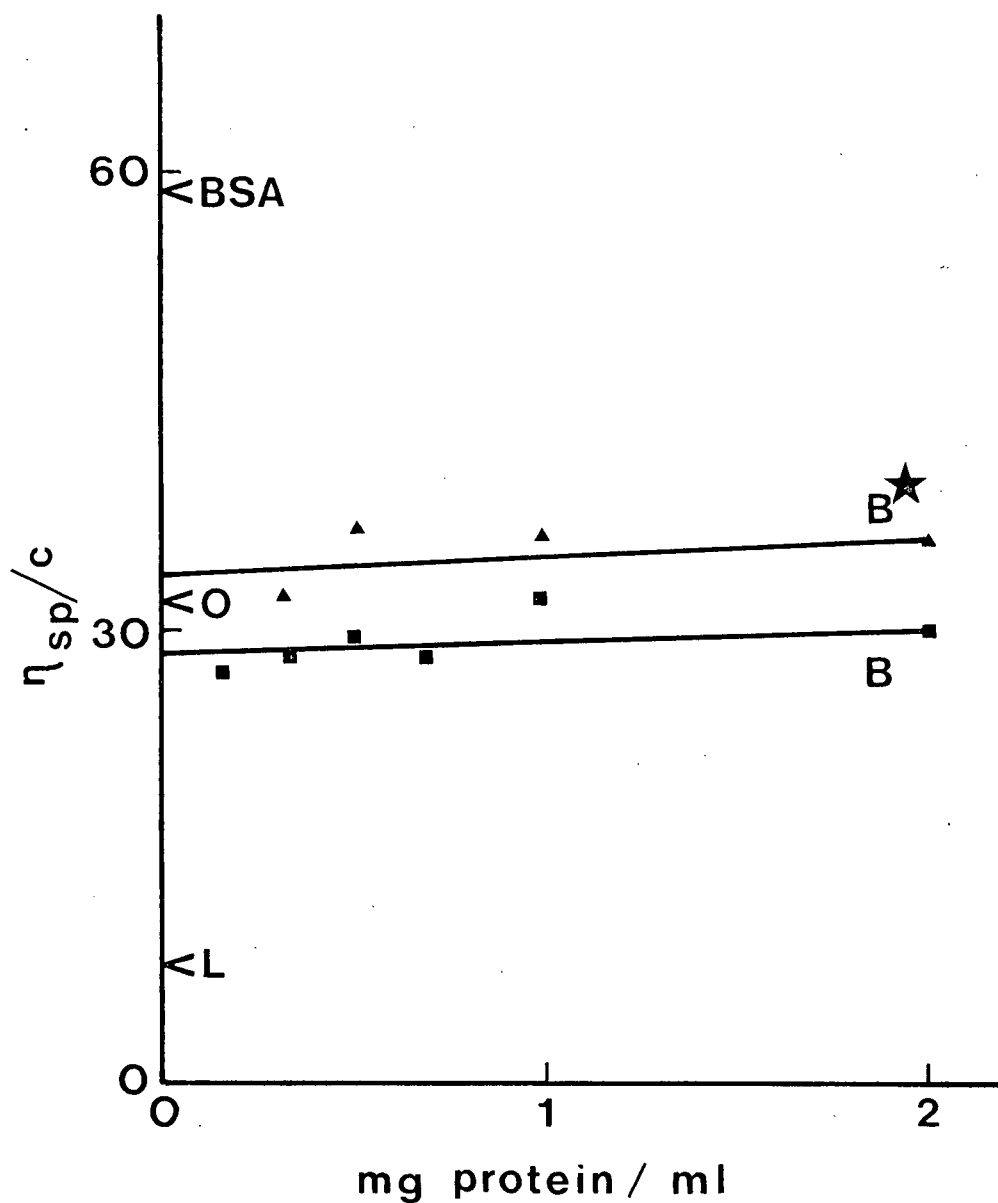


Fig. 19. Reduced viscosities (η_{sp}/c) of proteins B and B* at 25°C in 0.5% SDS, 0.1 M sodium phosphate buffer, pH 7.2. Reduced viscosities for standard proteins were determined under identical conditions and the extrapolated values are indicated by arrows. O, ovalbumin; L, lysozyme.

7.5 and 9.0 for proteins B and B*, respectively. These correspond to frictional ratios (f/f_0) of 1.4 and 1.5 for proteins B and B*. Using a molecular weight of 27,000 for the polypeptide chain as determined by Garten et al. from amino acid analysis and summation of the molecular weights of the peptides formed on cleavage with cyanogen bromide (87), absolute values for the dimensions of the protein-SDS complex could be determined. The protein-SDS complex of protein B had ellipsoid axes of 117 x 16 angstrom units while the protein B* complex had dimensions of 136 x 15 angstrom units.

The difference between proteins B and B* could be due to the increased asymmetry, an increased level of SDS binding, or a combination of both. To distinguish between these possibilities we have measured the amount of detergent bound to proteins B and B*. The results of SDS binding by equilibrium dialysis (Fig. 20) are presented in Fig. 21. Both proteins bound ca. 0.5g of SDS per gram of protein at concentrations of SDS up to 0.1%.

The amount of SDS bound to proteins B and B* above the critical micellar concentration (ca. 0.2% in 1 mM Tris-acetate buffer, pH 7.0), was determined by calculating (see Appendix) the molecular weight of the protein-detergent complex. The following equation can be applied to the protein-SDS complex (287):

$$s = \frac{M(1-\bar{v}\rho)}{6\pi\eta NR_s} \quad (1)$$

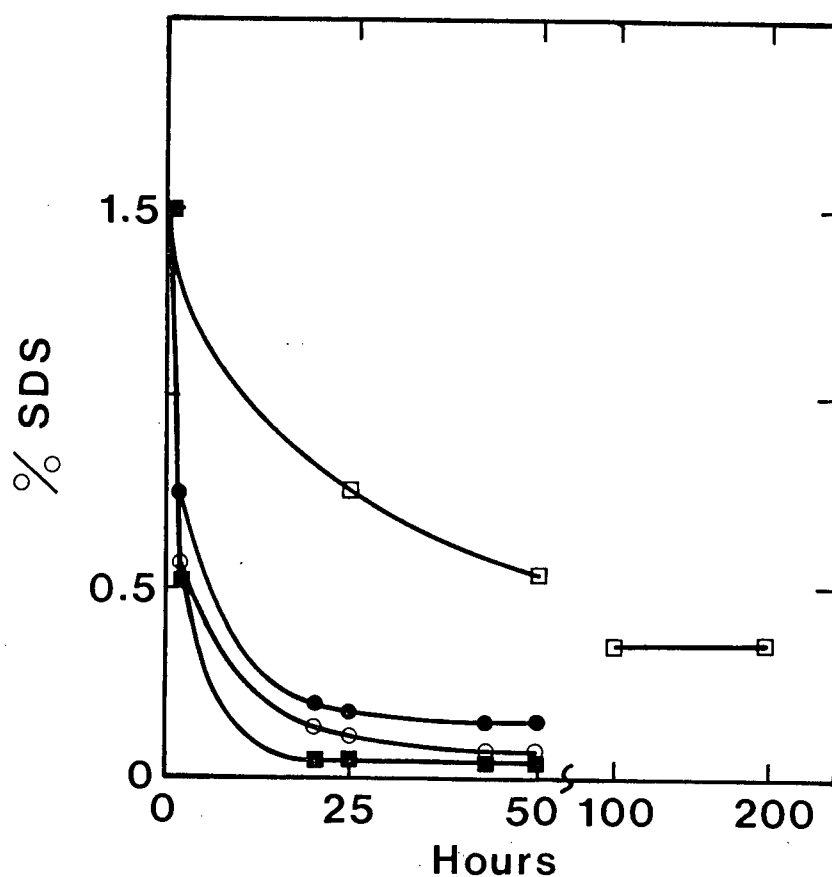
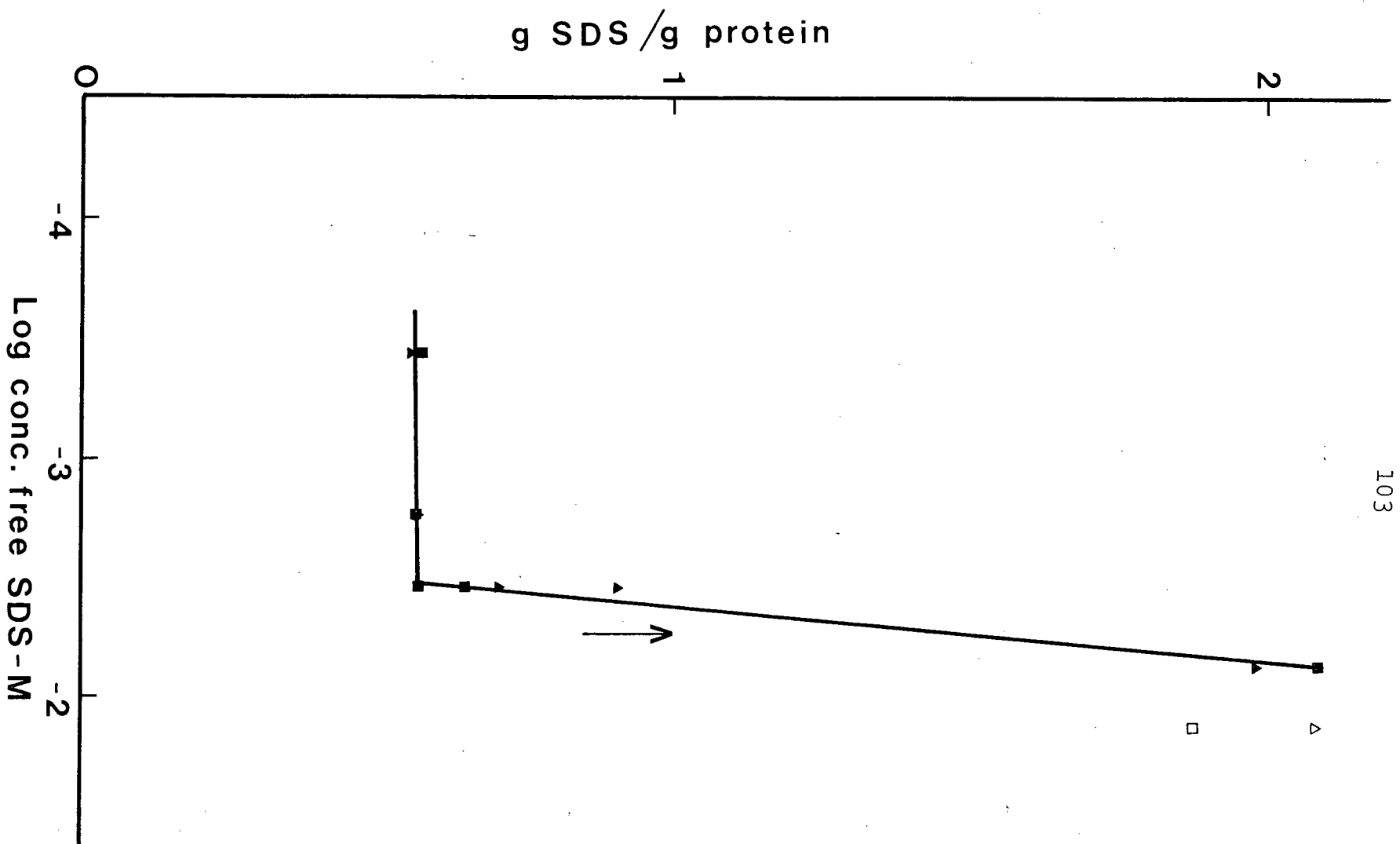


Fig. 20. Equilibrium dialysis of protein B. Protein samples (1 ml) were dialyzed at 22°C against 1 l of 1 mM Tris-acetate buffer, pH 7.0, containing 0.20% (□), 0.10% (●), 0.048% (○) and 0.008% (■) final concentration of SDS. Since the curves obtained for protein B* were indistinguishable from those shown for protein B, these points are not plotted.

Fig. 21. Binding of SDS to proteins B (■) and B* (▲) at 22°C in 1 mM Tris-acetate buffer, pH 7.0. Binding was determined by equilibrium dialysis except for the points indicated by the open symbols which were calculated from the molecular weights of the protein-SDS complexes as described in the text.



where M , R_s , and s are the molecular weight, partial specific volume, Stokes radius, and sedimentation coefficient, respectively, of the protein-SDS complex.

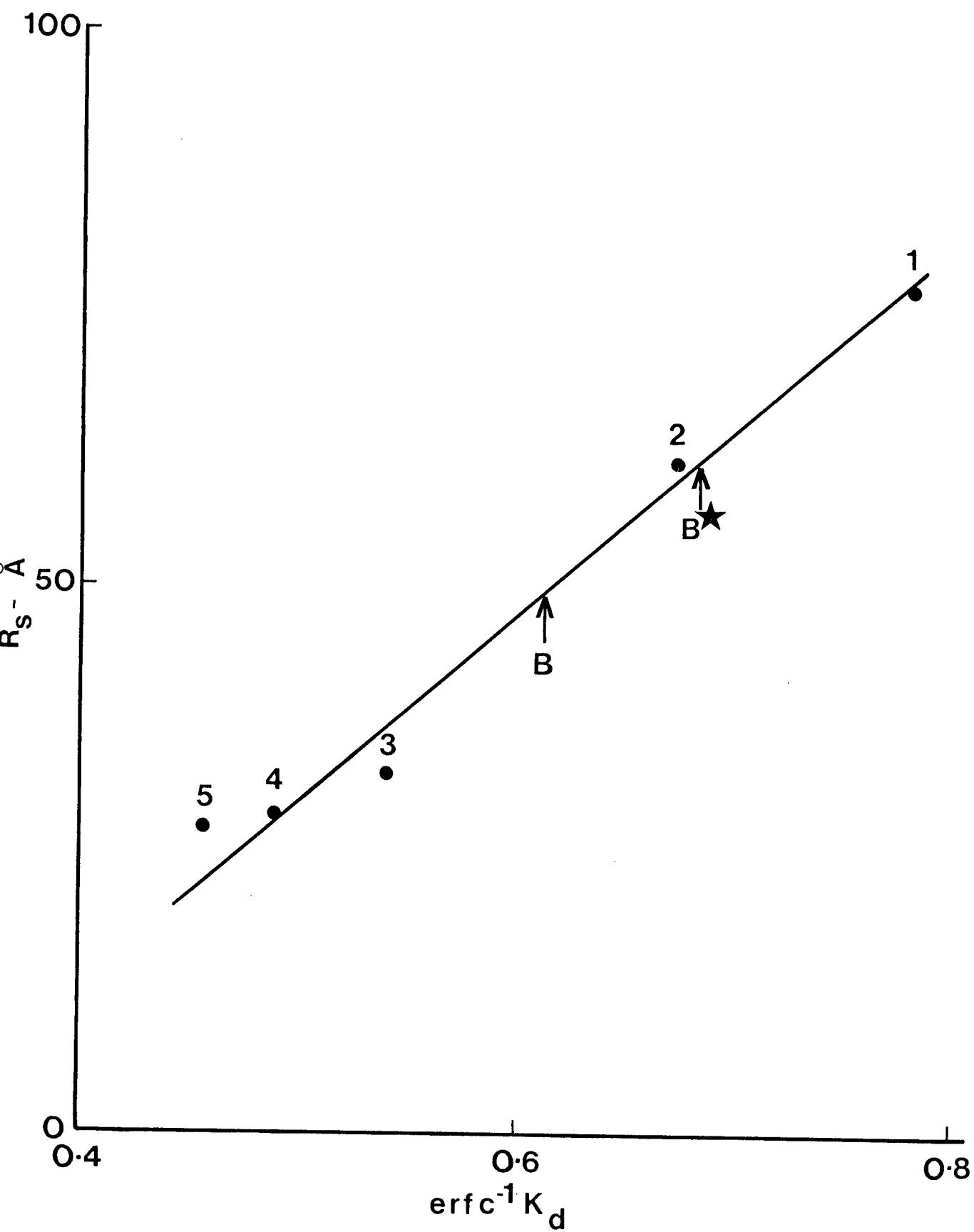
The Stokes radii of proteins B and B* were determined by gel filtration in the presence of 1% SDS (see Appendix). A plot of Stokes radius versus $\text{erfc}^{-1}k_d$ (288,289) is presented in Fig. 22. The values of Stokes radii for the standard proteins were taken from Reynolds and Tanford (152). The Stokes radius of protein B increased from 50 to 63 angstrom units upon conversion to form B*.

Sedimentation coefficients for proteins B and B* were determined by sucrose density gradient centrifugation at SDS concentration from 0.1 to 0.3%. The partial specific volumes of proteins B and B* were determined by sucrose density gradient centrifugation in H_2O and in D_2O -containing gradients (292). Assuming that the proteins bind the same amount of detergent in H_2O and D_2O , the partial specific volume () may be calculated (see Appendix) from:

$$\bar{v} = \frac{\frac{s_H \eta_H}{s_D \eta_D} - 1}{p_D \frac{s_H \eta_H}{s_D \eta_D} - p_H} \quad (2)$$

where subscripts H and D refer to values measured in H_2O and in D_2O , s is the measured sedimentation coefficient in a solution of density, p , and η , the viscosity determined at the half-distance of travel, r_{avg} . Results obtained at 0.170, 0.2%,

Fig. 22. Chromatography of proteins B and B* on Sepharose 6B in 1% SDS, 0.1 M sodium phosphate buffer, pH 7.2. Arrows indicate the elution positions of proteins B and B*. The Stokes radii of the standard proteins were taken from Reynolds and Tanford (152). Stokes radius (R_s) is plotted as a function of the inverse error function complement ($\text{erfc}^{-1}K_d$) (289). Standard proteins: 1, BSA; 2, ovalbumin; 3, β -lactoglobulin; 4, hemoglobin; 5, lysozyme.



0.3% SDS are given in Figs. 23, 24 and 25. The $s_{20,w}$ values for proteins B and B* at different concentrations of SDS were calculated (see Appendix) from:

$$s_{20,w} = s_{T,m} \frac{\eta_{T,m}}{\eta_{20,w}} \frac{(1-\bar{v}\rho_{20,w})}{(1-\bar{v}\rho_{T,m})} \quad (3)$$

The viscosities (η) of standard sucrose solutions in H_2O and in D_2O buffers, containing the specified concentrations of SDS, were measured at $20^\circ C$ with a Cannon-Manning viscometer. Densities (ρ) for the same solutions were determined by weighing 5.0 or 10.0 ml of the solution at $23^\circ C$. The determined values for sedimentation coefficient and partial specific volumes are presented in Table IV. The $s_{20,w}$ values are the means of the values obtained in H_2O and in D_2O gradients.

For the calculation of the molecular weight of the protein-SDS complex at concentrations of SDS above the critical micellar concentration, the $s_{20,w}$ value at 0.3% SDS was taken for both proteins B and B*. Substituting these values and the determined values for R_s into equation 1 gave molecular weights of 77,000 and 84,000 for the SDS complexes of proteins B and B*, respectively. The difference in molecular weight between the protein-SDS complex and the protein alone must be due to the bound detergent. Using a value of 27,000 for the molecular weight of protein B (87), the amount of SDS bound by proteins B and B* above the critical micellar concentration was 1.85 and 2.1 g per g of protein respectively.

That there is only a small difference in the amount of SDS

Fig. 23. Centrifugation of proteins B and B* in 5 to 20% sucrose gradients prepared in 0.1% SDS, 1 mM Tris-acetate buffer, pH 7.0. Panel A: gradient prepared in H₂O, panel B: gradient prepared in D₂O. The sucrose concentration of each fraction was determined from the refractive index.

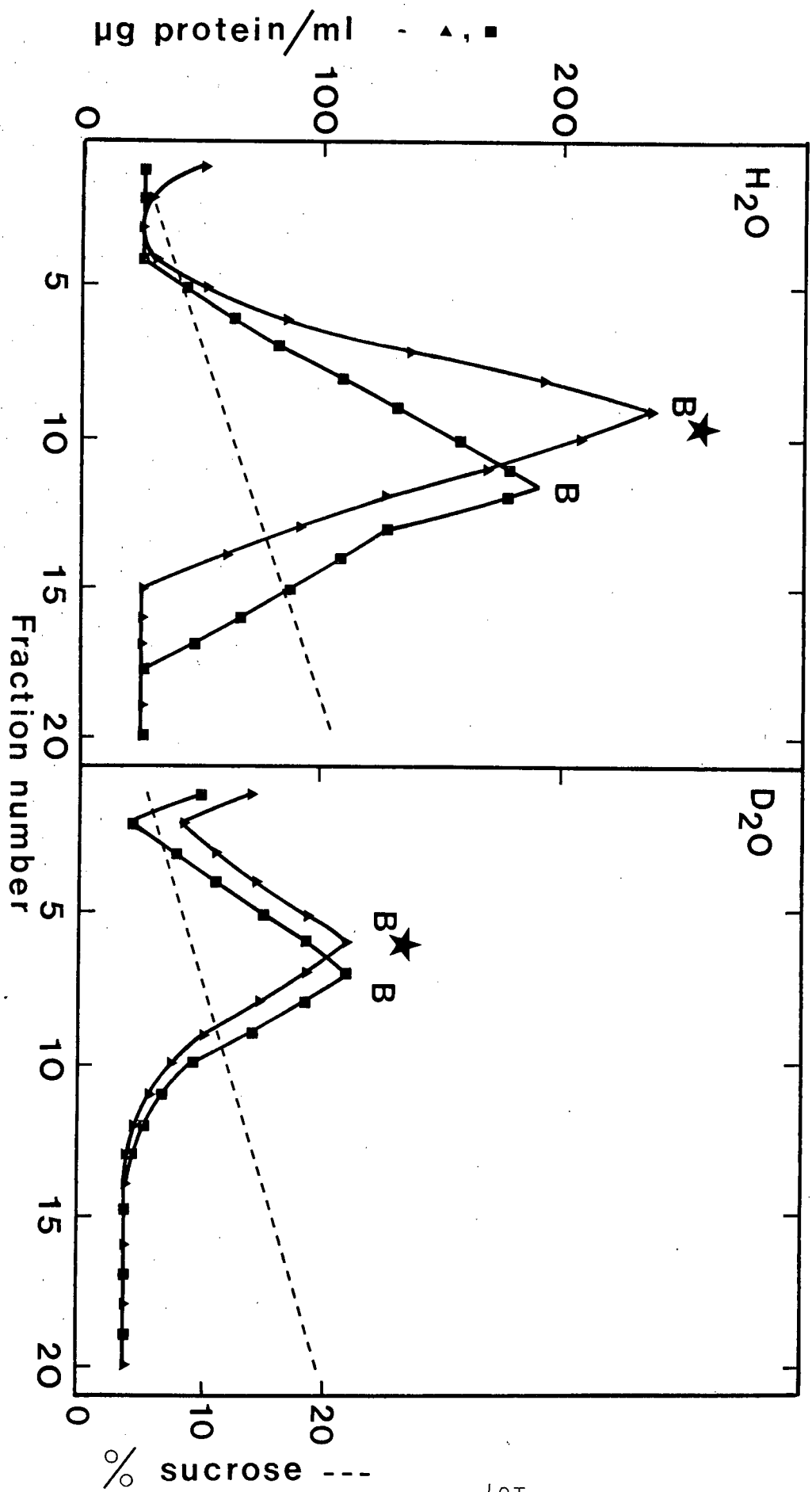


Fig. 24. Centrifugation of proteins B and B* in 5 to 20% sucrose gradients prepared in 0.2% SDS, 1 mM Tris-acetate buffer, pH 7.0. Panel A: gradient prepared in H₂O, panel B: gradient prepared in D₂O. The sucrose concentration of each fraction was determined from the refractive index.

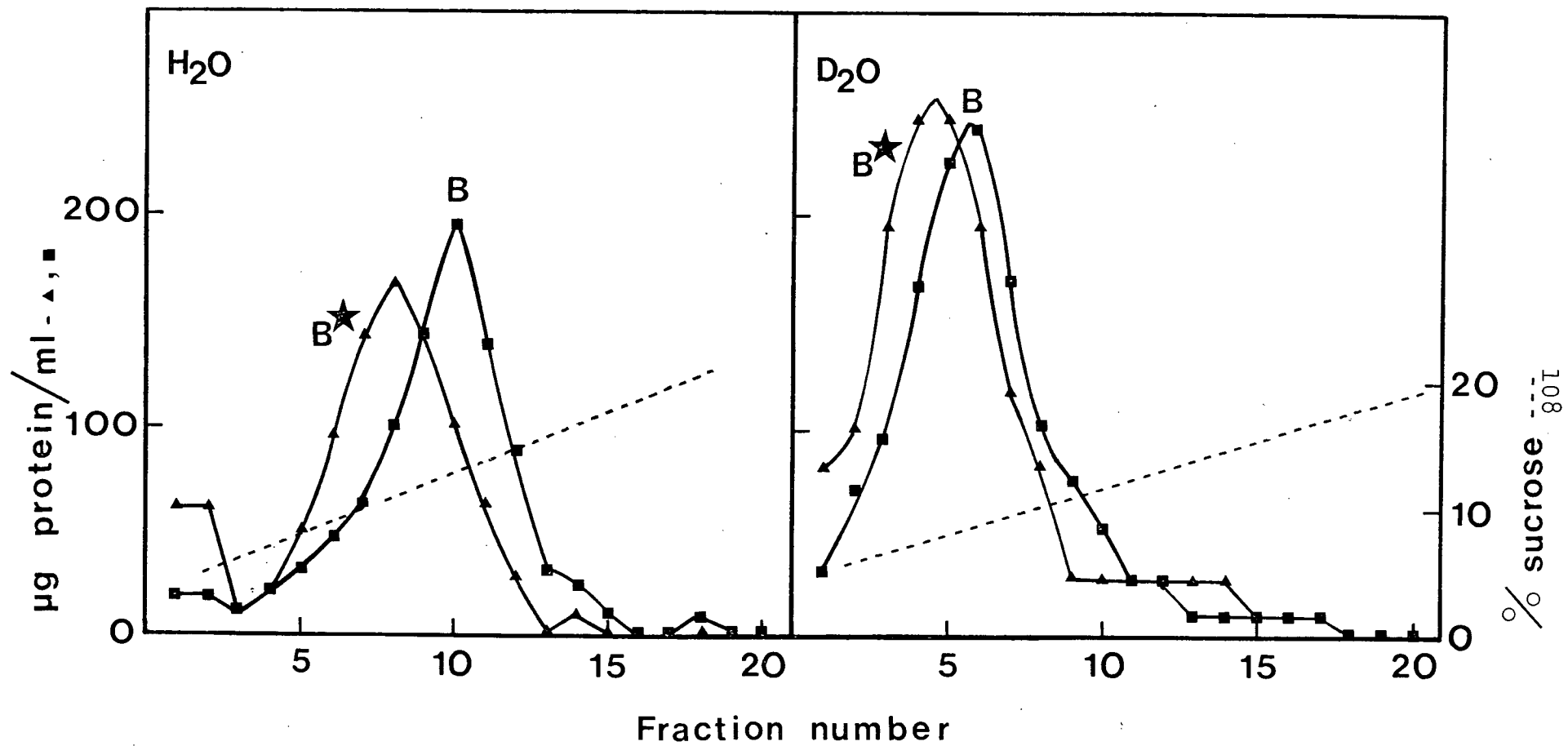


Fig. 25. Centrifugation of proteins B and B* in 5 to 20% sucrose gradients prepared in 0.3% SDS, 1 mM Tris-acetate buffer, pH 7.0. Panel A: gradient prepared in H₂O, panel B: gradient prepared in D₂O. The sucrose concentration of each fraction was determined from the refractive index.

