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AN ANALYSIS OF THE OUTER MEMBRANE OF *DESULFOVIBRIO VULGARIS* (WOOLICH)

L.KHAI SIEW

presented in partial fulfilment for

the degree of Doctor of Philosophy

to the

Council for National Academic Awards.

June 1987

City of London Polytechnic,

Old Castle Street, London, E1 7NT.

DECLARATION

I, L.Khai Siew, declare that whilst registered as a full-time candidate for the degree of Doctor of Philosophy, at the City of London Polytechnic, I was not registered for any other award of the CNAA or of a University. The work undertaken during this period was carried out by myself at the City of London Polytechnic, with guidance from Mrs. C. Gaylarde at the Polytechnic, and Dr. B. Herbert at the Shell Research Limited, Sittingbourne, Kent.

Advanced studies were undertaken in conjunction with my research programme in partial fulfilment of the degree of Doctor of Philosophy. These included a FEMS course on the Surface antigens of the enteric bacteria at the Institute of Public Health, Helsinki, Findland. I also presented research seminars to the academic staff at the City of London Polytechnic.

I attended several meetings of the Society for General Microbiology, and a meeting of the XIV International Congress of Microbiology. I have presented two poster communications at these meetings.

L.Khai Siew, June 1987.

ACKNOWLEDGEMENTS

I would like to thank Christine Gaylarde and Barry Herbert for their helpful advice and guidance on all matters. My thanks to my parents for financial support.

In particular, grateful thanks to Steve Fewings for his expert advice on all aspects of technical instrumentation, to Phil Cook and Aneal Mandal in the Department of Metallurgy and Materials, Electron Microscope Laboratory for the use of their instruments on those biological samples, Barry Saunderson for running pyrolysis mass spectrometer, to Patrick Foster for his help with photography, and Lee Pullen for help in plasmid DNA analysis.

My thanks to Mei in proof reading the text.

ABBREVIATIONS

BIS	N N′methylene bisacrylamide
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin in tris buffered saline
C+Fe	iron-rich
C-Fe	iron -deprived
EDTA	ethylenediaminetetraacetatic acid
Fe(II)	ferrous iron, (II) indicates the oxidation state of iron
Fe(III)	ferric iron, (III) indicates the oxidation of iron
Fe^{2+}	ferrous ion
Fab	antigen binding fragment of antibody
HPLC	high performance liquid chromatography
LPS	lipopolysaccharides
M.Wt.	molecular weight
Mops	4-morpholinopropanesulphonic acid
NC	modified Postgate's medium C
OM	outer membrane
Omp	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PYGC	pyrolysis gas chromatography
PYMS	pyrolysis mass spectrometric analysis
SDS	sodium dodecyl sulphate
sarkosyl	sodium lauroyl sarcosine
TEM	transmission electron microscopy
TEMED	N N N′N′-tetramethylene diamine
TBS-Tween 20	Tris buffered saline containing Tween 20
Tris	tris(hydroxymethyl) aminomethane

SUMMARY

D.vulgaris (Woolich) OMs can be extracted successfully and reproducibily from the cell envelopes by the selective solubilization of cell membranes by sarkosyl. Three Omps (Omps 1, 2 & 3) occur consistantly in the OMs of C-Fe, NC & C+Fe cells. NC & C+Fe OMs have, in addition, minor proteins absent from C-Fe OMs. Omp2 band is seen to be enhanced in C-Fe and NC OMs as indicated by PAGE. PAGE - immunoblotting of LPS indicates heterogeneity of these molecules.

Immunological analyses of NC, C-Fe & C+Fe cells using specific antisera indicate no differences among these cell-types. Neither are antigenic differences revealed by further analyses of OM Western blots by specific antisera. However, pyrolysis mass spectrometric (PYMS) analyses indicate that the cell-types are different. Further analyses show variations in the cell wall composition. The ability of this technique to differentiate a single bacterial species cultured under different growth conditions indicates its potential as a tool for numerical taxonomy of the sulphate reducers.

D.vulgaris(Woolich) does not produce extra Omps in response to iron deprivation. Instead, it increases the synthesis of Omp2 and LPS in its OM as reflected in the OM PAGE pattern and increased yields of LPS extracted from C-Fe cultures.

HPLC sugar analyses indicate the LPS contain N-glucosamine, possibly rhamnose and an unidentified sugar. A change in HPLC sugar pattern of the C+Fe LPS samples indicates interactions between Fe(II) and LPS. This interaction is substantiated by X-ray microanalysis of the elemental content extracted LPS. The evidence indicates that LPS may be involved in Fe(II) uptake by the cells.

LPS also takes part in the adhesion of *D.vulgaris* (Woolich) to mild steel surfaces. This is demonstrated by the data from experiments using anti-LPS Fab fragments. The presence of these antibodies reduces the number of cells adhering to mild steel coupons.

^{59}Fe-radiolabelling of OM components immobilized on nitrocellulose shows that Omps 1, 2 & 3 bind Fe(II). Blocking experiments using copper & magnesium indicate Omp1 to be Fe(II) specific while Omps 2 & 3 are not. The enhancement of Omp2 observed in C-Fe & NC OMs in PAGE analyses indicates Omp2 to be principal cation binder, whose synthesis is increased under iron deprivation.

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Elution profile of IgG fragments through CMC ion exchange column
Communications

Chapter One

INTRODUCTION

1.1. *Desulfovibrio*

Desulfovibrios are gram-negative motile vibrios found in a variety of aquatic and terrestrial habitats. They belong to a group of microorganisms known as the sulphate-reducing bacteria. Sulphate reducers are unique in that they conduct dissimilatory sulphate reduction, where sulphate acts as an oxidizing agent for the dissimilation of organic matter, as does oxygen in conventional respiration. Dissimilatory sulphate reduction is in many ways similar to conventional respiration. Essentially, it is an oxidative type of metabolism, with two non-oxidative processes; sulphate dissimilation and electron transport, followed by an oxidative process. A generalised scheme of dissimilatory sulphate reduction is illustrated in figure 1. A small amount of reduced sulphur is assimilated by the organism; virtually all is released into the environment as sulphide. They are important in the biological sulphur cycle (figure 2). Their main function in the cycle is to bypass assimilatory sulphate reduction and generate hydrogen sulphide. They do this in sufficient amount to support the growth of sulphide- and sulphur-oxidizing bacteria, creates a microbial ecosystem consisting of interdependent oxidizing reducing

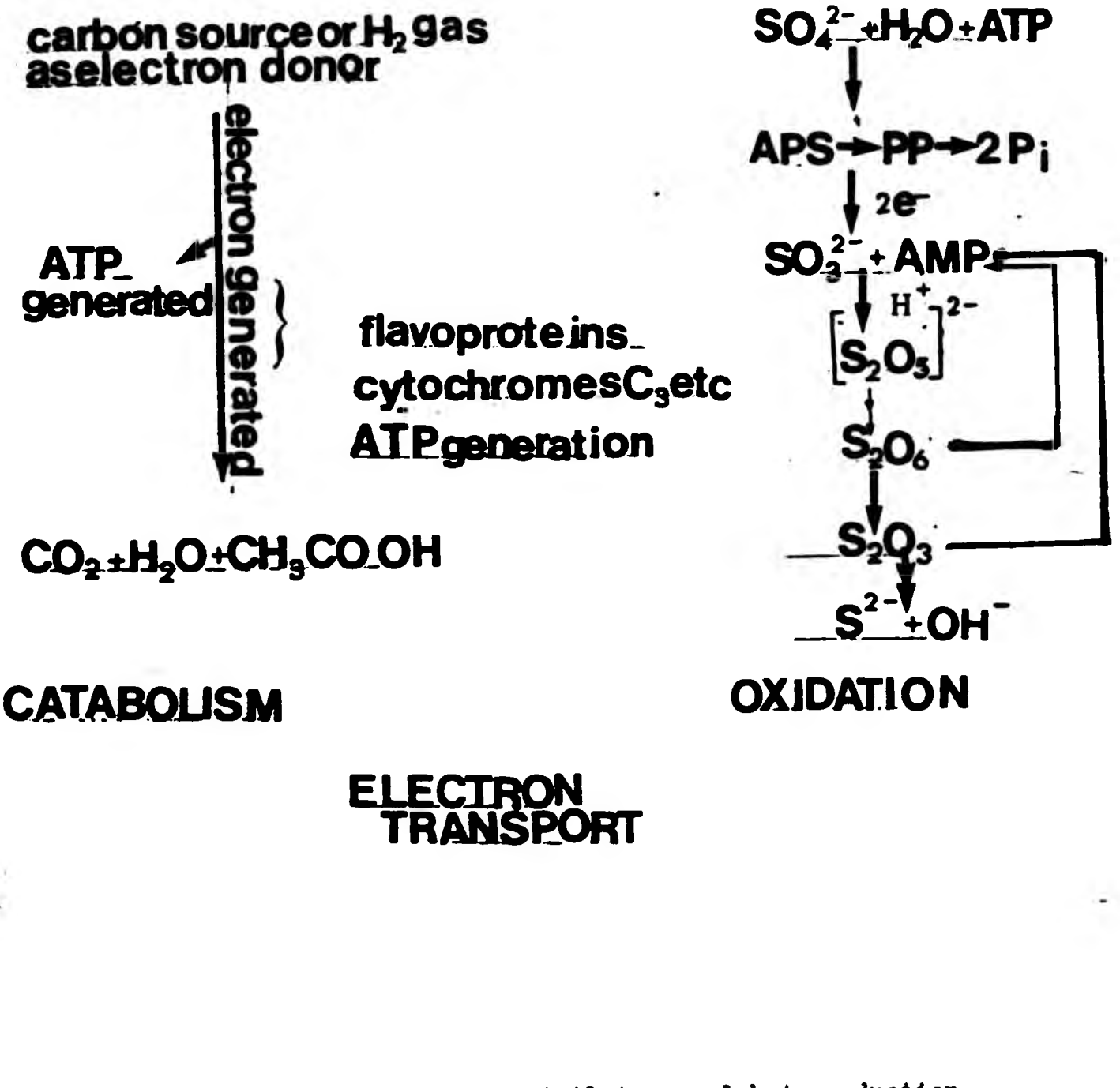
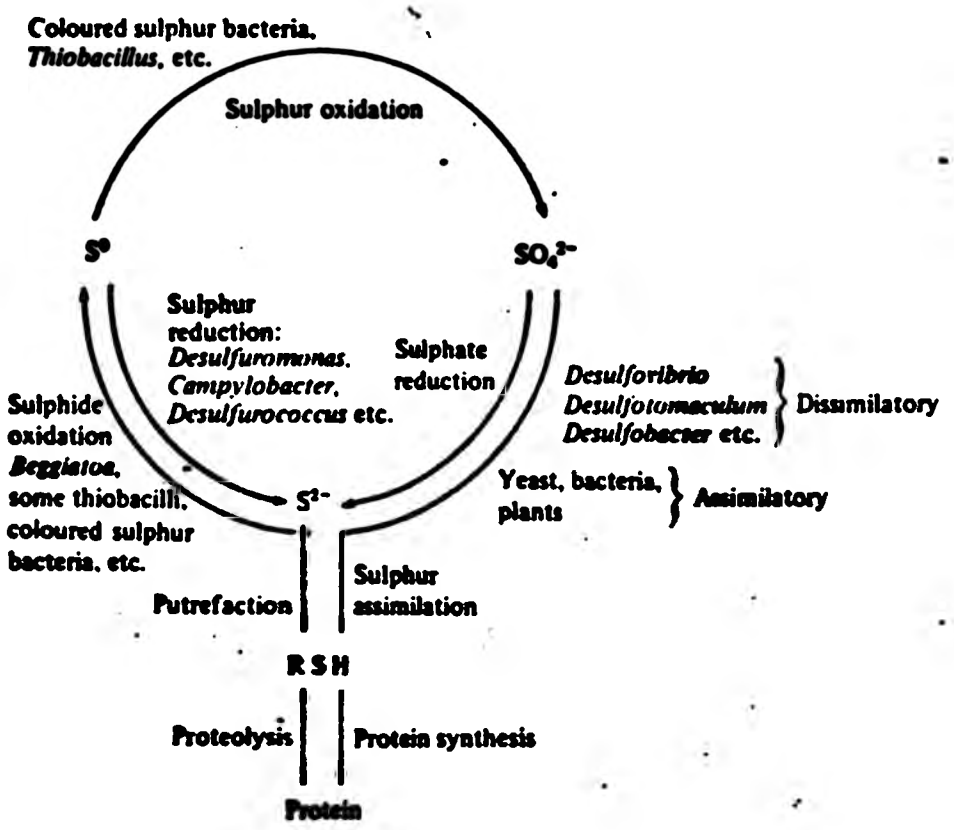


Figure 1. Generalised scheme for dissimilatory sulphate reduction. Carbon catabolism ceases at the acetate level of oxidation, and the oxidative step involves the removal of oxygen atom from sulphate and its reduction to sulphite. ATP is lost at this stage.

(Modified from Postgate, 1984)

Figure 2. The biological sulphur cycle.
(Postgate , 1984)

. The biological sulphur cycle. Sulphur (SO_4^{2-}) is reduced to sulphide (S^{2-}) by dissimilatory sulphate-reducing bacteria and provides substrates for sulphide-oxidizing bacteria which convert it, by way of elemental sulphur (S^0) back to sulphate. In assimilatory sulphate reduction, the sulphur of sulphate passes through the sulphide level of oxidation and becomes incorporated into an amino acid (RSH) before being built into plant as microbial protein. This is eaten by animals and the sulphur is eventually returned to the cycle as sulphide formed during the breakdown and putrefaction (by bacteria) of dead organisms.



bacteria, known as a sulfuratum. Their activities, either direct or through the sulphur cycle, have a variety of ecological and economic consequences.

Ecologically, sulphate reducers alter the environment to favour themselves and other sulphur bacteria by generating a reducing, anoxic environment through hydrogen sulphide generation. Consequently, aerobes are replaced by anaerobes, and organic material will be fermented rather than oxidized. In circumstances like brackish waters, coloured-non-sulphur bacteria and green algae will be replaced by coloured-sulphur bacteria and cyanobacteria, and perhaps thiobacilli. The establishment of sulphate-reducers in a habitat may alter the pH by removing sulphate from alkaline earths, the sulphides of which dissociate to form OH- and free hydrogen sulphide (Postgate,1984).

Economically, the damaging effects of sulphate reducers are enormous. A detailed coverage is beyond the scope of this report. They include tarnishing of domestic instruments, pollution of water and death of fish and birds; damage to human health, tourism and paint work (Widdle,1980). By far the most important is their ability to induce or enhance the corrosion of buried and immersed ferrous metals by their possible consumption of cathodic hydrogen and generation of hydrogen sulphide (Postgate,1984 & Miller & King, 1975). This tends to occur in anaerobic or intermittently aerobic environments such as oil fields and sewage work.

1.2. Corrosion and sulphate reducers

Corrosion of metals in aqueous environments is an electrochemical phenomenon (Miller & King. 1975). Two related, but distinct, processes occur on the surfaces of damp or immersed metals to give rise to the effects collectively referred to as corrosion. These are weight loss, generalised or in form of pitting attack, frequently associated with the formation of insoluble corrosion products which may be closely adherent- and protective to a greater or lesser degree- or loose and bulky. These processes are: i. anodic reaction where a metal passes into solution or water as cations leaving electrons, a negative charge, on the metal, until an equilibrium potential is established between metal and solution that is characteristic of the two. Formation of metal hydroxides or oxides is also possible in such situations. If such equilibrium is upset or not established because of the removal of electrons from a metal, then ionization and hydroxide formation continue. Thus if a base metal like iron immersed in an aqueous system is put in electronic contact with a more noble metal in tne same system, electrons will flow from the base metal (anode) to the less negatively charged noble metal (the cathode).

ii. Cathodic reaction- This depends on the situation. In the presence of oxygen at neutral pH, the electrons that flow to the cathode will be disposed of by the reaction between

oxygen and water to form hydroxide ions. In anaerobic conditions, these electrons react with hydrogen ion to form hydrogen (H_2). In either case, the resultant OH^- reacts with Fe^{2+} when the anode is the iron to form corrosive compounds.

Iron is a fairly base metal and thus easily becomes corroded. However, corrosion is not always due to the presence of a more noble metal in electronic and ionic contact with the iron or steel; variation in composition over surface structures, irregular crystalline structure, occurrance of millscale and other factors may result in surface differentiation into cathodic and anodic zones. Differential anodic and cathodic areas may also occur in metal surfaces immersed in aqueous environments of a high variability in ionic composition resulting in concentration cells. All these electrochemical processes can be initiated or augmented by microbial activities.

Corrosion brought about by sulphate reducers has three characteristics. (1) It occurs in anaerobic, intermittently or partially anaerobic environments. These include clay, waterlogged soils, polluted habours, area underneath marine encrustation, water beneath spirit in petroleum storage tanks, gas holder waters, and enclosed water circulating systems. "Tubercles" of mixed iron oxides on the inside of water pipes can habour sulphate reducers and permit corrosion from inside the pipe. (2) The corroded metals tend to be pitted indicating that corrosion is a non self-stifling process. Thus

breakdown of iron pipes takes the form of local perforation. (3) The metal at the point of corrosion is graphitized with the metallic iron completely removed leaving the graphite skeleton intact.

The mechanism by which sulphate reducers bring about corrosion is controversial. However, contributing factors such as cathodic depolarization of metal surfaces by the bacterial hydrogenase activities and the production of corrosive compounds have been suggested. In cathodic depolarization, the hydrogen film formed through the reaction of water and iron is removed by hydrogenase with a net formation of iron hydroxides and iron sulphide in the corrosion products. Hydrogenase is an enzyme which catalyses the interconversion of hydogen to hydrogen ions and vice versa. Iron sulphide itself is a corrosive compound and accelerates the corrosion process.

Gaylarde and Johnston (1980) showed that *Desulfovibrio vulgaris* adhered to metal surfaces and that this is important for corrosion. There seems to be an interaction between the surface of the organism and that of the metal surfaces.

1.3. Microbial adhesion

Over the last ten years microbial adhesion to submerged surfaces has received growing attention from both physical chemists and microbiologists (Fletcher and Marshall, 1982).

Most bacteria behave as colloidal particles

(Marshall,1980) because of their small size (approx. 0.4 - 4um in length), their density (only slightly greater than water) and their negatively charged surfaces at pH values occurring in natural habitats. Their ionogenic property may be exclusively acidic (carboxyl groups) with the bacteria having zero charge at low pH, or mixed acidic and basic (carboxyl and amino groups) with the bacteria having a positive charge reversal at low pH. These ionogenic properties of bacteria may be modified by the adsorption of multivalent cations, organic materials, or colloids at the surfaces. Thus bacterial cells can be considered in terms of classical colloid chemistry. However, the final analysis of the response of bacteria to conditions existing at solid-water interfaces is a function of both the colloidal and the biological properties of the cells.

Attachment of bacterial cells to surfaces provides cells with shelter thus protecting them from predation and lethal agents such as biocides. Additionally, submerged surfaces become coated with a conditioning layer providing a nutrient supply for growth. Interfaces occur at the surface of submerged solids. Interfaces are thermo-dynamically unstable due to the unsatisfied, potentially interactive surface molecules in both stationary solid and the bulk mobile aqueous phases (Fletcher, 1984). Solutes present in aqueous habitates vary from complex molecules (proteins, polysaccharides and nucleic acids) to simple substances such as sugars, amino acids and ions such as ammonium, nitrate and phosphate. Additionally, calcium, sodium

and hydrogen ions are present. Although nutrient adsorption and interaction at the substratum may be essential in bacterial nutrient accumulation in some situations, the transport of substrates from the bulk phase to the boundary at the interface is rate - limiting. Transport is enhanced by the flow of water and circulation in the bulk phases. Attached cells may be at an advantage if remaining stationary at the surface in a moving aqueous phase, because of the constant replenishment of nutrients and removal of wastes. In low nutrient situations, surfaces may provide an advantage by assisting the capture and/or uptake of scarce nutrients (Fletcher, 1984). *Desulfovibrio* is abundant in many habitats, some being nutrient-poor. The ability of *Desulfovibrio* cells to adhere to surfaces may help the survival of these organisms in such environments.

Microbial adhesion can be defined in terms of the energy involved in the formation of the adhesive junction (Marshall *et al.*,1984). Thus, the strength with which a microbial cell may be said to have adhered to a stratum can be qualified as the work required to remove the cell to its original isolated state. Most solid surfaces assume a net negative charge when immersed in water. Since the bacterial surface is also negatively charged, adhesion requires that the cells overcome the repulsive forces. Cations and a variety of macromolecular and colloidal materials are attracted to the solid-water interfaces. This spontanous adsorption of macromolecular conditioning films alter the charge, free energy and wetting

properties of the immersed surfaces (Baier, 1980). The modified surface charge will be a reflection of the ionogenic groups exposed at the surface of the conditioning film, which is composed of traces of macroorganic substrates. The onset of adhesion of microbial cells to surfaces, both animate and inanimate, and the mechanism by which they adhere, can be influenced directly by these or changes of these conditions. Thus the process depends on the environment. In addition, it has been shown that flow rate influences adhesion (Duddridge, personal communication). The mechanisms by which bacteria adhere to surfaces are non-specific attachment, polymer bridging and polymer consolidation. Non-specific attachment can be achieved by ionic, dipolar, or hydrogen bonding and by hydrophobic interactions. Polymer bridging and consolidation have been demonstrated for marine bacteria by Fletcher and Floodgate (1973). Other surface polymers which take part in adhesion processes have been described. For example, the lipopolysaccharides (LPS) of *Rhizobium* spp. adhere to the lectin of their appropriate legume host cells (Kamberger, 1979), lipoteichoic acids of *Streptococcus* adhere to human buccal epithelial cells (Ofek *et al.*, 1975) and type I fimbriae of *E.coli* attach to fungal, plant and animal cells (Ward & Berkeley, 1980). Adhesion of *Desulfovibrio* cells to metal surface has been reported (Gaylarde & Johnston, 1980). However, the mode of interaction is unknown. The study of the cell surface may contribute to the understanding of adhesion process.

1.4. The outer membrane of Gram negative bacteria

The cytosol of a bacterium is enclosed by a complex cell envelope, which usually consists of a cytoplasmic membrane and a peptidoglycan layer. Gram negative bacteria have in addition to the complex cell wall an outer membrane (OM), located on the outside of the peptidoglycan layer. This OM forms the physical and functional barrier between the cytoplasm and the environment. However, the OM is not always the outermost layer of the cells. Often it is covered by an additional layer (A-layer) consisting of a regular pattern of subunits usually proteinaceous in nature (Lugtenberg *et al.*, 1983). With the recent development of techniques for the isolation of the OM, its composition, structure, function and biogenesis have been extensively studied, especially in *E.coli* and *Salmonella typhimurium*. There is, however, little knowledge of the structure and function of the *Desulfovibrio* OM, the only published work being by Bradley and Gaylarde (1986) and Norqvist and Roffey (1985). The preliminary work of Bradley (1985) showed that the overall organisation of *Desulfovibrio* envelope conforms to that of the gram negative bacteria. i.e. the cell envelope consists of a cytoplasmic membrane, peptidoglycan and the OM.

1.4.1. Composition of the OM

The major constituents of the OM are proteins (including lipoproteins), lipopolysaccharides (LPS) and phospholipids (for

reviews, see Nikaido & Nakae, 1979; Lugtenberg & Van alphen, 1983).

1.4.1.1. Phospholipids

In the *Enterobacteriaceae*, the phospholipid composition of the OM is very similar to that of the cytoplasmic membrane, containing mainly phosphatidylethanolamine, some phosphatidylglycerol and very small amounts of diphosphatidylethanolamine (cardiolipin) (Osborn,*et al.*, 1972). In two species of *Desulfovibrio*, *D.desulfuricans* Norway and *D.vulgaris*, the pattern of phospholipids is typical of gram negative bacteria, consisting of 61-72% phosphatidylethanolamine, 20-21% phosphatidylglycerol and small amounts of cardiolipins. *D.desulfuricans* has in addition, 11% phosphatidylserine (Makula & Finnerty, 1974). For a detailed account of the structure and distribution of phospholipids in bacteria, the review of Goldfine (1982) should be consulted.

In the OM, phosphatidylethanolamine is the major constituent (Osborn *et al.*, 1972; Jones *et al.*, 1977 & Lugtenberg *et al.*, 1983). The relative abundance of this compound is affected by factors such as growth conditions (e.g. temperature, supplementation of medium with fatty acids) and mutations. It has been reported by Lugtenberg *et al.* (1976) that in various strains of *E.coli* the relative abundance of phosphatidylethanolamine changes when the fatty acid composition in the medium is altered. The reason for the enrichment of phosphatidylethanolamine in the OM is probably that it forms a more stable bilayer with LPS (Fried *et al.*,

1978).

The major fatty acids in *E.coli* OM phospholipids are palmitic [C16:0] and cis-vace nic [C18:1] and cyclopropane derivatives (Cronon *et al.*, 1972). Detailed studies have shown that the phosphatidylethanolamine of the OM contains more saturated fatty acids (Lugtenberg *et al.*, 1976) and less molecular species with only two unsaturated fatty acids than its counterpart in the cytoplasmic membrane (Ishinaga *et al.*, 1979). In *Desulfovibrio* OM it is found that there are four major fatty acids; C18:0, anti-iso C17:0, C16:0 and one unidentified (Bradley, 1985).

1.4.1.2. Lipopolysaccharides (LPS)

LPS, characteristic for gram negative bacteria, are amphipathic molecules, located on the outer leaflet of the OM (Nikaido & Nakae, 1979). To date, the LPS of *S.typhimurium* and *E.coli* are probably the most thoroughly investigated LPS and so the results obtained from these macromolecules may form the basis to which results of LPS analyses in other bacterial families may be compared. The LPS molecules have a hydrophobic part, the lipid A, and a hydrophilic part consisting of an oligosaccharide core which is usually substituted by the O antigen (O Ag), the latter being a polymer of repeating carbohydrate units. A schematic representation of the structure is given in figure 3. LPS structures differing from that of *Enterobacteriaceae* have been reported (Flesher & Insel, 1978).

Colonies of strains with or without the O Ag often have a

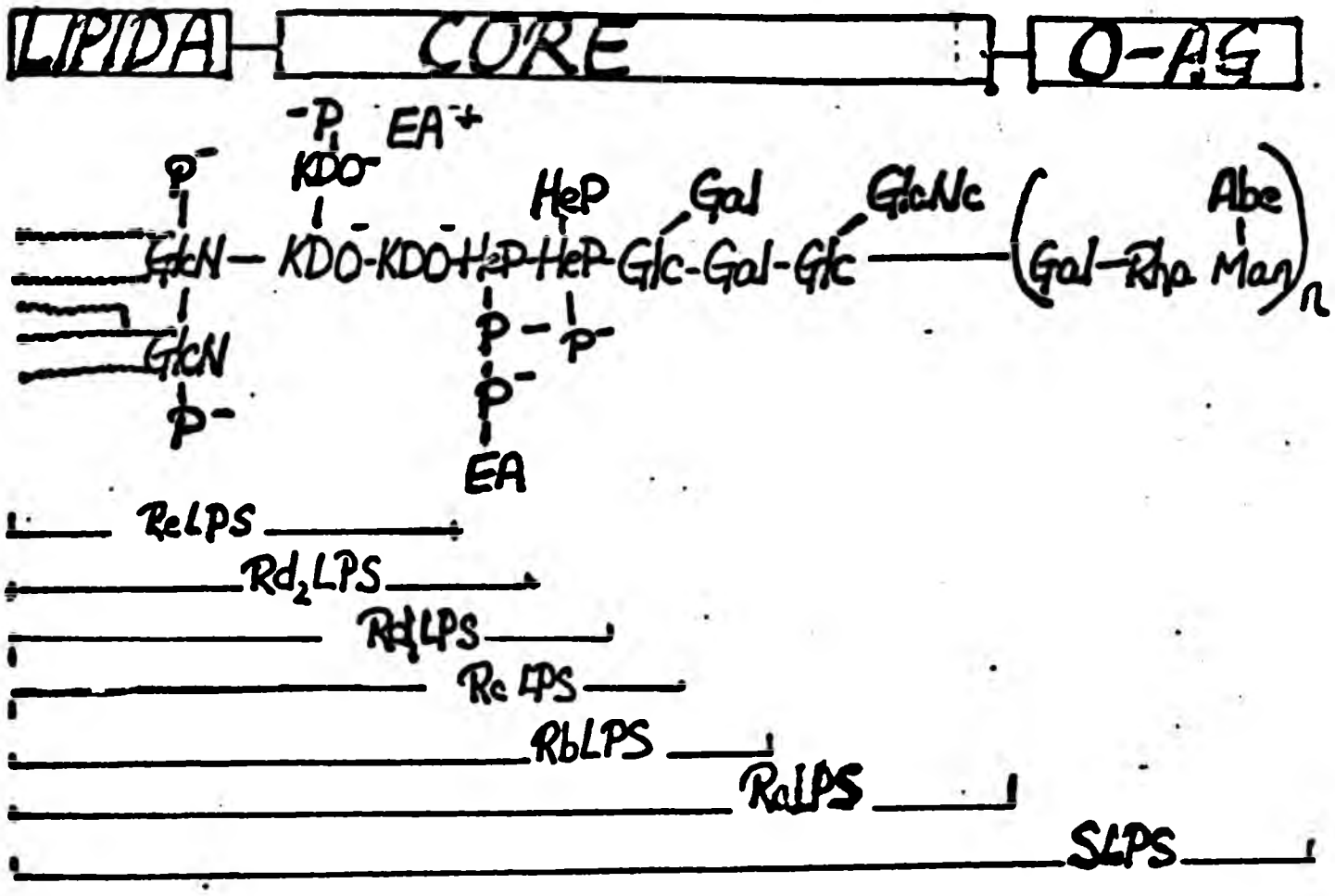


Figure 3. Schematic representation of the chemical structure of LPS from S.typhimurium.
wavy lines = long chain fatty acids (C_8 - C_{16})

n = no. of repeating unit in the O-side chain
S LPS = wild type LPS (smooth)
Ra to Re LPS = mutant chemotypes
Rd to Re LPS = deep rough mutants
P = phosphate group
EA = ethanolamine
Glc = D-glucose
Abe = abequose
Man = mannose
Rha = L- rhamnose
Gal = D- galactose
GlcNAC = N-acetyl-O-glucosamine
Hep = L- glycero-D-mannoheptolose
KDO = 3-deoxy-D-manno-octulosonic acid

smooth (S) or rough (R) appearence respectively. Thus one speaks of S and R strains and of S and R types of LPS. However, there are no reports of rough colonies of *Desulfovibrio*. Among the *Enterobacteriaceae*, the structure of the lipid A-core region is rather well conserved, but that of the O Ag has been subject to evolutionary changes. Many reviews have been written on LPS in general (Galanos *et al.*, 1977; Wright *et al.*, 1971), physical (Shands, 1971) and biological (Orskov *et al.*, 1977; Luderitz *et al.*,1982) properties and on their genetics (Makela *et al.*, 1969; Stocker *et al.*, 1971) and synthesis (Osborn, 1979). Therefore a brief description only will be presented here.

1.4.1.3. Lipid A

The lipid A of *Enterobacteriaceae* is a glycolipid which contains a *B*-(1,6) linked glycosamine disaccharide unit which carries a phosphate residue in position 1 and a phosphate or pyrophosphate residue in position 4' and also approximately six fatty acyl chains (Rosner *et al.*, 1979; Wollenweber *et al.*, 1982). Ligands such as glycosamine, ethanolamine and various ions can be attached non-convalently. Among the fatty acids, *B* hydroxy acids are abundant. Most of the fatty acids present in the lipid A are relatively short chained [C8-C14]. The amide-linked *B*-hydroxy fatty acids are specific to LPS.

Lipid A backbones different from that of the *Enterobacteriaceae* do exit. For instance, in *Chromobacterium* there is extensive substitution of the phosphate groups (Galanos

et al., 1977). Thus the lipid A backbone contains a chain of four amino sugars which are linked together by one glucosidic linkage and two phosphate bridges, and the absence of a non-reducing end is due to the trihalose-like structure of terminal - GlcN'- -→-P-←-GlcN unit.

1.4.1.4. Core region

The LPS core region in *Enterobacteriaceae* contains 3-deoxy-D-manno-octulosonic acid (KDO) and L-glycero-D-mannoheptolose sugars, both of which are specific to LPS. In addition, it contains a number of more common sugars like glucose, galactose and N-acetyl-D-glucosamine (Galanos *et al.*, 1977; Osborn, 1969; Luderitz *et al.*, 1971). *Desulfovibrio* LPS apparently does not contain KDO (Bradley, 1985). Other species lacking KDO are known.Within the *Enterobacteriaceae* mutants completely missing KDO can be isolated, this is conditionally lethal (Lehmann *et al.*, 1977; Lehmann *et al.*,1977a). The core structure described above is probably characteristic of *Enterobacteriaceae*, since core structures without either KDO or heptose or both have been found. Examples are *Actinobacter* spp., *Pseudomonas* spp., *Spirillum serpens* and *Vibrio cholera* (Galanos *et al.*, 1977). More recently, Howard and Buckley (1985) reported that the LPS of *Aeromonas hydrophilia* had no KDO.

1.4.1.5. O-side chain (O-antigen, O-Ag)

The O-side chains contain the immunodeterminant structures against which the anti-O antibodies formed during infection or

immunization are directed (Luderitz *et al.*, 1966). Each bacterial serotype synthesises a unique LPS, characterized by a specific composition and structure of the O-chain and by an individual O antigenicity, a property used in the serotyping of strains and substrains. The side chains are composed of repeating units of identical oligosaccharides (Robbins & Uchida, 1962). The LPS is heterogenous due to the different constituents in the repeating units. In some cases, the repeating units may contain an oligomer of a single type though in a distinct linkage sequence, so that repeating units can still be recognised. In these cases, the O-chain represents the homopolysaccharide such as mannan in *E.coli* O9 (Prehm *et al.*, 1976). The number of units may vary within cells from a single culture from one to more than forty (Goldman & Leive, 1980; Munford *et al.*, 1980; Palva & Makela, 1980). *Desulfovibrio* has been reported to contain a particularly short O-side chain (Bradley, 1985). The O-Ag sugars can be substituted with O-acetyl groups, phosphate, amino acids, or even ethanolamine triphosphate. So the O-chains contribute to the net surface charge of the bacterial cells and must be important in adsorption of cells to charged particles and surfaces. Some LPS molecules have been shown to contain no O-chains (eg. in *E.coli* K12; Prehm *et al.*, 1976).The production of O-chains within a given strain can be influenced by the growth conditions McConnell & Wright (1979) and their structure and composition can be altered by certain prophages (Losick &

Robbins, 1967) and plasmids (Derylo *et al.*, 1975).

1.4.1.6. Proteins

The protein composition of the OM is very diverse, but unlike the cytoplasmic membrane it is poor in enzyme activities (Osborn *et al* ., 1972). The study of OM proteins was made feasible by the development of techniques for OM isolation and sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE). The following description is a brief survey of Omps (outer membrane proteins), mainly from the enteric group of bacteria.

1.4.1.7. Enzymes

Although few have been reported, enzymes are present in the OM. The first enzyme to be isolated, in *E.coli* OM, was a phospholipase A with a molecular weight of 28000 (Scandella *et al.*, 1971; Nishijima *et al.*, 1977). Lysophospholipase, lysophosphatidic acid phosphotase and UDP-glucose hydrolase were subsequently found to occur in this membrane by Osborn *et al.*(1974). Proteolytic activities are also found. More recently, an alkaline phosphatase has been reported to occur in the OM of *Lysobacter enzymogenas* (Tigerstrom & Stelmaschik, 1986). Enzymes located in the OM have been reported to be responsible for, among other things, conversion of alkaline phosphatase to the mature form (Inouye & Beckwith, 1977), modification of the ferric enterobactin receptor (Fiss *et al.*, 1979) and cleavage of bacteriophage precoat protein to coat protein (Wicker, 1980). However, the number of enzymes

responsible for such activities present in the OM is unknown (Lugtenberg *et al.*, 1983). A peptidoglycan hydrolytic enzyme, N-acetylmuramyl-L-alanine amidase has been suggested to be loosely associated with the OM by Wolf-Normark (1976) and Heller (1979) has found a OM-bound penicillinase in *Serratia marcescens* OM. No enzymic activities have been reported, however, in *Desulfovibrio* OM.

1.4.1.8. Lipoproteins

Braun's lipoprotein was the first lipoprotein to be purified from *E.coli* and was investigated by Braun *et al.* (1969). It contains 58 amino acid residues and is covalently bound to the carbonyl group of every tenth and twelfth diaminopimelic acid residue of the peptidoglycan through the *E*-NH group of its C-terminal lysine residue. However, a majority of these proteins occur free within the membrane (Inouye *et al.*, 1972). These proteins are not essential for the survival of the bacteria. However, mutants with a deletion of lipoproteins are unable to grow in the rod shaped form and require high concentrations of magnesium and calcium ions, suggesting that these proteins are needed for the maintenance and determination of the rod shape, in stabilization of the OM and in anchoring the OM to the peptidoglycan layer (Sonntag *et al.*, 1978, Wensink & Witholt, 1981). The lipoproteins are rich in *L*-helix structures (Braun *et al.*, 1976, Lee *et al.*, 1977),

with all the hydrophobic amino acid residues regularly arranged in an alternating 3 to 4 pattern of repeating units. As 3.6 residues make up one turn, all the hydrophobic residues can be aligned as two series on one face of the helical rod (Braun, 1975, Inouye, 1974) (see figure 4). Presumably this is important in molecular interactions within the membrane.

Lipoproteins have also been found in non-enteric organisms like *Ps. aeruginosa* (Mizuno *et al.*, 1979) and *Rhodopseudomonas sperοides* (Baumgartner *et al.*, 1980). Probably they are normal components of the OM and may be expected to be present in *Desulfovibrio*.

Peptidoglycan-associated lipoproteins are closely but non-covalently associated with peptidoglycan and have been found to occur in *Proteus mirabilis* (Mizuno, 1979) and *E.coli* (Ichihara *et al.*, 1981).

1.4.1.9. Omp A protein

Outer membrane protein A, found in *E.coli*, is a heat modifiable protein in that its apparent molecular weight on PAGE gels is higher in the heat-modified form than in the non-denatured form. It has a high *B*-structure content (Nakamura *et al.*, 1976). It acts as a receptor in F-pilus mediated conjugation (Havekes *et al.*, 1976) and its function here is to stabilize the mating conjugates (Achtmam & Skurray, 1977). Together with Braun's lipoprotein, Omp A protein is somehow involved in maintaining both the structural integrity of the OM and rod shape of the bacteria (Sonntag *et al.*, 1978). From

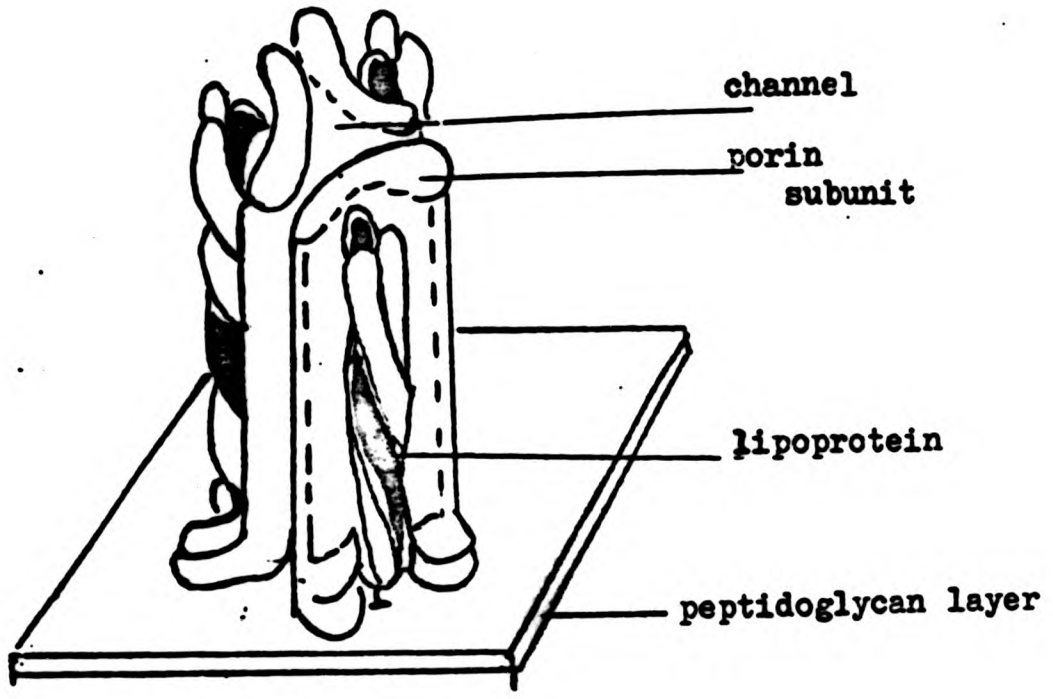


Figure 4. Illustration of the lipoprotein in association with pore protein e.g. OMP A. (Di Rienzo et.al., 1978)

enzymatic cleavage experiments Bremer *et al.* (1982) proposed that it has two domains, the N-terminal domain (180 residues) located in the membrane and the second domain of 55 residues located in the periplasmic space.

1.4.1.10. Peptidoglycan-associated general diffusion pore proteins

This is a large family of proteins found in enteric bacteria. They may occur in the OMs of other bacteria such as *Neiserria gonorrhoae* (Douglas *et al.*, 1981). They may include those proteins induced by certain growth conditions (Overbeeke *et al.*,1980; Hancock *et al.*, 1980) or coded for by a prophage (Reeves *et al.*,1979; Schnaitman *et al.*, 1975) or by plasmid (Iyer, 1979; Achtman *et al.*, 1977a). These proteins function as general pores. They were discovered by the purification of the OM components responsible for the generation in phospholipid - LPS liposomes of aqueous pores through which molecules with a molecular weight of about 600 daltons could pass. Examples of such molecules are galactose, glucosamine, glucose-1-phosphate, and lysine. However, these proteins are impermeable to polyethylene glycol with a molecular weight of 1540. These proteins were named porins by Nakae (1976). They occur in the OM as trimers spanning the bilayer (see figure 5). Porins are rich in *B*-structures (Schnaitman, 1974; Tokunaga *et al.*, 1979) and bind relatively little SDS (Schnaitman, 1974; Nakae, 1976a; Tokunaga *et al.*, 1979). They act as non-specific molecular sieving channels through which hydrophilic solutes

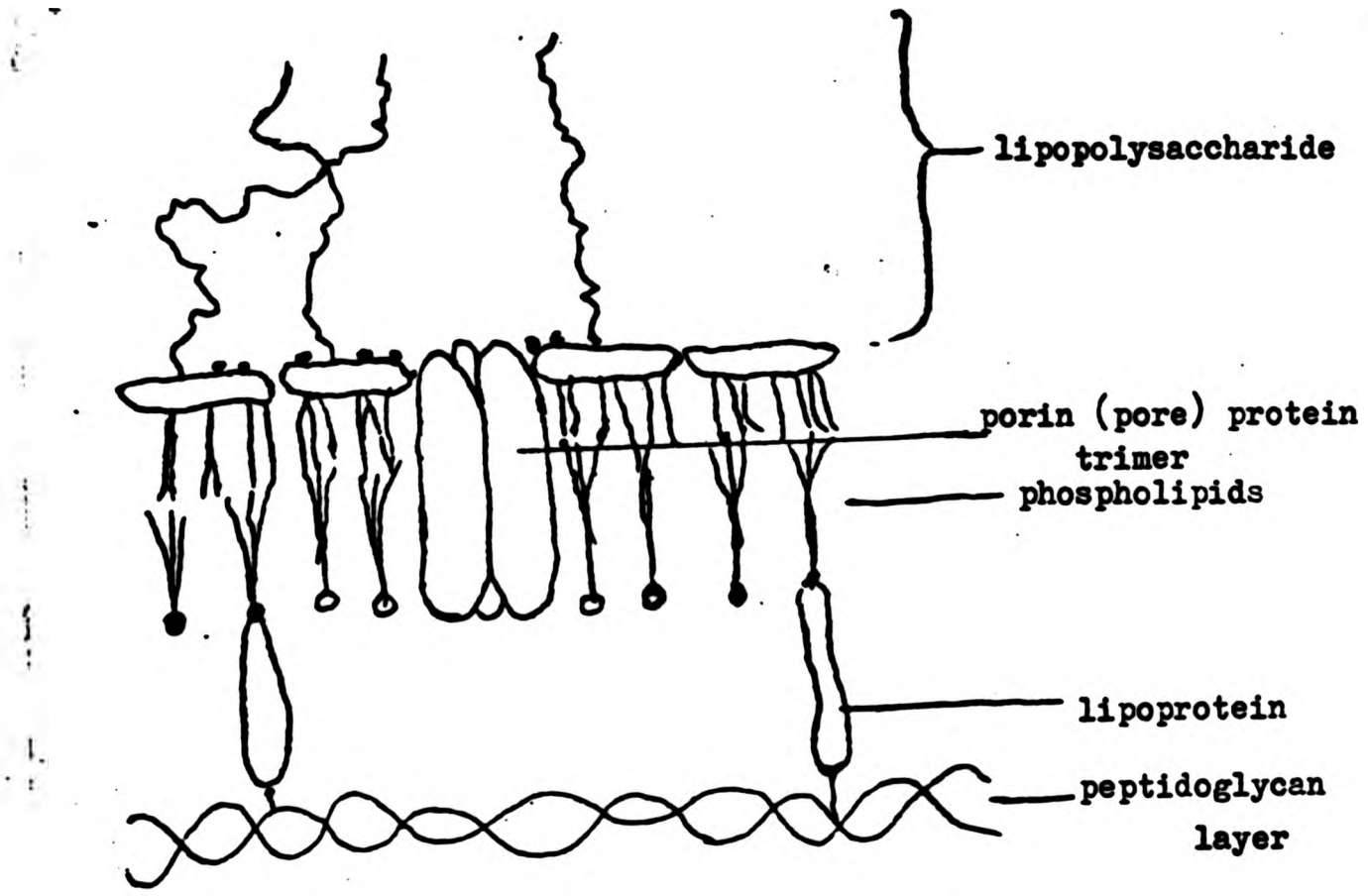


Figure 5. Position of pore protein trimer in the membrane.

diffuse. In *Enterobacteriaceae* the size limit for solutes is 600-700 daltons (Nikaido, 1979). In *Pseudomonas* spp. the exclusion limit is approximately 6000 daltons (Hancock & Nikaido, 1978; Hancock *et al.*, 1979). This probably reflects the ability of *Pseudomonas* to utilize a large variety of nutrients including high molecular weight compounds.

Some channels, for example those formed by PhoE proteins in *E.coli*, have a preference for certain substrates due to the presence of recognition or binding sites, presumably at the entrance of the pore (Overbeeke & Lugtenberg (1982). PhoE is induced in wild-type cells by phosphate limitation (Overbeeke *et al.*, 1980) as a component of a series of proteins to scavenge traces of phosphate from the environment (Wanner *et al.*, 1981).

1.4.1.11. Proteins with specific substrate-binding capability

These include vitamin B12-binding protein and siderophore-binding proteins which take up chelated ferric iron ions in *E.coli* (Neilands, 1982). Another such substrate-specific pore protein is LamB, specific for maltose and maltodextrins.

1.4.1.12. Protein associated with adsorption to surfaces

Recent work shows that there is a family of proteins in *N.gonorrhoeae*, namely protein II, which contribute to the specific attachment of the bacteria to cell surfaces (Watt, 1980). Whether the OMPs in *Desulfovibrio* contribute to the attachment of *Desulfovibrio* cells to metal surfaces is not known.

To summarize, known functions of OM proteins are:-

i. enzymic activities (few)

ii. stabilization of OM structure (OmpA & lipoproteins)

iii. receptor activity (OmpA in conjugation: lambda receptor protein)

iv. membrane permeability: non-specific pores (porins)

v. specific uptake systems (siderophore receptors; T6-receptor protein

vi. specific attachment to surfaces (protein II of *N.gonorrhoaeae*).

1.4.2. Structural organization of the outer membrane

The OM composition just described may be altered by factors such as genetic mutation and growth conditions. Genetic mutations involving loss or reduction in amount of a protein are compensated by an increase in the production of other constituents such as LPS (Gmeriner & Schlecht, 1980, Van Alphen *et al.*,1977 & Schweizer *et al.*, 1976), phospholipids (Smit *et al.*, 1975 & Van Alphen *et al.*,1977), and of other proteins (Van Alphen *et al.*, 1976, Chai & Foulds, 1977). Growth conditions such as availability of iron will alter the protein profile of the OM. This has been reported in a pathogenic strain of *E.coli* (Griffiths *et al.*, 1983) and in *V.vulnificus* (Chart & Griffiths, 1985). Such abnormalities will lead to a reorganization of the OM components. Nevertheless, the overall structure still conforms to the fluid mozaic model of biological membranes.

On the basis of experimental evidence available thus far, the OM appears to be an assymmetrical bilayer with LPS and phospholipid molecules as the major constituents (figure 6). The majority of phospholipids form the monolayer of the OM inner face (Kamio & Nikaido, 1976, Nikaido *et al.*, 1977), while LPS occurs exclusively on the outer face (Muhlradt *et al.*, 1975). The LPS molecules anchor themselves in the membrane by hydrophobic interactions between the lipid A portion and the phospholipids, leaving the hydrophilic portion protruding out of the membrane. It seems that LPS occur in clusters, since newly synthesized molecules do not mix with old ones (Leive, 1977).

As for proteins, the OmpA trimers span the OM, are partially exposed at the surface and at the same time protrude into the space between the OM and the peptidoglycan Furthermore, they are found to be associated with lipoproteins and may also interact with the peptidoglycan layer (Sonntag *et al.*, 1978). Other trimeric pore proteins, such as LamB, form, together with the LPS, wedge- shaped structures which are embedded in the OM. It is not known whether they interact with peptidoglycan. Some lipoproteins have been shown to interact with other proteins (e.g. OmpA). However, the majority of lipoproteins occur in the free form, only one third of them being covalently bound to the peptidoglycan. Antibodies directed against these proteins do not react with intact wild-type cells (Braun *et al.*, 1976), indicating that

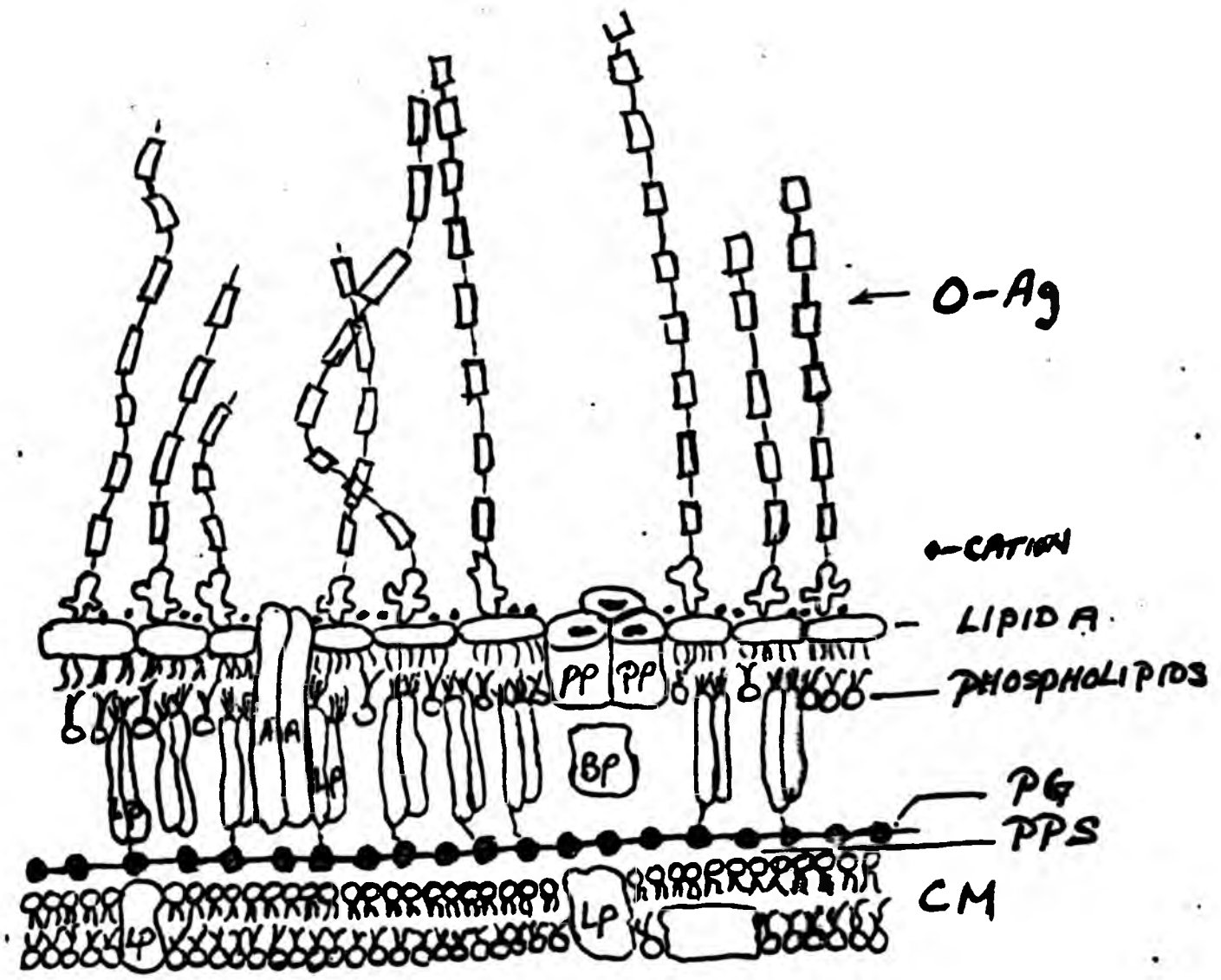


Figure 6. Molecular organization of the outer membrane of Enterobacteriaceae (wild type).
The likely positions of the OM constituents are schematically illustrated. Only three types of proteins have been shown, namely pore protein, Omp A and lipoprotein (LP). The pore protein drawn here is LamB protein (PP). Although it has been drawn without showing any interaction with peptidoglycan, such an interaction cannot be excluded. O-antigen side chains of the LPS are much longer than visualized, although some may occur as short chains. Other aspects of the cell envelope like the periplasmic space (PPS) with a nutrient-binding protein (BP), the peptidoglycan layer (PG) and the cytoplasmic membrane (CM) with a carrier protein (CP) involved in transport have also been drawn.

(From Lugtenberg et al., 1983)

lipoproteins are not exposed at the cell surface.

The integrity and correct functioning of the OM relies on the interaction of these components and ionic interactions between LPS and external cations.

Table 1 summarises the current knowledge of the OMs of sulphate reducers and other gram-negative bacteria.

1.4.3. Aims of this study

The economic effects of sulphate reducers have led to an accumulation of knowledge of their physiology. However, little is known about their OMs. Sulphate reducers have a high requirement for iron for growth, and thus, an efficient iron uptake system must be present. Studies in pathogenic organisms such as *E.coli* show that these bacteria have an elaborate enzyme system for the synthesis of siderophores and their receptors in the OM in response to iron limitation.

This investigation aims to answer the following questions:-

1. Can *Desulfovibrio* grow in iron limited conditions?
2. Do they exhibit phenonmena observed in other gram-negative when subjected to iron limitation?
3. Any iron-specific receptor proteins present in the OM? If so, which protein species is responsible for iron binding?

It has been noted that LPS in the OM of *E.coli* interact with metal cations (Schindler & Osborn, 1979). The present study also aims to address the following questions:-

4. Do LPS molecules play a role in iron uptake? If so, in what

Table 1 Summary of the current knowledge of the OMs of sulphate reducers and other gram-negative bacteria.

OM components	non sulphate reducing gram-negatives	sulphate reducers
LPS	studies in E.coli and Salmonella well established heterogenous in polysaccharide chain lengths, endotoxins	?
phospholipids	in enteric bacteria phosphatidylethanolamine phosphatidylglycerol diphosphatidylethanolamine	D.vulgaris phosphatidylethanolamine phosphatidylglycerol diphosphatidylethanolamine D.desulfuricans Norway as above and phosphatidylserine
proteins		
enzymes	B-latamase (enteric bacteria) penicillinase (S.marcesens) phospholipase A (E.coli) alkaline phosphotase (L.enzymogens)	?
lipoproteins	present in E.coli " " Ps. aeruginosa " " Proteus mirabilis	?
protein involved in F-pillus conjugation	Omp A (E.coli)	?
general pore proteins	OmpC & OmpF (E.coli K 12) protein I (N.gonorrohoae)	?
derepressable pore proteins	PhoE in E.coli FeC & FepA in E.coli	?
substrate specific pore proteins	LamB - maltose, maltodextins vitamin B_{12} binding protein in E.coli	?
protein for attachment	protein II in N. gonorrohoae	?

chemical structure does the iron-specific binding of LPS reside?

5. In view of the importance of adhesion in the corrosion process are LPS molecules involved in the adhesion of *Desulfovibrio* to ferrous metal surfaces ?

Chapter Two

MATERIALS AND METHODS

Chemicals used in this project were purchased, except where specified, from BDH.

2.1. Cultivation and growth

The species used in this investigation was *Desulfovibrio vulgaris* strain Woolich (NCIB 8451). This was originally isolated from slime covering corrosion test specimens in the River Thames at Woolich, London, in 1960. The organism was grown routinely in modified Postgate's medium C (NC) (See table 2). In the "iron-deficient" cultures, the cells were cultivated in this basic medium without added iron and citrate, giving C-Fe cultures. In some cultures, a 4 x 1.5cm mild steel coupon was added to the basic medium to give the iron-rich (C+Fe) cultures. Maintenance cultures were grown in either of the above modified media in 20ml Universal bottles at 30°C. For extraction, cells were inoculated from the maintenance cultures into 500ml batch bottles and incubated at 30°C for 4 days to a late log phase. With the C+Fe cultures, the inoculum size was 10ml, whilst with the C-Fe cultures, the inoculum size was as much as 30ml. Presumably the presence of H_2S from the inoculum generated a redox potential of approximately -100mA

Table 2. Modified Postgate's medium C

KH_2PO_4	0.50g
NH_4Cl	1.00
$CaCl_2$. 6 H_2O	0.06
$MgSO_4$. 7 H_2O	0.06
Na_2SO_4	4.50
sodium lactate	6.00
sodium tricitrate	2.50
yeast extract (Oxoid)	0.50
$FeSO_4$ .7 H_2O	0.004
distilled water	1 litre

The pH was adjusted to pH7.7. After autoclaving at 121 C for 15 minutes, the pH dropped to pH7.5.
This is a pale yellow, transparent medium.
The amount of yeast extract and citrate used is half the amount suggested by Postgate. However, recently in his new monograph on sulphate reducing bacteria (Postgate, 1984), he suggested that the sulphate reducers in laboratory growth conditions can tolerate a citrate level as low as 0.3g/litre. Hence the majority of the cultures were grown at this concentration. -

Despite the use of these recommended medium, sometimes growth does not occur (see results).

to allow growth. Sulphate reducers require a redox potential of at least -100mA for growth to occur (Postgate,1984). Purity checks were made by plating out on to nutrient agar plates at inoculation and incubating both aerobically and anaerobically. Plates were observed for growth over a period of ten days. Bacteria numbers were determined by haemocytometer counts, as viable counts were unreliable.

2.2.1. Outer membrane preparation

The study of the outer membrane (OM) was made possible after Miura and Mizushima devised their buoyant density method in 1969. In their study, *E.coli* cells were disrupted by osmotic lysis of lysozyme-ethylenediaminetetraacetic acid (EDTA) spheroplasts and membranes separated out by equlibrium sucrose density gradient centrifugation. The OM fraction, being denser, occurred in the lower gradient band. This method was closely reviewed by Osborn *et al.*, in 1972 and this became the standard reference method for OM isolation from Gram negative bacteria.

Another method for fracturing cells is mechanical rupture by the French pressure cell, originally developed by Schnaitman (1970). In this method, intact cells are disrupted by shear and tear forces and the OM-peptidoglycan complex separated by equilibrium gradient centrifugation. Large quantities of materials can be handled by this method and discontinuous density gradient steps can be used. However, EDTA is still present in the gradient and this may interfere with subsequent analytical studies. Both Osborn and Schnaitman's methods are

applicable to Gram negative bacteria with reproducible results, particularly with *E.coli* and *S. typhimurium*. Sometimes the OM can be purified by cell disruption and repeated centrifugation. This method has been used by Thornly *et al.*(1973) for *Actinobacter* spp. and by Hurlbert *et al.*,(1983) for *Ps.syringae*.

In the case of *Desulfovibrio*, the Osborn method is unworkable, despite the efficient formation of spheroplasts even on varying the method by the addition of lysozyme, spheroplast lysis by osmotic shock or lengthening of the gradient system (Bradley, 1985). However, the OM can be isolated from the cells by other means. These are based on treatment with detergents such as Triton X 100 and sodium lauroyl sarcosinate (Sarkosyl). Filip *et al.*, (1973) reported that Sarkosyl could selectively solubilise the cytoplasmic membrane of certain Gram negative bacteria and this has since been used by many workers (Spratt, 1977; Lambert & Booth, 1982). Based on this principle, the OM of *Desulfovibrio* has been successfully isolated by Bradley (1985). The method adopted here is a modified version of that used by De Pamphilis (1971) employed by Bradley (1985).

Cells were harvested from 1 litre batch cultures at 3000g for 30 minutes. They were washed in 100ml cold Mops buffered saline (0.15M NaCl, 50mM 3-N-morpholino propane-sulfonic acid) pH 7.4 and the pellet after centrifugation resuspended in 20ml Mops buffer and sonicated in an MSE soniprep 150 for 10 x 1

minute bursts at 16u amplitude with 30 second intervals. Any unbroken cells and remaining culture debris was removed by centrifugation at 3500g for 10 minutes. The supernatant was incubated with 20%w/v Sarkosyl (Sigma)(1ml per 10ml supernatant) at room temperature for 30 minutes with occasional mixing. The unsolubilised OM was pelleted at 35000g for 30 minutes and washed in buffer. The final pellet was suspended in a small volume of distilled water, freeze-dried and stored at -70°C if necessary.

To investigate whether ferrous ions interfered with the solubilisation of the OM, the above method was modified as follows:-

The washed cells were resuspended in 20ml Mops,vortexed and the suspension split into two equal portions. To one portion, 1mg ferrous sulphate and 1mg sodium ascorbate were added, while to the other portion only sodium ascorbate was added. Each suspension was mixed and incubated either on ice or at room temperature for 15 minutes, (see section 4.1.) after which the cells were sonicated and treated as above.

2.2.2. Lipopolysaccharides extraction

Lipopolysaccharides (LPS) can be extracted from the OM complex by various mild procedures (Wilkinson, 1977). However, the efficiency of extraction depends on

a) the species of organism

b) the nature of the starting materials (e.g., cell walls or whole cells)

c) the method used.

An extract starting from intact cells will inevitably be contaminated with other cellular components such as nucleic acids and glycan. Hence it is better to extract LPS from isolated cell walls or cell envelopes. With isolated cell envelopes, there may be incomplete dissociation of the LPS from the complex (Geyer *et al.*, 1979). Thus it is best to use isolated cell walls.

Extraction with cold aqueous trichloracetic acid yields LPS complexed with protein and phospholipid. This complex can be split with hot aqueous phenol, the LPS being located in the upper aqueous phase on cooling. The hydrophobic portion will remain in the lower phenol phase. This is the most effective method for obtaining LPS almost free of contaminants. However, some lipophilic LPS such as those of rough mutants with short O side chains may remain in the phenol phase. For increased yields of such LPS, the selective phenol-chloroform-petrol method of Galanos *et al.*,(1969) can be used.

In this investigation, cell walls were prepared from one litre batch cultures as described in 2.2.1. The cell wall pellet was placed in 45% w/v aqueous phenol for 10 minutes at 68°C with occasional mixing. At this temperature, the mixture existed in one phase. After incubation, the mixture was allowed to cool to 4°C at which temperature a biphasic system formed. The upper aqueous phase was removed and dialysed

exhaustively against distilled water to remove the phenol. The extract was freeze-dried and stored at -70°C for use.

Although LPS extracted by this method are relatively pure, it may contain cations such as sodium, potassium, calcium, and, in the case of *Desulfovibrio*, ferrous ions. It is known that extracted LPS irrespective of the extraction method used contains cations and other low molecular weight molecules. To improve purity, it is necessary to remove such molecules and this could be achieved by electrodialysis (Galanos & Luderitz, 1975).

Some of the LPS in this study were subjected to electrodialysis prior to further analysis.

2.2.3. Electrodialysis

The LPS solution (3-5mg/ml) was placed in dialysis tubing with both ends sealed. The tubing was put in the electrophoresis tank containing distilled water. Heat is generated during the course of electrodialysis and so the apparatus was cooled by circulating ice-cold water. The cooling water was provided by a rubber tubing connected to a mini motor pump and the tank, with the tubing coiled inside an ice bucket containing ice. Water was changed 2-3 times in the electrophoresis tank during the procedure. The progress was monitored by current changes which altered from 12-20mA to 4-5mA at the end of the process (4-4.5 hours). LPS were then removed from the tubing and hydrolysed for sugar analysis or used for other purposes.

2.2.4. Plasmid isolation

Plasmid isolations were performed according to Cannon (1980) with some modifications. Cells (500ml) were grown to early log phase. Absolute ethanol (15ml) containing 0.501g chloramphenicol was added to the culture. After overnight incubation, the cells were harvested at 3000g for 30 minutes and washed three times in a weighed tube in 1 M TrisHCl pH8. The pellet was resuspended in 1 M TrisHCl pH8 and freeze-dried. For extraction, 100mg of cells were suspended in 0.5ml 1M TrisHCl pH8 in a sterile 50ml beaker and 9.5ml of freshly prepared lysing solution pH12.5 (0.5g SDS in 95ml of 50mM EDTA pH8 , pH adjusted to 12.5 with 3M NaOH) was added to the suspension. This was stirred for 1.5 minutes and incubated in a water bath at 34°C for 2.5 minutes. A small amount of proteinase K (Sigma, kind gift of Dr. Brownson, Dept. of Biological Studies, City of London Polytechnic) was added to the mixture and this was incubated overnight at room temperature. A volume (0.6ml) of 2 M TrisHCl pH7 was added and the solution stirred for 1.5 minutes. Then 320mg of NaCl were added and 10ml of phenol previously saturated with 2mM Tris and 2mM EDTA pH8. The mixture was centifuged at 2000g at 4°C for 10 minutes. The upper phase was drawn off and placed in a 50ml beaker, 1/10 volume of 3M sodium acetate pH8 added and then 2 volumes absolute ethanol at -20°C. The mixture was incubated overnight at -20°C. the white precipitate which formed was removed by low speed centifugation (1000g) at 4°C. The plasmids

were obtained by centifuging the supernatant at 12000g at 0°C for 10 minutes. After decanting the supernatant, the pellet was resuspended in 100-200ul of Tris-sodium EDTA saline pH8. Before resuspension, the tubes were drained on paper towels for 30 minutes. The DNA was ready for electrophoretic analysis.

2.2.5. Antibody production

New Zealand white rabbits were immunized as follows:-

a) Immunogen = intact cells with adjuvant

Three rabbits were primed with a dose of 10^9 cells in Freund'complete adjuvant (FCA, Difco) administered by subcutanous route (s.c.) in the haunches. Each rabbit was primed with a specified cell-type. The response was monitored 2 weeks after priming by the serial doubling dilution tube test. (See Section 2.3.3) A further booster was given at this time by s.c. injection of 10^9 cells in Freund's incomplete adjuvant (FIA). The antibody titre in the serum was monitored by further tube agglutination tests and when hyperimmunization was achieved no further immunization was given. The serum was stored at -20°C for further use.

b) Immunogen = extracted C-Fe LPS

1. LPS (5mg) was reconstituted in distilled water and injected into a rabbit s.c. in each haunch. The response was tested by the interfacial ring test and gel diffusion 2 weeks after the challenge. The omission of the adjuvant in the immunogen preparation was to check for activity of the LPS alone. It was known that LPS molecules were potential endotoxins. No adverse

reaction was observed.

2. LPS (5mg) was reconstituted in 1ml of distilled water. To this, FIA (1ml) was added slowly with agitation until the mixture became creamy in appearance. The mixture was injected into a rabbit s.c. in each haunch. The response was monitored by gel diffusion and slide agglutination (Hudson & Hay, 1980).

3. In this protocol, FIA(1ml) was added slowly with agitation to the LPS solution containing 1ug ferrous sulphate. The final mixture was injected into the rabbit's haunches via the s.c. route. The response was monitored as in (2).

4. LPS suspension (1ml) in 1ml FCA was administered into a rabbit by the s.c. route as above.

2.2.6. Preparation of antigen binding fragments of IgG (Fab)

a). IgG purification

IgG was purified from antiserum raised against C-Fe LPS by salt precipitation using 32% w/v Na_2SO_4 (Phillips *et al.*, 1984). An equal volume of Na_2SO_4 (32% w/v) was added drop-wise to the serum with continuous stirring. The mixture was left to stand at room temperature for 30 minutes, and then centrifuged to harvest the precipitate which was redissolved in 30ml of PBS(phosphate buffered saline, pH7.2 (0.15M containing 8g/L NaCl, 0.2g/L KCl, 0.2g/L KH_2PO_4), and dialysed against PBS, 0.15M pH7.2 until sulphate free. The presence of sulphate ions was monitored by the addition of acidified $BaCl_2$ solution to a sample of dialysate. When no precipitate formed, dialysis was stopped. The product was lyophilized.

b) papain digestion of IgG

The procedure employed here was that of Johnstone and Thorpe (1982) with slight modification. Papain (1mg) (Sigma P 4762) was dissolved in 100ul of 0.1M Na_3PO_4 buffer pH7 containing 0.01M cysteine and 2mM EDTA. The papain containing buffer (50ul) was quickly mixed with 50mg of IgG dissolved in 3ml of Na_3PO_4 buffer pH7. The final mixture was swirled gently and incubated at 37°C for 16 hours. After incubation, the mixture was dialysed at 4°C against distilled water and then against 0.01M sodium acetate buffer pH5.5 (three changes of 500ml). The dialysate was lyophilized and reconstituted in 1ml of 0.01M acetate buffer pH5.5 and loaded onto a carboxyl methyl cellulose column. The sample was eluted out with a linear gradient of 200ml from 0.01M to 0.9M acetate buffer pH5.5. Fractions (5ml) were collected and their absorbance at 280nm monitored. The elution profile contained 3 peaks (see appendix). Each was concentrated by freeze-drying. The presence of Fab fragments was detected by immunoelectrophoresis using goat - anti rabbit Fab serum (Miles Scientific, UK).

c) Immunoelectrophoresis

The freeze-dried IgG fragments were reconstituted in 1ml of distilled water and dialysed against distilled water overnight at 4°C to remove acetate before immunoelectrophoresis. The analysis was carried out according to Hudson and Hay (1980), using 0.02% barbitone buffered agar coated slides. Samples (10ul) were loaded into the sample wells and electrophoresed for 2 hours at a current of 8mA. At the end of the run, slides

were removed from the electrophoretic tank. A aliquot of 20ul of 1/16 goat - anti rabbit Fab serum was placed in the trough and incubated overnight at 4°C in a moist chamber. Whole rabbit serum and IgG purified by Na_2SO_4 were also tested for their ability to react with goat - anti rabbit Fab serum.

2.3. Analytical techniques

2.3.1. Negative staining of *Desulfovibrio* cells (Mercer & Birbeck, 1972) for electron microscopy

Cells to be used for transmission electron microscopy were harvested, spin washed as in section 2.2.1. and resuspended in Mops. Aliquots of this suspension (50ul) were mixed with 50ul of 2% w/v phosphotungstic acid (TAAB,England) pH 7. Aliquots of the mixture (10ul)was placed on to a formvar coated copper grid (TAAB) and left for 5 minutes. Excess liquid was removed by means of tissue paper. The deposit was air dried, and examined in an AEI 6B transmission electron microscope operating at an accelerating voltage of 80KV.

2.3.2. Electron microscopic examination of extracted LPS

The procedure used here was that of Amano & Fukushi (1984). One drop of 1mg/ml LPS suspension in distilled water was placed on a carbon-strengthened formvar grid using a pasteur pipette. The suspension was air-dried on the grid, after which an equal volume of staining reagents (3% uranyl

acetate, 3% phosphotungstic acid and 2% ammonium molybdate (TAAB)} was deposited directly on to the prepared grid. After staining at room temperature for 15 minutes, the excess stain was removed with filter paper and the grid allowed to dry. The specimens were examined with an AEI 6B transmission electron microscope operating at an accelerating potential of 100KV.

2.3.3. Scanning electron microscopic-X-ray microanalysis

The basis of microanalysis is the incorporation of X-ray detectors into an electron microscope (Erasmus,1978). These together provide a link between morphology and biochemistry, and allow the possibililty of analysing elements present in defined areas of biological samples. The techniques can be used to analyse fluid samples obtained directly from the biological environment or solubilized samples. The principle of the technique involves ionization of atoms in the material and measurement of the excess energy released when electrons drop back in to fill the inner shell of the atom. The difference between the energy levels of the shells is the X-ray energy, characteristic of an element. These characteristic X-rays convey information on the identity and quantity of the atom emitting the photons, seen as peaks in the spectrometer. The spectrometer employed in microanalysis can be either a wavelength- or energy-dispersive analyser. Wavelength-dispersive analysis, though more sensitive than energy-dispersive, is slow and laborious, since it can only

analyse one element at a time. The energy-dispersive system gives a simultanous display of all elements present in the sample and thus provides a rapid method of analysis. Since energy-dispersive detection is used in this analysis, a brief account of this system is described.

The analyser consists of a detector, field effect transistor, and an amplifier. X-rays emitted by the sample enter the detector, a silicon semi-conductor. Each X-ray is absorbed by the conductor and its energy is used to raise electrons to the conduction band. The number of electrons is proportional to the X-ray energy. These conduction electrons are then collected by an applied high voltage and the detector is ready within a fraction of a microsecond for the next X-ray to enter. The electron charge for each X-ray is accumulated in a field effect transistor closely connected to the detector providing a small voltage pulse. These pulses are amplified. Each pulse has a magnitude linearly proportional to the energy of the X-ray that produces it. The height of each pulse is measured by allowing it to charge up a small capacitor and measuring the time required for it to discharge on a very high frequency clock. This time is used as an address in a memory to store a count for that X-ray, so that a complete spectrum is built up with X-ray energy on the horizontal scale, and the number of photons counted at each energy on the vertical axis. The location of peaks in the spectrum thus identifies their energy and hence the element from which the X-rays came.

The size of the peak gives the number of X-rays from that element, which can be used to obtain quantitative information. In this investigation, LPS extracted from cells grown under different conditions of iron availability was deposited onto the surface of double-sided - sellotape-coated aluminium stubs. The stubs were then carbon-coated to enhance the surface conductivity of the samples. The samples were subsequently analysed in the scanning electron microscope (SuperMini-SEM, International Scientific Instruments) equipped with an energy-dispersive X-ray analyser (Lewell electronics Ltd., UK) and Princeton gamma-Tech system 4 computer. The samples were scanned at an acceleration voltage of 15KV and a magnification of 1000. The beam current used for the X-ray analysis was between 5 & 6 namps with a working distance of 23mm (distance between sample and detector). The X-rays were collected for 100 seconds. Three replicate analyses were performed on each sample. The counts of elements in a sample were expressed as a percentage of the total counts for all elements.

2.3.4. Electrophoretic techniques

Many of the amino acid side chains of a protein in solution are capable of undergoing ionization, thus becoming electrically charged. By this process, a protein molecule may become cationic or anionic according to the sum of the various charges on it. the separation of molecules by their size and charge properties is the process of electrophoresis. The electrophoretic mobility of proteins undergoing electrophoresis

is affected by:

a) the charge on the protein which dictates the direction of the migration and directly influences the velocity.

b) the strength of the electric field

c) frictional forces, which oppose migration, i.e. the viscosity of the medium through which electrophoresis is occurring.