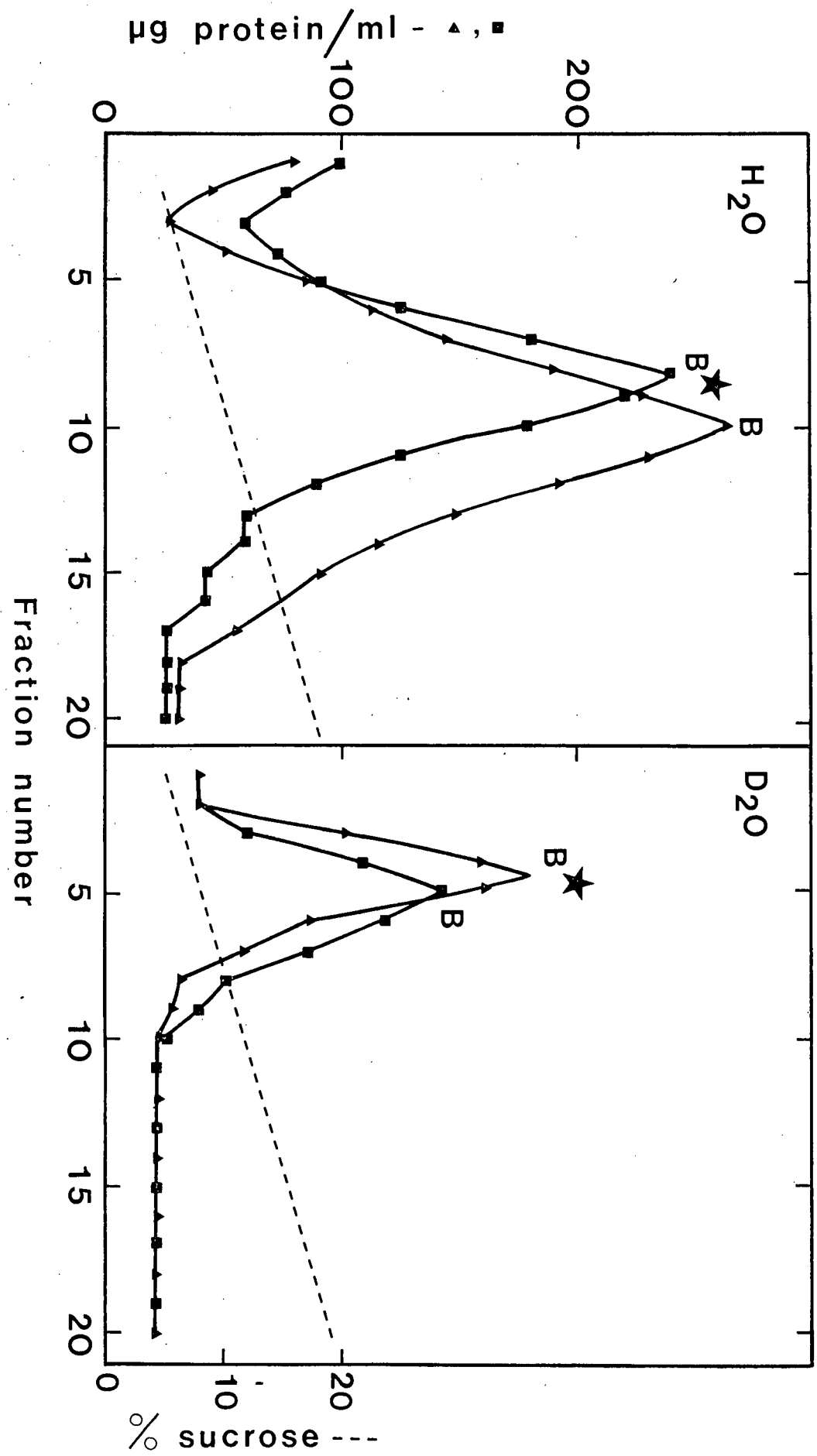


Table IV

Partial specific Volumes and sedimentation coefficients of proteins B and B* at different concentrations of SDS

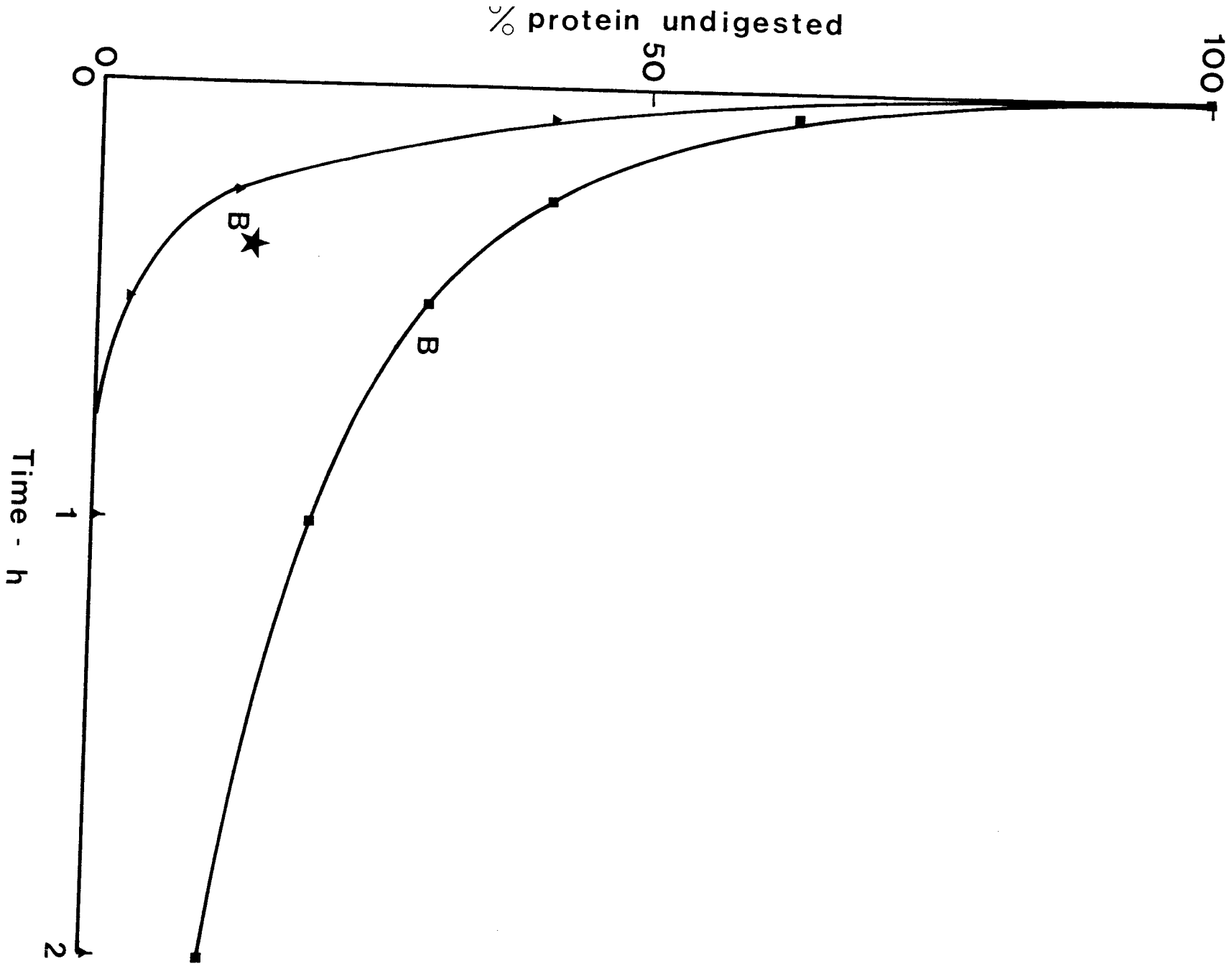
SDS (% w/v)	B		B*	
	\bar{v}	$S_{20, W}$	\bar{v}	$S_{20, W}$
0.1	0.772	3.40	0.753	2.73
0.2	0.776	2.78	0.772	2.20
0.3	0.793	2.83	0.797	2.40

bound to proteins B and B* is further substantiated by their partial specific volumes. The partial specific volumes of both proteins B and B* increased with increasing SDS concentrations (Table IV) indicating that more detergent was bound at higher SDS concentrations as has been found with other proteins (290,291,293). However, above the critical micellar concentration there was no significant difference between the partial specific volumes of proteins B and B*. At all SDS concentrations examined, the sedimentation coefficient of protein B was always higher than form B* (Table IV). There was no obvious relationship between the sedimentation coefficient and the concentration of SDS as has also been observed by Nelson (294).

The similar size of the protein-SDS complexes of proteins B and B* and the relatively small differences in the amount of detergent bound indicates that the apparent increased molecular weight of protein B compared to form B* is not due to increased binding of detergent but rather is due to the increased asymmetry of the protein B* molecule. This would account for the higher Stokes radius and the lower sedimentation coefficient of protein B*.

A protein on unfolding will become more susceptible to degradation by proteolytic enzymes (204). We therefore examined the susceptibility of proteins B and B* to digestion by pronase in 1% SDS. As can be seen from Fig. 26, protein B* was degraded more rapidly than protein B when a pronase to protein ratio of 1:200 was used. These results suggest that protein B* is indeed more unfolded than protein B.

Fig. 26. Digestion of proteins B and B* by pronase in 1% SDS, 10 mM Tris-HCl buffer, pH 7.5. The proteins were incubated at 37°C at a protease to protein ratio of 1:200. Samples were removed at intervals up to two hours and digestion was stopped by adding a 100-fold excess of phenylmethanesulfonyl fluoride followed by heating at 100°C for 5 min in the presence of 0.1% α -mercaptoethanol. Samples were made 4 M in urea and then examined by SDS-polyacrylamide gel electrophoresis. The gels were stained for protein with Coomassie Blue and scanned at 550 nm using a Gilford Model 240 spectrophotometer. The relative areas of the peak corresponding to protein B* were determined by weighing.



Since the results presented above have indicated that a marked shape change occurs upon heating, optical methods were employed to determine the effect on protein conformation. In all samples examined, protein B* had an increased ultra-violet absorption at 275 nm over protein B (Fig. 27). This was most clearly seen in the difference spectra between proteins B* and B shown in the lower panel of Fig. 27. The increased absorption due to aromatic amino acids indicates that there was an unfolding of the polypeptide chain upon heating.

ORD and CD spectroscopy have been extremely useful in studies of protein conformation in biological membranes and in detergent solutions (152,295). The ORD and CD spectra (Fig. 28, panel A) of the two proteins did not differ greatly but both had a form that is typical of protein-SDS complexes with a negative trough at about 233 nm (152). Reynolds and Tanford (152) have found that increased binding of SDS decreases the magnitude of this trough. Thus, the identical nature of the spectra for proteins B and B* supports the finding that these proteins did not differ greatly in the amount of bound detergent.

The CD spectra for proteins B and B* in 0.2% SDS are shown in panel B. Protein B was slightly more optically active in the 250-300 nm region of the CD spectrum while only small differences could be seen in the 190-250 nm region. This suggests that protein B* has a more disordered structure than protein B. The CD spectra are consistent with a helical conformation for the protein-SDS complex. As Tanford (287) has suggested, small differences in optical properties of protein-

Fig. 27. Panel A: ultraviolet absorption spectra of proteins B and B* at 22°C in 0.2% SDS, 1 mM Tris-acetate buffer, pH 7.0. The scan for protein B* is displaced upwards by 0.1 absorbance unit. Protein concentration, 0.7 mg/ml. Panel B: ultraviolet difference spectrum of protein B* versus B. The same samples from panel A were used.

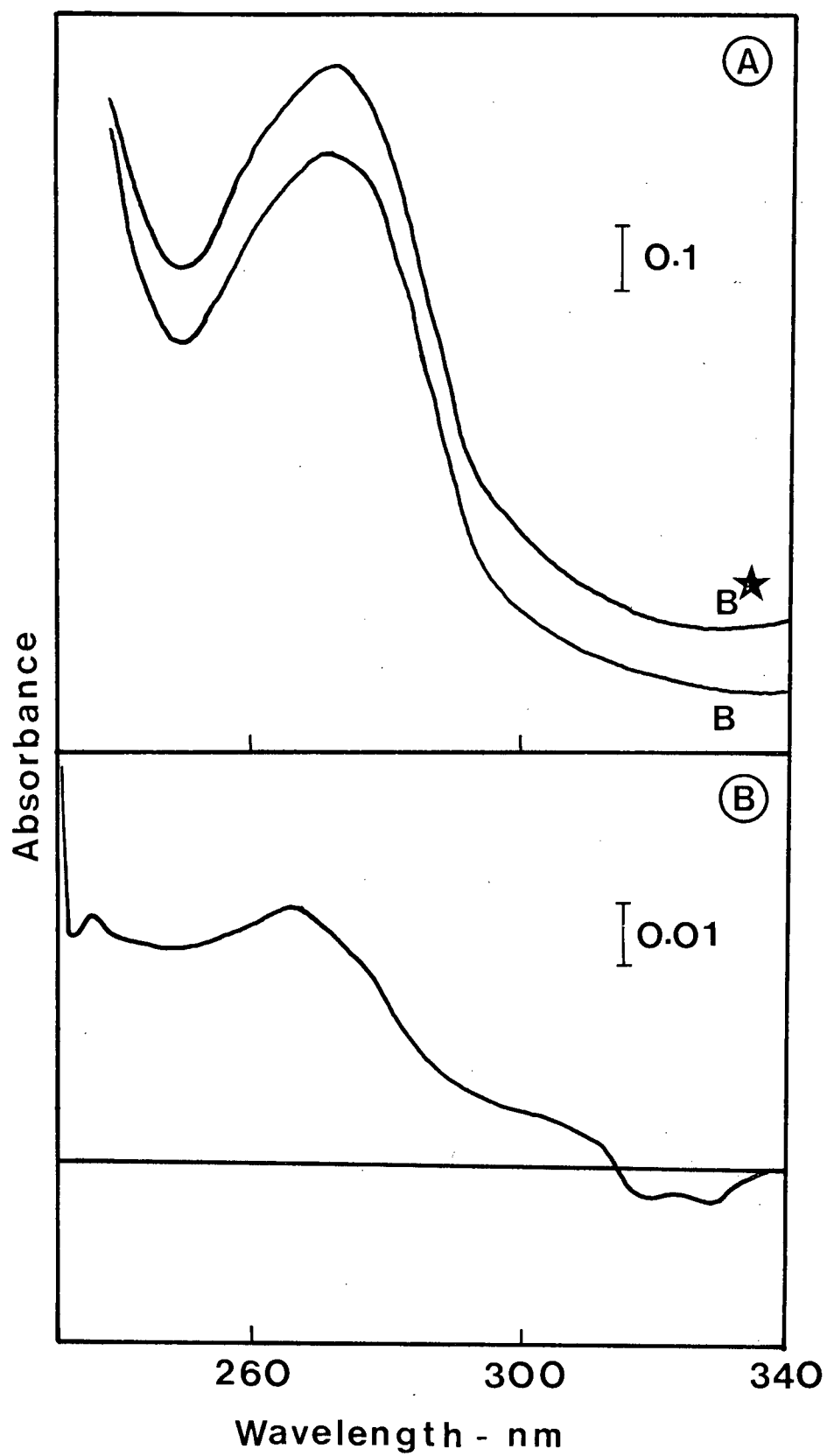
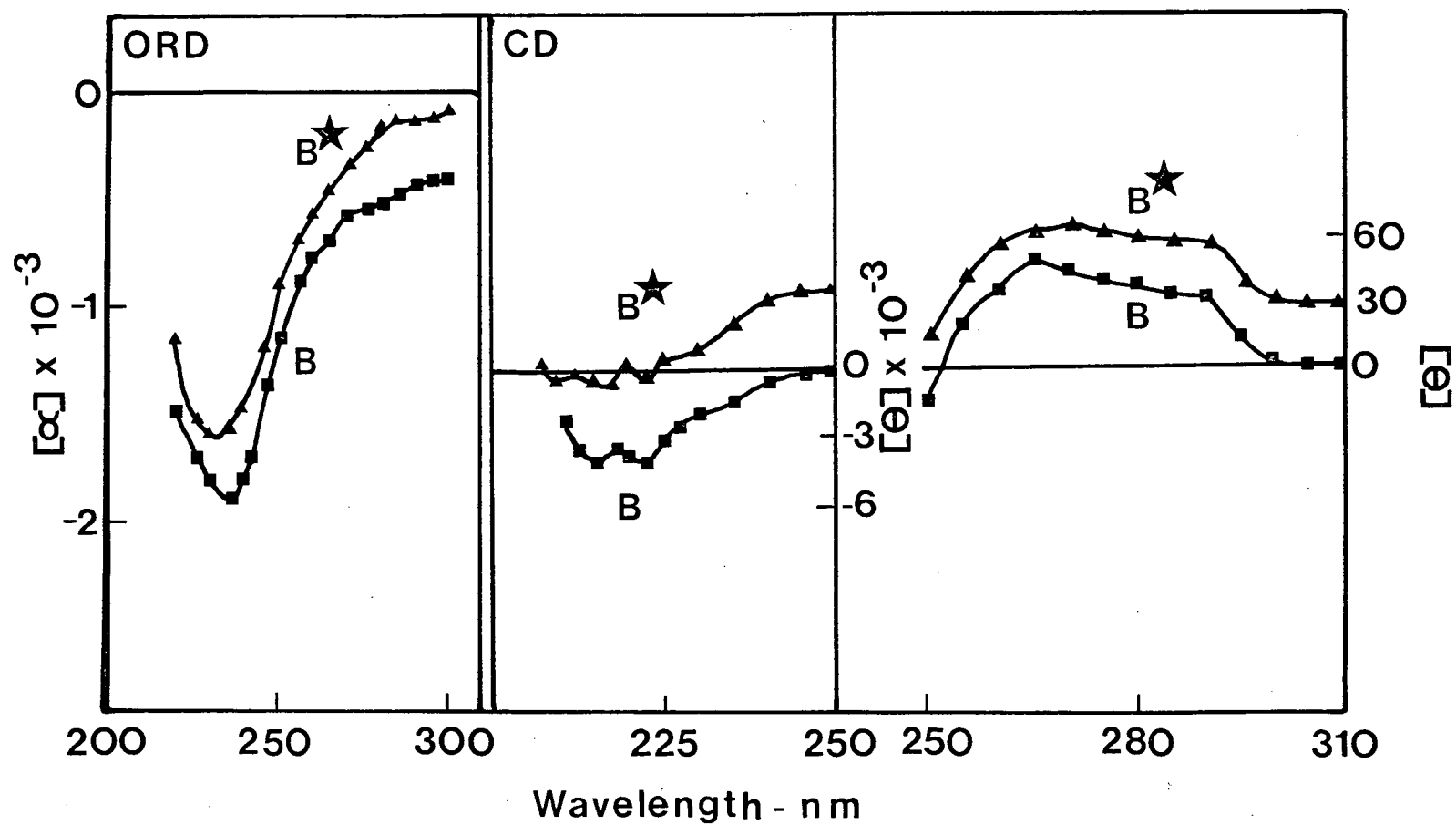


Fig. 28. Panel A: ORD spectra of proteins B and B* at 25°C in 0.2% SDS, 1 mM Tris-acetate buffer, pH 7.0. For clarity the spectrum of protein B is displaced downwards by 300 degrees. Protein concentration, 0.7 mg/ml. Panel B: CD spectra of proteins B and B*. The spectra of protein B* are displaced 30 and 3000 degrees $\text{cm}^2 \text{decimole}^{-1}$ upwards in the near uv and far uv regions, respectively. A 1 cm cell was used in the near uv region, while a 0.1 mm pathlength was used in the far uv region. The same samples as in the ORD spectra were used.

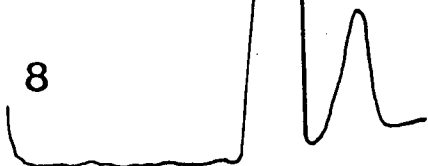
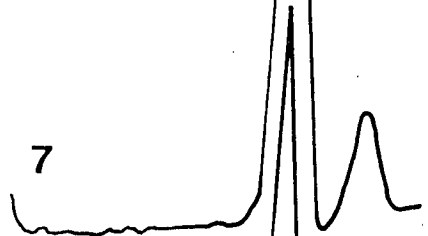
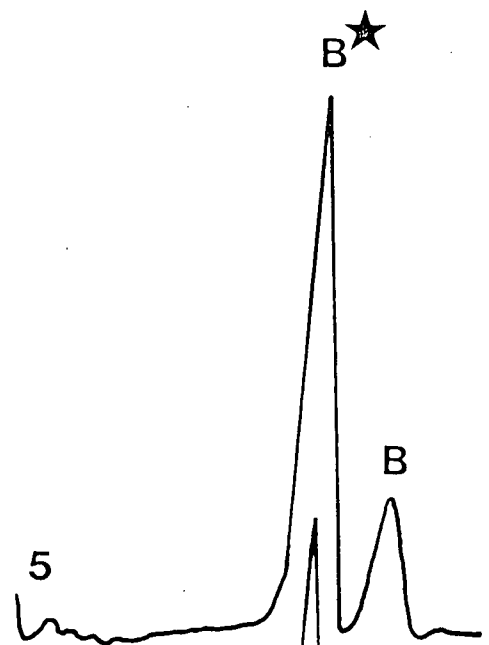
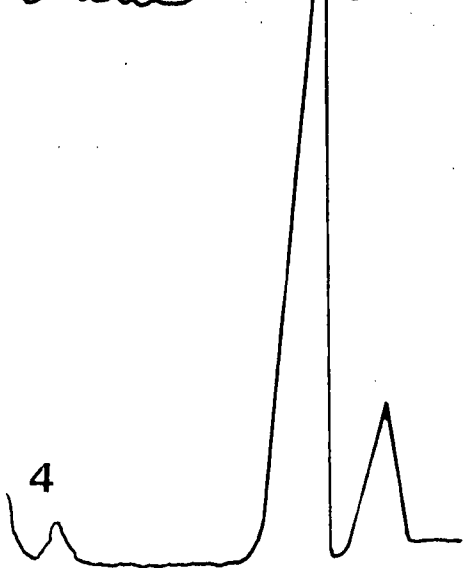
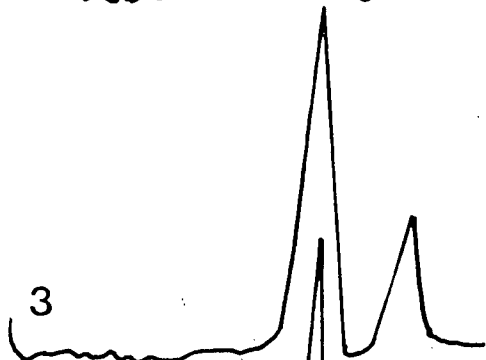
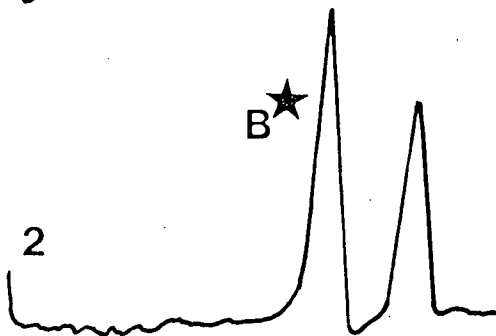
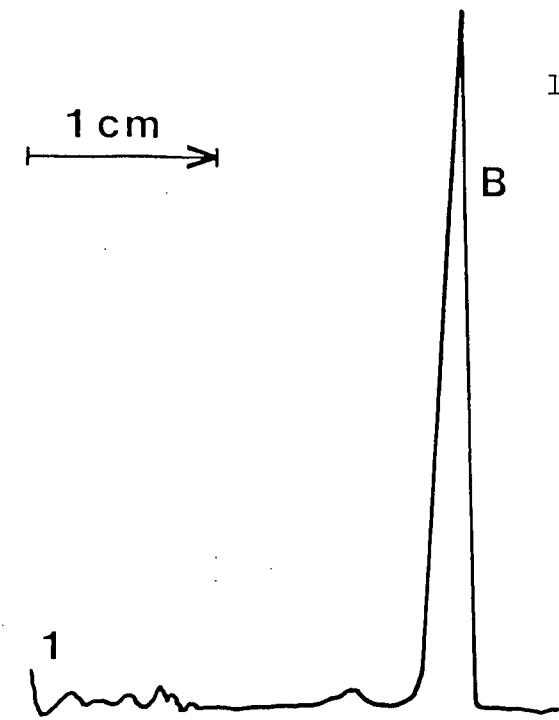


detergent complexes may not reflect the extent of conformational change that might occur upon heating in SDS-containing solutions.

The results above indicate that heating of protein B in SDS-containing solutions causes unfolding of the polypeptide chain without a significant change in the amount of detergent bound. SDS is required to solubilize protein B from the membrane, however, it is not clear if it is required for the conversion of protein B to form B*. This problem was investigated as follows.

Weber and Kuter (144) have shown that complete removal of SDS from protein may be accomplished by ion exchange in the presence of 6M urea in 0.05 M Tris-acetate buffer (pH 7.8). SDS was removed from both proteins B and B* to a level undetectable by the methylene blue assay by passage through a small column of this resin. This assay detects both free and bound SDS (169). Protein B, free of SDS, was heated in the Tris-urea buffer at 100°C for up to 15 min. Samples were removed at 0, 2, 5, and 10 min and were made to a concentration of 1% in SDS immediately. The samples were examined for the extent of conversion of protein B to form B* under these conditions by submitting the samples to SDS polyacrylamide gel electrophoresis without further heating. As seen in Fig. 29, scans 1-4, protein B was progressively converted to form B* upon heating in the absence of SDS. This change was correlated with an increased light absorption in the ultraviolet region similar to that previously observed when heating was carried out in the

Fig. 29. Effect of heating protein B in the absence of SDS. SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (90). Gels were stained and scanned as described in Fig. 17. The experiment was performed as described in the text.