Some of these factors relate directly to properties of the proteins themselves, so that under any one set of conditions of electric strength, pH, etc., two proteins of similar size and shape but of different amino acid composition and therefore charge will have different electrophoretic mobilities. The process of electrophoresis, then, has the potential to separate such proteins from one another for the purpose of analysis or purification.

2.3.4.1. SDS-PAGE

This gives information about the number and types of proteins present in a mixture, their abundance, and a measure of their molecular weights. SDS (sodium dodecyl sulphate) is an anionic detergent that has a polar sulphate group linked to a non-polar aliphatic chain. It is reacted with proteins before electrophoresis. With the exception of a few structural proteins, protein-SDS complexes are soluble and under the influence of an electrical field will migrate through a polyacrylamide gel towards the anode, generally at a rate inversely proportional to the logarithm of their molecular

weight. This relationship may be used to determine the molecular weight of an unknown protein when run together with proteins of known molecular weights.

SDS-PAGE can be used to analyse other macromolecules such as LPS. Jann *et al.* (1975) have used such technique to analyse heterogenous polysaccharide chain lengths in bacterial LPS.

In this study, slab gels were prepared from solutions shown in table 3 and set in a clean glass plate of dimensions 20 x 15cm² using 1.5mm perpex spacers. Routine runs were on gels of 15% acrylamide, 0.6% BIS containing 0.37M Tris/HCl buffer pH8.6 and 0.13% SDS. Gels were polymerized 2-3 hours, after which 2-3cm stacking gel of 3.5% acrylamide, 0.2% BIS containing 0.125M Tris/HCl buffer pH6.8 and 0.1% SDS was cast above the separating gel or running gel. Sample slots were made within this gel using a perspex "comb".

Freeze-dried samples or standards of known protein concentration were taken up in the solutions listed in table 4. This was mixed well and heated to 100°C for 10 minutes.Dried extracted LPS samples were treated similarly.

Formed gels were clamped as pressed on the greased perspex electrophoresis tank. The tank buffer was placed in both reservoirs. This was Tris/glycine buffer pH8.3 containing 0.025M Tris/0.0192M glycine and 0.1% SDS. Samples were applied to the gel by micropipette. The volume applied varied according to the concentration used. The gel was then run in

table 3. Solutions for gel formation

solutions	running gel	stacking gel
50% acrylamide 2% 'BIS'	9ml	1ml
2.2M tris/Cl^- pH 8.6	4.95	-
1.25M tris/Cl^- pH 6.8	-	1
20% SDS	0.2	0.05
0.5% TEMED	1.5	0.5
1.5% ammonium persulphate	1.5	0.5
water	12.85	6.95
total volume	30.00 ml	10.00 ml

table 4. Solutions for sample preparations

distilled water containing sample	500ul
0.5M tris/Cl^- pH 8.6 + 6.1mM EDTA	100
10% mercaptoethanol	100
20% SDS	100
0.001% bromophenol blue/glycerol	200
total volume	1000 ul

the constant voltage mode of a "Vokam" (Shandon Southern Ltd.) transformer, SAE 2761. A voltage (10v) was used to equilibrate the gel and for sample application. Then 100v was used for the initial "stacking ", followed by 300v for the separating gel. Towards the end of the run, for the last 10cm of the gel, the constant voltage mode was switched to constant current until the end of the run. This was to sharpen the bands. The complete run took about 3.5-4 hours During the run, the gel was cooled by a cold air fan over the plate. The gel was then removed and stained immediately.

Staining procedures 1. Page Blue 83 : Gels were immersed in 0.05% Page Blue, 50% methanol, 10% acetic acid, overnight. Several changes are necessary as all SDS must be removed before dye-binding can take place. Destaining was achieved by using 7.5% methanol until a clear background was obtained. Protein positive bands appear bright blue.

2. Silver staining: This is an extremely sensitive stain. There are several versions of the silver stain. The procedures used here were based on those of Morrisey (1981) and Hemplemann *et al.* (1984). In Morrisey's method, the gel was fixed in 15% acetic acid and 10% methanol overnight, after which it was soaked in distilled water for at least 2 hours with changes of water. The gel was then rinsed and soaked in 5ug/ml dithiothreitol for 30 minutes. After a thorough rinse, the gel was soaked and developed with 50ul of 37% formaldehyde in 200ml of 3% sodium carbonate until the desired staining

level was achieved. The reaction was stopped with 10ml of 2.3M citric acid and shaking for 10 minutes. During these preceeding steps, the gel was constantly agitated on a shaker. The stained gel could be stored overnight in 0.05% sodium carbonate in the dark.

Recently a new version of the silver stain has been developed (Hemplemann *et al.*,1984). This new method reduces the background staining. The procedure described below was a modified version of Hemplemann *et al.* (1984).The gel was soaked in 200ml of each of the following solutions in sequential order. All the solutions were prepared in doubled distilled water. The gel was prefixed in 10% (w/v) trichloroacetic acid for 30 minutes, followed by ethanol/acetic acid/water (20:3:75) containing 0.002% (w/v) dithiothreitol for 30 minutes. This was the most critical step of the whole procedure. Then the gel was immersed in 0.5%(w/v) potassium dichromate for 5 minutes and water for 5 minutes. It was then immersed in 0.1% (w/v) silver nitrate for 10 minutes followed by 1 minute in water. The gel was transferred to a freshly prepared solution of 3% (w/v) sodium carbonate containing 0.02% (w/v) formaldehyde and left there for 7 minutes. For storage 1% (w/v) aqueous acetic acid is used.

2.3.4.2. Plasmid DNA analysis

The extracted plasmid DNA was mixed with 1/5 volume of sample buffer containing 7M urea, 0.1mM EDTA, 50% glycerol and 0.1% bromophenol blue. The mixture was mixed well and loaded on to

a 2% agarose gel. The gel was run in an electrophoresis tank for 18 hours at a constant current of 30mA. The tank buffer was tris base-borate - EDTA pH8. Ethidium bromide (5ul of 10mg/ml for every 10ml of buffer) was incorporated into the buffer just before use. At the end of the run, the bands were visualized with a Transilluminator (Genetic Research Instruments Ltd.) at 340nm and photographed.

2.3.4.4. Electroblotting (Western blotting)

Polyacrylamide gel electrophoresis has proved to be a powerful analytical tool for complex mixtures of proteins. Despite the advances in resolving power, it still does not allow further analysis of the separated proteins as these are embedded in the gel matrix and are relatively inaccessible. Although methods such as elution of bands (Bustein *et al.*,1978) or direct *in situ* analysis using antisera (Adari *etal.*,1978) or protein probes (Snabes *et al.*, 1981) have been developed, they are time-consuming and relatively insensitive resulting in a loss of resolution. The appreciation that procedures used in DNA blotting, pioneered by Southern (1975), could be applied to proteins has led to a major advance in protein analysis.

Blotting is the transfer of macromolecules from gels to an immobilising matrix. By such a technique proteins become accessible for further analysis using probes. Electroblotting was first described by Towbin *et al.*,(1979). Proteins were driven out of the gel on to a filter of nitrocellulose (NC), 0.45um pore size. Burnette (1981) reported that nitrocellulose

with a 0.2um pore size gave better binding to smaller proteins. Although this active transfer of proteins provided a relatively higher efficiency than non-electrical transfer, the rate of transfer is a function of molecular weight, the larger molecules being transferred more slowly. Thus the precise running conditions for such a process depend on the molecular weight profile of the protein mixture. Other factors which might affect transfer efficiency are the gel system used (Towbin *et al.*,1979; Burnette,1981) and the nature of the transfer buffer. In order to ensure the most efficient transfer of proteins from gel to the filter, it is necessary to allow sufficient running time and to ensure maximum solubility of the proteins under investigation by adjusting the composition and pH of the buffer.

The principal steps in the electroblotting process are depicted in figure 7. Molecules such as LPS can also be transferred from polyacrylamide gels on to nitrocellulose filters (Bradbury *et al.*,1984).

In this investigation, the transfer sandwich was assembled as in figure 7 and placed in the transfer tank with the nitrocellulose facing the anode, as molecules separated in SDS-PAGE had an overall negative charge. The buffer used was Tris-glycine-methanol (25mM, 192mM. 20% (v/v) respectively, pH 8.3). The transfer was run at 35v for 30 minutes, followed by 3 hours at 60v (Karch *et al.*, 1984). At the end of the run, the nitrocellulose filter was either analysed immunologically

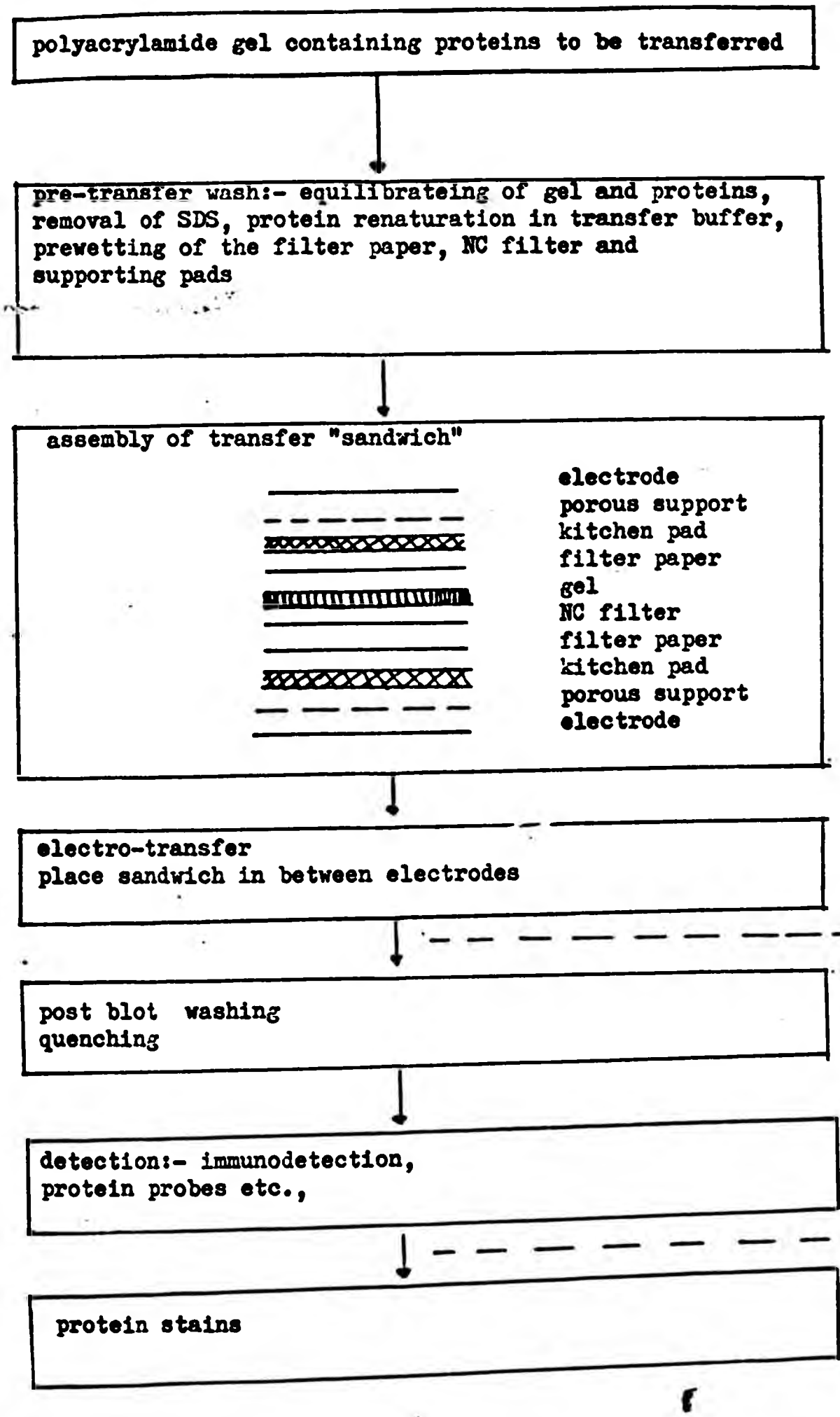


Figure 7. Principal steps of electroblotting

(section 2.3.8.ii) or used for functional studies (section 2.4.1).

2.3.5. Pyrolysis mass spectroscopy (PYMS)

Pyrolysis is the break down of complex materials in an inert atmosphere to produce a series of volatile, lower molecular weight substances using heat alone. In PYMS, these fragments are subsequently detected and analysed using a mass spectrometer. A similar technique is pyrolysis gas chromatography (PYGC) which uses gas chromatography as the detector system.

The first report of PYMS in analysing biological materials was that of Zemany (1952), who analysed albumin and pepsin. Reiner (1965) showed that PYGC could be used as an aid to bacterial identication. The problem of PYGC in earlier analytical pyrolysis was reproducibility. This led to the development of PYMS, which is more reproducible, faster and more easily automated than PYGC.

The major contribution in the analysis of biological materials by PYMS is that of Meuzelaar and Kistemaker. In 1973, they developed a pyrolysis mass spectrometer specifically for fingerprinting complex samples such as bacteria. The mass spectra produced by PYMS can be treated as chemical profiles or "fingerprints" of the organisms. Additionally, it can be used for identication and discrimination by comparing the data generated with the stored data in computer data banks (Borst *et al.*, 1978), or for studying inter and intra species

relationships. The latter is achieved by the employment of multivariate analysis and the associated statistical techniques.

The characterization of bacteria using the PYMS technique has been reported. For example, Wietin *et al.* (1981, 1982) used the method in characterization of mycobacteria. Their study has progressed beyond simple identification and differentiation of strains to factor analysis of mass spectra. The differences in the spectra due to the presence or absence of specific chemical compounds can be elucidated and correlated with chemotaxonomic data.

a. Principles of PYMS

PYMS can be performed in two ways. One is DPMS, direct probe mass spectrometry, as employed by Gutteridge and Puckey (1982). In this method, samples are thermally degraded adjacent to the ion source of the mass spectrometer using a ballistically heated probe. The other is curie-point pyrolysis using a curie-point pyrolyser linked to a mass spectrometer. The pyrolysate is passed through an empty glass chromatography column and jet separator before entering the mass spectrometer.

After pyrolysis, the pyrolysate mixture is ionized to become amenable to mass analysis. The ionization procedure most commonly used is electron impact ionization (EI). In EI, the pyrolysate is bombarded by electrons from the ion source causing electrons to be removed from the pyrolysate fragments,

producing positively charged ions. These are accelerated across a potential difference into the mass spectrometer where they are separated according to their mass/charge (m/z) ratio. Mass analysis is carried out by passing ions through either an electric field, in the case of a quadrupole mass spectrometer, or magnetic field, in the case of a magnetic sector mass spectrometer. Separation of the ions relies on the fact that ions of different masses will be deflected by different amounts and will pass through the field at different rates. Once separated, the ions are detected by either ion counting or current monitoring. Upon detection, the ions are recorded by a signal averager or by a computer. The mass spectra are then ready for data analysis.

The method employed in this study is the DPMS used by Gutteridge and Puckey (1982) with modifications.

b. Sample preparation for PYMS

Cells from 4-day old culture were used. Triplicates of 50ug of washed lyophylised cells were placed in a clean quartz glass tube which was then inserted into a stainless steel probe. In addition, cell walls extracted from such cells were analysed.

c. Mass spectrometry

The analyses were carried out in a Jeol DX 300 mass spectrometer with EI equipped with a Texas computer. The spectrometer was operated at an electron energy of 70eV with a multiplier current of 1.2Kv and a source temperature of 210°C.

After insertion into the high vacuum of the equipment, the probe temperature was programmed to increase at 64°C/minute from ambient to 300°C. The spectra were recorded onto a magnetic disc over the mass range m/z 30 to 510 m/z at a scan speed of 2 second. Since the temperature of the probe was programmed, the total ions detected by the mass spectrometer were recorded and displayed as a function of time in an ion current profile. In order to obtain a spectrum the total ions between the beginning and the peak of the pulse were averaged, and then the background (an average of 10 scans taken in the range 15-5 scans before the start of the pulse) was subtracted.

d. Analysis of data

The data were analysed by simple comparison of the ion counts. In this process, the data were first averaged to give mean value for each ion peak in the spectra. These were then compared in turn by calculating the % difference using the means of each sample type. Furthermore, the data were analysed by the characteristicity calculation of Eshuis *et al.* (1977).

2.3.5. ^{125}I-radiolabelling of outer membrane proteins

The cell membrane allows the communication with its environment. Consequently, the macromolecules on the surface are important in communication with the neighbouring cells or the environment for nutrient uptake, transport of metabolites and DNA transfer. The technique of radioiodination is a useful method of labelling surface proteins for their study. Several methods are available for radioiodination. These

include lactoperoxidase-catalysed iodination; glucose-glucose oxidase mediated peroxidase-iodination; chloramine T and Iodo-Gen (1,3,4,6,-tetrachloro-3 ⅄,6 ⅄ -dephenylglycouril) methods. The lactoperoxidase system is selective in that only those tyrosine residues which can form an enzyme subtrate complex with the peroxidase will be iodinated. Only tyrosine and histidine form stable derivatives as a result of peroxidase-catalysed iodination (Morrison,1980). The residue which will be iodinated by lactoperoxidase must have the proper geometric position, while other methods are influenced by reactivity only. The reactivity depends upon the microenvironment of the tyrosine. There is an inverse relationship between the extent of tyrosine iodination and the dielectric constant of the microenvironment. In this method, steric factors also influence iodination, since the relatively large iodine atoms may be blocked in either the production of monoiodotyrosine or the formation of diiodotyrosine.

Cushley *et al.*(1983), comparing iodination by lactoperoxidase and glucose-glucose oxidase methods, suggested that the latter provided a more specific labelling of proteins.

Chloramine T, though claimed to be simple and rapid, with a minimal exposure of personnel to radioactivity (McConahey & Dixon,1980), is of limited use. The major disadvantages of this method are the possibility of protein denaturation caused by this strong oxidizing agent and the requirement for a strong reducing agent to terminate the reaction.

Iodo-Gen, a water insoluble compound, has recently been introduced as a gentle effective iodinating agent for soluble and membrane proteins (Fraker & Speck, 1978, Markwell, 1981). The technique is gentle and simple to perform. Reaction vessels can be prepared in advance and stored. The Iodo-Gen mediated reaction is unaffacted by denaturing conditions or the presence of enzymatic inhibitors in reaction mixture, and requires no terminator to stop the reaction. Termination of the reaction is achieved by the removal of cells from the reaction vessels.

In this study, the Iodo-Gen method (Markwell,1981) was used for radioiodination of *Desulfovibrio* outer membrane proteins.

a. Coating of dishes with Iodo-Gen

Iodo-Gen (100ug) (Pierce Chemical, kind gift of Dr. A. Norqvist, Division of Microbiology, National Defence Research Institute, Sweden) was dissolved in 1ml of $CHCl_3$ (analar) in clean glass dish (diameter,9cm). The solution was mixed and evaporated slowly under a stream of oxygen free nitrogen gas (BOC) in a fume cupboard. The dried Iodo-Gen coated dishes were placed in a dessicator until use (approximately 1 hour).

b. Iodination

Four-day-old cultures (500ml) of C-Fe and C+Fe cells were used. They were harvested and spin-washed as in section 2.2.1. The washed cells were resuspended in 10ml of Mops containing 4.8×10^{14}/ml and 4×10^{14}/ml C-Fe and C+Fe cells respectively. The coated dishes were rinsed with Mops to remove any flakes of

Iodo-Gen not adequately fixed on the surface. The cell suspensions were individually placed in the Iodo-Gen coated dishes in the fume cupboard. 200uCi of ^{125}I (as $Na^{125}I$ carrier free, Amersham, UK) was added. The suspension was gently agitated for 15 minutes at room temperature. The reaction was terminated by transferring the cells into a Universal bottle to which 0.25mM NaI was added for safe handling. Cells were spun down in a capped Universal bottle in a bench centrifuge, washed twice more in Mops containing 0.25mM NaI, and finally resuspended in 10ml Mops. The OM was extracted by the sarkosyl procedure as described in section 2.2.1 and subjected to SDS-PAGE analysis as in section 2.3.4.1. After electrophoresis, the OM proteins were electroblotted onto a nitrocellulose filter membrane for autoradiography. The filter was exposed to Kodak X-omat RP1 film shielded by intensifying screens 18x24cm(Philips) for a fortnight at -20°C.

c. Modifications of the Markwell procedure of iodination

1. Reduction of incubation time

Iodo-Gen (100ug) was used as the catalyst. The cells were incubated with 200uCi ^{125}I in the Iodo-Gen coated dish for 8 minutes at room temperature. They were then washed as above and resuspended in 10ml of Mops. The suspension was vortexed and 2ml was removed for whole cell solubilization for PAGE analysis. The remainder was used for OM extraction and PAGE

analysis.

2. Modification of Sullivan & Williams (1982)

Here, the cells were exposed to ^{125}I (200uCi) for 4.5 minutes before proceeding to OM extraction and electrophoresis. Again a small portion of the radiolabelled cell suspension was retained for whole cell solubilization. NC cells were also analysed, and the film was developed after a fortnight's exposure at -20°C.

3. Reduction of quantity of Iodo-Gen and incubation time

This was based on the method of Sullivan & Williams (1982). Here all three cell types were analysed. Iodo-Gen (50ug) in $CHCl_3$ was coated onto the walls of clean scintilation vials. The cell suspension was exposed to ^{125}I (200uCi) for 45 seconds at room temperature with gentle swirling. The cells were washed once in Mops containing 10mM NaI and then in Mops + 5mM NaI in capped Universal bottles. Two ml of the final suspension was removed for whole cell analysis, and the reminder was used for OM extraction. The film was exposed to Kodak X-omat RPI film at -20°C for one month before development (extended time was due to low radioactivity).

2.3.7.Acid hydrolysis and high performance liquid chromatography (HPLC)

This was employed for sugar analysis.

The advantages of HPLC over other forms of chromatography are the high speed of resolution obtainable and the ability to use minute quantities of material. Though the layout is

superficially similar to the more traditional chromatographic techniques, it is different in a number of details. The apparatus consists of a narrow bore tubing (1/64" internal diameter) and fine column packings (5-20u particles), which gives the high resolution separations, leading to considerable resistance to the flow of solvent through the system. To overcome this back pressure, solvent is pumped through the column under high pressure, usually between 500 and 2,000psi. Thus this technique is sometimes known as high pressure liquid chromatography.

The most important component of a HPLC system is the packing material in the column. The separation depends on the relative importance of the competing attractions of the packing materials, or stationary phase, for the sample. These interactions can be modified by alteration of the composition of the mobile phase, as in gradient chromatography, or by changing the flow rate (i.e. pressure) or the temperature at which the chromatography is performed. It is important that the solvents used are highly purified since any impurities present may affect the column or interfere with the detector system. Furthermore, it is essential that the solvents be "degassed" before use as "gassing" tends to occur in most pumps. The presence of air bubbles in the solvent can alter the resolution of the column and interfere with the continuous monitoring of the column effluent.

For this investigation, dried LPS samples, extracted either

from C-Fe or C+Fe cultures, were dissolved in 1ml distilled water. One ml of 1N HCl was added to the solution and the mixture transferred into ampoules which were sealed with a bunsen flame. The sugar constituents of the extracted LPS were released by a strong acid hydrolysis *in vacuo* at 100°C for 4 hours in a preheated oven. Some electrodialysed LPS (C-Fe) was incubated with 1ug/ml $FeSO_4$ for 30 minutes prior to acid hydrolysis.

Hydrolysed sugar samples were passed through a SEP-PAK (Waters) C18 cartridge, which was first activated with 2ml methanol followed by 4ml distilled water. Sugars were eluted immediately from the cartridge, leaving the remaining lipids and/or hydrophobic contaminants on the cartridge. Sugar samples were then freeze-dried and taken up in (50ul) of mobile phase.

A Waters HPLC system was utilised for the separation. This incorporated a U6K injector, 6000A solvent delivery system, 401 refractive index detector and a Delsi integrator. The column was a Waters uBondapak amino column as a radial compression separation system (RCSS) cartridge. The mobile phase was an acetonitrile/water (80:20) isocratic system at a flow rate of 1.8ml per minute. Acetonitrile was HPLC grade (Rathburn). The water was double distilled and was further purified by passing through a Millipore "Norganic"(trace organic removal) cartridge and filter membrane. All the solvents were degassed by evacuation.

2.3.8. Serological Analysis

a. Agglutination

This was performed either in the traditional Durham tubes or in microtitre plates (U-shape, Cooke, England). Serial doubling dilutions of sera (0.5ml) in Mops buffer were made in tubes. The appropiate cells (15ml) were washed once in Mops and resuspended in 5ml Mops. An aliquot of 50ul of cell suspension (1-2 $\times 10^7$/ml) was added to each tube and the tubes mixed by hand and incubated in a 37°C water bath for 4 hours. The end point was read as the last tube showing a clear supernatant and an agglutination lawn at the bottom. For the purpose of conserving antisera, microtitre plates were employed in the later tests. Serial doubling dilutions of antisera (50ul) were prepared in Mops. C+Fe cells were first harvested and resuspended in 0.85% NaCl. They were then centrifuged at 1000g at 4°C for 8 minutes. This was to remove excess FeS from the suspension, a modification of the procedure employed by Singleton *et al.* (1985).The supernatant was centrifuged at 3500g for 20 minutes to pellet the cells and these were resuspended in 5ml Mops. To each well containing diluted antiserum 50ul cell suspension (1-2$\times 10^7$/ml) was added. The plates were gently shaken by hand and incubated at 4°C overnight. In both methods, controls were prepared by suspending cells in Mops to check for autoagglutination and in pre-immune serum to check for any nonspecific reactions.

b.Passive heamagglutination (PHA)

This test was carried out in three stages: 1) deacylation of LPS 2) sensitisation of sheep red blood cells (SRBC) and 3) agglutination.

1. Deacylation

This treatment enhances or allows the coating of (SRBC) with LPS. LPS (10mg) was incubated in 1ml of 0.25M NaOH for 1 hour at 56°C. After incubation, the mixture was allowed to cool to room temperature and centrifuged (2000g) at 4°C for 15 minutes. The supernatant was neutralized with 1ml of 1N acetic acid and dialysed against distilled water overnight with changes of water. The dialysate was lyophilized.

2. Sensitisation of SRBC 500ul of SRBC in Alsever's solution was pelleted at 2000g for 5 minutes and the cells were washed three times in 5ml of Mops. 25ul of the pellet was suspended in 5ml of Mops containing 125ug of alkali-treated LPS. The mixture was gently shaken and incubated at 37°C for 30 minutes with occasional shaking. Unbound LPS was removed by centifugation. The sensitised cells were washed three times in Mops and finally resuspended in 5ml of Mops to give a 0.5% suspension. A control suspension was prepared by suspending 25ul of unsensitised cells in 5ml Mops.

3. Agglutination

Serial doubling dilutions of the appropriate antiserum (50ul) in Mops were made in microtitre plates with U-shaped wells. Sensitised cells (50ul) were added to each well and the plates gently shaken. After incubation at 37°C for 4 hours,

the plates were incubated at 4°C overnight. Negative results appeared as tight buttons of cells at the base of the wells.

c.Immunological analysis of electroblots

After transfer of macromolecules from the SDS-PAGE gels, the blot was quenched with bovine serum albumin (BSA) (2% w/v in Tris buffered saline: 10mM Tris, 0.9% NaCl, (TBS) pH7.4) for 1 hour followed by an overnight incubation with 1/30 dilution of anti-*Desulfovibrio* antibodies in 1% BSA-TBS pH7.4 at room temperature. The blot was washed twice, each time by soaking in 100ml TBS-Tween 20 (0.05%) (2x40 minutes). It was then overlayed with anti-rabbit IgG conjugated to peroxidase (Sigma) (80ul in 15ml 1% BSA-TBS pH7.4) and incubated at room temperature for 2 hours, followed by washing in 100ml TBS-Tween 20 (0.05% v/v) (2 x 40 minutes). After a brief rinse in tap water, the blot was incubated with the substrate for 25-35 minutes in a dim light. The substrate solution: 4-chloro-1-naphthol, which was light sensitive, was prepared fresh in 10ml of ice-cold methanol mixed with 50ml TBS pH7.4 containing 40% hydrogen peroxide.

2.4. Functional studies

2.4.1. ^{59}Fe -radiolabelling of electroblots

Bowen *et al.* (1980) reported that DNA-binding proteins immobilised on nitrocellulose filters by blotting retained their DNA-binding ability as indicated by (^{32}P)DNA-binding. Thus it should be feasible to radiolabel *Desulfovibrio* Omps or

LPS by a similar method.

The blot was air dried at room temperature overnight. It was then transfered to a mixture of 10ml Mops and 50ml 10mM Na ascorbate containing 200ul ^{55}Fe (approximately 10.5uCi), agitated on ice for 1 hour, washed thoroughly with distilled water,blotted dry with a Whatman filter paper and air dried. The blot was then exposed to Kodak X-omat RP1 film shielded by intensifying screens 18x24cm^2 (Philips) for 1.5 months,(2 weeks at room temperature and 4 weeks at 4°C), or at -20°C for 6 weeks.

In attempts to reduce non specific binding of ^{55}Fe to the blot, quenching of the blot with 30ml of NaCl (8.965g/L), or 30ml of 0.5% polyvinylpyrrolidone (approximately 24,500 M.WT.,Koch-light Lab Ltd.) 30 minutes prior to labelling was performed.

Other modifications included the incubation of the blot with ^{55}Fe in the presence of 2mg $MgSO_4$, 2mg or 4mg $CuSO_4$ or incubation of the blot post ^{55}Fe labelling with 60ml of distilled water containing 10mg$CuSO_4$. In all cases, the blots were washed thoroughly with distilled water, then blotted dry and autoradiographed.

2.4.2. *In vivo* ^{55}Fe labelling of OM components

A 3-4 day old C-Fe culture (250ml) was incubated with 30ul of ^{55}Fe (activity, 0.047 mCi) at 30°C for 1 hour. The cells were subsequently harvested, washed and resuspended in 10ml of Mops. They were then used either for LPS or OM extraction as

described in sections 2.2.1. & 2.2.2.. The extracts were electrophoresed in SDS-PAGE and blotted on to a nitrocellulose filter. The latter was autoradiographed at -20°C for 4 weeks.

2.4.3. Adhesion

The method adopted for this study was a resin replica technique previously employed by Birkby & Preece (1981) for leaf surfaces. An acrylic resin emulsion was allowed to dry on the metal coupon surface. It could be peeled off to produce a transparant replica of the surface with any bacterial cells embedded in it and these could be stained for light microscopic examination.

a. Grease-free coupons (4x1.5 cm^2) were introduced into Universal bottles containing 20ml of filtered tap water. To each bottle, 1ml of either NC or C-Fe cells (approximately 10^6/ml) was added . They were incubated at 30°C for 15 minutes removed, rinsed in distilled water and blotted dry with tissue. A layer of resin (Ac634, Spectrum Colour, Horsham, Sussex, UK) was pipetted over the surface. When set, the resin was peeled off, stained with phenolic aniline blue and examined under the light microscope. Ten random fields (400 um^2) (x100 objective) defined by an eye piece grid were examined and cells lying within four squares area at the top right hand corner counted.

b. To analyse the role of LPS in the adhesion process, anti LPS abs in form of Fab was used as an inhibitor of the process.

Fab fragments prepared as described in sections 2.2.6. were first tested for their Ag binding ability. This was done

by incubating 10ul of cell suspension for 1/2 hour at 30°C and then testing for agglutination by anti-Fab abs using the slide technique.

C-Fe cells (200ml) were harvested and resuspended in 10ml of fresh C-Fe medium. 1ml of Fab fragment was added to the suspension, mixed gently and incubated at 30°C for either 2 hours or 1/2 hour. At the end of incubation, the cell number was determined by a haemocytometer count. An inoculum of 10^6/ml of cells was inoculated into Universal bottles containing 20ml of filtered tap water and a metal coupon. The procedure described under (a) above was repeated.

RESULTS AND DISCUSSIONS

Chapter Three

GROWTH, MORPHOLOGY AND CHEMICAL STRUCTURE OF C+Fe, C-Fe AND NC CELLS

3.1. Growth

Iron concentrations in the growth media are 1.45uM for C-Fe, 17.2uM for NC and 353uM for C+Fe, after three days of incubation with a mild steel coupon (Bradley, 1985).

Desulfovibrio species have a high requirement for iron for growth (Postgate, 1984). However, they can be grown at the low iron concentrations quoted above. Figure 8 shows that the growth rates of NC and C-Fe cultures differred. For NC, the doubling time is 2 days, while for C-Fe it is 2.40 days. Occasionally, no growth is observed in C-Fe and NC media. The growth rate of the cells in C+Fe is not determined because the presence of FeS particles made accurate cell counts impossible.

3.2. Morphology

C+Fe cultures appear black, while NC and C-Fe are dull green and buff respectively. The colour of C+Fe cultures is due to the FeS precipitate formed in the medium.

When *Desulfovibrio* cells are examined in the transmission electron microscope, both C+Fe and NC cells appear uniformly

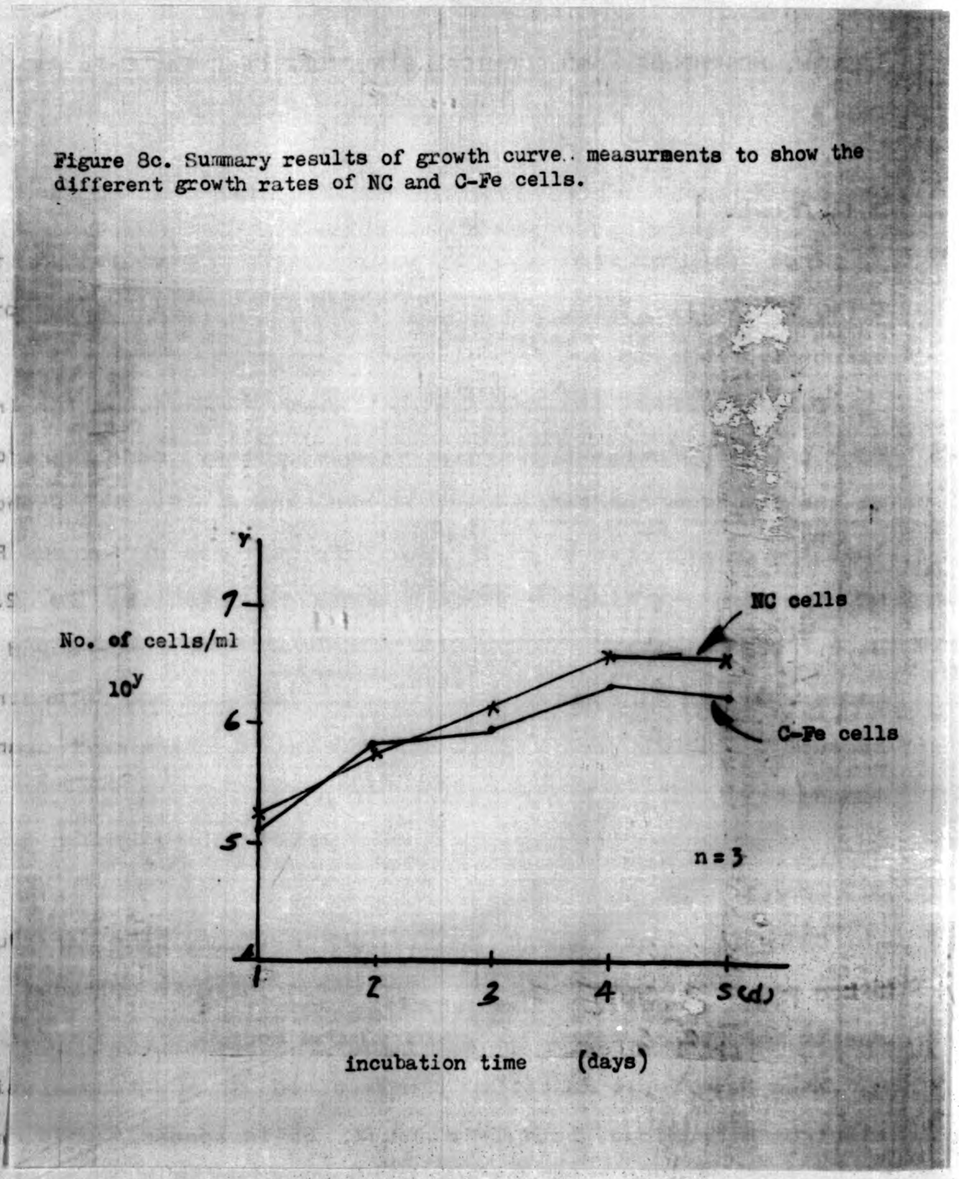
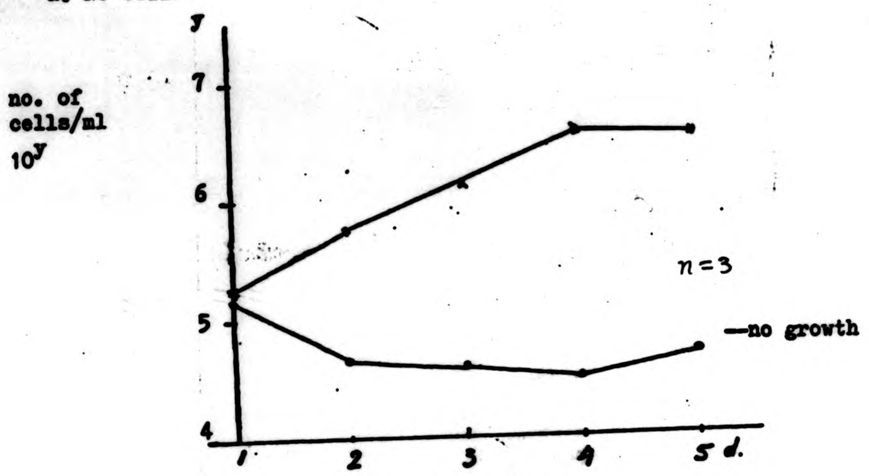


Figure 8c. Summary results of growth curve measurments to show the different growth rates of NC and C-Fe cells.