1 cm
→

presence of SDS.

In order to test for the reversal of this conversion the remainder of the heated mixture was allowed to cool at 22°C for 48 h. Samples removed at various time intervals showed no reconversion of form B* to protein B (Fig. 29; scans 5-8). Moreover removal of SDS from the protein B*-SDS complex did not result in reformation of protein B. These studies indicate that bulk SDS is not required for the conversion of protein B to form B* and that the irreversible nature is not due to the binding of large amounts of SDS. However, the possible involvement of a few molecules of SDS still remaining bound to the protein, but undetectable by the detergent assay method, cannot be ruled out.

It appears that protein B, as extracted from the outer membrane of E. coli by 0.5% SDS at 37°C, contains some native structure and that this structure is lost upon heating. This process will occur in the absence of SDS and involves the unfolding of the polypeptide chain. Although further binding of SDS may occur to a small extent it is unlikely that the apparent increase in the molecular weight is due to a greater amount of bound detergent.

Effect of growth conditions on the outer membrane proteins

Schnaitman (91) reported that the synthesis of protein 2 (Table I) was under catabolite repression. The effect of the carbon source on the formation of the proteins of the outer membrane of E. coli NRC 482 was studied. Cells were grown on

inorganic salts medium containing glucose, glycerol, succinate or acetate, or on casein amino acids or trypticase soy broth and harvested during the exponential and stationary phases of growth (Fig. 30). The outer membrane proteins were isolated and resolved on SDS-polyacrylamide gels (Fig. 31). Protein A₁ predominated over protein A₂ in cells grown on media containing glucose (Fig. 31, Glc and TSB). The level of protein A₂ relative to protein A₁ increased in cells grown on glycerol, succinate, acetate and casein amino acids (Fig. 31). This suggests that protein A₂ is equivalent to Schnaitman's protein 2.

Purification of protein A

Protein A is tightly associated with the peptidoglycan. Extraction of the cell envelope with SDS at 60°C leaves an insoluble residue of protein A bound to the peptidoglycan (Fig. 6). The protein can be extracted from this complex with SDS at 100°C. In cells grown on glucose both proteins A₁ and A₂ were associated with the peptidoglycan, protein A₁ being the predominant protein (ca. 75%) (Fig. 32, upper scan). In cells grown on casein amino acids, where protein A₂ predominated (ca. 75%), again both proteins A₁ and A₂ were associated with the peptidoglycan (Fig. 32, second scan). Extracts of protein A, derived from glucose and casein amino acid-grown cells were further purified by gel filtration in the presence of SDS (Fig. 33) for amino acid analysis and cyanogen bromide cleavage. The extract containing protein A from glucose-grown cells was consistently found to contain more carbohydrate than the protein

Fig. 30. Growth of E. coli NRC 482 on different carbon sources at 37°C with vigorous aeration. Glc, glucose; Gly, glycerol; Succ, succinate; CAA, casein amino acids; TSB, trypticase soy broth; Acet, acetate. Arrows indicate the time at which the cells were harvested.

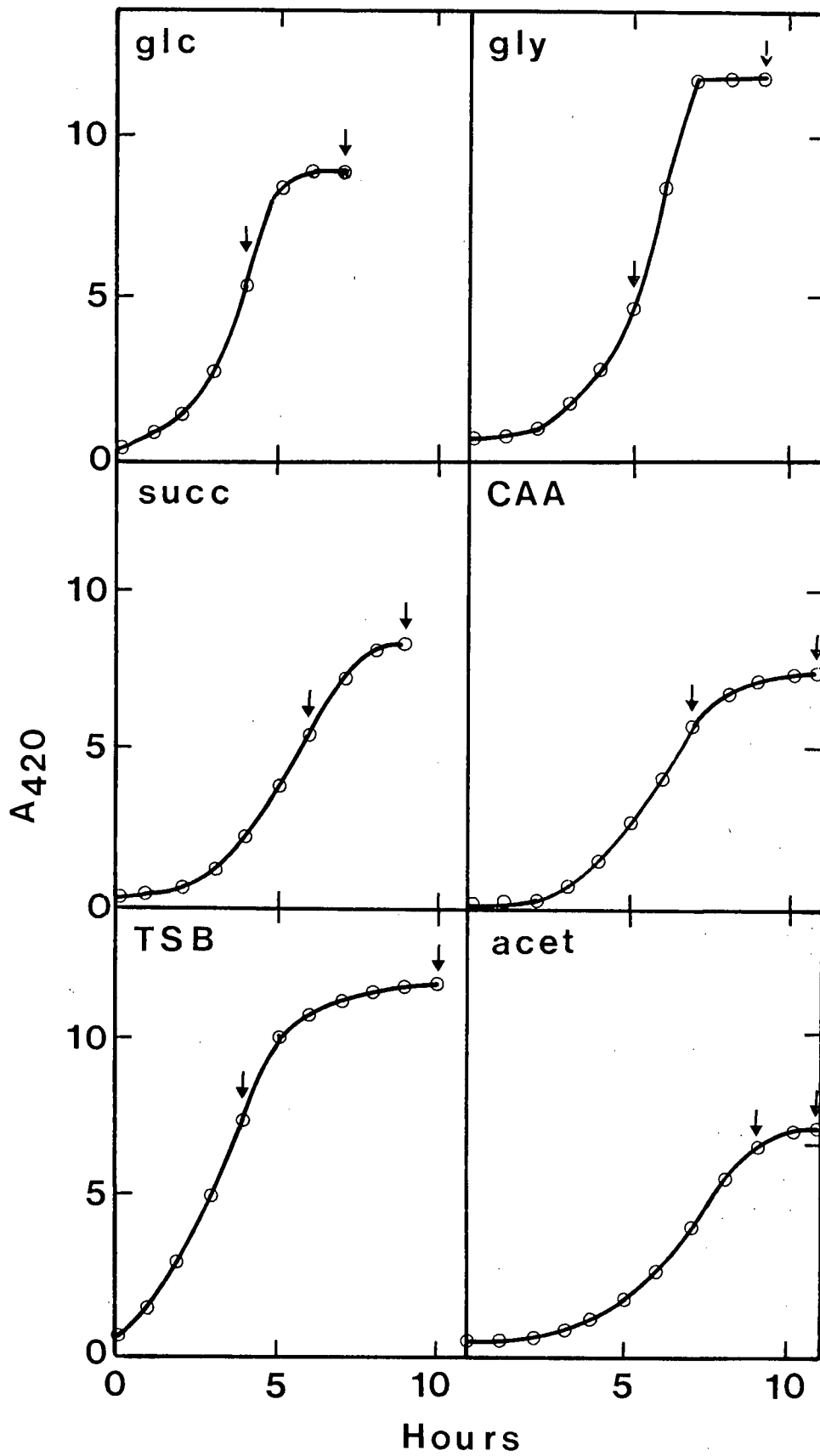
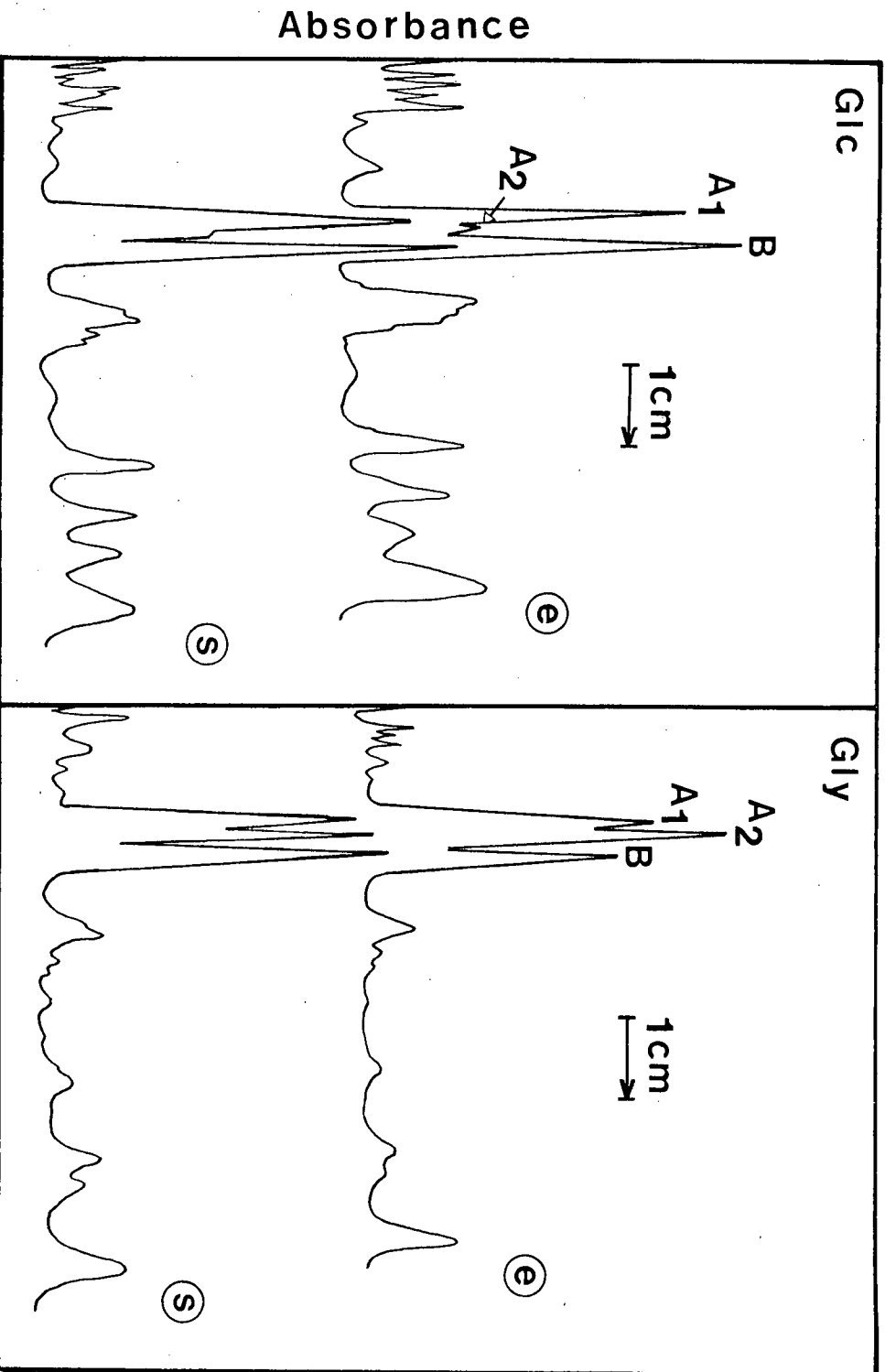
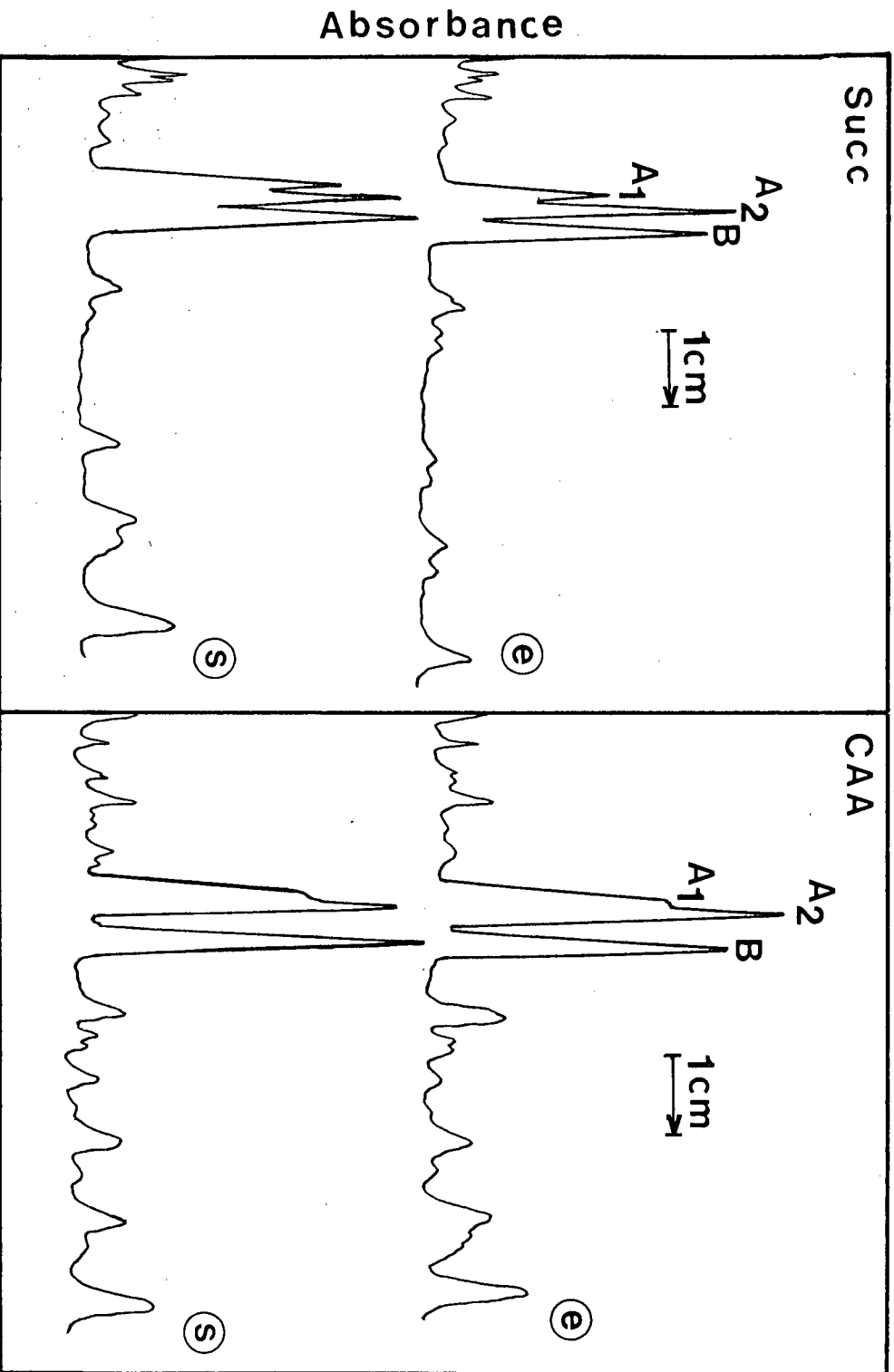


Fig. 31. Effect of growth conditions on outer membrane proteins. Scans of SDS-polyacrylamide gels of outer membrane proteins prepared from cells grown on glucose, Glc; glycerol, Gly; succinate, Succ; casein amino acid, CAA; trypticase soy broth, TSB and acetate, Acet, and harvested during the exponential (e) and stationary (s) phases of growth. Gels were stained with Coomassie Blue and scanned at 550 nm.





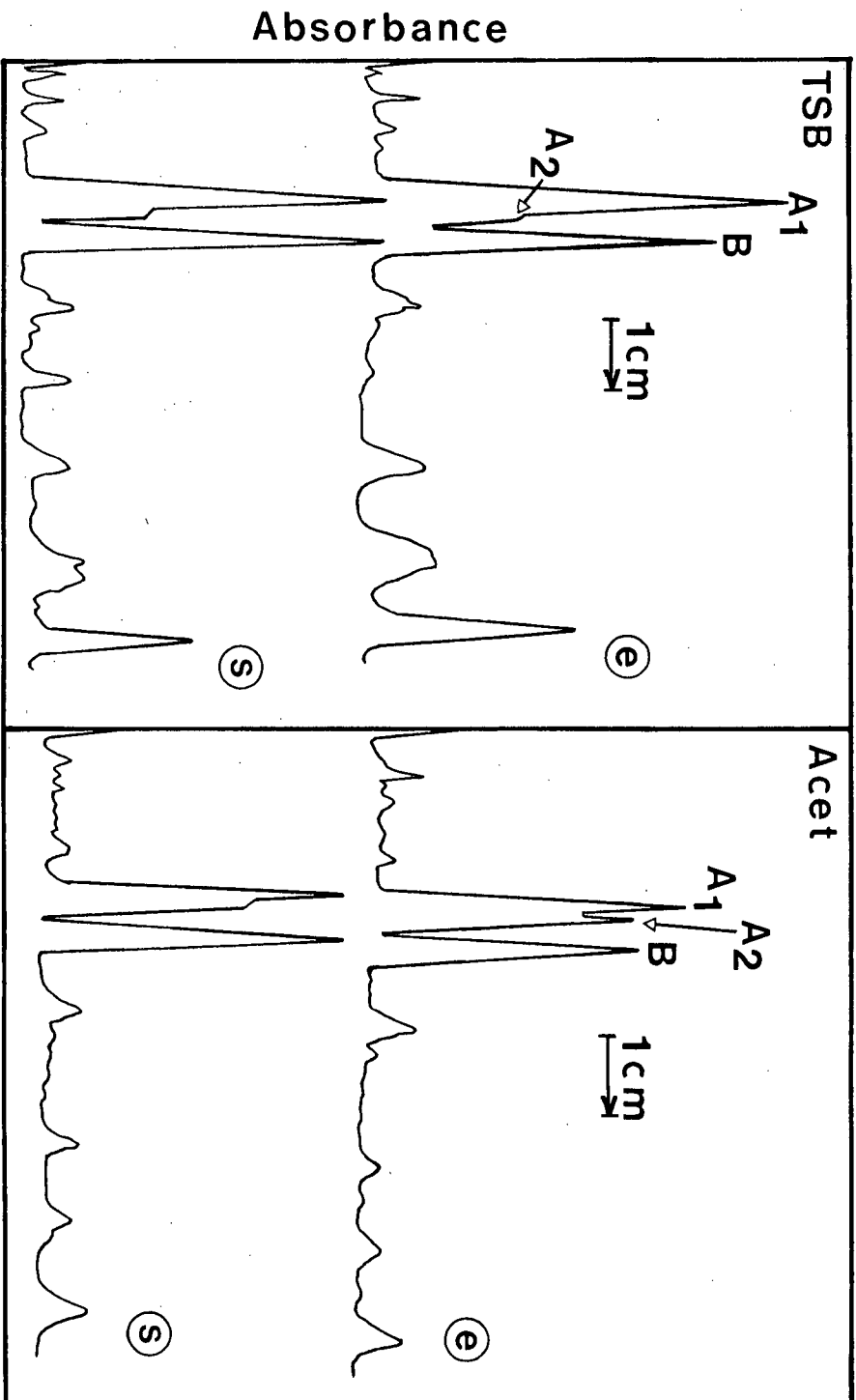
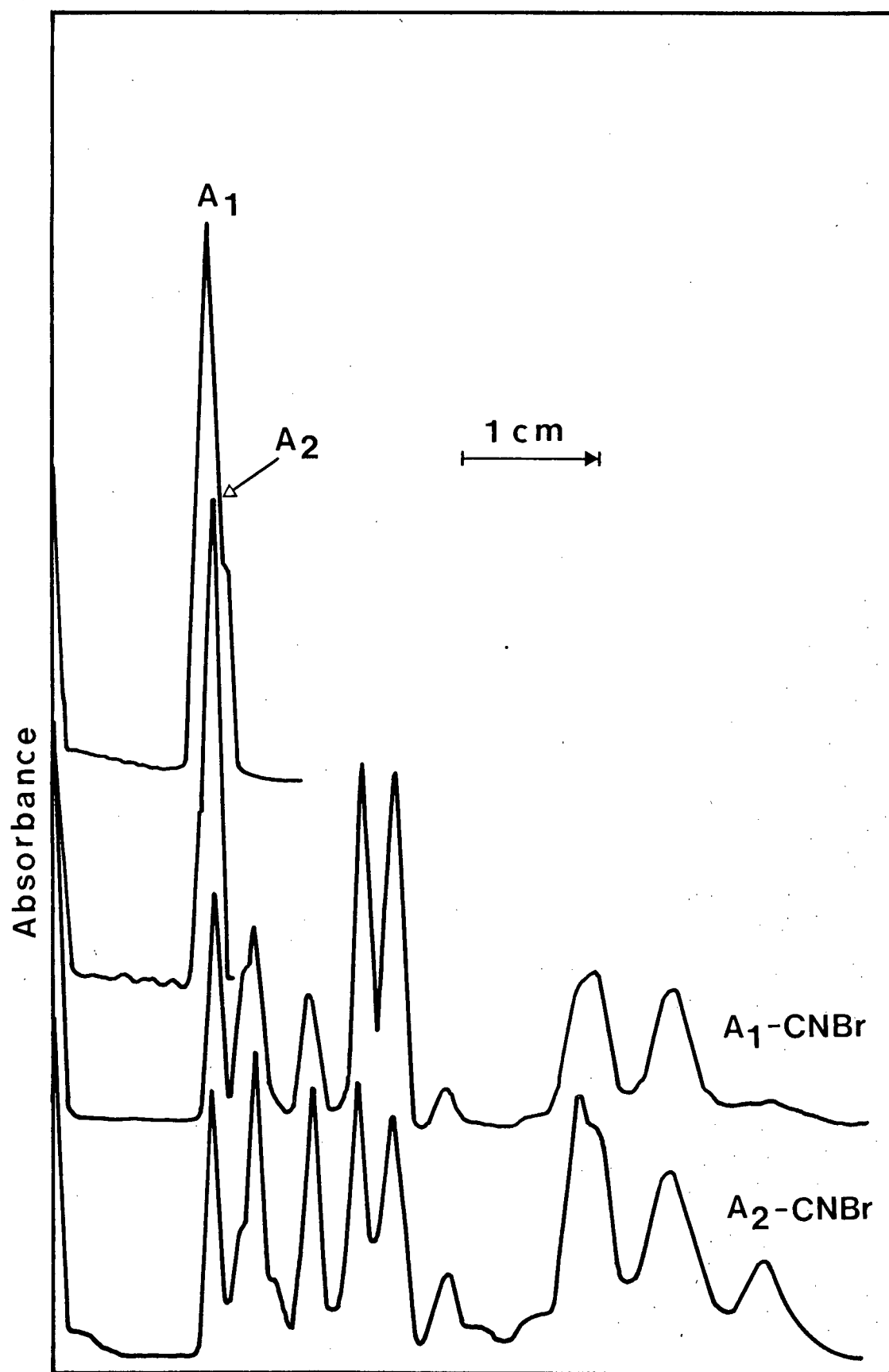


Fig. 32. Cyanogen bromide cleavage of proteins A₁ and A₂. The two lower gel scans show the separation of the cleavage products. Gel scans of the preparations of proteins A₁ and A₂ are given for reference. SDS-gels (12.5% acrylamide) were run with a phosphate buffer system at an alkaline pH, stained with Coomassie Blue, and scanned at 550 nm.



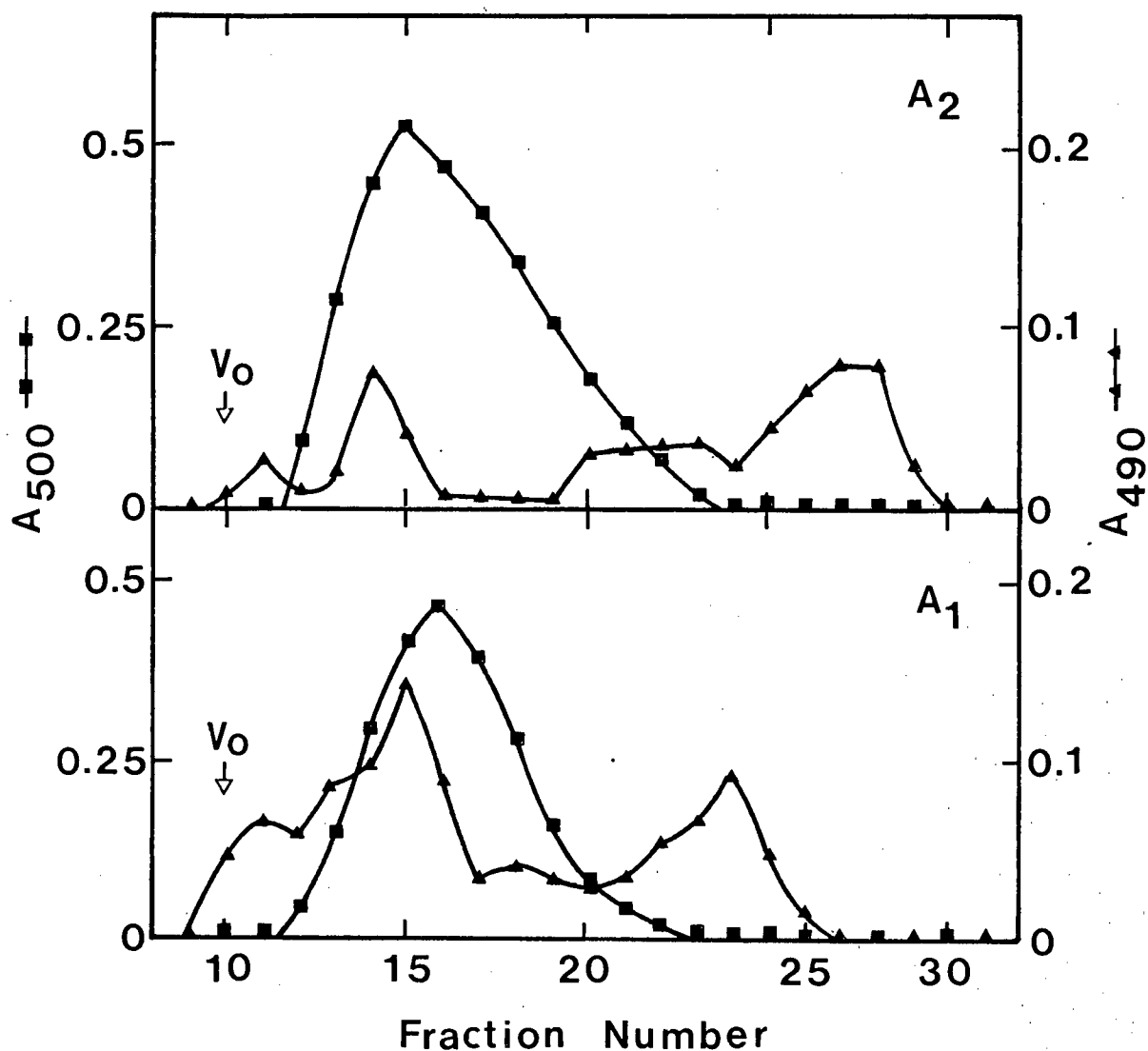


Fig. 33. Gel filtration chromatography of proteins A₁ and A₂ on a column of Bio Gel P150 (1 x 45 cm) equilibrated with 0.1M sodium phosphate buffer, pH 7.2, containing 1% SDS. Samples (1 ml) were dissolved to 3 mg protein/ml in column buffer containing 0.1% 2-mercaptoethanol and heated at 100°C for 5 min before applying to the column. Fractions (0.9 ml) from the column were collected and assayed for protein (■) and carbohydrate (▲). V_o, void volume.