Figure 8. Growth curves of NC and C-Fe cells

a. NC cells



b. C-Fe cells

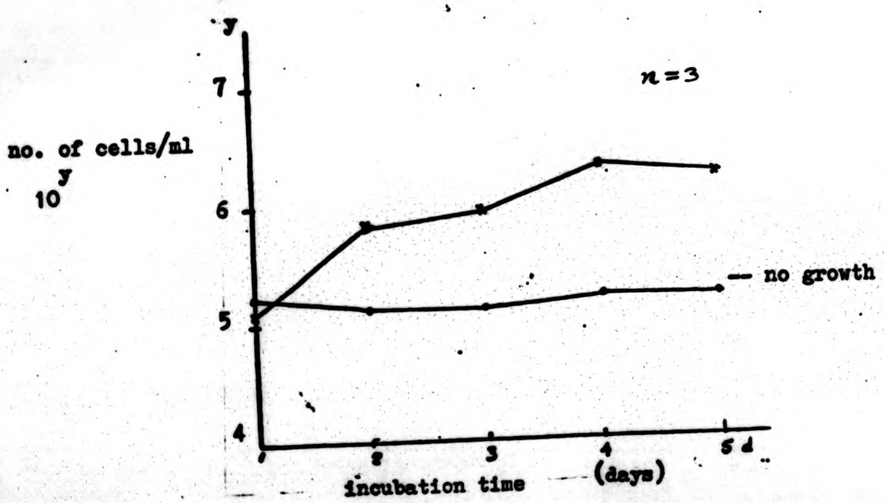


figure 8c. Summary results of growth curve measurements to show the different growth rates of NC and C-Fe cells.

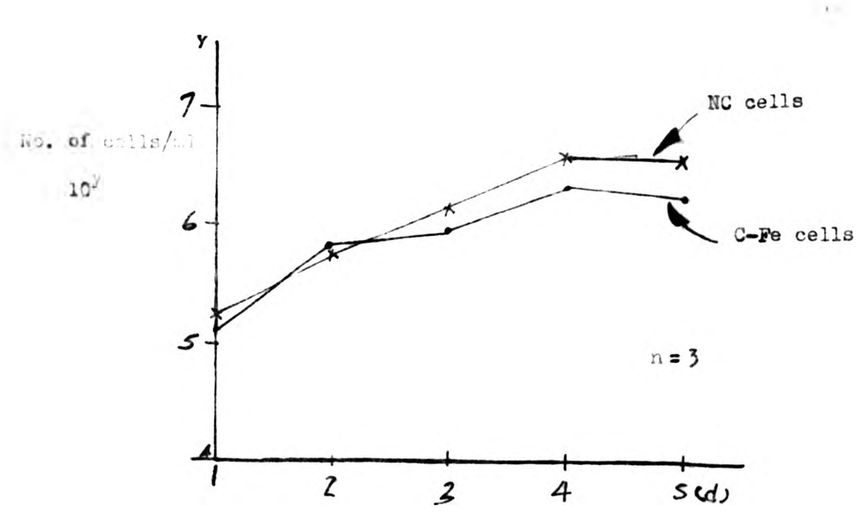
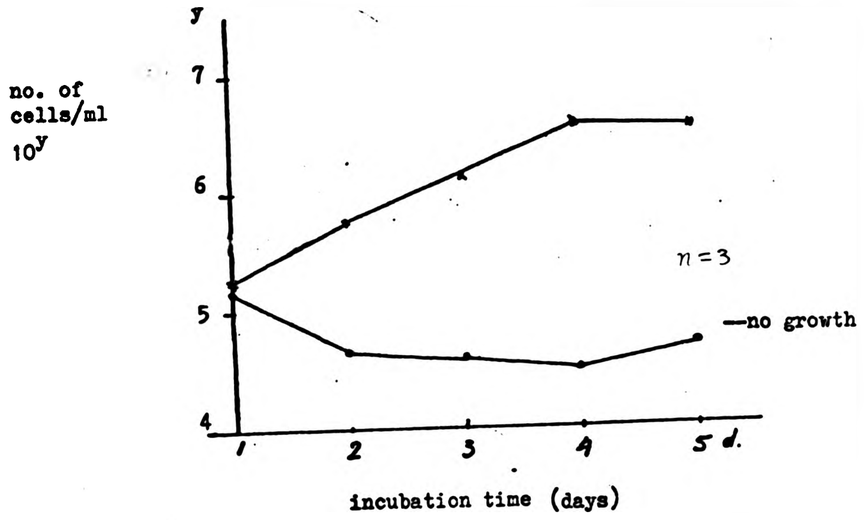


Figure 8. Growth curves of NC and C-Fe cells

a. NC cells



b. C-Fe cells

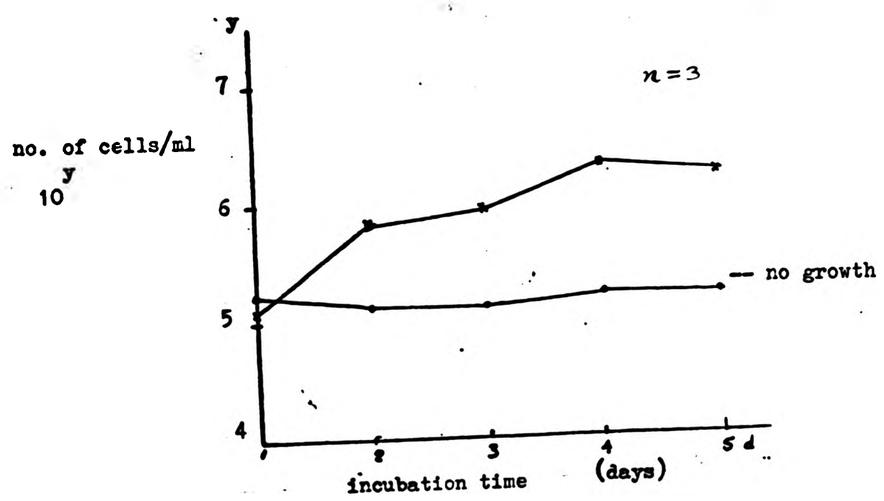


Plate 1. Electron micrograph
of C+Fe cell.
(magnification 40K)
bar= 0.01um

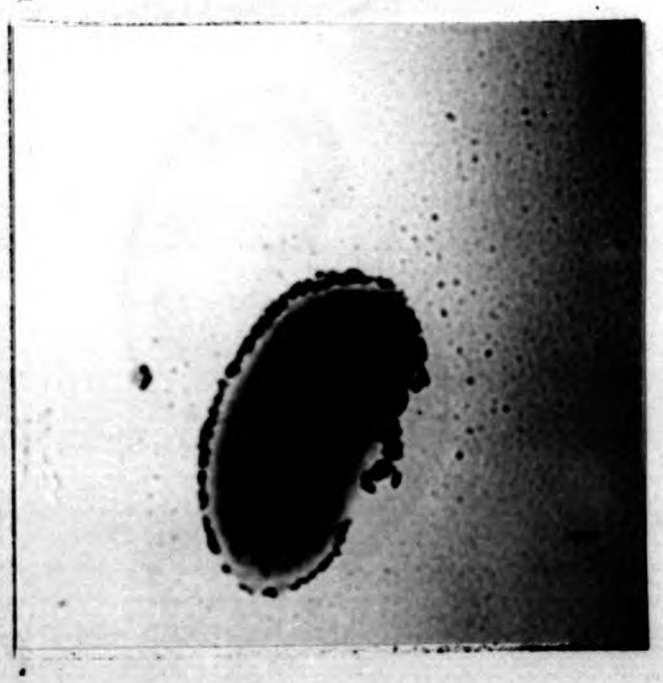


Plate 2. Electron micrograph
of NC cell.
(magnification 25K)
bar = 0.1um

Plate 1. Electron micrograph
of C+Fe cell.
(magnification 40K)
bar= 0.01um

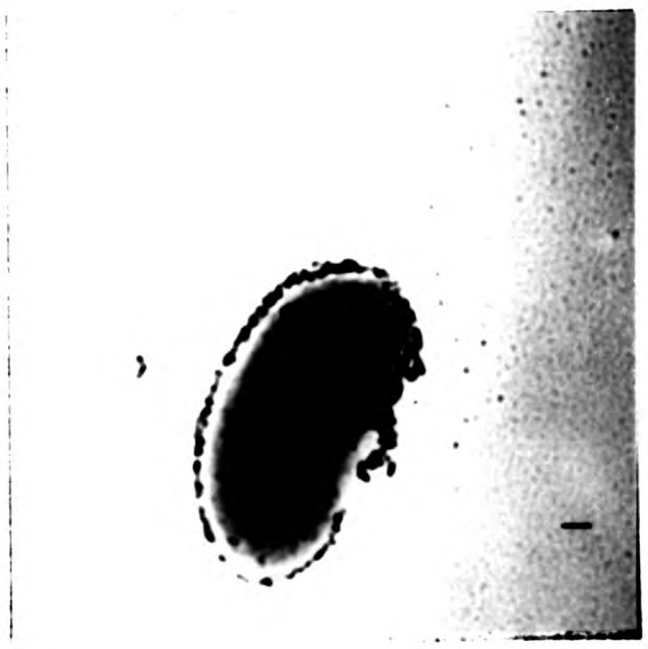


Plate 2. Electron micrograph
of NC cell.
(magnification 25K)
bar = 0.1um

Plate 3. Electron micrograph
of C-Fe cell.
(magnification 25K)
bar = 10um

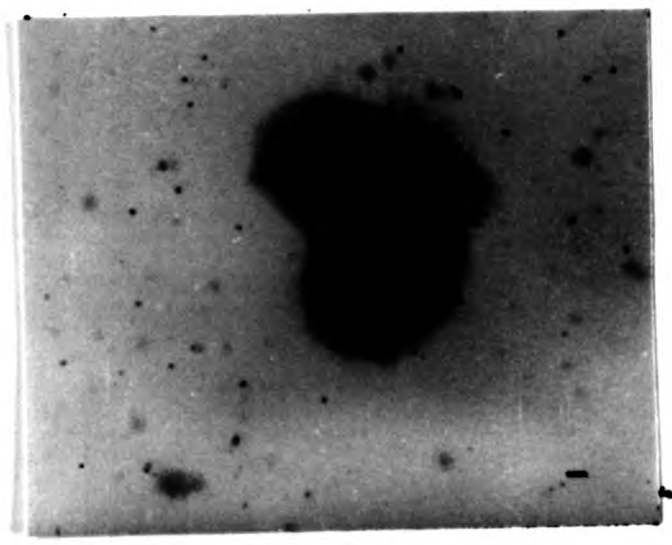


Plate 4. Electron micrograph
of C-Fe cell.
(magnification 16K)
bar = 0.1um

Plate 5. Electron micrograph
of C-Fe cell.
(magnification 40K)
bar = 0.1um

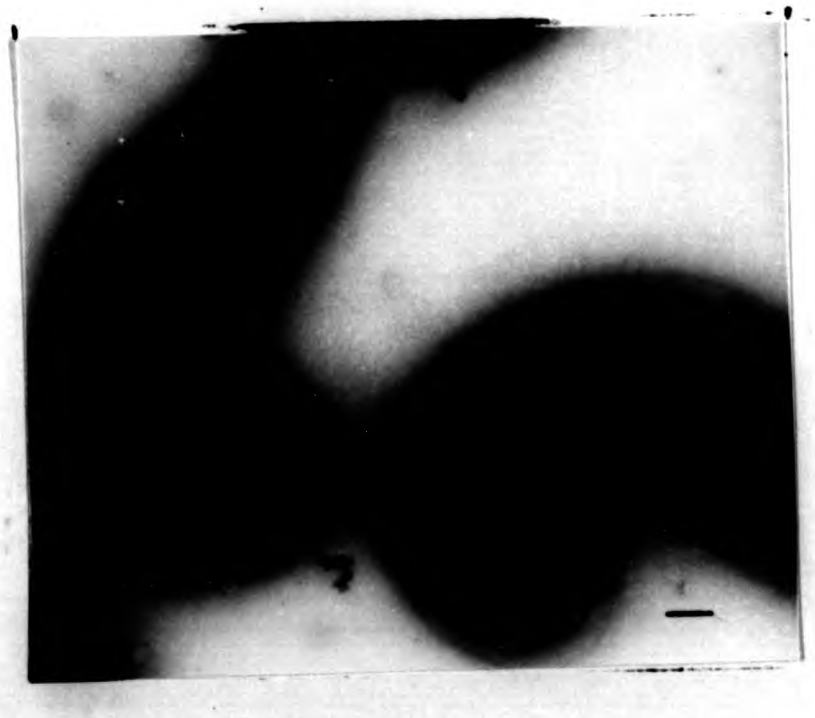


Plate 3. Electron micrograph of C-Fe cell. (magnification 25K) bar = 10um

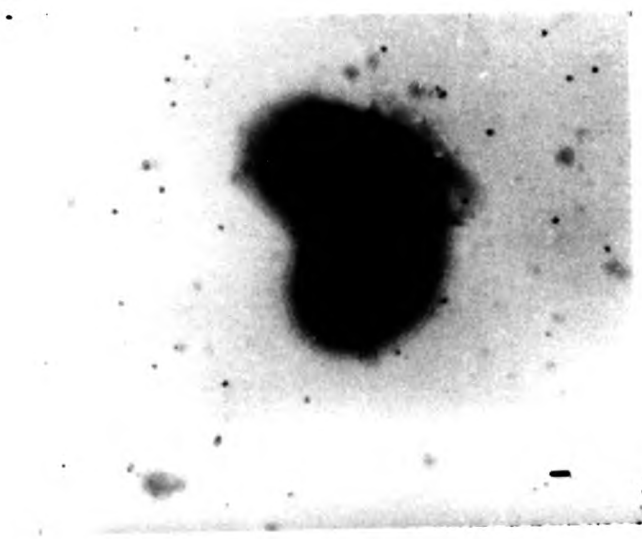


Plate 4. Electron micrograph of C-Fe cell. (magnification 16K) bar = 0.1um

Plate 5. Electron micrograph of C-Fe cell. (magnification 40K) bar = 0.1um

vibrioid in shape. They also harbour electron dense partic' on their surfaces (plates 1 & 2). C-Fe cells on the contrary, are pleomorphic and have a relatively clean surface (plates [illegible] to [illegible]). This morphological variation, frequently seen in stressed cells, could reflect an altered metabolism. To assess this possibility, pyrolysis mass spectrometry of the cells is performed.

7.3. Pyrolysis-mass spectrometry [illegible]

The total ions detected by the mass [illegible] displayed as a function of time in an ion current profile (figure 9). The mass spectra of these analyses are shown in figures 10,11,& 12. The differences indicated in the spectra by arrows are based on the numerical data obtained from the spectrometer. An example of such data is illustrated in figure 13. When the data are examined pairwise by % difference between the means, NC cells are found to be intermediate in [illegible] ion intensities between C+Fe and C-Fe cells. When C+Fe and C-Fe are compared, the overall ion intensities in the C+Fe spectra are found to be higher than C-Fe with ions of mass 65 and 94 specifically present in C+Fe and not in either NC or C-Fe. Ion of mass 128 in C-Fe is absent in C+Fe. The major differences between the mass spectra of C+Fe and C-Fe cells lie in the upper and lower regions of the spectra. C+Fe give rise to more ions with lower masses ranging from 50 to 170, whilst C-Fe give rise to more ions at a higher mass range between 170

Figure 9. Ion current profiles obtained by PYMS analyses of whole cells.

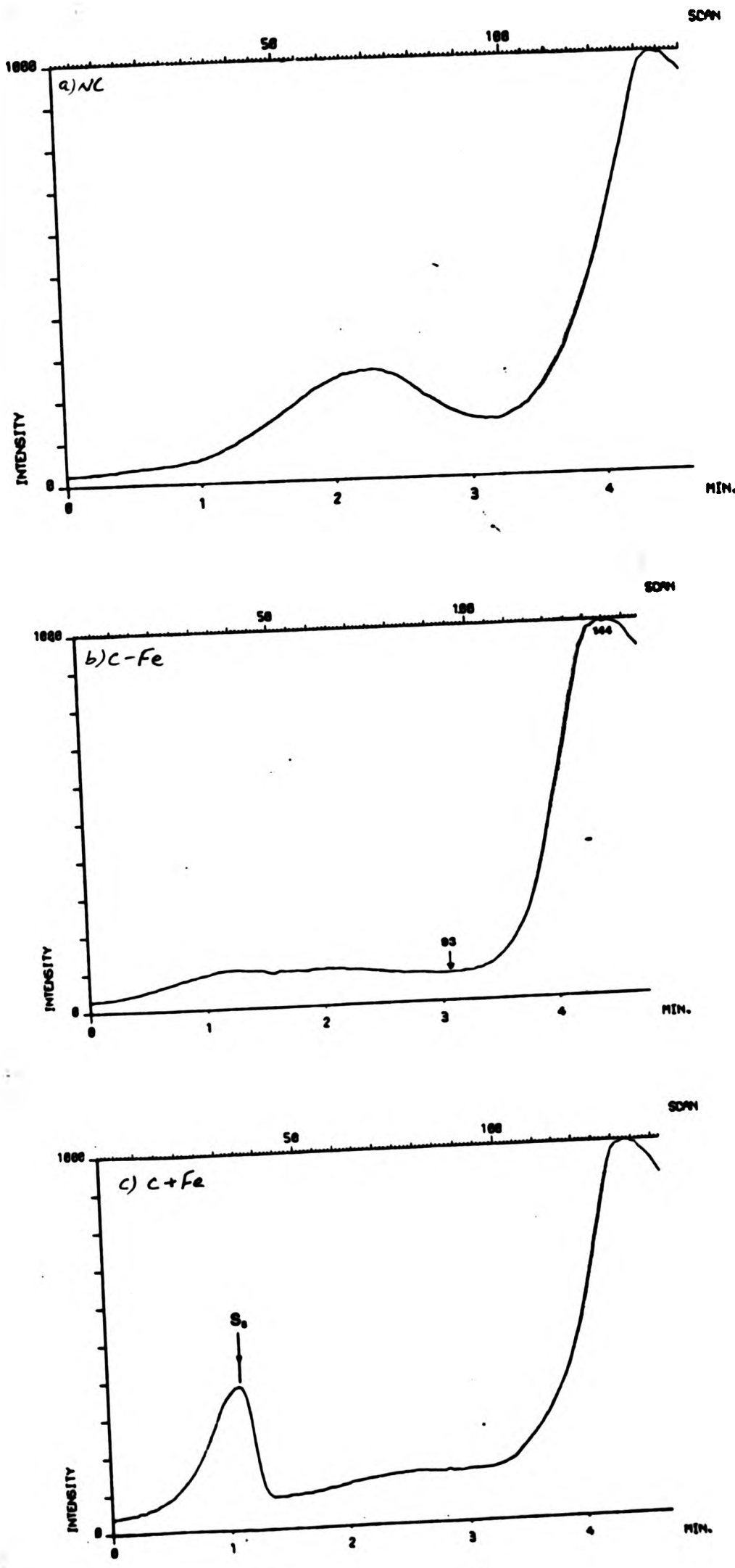


Figure 10. Mass spectrum of C+Fe cell pyrolysates. Arrows indicate the differences between C+Fe & C-Fe cells. These ions are lower than their counterparts in C-Fe cells.

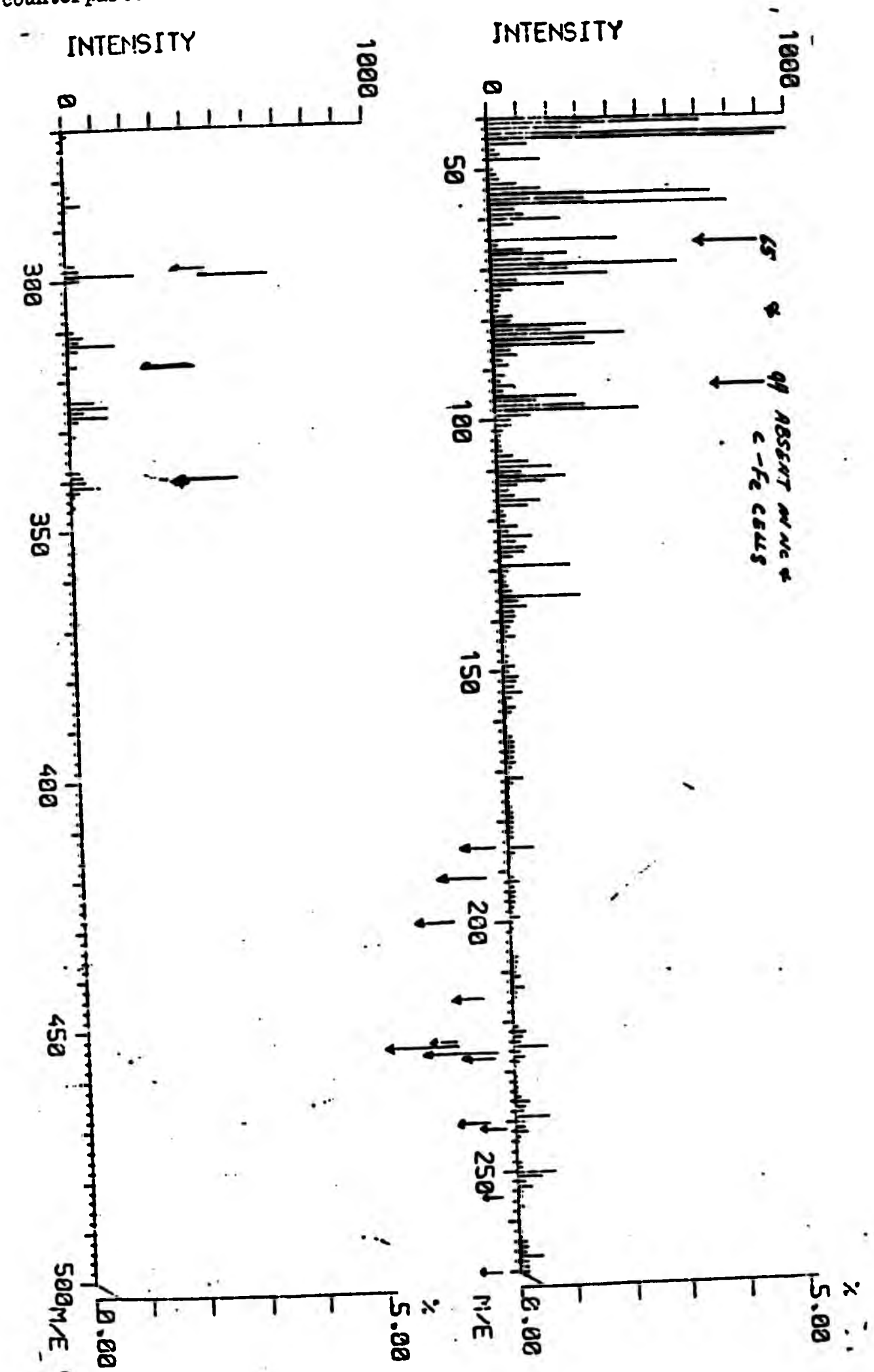


Figure 11. Mass spectrum of C-Fe cell pyrolysates. Arrows indicate differences between C-Fe & C+Fe cells. The intensities of these ions are lower than their counterparts in C+Fe.

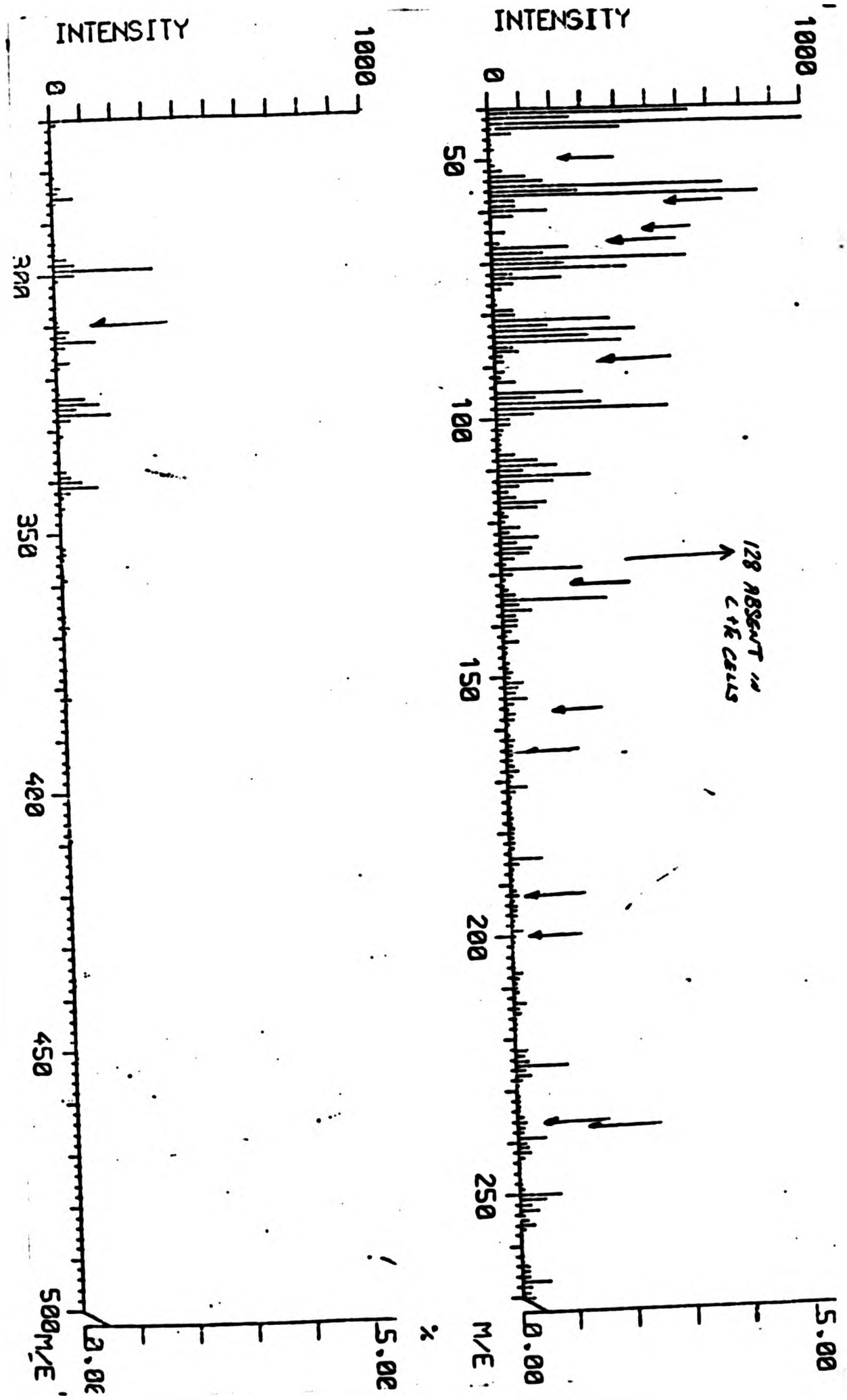


Figure 12. Mass spectrum of NC cell pyrolysates. ↓ indicates the intensities of these ions are lower than their counterparts in C-Fe. ▮ indicates the intensities of these ions are lower than their counterparts in C+Fe. In both cases the differences are at least 30%.

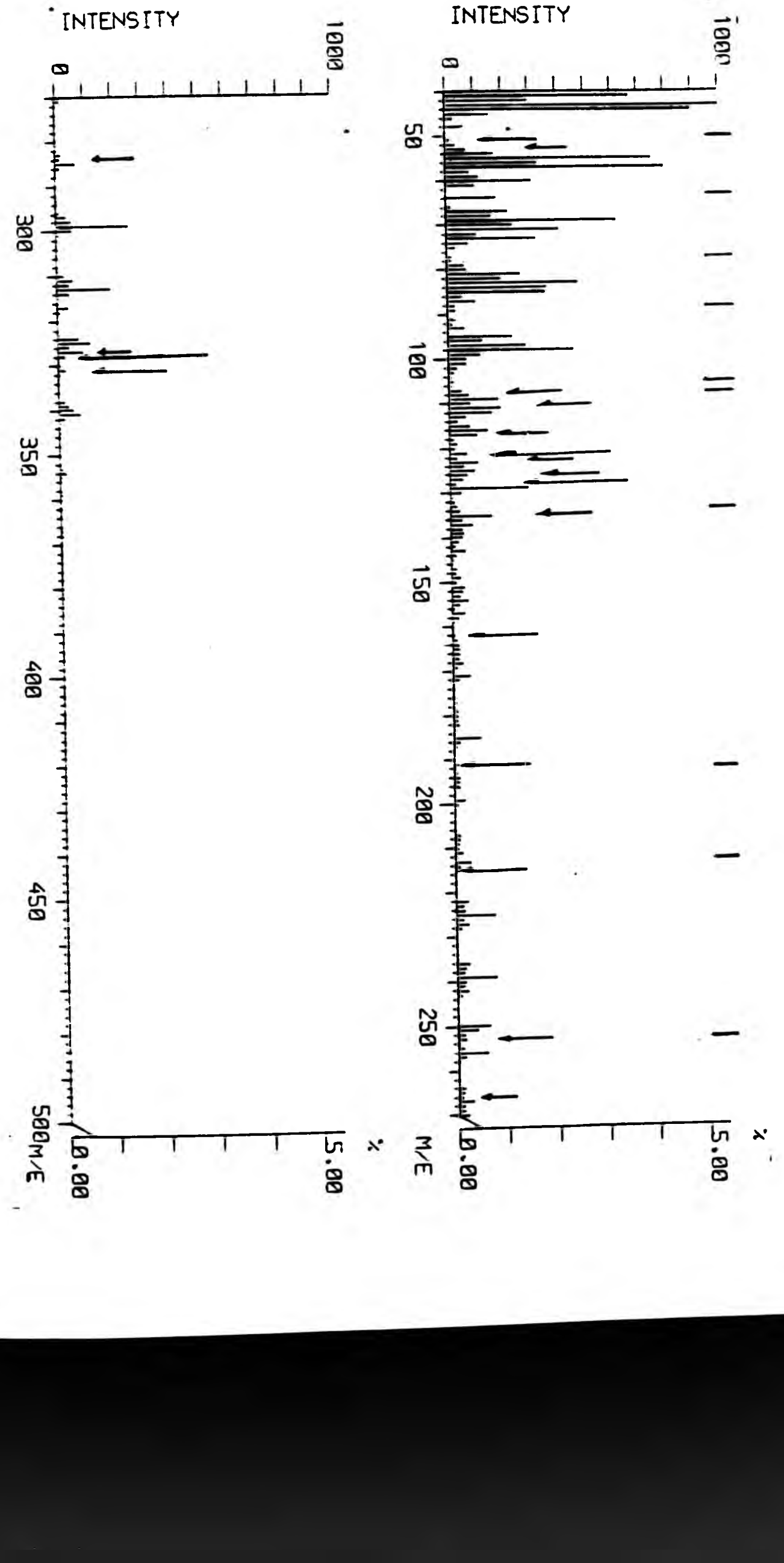
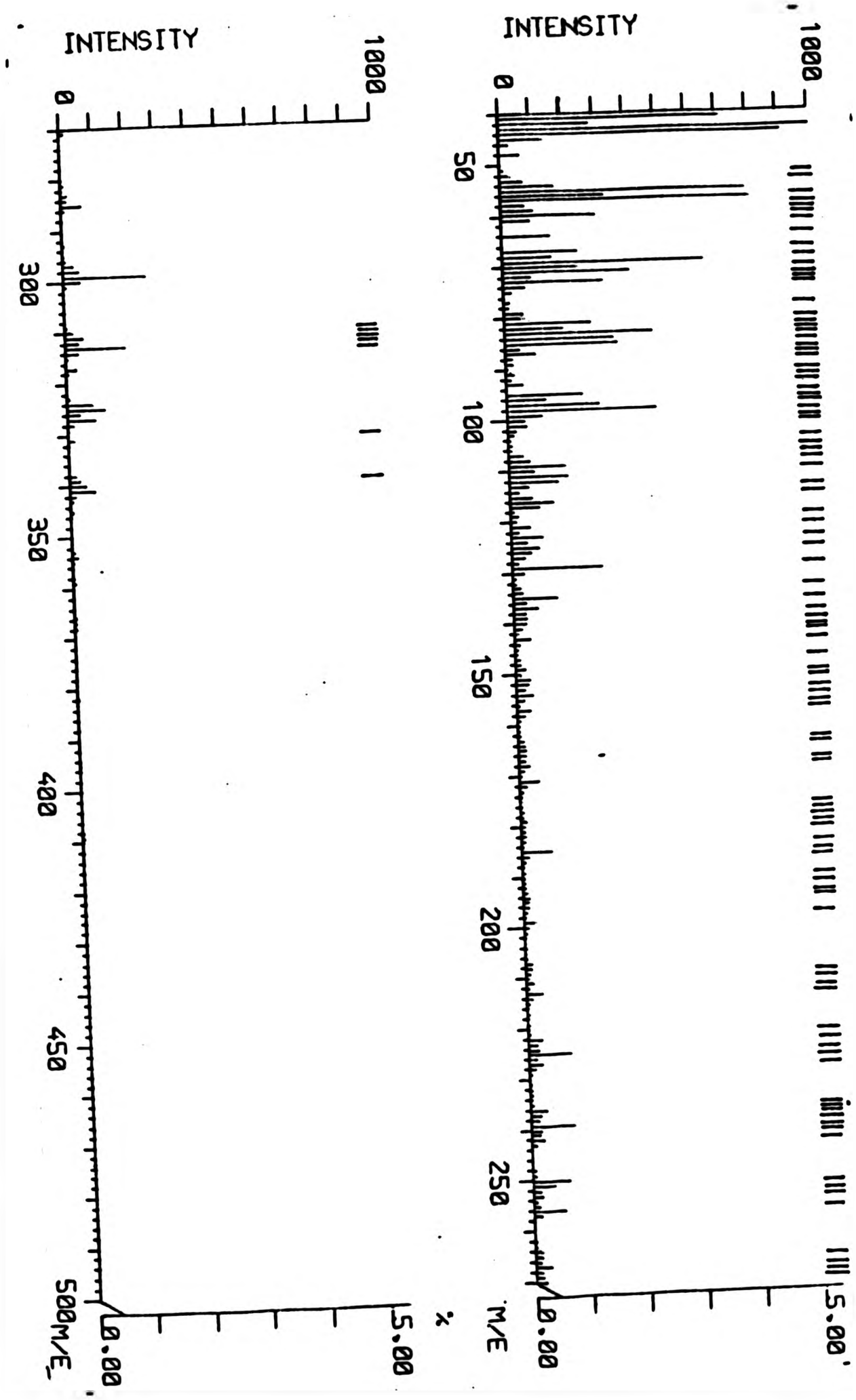


Figure 13. An example of the data obtained from mass spectrometric analyses of the pyrolysates.

MASS SPECTRUM) (85 TO 90) - 1.00(30 TO 40)
SAMPLE:DESULFOVIBRIO
NOTE :SJC NO A39/D :PROBE 50 DEG/MIN
BASE PEAK : M/E 41.0 INT. 45.3

M/E	RAW INT.	R.INT.	SIGMA(%)	M/E	RAW INT.	R.INT.	SIGMA(%)	M/E	RAW INT.	R.INT.	SIGMA(%)
33.0	1.2	26.9	0.23	75.0	0.4	10.7	0.09	114.0	1.2	28.2	0.24
36.0	3.9	86.1	0.75	76.0	0.5	11.4	0.10	115.0	3.0	68.0	0.59
37.0	0.2	5.3	0.04	77.0	1.8	40.4	0.35	116.0	1.3	28.9	0.25
38.0	1.1	26.2	0.22	78.0	0.4	10.7	0.09	117.0	0.1	3.3	0.02
39.0	7.0	156.2	1.36	79.0	2.6	58.5	0.51	118.0	0.5	11.4	0.10
40.0	4.6	103.7	0.90	80.0	1.1	24.9	0.21	121.0	2.3	52.5	0.45
41.0	45.3	1000.0	8.75	81.0	12.7	282.1	2.46	122.0	0.8	19.5	0.17
42.0	8.0	177.1	1.54	82.0	4.8	107.7	0.94	123.0	2.1	48.4	0.42
43.0	32.2	711.1	6.22	83.0	14.0	310.4	2.71	124.0	1.0	22.8	0.20
45.0	7.6	168.3	1.47	84.0	5.8	128.6	1.12	125.0	2.1	47.8	0.41
46.0	0.8	19.5	0.17	85.0	5.0	111.7	0.97	126.0	0.8	18.8	0.16
50.0	0.4	9.4	0.08	86.0	0.5	12.1	0.10	127.0	0.8	18.8	0.16
51.0	0.7	17.5	0.15	87.0	5.4	119.1	1.04	128.0	0.1	2.6	0.02
52.0	0.2	6.0	0.05	88.0	0.8	18.8	0.16	129.0	6.6	146.1	1.27
53.0	3.7	82.8	0.72	89.0	0.4	9.4	0.08	130.0	0.7	16.8	0.14
54.0	6.0	133.3	1.16	91.0	0.2	5.3	0.04	132.0	0.2	4.7	0.04
55.0	37.3	824.9	7.21	92.0	0.1	2.6	0.02	133.0	0.2	5.3	0.04
56.0	12.2	270.0	2.36	93.0	2.9	65.3	0.57	134.0	0.2	6.0	0.05
57.0	23.2	513.1	4.49	94.0	1.8	39.7	0.34	135.0	1.3	29.6	0.25
58.0	1.0	23.5	0.20	95.0	5.2	115.1	1.00	136.0	1.3	30.3	0.26
59.0	1.8	41.0	0.35	96.0	4.3	95.6	0.83	137.0	2.2	49.1	0.43
60.0	21.6	477.4	4.17	97.0	9.6	212.1	1.85	138.0	1.2	27.6	0.24
61.0	5.0	110.4	0.96	98.0	5.5	121.8	1.06	139.0	1.5	33.6	0.29
62.0	0.1	2.6	0.02	99.0	2.1	47.8	0.41	140.0	0.7	16.8	0.14
63.0	0.2	4.7	0.04	100.0	0.8	18.1	0.15	141.0	0.7	15.4	0.13
64.0	0.3	7.4	0.06	101.0	2.8	61.9	0.54	142.0	0.3	6.7	0.05
65.0	1.1	25.5	0.22	102.0	0.7	16.8	0.14	143.0	1.5	33.6	0.29
66.0	0.6	13.4	0.11	103.0	0.3	6.7	0.05	144.0	0.2	5.3	0.04
67.0	9.8	216.8	1.89	104.0	0.3	6.7	0.05	147.0	0.6	14.8	0.12
68.0	7.2	159.5	1.39	105.0	1.4	32.3	0.28	148.0	0.6	13.4	0.11
69.0	33.9	748.1	6.54	106.0	0.7	17.5	0.15	149.0	6.2	138.7	1.21
70.0	8.5	188.5	1.64	107.0	2.5	55.8	0.48	150.0	0.9	20.8	0.18
71.0	9.0	198.6	1.73	108.0	2.1	47.8	0.41	151.0	0.8	18.1	0.15
72.0	0.7	16.1	0.14	109.0	4.3	94.9	0.83	152.0	0.7	16.8	0.14
73.0	19.8	439.0	3.84	110.0	1.6	35.6	0.31	153.0	0.7	15.4	0.13
74.0	2.0	44.4	0.38	111.0	1.0	23.5	0.20	154.0	0.7	15.4	0.13
				112.0							
				113.0							

Figure 14. Illustration of the result of characteristicity analysis of the cell PYMS data. 1 indicates the ions picked up by the analysis to be differential among cell-types.



~440. The intensities of these differential ions have a % difference of ~ 70%. When NC and C-Fe are compared, there are few differences, although again many differences occur in the low mass range. When NC and C-Fe [illegible], the major differences lie between 100 and 150 mass ranges. Small differences in ion intensities are seen in the 170 - 330 mass ranges.

When these data are analysed by characteristicity analysis which take into account both intra-cell type and inter-cell type variations, a pattern of generalised difference is obtained (figure 14). This seems to represent a compilation of differences picked up by % difference analysis.

The data suggest that C-Fe cells have an increased quantity of high mass fragments. In an attempt to identify the cellular location of the differences, cell walls are analysed by PYMS.

3.1. PYMS of cell walls

Cell walls prepared by sonication and ultracentrifugation are subjected to PYMS analyses under the same conditions as the whole cells. The results are illustrated in figures 15 to 18, with characteristicity results in figure 19. Figure 15 shows the ion current profiles of the three wall-types. When the mass pyrogramme/spectra of walls are compared with those of cells, there is a decrease in intensities of the wall ions. This decrease in the ion intensities perhaps indicates

Figure 15. Ion current profiles obtained by FYMS analyses of cell walls.

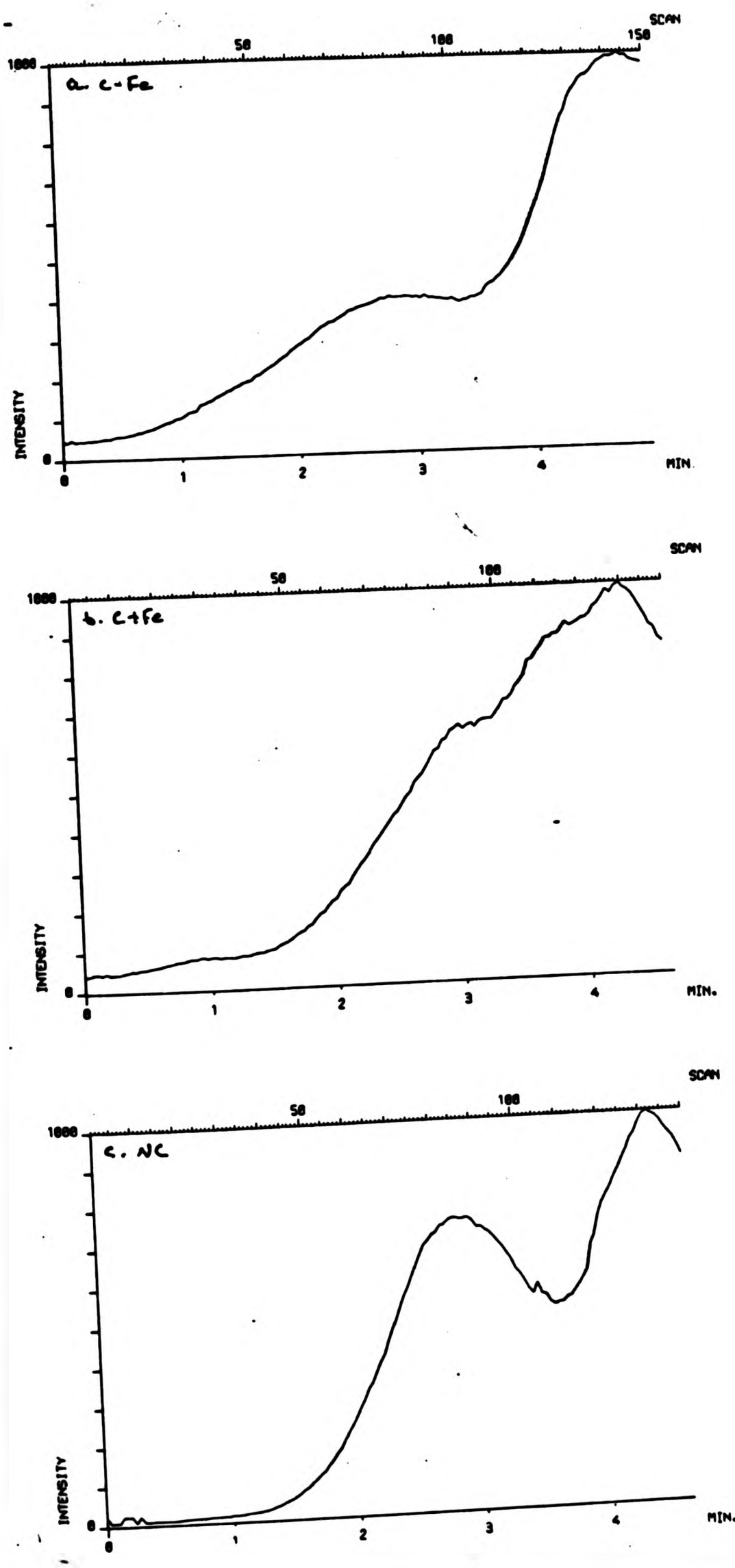


Figure 16. Mass spectrum of C+Fe cell wall pyrolysates. Arrows indicate the intensities of these ions are higher than their couterparts in C-Fe walls by 30% or more.

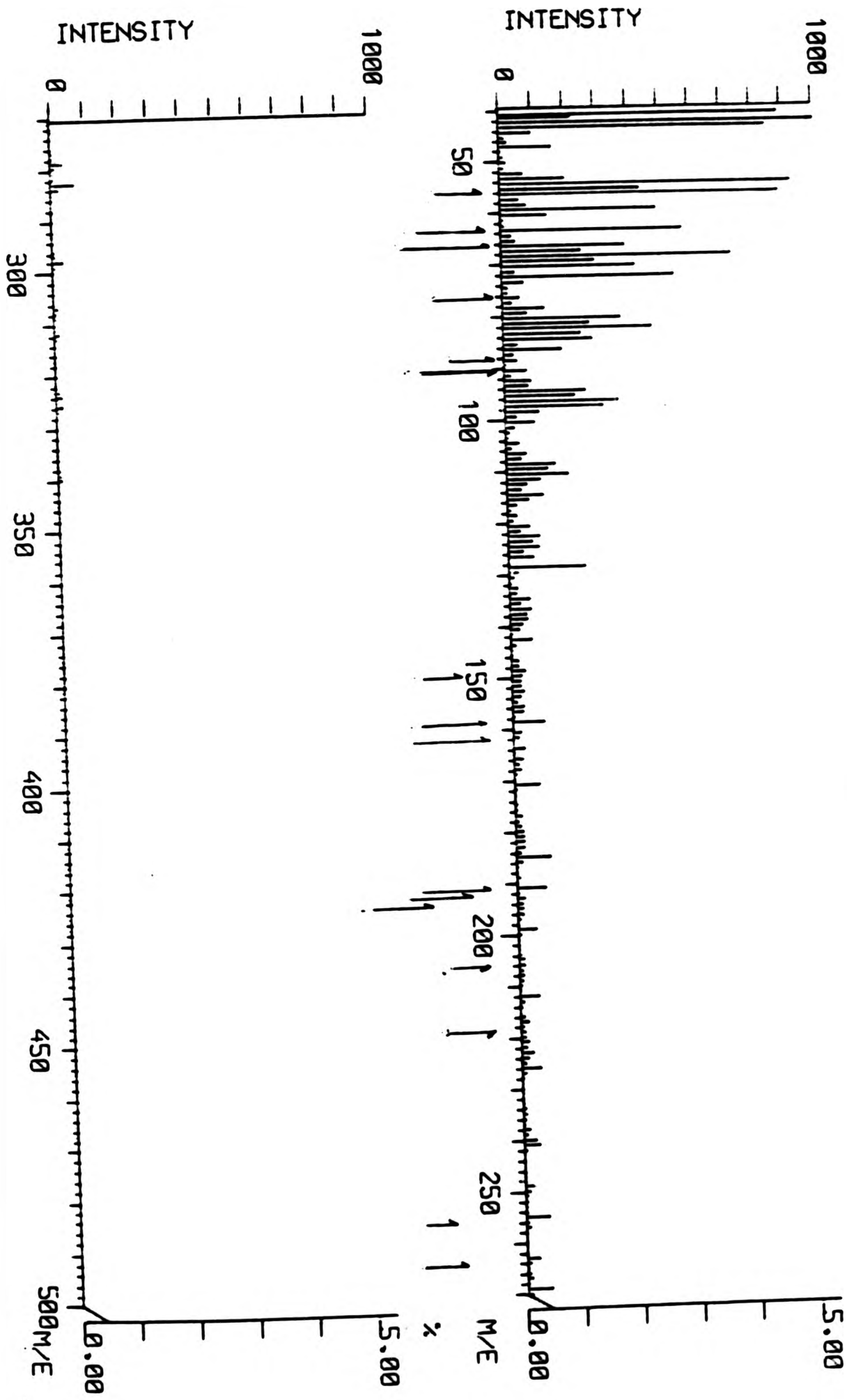


Figure 17. Mass spectrum of C-Fe cell wall pyrolysates. Arrows indicate the intensities of these ions are higher than their counterparts in C-Fe walls by 30% or more.

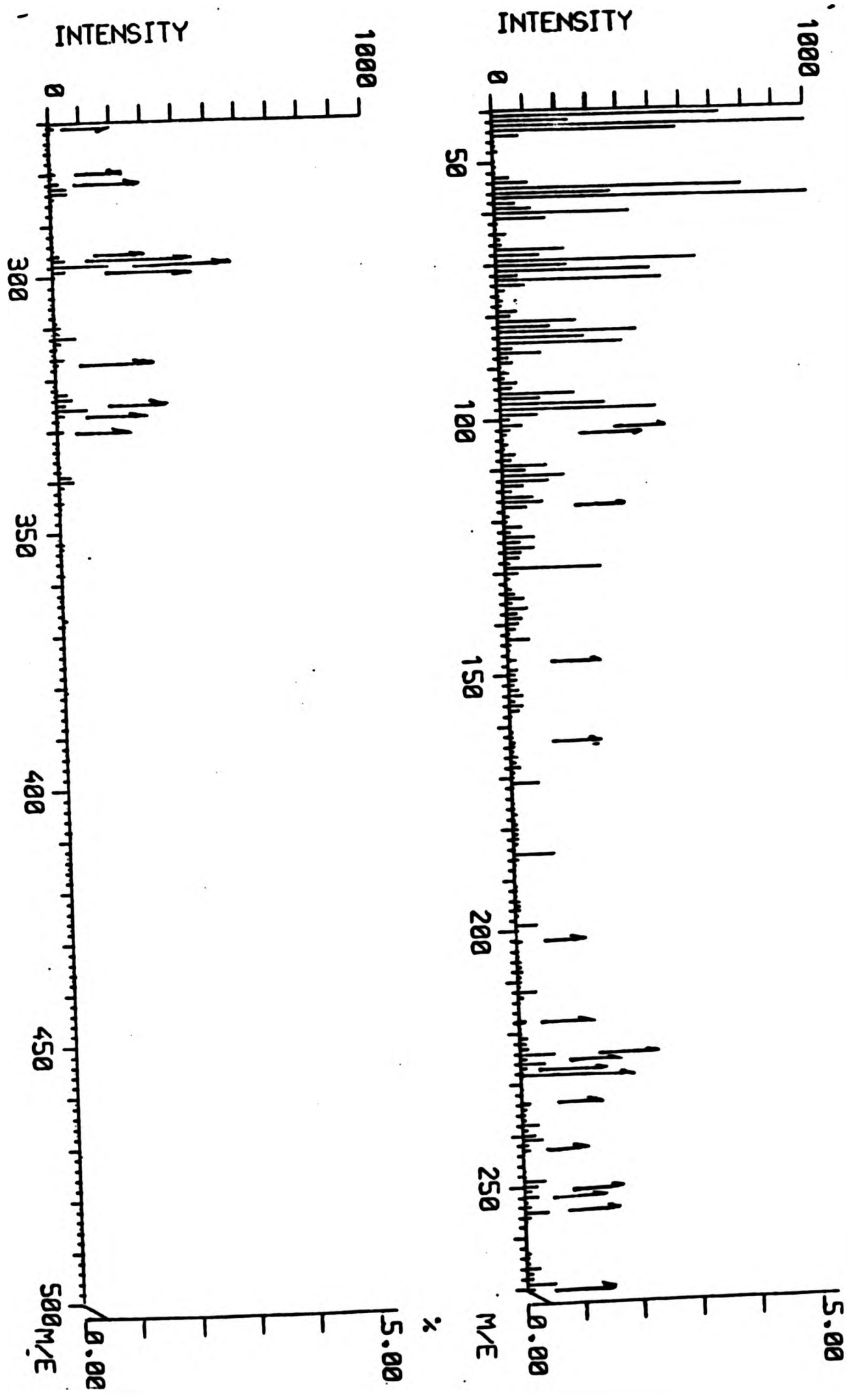


Figure 18. Mass spectrum of NC cell watt pyrolysates. † indicates the intensities of these ions are lower than their counterparts in C+Fe. ‡ indicates the intensities of these ions are lower than their C-Fe counterparts. In both cases the differences are at least 30%.

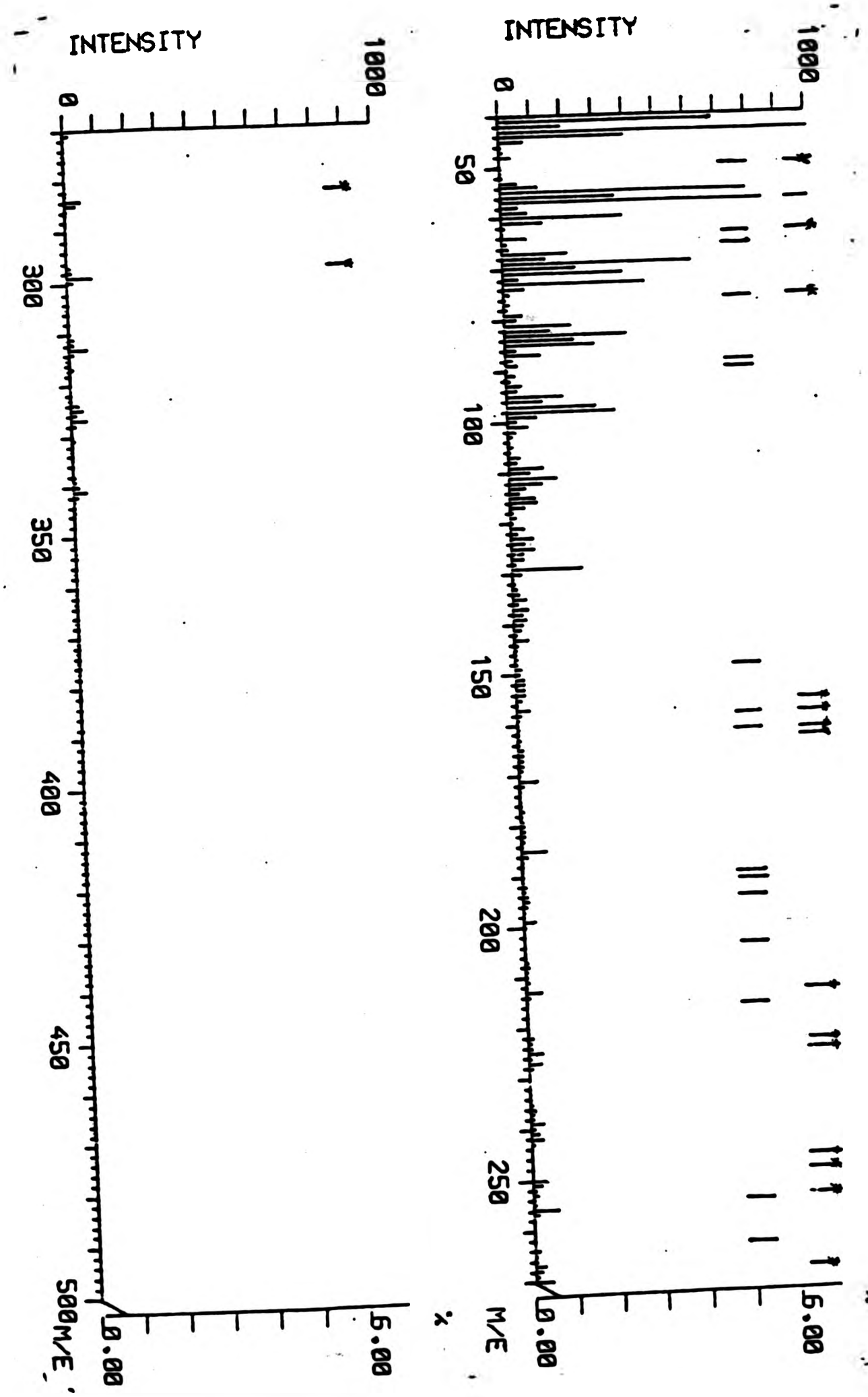
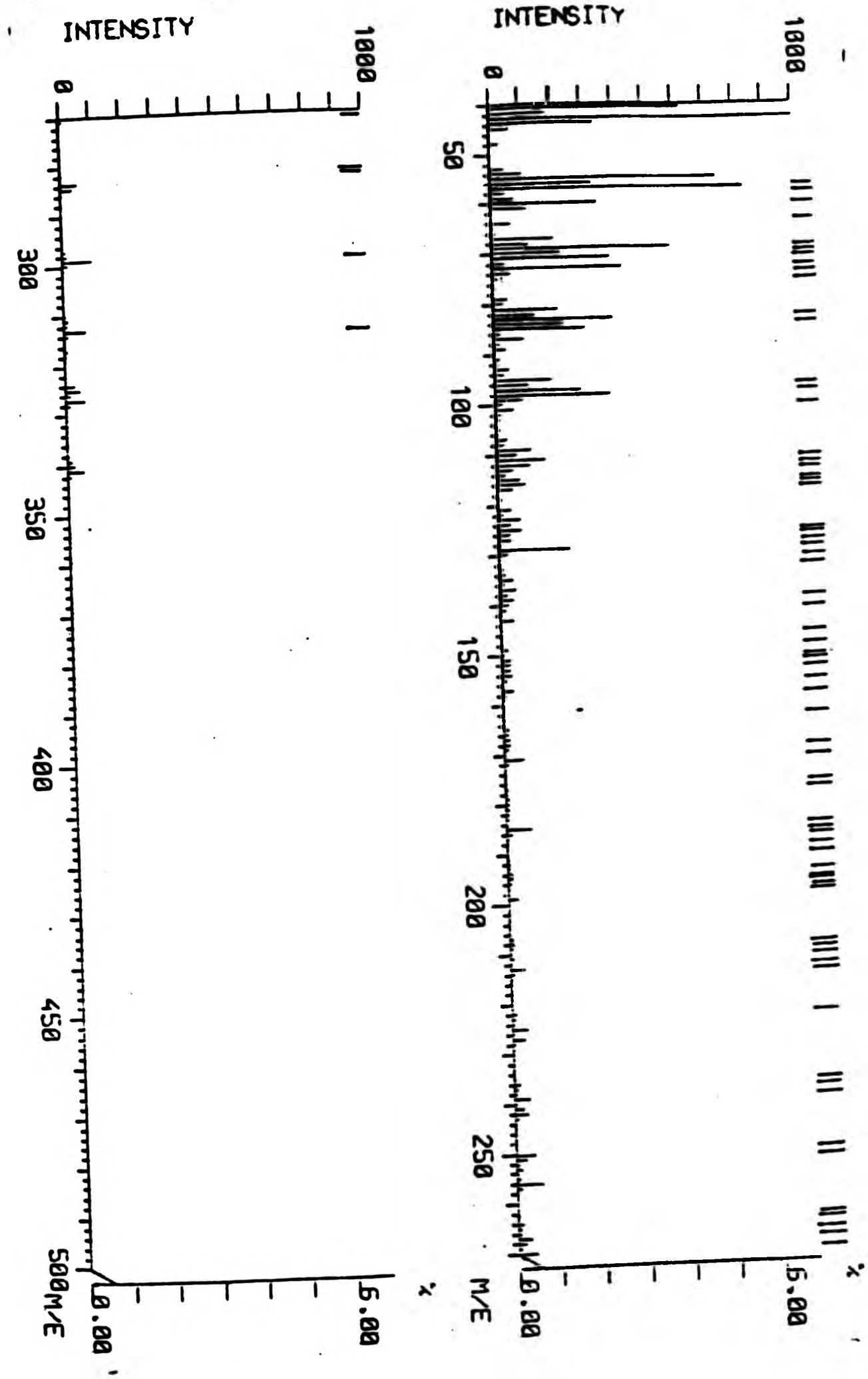


Figure 19. Illustration of the results of characteristicity analysis of the wall PYNS data. 1 indicates the ions picked up by the analysis to be differential among cell-types.



the loss of intracellular components which give rise to ions of the same mass. Similar observations have been reported in analyses of *B.subtilis* (Boon *et al.*,1981). In this organism the patterns of dissimilarity among the wall-types generally matched with those shown by the whole cells. If the ions picked up as indicators of differences between cell wall composition of the three cell-types are the same as those in the whole cell spectra, the differences observed in the whole cells could be due to the variations in wall composition.

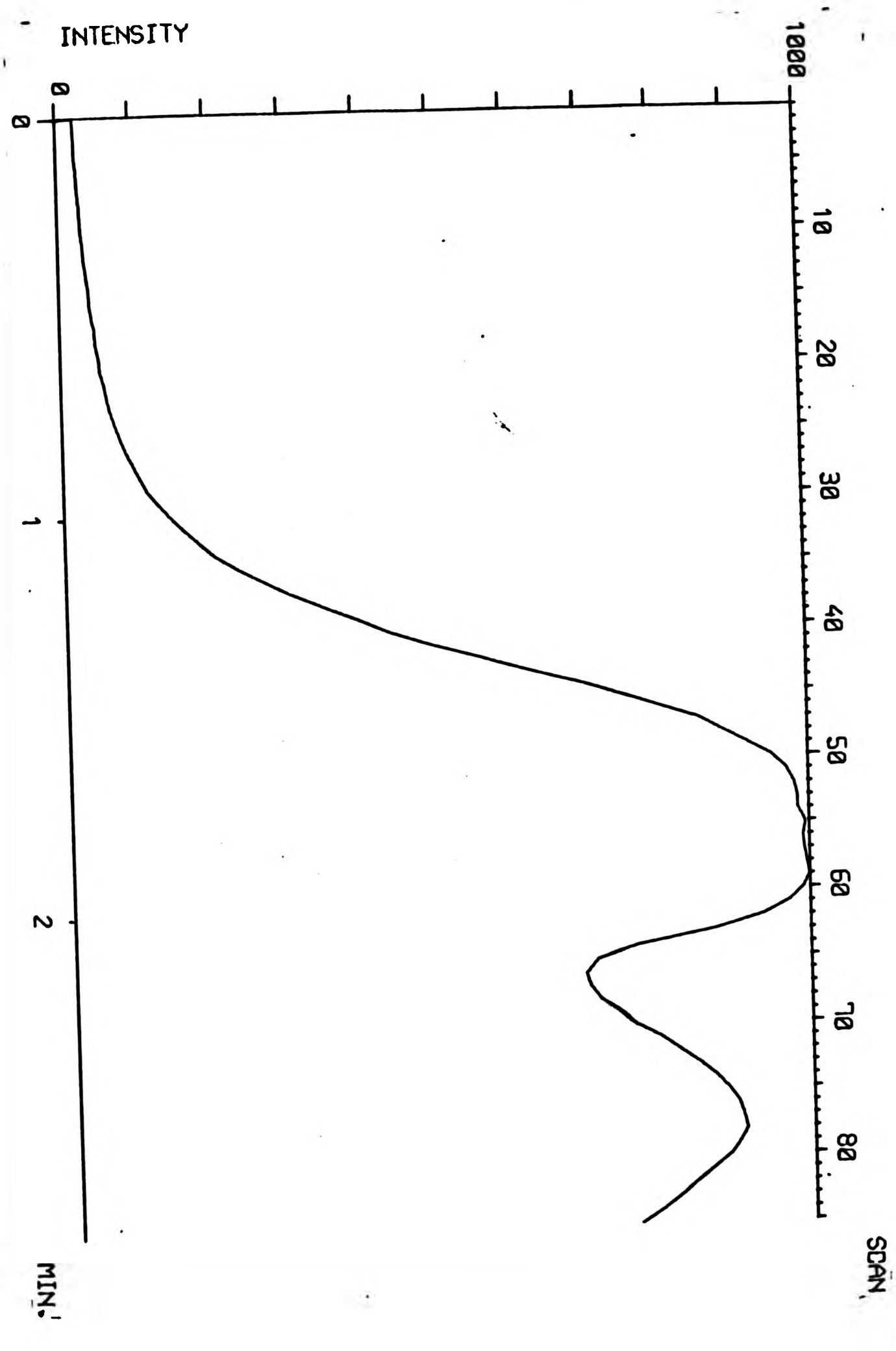
It has been reported by Boon *et al.*(1981) that the mass spectrum of peptidoglycan shows qualitative resemblances to the spectrum of chitin (Meuzelaar *et al.*,1974, and Weyman,1977, cited by Boon *et al.*,1981). Two series of ions characteristic of amino sugars have been identified in the mass spectra of chitin and peptidoglycan by these workers. They are m/z 67, 81, 95, 109, 123, 137, 151 and m/z 69, 83, 97, 111, 125, 139 and 153. These ions are also present in the pyrolysates of *Desulfovibrio* cells as reported here.

Ions with m/z in the 200-300 region have been suggested to be lipid-containing compounds (personal communication, C.Gutteridge, Cadbury Schwepps, Reading). If this is the case, then it seems that C-Fe cells produce more lipid-containing compounds in their cell walls than C+Fe or NC cells. These lipid-containing compounds may represent LPS. If this is true, than this may explain the increased yield in the LPS extracted from C-Fe cultures (see chapter four). Attempts to define the

ions contributed by LPS have been unsuccessful. The major problem is a technical one. Figure 20 shows that the ion current profile produced by extracted LPS is very irregular. This makes the designation of background difficult. It has not been possible to improve on this result. The double peak seen in the profile could represent the portions of LPS (lipid and polysaccharide). Alternatively, it may be caused by thermal lag, where the first peak (ions produced at a lower temperature) represent partially degraded LPS molecules. Analyses of isolated lipid from extracted LPS may be a better approach to solve this problem.

The PYMS data obtained from both whole cells and isolated cell walls suggest that C-Fe cells have altered cell walls. This difference may lie in the altered OM. OM components (e.g. OmpA and lipoproteins) are somehow involved in the maintenance or determination of cell shape (Lugtenberg, 1981). The altered shapes seen in C-Fe cells may be due to altered OM composition. An analysis of the OM will be appropriate to substantiate this hypothesis.

Figure 20. Ion current profile obtained by PYMS analysis of LPS.



Chapter Four

ANALYSIS OF THE OUTER MEMBRANE

The extracted membranes from the three cell-types (NC, C-Fe and C+Fe) differ from one another in appearance. C-Fe OM appeared buff in colour, while the other two are black. Blackening is due to the presence of adsorbed FeS particles, presumably the electron dense particles seen in TEM. The adsorption of FeS to the surface of *Desulfovibrio* cells has previously been reported by Findley and Agagi (1968).

The amount of LPS extracted from C-Fe and C+Fe cultures varies. C-Fe produces higher yield despite the poorer growth of these cells. Similar observations on yield of extracted LPS were reported by Bradley (1985). The observed differences in yield of extracted LPS probably indicates a change in the OM structure. This should be demonstrated by PAGE and serological analyses.

4.1. PAGE profile of OM components

The protein profiles of OMs extracted from the three cell-types are shown in plates 6 and 7. The technique employed is reproducible. C-Fe OM differs from the other two in having a reduced number of peptide bands. Three major protein bands (Omp1, Omp2 and Omp3) seen in C+Fe OM are

Plate 6. PAGE profile of OMs stained with PAGE BLUE 83.

lane 1 standards
" 2 C-Fe OM
" 3 NC OM
" 4 C+Fe OM

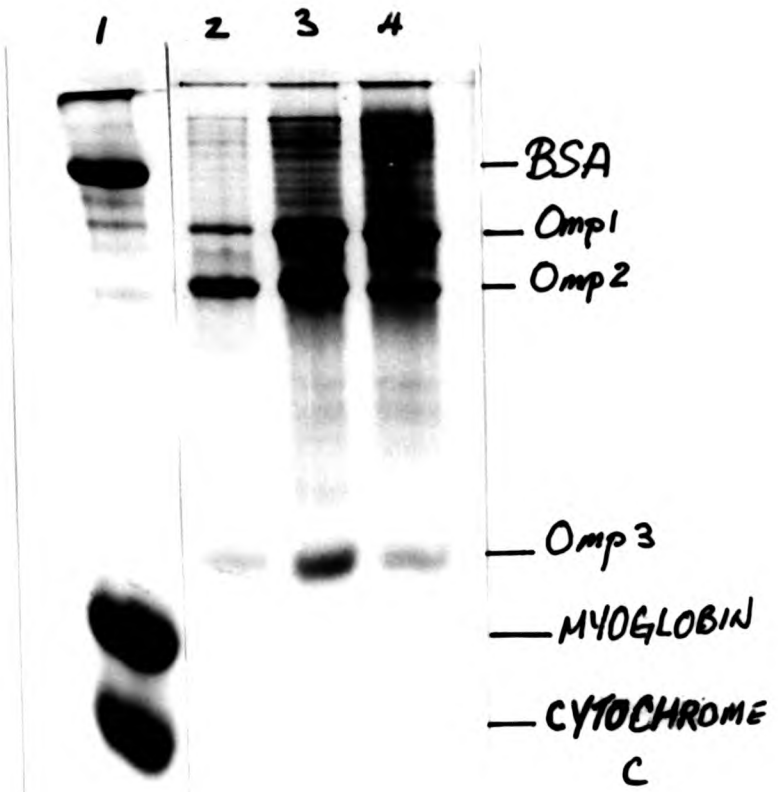


Plate 7. PAGE profile of OMs stained with PAGE BLUE 83. This plate shows that the band pattern is reproducible.

lane 1 standards
" 2 C-Fe OM
" 3 C+Fe OM
" 4 C+Fe OM
" 5 NC OM
" 6 NC OM

Replicates are from different cultures.

Plate 6. PAGE profile of OMs stained with PAGE BLUE 83.

lane 1 standards
" 2 C-Fe OM
" 3 NC OM
" 4 C+Fe OM

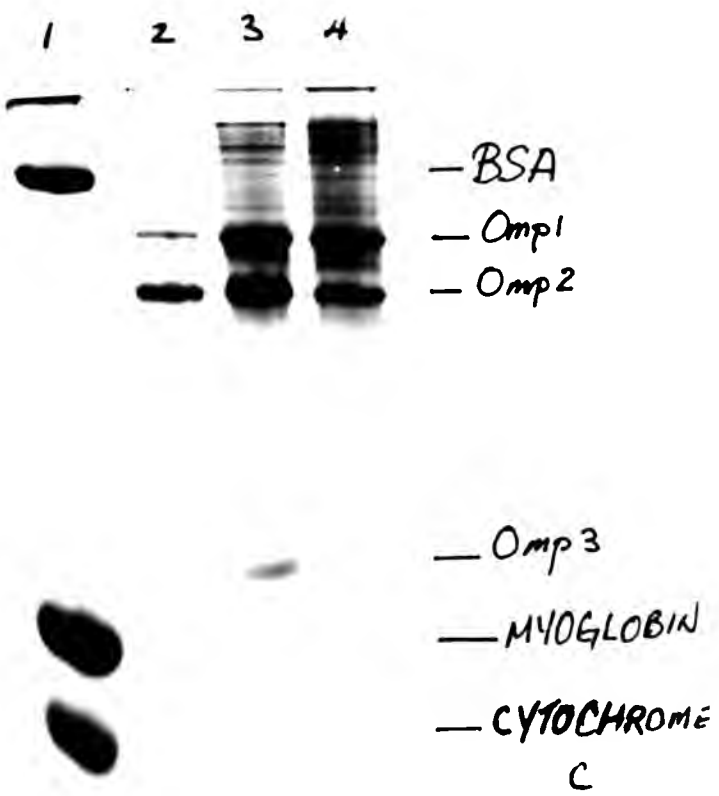


Plate 7. PAGE profile of OMs stained with PAGE BLUE 83. This plate shows that the band pattern is reproducible.

lane 1 standards
" 2 C-Fe OM
" 3 C+Fe OM
" 4 C+Fe OM
" 5 NC OM
" 6 NC OM

Replicates are from different cultures.

retained in the C-Fe OM. They have molecular weights of approximately 56.2K, 46.8K and 20.8K as determined by a logrithmic molecular weight/relative mobility plot (figure 21). The Omp1 band in C+Fe and NC OMs appears to be denser than that in C-Fe OM: the double banding appearance has been noted by Bradley (1985). Occasionally, some of the higher molecular weight bands are also retained in C-Fe cells. This suggests that the Fe level might have been higher than that normally present in C-Fe medium. There seems to be a slight enhancement in the quantity of Omp2 in both NC and C-Fe OMs (more clearly shown in plate 8). Furthermore the silver stain (plate 8) reveals bands not shown by page blue 83. The OM profiles from the different cell-types implies that these cells have different OM compositions. The differences noted in PYMS spectra for C+Fe and C-Fe cells (chapter three) could be partially due to these changes in the OM structures. However, it is not possible to exclude artifacts such as interference by ions with detergent action in the OM extraction procedures. To investigate this possibility, C-Fe cells were incubated with $FeSO_4$ at 0°C for 15 minutes prior to sonication and sarkosyl solubilisation. The results do not seem to indicate any interference by Fe(II) ions on sarkosyl action, since the OM extracted from C-Fe cells treated with $FeSO_4$ does not give C+Fe/NC OM PAGE patterns (results not shown). When the OMs extracted from C-Fe cells incubated with Fe(II) at room temperature prior to OM extraction were analysed by PAGE, they

Figure 21. Plots of $\log_{10}$ molecular weights of standard proteins against their relative mobilities, showing positions of 3 major Omps.