A extract from casein amino acid-grown cells. The bulk of carbohydrate was found not to co-elute with the protein (Fig. 33) and is therefore likely a contaminant solubilized from the peptidoglycan.

Amino acid composition of proteins A₁ and A₂

The amino acid compositions of the protein A fractions from cells grown on glucose (mainly protein A₁) and on casein amino acids (mainly protein A₂) are shown in Table III. The compositions of proteins A₁ and A₂ were very similar which is in agreement with their similar disposition in the outer membrane. Like protein B, protein A was enriched in acidic amino acids, was only moderately hydrophobic, and contained little cysteine. Proteins A and B are clearly different polypeptides as indicated by the differences in the content of histidine, methionine, tyrosine, phenylalanine and proline residues.

Cyanogen bromide cleavage of protein A

Cyanogen bromide cleavage of preparations enriched in proteins A₁ and A₂ gave similar cleavage products as determined by SDS-polyacrylamide gel electrophoresis (Fig. 32, lower two scans). The migration positions of the peptides derived from proteins A₁ and A₂ were identical, however the amount of polypeptide in each peak was different. This was probably due to incomplete cleavage of the protein since the sum of the molecular weights of the cleavage products exceeded the molecular weight of protein A. Since the cyanogen bromide cleavage

patterns and the amino acid compositions of proteins A₁ and A₂ were similar, they may be two forms of the same polypeptide.

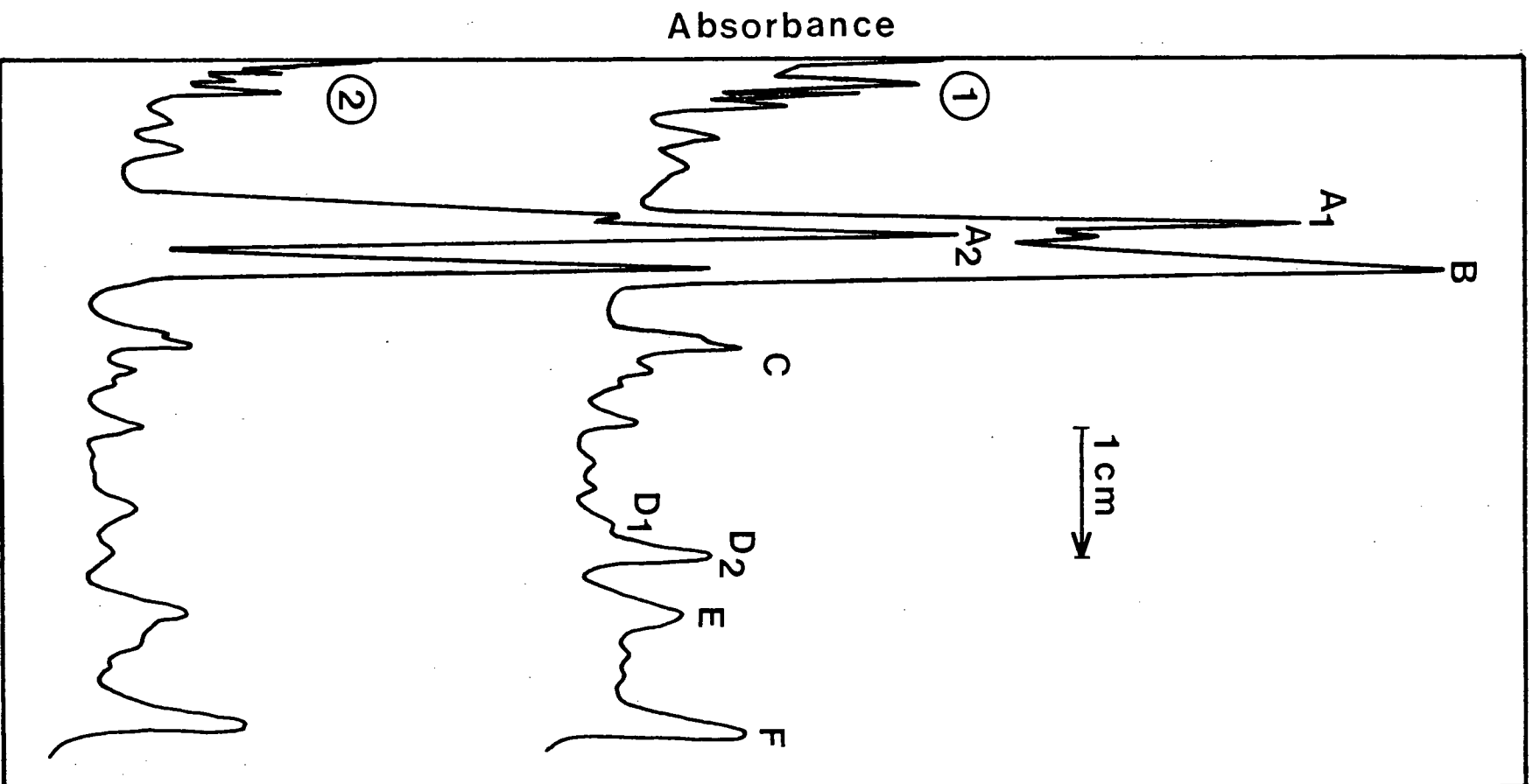
Organization of proteins in the outer membrane

The organization of the proteins in the outer membrane was examined by proteolytic digestion, covalent labelling and crosslinking techniques which have been successfully applied to the study of other membrane systems (234,235,296-298). The outer membrane of E. coli is a useful system for the study of membrane structure because of the relatively simple protein composition. In addition, the components of the outer membrane can be altered nutritionally or by mutation and the effect of the alteration on membrane structure can be studied.

Effect of proteolytic enzymes on the proteins of the outer membrane in intact cells

Treatment of intact cells with pronase, trypsin or chymotrypsin did not alter the protein profiles seen in Fig. 34 for cells grown on glucose or on casein amino acids. Since the carbohydrate chains of the lipopolysaccharide might be restricting the accessibility of the outer membrane proteins to the proteolytic enzymes, the cells were also pretreated with 5 mM EDTA for 30 min. This resulted in the loss of one-third of the lipopolysaccharide from the outer membrane (Fig. 35). This loss was not accompanied by the loss of any of the major proteins from the outer membrane, nor were the proteins any more susceptible to pronase digestion. Thus, the EDTA-

Fig. 34. Control scans of SDS-gels in studies on the effect of proteolytic digestion on the outer membrane proteins in cells. Coomassie Blue stained SDS-polyacrylamide gels of the outer membrane proteins prepared from cells grown on glucose (scan 1) or casein amino acids (scan 2) were scanned at 550 nm. The alkaline buffer system was used for electrophoresis.



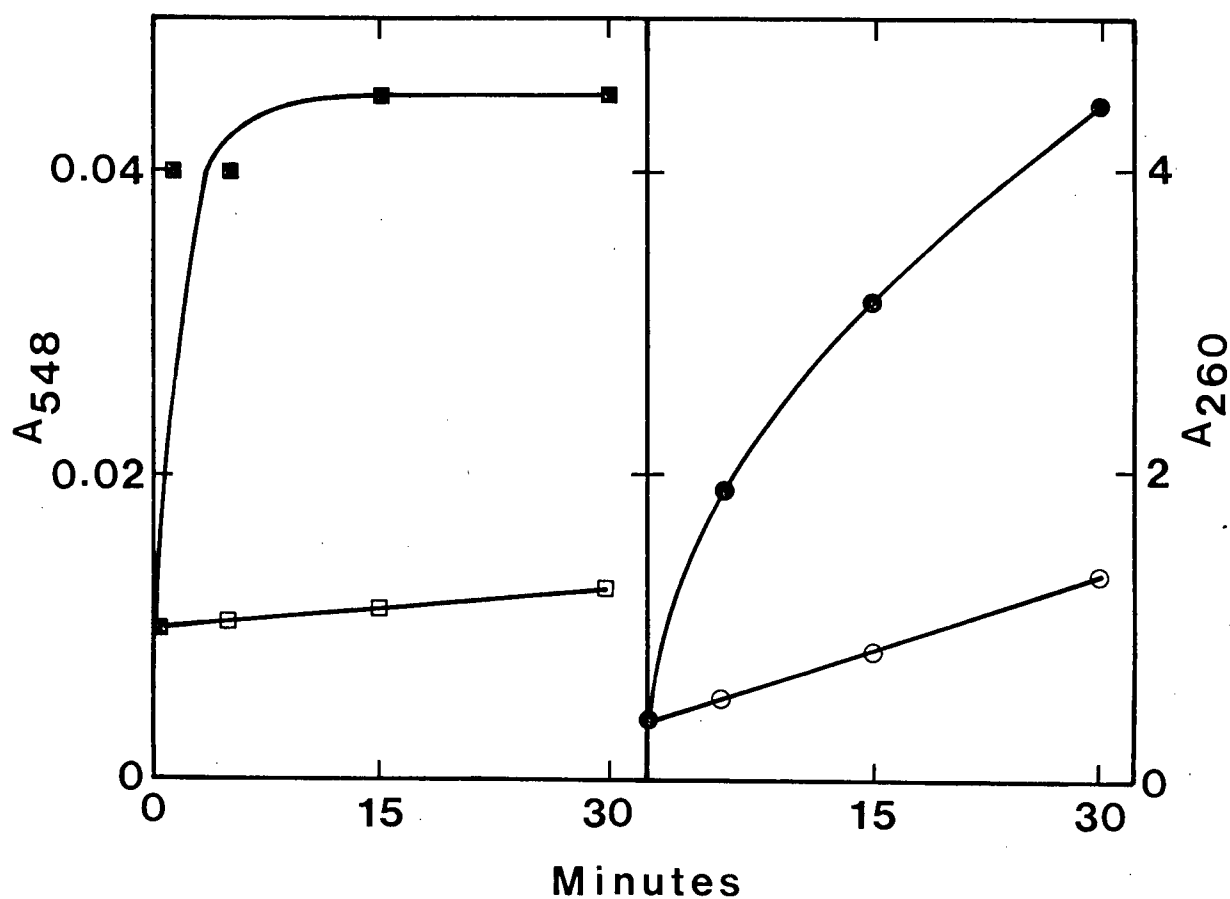


Fig. 35. Release of lipopolysaccharide from cells by EDTA. Cells were suspended (1g/8 ml) in 0.1M Tris-HCl buffer, pH 8.0, containing either 10 mM MgCl₂ (control, open symbols) or 5 mM EDTA (closed symbols) and incubated at 37°C for 30 min. Samples (1 ml) were removed, MgCl₂ was added (10 mM, final concentration) to the EDTA-treated samples and the cells were recovered by centrifugation at 10,000 x g for 5 min at 4°C. The absorbance at 260 nm and lipopolysaccharide content of the supernatant were determined. Cells for pronase digestion were resuspended (1 g/10 ml) in 0.1 M Tris-HCl buffer, pH 8.0, containing 35 mM MgCl₂.

releasable fraction of the lipopolysaccharide cannot be responsible for the resistance of the outer membrane proteins in intact cells to digestion by pronase.

The possible protection of the outer membrane proteins by the non-releasable fraction of the lipopolysaccharide was examined in a heptose-deficient mutant of E. coli. Strain NS-1 lacks the core region of the lipopolysaccharide and, as previously shown (179), contains drastically reduced amounts of proteins A and B (Fig. 36, sample 2) when compared to the parent strain, JE 1011 (sample 1). Treatment of intact cells of both the parent and the mutant with pronase resulted in the loss of only a few higher molecular weight proteins from the outer membrane (samples 3 and 4). When isolated cell walls, consisting of outer membrane and peptidoglycan, prepared from these strains were treated with pronase there was a marked change in the protein profile of the outer membrane (samples 5 and 6). Protein A was resistant to digestion but protein B was cleaved to a pronase-resistant fragment, protein Bp, with a molecular weight of about 20,000. The smaller amounts of these proteins in the mutant behaved similarly. The loss of proteins A and B, and the modified lipopolysaccharide structure, do not alter the arrangement of the outer proteins in the outer membrane.

Effect of proteolytic enzymes on the proteins in isolated membrane preparations

In contrast to the results with whole cells, protease treatment of envelope preparations resulted in extensive diges-

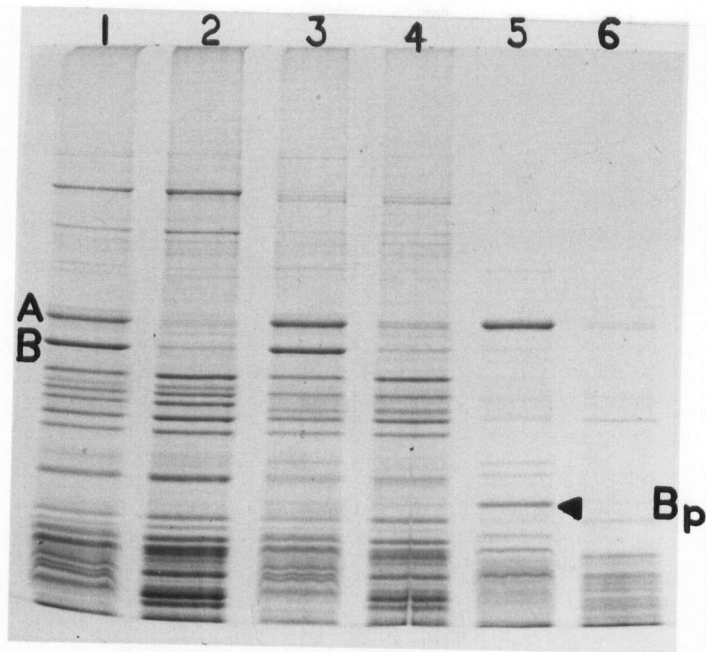


Fig. 36. Effect of alteration in lipopolysaccharide structure on the digestion of outer membrane proteins by pronase. Outer membrane proteins were resolved by sodium dodecyl sulfate gel electrophoresis on 0.75 mm thick 12% polyacrylamide slabs. 1, 2, outer membrane proteins from untreated cells of JE 1011 and heptose-deficient mutant NS01, respectively; 3, 4, outer membrane proteins from pronase-treated cells of JE 1011 and NS-1, respectively; 5, 6, outer membrane proteins from pronase-treated cell walls of JE 1011 and NS-1, respectively.

tion of certain outer membrane proteins. The envelope preparation following proteolytic digestion was extracted with Triton X-100 in the presence of Mg^{2+} in order to remove proteins of the inner membrane. The outer membrane proteins were then solubilized from the extracted preparation with 1% SDS at $100^{\circ}C$ for examination by polyacrylamide gel electrophoresis. The results were confirmed using isolated outer membranes prepared from spheroplasts by the method of Mizushima and Yamada (77) in order to eliminate the possibility that Triton X-100 had removed proteins or digestion products from the outer membrane.

The kinetics of digestion by pronase of the outer membrane proteins in envelopes prepared from glucose-grown cells are shown in Fig. 37. The rates of digestion of the different proteins varied greatly. Both proteins A_1 and A_2 were resistant to digestion. In cells grown on casein amino acids in which protein A_2 was predominant, the same resistance to digestion was observed. The loss of protein B occurred with the concomittant production of a pronase resistant fragment, protein Bp. This suggests that protein B is partially exposed from the membrane. The fragment must be protected by its arrangement in the membrane. Proteins of the C group which were readily degraded are probably at the surface of the outer membrane. The plateau seen for protein F, the free form of the lipoprotein may indicate that there are two populations of this protein in the membrane. The kinetics of digestion of the outer membrane proteins by trypsin (Fig. 38) and chymotrypsin (Fig. 39) were

Fig. 37. Kinetics of digestion of outer membrane proteins by pronase. Cell envelope was incubated at 37°C with pronase at an enzyme:protein ratio of 1:25. Samples were removed at intervals and the outer membrane proteins were prepared. The proteins were resolved by SDS-gel electrophoresis and quantitated from absorbance scans of the stained gels by weighing. The proteins were identified as in Fig. 32. Protein Bp is the pronase-resistant fragment derived from protein B.

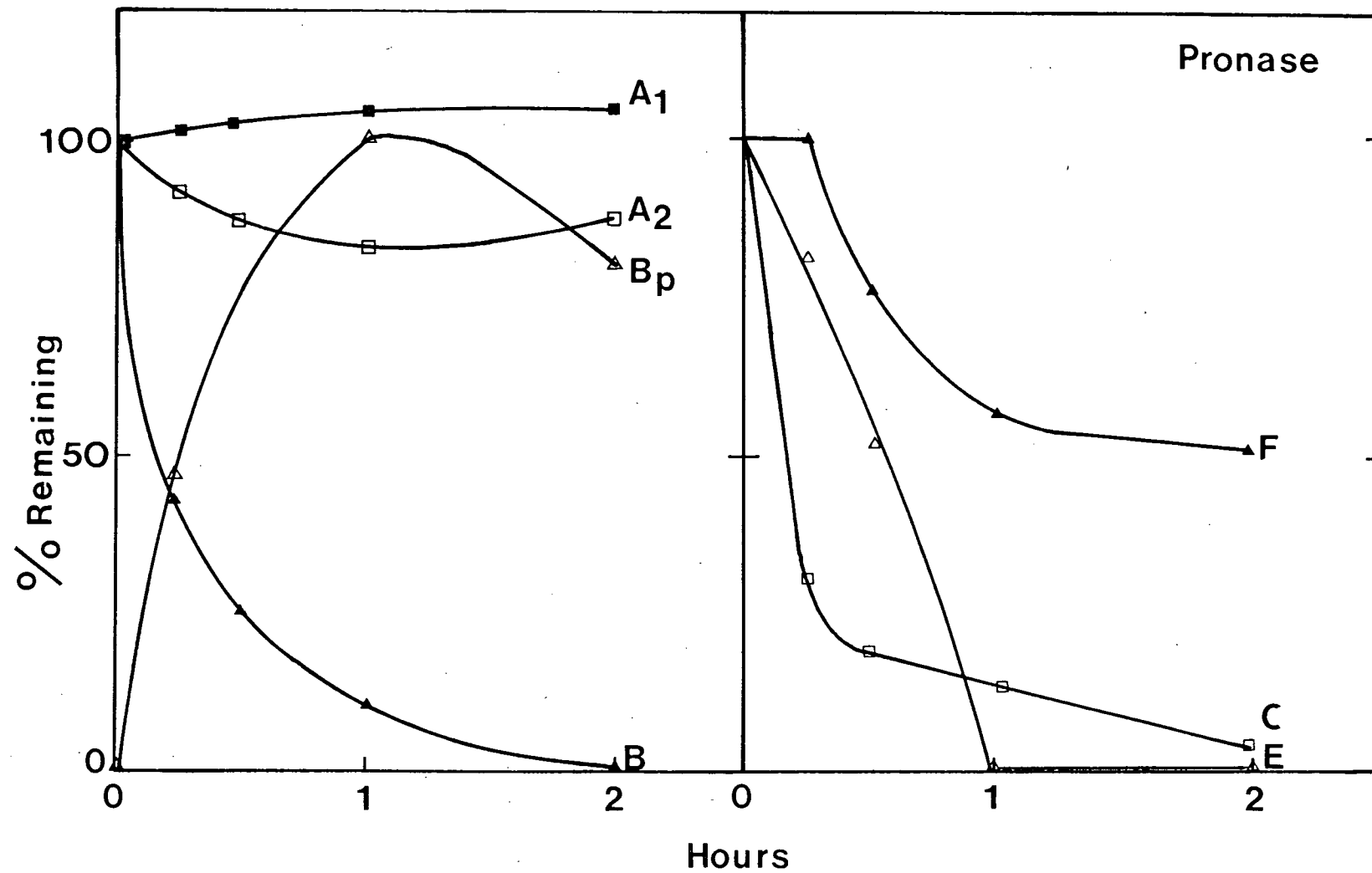


Fig. 38. Kinetics of digestion of outer membrane proteins by trypsin. Cell envelope was incubated at 37°C with trypsin at an enzyme:protein ratio of 1:25. Samples were removed at intervals and the outer membrane proteins were prepared. The proteins were resolved by SDS-gel electrophoresis and quantitated from absorbance scans of the stained gels by weighing. The proteins were identified as in Fig. 32. Protein B_t is the trypsin-resistant fragment derived from protein B.

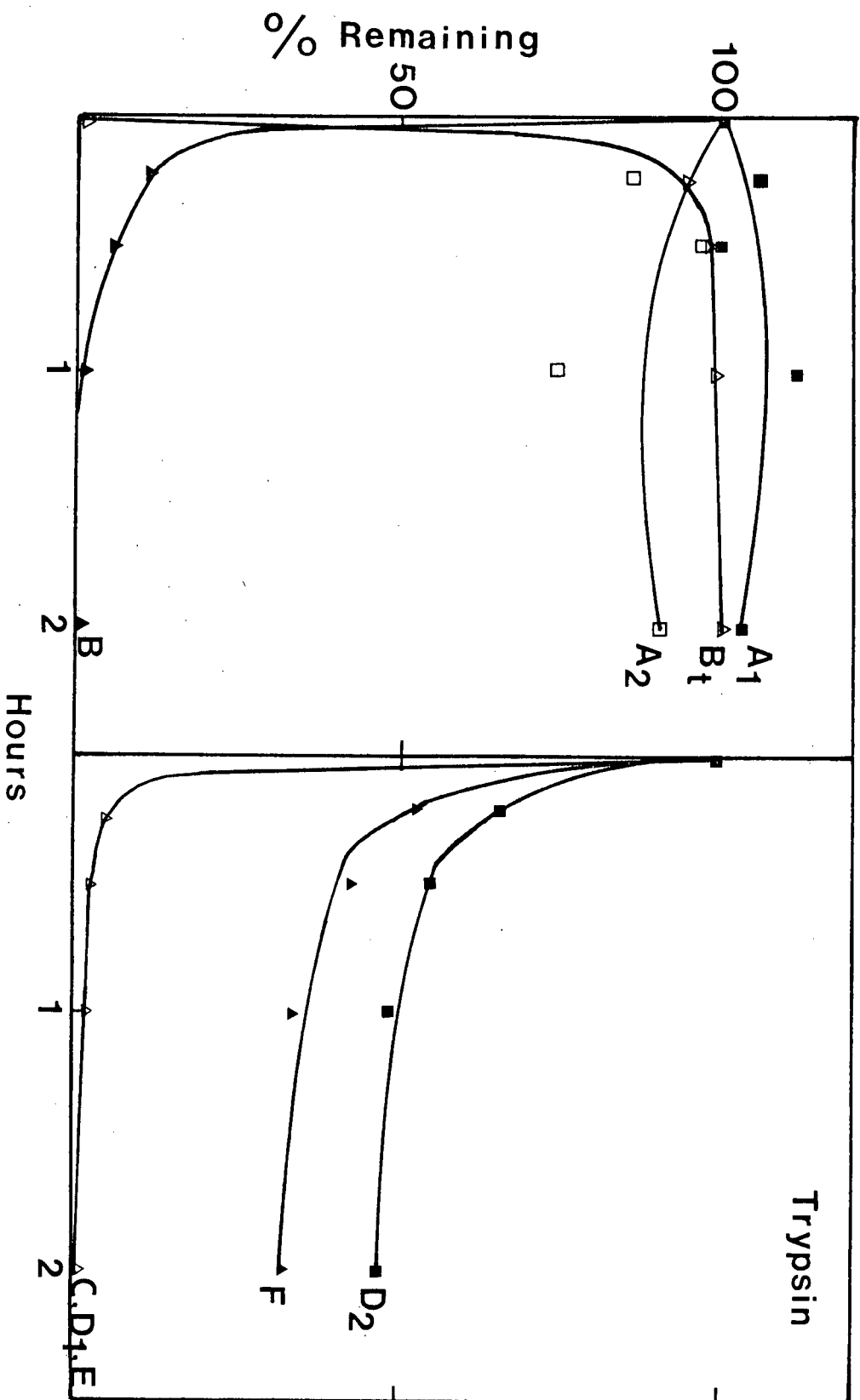
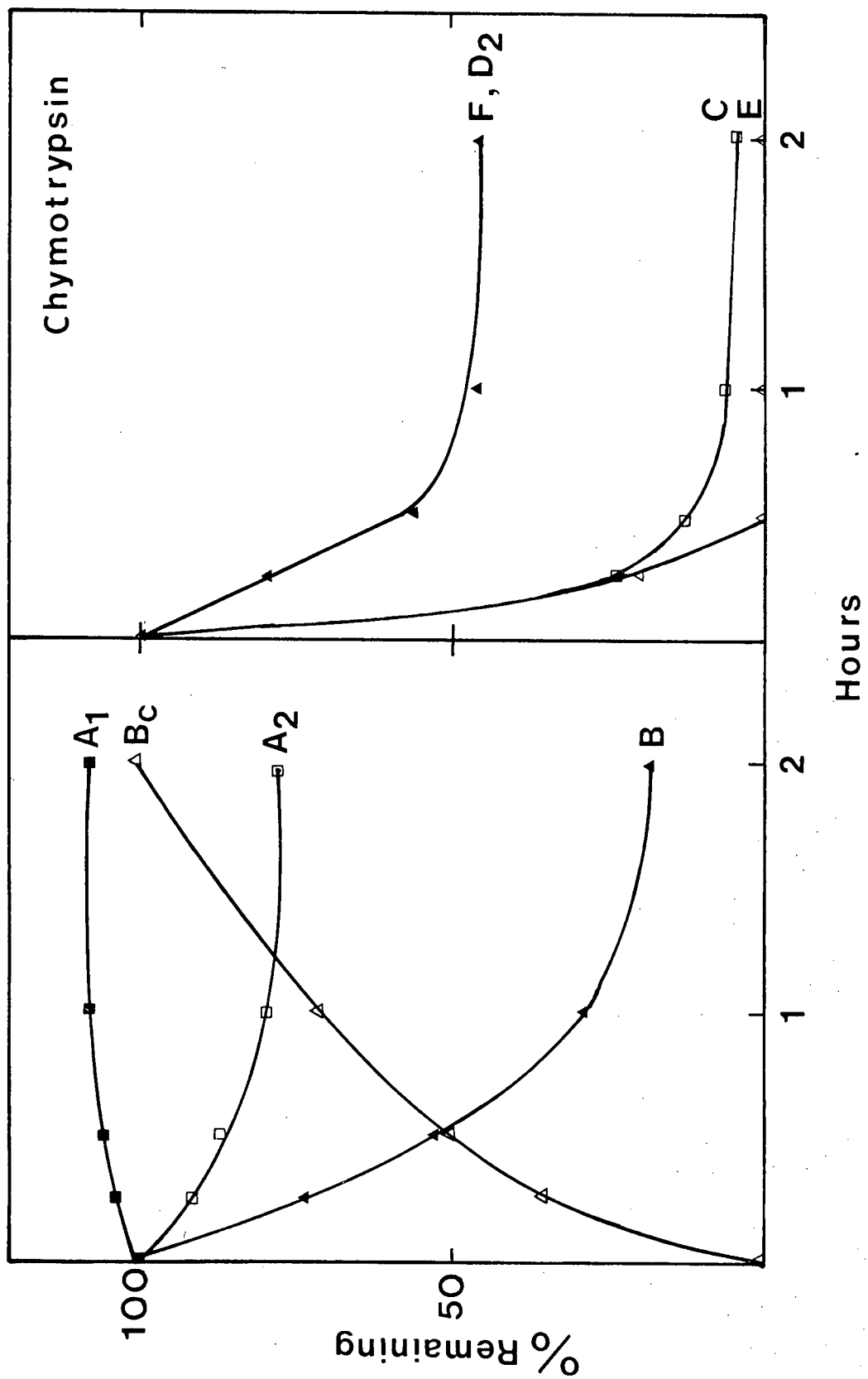


Fig. 39. Kinetics of digestion of outer membrane proteins by chymotrypsin. Cell envelope was incubated at 37°C with chymotrypsin at an enzyme: protein ratio of 1:25. Samples were removed at intervals and the outer membrane proteins were resolved by SDS-gel electrophoresis and quantitated from absorbance scans of the stained gels by weighing. The proteins were identified as in Fig. 32. Protein B_c is the chymotrypsin-resistant fragment derived from protein B.