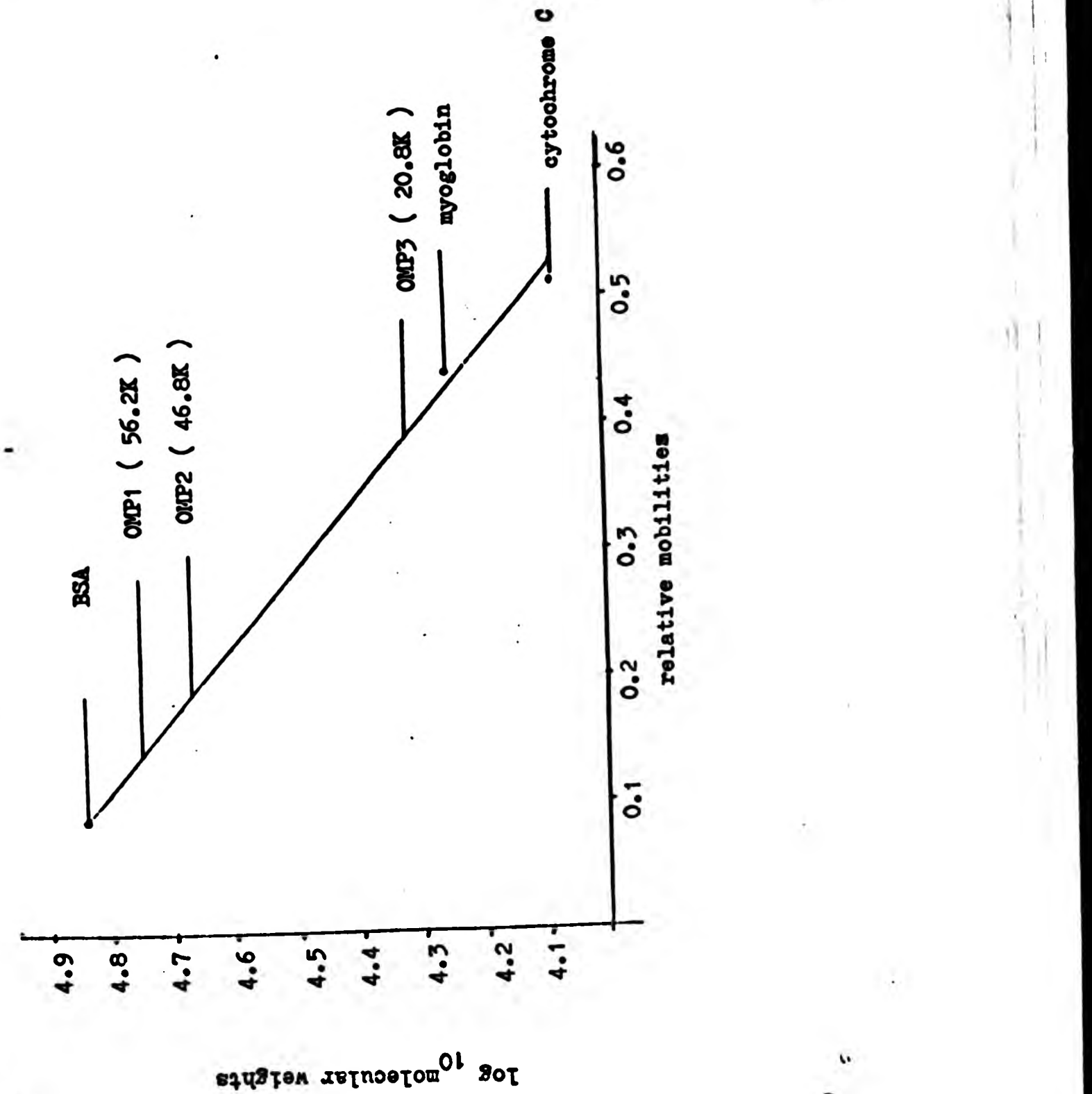
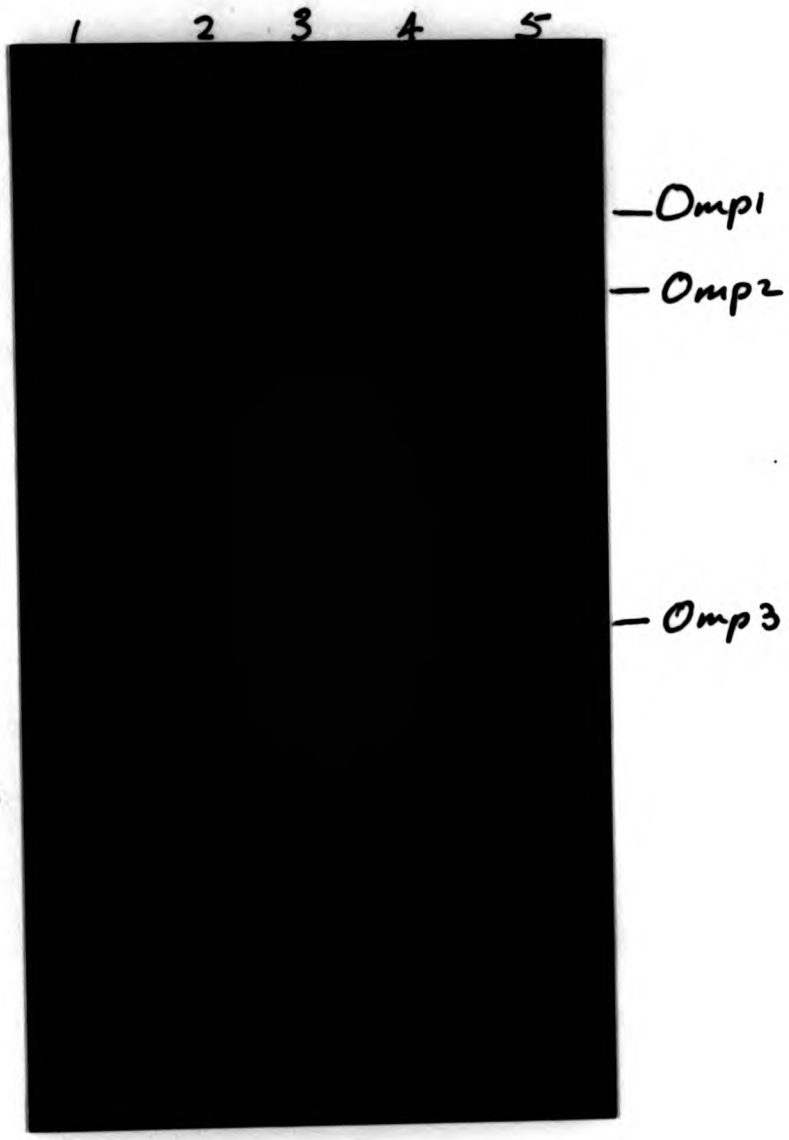
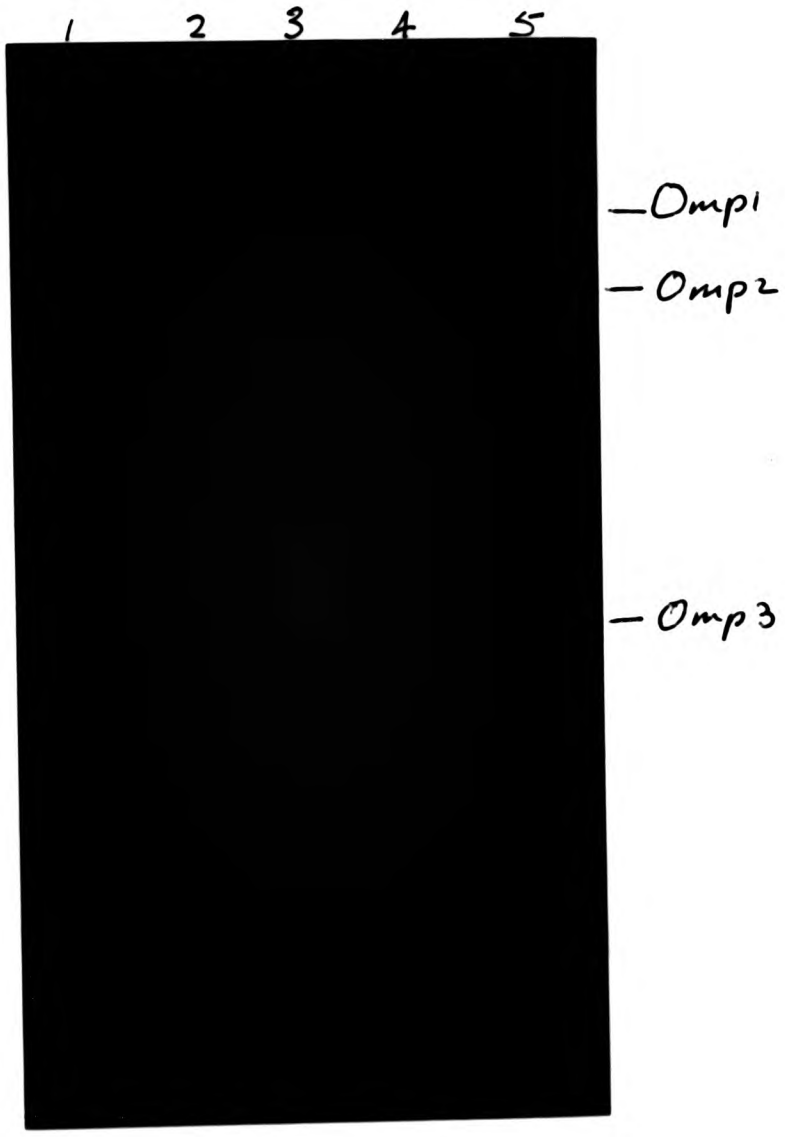


Plate 8. PAGE profile of OM stained with silver nitrate.



lane 1 standard proteins
" 2 C-Fe OM
" 3 NC OM
" 4 NC OM
" 5 C+Fe OM

Plate 8. PAGE profile of OM stained with silver nitrate.



lane 1 standard proteins
" 2 C-Fe OM
" 3 NC OM
" 4 NC OM
" 5 C+Fe OM

Plate 8. PAGE profile of OM stained with silver nitrate.

lane 1 utandard proteins
" 2 C-Fe OM
" 3 NC OM
" 4 NC OM
" 5 C+Fe OM

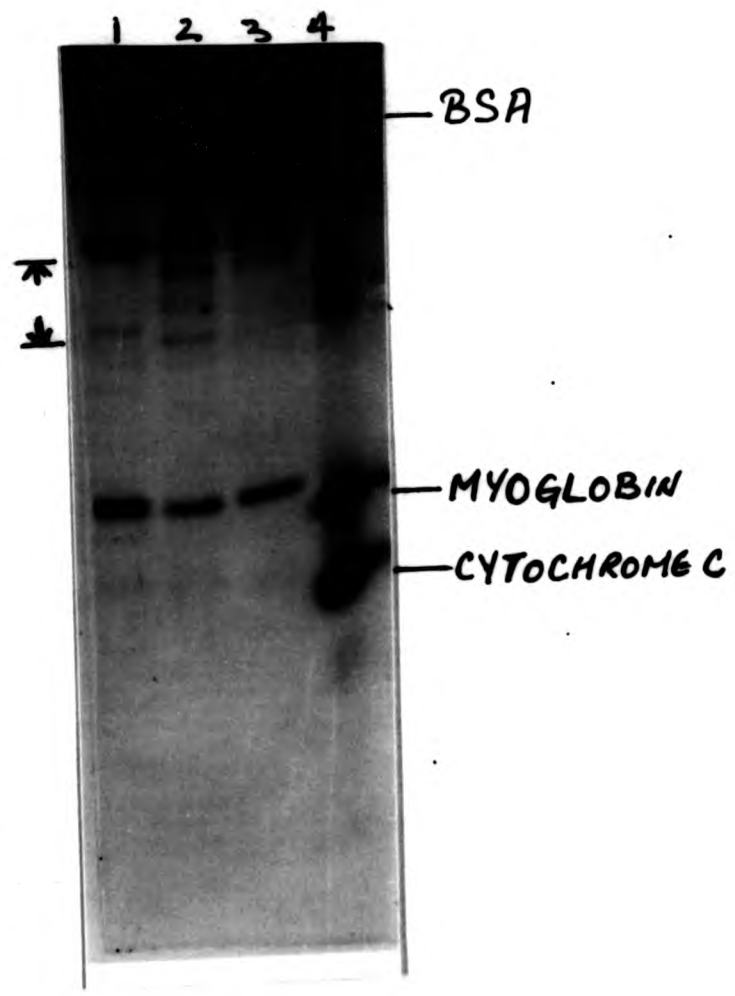
showed a partial restoration of the C+Fe/NC OM pattern, and the intensity of Omp1 band appeared to be greater (plate 9). This result suggests that Fe "protects" some Omps from detergent solubilisation. Also, it indicated an interaction between Omps and Fe(II). The negative result obtained at 0°C suggests that the interaction requires energy.

The partial resemblence of Omp pattern in "protected" C-Fe OM to C+Fe/NC OM profile means there are minor differences in the OMs of C-Fe and C+Fe/NC. The observed pleomorphism in C-Fe cells could be due to the reduced structural OM components in the C-Fe OMs. It is known that *E.coli* mutants lacking Braun's lipoproteins are unable to grow in the rod form (Sonntag *et al.*, 1978), and it would be interesting to identify the nature of the missing proteins in the C-Fe OM. LPS could be increased to compensate for this decreased proteins content. Goldberg and Nikaido (unpublished results cited by Nikaido and Vaara, 1985) found that there was a 20-30% increase in LPS in *S.typhimurium* deep rough mutants with subnormal OM protein levels. This could explain the higher LPS yield in C-Fe cells. However, Smit *et al.*(1975) reported unchanged LPS content in such mutants.

In PYMS wall analyses, however, a standard quantity of specific wall was used for the studies. The results suggest that there is a difference between C-Fe wall and the other two types and the increased level of ions of mass 200-300 suggests that lipid-containing molecules are higher in C-Fe walls.Thus

Plate 9. PAGE profile of "Fe-protected"
C-Fe OM stained with PAGE BLUE 83.

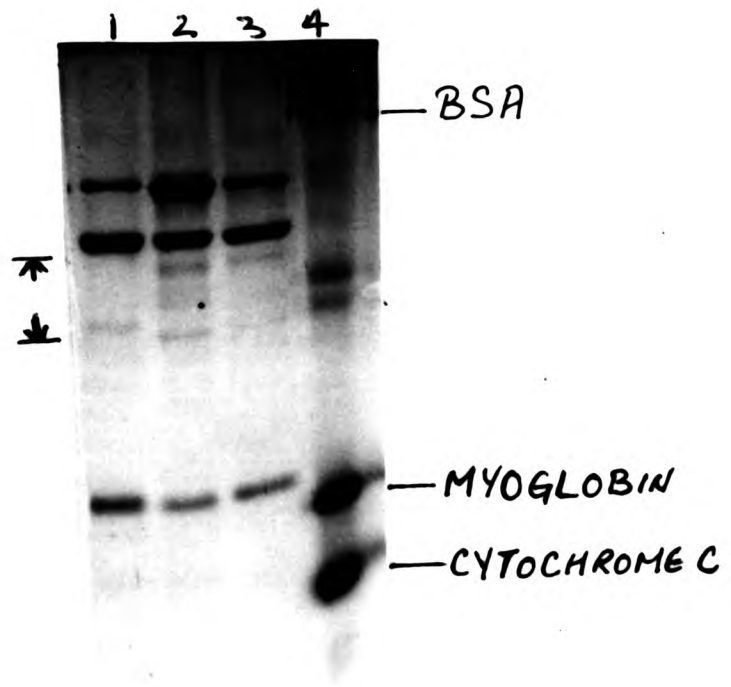
lane 1 C-Fe OM
" 2 "protected C-Fe OM
" 3 unprotected C-Fe OM
" 4 standard proteins



Arrowed area indicates extra bands in lane 2.

Plate 9. PAGE profile of "Fe-protected"
C-Fe OM stained with PAGE BLUE 83.

lane 1 C-Fe OM
" 2 "protected C-Fe OM
3 3 unprotected C-Fe OM
" 4 standard proteins



Arrowed area indicates extra bands in lane 2.

detected LPS differences may be real.

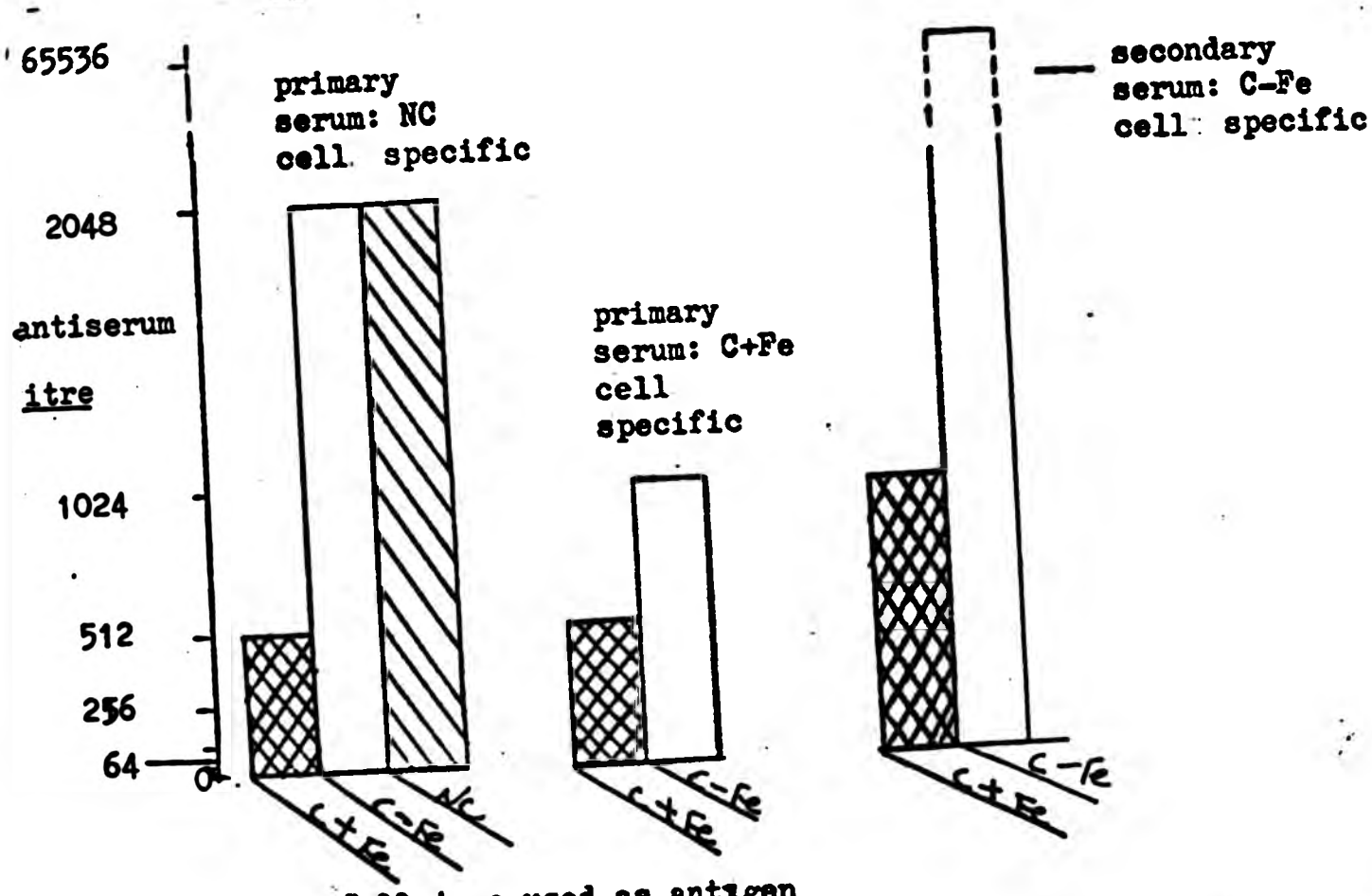
One-dimensional SDS-PAGE does not allow polypeptides of the same mobility to be classified as identical. A second approach to study surface molecules is serological analysis using specific antisera.

4.2. Analysis of antisera by agglutination

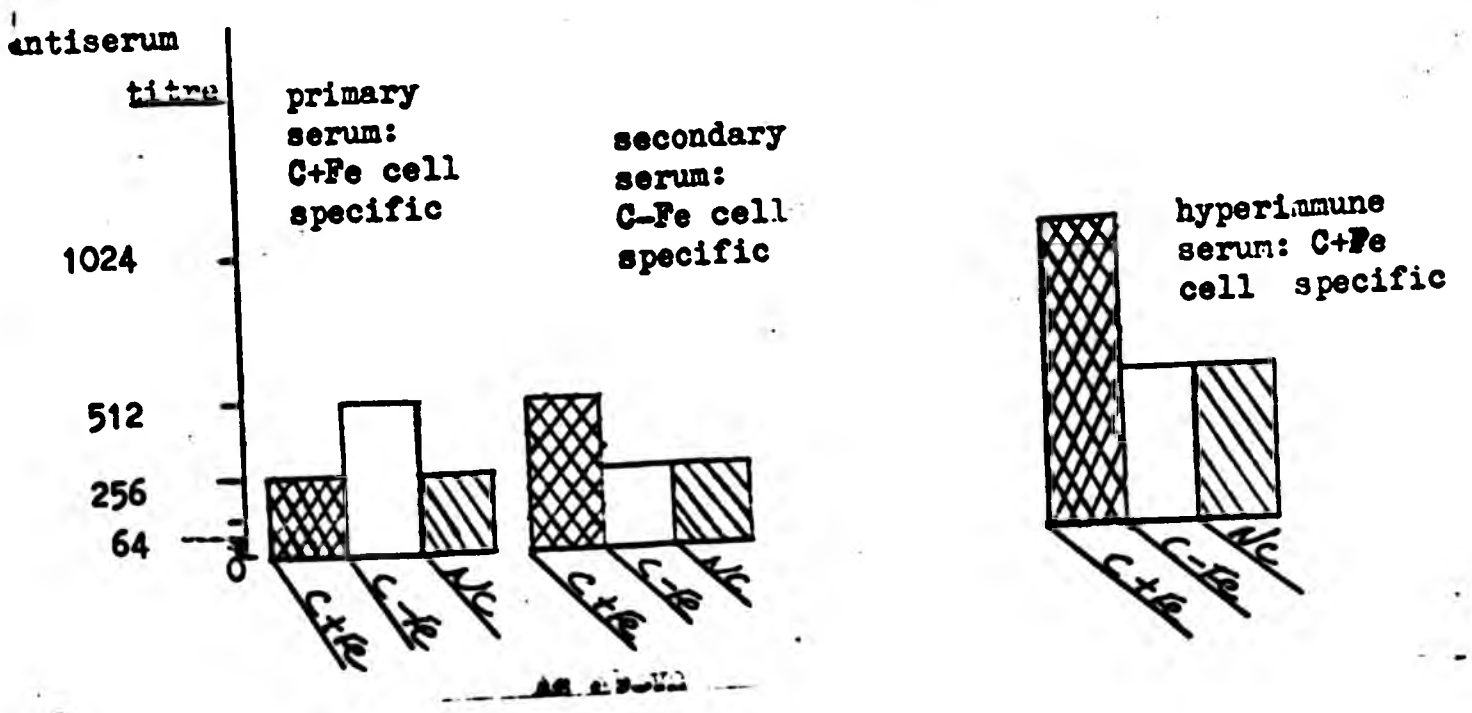
The results illustrated in figure 22 show titres obtained from assays performed in Durham tubes and microtitre plates at various times. When any particular antiserum is used to test against other cell types, the results obtained seem to suggest that there are no major differences between C-Fe and NC cells. However, there is a lower titre in Durham tube agglutinations for C+Fe cells. The slight difference in titre suggests only minor variations, if any. However, the presence of FeS on the C+Fe cell surface might interfere with the antigen-antibody reaction, which depends upon the stereo-conformations of the reactants. It is possible that FeS, together with other ionic species, might form pockets of colloids on the surface in such a way that antibodies do not have access to the surface antigens. This is not impossible, since C+Fe cells are only washed once before the test.

Microtitre plate results differ from the above in that C+Fe cells produce a higher titre than C-Fe (512 as compared with 1024), when used as antigen against anti-C-Fe and anti-LPS antisera. In contrast, when anti-C+Fe serum is used

Figure22. a. Histograms showing titres of different antisera and their relative titres against individual cell-types (results obtained from Durham tubes).



b. Histograms showing titres obtained from microtitreplate assays.



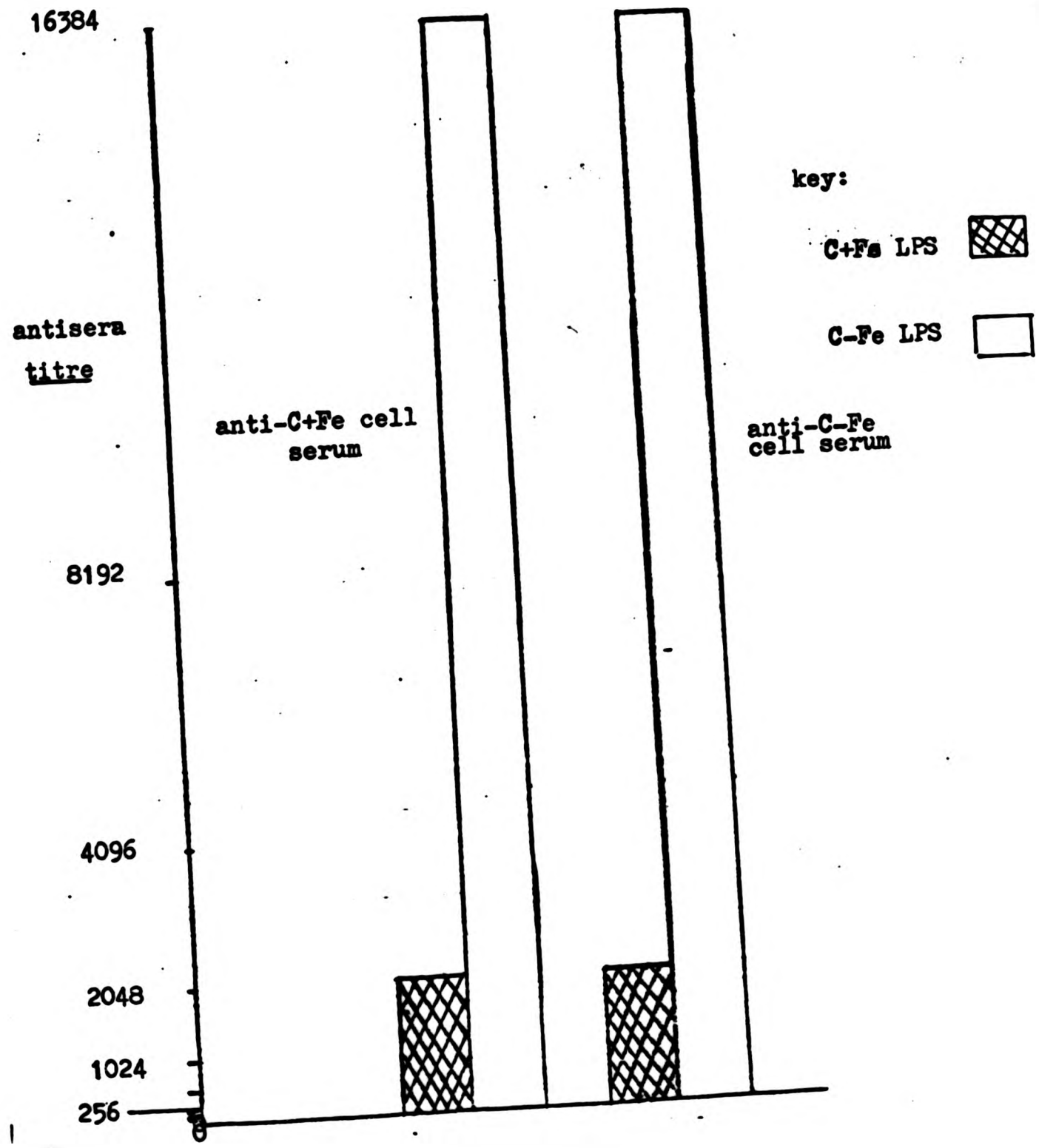
against non-homologous cell types, C-Fe cells give a higher titre (512), while those of C+Fe and NC are the same (256). The disagreement here is probably due to (a) variation in cell density in the antigen suspension and (b) in this set of assays, C+Fe cells are washed in 0.85% aqueous NaCl to remove excess FeS prior to use (Singleton *et al.*, 1985). Here, titres of C+Fe and NC in the test using anti-C+Fe serum are identical (256), but lower than for C-Fe cells (512). However, when anti-C-Fe and anti-C-Fe LPS sera are used against the three cell types, C+Fe cells give a higher end point (512 & 1024 respectively) than the other two cell types, which yield similar end points of 256 and 512 respectively.

Together with the data from Durham tube assays, the results imply that the surface antigens, as far as those expressed by these three cells are concerned, are probably identical. The relatively large difference observed in Durham tube assays (especially with C-Fe serum) is probably caused by excess FeS in the suspension used for the assay. Passive haemagglutination results (fig.23) suggest only minor variations between the two cell types. In this test the differences in titre can be explained by the presence of adsorbed ferric ions on the C+Fe LPS. This is observed as a brown colouration.

Immunoblotting and chemical analyses of the LPS should help to clarify the picture.

4.3. Immunoblots

Figure 23. Histograms showing titres obtained from passive heamagglutination assay using anti-C-Fe & anti-C+Fe antisera.



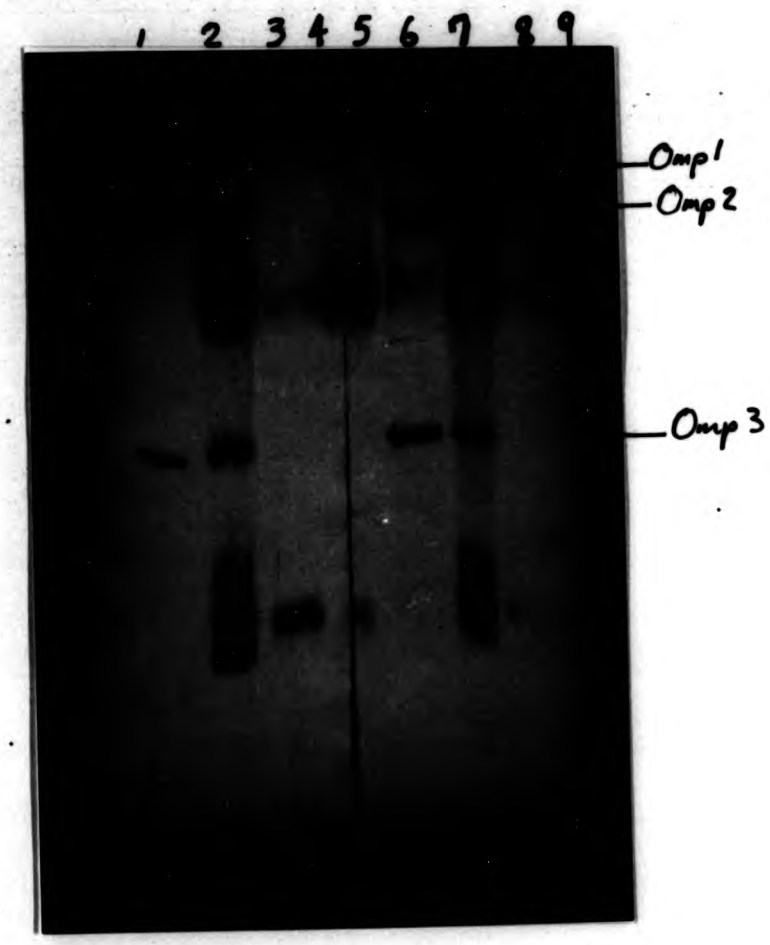
The results of the immunoblots are shown in plates 10, 11, & 12. For lanes 2 to 4, the first antibodies used are C+Fe specific and for lanes 5 to 9, the first antibodies are C-Fe specific. Neither of the antisera differentiates the two different OMs and LPS used, except that the Omp1 bands are rather weak (plate 10). Plate 11 shows the reverse side of the blot showing lanes 5 to 9. This is to show the LPS bands not clearly shown in plate 10. In addition, radio-immunodetection of OM bands using ^{125}(I)-anti-rabbit IgG revealed no significant antigenic differences among expressed surface antigens (plate 12).

The immunological data thus far does not indicate new OM components in C-Fe cultures. Although *Desulfovibrio* requires Fe(II) for growth, it survives when subjected to iron deprivation. Unlike *Pseudomonas* in ferric-deficient conditions (Meyer *et al.*, 1979), it does not produce any new molecules in its OM. Rather *Desulfovibrio* appears to increase the production of Omp2 (section 4.1.). This molecule may therefore, be important in iron uptake by the cells.

4.4. ^{125}I-radiolabelling of outer membrane proteins

The results obtained are illustrated in plates 13 to 17. Plate 13 shows the PAGE profiles of OMs and whole cells obtained from these experiments. Three Omps (Omp1, 2 & 3) are labelled irrespective of the length of incubation time (15 minutes, 8 minutes, 4.5 minutes 45 seconds) Multiple labelled bands are also observed in the OMs extracted from cells incubated with ^{125}I for a period of 15, 8 and 4.5 minutes. Even

Plate 10. Immunoblots of C-Fe and C+Fe OMs and LPS analysed with rabbit anti-C+Fe and anti-C-Fe antisera.

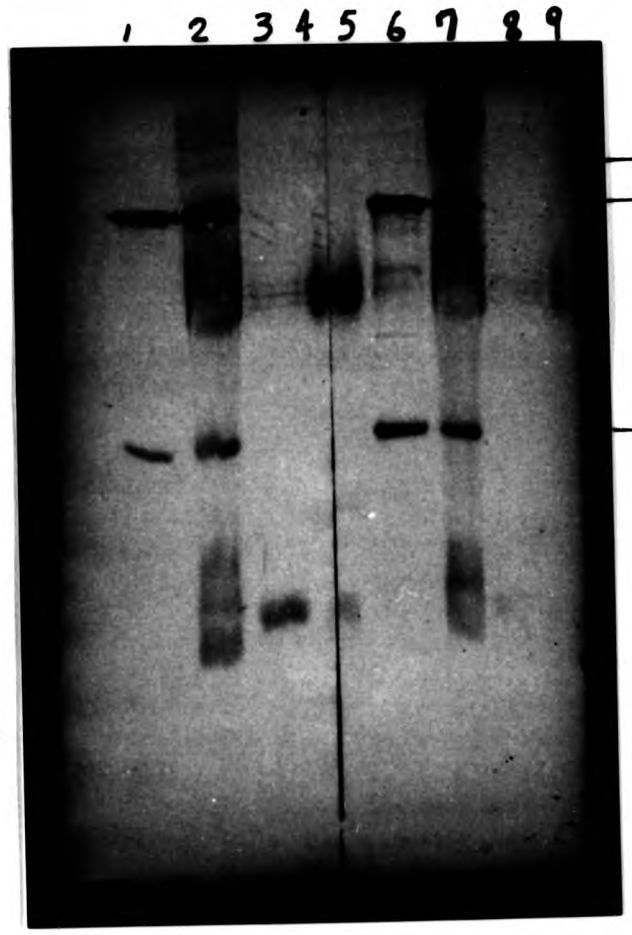


lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe LPS
" 4 C+Fe LPS
" 5 C+Fe LPS

lane 6 C-Fe OM
" 7 C+Fe OM
" 8 C-Fe LPS
" 9 C+Fe LPS

lanes 2-4 probed with anti-C+Fe serum.
lanes 5-9 probed with anti-C-Fe serum.

Plate 10. Immunoblots of C-Fe and C+Fe OMs and LPS analysed with rabbit anti-C+Fe and anti-C-Fe antisera.



lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe LPS
" 4 C+Fe LPS
" 5 C+Fe LPS
" 6 C-Fe OM
" 7 C+Fe OM
" 8 C-Fe LPS
" 9 C+Fe LPS

lanes 2-4 probed with anti-C+Fe serum.
lanes 5-9 probed with anti-C-Fe serum.

Plate 11. The reverse side of the blot analysed with anti - C-Fe serum showing the LPS band not clearly shown in the C-Fe OM in plate 9.

lane 1 C+Fe LPS
" 2 C-Fe LPS
" 3 C+Fe OM
" 4 C-Fe OM
" 5 C+Fe LPS

Plate 12. Radioimmunoblot using ^{125}I-goat anti-rabbit IgG.

lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe OM
" 4 C+Fe OM

The 1st antibodies used for lanes 1 and 2 are C-Fe specific.

The 1st antibodies used for lanes 3 and 4 are C+Fe specific.

This immunoblot was kindly performed by Dr. Chart of NIBSC. HOlly Hill, Hampstead, London.

Plate 11 . The reverse side of the blot analysed with anti - C-Fe serum showing the LPS band not clearly shown in the C-Fe OM in plate 9.

lane 1 C+Fe LPS
" 2 C-Fe LPS
" 3 C+Fe OM
" 4 C-Fe OM
" 5 C+Fe LPS

Plate 12. Radioimmunoblot using ^{125}I-goat anti-rabbit IgG.

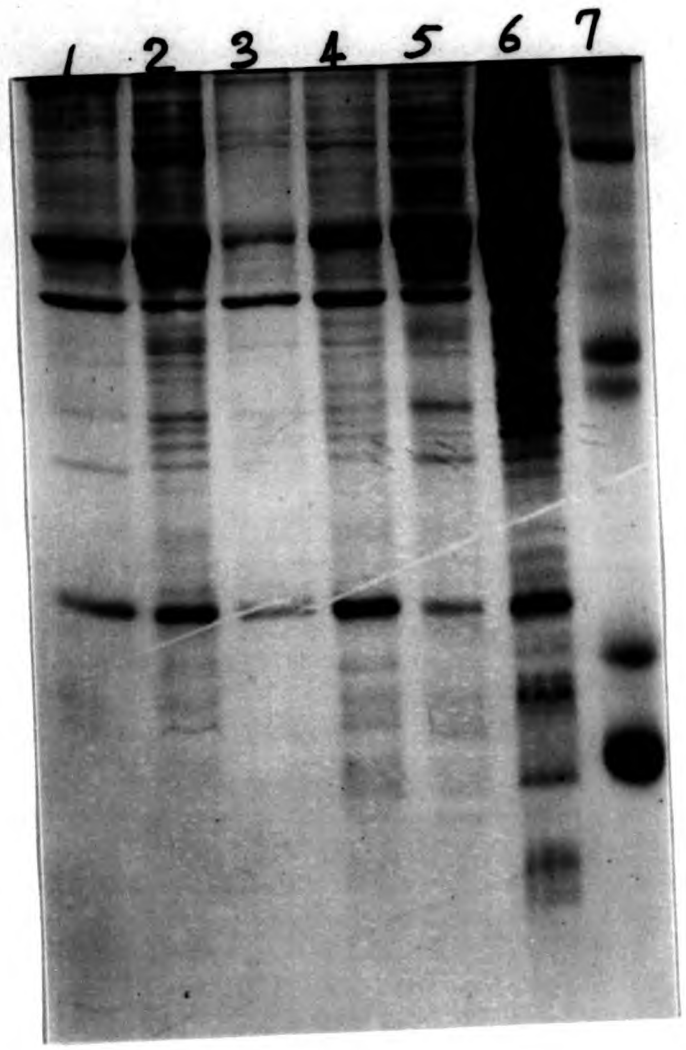
lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe OM
" 4 C+Fe OM

The 1st antibodies used for lanes 1 and 2 are C-Fe specific.

The 1st antibodies used for lanes 3 and 4 are C+Fe specific.

This immunoblot was kindly performed by Dr. Chart of NIBSC. HOlly Hill, Hampstead, London.