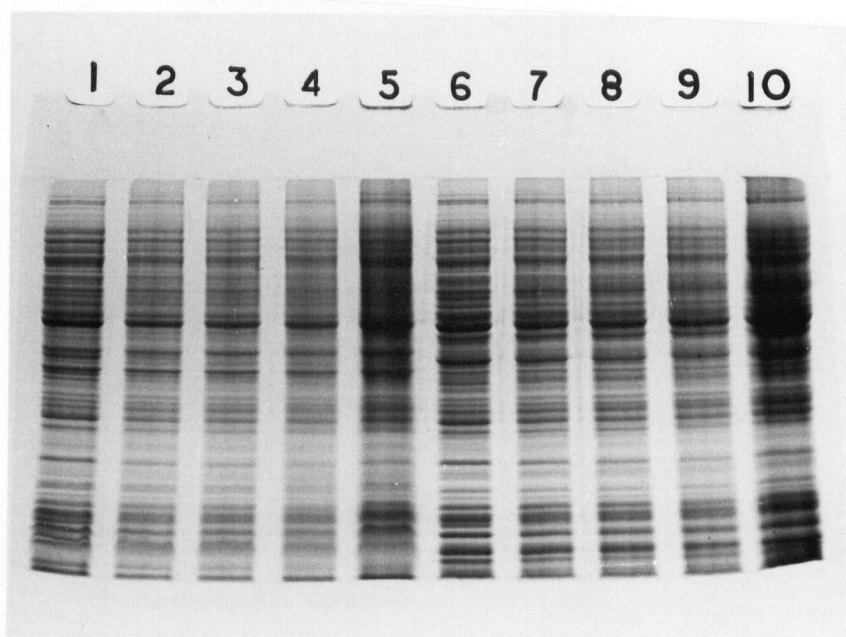
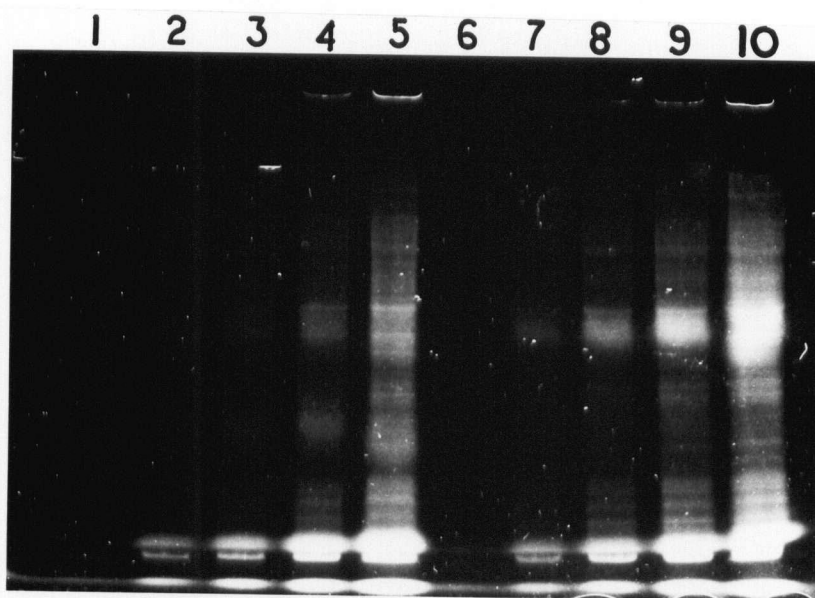
similar to those obtained with pronase. However, the fragments derived from protein B by trypsin and chymotrypsin treatment both had a molecular weight of 25,000. In addition, the kinetics of digestion of protein D₂ suggests that this protein may not be accessible at the surface of the membrane.

Labelling of outer membrane proteins with fluorescamine

The exposure of proteins at the surface of membranes can also be determined by covalent labelling with non-penetrating reagents (235). Fluorescamine reacts rapidly with amino groups to give a fluorescent label on protein molecules (299). It has been used to label the surface proteins of BHK cells (300) where its high reactivity results in the labelling only of exposed proteins. We have confirmed that cytoplasmic proteins are not labelled when intact cells of E. coli are treated with fluorescamine. Labelling of cell envelope components in both E. coli JE 1011 and its heptose-deficient mutant NS-1 were found after treating intact cells with fluorescamine (Fig. 40).

Isolated outer membrane or cell wall (outer membrane-peptidoglycan) preparations were also treated with fluorescamine. The proteins were then extracted with SDS and examined by polyacrylamide gel electrophoresis. As shown in Fig. 41, although all of the outer membrane proteins could be labelled by this reagent, the extent of reaction varied. The bands migrating around the dye front which are not associated with a particular protein were not identified, but one of these

Fig. 40. Slab gel electrophoresis of fluorescamine-labelled intact cells of E. coli JE 1011 (Samples 1-5) and its heptose-deficient mutant NS-1 (Samples 6-10). Cells were suspended (1:1g/10 ml) in 0.2M triethanolamine buffer, pH 8.5 and treated with various levels of fluorescamine at 22°C. After 15 sec the samples (100 μ l) were solubilized by the addition of 2 volumes of SDS-electrophoresis buffer followed by heating at 100°C for 3 min. Samples (10 μ l) 1 and 6, control; 2 and 7, 100 μ g fluorescamine; 3 and 8, 250 μ g fluorescamine; 4 and 9, 500 μ g fluorescamine. Samples (25 μ l) 5 and 10; 500 μ g fluorescamine. The slab gel (12% acrylamide) was photographed under ultraviolet light (upper photograph) and then stained for protein with Coomassie Blue (lower photograph).



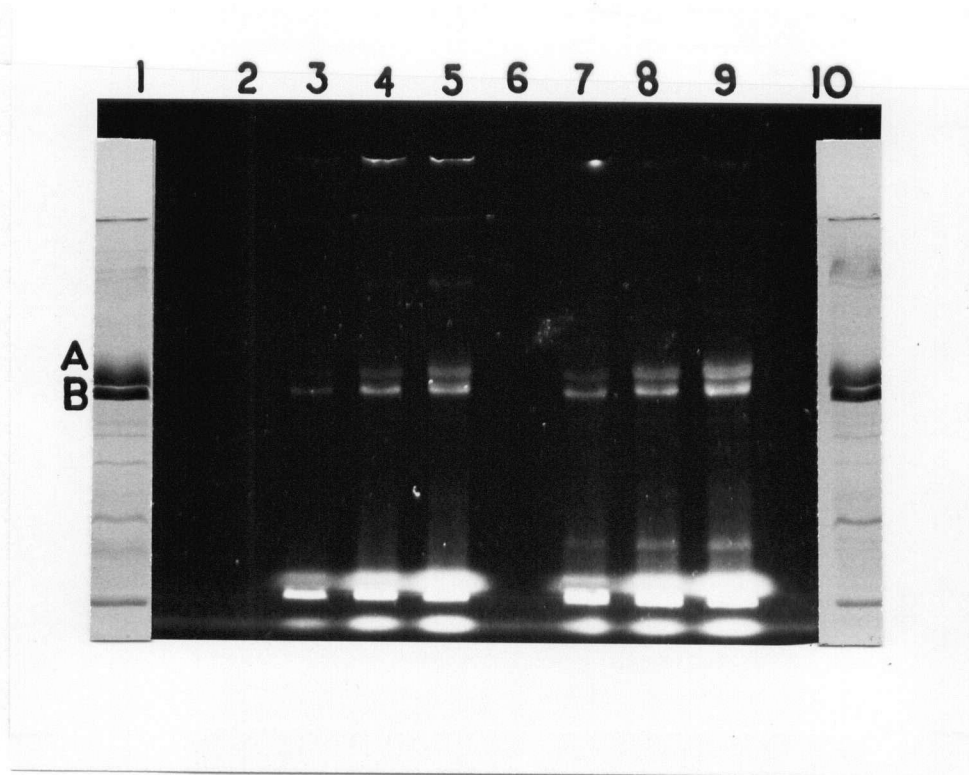


Fig. 41. Slab gel electrophoresis of fluorescamine-labelled outer membrane proteins. Samples ($100\mu\text{l}$) of cell wall (samples 1-5) and outer membrane (samples 6-10) preparations containing about 1 mg/ml of protein in 0.2 M triethanolamine buffer, pH 8.5, were treated with various levels of fluorescamine at 22°C . After 15 sec the samples were solubilized by the addition of 2 volumes of 1% SDS in the electrophoresis sample buffer and heated at 100°C for 3 min. Samples 2 and 6, control; 3 and 7, $50\mu\text{g}$ fluorescamine; 4 and 8, $100\mu\text{g}$ fluorescamine; 5 and 9, $500\mu\text{g}$ fluorescamine. Samples 1 and 10 are control samples stained with Coomassie Blue. Samples 2-9 were photographed under ultraviolet light. The concentration of polyacrylamide gel was 12%.

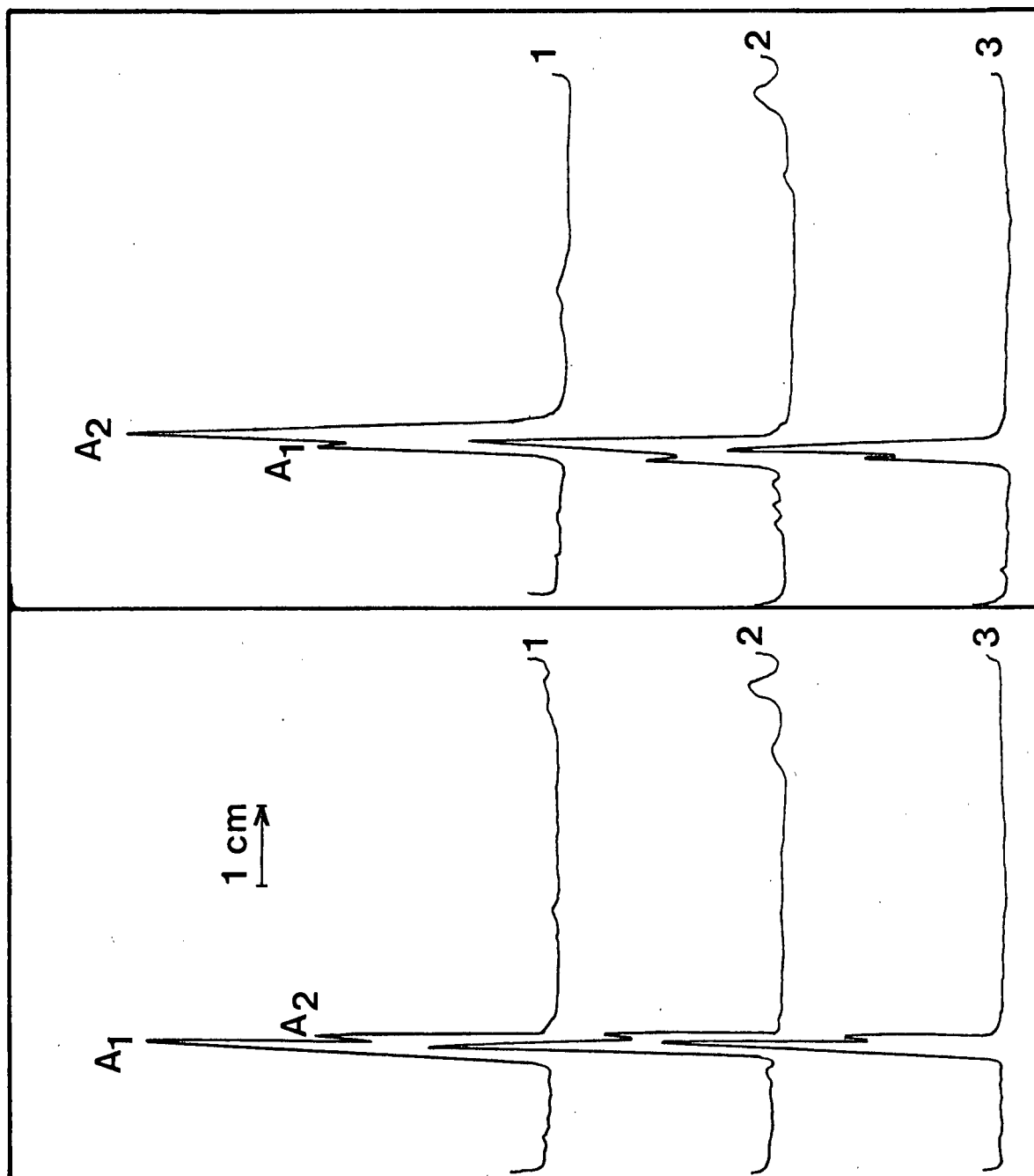
products is probably labelled phosphatidylethanolamine. Protein B reacted more readily with the reagent than protein A although both proteins were present in about equal amounts in the membrane. This result confirms that obtained by pronase which suggests that protein B is more exposed at the membrane surface than protein A.

Association of oligomers of protein A with the peptidoglycan

Protein A is tightly associated with the peptidoglycan. In cells grown on glucose both proteins A_1 and A_2 were associated with the peptidoglycan, protein A_1 being the predominant protein (Fig. 42, scan 1, left). In cells grown on casein amino acids, where protein A_2 predominated, again both proteins A_1 and A_2 were associated with the peptidoglycan (Fig. 42, scan 1, right).

Digestion of these protein A-peptidoglycan complexes, with pronase did not result in any loss of proteins A_1 or A_2 as similar protein profiles to those seen in Fig. 42, scans 1, were obtained. Intact peptidoglycan was not responsible for the resistance of these proteins to digestion since preincubation of the complex with lysozyme (Fig. 42, scans 2) prior to the addition of pronase did not result in digestion (Fig. 42, scans 3). Since this resistance to digestion was lost on extraction of the proteins into SDS, it appeared that interaction between different molecules of protein A might account for this phenomenon. Therefore, an attempt was made to isolate oligomers of protein A.

Fig. 42. Resistance of proteins A₁ and A₂ to pronase digestion. Protein A - peptidoglycan complexes were prepared from cells grown on glucose (left panel) or casein amino acids (right panel). The proteins were extracted from the complexes with 1% SDS at 100°C after the following treatments: 1, none; 2, complexes with lysozyme (40 μ g/mg protein); 3, complexes pretreated with lysozyme and then with pronase (1 mg/25 mg protein A). The proteins were resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The stained gels were scanned at 550 nm.



Extraction of the protein A-peptidoglycan complex with chaotropic agents such as 6 M urea, did not solubilize any protein at 37°C, although at 100°C protein A was completely removed from the peptidoglycan (Fig. 43). The protein in these extracts was present as a monomer as shown by gel filtration (Fig. 44) and electrophoresis on polyacrylamide gels in the presence of 6 M urea (Fig. 44). Similar results were obtained with 4 M guanidinium hydrochloride or 4 M guanidinium thiocyanate.

Pretreatment of the protein A-peptidoglycan complex with lysozyme at room temperature, followed by the addition of urea to a concentration of 6 M, produced a complex of protein, possibly still associated with fragments of peptidoglycan, which was excluded from a column of Sepharose 6B equilibrated with 50 mM Tris-acetate buffer, pH 7.5, containing 6 M urea (Fig. 45). The complex yielded monomers of protein A when heated at 100°C for 5 min.

Isolated outer membrane prepared from spheroplasts (77) was completely solubilized by 1% SDS at room temperature although it was little affected by 6 M urea. Protein A existed as oligomers in the detergent extract as shown by gel filtration in the presence of sodium dodecyl sulfate (Fig. 46, lower profile). Protein A was eluted in fractions 49-51 as judged by SDS-polyacrylamide gel electrophoresis of heated samples from each column fraction. This elution position corresponds to a molecular weight of 60,000. However, this value is probably lower than the true molecular weight of the oligomer since

Fig. 43. SDS-polyacrylamide gel scans of protein A extracted from protein A-peptidoglycan complexes by 1% SDS at 100°C for 15 min (scan 1), 6 M urea at 100°C for 15 min (scan 2), 6 M urea at 37°C for 1 h (scan 3). Peptidoglycan was removed by centrifugation at 100,000 x g for 1 h at 15°C. All samples for electrophoresis were made 1% in SDS and 6 M in urea and heated at 100°C for 5 min. Gels (10% acrylamide) were run in a phosphate buffer system at neutral pH, stained with Coomassie Blue and scanned at 550 nm.

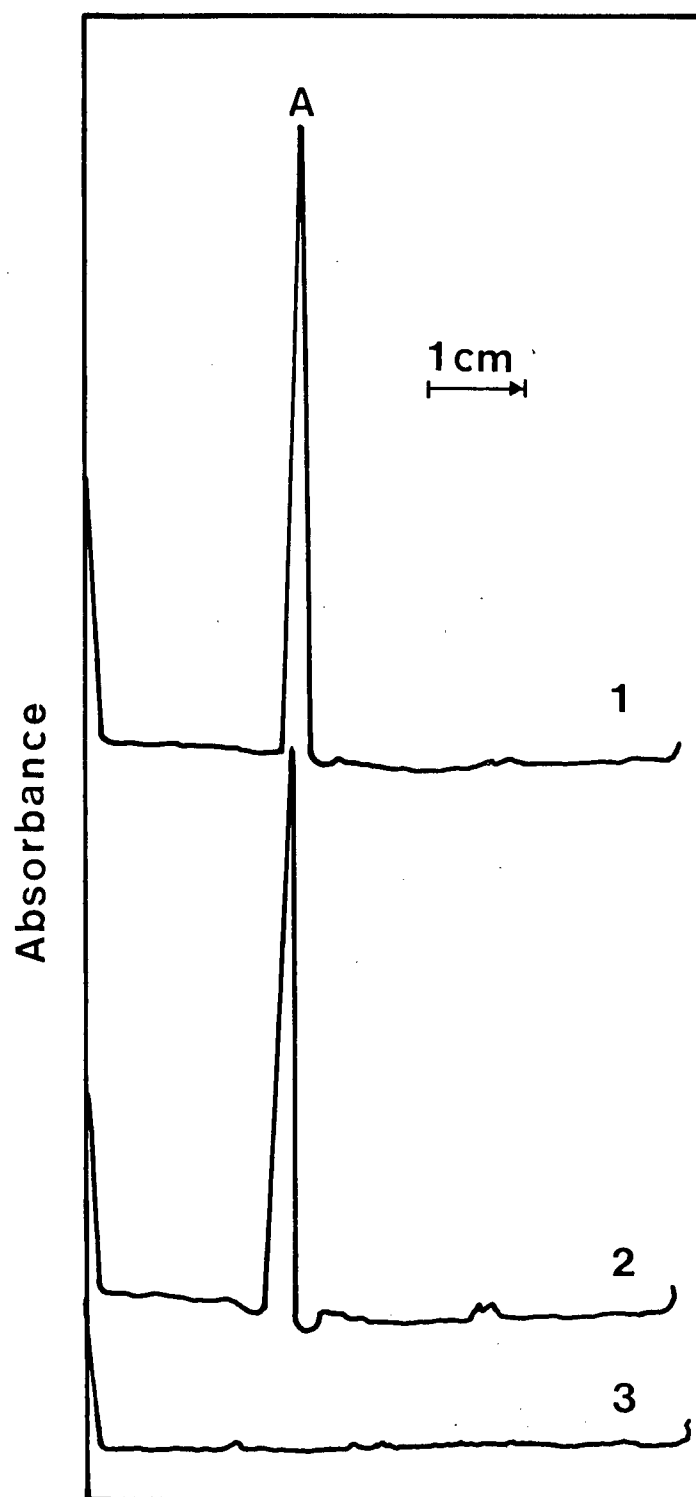


Fig. 44. Extraction of protein A-peptidoglycan complexes, prepared from glucose-grown cells, with 6 M urea in 50 mM Tris-acetate buffer, pH 7.5 at 100°C for 15 min. Peptidoglycan was removed by centrifugation at 120,000 x g for 1 h at 15°C. Panel 1, scan of a 5% polyacrylamide gel run in the presence of 6 M urea in a Tris buffer system. The gel was stained with Coomassie Blue and scanned at 550 nm. The direction of migration was towards the anode. Panel 2 shows the elution profile of protein A from a column (1 x 37 cm) of Sepharose 6B, equilibrated with 50 mM Tris-acetate, pH 7.5, containing 6 M urea. The sample (1 ml) in the column buffer (1 mg protein/ml) was applied to the column and fractions (0.9 ml) were collected and the protein content determined (■). The elution positions of Blue Dextran (V_0), bovine serum albumin (BSA) and ovalbumin (Oval) are indicated.

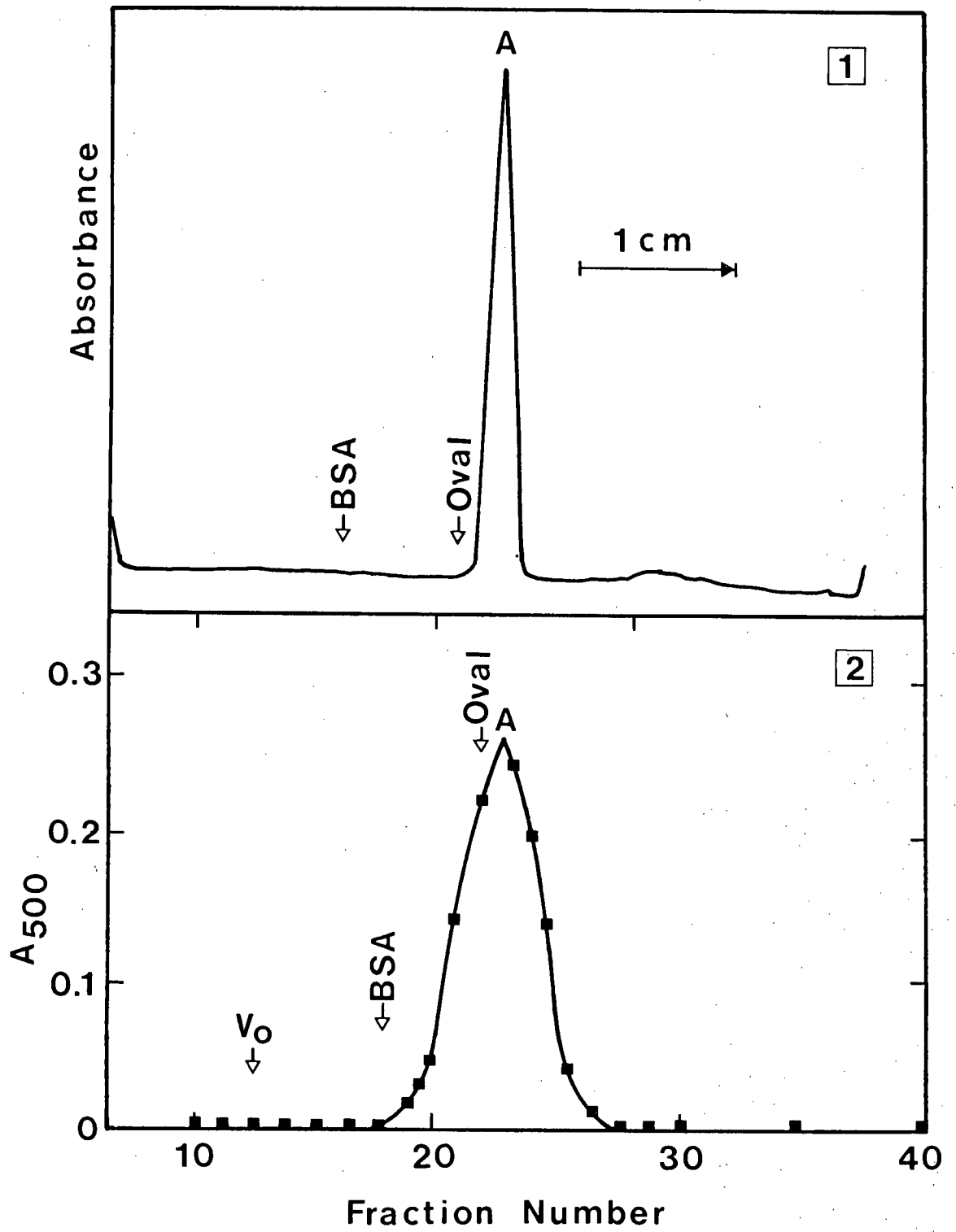


Fig. 45. Gel filtration of protein A oligomers. Protein A-peptidoglycan complexes derived from glucose-grown (A_1) and casein amino acid-grown (A_2) cells were digested with lysozyme (10 μ g/mg protein) for 15 h at 37°C in 50 mM Tris-acetate buffer, pH 7.5. Urea was added to a concentration of 6M and the sample (1 ml, containing ca. 1 mg of protein) was applied to a column (1 x 37 cm) of Sepharose 6B, equilibrated with Tris-GM urea buffer. Fractions (0.9 ml) were collected and the protein content determined. Absorbance was measured at 500 nm. Open symbols, sample incubated at 37°C for 1 h prior to application to column; closed symbols, sample heated at 100°C for 15 min prior to application to column.