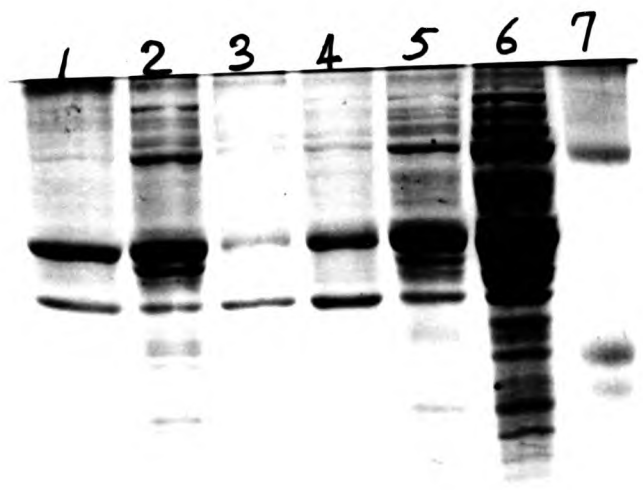
Plate 13. PAGE profile of OMs and whole cel s obtained from cells used in ^{125}I-radiolabelling experiments. (See plate 14)



lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe OM
" 4 C+Fe OM

lane 5 C-Fe whole cell
" 6 C+Fe whole cell
" 7 standards

Plate 13. PAGE profile of OMs and whole cel s obtained from cells used in ^{125}I-radiolabelling experiments. (See plate 14)



lane 1 C-Fe OM
" 2 C+Fe OM
" 3 C-Fe OM
" 4 C+Fe OM

lane 5 C-Fe whole cell
" 6 C+Fe whole cell
" 7 standards

Plate 14. Autoradiograph of ^{125}I radiolabelled OM (15 minutes incubation time).

lane 1 C+Fe OM replicates
" 2 C+Fe OM
" 3 C-Fe OM replicates
" 4 C-Fe OM

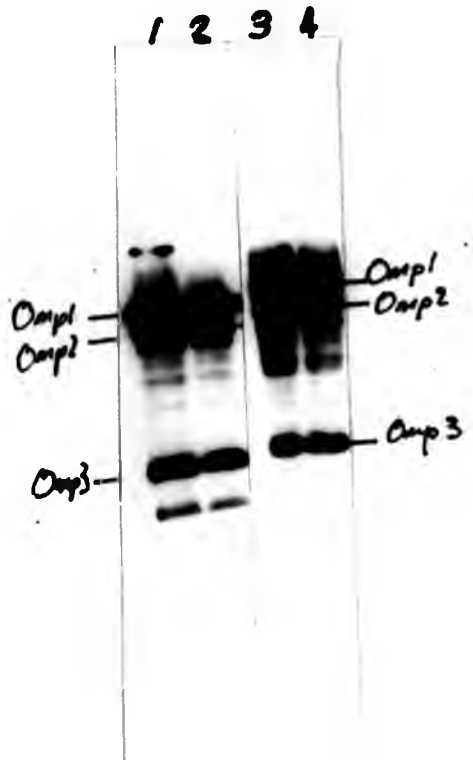


Plate 15. Autoradiograph of ^{125}I radiolabelled OM and C-Fe whole cells (8 minutes incubation time).

lane 1 C-Fe OM
" 2 C-Fe whole cell
" 3 C-Fe OM - replicate of 1 with a higher loading

Plate 14. Autoradiograph of ^{125}I radiolabelled OM (15 minutes incubation time).

lane 1 C+Fe OM replicates
" 2 C+Fe OM
" 3 C-Fe OM replicates
" 4 C-Fe OM

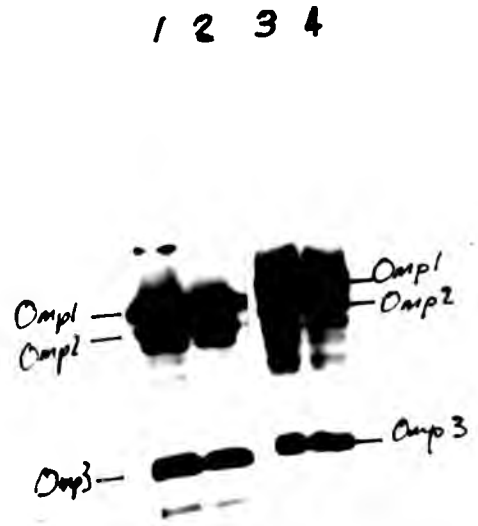


Plate 15. Autoradiograph of ^{125}I radiolabelled OM and C-Fe whole cells (8 minutes incubation time).

lane 1 C-Fe OM
" 2 C-Fe whole cell
" 3 C-Fe OM - replicate of 1 with a higher loading

Plate 16. Autoradiograph of ^{125}I radiolabelled Omps and whole cells (4.5 minutes incubation time).

lane 1 C-Fe OM
" 2 C-Fe OM replicates
" 3 C-Fe whole cell
" 4 C+Fe OM replicates
" 5 C+Fe OM
" 6 C+Fe whole cell

Plate 17. Autoradiograph of ^{125}I radiolabelled Omps and whole cell (incubation time = 45 seconds).

lane 1 C-Fe whole cell
" 2 NC OM
" 3 NC whole cell
" 4 C+Fe OM

Plate 16. Autoradiograph of ^{125}I radiolabelled Omps and whole cells (4.5 minutes incubation time).

lane 1 C-Fe OM
" 2 C-Fe OM replicates
" 3 C-Fe whole cell
" 4 C+Fe OM replicates
" 5 C+Fe OM
" 6 C+Fe whole cell

Plate 17. Autoradiograph of ^{125}I radiolabelled Omps and whole cell (incubation time = 45 seconds).

lane 1 C-Fe whole cell
" 2 NC OM
" 3 NC whole cell
" 4 C+Fe OM

more labelled bands are seen in the autoradiographs of whole cell preparations obtained from experiments with an incubation time of 8 and 4.5 minutes. (No 15 minutes incubation was performed). Thus the labelling is not specific to outer membrane proteins. Surface specific labelling in the Iodo-Gen procedure depends on the surface- exposed substrate being in excess over the iodinating species generated. It also depends on the rate of iodination being greater than the rate of diffusion of the iodinating agent through the membrane, and on the restriction of the catalyst to the walls of the reaction vessels (Markwell & Fox, 1978, cited by Loeb *et al.*, 1983). The non specific labelling observed in this study could well be due to the generation of free iodinating species such as I_2. Iodo-Gen can mediate I_2 generation. A yellow colouration in the reaction mixture was observed in the first two iodination procedures (15 minutes & 8 minutes incubation time in the presence of 100ug Iodo-Gen). This could have been I_2. Non-specific surface labelling has been reported for *H.influenzae* OMs by Loeb and Smith, (1983). They used 4ug Iodo-Gen, 19.5mCi ^{125}I with incubation for 16 minutes at 0°C. This is a much higher level of radioactive iodine than used in the present study. They also reported that labelling using the peroxidase methods produced non-specific labelling, and it may be that either the conditions employed or the OM of *H.influenzae* were sufficiently unusual to result in overall labelling of cell proteins. Sullivan and Williams (1982) were

able to restrict the labelling of Omps in *N.gonorrhoeae* by the Iodo-Gen procedure using 50ug Iodo-Gen, 0.5mCi ^{125}I, 45 seconds, room temperature). The results reported here agree with Sullivans and Williams (1982) that specific labelling of Omps can be achieved by strictly controlled experimental conditions. These results show that three Omps (1,2 & 3) are exposed to the surface, regardless of the experimental conditions employed in this study.

For a better indication of these Omps which are exposed at the surface, an antibody-labelling procedure is more appropriate. Attempts to adopt this approach were abandoned due to the poor quality of the antisera obtained, after immunization of rabbits with Omps 1,2 and 3 extracted from PAGE gels.

Chapter Five

PHYSICOCHEMICAL PROPERTIES OF LPS

Desulfovibrio LPS was analysed by transmission electron microscopy, for sugar content by HPLC and for its possible heterogeneity by SDS-PAGE.

5.1. Ultrastructure of LPS

The freeze-dried extracted LPS appear as a fluffy powder to the naked eye. This is white for C-Fe, light yellow for NC and light brown for C+Fe LPS. All form opalescent solutions when dissolved in distilled water, but clear solutions in the presence of 0.5N HCl. Opalescence is due to the aggregation of LPS molecules (Wilkinson,1977) and HCl enhances the solubility by interferring with aggregate formation.

The electron micrograph of C-Fe LPS suspended in distilled water revealed that they form aggregates of twisted ribbons (plates 18) which have rounded ends. It has been reviewed in the literature that bacterial LPS occur in various forms (e.g., disc, ribbons, vesicles) depending on the method of extraction and the parental organisms (Wilkinson, 1977). Recently Kato *et al.* (1985) showed that an R-form LPS from *Klebsiella* sp. had the ability of *in vitro* self-assembly into a hexagonal lattice structure in the presence of magnesium ions.

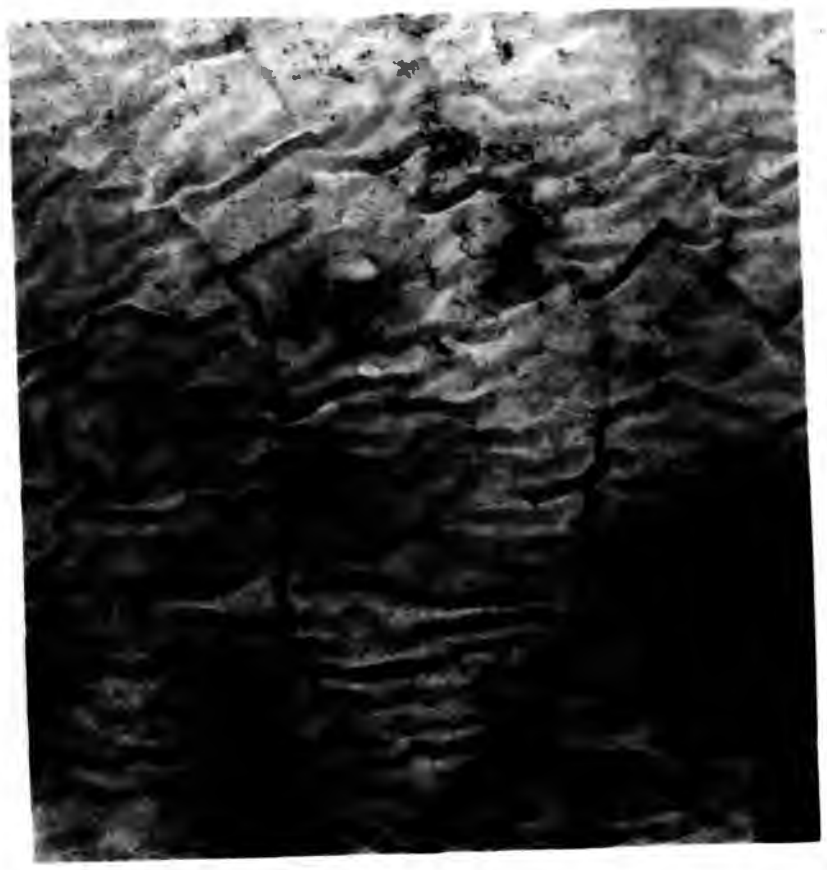
Plate 18. Electron micrograph
of C-Fe LPS.
(magnification 50K)
bar = 0.1um

Plate 19. LPS PAGE profile.

lane 1 electrodialysed C-Fe LPS
" 2 electrodialysed C+Fe LPS
" 3 C-Fe LPS
" 4 C+Fe LPS
Numbers at the side are the positions of the protein markers (see plate 6 for details).

Arrow indicates the extra band in C-Fe LPS.

Plate 18. Electron micrograph of C-Fe LPS.
(magnification 50K)
bar = 0.1um



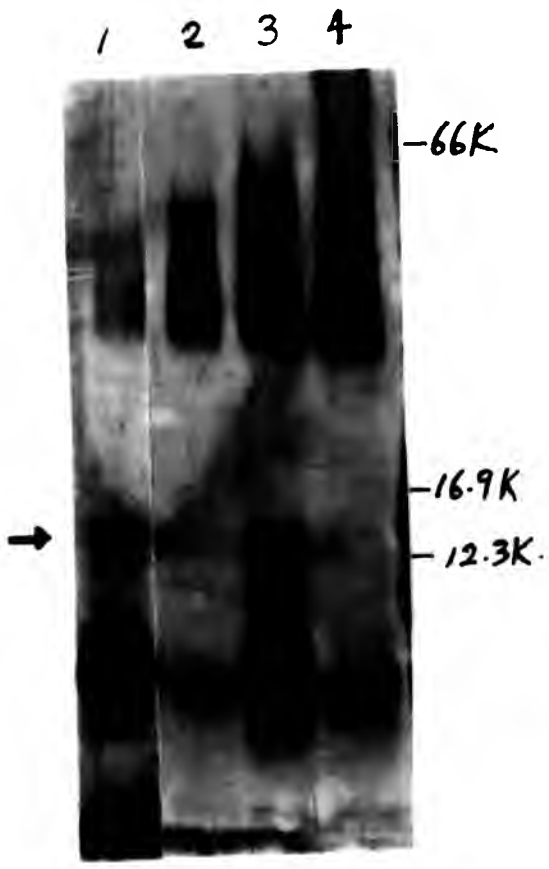


Plate 19. LPS PAGE profile.

lane 1 electrodialysed C-Fe LPS
" 2 electrodialysed C+Fe LPS
" 3 C-Fe LPS
" 4 C+Fe LPS
Numbers at the side are the positions of the protein markers (see plate 6 for details).

Arrow indicates the extra band in C-Fe LPS.

The self-association of LPS molecules is due to hydrophobic, metal cation mediated interactions and hydrogen bonding (Wilkinson, 1977). The observed aggregation of C-Fe LPS in *Desulfovibrio* might be due to these interactions.

5.2. PAGE

Analysis of LPS by PAGE has been performed by workers such as Jann *et al.* (1975), Hurlbert & Hurlbert (1977) and Barton-Willis *et al.* (1984) using cells of the *Enterobacteriaceae*, *Citobacter*, *Ps.syringae* and *Corynebacterium vinosum*. A similar analysis of *Desulfovibrio* LPS has been reported by Bradley (1985). The results of the present study are shown in plate 19. There is no major difference observed between the C-Fe and C+Fe LPS (lanes 1 & 2). Both have two broadly diffused bands and there is an extra band in C-Fe. This is not shown by Bradley (1985). Depending on the loading, multiple bands may be seen within the more slowly moving broad band (see immunoblot, plate 10). When LPS samples were electrodialysed to remove cations prior to analysis by PAGE, the overall pattern was unchanged but there was a decrease in the intensity of all bands, probably due to the reduced yield after electrodialysis.

The banding seen here represents the heterogeneity of the LPS. It was proposed by Jann *et al.* (1975) that LPS produced PAGE band patterns corresponding to various chain lengths of LPS. Support for this notion came from the work of Palva & Makela (1980), Hitchcock & Brown (1983), Barton-Willis *et*

al.(1984) and Ohta *et al.* (1985). The immunological analysis of *Desulfovibrio* LPS by blotting on nitrocellulose also indicates heterogeneous chain lengths in *Desulfovibrio* LPS. Immunoblotting of LPS has been reported by Bradbury *et al.* (1984) and by Karch *et al.* (1984). They concluded that the multiple bands in PAGE gels demonstrated the heterogeneous nature of the LPS. *Desulfovibrio* LPS is also heterogeneous and is composed of molecules of different polysaccharide chain lengths, represented by the different bands. The multiple LPS bandings demonstrated in immunoblots indicate that the LPS are not in fact short chained molecules as was suggested by Bradley (1985).

The extra band observed in the C-Fe samples does not represent a polysaccharide chain absent from C+Fe, but the disintegration of higher molecular weight chains to smaller units. Evidence for this conclusion comes from the examination of electrodialysed LPS by PAGE (plate 19). In C-Fe samples (lanes 1-3) a change in band pattern is seen. There is an increase in band intensity in the fast-moving band and a decrease in the slower (i.e., higher molecular weight) band. However, electrodialysed C+Fe LPS does not produce a pattern different from the non-electrodialysed counterpart. It seems that cations (Fe^{2+}) have a role in maintaining the integrity of the LPS structure. Electrodialysis fails to remove all Fe^{2+} ions from C+Fe LPS, hence allowing the maintenance of polysaccharide chain length.

These results, together with those from the immunoblot (plate 10) lead to the conclusion that the dissimilarity in band pattern between C-Fe and C+Fe LPS is due to the relative instability of C-Fe LPS because of the absence of cations (Fe^{2+}) and not due to different polysaccharide chain units containing different antigenic determinents.

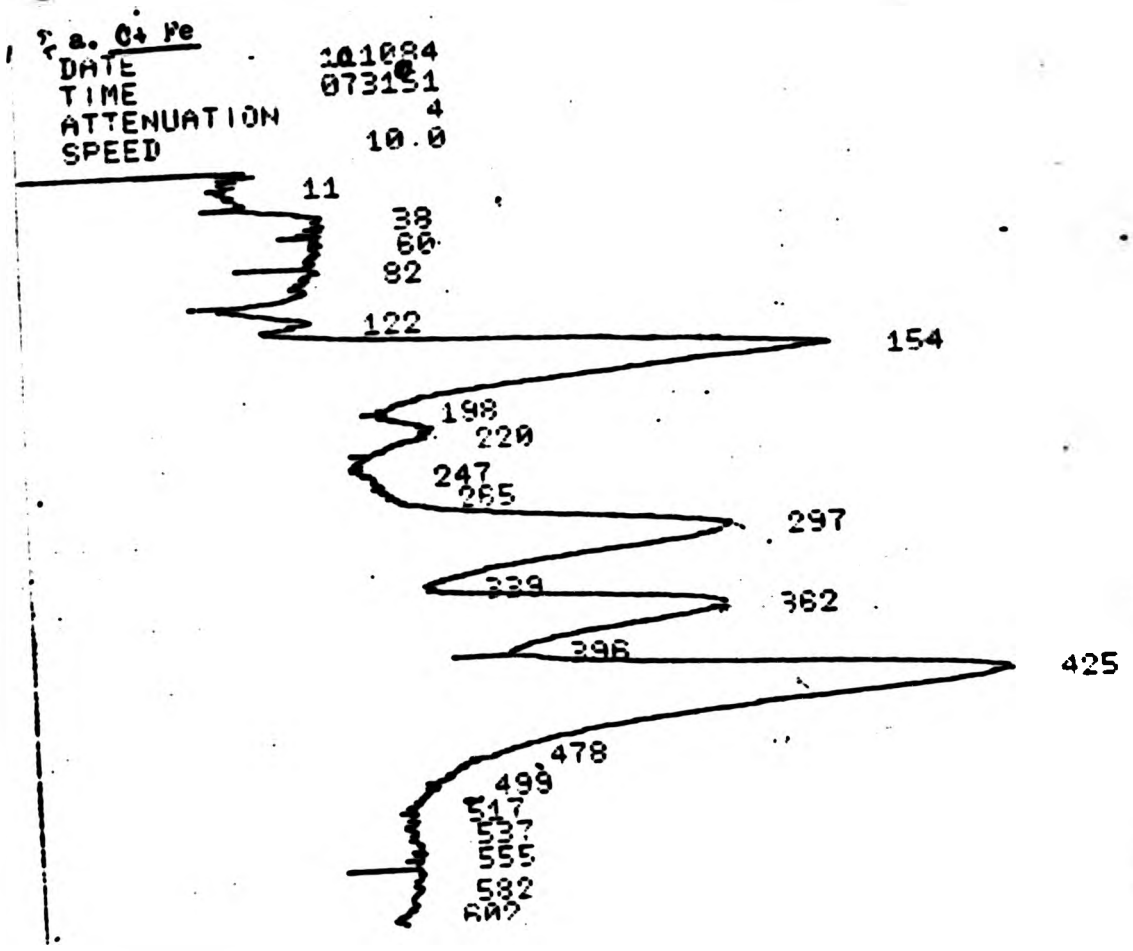
Sugar analysis by HPLC was attempted to add further evidence to this conclusion.

5.3. HPLC

Sugars separated from by HPLC were analysed using a refractive index detector. The results are shown in figures 24 and 25, with standards in figure 26. One of the problems encountered in this analysis is drifting of the baseline, making identification of peaks difficult. Nevertheless, N-glucosamine is identified and a second peak is tentatively assigned to rhamnose. Other sugars are not identified. Attempts at analysing their trimethylsily alditol derivatives using GC columns (SE52 & OV17) were unsuccessful. Further attempts were made at analysing these derivatives using a general purpose and SE30 wall coated glass capillary column. These were also unsuccessful. The major problem in these analyses lies in inadequate resolution.

When HPLC chromatograms of C+Fe and C-Fe samples (Fig.24) are compared, they are seen to differ by one extra peak (362) in the C+Fe sample. A small shoulder associated with peak 291

Figure 24. Chromatograms of sugars released from LPS samples.



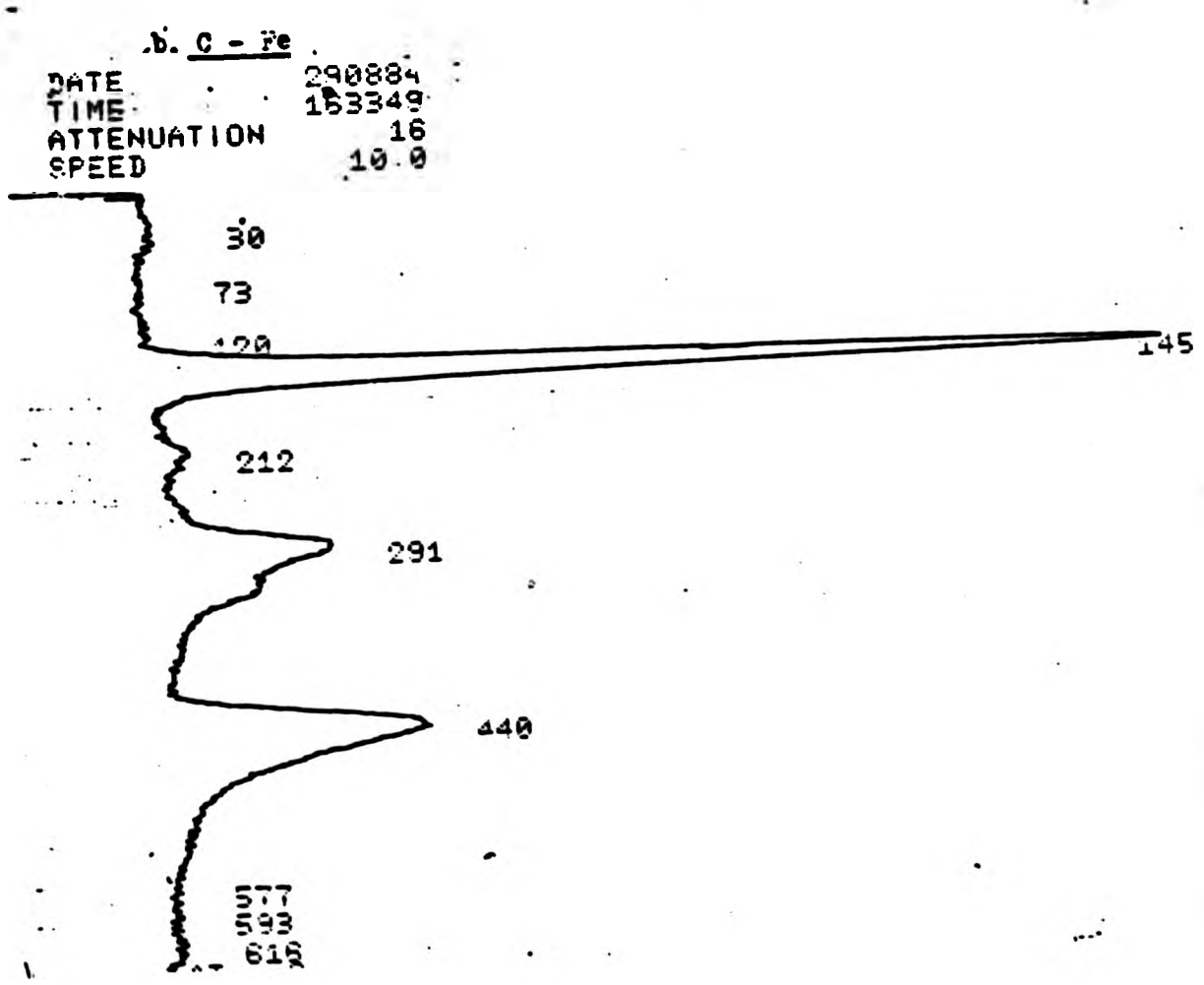


Figure 25. Chromatograms of sugars from electrodialysed LPS samples.

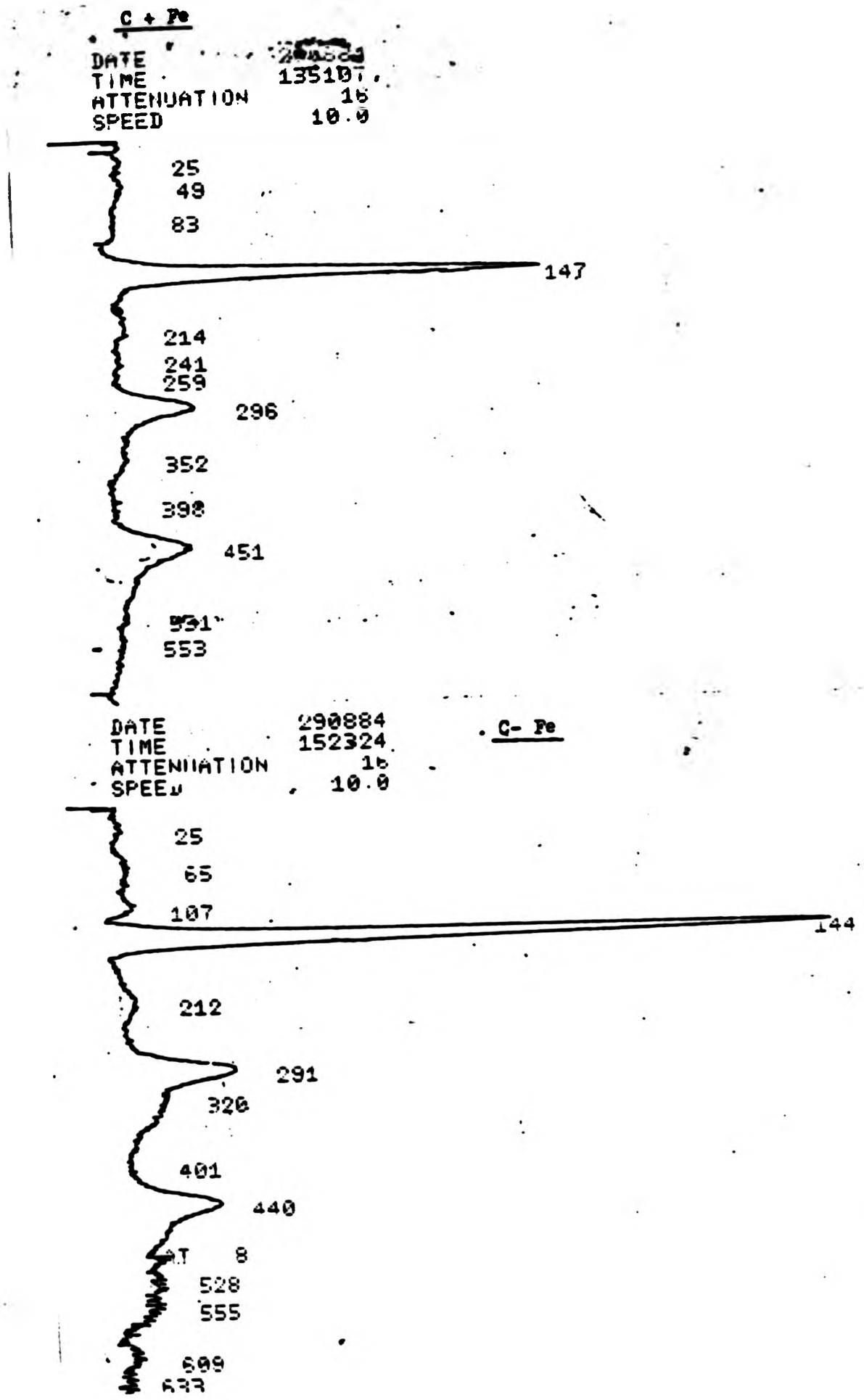
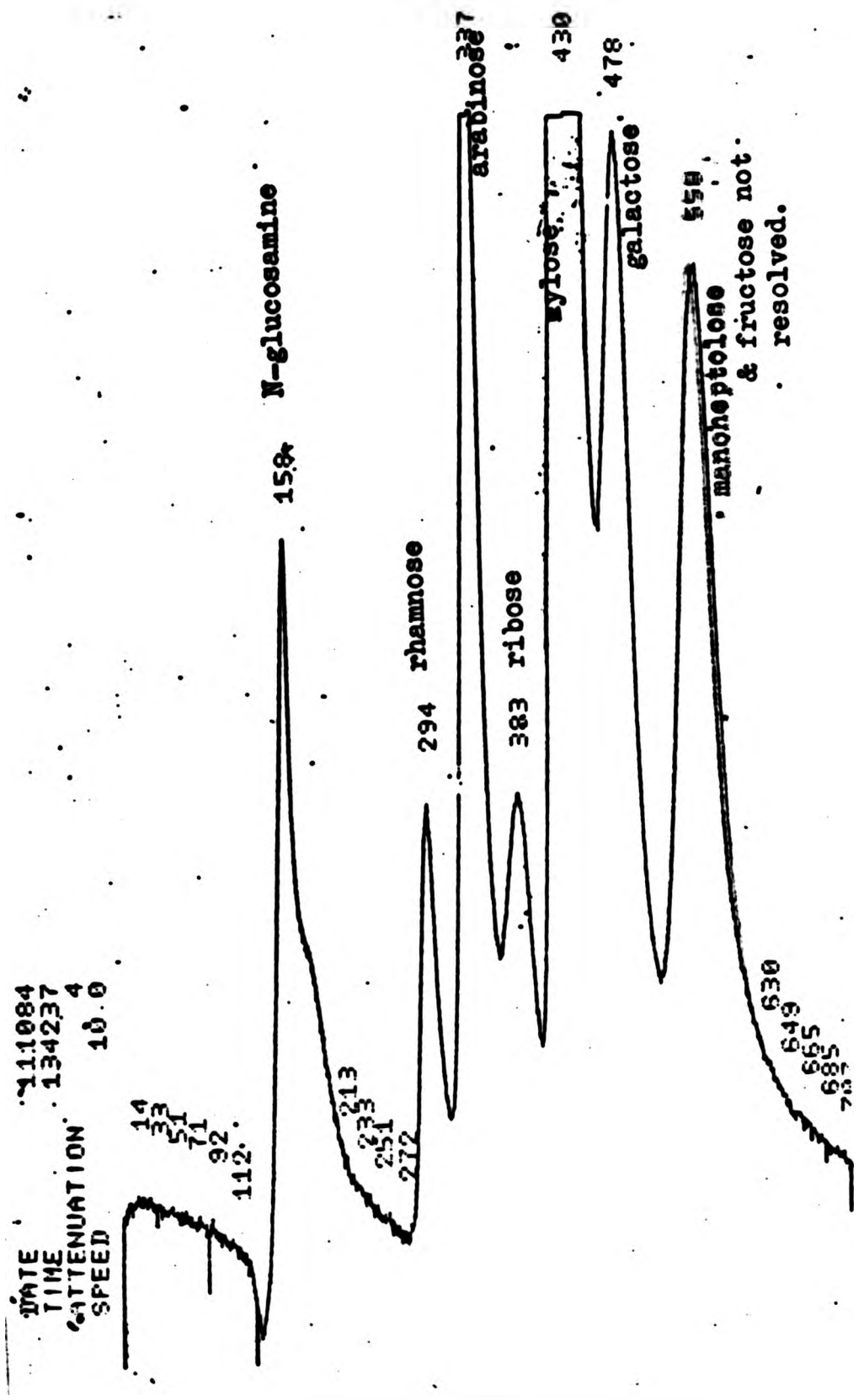


Figure 26. Chromatogram of the sugar standards used in HPLC analysis.



in the C-Fe chromatogram could be a much reduced version of this peak. When C+Fe LPS samples are electrodialysed prior to HPLC analysis, they produce chromatograms similar or identical to that of non-electrodialysed C-Fe LPS (fig.25). These results suggest that the ferrous ions present in the iron-rich samples interact with the column material or with one of the sugar moieties (possibly the peak with retention time 420-450sec.), thus modifying its structure to produce peak 362. To verify this hypothesis, electrodialysed C-Fe LPS was incubated with 1ug/ml ferrous sulphate for 30 minutes prior to hydrolysis. The result is shown in figure 27. Peak 362 reoccurs in these samples producing a chromatogram similar to that produced by C+Fe extracts. It is therefore likely that the extra peak (362) in the C+Fe chromatogram is due to interaction between ferrous ions and the sugar represented by peak 420-450. This modified moiety is perhaps responsible for the minor variation in antibody titre observed in the agglutination tests (chapter four). In addition, this result suggests that this particular sugar (420-450) occurs in the iron-binding site of the LPS molecule or at the linkage site where Fe^{2+} ions stabilise the polysaccharide chains. The HPLC results, in combination with PAGE results, implies that the LPS of C+Fe and C-Fe are identical, or show only minor variations in a sugar induced by Fe^{2+} ions.

Figure 27. Chromatogram of sugars from electrodialysed C-Fe LPS reincubated with 1ug/ml of $FeSO_4$ ½hr. prior to acid hydrolysis.

Chapter Six

Detction of plasmid DNA

The analysis of plasmid DNA was carried out to determine whether the Omps in *D.vulgaris* (Woolich) were encoded by chromosomal or plasmid-borne genes. Plate 20 shows the result of agarose gel electrophoresis of DNA extracted from C+Fe and C-Fe cells. A direct comparison is difficult because of the unequal loading of the two samples, but it appears that there is no difference between the two. Five plasmids are obviously present, along with some RNA (the fast-moving band). Postgate *et al.* (1984) found a number of plasmids of molecular weights 40 to 130MDal in *Desulfovibrio* spp., but they were unable to assign functions to any of them. The conditions under which their preparations are run are different from those in this experiment and the plasmids detected here have lower molecular weights as shown in plate 20. The band observed in lane 1 is a partially digested lambda phage DNA.

A second run of the samples in 1% gel with an increased loading of the C+Fe sample (approximately twice that used in the first run) does not produce any additional information and the fifth plasmid band is lost (plate 21). C+Fe and C-Fe cells still appear to be identical in their plasmid content as shown in plate 20.

Plate 20. Plasmid DNA analysed by agarose gel electrophoresis using 2% gel.

lane 1 partially digested lambda DNA
" 2 C-Fe plasmids
" 3 C+Fe plasmids

Plate 21. Plasmid DNA analysed by agarose gel electrophoresis using 1% gel.

lane 1 C-Fe plasmids
" 2 C+Fe plasmids
" 3 partially digested lambda phage DNA
" 4 HindIII digest of lambda phage DNA.

(Gels were run by Lee Pallen, Dept. of Biolog. Studies, City of Lond. Polytechnic)

Plate 20. Plasmid DNA analysed by agarose gel electrophoresis using 2% gel.

lane 1 partially digested lambda DNA
" 2 C-Fe plasmids
" 3 C+Fe plasmids

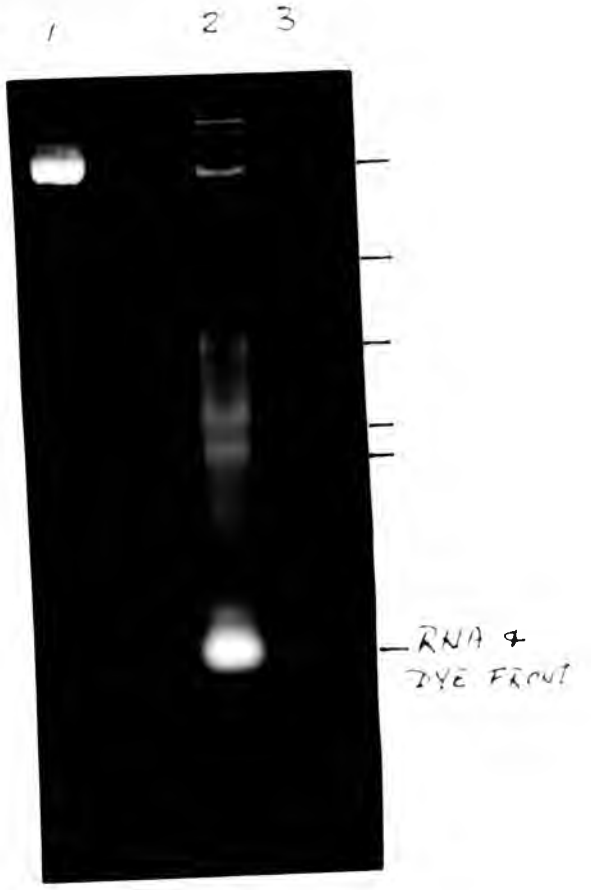
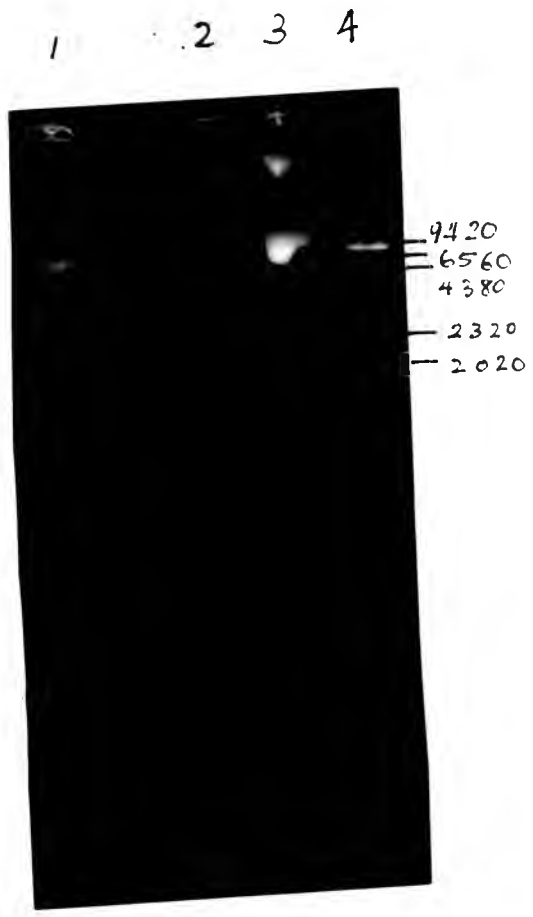


Plate 21. Plasmid DNA analysed by agarose gel electrophoresis using 1% gel.

lane 1 C-Fe plasmids
" 2 C+Fe plasmids
" 3 partially digested lambda phage DNA
" 4 HindIII digest of lambda phage DNA.