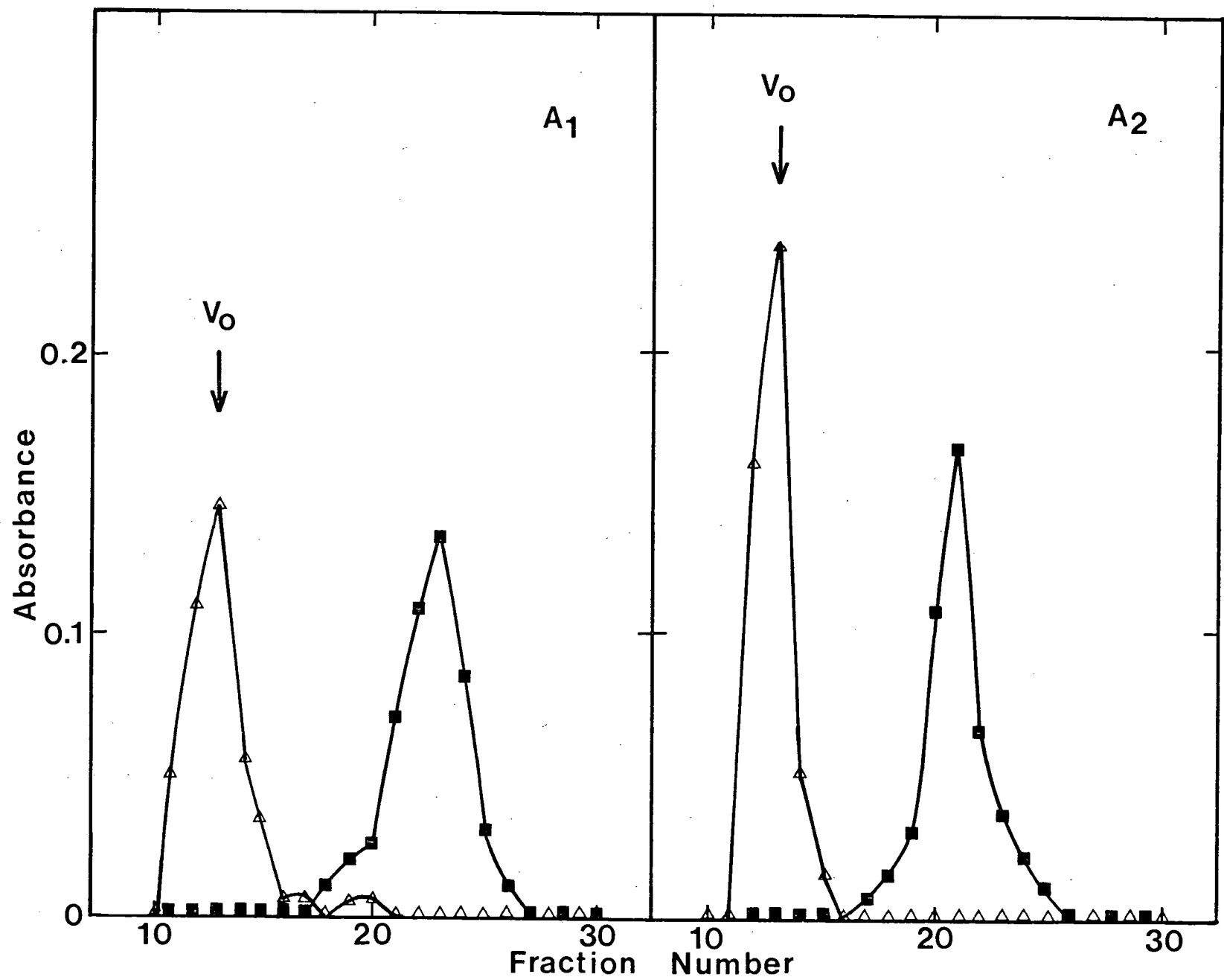
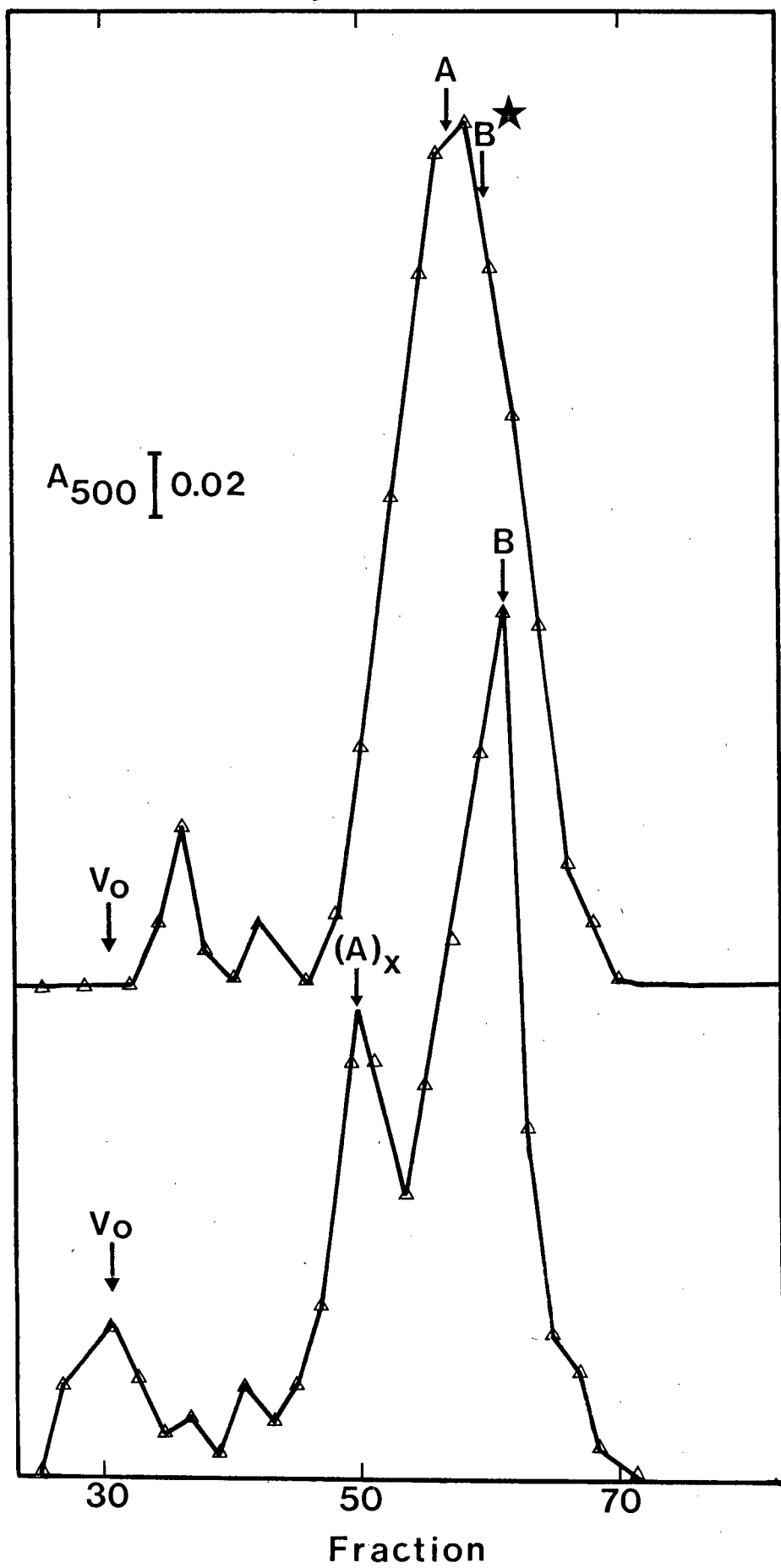


Fig. 46. Gel filtration of proteins solubilized from isolated outer membrane by SDS at 22°C (lower profile) and at 100°C (upper profile). The proteins were resolved on a column of Sepharose 6B (1 x 37 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2. Fractions (0.9 ml) were collected and the protein content was determined by the method of Lowry et al. (171). A, (A)x, B and B* indicate the elution positions of protein A oligomer, protein B, and heat-modified protein B, as determined by analysis of the fractions by SDS-polyacrylamide gel electrophoresis.



protein A does not readily bind sodium dodecyl sulfate (80) and so would have a smaller Stokes radius compared to the molecular weight markers which would bind up to 1.4 g detergent/g protein (152,290). Moreover, the molecular weight markers would be retarded to some extent on the column due to the asymmetry of their SDS complexes (152). Protein B migrated as the non-heat modified form (fractions 60-62) with a molecular weight of 25,000. When the extract was heated at 100°C prior to chromatography protein B was converted to the heat-modified form (protein B*) and eluted at a position corresponding to a molecular weight of 33,400 (Fig. 43, upper curve). Oligomers of protein A were disaggregated into the monomer and eluted in fractions 56 and 57 corresponding to a molecular weight of 43,000. The molecular weight determined by SDS-polyacrylamide gel electrophoresis was 37,000.

Protein A, as monomeric subunits in SDS or urea was completely sensitive to digestion by pronase. Removal of the denaturant by dialysis against 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM MgCl₂, in the presence or absence of peptidoglycan, did not restore resistance to digestion. Thus, the conditions required for dissociation of the protein A-peptidoglycan complex must irreversibly denature the protein.

Reassociation of monomeric protein B and the formation of protein Bp

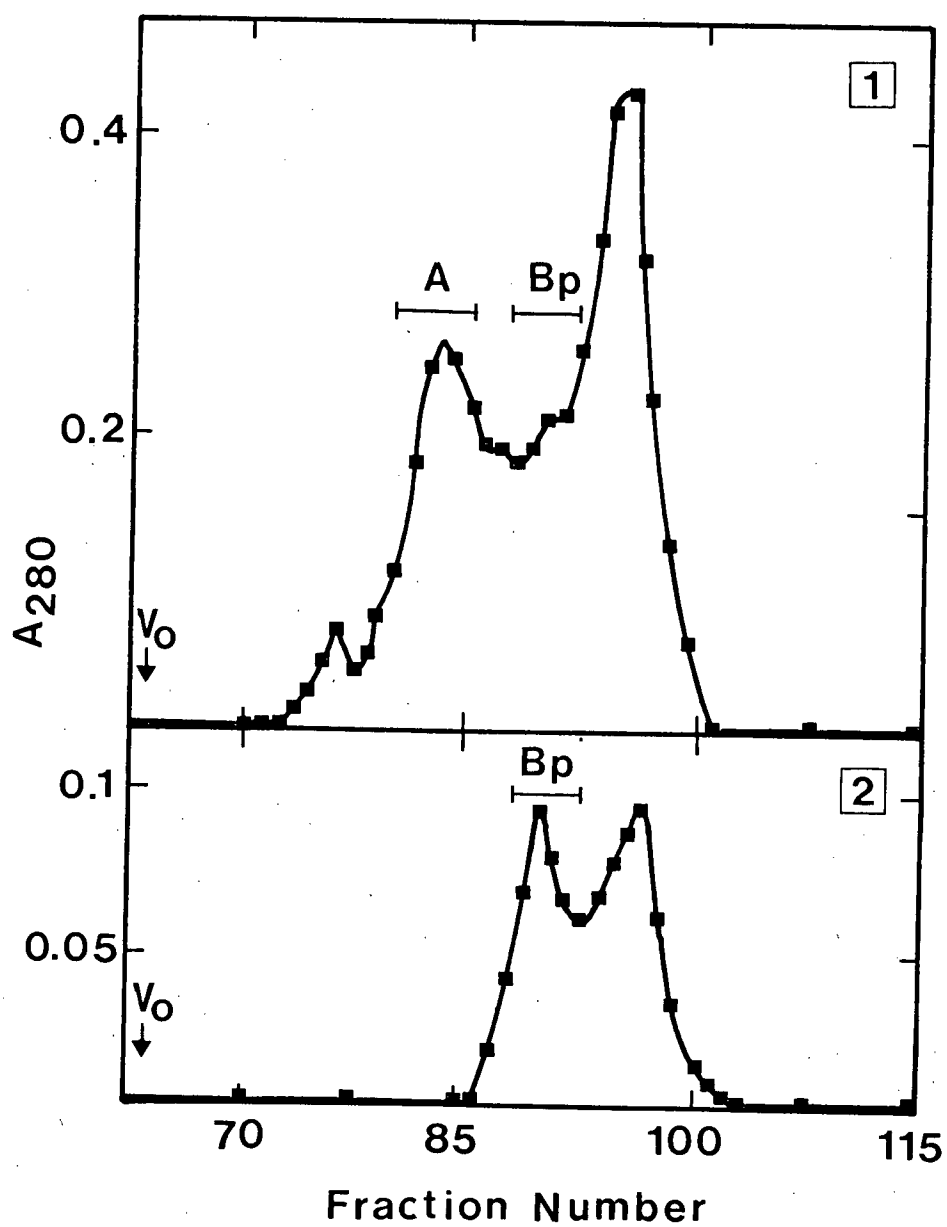
A portion of protein B resistant to digestion by pronase remains in the membrane following treatment with pronase.

Whereas, protein B was readily extracted from the membrane with SDS at 37°C, higher temperatures (about 100°C) were required to solubilize protein Bp. Protein Bp was isolated and purified by gel filtration in the presence of SDS (Figs. 47,48). Its amino acid composition (Table III) was not enriched in hydrophobic amino acids when compared to protein B in spite of the increased difficulty in solubilizing it from the membrane. Thus, its interaction with the other components of the membrane may not be primarily hydrophobic, although a small hydrophobic sequence cannot be excluded. Pronase treatment must remove a portion of the amino-terminal sequence of protein B since protein Bp contained an amino-terminal valine residue (Fig. 16) whereas the amino-terminal amino group of the polypeptide chain of protein B was not available for reaction (Fig. 16).

Further evidence on the nature of the interactions of protein B in the membrane was obtained from reconstitution studies. The criterion we used to show that protein B had been reassociated to a similar state to that in the native membrane was the formation of protein Bp upon digestion with pronase. Protein B was freed of detectable phospholipid and lipopolysaccharide for these experiments.

Digestion of protein B in the presence of SDS produced numerous small fragments which migrated near the dye front on SDS-polyacrylamide gel electrophoresis. When the detergent was removed by dialysis for 24 h against a 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM MgCl₂, a precipitate of re-

Fig. 47. Purification of protein Bp by gel filtration in the presence of SDS. Pronase-treated cell wall was extracted twice with SDS, first at 37°C for 1 h, then at 100°C for 15 min. Peptidoglycan was removed by centrifugation at 120,000 x g for 1 h. The second extract was dialyzed against distilled water, freeze-dried, and then dissolved to 2 mg protein/ml in 10 ml of column buffer, containing 0.1% 2-mercaptoethanol. After heating at 100°C for 5 min, the sample was applied to a column of Sephadex G100 (5 x 40 cm) connected in series to a column of Sephadex 6B of the same dimensions. Proteins were eluted with 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. Fractions (10 ml) were collected, the absorbance measured at 280 nm and the protein content determined by SDS-gel electrophoresis. Fractions (88-92, profile 1) containing protein Bp were pooled and reappplied to the same column system. Fractions (89-93, profile 2) containing protein Bp were pooled, dialyzed and freeze-dried.



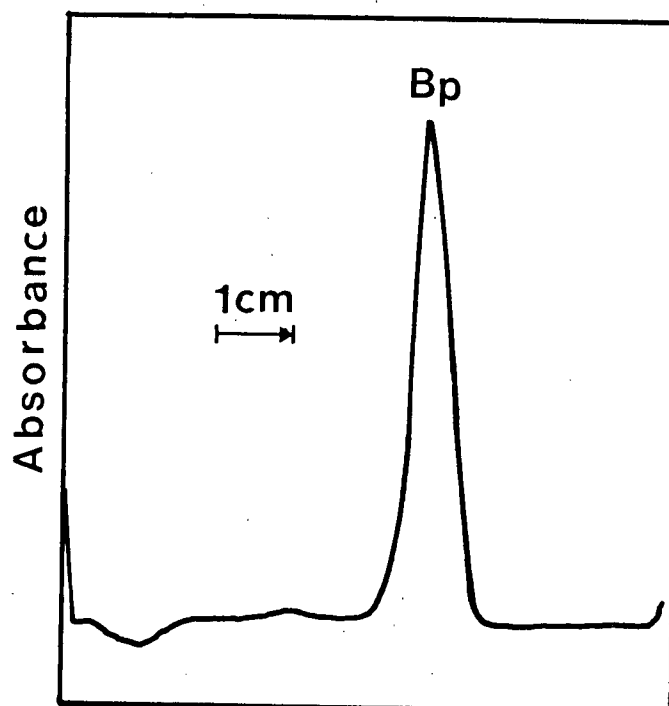


Fig. 48. SDS-polyacrylamide gel scan of purified protein Bp run in system 1. The gel (10% acrylamide) was stained with Coomassie Blue and scanned at 550 nm.

associated protein B was formed. Treatment of this material with pronase gave a fragment with a molecular weight of about 20,000 (Fig. 49, scan 3) which appeared to be similar to protein Bp prepared by digestion of the native membrane. If the MgCl_2 was replaced by 20 mM EDTA in the dialysis buffer, pronase digestion of the precipitated protein B yielded multiple digestion fragments (Fig. 49, scan 2). These results suggest that in the presence of divalent cations protein B will re-associate to a state similar to that in the native membrane.

The conditions for reassembly were further examined (Table V). The amount of protein Bp produced by pronase digestion of protein B reassociated in the presence of MgCl_2 was taken as 100%. No intact protein B remained under the digestion conditions used (Fig. 49, scan 3). Dialysis of protein B in SDS against buffers containing manganous or calcium ions produced yields of protein Bp similar to that formed with magnesium ions. If the sample of protein B was frozen, lyophilized, or converted to form B* by heating (4), prior to attempted reassembly, no pronase-resistant digestion fragments were produced. This supports the previous suggestion that protein B as isolated from the membrane with SDS retains some native conformation which is lost upon heating.

The importance of protein-protein interactions in the protection of a portion of the protein B molecule from digestion by pronase was supported by the following observation. Addition of purified lipopolysaccharide or soy bean phospholipids in up to a 10-fold and 50-fold excess by weight over

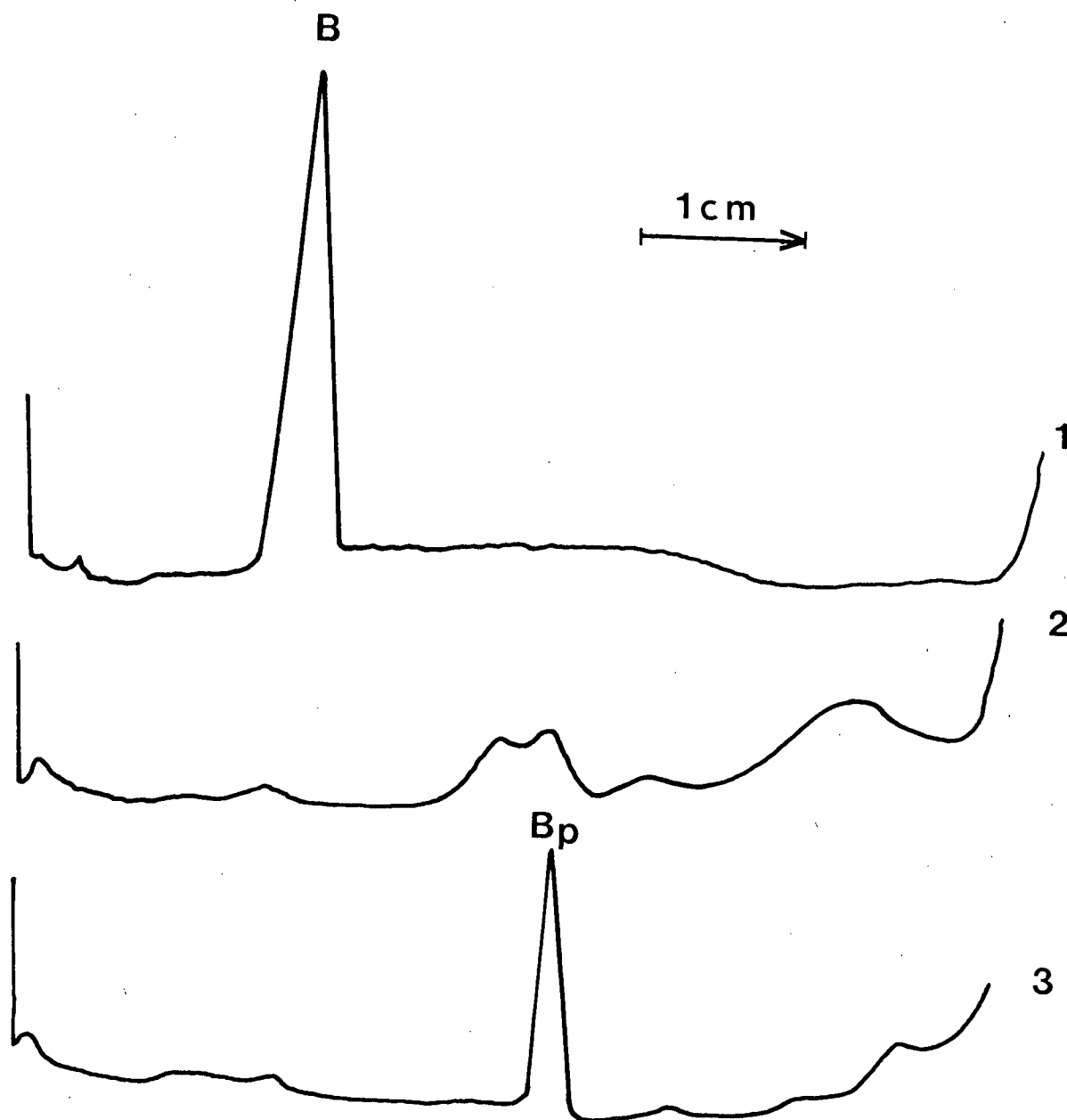


Fig. 49. Production of protein Bp by pronase digestion of reassociated protein B. Coomassie Blue-stained SDS-polyacrylamide gels were scanned at 550 nm. 1, purified protein B; 2, effect of pronase (1 mg/25 mg protein B) on protein B which had been dialyzed for 24 h against 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM EDTA; 3, effect of pronase on protein B which had been dialyzed against 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM $MgCl_2$.

Table V

Conditions for reassociation of protein B

Treatment	% Bp	number of pronase-resistant peaks
No dialysis	20	multiple
Dialysis with Mg ²⁺	100	single
Mn ²⁺	97	single
Ca ²⁺	158	single
EDTA	31	multiple
Protein B: Frozen	0	none
Lyophilized	0	none
heated	0	none
Lipopolysaccharide added	100	single
Phospholipid added	83	single

Protein B (1 ml) in 1% SDS was dialyzed against 1 l of 10 mM Tris HCl, pH 7.5, containing either 20 mM MgCl₂, MnCl₂, CuCl₂ or EDTA at 22°C for 24 h. After dialysis, the preparation was treated with pronase at an enzyme:protein ratio of 1:25 for 2 h at 37°C. The amount of protein Bp produced under these conditions after dialysis against Tris buffer containing 20 mM MgCl₂ was taken as 100%. In some experiments protein B was frozen, lyophilized, or heated at 100°C for 5 min, or lipopolysaccharide or phospholipid was added prior to dialysis (see text).

protein B, respectively, prior to the dialysis step did not increase the yield of the pronase resistant fragment.

Crosslinking of protein A-peptidoglycan complexes

The evidence presented above suggests that extensive protein-protein interactions occur in the outer membrane. The relationships of the outer membrane proteins to one another was examined by the use of crosslinking agents and two-dimensional SDS-polyacrylamide gel electrophoresis.

Protein A₁- and protein A₂-peptidoglycan complexes prepared from glucose- and casein amino acid-grown cells, respectively, were treated with increasing amounts of the crosslinker DSP. As shown in Fig. 50, both proteins A₁ and A₂ crosslinked to form dimer, trimer, and higher oligomers. The trimer was always produced in excess over the tetramer which suggests that protein A might be organized as trimers in the membrane. Although proteins A₁ and A₂ were not well resolved by the gel system the content of A₁ and A₂ in the two preparations was confirmed by SDS electrophoresis at an alkaline pH (79).

Crosslinking of protein A to oligomers in protein A-peptidoglycan complexes was also observed with glutaraldehyde (Fig. 51).

Since the protein A-peptidoglycan complexes also contain lipoprotein (protein F) covalently bound to the peptidoglycan (44), it was possible that protein A could be crosslinked to the peptidoglycan through this protein. This possibility was examined as described in Fig. 52. The protein A-peptidoglycan

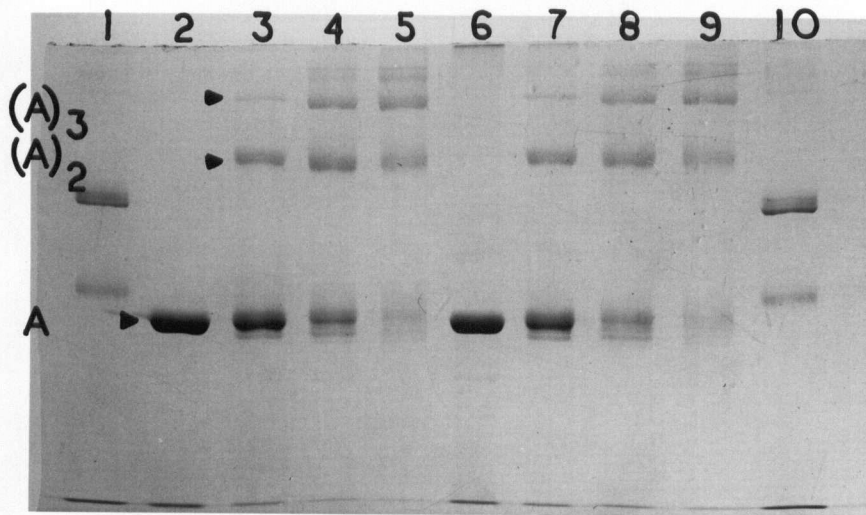


Fig. 50. Crosslinking of protein A-peptidoglycan complexes with DSP. Samples (100 μ l; 0.1 mg protein) of protein A-peptidoglycan complexes prepared from glucose-grown (samples 2-5) and casein amino acid-grown cells (samples 6-9) were treated with 0 μ g (samples 2 and 6), 40 μ g (samples 3,7), 200 μ g (samples 4,8), and 600 μ g (samples of 5,9) of DSP. The samples were solubilized with 9 volumes of SDS-electrophoresis sample buffer and then resolved on 9% polyacrylamide gel slabs. Samples 1 and 10 contain as molecular weight markers bovine serum albumin (66,000), ovalbumin (46,000), and lysozyme (14,400).

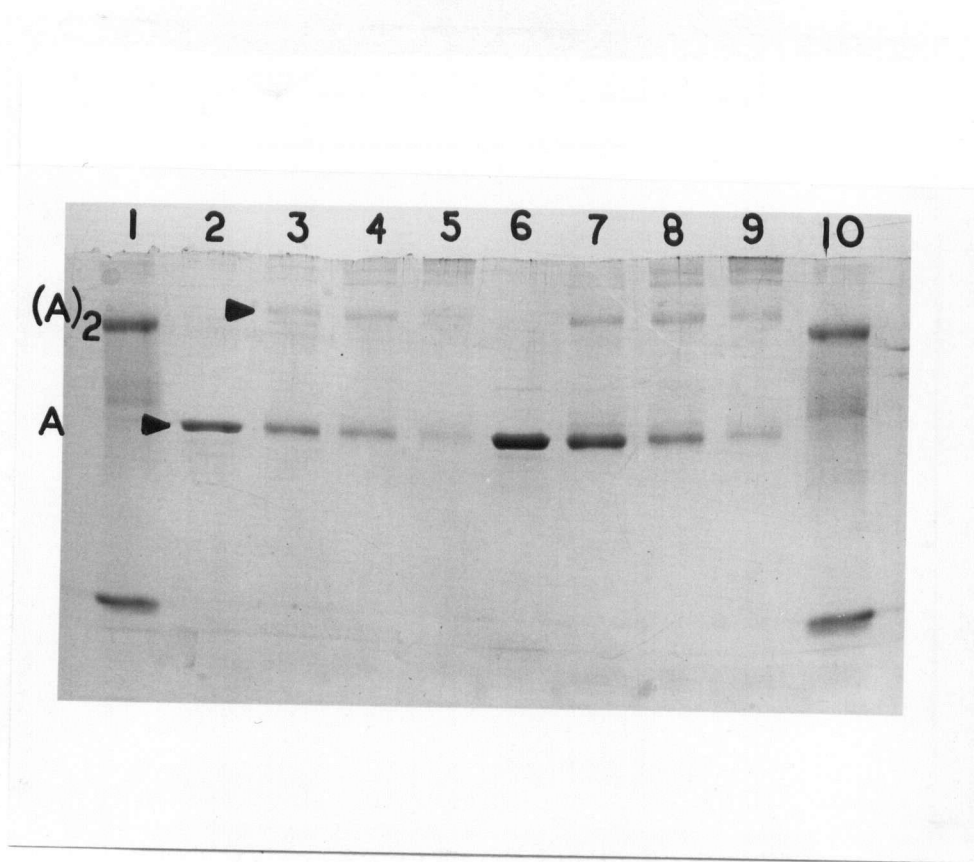
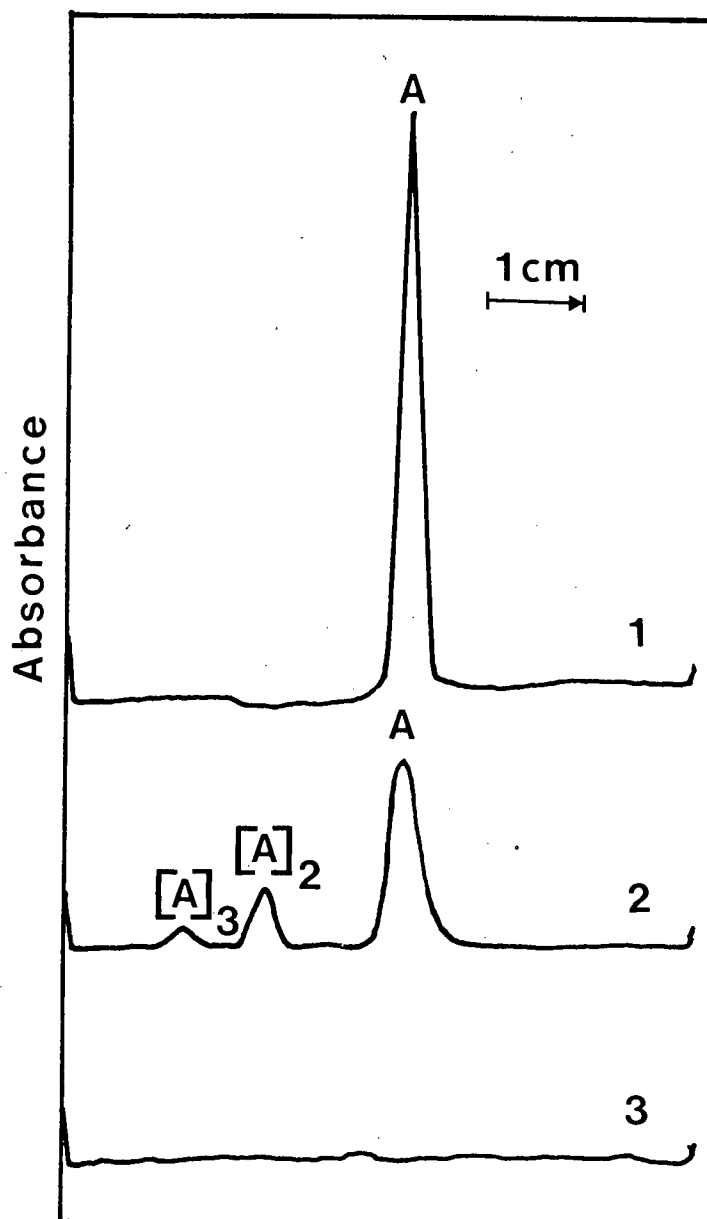


Fig. 51. Crosslinking of protein A-peptidoglycan complexes with glutaraldehyde. Samples (100 μ l; 0.1 mg protein) of protein A-peptidoglycan complexes prepared from glucose-grown (samples 2-5) and casein amino acid-grown cells (samples 6-9) were treated with 0% (v/v) (samples 2 and 6), 0.05% (samples 3 and 7), 0.1% (samples 4 and 8) and 0.5% (samples 5 and 9). Final concentration of glutaraldehyde in 0.2 M triethanolamine buffer, pH 8.5. The samples were solubilized with 9 volumes of SDS-electrophoresis buffer and then resolved on 9% polyacrylamide gel slabs. Samples 1 and 10 contain as molecular weight markers bovine serum albumin (66,000), ovalbumin (46,000) and lysozyme (14,400).

Fig. 52. Lack of crosslinking of protein A to the peptidoglycan layer by DSP treatment of protein A-peptidoglycan complexes. The protein A-peptidoglycan complex, prepared from glucose-grown cells was suspended to 0.3 mg protein/ml in 10 ml of 0.2 M triethanolamine buffer, pH 8.5. DSP (5 mg) was added from a stock solution (30 mg/ml in dimethyl sulfoxide). After 1 h at 22°C, 2.5 ml of 1 M Tris-HCl buffer, pH 8.5, was added and the complex was recovered by centrifugation at 100,000 x g for 1 h at 15°C. The crosslinked complex was extracted with 1% SDS at 100°C for 15 min. The peptidoglycan was recovered by centrifugation and reextracted by 1% SDS, 1% 2-mercaptoethanol at 100°C for 15 min. The peptidoglycan was removed by centrifugation. A control experiment without added DSP was performed in parallel. Scan 1, SDS-polyacrylamide gel of protein A extracted by SDS from control peptidoglycan complexes. Scan 2, SDS-polyacrylamide gel of crosslinked protein extracted from DSP-treated peptidoglycan complexes. Scan 3, SDS-polyacrylamide gel of second SDS extract of DSP-treated peptidoglycan complexes. All samples were made 10% in glycerol, heated at 100°C for 5 min and then resolved in 5% polyacrylamide gels run with a phosphate buffer system 1 at neutral pH. Gels were stained with Coomassie Blue and scanned at 550 nm.



complex was first reacted with DSP and then extracted with 1% SDS. Uncrosslinked protein A is extracted by SDS at 100°C (scan 1) as were its crosslinked oligomers (scan 2). The peptidoglycan, and any proteins linked to it, was reextracted as before but in the presence of 1% 2-mercaptoethanol to cleave the crosslinking agent. No further protein A was released (scan 3) indicating that it had not been crosslinked to the bound lipoprotein or directly to the peptidoglycan.

Crosslinking of outer membrane

Cell wall, composed of outer membrane and peptidoglycan, and isolated outer membrane preparations were treated with different levels of DSP (Fig. 53). Proteins A and D₂ (molecular weight 18,000) were less readily crosslinked than proteins B and F, the free form of the lipoprotein. The ability of the proteins to be crosslinked is determined by the degree of exposure of suitably disposed amino groups. Thus, protein B, which was most readily crosslinked, was also most reactive with the amino group reagent fluorescamine and was readily digested by pronase. In contrast, protein A, which was less readily crosslinked in outer membrane preparations, reacted more slowly with fluorescamine and was resistant to digestion by pronase.

The effect of DSP crosslinking of pretreating the outer membrane with pronase was studied. Pronase cleaves protein B to a fragment, protein Bp, which remains embedded in the membrane, but does not digest protein A. In the pronase-

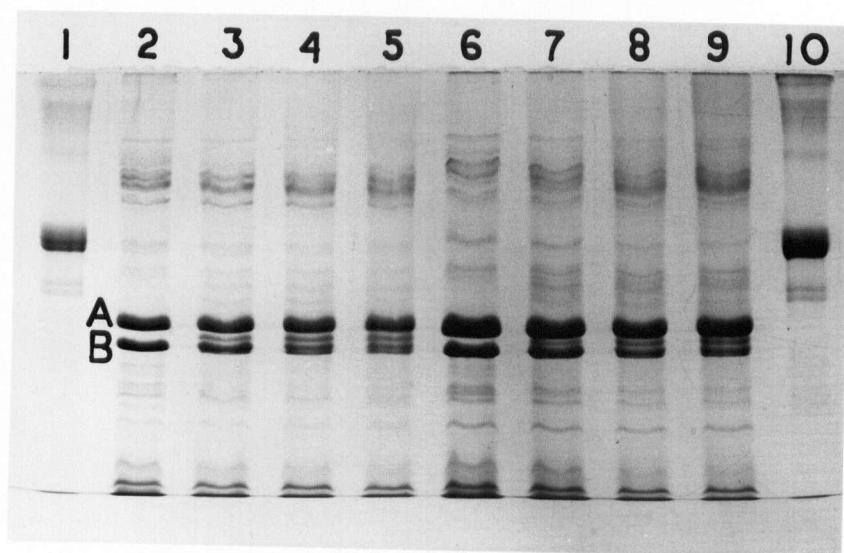


Fig. 53. Crosslinking of cell wall and outer membrane with DSP. Samples (100 μ l; 0.1 mg protein) of cell wall (samples 2-5) and outer membrane (samples 6-9) prepared from glucose-grown cells were treated with 0 μ g (samples 2 and 6), 20 μ g (samples 3 and 7), 40 μ g (samples 4 and 8) and 60 μ g (samples 5 and 9) of DSP. The samples were solubilized with two volumes of SDS electrophoresis sample buffer and resolved on 7.5% polyacrylamide gel slabs. Samples 1 and 10 contained as molecular weight markers bovine serum albumin (66,000), ovalbumin (46,000), and lysozyme (14,400).

treated membrane protein A was crosslinked to the dimer and trimer. In contrast to intact protein B, protein Bp was not readily crosslinked (Fig. 54). Thus, the functional groups responsible for the crosslinking of protein B must be situated on the exposed portion of this protein. This supports the view that the accessibility of the reactive groups on the protein are important in determining its ease of reaction with the crosslinker.

The formation of the dimer and trimer of protein A occurred when relatively high levels of DSP (2 mg/mg protein) were reacted with the outer membrane. This was confirmed by two-dimensional SDS-polyacrylamide gel electrophoresis in which the crosslinked products separated in the first dimension were cleaved by 2-mercaptoethanol prior to entering the separating gel of the second dimension. The cleaved products could then be identified from their characteristic rate of migration (Fig. 55). The formation of the dimer and trimer of protein A by crosslinking with both the outer membrane and the protein A-peptidoglycan complex suggests that protein A is arranged in the same way in both preparations. At the higher level of crosslinker, protein B gave a high molecular weight complex that penetrated the 4% stacking gel but did not enter the 7.5% polyacrylamide gel of the first dimension. Crosslinking of protein A to protein B was never observed.

Interaction of proteins B and F with peptidoglycan

When the cell wall (outer membrane-peptidoglycan) pre-

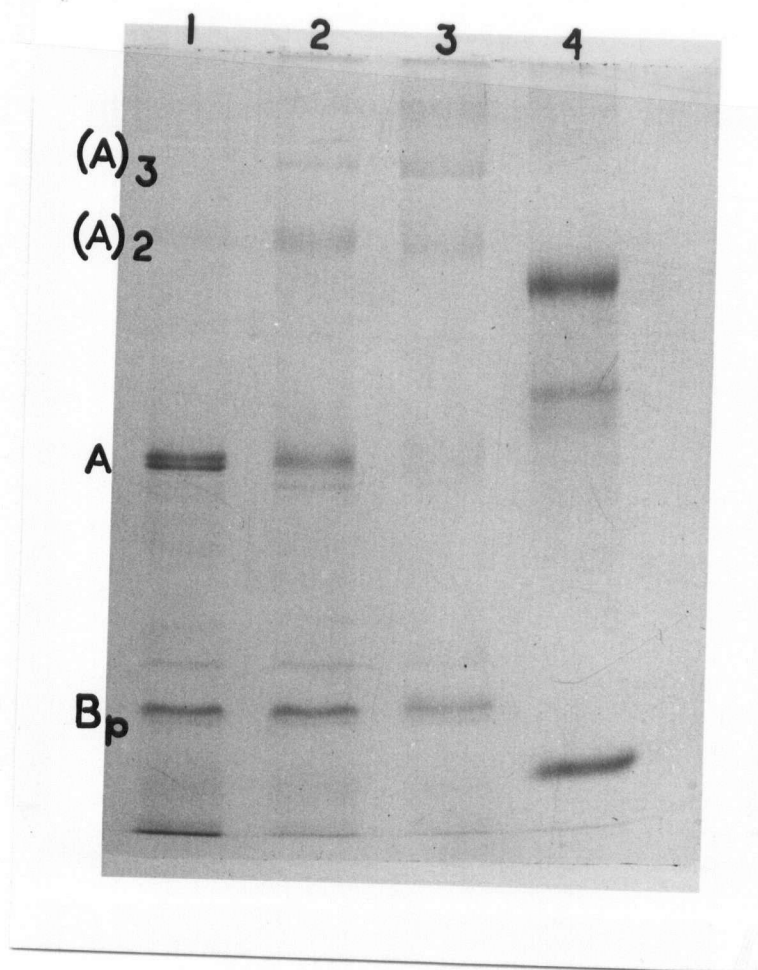


Fig. 54. Crosslinking of pronase-treated outer membrane with DSP. Outer membrane was treated with pronase at an enzyme: protein ratio of 1:25 for 2 h at 37°C. The outer membrane was recovered by centrifugation at 120,000 x g for 1 h and resuspended to about 1 mg of protein/ml in 0.2 M triethanolamine buffer, pH 8.5. Samples (100 μ l; 0.1 mg protein) of digested membrane were treated with 0 μ g (sample 1), 200 μ g (sample 2), and 600 μ g (sample 3) of DSP. The samples were solubilized with nine volumes of SDS electrophoresis sample buffer and then resolved on 9% polyacrylamide gel slabs. Sample 4 contained as molecular weight markers bovine serum albumin (66,000), ovalbumin (46,000), and lysozyme (14,400).

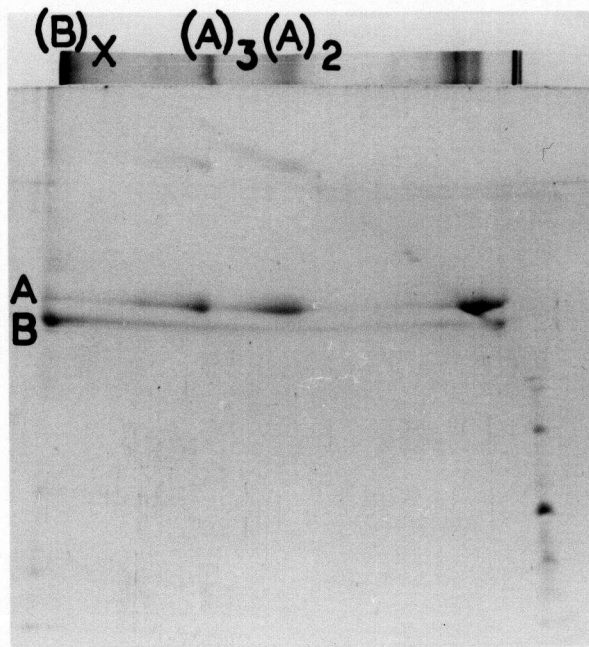


Fig. 55. Two-dimensional gel of products from crosslinked outer membrane proteins. A sample ($100\mu\text{l}$; 0.1 mg protein) of outer membrane was treated in the $200\mu\text{g}$ of DSP then solubilized with two volumes of SDS electrophoresis sample buffer, and resolved on a first dimension 7.5% polyacrylamide gel. The crosslinked products were cleaved in the second dimension by 2-mercaptoethanol and resolved on a 12% polyacrylamide gel. The positions of monomer (A), dimer $(A)_2$, and trimer $(A)_3$, of protein A, and the high molecular weight oligomer of protein B, $(B)_x$, are indicated. A stained first dimension gel is placed along the top of the gel slab.

paration was treated with DSP under identical conditions to those used in the previous experiment with the isolated outer membrane, a similar two-dimensional gel pattern was obtained (Fig. 56). However, it was consistently observed that a much larger crosslinked complex of protein B was formed which could not enter the 4% stacking gel in the first dimension. In order to determine if this was due to the crosslinking of protein B to the peptidoglycan, either directly or through another protein, the following experiment was performed. The cell wall preparation was crosslinked with DSP and then extracted with a buffer containing 1% sodium dodecyl sulfate and 10% glycerol in 62.5 mM Tris-HCl, pH 6.8, at 100° for 3 min. The insoluble peptidoglycan and attached proteins were reextracted with this buffer but containing 1% 2-mercaptoethanol. A control experiment was carried out in which the crosslinker was omitted. As a further control, the isolated outer membrane was taken through the above procedure. The results of this experiment are shown in Fig. 57. In the absence of DSP the first extract of the cell wall contained all of the outer membrane protein (sample 1). The first extract of the crosslinked cell wall showed the presence of the dimer and trimer of protein A (sample 2) which could be cleaved by the addition of 2-mercaptoethanol (sample 3). The second extract of the non-crosslinked sample contained virtually no proteins (sample 4). However, the second extraction of the crosslinked cell wall with the 2-mercaptoethanol-containing buffer released proteins B and F from the peptidoglycan (sample 5). In contrast to cell

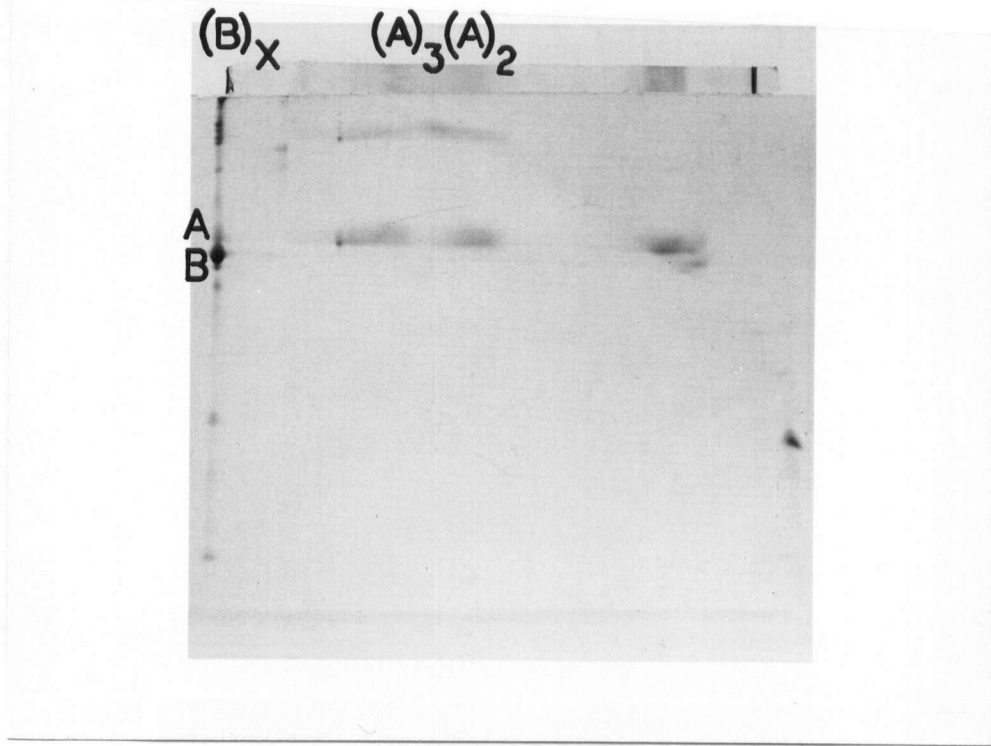


Fig. 56. Two-dimensional gel of products from crosslinked cell wall. A sample of cell wall, consisting of outer membrane and peptidoglycan was treated as described in Fig. 52. The positions of monomer (A), dimer $(A)_2$, and trimer $(A)_3$, of protein A, and the high molecular weight oligomer of protein B, $(B)_x$ are indicated. A stained first dimensional gel is placed along the top of the gel slab.

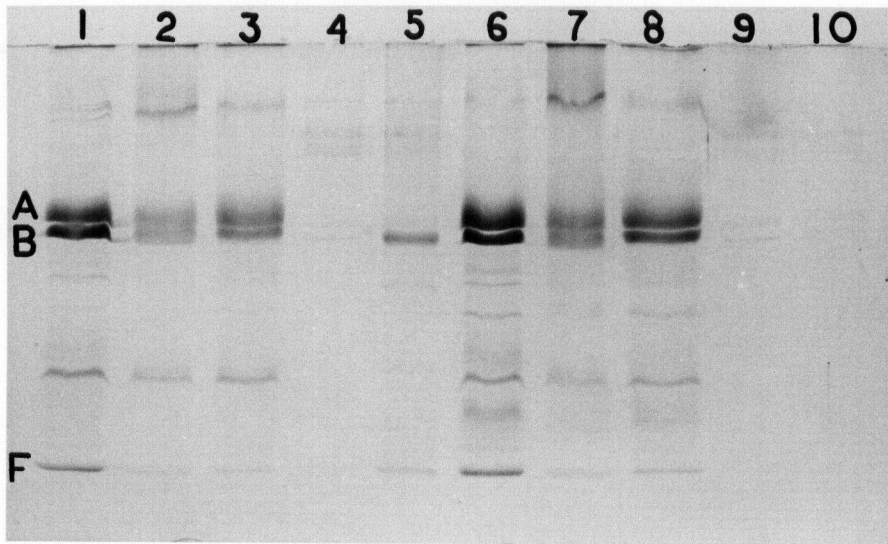


Fig. 57. Crosslinking of proteins B and F to the peptidoglycan layer. Preparations (1 ml; 1 mg protein) of cell wall (samples 1-5) and outer membrane (samples 6-10) were treated with 2 mg of DSP for 30 sec. After addition of excess Tris-HCl, pH 8.5, two volumes of SDS electrophoresis sample buffer without 2-mercaptoethanol were added and the proteins were solubilized by heating at 100°C for 3 min. The supernatant (first extract) was removed after centrifugation of the mixture at 120,000 x g for 1 h at 15°C and the pellet was re-extracted with three volumes of sodium dodecyl sulfate electrophoresis sample buffer containing 1% 2-mercaptoethanol (second extract). The peptidoglycan was removed by centrifugation at 120,000 x g for 1 h at 15°C. Control preparations (1 ml) untreated with DSP were carried through the same procedure. Samples 1 and 6, first extract of control preparation; samples 2 and 7, first extract of DSP-treated preparation; samples 3 and 8, same as samples 2 and 7 but treated with 2-mercaptoethanol prior to electrophoresis; samples 4 and 9, second extract of control preparation; samples 5 and 10, second extract of DSP-treated preparation.

wall, all the crosslinked products of the isolated outer membrane was readily solubilized in the first extract (samples 6-8) as the second extract did not contain any proteins (samples 9,10).

The results indicate that proteins B and F can be crosslinked to the peptidoglycan. This might occur by linking to a free amino group of the peptidoglycan itself or by linking to the bound lipoprotein. However, the linking of protein B or protein F to the bound lipoprotein or peptidoglycan could be indirect through the other protein. In this case a crosslinked product containing both proteins B and F should be formed. Fig. 58 shows the effect of treating a cell wall preparation with DSP at a relatively low level (0.4 mg DSP/mg protein). Besides a dimer of protein A and one of protein B, a crosslinked product with a molecular weight of about 40,000 and containing one molecule each of proteins B and F was detected. This product gave two spots of both proteins B and F on the gel following cleavage with 2-mercaptoethanol. This is probably due to the presence of internal crosslinking such that one or both of the proteins retains a more compact conformation on denaturation with SDS which would affect its rate of migration on electrophoresis. A small amount of internally crosslinked protein A, which comigrated with protein B in the first dimension, can also be seen in Fig. 58.

The presence of a dimer of protein F was detected when crosslinked material similar to that used in the previous

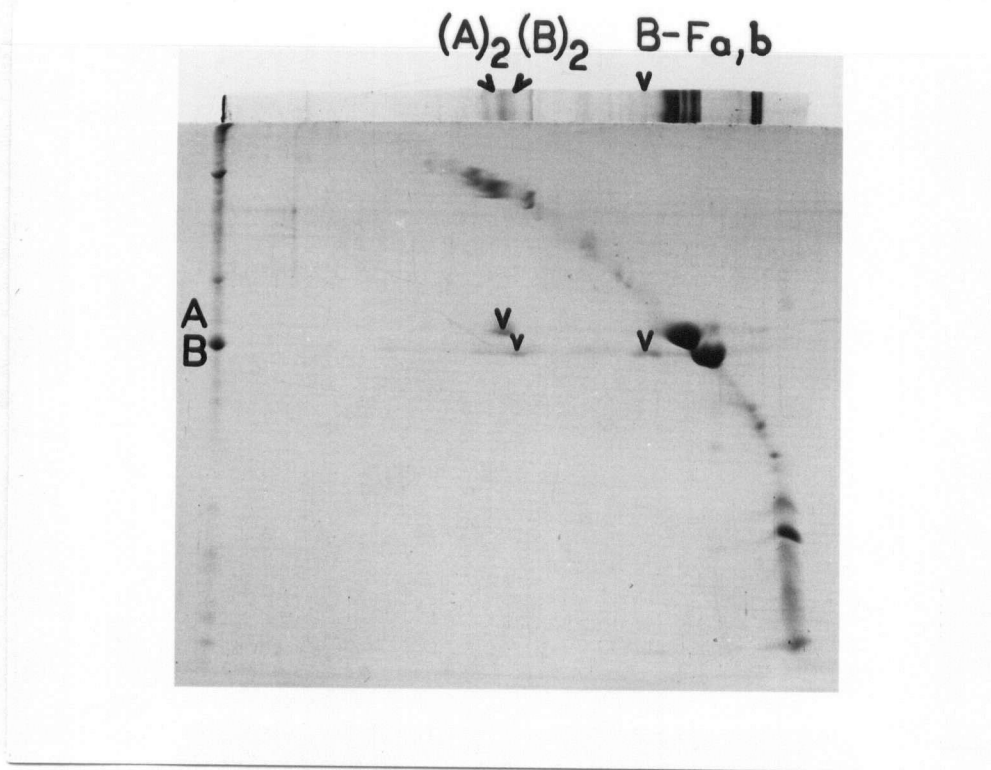


Fig. 58. Two dimensional gel of products from crosslinked cell wall. A sample (100 μ l; 0.1 mg protein) of cell wall was treated with 40 μ g of DSP, solubilized with two volumes of SDS electrophoresis sample buffer, and resolved on a 7.5% polyacrylamide gel. The crosslinked products were cleaved in the second dimension with 2-mercaptoethanol and resolved on a 12% polyacrylamide gel. The positions of the crosslinked products containing both proteins B and F ($B-F_a$, molecular weight, 42,200; and $B-F_b$, molecular weight, 40,300), and the dimers of protein A ($(A)_2$, molecular weight, 80,000) and protein B ($(B)_2$, molecular weight, 70,500) are indicated. A stained first dimension gel is placed along the top of the gel slab.

experiment was run on two-dimensional gels containing higher concentrations of polyacrylamide (Fig. 59).

These results indicate that at least some of the molecules of protein B and protein F in the outer membrane must be in close proximity to one another, and that there may be groups of molecules of protein F as suggested by Inouye (133). These results do not prove that either protein B or F is linked through the other to the peptidoglycan layer but they are not inconsistent with this possibility.

The results of proteolytic digestion, covalent labelling and crosslinking have been integrated to give a model for the organization of the proteins of the outer membrane of E. coli. This model is presented in the Discussion section.

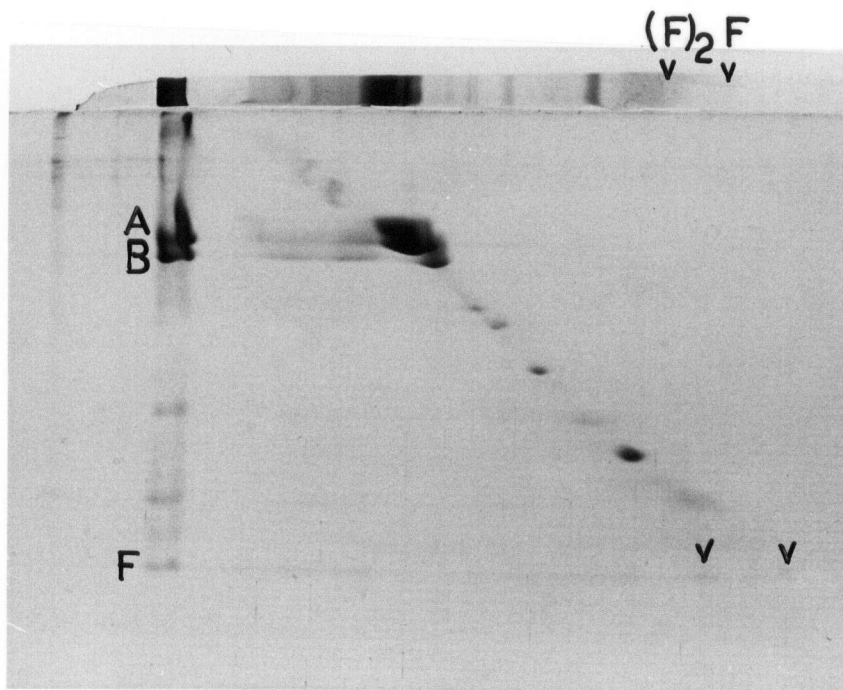


Fig. 59. Two dimensional gel of products from crosslinked cell wall. A crosslinked preparation of cell wall similar to that described in Fig. 6 was separated on a first dimension 12% polyacrylamide gel to resolve low molecular weight cross-linked products. The crosslinked proteins were cleaved in the second dimension with 2-mercaptoethanol and resolved in a 15% polyacrylamide gel. The position of the crosslinked products of proteins B and F (B-F) and the dimer of protein F, $((F)_2)$ are indicated. A stained first dimension gel is placed along the top of the gel slab.

DISCUSSION

Properties of the outer membrane proteins

Protein B

Protein B is released from the outer membrane by detergent treatment and may therefore be classified as an integral membrane protein (156). This protein has an amino acid composition that appears to be characteristic of membrane proteins. Namely, a low content of cysteine, an excess of acidic amino acids over basic, and a moderate content of hydrophobic amino acids (204). The amino acid composition of protein B presented in this thesis agrees with that determined by Henning's group for protein II* from E. coli B/r (87). This suggests that protein B and Henning's protein II* are the same protein (Table I).

Schnaitman has shown that protein 3 from E. coli O 111 is composed of two different polypeptides, both of which are heat-modifiable (88). The single band on SDS-gels found before and after heating, the kinetics of conversion of protein B to form B*, the cyanogen bromide cleavage pattern, the amino terminal analysis, the production of single protease-resistant fragments by pronase, trypsin and chymotrypsin treatment of the outer membrane, all suggest that protein B from E. coli NRC 482 is a single polypeptide. The same conclusion was reached by Henning for the heat-modifiable protein from E. coli B/r. The difference in Schnaitman's results may be

because the cells he used were grown on succinate rather than glucose or because of differences in the strain used.

The cyanogen bromide cleavage pattern obtained for protein B on SDS-gels differs from that reported by other workers. Henning's group found that protein II* contains five methionine residues and cyanogen bromide treatment resulted in the production of five major fragments with molecular weights of 14,000, 11,000, 9,000, 2,000, 1,000 as determined by SDS-gel electrophoresis (87). Schnaitman (88) separated protein 3 by ion-exchange chromatography into two polypeptides, 3a and 3b that differed in their cyanogen bromide cleavage patterns. Protein 3a gave two major peptides (M.W. 22,000 and 12,000) and a large number of smaller peptides on SDS-gels after cyanogen bromide cleavage. The SDS-gel pattern for the cyanogen bromide cleavage products of protein 3b showed two broad bands with different molecular weights from that determined for the fragments derived from protein 3a. The relationship of the two major peptides (M.W. 18,000 and 15,000) obtained by cyanogen bromide treatment of protein B to these results is not clear. The difference may be accounted for by the use of different gel systems or differences due to strain or growth conditions.

No amino-terminal derivative was detected for protein B. The nature of the blocking group was not determined, however, it may be carbohydrate (88) or perhaps a pyrrolidine carboxylic residue. Henning's group reported that protein II* has an amino-terminal alanine (87). However, no further de-

rivatives were released by subsequent Edman degradations. The heat-modifiable protein characterized in this thesis may differ from the protein characterized by Henning's group by the amino-terminal amino acid.

Protein B (M.W. 28,500) was converted to a higher molecular weight form B* (M.W. 33,400) upon heating. The temperature-dependent conversion of protein B to B* accounts for the multiple protein bands observed by a number of authors (81,82,177). These workers had heated membrane extracts at 70°C for 20 min. This results in the conversion of about 25% of protein B to form B*. Peak 7 of Inouye and Yee (82), peak C of Schnaitman (81) and band D of Koplow and Goldfine (177) represent unconverted protein B, while peak 6 of Inouye and Yee, peak B of Schnaitman and band C of Koplow and Goldfine correspond to form B* (Table I). Protein 3 of Schnaitman (88) and II* of Henning show similar heat-modifiable characteristics to protein B and may be the same protein.

Schnaitman (81) suggested that the conversion of protein B to B* was due to unfolding of the protein causing an increased level of SDS binding and conversion to the rigid rod conformation proposed by Reynolds and Tanford (152) for SDS-protein complexes. It was confirmed that protein B unfolds irreversibly upon heating, however no further binding of SDS occurred. This suggests that protein B as isolated from the membrane contains some native structure which is lost upon heating. This is confirmed by the observation that protein B, but not form B* is able to reassociate into an arrangement similar to

that found in the native membrane.

The true molecular weight of protein B is unknown. Henning and coworkers have determined from amino acid analysis and by summation of the molecular weights of the peptides formed on cleavage with cyanogen bromide, that the molecular weight of protein B is 27,000, closer to the molecular weight of the unmodified form (87). Molecular weight determinations by SDS-polyacrylamide gel electrophoresis are subject to error (204) as are determinations based on minimal chemical molecular weights by amino acid analysis. The true molecular weight of protein B may be closer to the modified form since heating would cause the protein to attain a more extended structure with loss of all native structure. Conversion to a rigid rod conformation would make comparisons of protein B to standard proteins in SDS more meaningful. Further studies on this protein are required before its molecular weight can be established with certainty.

Protein A

Protein A is firmly attached to the peptidoglycan and as such is resistant to proteolytic digestion. Like protein B, it is only moderately hydrophobic, has a low content of cysteine and contains more acidic residues than basic. The amino acid composition of protein A agrees with that determined by Rosenbusch (80) for the matrix protein and by Henning's group for protein 1 suggesting that they are the same protein (Table I). The matrix protein contains four methionine residues,

however it is not readily cleaved by cyanogen bromide. This results in the production of peptides, the molecular weight of which, when summed, greatly exceed that of the intact protein. The incomplete cleavage makes comparisons of SDS-gel patterns of separated fragments from different laboratories difficult.

E. coli NRC 482 contains two different matrix proteins, A₁ and A₂ that can be resolved by SDS-polyacrylamide gel electrophoresis at an alkaline pH (system 2). Both are associated with the peptidoglycan, have similar amino acid compositions, and give similar spectrum of peptides on SDS-gels after cyanogen bromide cleavage, suggesting that they may be two forms of the same polypeptide. Proteins 1 and 2 of Schnaitman are likely equivalent to proteins A₁ and A₂ (Table I). This is supported by the finding that like Schnaitman's protein 2, protein A₂ is under catabolite repression. No protein equivalent to protein A₂ is found in most E. coli strains including K 12 (91,140), JE 1011 (179) and B/r (91,149). E. coli B/r does however contain two forms of the matrix protein, Ia and Ib that differ in only one region of the polypeptide chain (142). The relationship of proteins Ia and Ib to protein A₁ is not clear, however it is likely that one of the forms of protein I is the same as protein A₁.

It is apparent that the matrix protein varies with strain and growth conditions. The relationship of these variations to the function of the matrix protein as a passive pore through the outer membrane remains to be elucidated.

Organization of proteins in the outer membrane

The experiments described in the second part of this thesis have been primarily directed towards understanding the arrangement of the proteins in the outer membrane of E. coli and their relationship to some of the other components of this membrane.

Attempts to use intact cells in these studies met with limited success. Whereas pronase readily digested certain proteins of the outer membrane in isolated cell envelope or outer membrane preparations, the major proteins in intact cells were unaffected by this treatment. Removal of a fraction of the lipopolysaccharide by EDTA treatment did not result in the loss of any major outer membrane proteins from intact cells and these proteins remained insensitive to digestion. Thus, there does not appear to be an intimate relationship between this pool of lipopolysaccharide and the major outer membrane proteins. However, the possibility that the non-releasable portion of the lipopolysaccharide might be protecting the proteins from digestion was examined using a heptose-deficient mutant which the O-antigen and core regions of the lipopolysaccharide were absent. There was no change in the pattern of digestion in this mutant. Moreover, in spite of the lower levels of proteins A and B found in mutants of this type (86,177,179) there was no change in the behaviour of the other outer membrane proteins. This suggests that these proteins in the mutant must be arranged in a similar way to those in the parent strain, and that the resistance of

the proteins in intact cells was not due to the lipopolysaccharide. The possibility that resistance was due to the unavailability of certain groups at the surface of the membrane is supported by the finding that dansyl chloride was unable to label cell surface proteins in both rough and smooth strains of Salmonella typhimurium (238). However, tyrosine residues are exposed at the surface of the membrane in both this organism and in Pseudomonas facilis since lactoperoxidase-catalyzed iodination of proteins of molecular weights 16,000 and 14,000, respectively, occurred in intact cells of these organisms (58,59). Also, the ability to label surface components of intact cells of JE and NS-1 suggests that reactive groups are available on the surface of the cell.

The use of cell envelope, cell wall (outer membrane-peptidoglycan) and isolated outer membrane preparations gave a clearer understanding of the arrangement of the outer membrane proteins. The similarity in response of the proteins to proteolytic digestion, labelling and crosslinking in the different preparations argues against a massive artifactual rearrangement of the outer membrane proteins in subcellular preparations.

Recent observations, including those presented in this thesis have permitted an updating of the models of the organization of outer membrane components (Figs. 5a-i). The model presented in Fig.60 summarizes the results of proteolytic digestion, covalent labelling and crosslinking studies. This model is schematic only and does not rule out

alternative arrangements of the proteins.

Lipopolysaccharide is distributed on the outside of the bilayer with the polar carbohydrate chains extending into the medium (45). The EDTA-releasable fraction of the lipopolysaccharide is stabilized by divalent cations such as magnesium and calcium (38,61) and probably does not interact primarily with protein. The phospholipids are distributed mainly in the inner layer of the bilayer or are covered by proteins when in the outer layer as proposed recently by Nikaido (107,282).

Proteins C, D₁ and E are probably exposed at the surface of the membrane since they are readily extracted with sodium dodecyl sulfate and are digested by pronase. Protein D₂, which is resistant to digestion and is not readily crosslinked, must be protected by its arrangement in the membrane.

The bound lipoprotein extends from the peptidoglycan into the outer membrane (44). If the free and bound form of the lipoprotein form a channel (Figs. 5 g and i) as proposed by Inouye (130) then they will interact with the hydrophobic regions of the membrane. The existence of groups of protein F molecules is supported by the detection of a crosslinked dimer of protein F. The apparent lack of reaction of the lipoprotein with antibodies at the surface of intact cells (123) does not necessarily exclude the penetration of the outer membrane by the lipoprotein complex. Its external surface might be protected from interaction with the antibody by lipopolysaccharide or other proteins. This is supported by the finding that protein F, the free form of the lipoprotein,

like protein B can be digested at the surface of isolated outer membrane preparations but not in intact cells.

Alternatively, single lipoprotein molecules might span the distance from the peptidoglycan to the outer membranes illustrated in most models (Figs. 5c,d,e, and f). However, it appears from sequence analysis that the function of the lipoprotein is to serve as an interface between a hydrophobic and a hydrophilic zone. Channel models would fit this concept of lipoprotein function. A lipoprotein complex proposed by Inouye would not be located in the periplasmic space (Fig. 5h) since the hydrophobic amino acid residues would have to be in contact with the water of the periplasmic space.

The inability of proteases to digest proteins A₁ and A₂ may not be due to the absence of these proteins at the surface of the membrane since they probably act as receptors for certain bacteriophages (91,98,105). Rosenbusch (80) has shown that molecules of protein A are arranged in a regular array on the peptidoglycan in isolated protein A-peptidoglycan complexes. Protein A was not digested by pronase in these preparations, but it was readily digested when solubilized. This observation was confirmed both with complexes containing primarily protein A₁ and with complexes of protein A₂. Thus, the pronase resistance of these proteins is probably a function of the folding of the protein molecule or of the interaction between it and other molecules of protein A. Moreover, proteins A₁ and A₂ must be similarly disposed in the outer

membrane. This is supported by their similar amino acid compositions. Protein A also can form channels through lipopolysaccharide-phospholipid vesicles (134). These properties suggest that protein A spans the outer membrane in conflict with Inouye's model (Fig. 5i).

Rosenbusch (80) using electron microscopy has suggested that protein A forms a lattice structure with hexagonal symmetry on the outer face of the peptidoglycan. The results in this thesis also indicate that molecules of protein A must interact with one another since oligomers of this protein were isolated although the number of subunits in the oligomer could not be determined.

Protein A can be crosslinked to the dimer and trimer but no linking to other protein has been detected. Thus, protein A is probably restricted to patches in the membrane and the channel might be formed by the association of three molecules of this protein. This would be consistent with the morphological results of Rosenbusch (80) who suggested that 1 to 3 molecules of protein A form a unit, and with the isolation of oligomers of this protein. Surprisingly, no crosslinking of protein A to the peptidoglycan was detected.

Protein B appears to be exposed at the surface of the membrane since about 25% of the molecule could be removed by pronase digestion to leave a fragment of 20,000 molecular weight embedded in the membrane. Henning and coworkers (131, 136) have found that trypsin digestion of protein B in ghosts gave a fragment with a molecular weight of 25,000. A similar

fragment was produced by digestion with chymotrypsin. The exposure of protein B at the surface was confirmed by covalent labelling with fluorescamine. Protein B was more readily labelled than protein A in agreement with their apparent degree of exposure at the surface of the membrane determined with proteolytic digestion.

The exposed portion of protein B is responsible for the ease with which this protein is crosslinked to itself to form high molecular weight aggregates. The presence of protein-protein interactions between molecules of protein B is supported by the divalent cation-dependent reassociation of this protein which can occur in the absence of phospholipid and lipopolysaccharide. However, the portion of the native structure which is retained when protein B is solubilized with sodium dodecyl sulfate at 37°C is necessary to ensure the reassociation of protein B into structures, which on the basis of cleavage with pronase to yield protein Bp, may resemble the arrangement of protein B in the native outer membrane. Protein Bp has an amino acid composition which is not markedly more hydrophobic than protein B. This would be consistent with the primary interaction of protein B with components of the membrane being other than hydrophobic.

Although most of the interactions which we have found are between molecules of the same protein, as also seems to be the case with the erythrocyte membrane (296), an association of protein B with protein F was detected. These proteins can be crosslinked to the peptidoglycan possibly through the

lipoprotein which is covalently bound to the peptidoglycan. The association of proteins B and F with the bound lipoprotein would restrict their mobility in the membrane. The non-covalent interaction of protein A with the peptidoglycan might also have the same effect. Thus, the lateral mobility of the outer membrane proteins is likely to be restricted. This could result in the existence of separate regions of protein in the membrane. The absence of detectable interactions between protein A and B would support this hypothesis. Thus, as Henning and coworkers have suggested (13), the Singer-Nicholson model of membrane structure (240) may not apply to the outer membrane of E. coli.

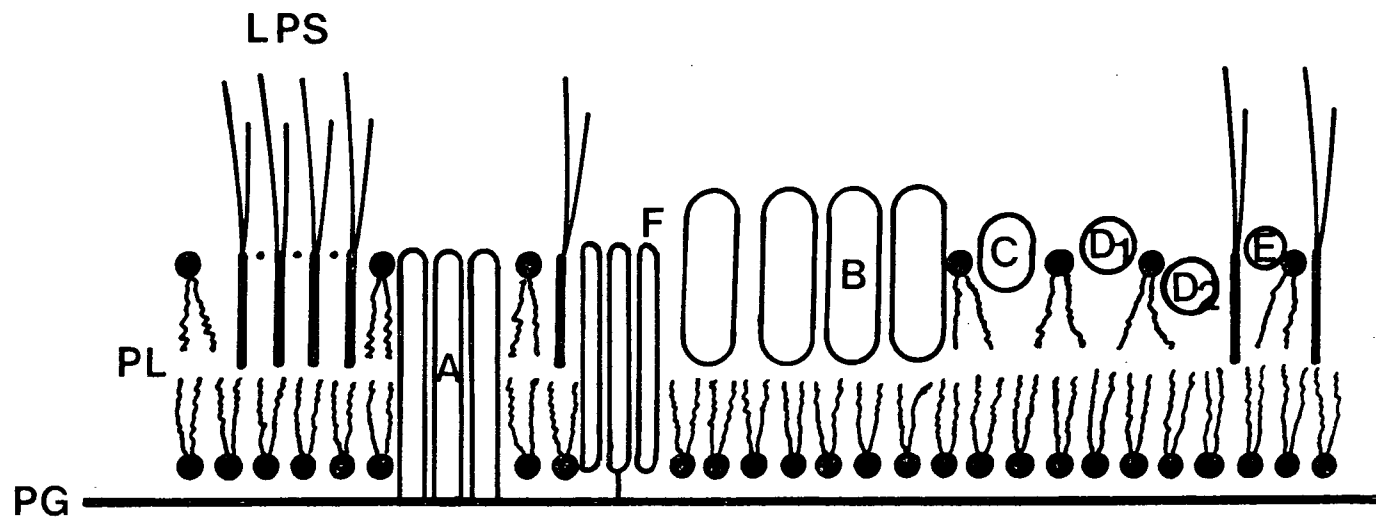


Fig.60. Schematic model for the arrangement of proteins in the outer membrane of *E. coli*. Divalent cations (•), phospholipid (PL), lipopolysaccharide (LPS), peptidoglycan (PG) and proteins A to F are indicated.

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Appendix

Calculation of intrinsic viscosity (286)

The relative viscosity (n_r) of a solution is the ratio of solution to solvent viscosity:

$$\frac{n_s}{n_o} = 1 + \nu \phi$$

where ν is a shape factor and ϕ is the fraction of a solution volume occupied by solute. The shape factor is a function of the axial ratio of the solute particle. The specific viscosity (n_{sp}) is a measure of the fractional change in viscosity produced by adding solute:

$$n_{sp} = \nu \phi$$

therefore
$$n_{sp} = n_r - 1$$

since
$$\phi = C\bar{v}$$

where C is grams of solute per milliliter of solution and \bar{v} is the partial specific volume of the solute, then

$$n_{sp} = \bar{v}$$

and
$$\frac{n_{sp}}{C} = \bar{v}$$

The limit of n_{sp}/C as $C \rightarrow 0$ is the intrinsic viscosity n .

Experimentally, the relative viscosity (η_r) is determined at a number of solute concentrations as

$$\frac{\eta_s}{\eta_o} = \frac{t_s}{t_o} \frac{\rho_s}{\rho_o}$$

where $\rho_{s,o}$ are the densities of the solution and solvent respectively. The intrinsic viscosity is determined by plotting η_{sp}/C as a function of C and extrapolated to infinite dilution ($C = 0$).

Calculation of the dimensions of a protein-SDS complex
(152,286)

The intrinsic viscosity is related to the hydrodynamic volume of a particle by:

$$\eta = \nu (\bar{v}_2 + \delta_1 \bar{v}_1 + \delta_2 \bar{v}_2^0)$$

where ν is the simha shape factor, \bar{v}_2 is the partial specific volume of the protein, δ_1 is the g of H_2O per g of protein, \bar{v}_1^0 is the specific volume of water, δ_2 is the g of SDS per g of protein and \bar{v}_2^0 is the specific volume of SDS. The following values were taken from Reynolds and Tanford (152):

$$\bar{v}_2 = 0.725 \text{ cc/g}$$

$$\delta_1 = 0.9 \text{ assuming 1.4 g of SDS per g}$$

$$\bar{v}_1^0 = 1 \text{ cc/g}$$

$$\delta_2 = 1.4 \text{ g of SDS per g of protein}$$

$$\bar{v}_2^0 = 0.886 \text{ cc/g}$$

Assuming a prolate ellipsoid, the axial ratio (a/b) was determined from the simha relationship (286) between the shape factor and a/b . The absolute values for the radii of the particle was calculated from the known particle volume ($\frac{4}{3} \pi ab^2$) where

$$\left(\frac{4}{3} a/b\right)b^3 = \frac{M}{N} (\bar{v}_2 + s_1 \bar{v}_1^0 + s_2 \bar{v}_2^0)$$

therefore

$$b = \sqrt[3]{\frac{M}{N} \cdot \frac{n}{v} \cdot \frac{3}{4} \cdot \frac{1}{\pi} \cdot \frac{b}{a}}$$

Calculation of Stokes radius of a protein-SDS complex (152, 288,289)

Ackers (289) has shown that the elution of a solute moving through a porous gel chromatographic column depends on the Stokes radius (R_s) of the solute particle:

$$R_s = A + B \operatorname{erfc}^{-1} K_d$$

where A and B are constants of the particular gel and the distribution coefficient:

$$K_d = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume of the solute, V_t , the total volume of the column and V_o , the void volume of the column. $\operatorname{Erfc}^{-1}K_d$ is the inverse of the complement of the standard error function.

The fraction of the interior volume of the gel available for distribution of the solute (K_d) is defined as the area under a random distribution probability curve for particles the same size as the solute and greater. Mathematically, this is the complement of the standard error function.

Calculation of partial specific volume of a protein-SDS complex (292)

The sedimentation coefficient of a particle at the position r_{avg} is defined as

$$S_{T,M}(r_{avg}) = \frac{(r-r_0) / t}{2 r_{avg}}$$

where $S_{T,M}$ is the coefficient at a given temperature in a given medium; $r_{avg} = (r_0 + r)/2$ where r_0 is the distance of the applied sample from the center of rotation (4.96 cm) and r is the distance of the sample at time t and ω is the angular velocity of the rotor ($\omega^2 t = 17.4 \times 10^4 \text{ scc}^{-1}$). The $S_{20,w}$ values were calculated from:

$$S_{20,w} = S_{T,M} \frac{n_{T,M}}{n_{20,w}} \cdot \frac{(1-\bar{v}\rho_{20,w})}{(1-\bar{v}\rho_{T,M})}$$

where \bar{v} is the partial specific volume of the protein-SDS complex. $n_{T,M}$ and $\rho_{T,M}$ are the viscosity and density respectively at r_{avg} while $n_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20°C. The viscosities and densities of standard sucrose solutions in H_2O and D_2O were measured by

viscometry and be weighing known volumes respectively. The % (w/w) sucrose was determined from the refractive index and the viscosity and density at r_{avg} was estimated from standard plots of viscosity and density as a function of % (w/w) sucrose respectively.

The partial specific volume (v) was calculated from

$$\bar{v} = \frac{\frac{S_H n_H}{S_D n_O} - 1}{\rho_D \frac{S_H n_H}{S_D n_O} - \rho_H}$$

where subscripts H and D refer to values measured in H_2O and D_2O , s is the determined sedimentation coefficient in a solution of density, ρ , and n , the viscosity determined at the half-distance of travel, r_{avg} .

Calculation of the molecular weight of a protein-SDS complex (287)

The molecular weight of a protein-SDS complex

$$M = M_p (1 + \delta_d)$$

where M_p is the molecular weight of the polypeptide and δ_d is the g of SDS bound per g of protein. The following equation can be applied to protein-SDS complexes:

$$S = \frac{M (1 - v\rho)}{6 \pi n N R_s}$$

where M , \bar{v} , R_s , and s are the molecular weight, partial specific volume, Stokes radius, and sedimentation coefficient respectively, of the protein-SDS complex. The Stokes radius was determined by gel filtration in the presence of SDS. The partial specific volumes of protein-SDS complexes were determined by sucrose density gradient centrifugation in H_2O and D_2O -containing gradients.

Publications

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