(Gels were run by Lee Pullen, Dept. of Biolog. Studies, City of Lond. Polytechnic)

This result indicates that any differences between iron-rich and "iron-deficient" cells of *Desulfovibrio* are unlikely to be due to the loss of plasmids. Tolmasky *et al.*,(1985) suggested that an Omp involved in Fe uptake in *Vibrio anguillarum* was plasmid coded. However, no extra Omps are seen in the OMs of C-Fe cells. This suggests the iron uptake system in *Desulfovibrio* is different from *V. anguillarum* or pathogenic *E.coli*. The results obtained here suggest that changes in C-Fe OMs are probably due to changes in the expression of genes encoding the Omps.

Chapter Seven

Functional studies

7.1. Fe(II) binding studies [Interactions of OM components with Fe(II)]

7.1.1. ^{55}Fe-labelling of blots

Bowen *et al.* (1980) reported that DNA-binding proteins could be detected by protein blotting techniques. In this study, a similar method was employed to immobilize *Desulfovibrio* OM materials to nitrocellulose and probe with ^{55}Fe. The major problem with this approach is that ^{55}Fe bound to the nitrocellulose filter non-specifically. Despite this difficulty, Omp1 and Omp2 bands are observed to be labelled in the autoradiograph (plate 22). In these experiments, NaCl is present in the probing solution in the form of Mops buffer. This solution also contains 100mM sodium ascorbate and 200ul of ^{55}Fe (approximately 105uCi). The concentration of NaCl in Mops is 8.765g/L. This incubation of a low concentration of NaCl with ^{55}Fe does not stop the nonspecific binding of ^{55}Fe (background) on to the nitrocellulose membrane. In an attempt to improve the resolution of the technique, the post- blot nitrocellulose membrane is quenched with either 30ml of physiological saline (8.765g/L) or 30ml of 0.5% polyvinylpyrolidone (PVP)(approximately 24,500 m.wt.), 30

Plate 22. Autoradiograph of ^{55}Fe radiolabel of OM components blotted on nitrocellulose membrane.

No quenching agents used prior to labelling procedure.

Plate 23. Autoradiographs of ^{55}Fe radiolabel of OM components blotted on nitrocellulose membrane, quenched with quenching agents prior to radiolabelling.

a. physiological saline
b. 0.5% PVP (polyvinylpyrrolidone)

Plate 22. Autoradiograph of ^{55}Fe radiolabel of OM components blotted on nitrocellulose membrane.

No quenching agents used prior to labelling procedure.

Plate 23. Autoradiographs of ^{55}Fe radiolabel of OM components blotted on nitrocellulose membrane, quenched with quenching agents prior to radiolabelling.

a. physiological saline
b. 0.5% PVP (polyvinylpyrrolidone)

minutes prior to labelling. In the case of NaCl, there is a reduction in the background. Three bands corresponding to Omps 1, 2 and 3 are labelled and no LPS bands. With PVP, though there is some reduction in the background, no bands are seen in the autoradiograph (plate 23). Negative results in the case of PVP quenching are probably due to the binding of PVP to the protein bands. It seems that inorganic charged ions are better agents for the quenching process. These results indicate that Omps 1, 2 and 3 are Fe(II) receptors. They may play a part in the Fe(II) uptake by the cells.

To improve the resolution of the autoradiographs the post blotted membrane is incubated with ^{55}Fe in the presence of either $MgSO_4$ or $CuSO_4$. The results are illustrated in plate 24. In all cases, there is a reduction in the non-specific binding of ^{55}Fe on the membrane. These results indicate that divalent cations are better than Na^+ as a quenching agent. When 10mg $CuSO_4$ is used as quenching agent no ^{55}Fe bound to the blot (plate25). The results also show that the three Omp bands, but not the LPS bound ^{55}Fe. There is a differentiation in the labelling intensities in the autoradiographs processed under different quenching conditions. In the case of Mg, there is a reduction in the labelling of Omp2 band, but not of Omps 1 and 3. The presence of 4mg $CuSO_4$ resulted in a pronounced reduction in the labelling of Omps 2 and 3 but not Omp 1. The observations shown here illustrate that cations such as Mg and Cu can reduce the Fe(II) binding process, Cu being a stronger

Plate 24. Autoradiographs of ^{55}Fe radiolabelling of OM components blotted on nitrocellulose membrane where labelling was performed in the presence of:-

a. $MgSO_4$

b. $CuSO_4$

Plate 25. Autoradiograph of ^{55}Fe radiolabelling of OM components blotted on nitrocellulose membrane where the post-labelled blot was quenched with 10mg $CuSO_4$.

Plate 24. Autoradiographs of ^{55}Fe radiolabelling of OM components blotted on nitrocellulose membrane where labelling was performed in the presence of:-

a. $MgSO_4$

b. $CuSO_4$

Plate 25. Autoradiograph of ^{55}Fe radiolabelling of OM components blotted on nitrocellulose membrane where the post-labelled blot was quenched with 10mg $CuSO_4$.

inhibitor than Mg. The differential inhibition of the three bands by Mg implies that Omp 2 may be involved in Mg uptake by the cells.

Since Omp 2 band is enhanced in C-Fe and NC OMs subjected to PAGE (see chapter four), it seems likely that Omp 2 is a Fe(II) receptor. The fact that binding of ^{55}Fe to this protein band is inhibited by Mg and Cu(II) suggests that Omp 2 may be a divalent cation binding protein rather than specific for Fe(II). It has been reported that wild type *E.coli* can be induced by phosphate limitation to synthesize PhoE protein (Overbeeke & Lugtenberg, 1980) as a component of a series of proteins to scavenge traces of phosphate from the environment (Wanner *et al.*, 1981). It is possible that *Desulfovibrio* cells respond to cation (including iron)limitation by increasing the synthesis of Omp2.

It is possible that Omps 1 and 3 are also iron chelating proteins, since they are shown to bind ^{55}Fe. If this were the case, an increase in synthesis in C-Fe and NC cells might be anticipated. However, no such effect is seen (chapter four & Bradley, 1985). Omp1 appears to be relatively iron-specific, since iron binding by this protein is not inhibited by copper or magnesium. It may be that Omp1 is a specific iron chelator, but that its increased synthesis in C-Fe cells is not necessary because of the additional iron bound by the non-specific cation chelators Omps 2 and 3.

Lps are not demonstrated to interact with ^{55}Fe by these

experiments. No intensely labelled bands appear to correspond with the LPS PAGE band pattern. This could imply that LPS does not bind Fe. However, Bradley *et al.* (1985) using an indirect method demonstrated that *Desulfovibrio* LPS interacted specifically with Fe(II). It could be that labelling of western blots is not sufficiently sensitive to detect this interaction or that the results of the indirect technique used by Bradley *et al.* (1985) did not imply a specific Fe-LPS interaction.

7.1.2. *In vivo* ^{55}Fe-radiolabelling of *Desulfovibrio* cells

The results illustrated in sections 5.3. and 7.1.1. indicate the interactions of OM components with Fe(II). To analyse the interaction in whole cells, four-day old C-Fe cells were incubated with 30ul of ^{55}Fe for 1 hour at 30°C, and the OM extracted for PAGE separation, electroblotting and autoradiography. Radioactivity is detected in the extracts using a Geiger counter (Mini instrument Ltd.,England). However, the results obtained in the autoradiographs are negative. The negative results may be due to a number of factors. It may mean that the level of isotope used is inadequate for the labelling process to occur. However,activity is detected in the extracts. The presence of EDTA in the sample buffer employed for sample preparation used in PAGE plus the electrical current may remove the bound ^{55}Fe form the OMPs. In a modified experiment, the extracted OM is prepared in Tris/HCl buffer without EDTA. Instead of electroblotting, the gel strip was dried and autoradiographed. To minimize the possibility of

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further losses of bound iron due to electrical forces, the electroblotting step was omitted in this experiment. However, the negative results obtained indicate any removal of bound Fe by EDTA is negligible.

The lacking of detectable radioactivities in the OM samples could be due to insufficient incorporation of label. Alternatively, the failure to detect radiolabelled bands in autoradiographs may suggest the Fe-binding process is transient. In this case, it would be difficult to capture the ^{55}Fe bound to the OM at the time of extraction. If this is true, then the three Omps labelled in *in vitro* study may function as the receptors in the OM, whence the bound Fe would be transferred into the cell for use.

No binding of ^{55}Fe to LPS is detected by this method.

7.1.3. X-ray dispersive study of LPS

LPS extracted from the three cell-types and electrodialysed C+Fe LPS were analysed by X-ray dispersive analysis. An example of a spectrum obtained in this study is illustrated in plate 26 and figure 28 illustrates the cations detected expressed as % total.

Electrodialysis did not remove cations effectively, only Fe and a small amount of Mg being removed. It is reported that cations tightly associated to *E.coli* LPS cannot be removed by electrodialysis (Coughlin *et al.*, 1981). The data presented here indicate such tight interactions between LPS and Fe in *D.vulgaris* (Woolich) The amount of Fe detected in NC and C+Fe

Plate 26. An example of a spectrum obtained from X-ray microanalysis of elemental contents of LPS samples. The spectrum shown here is that of C+Fe L^{PS}.

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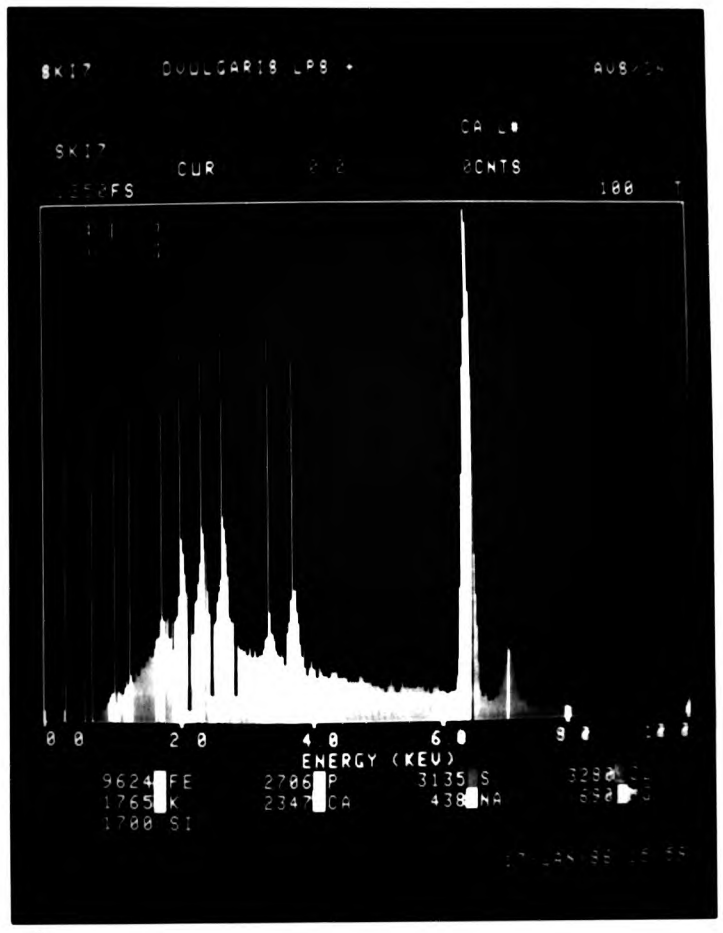
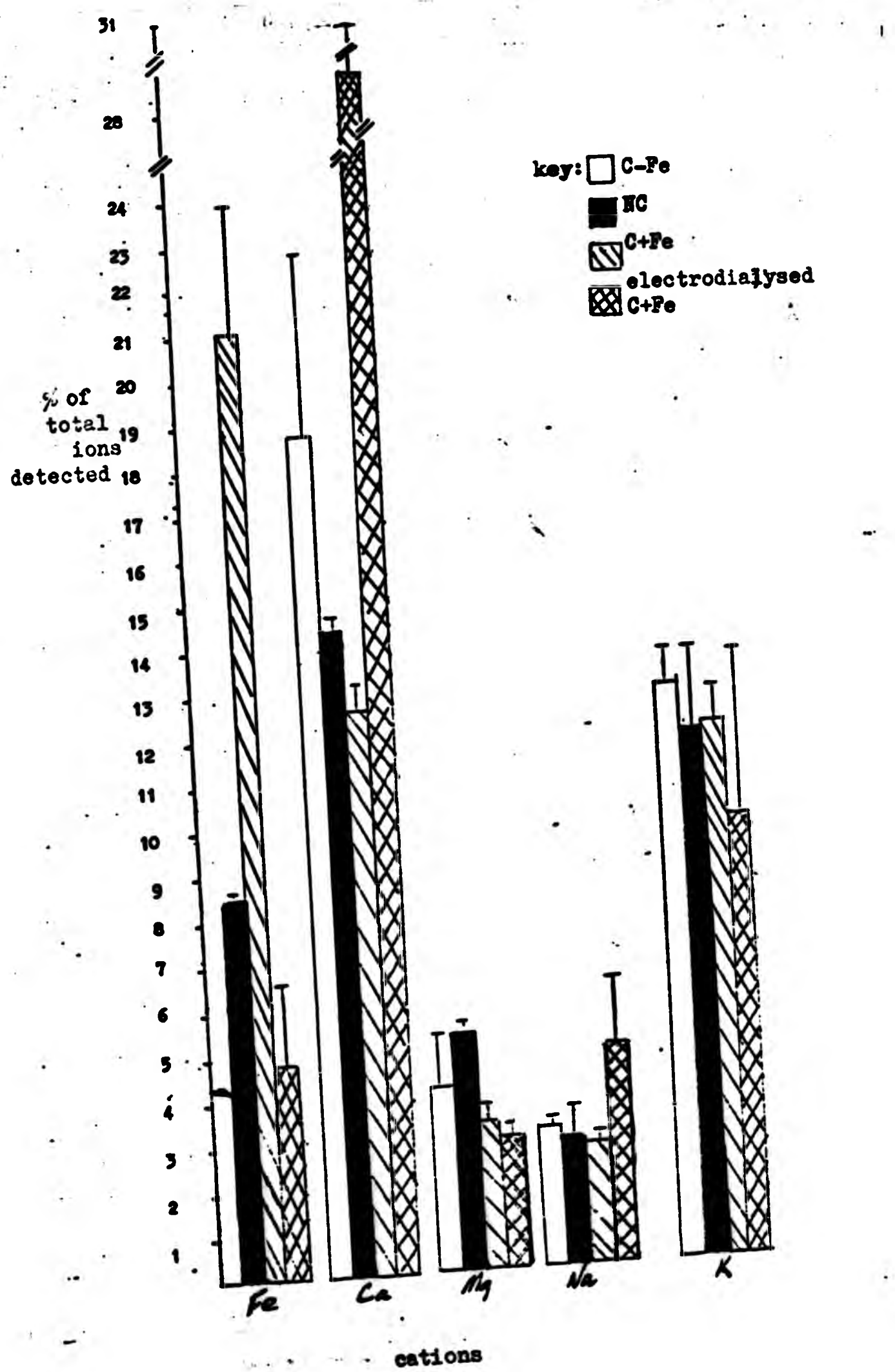


Figure 28. Histograms showing cations detacted by X-ray microanalysis of LPS extracted from the different cell-types, expressed as % of total ions counted. Electrodialysed C+Fe LPS was analysed similarly.



LPS is considerably greater than that in C-Fe, as expected. These differences are mirrored by Ca levels, which are higher in C-Fe LPS, but not by Mg ions, whose levels are not significantly different in any extract (figure 28). Mg and Ca have different coordination geometries and may occupy different anionic sites in the LPS moiety. Mg and Ca appear to be critical in stablizing pure LPS domains within the OM in *E.coli* (Coughlin *et al.*, 1983) and could function similarly in *Desulfovibrio* LPS. The inverse relationship between Fe and Ca indicates that they may have a similar mode of action with LPS. When Fe is removed by electrodialysis from C+Fe LPS, there is an increase in the quantity of Ca. Ca may be a substitute for Fe in neutralizing the charges at the anionic locus. The incomplete removal of Fe from C+Fe LPS by electrodialysis suggests that of the Fe detected in C+Fe LPS, only a small proportion is tightly bound. The vast majority of the Fe, which is loosely bound to the LPS,acts as a focus for accumulation of Fe^{2+} from the environment prior to its uptake by proteins. The more tightly bound Fe probably serves to maintain the integrity of the LPS molecules, as suggested by evidence from PAGE. As to its role in maintaining the integrity of the LPS within the OM is not suggested by these results. However, the results of Bradley *et al.* (1984) indicates the maintenance of LPS in the OM by cation binding.

The quantity of monovalent K^+ present in the three LPS types appears to be similar. This is also true for Na^+. When

the quantity of these ions is compared, there is a larger amount of K^+ present in the LPS, indicating a preference of the LPS molecules for K^+. A small quantity of K^+ is removed by electrodialysis, but is replaced by Na^+. This suggests that K^+ and Na^+ may bind to the same anionic sites in the LPS molecules. K^+ has an ionic radius of 1.33A while Na^+ has an radius of 0.98A. It may be that similarity in the ionic radii allows these ions to bind to the LPS at the same locus.

7.2. Cell adhesion to metal surfaces

The results of the adhesion experiments are shown in figure 29. These results show that *Desulfovibrio* adhered to metal surfaces and that the technique employed is suitable for the study of this process.

When the results of the first set of experiments carried out in the absence of antibodies, are analysed by the Student t-test, a t value of 1.74 is obtained. This indicates that there is no significant difference between the two cell-types (A t value of 2.00 would be significant at the 0.05% level). It seems that there is no significant difference between the adhesion of NC and C-Fe cells to metal surfaces.

In experiments designed to test the effect of anti-LPS on cell adsorption, C-Fe cells were incubated with anti-*Desulfovibrio* LPS Fab for either 0.5 hour or 2 hours before being introduced to a steel coupon. The results are illustrated in figure 30. The time of cells incubation with Fabs does not

Figure 29. Results of adhesion studies using the replica technique. Number of adherent NC and C-Fe cells on 400um^2 of a mild steel coupon.

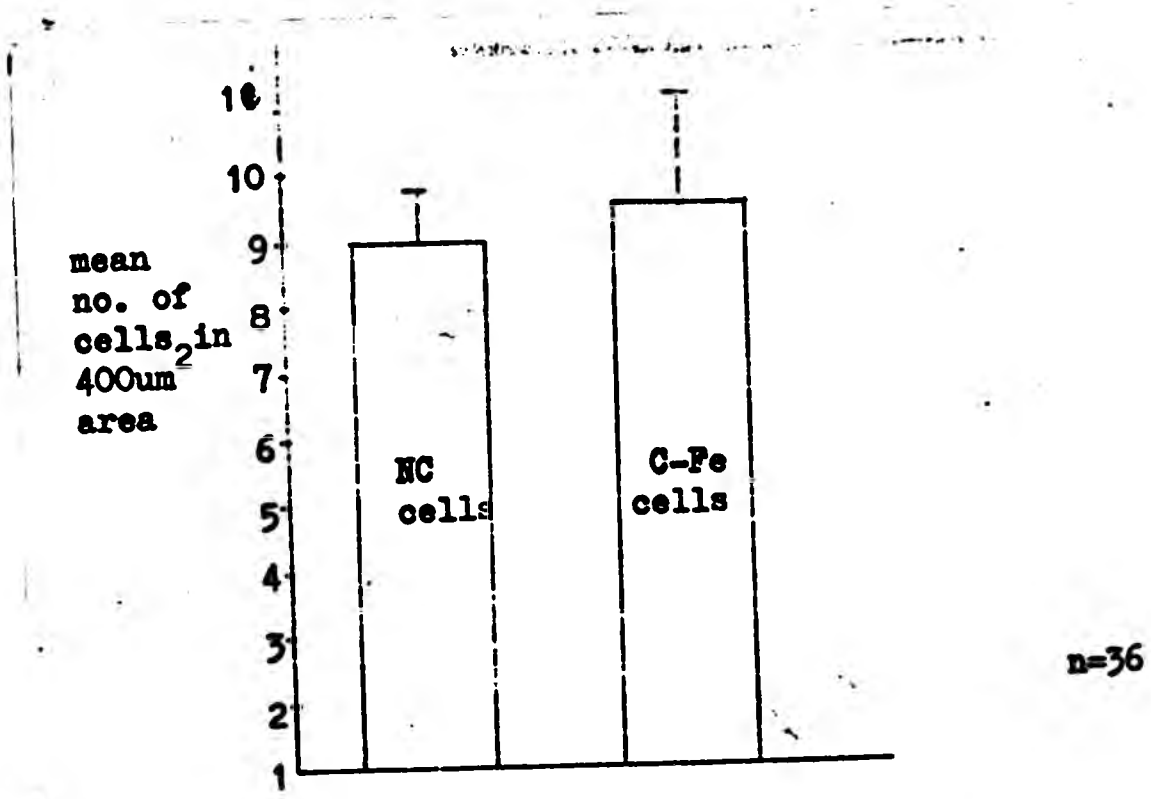
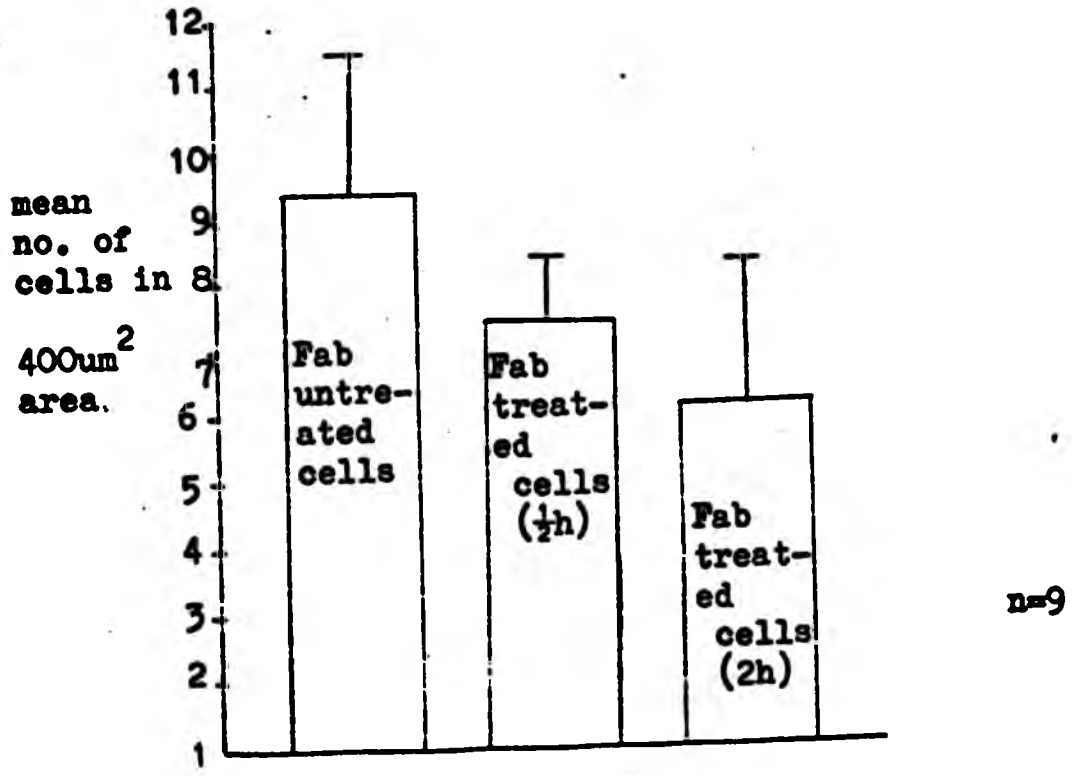


Figure 30. C-Fe cells adhering to a mild steel coupon (400um^2) in the presence and absence of anti-LPS Fab fragments.



seem to affect the results of the experiments as indicated by t-test (t=1.63). At 0.05% level, a t value of 2.11 will be significant. The experiments performed in this analysis are arranged in sets of 9 replicates. In the case of Fab non-treated NC and C-Fe cells, there are 4 sets of 9 replicates while in the case of Fab-treated C-Fe cells, there is only one set of 9 replicates for each Fab incubation time. For comparison of adhesion to metal surfaces between Fab-treated and non-Fab-treated cells, one set of non-treated C-Fe cells is chosen by means of a random table. The number of adherent Fab untreated cells detected is 93/400um^2. In the Fab treated cultures, the number of adherent cells detected after 0.05 hour incubation is 74 and after 2 hours the number is 61/400um^2. The reduction in the number of adherent cells being detected in the presence of anti-LPS Fab indicates that LPS plays a role in the adhesion process. The level of reduction (39% and 23% for 2 hours and 0.5 hour respectively) in the number of anti-LPS Fab-treated cells adhering to the metal surface suggests that LPS is not the sole factor involved in the adhesion process. Other factors such as ionogenic properties may also play a part in the interaction. Most bacteria behave as colloidal particles (Marshall,1980) because of their small size, their density and their negatively charged surfaces at pH values occurring in natural habitats. Their ionogenic properties may be exclusively acidic (carboxyl groups) with the bacteria having zero charge at low pH, or

mixed acidic and basic (carbonyl and amino groups) with the bacteria having a positive charge reversal at low pH. The ionogenic properties of bacteria may be modified by the adsorption of multivalent cations, organic materials or colloid at the surfaces. Ionogenicity of the surfaces may facilitate bacterial adhesion via non-specific attachment in the initial stage of the adhesion process. The adsorbed anti-LPS Fab may alter the ionogenicity of the Fab-treated cells. If this is the case, then it may contribute to the observed results in the adhesion behaviour of the Fab-treated cells.

Chapter Eight

Summary Discussion

D.vulgaris (Woolich) cells respond to Fe(II) restriction by an alteration in metabolism. This is reflected in their growth rates, morphologies, and OM compositions as revealed by PAGE analyses. The variability exhibited by cells grown in media of different Fe availabilities is further demonstrated by PYMS analyses of whole cells and their corresponding cell walls. Iron-deplete cells grow at a slower rate than NC cells. A similar retarded growth rate has been reported by Norqvist *et al.*, (1978) in *N.gonorrhoeae* subjected to Fe-restriction. Morphologically, C-Fe cells appear to be pleomorphic in nature, unlike their Fe-replete counterparts which are uniformly vibrioid. Pleomorphism is associated with their altered OM composition as shown by PAGE. However, when the OM is extracted from C-Fe cells and reincubated with Fe prior to detergent solubilization and analysed by PAGE, a partial restoration of the C+Fe/NC OM PAGE pattern is observed. This indicates the "protective " role of Fe in the solubilization of some Omps by detergent. The Omp1 band in "Fe-protected" C-Fe OM in PAGE gels appears to be denser than in "unprotected" OM. Bradley (1985) postulated from the observation of resistance to protease digestion, 2% SDS-solubilization at 60°C and partial removal

by either acetate or EDTA extraction that the Omp1 band is due to the co-migration of two proteins(Omp1a & Omp1b; Omp1a is protease susceptible & acetate extractable, while Omp1b is not susceptible to either of these treatments). The observed enhancement in the band intensity of Omp1 might indicate that these two proteins of similar M.Wt. co-migrate in the SDS-PAGE system employed, one protein being more susceptible to detergent solubilization in the absence of iron. Though the PAGE results of the "Fe-protected" C-Fe OM demonstrate that the simplified PAGE pattern seen in the "unprotected" C-Fe OM is partially artifactual, the results of PYMS cell wall analyses confirm the altered C-Fe OM composition. There is an increase in yield of LPS from C-Fe cells, presumably to compensate the altered protein synthesis. Rather than producing new OM components to scavenge the iron essential for growth as in *Pseudomonas* when subjected to Fe(III) restriction (Meyer *et al.*, 1979), *Desulfovibrio* switches off the synthesis of many Omps and increases the production of Omp2. Derepression of the synthesis of iron-regulated proteins reported to occur in *V.vulnificus* (Chart & Griffiths, 1985) and in *Pseudomonas* (Meyer *et al.*, 1979) does not seem to occur in *Desulfovibrio*. Plasmid analyses of C+Fe and C-Fe cells suggest that the altered OM pattern is not due to loss of plasmid genes.

No difference in plasmid content is seen between cells grown in iron-enriched (C+Fe) or iron-deplete (C-Fe) medium. Omp2 is shown by ^{59}Fe incubation of western

blots from SDS-PAGE gels of OMs to bind Fe(II). This technique also shows the ability of Omps 1 and 3 to bind ^{55}Fe. However, there is no observed increase in Omps 1 and 3 content in C-Fe OM. All three proteins are shown by ^{125}I-radiolabelling to be exposed to the surface. It is suggested that they may be involved in the transport of iron through the OM. The fact that Omps 2 and 3 only can be inhibited from binding Fe by magnesium and copper suggests that these proteins are cation chelators in the OM. Omp2 is probably the major Fe(II) chelator, since there is an enhancement in its synthesis in C-Fe and NC OMs. Fe-binding by Omp1 is not inhibited by other cations and this may be the iron specific iron transport protein. Attempts to demonstrate iron-Omp interactions *in vivo* were unsuccessful. These suggest that proteins may be denatured by the denaturing action of SDS-PAGE or the Omp-iron interactions are transient processes. If these proteins are involved in iron transport, then it would be expected that the binding would be reversible.

Immunoblot analyses confirm the alteration in the OM composition on Fe(II) depletion. In spite of changes in the OM contents, there appears to be little immunological distinction between C-Fe, C+Fe, and NC cells. However, a change in LPS structure is apparent from HPLC analysis and this could contribute to the slight immunological differences noted. The HPLC results indicate an interaction between iron and a sugar moiety in the LPS molecules, suggesting that the latter may be involved in Fe(II) uptake by the cells. This suggestion is

supported by the observation that C-Fe cells contain increased amount of LPS, as indicated by the relative yields of these macromolecules when extracted from cells grown under Fe-deplete and Fe-rich conditions. Cation -LPS interaction is successfully demonstrated by X-ray dispersive microanalysis. The cations are iron, calcium, magnesium, sodium and potassium. The inability of electrodialysis to remove these ions, except for some iron, indicates that they are tightly bound to LPS. It is possible that the integrity of this macromolecule relies on the neutralization of charges by these ions. The relative ease of removal of some iron from the C+Fe LPS by electrodialysis indicates that this interaction differs from that between LPS and other ions. The firmly bound iron may help to maintain the integrity of the LPS and OM, while the less tightly bound form may be taken into the cells.

Sols of metals, sulphur or metallic sulphide particles usually carry negative charges, depending on the colloid first formed (Shaw, 1970). Sulphides preferentially adsorb ions which are in common to them. i.e. FeS preferentially adsorbs Fe^{2+} and $S^{=}$. Thus the adsorbed FeS particles seen in the surface of *Desulfovibrio* cells may further provide a nucleus for Fe^{2+} accumulation. The constant removal of Fe^{2+} by *Desulfovibrio* metabolism upsets the equilibrium of the colloidal system leading to the uptake of more Fe^{2+} from the environment. Thus these two mechanisms of iron acquisition (uptake by LPS & colloidal accumulation) render the synthesis of siderophores

and siderophores receptors system in the OM unnecessary in *Desulfovibrio*. Additionally, the iron present in the environment where *Desulfovibrio* normally occurs is in the reduced state,which is more soluble than Fe(III), hence the secretion of specific iron chelators by the cells is not required.

The adhesion of *Desulfovibrio* cells to metal surfaces is successfully demonstrated by the replica technique. Changes in the OM composition do not appear to affect the adhesive properties of C-Fe and NC cells. The results indicate that there is no significant difference between numbers of NC and C-Fe cells adhering to mild steel surfaces over 15 minutes. Treatment of C-Fe cells with anti-LPS Fab fragments partially inhibits the ability of the cells to adhere to the metal surface. This suggests LPS may play a part in the initial process of adherence of *Desulfovibrio* cells to metal. In addition, the adsorbed FeS particles and Fe^{2+}/FeS and other ionic species on the surface of *Desulfovibrio* may ultimately interact with metal surfaces in the adhesion process in the natural environment. Adhesion of bacterial cells requires (i) the initial modificaton of the physico-chemical properties of the metal surfaces, a reversible phase during which the bacteria are held at the surface by a balance of van der Waals attractive forces and the electrical repulsive energies of the two surfaces, and (ii) consolidation of the bacterial attachment, a time-dependent irreversible phase (Fletcher &

Floodgate, 1973). Nothing is known about the adsorption of *Desulfovibrio* cells to metals, but this process has been shown to be important in corrosion (Obuekwe *et al.* 1981 & Gaylarde & Johnston 1980). The close proximity of *Desulfovibrio* cells to the metal allows the dynamic ionic fluxes in the colloidal system to influence the physico-chemical properties of the metal surfaces. Recent work by Daumus *et al.* (1987) suggests that *Desulfovibrio*, a hydrogen - consuming sulphate reducer, induces changes in the physico-chemical properties of steel coupons by direct contact of cells with the metal.

An important application of this work could be the development of new techniques for controlling *Desulfovibrio*-induced problems in industry. An understanding of the adhesion process may help in devising control procedures against corrosion. Further studies on the mechanisms of iron uptake employed by *Desulfovibrio* may lead to alternative strategies for combating these organisms.

Table 5 shows how this study has added to our knowledge of the OM of *D.vulgaris* (Woolich).

Figures (31, 32 & 33) depict the current understanding of *D.vulgaris* (Woolich) cell outer membrane, incorporating the results of this study and speculations derived from it.

Table 5. Additional knowledge of OM of D.vulgaris (Woolich) gained from this study and that of Bradley (1985). This should be compared with table 1.

OM components	physical & chemical properties	functions
LPS	heterogenous in polysaccharide chain lengths polysaccharide containing N-glucosamine & possibly rhamnose	interacts with cations in particular with Fe(II) ions possibly play a role in Fe(II) uptake by cells play a role in adhesion of D.vulgaris (Woolich) to mild steel surfaces
phospholipids	not studied	
proteins		
enzymes	not studied	
lipoproteins	not studied	
Omp1a	acetate extractable M.Wt. 54-55K	probably structural protein function unknown
Omp1b	acetate inextractable (Bradley) exposed to surface M.Wt. 54K	specific Fe(II) binder in the OM probably pore protein
Omp2	exposed to surface synthesis enhanced in Fe - deprived conditions M.Wt. 45K	cation binder binds Fe(II) and other cations such as Mg.
Omp3	exposed to surface M.Wt. 18K	Fe(II) binder
derepressable protein with Fe(II) binding specificity	not found	
molecules for attachment to surfaces	LPS	

Figure 31. Speculative interactions of Fe(II) ions and OM components. LPS, Omp1b and Fe(II) ions (not drawn to scale).

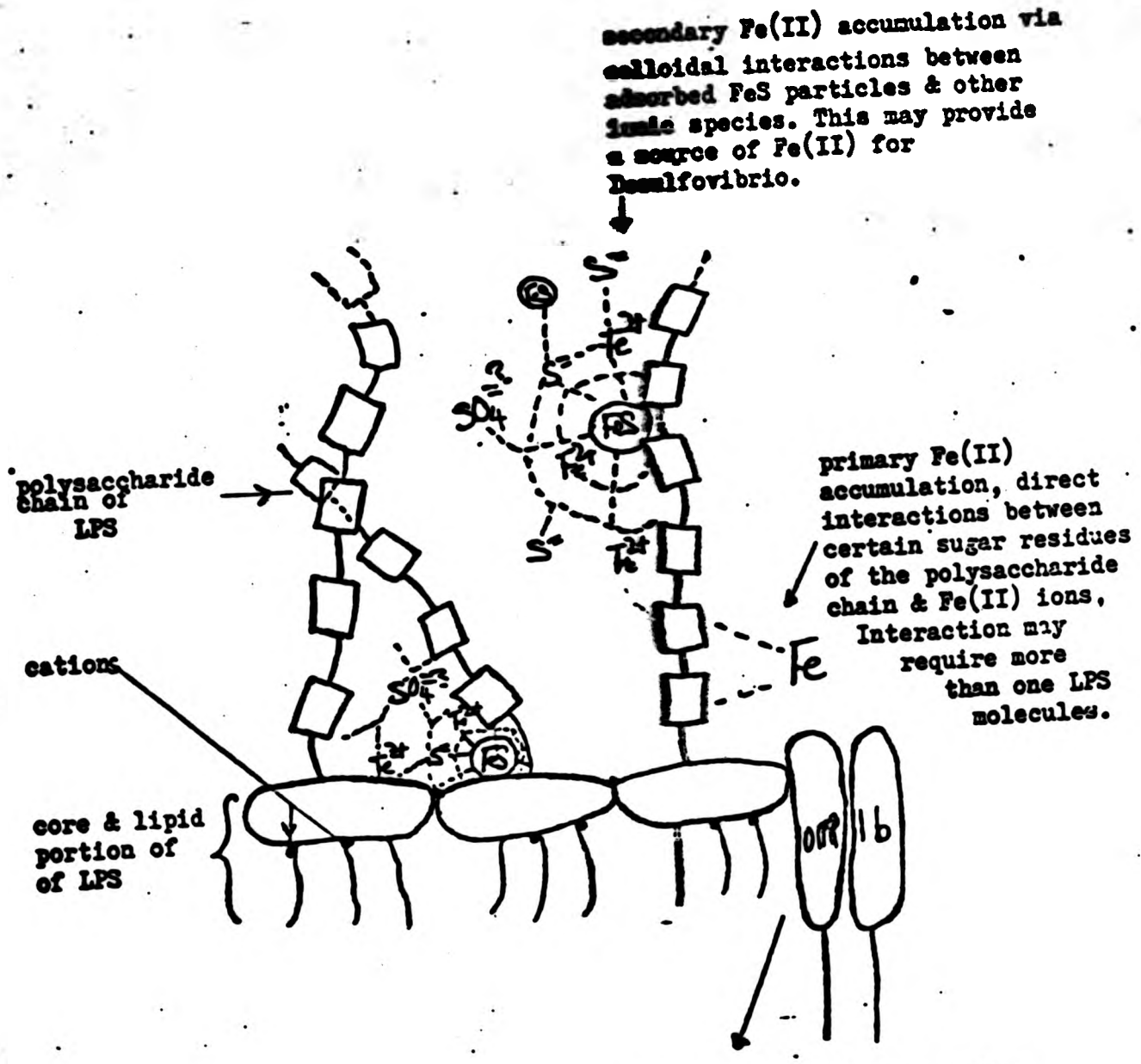


Figure 52. Speculative interactions of other Omps with Fe(II) ions

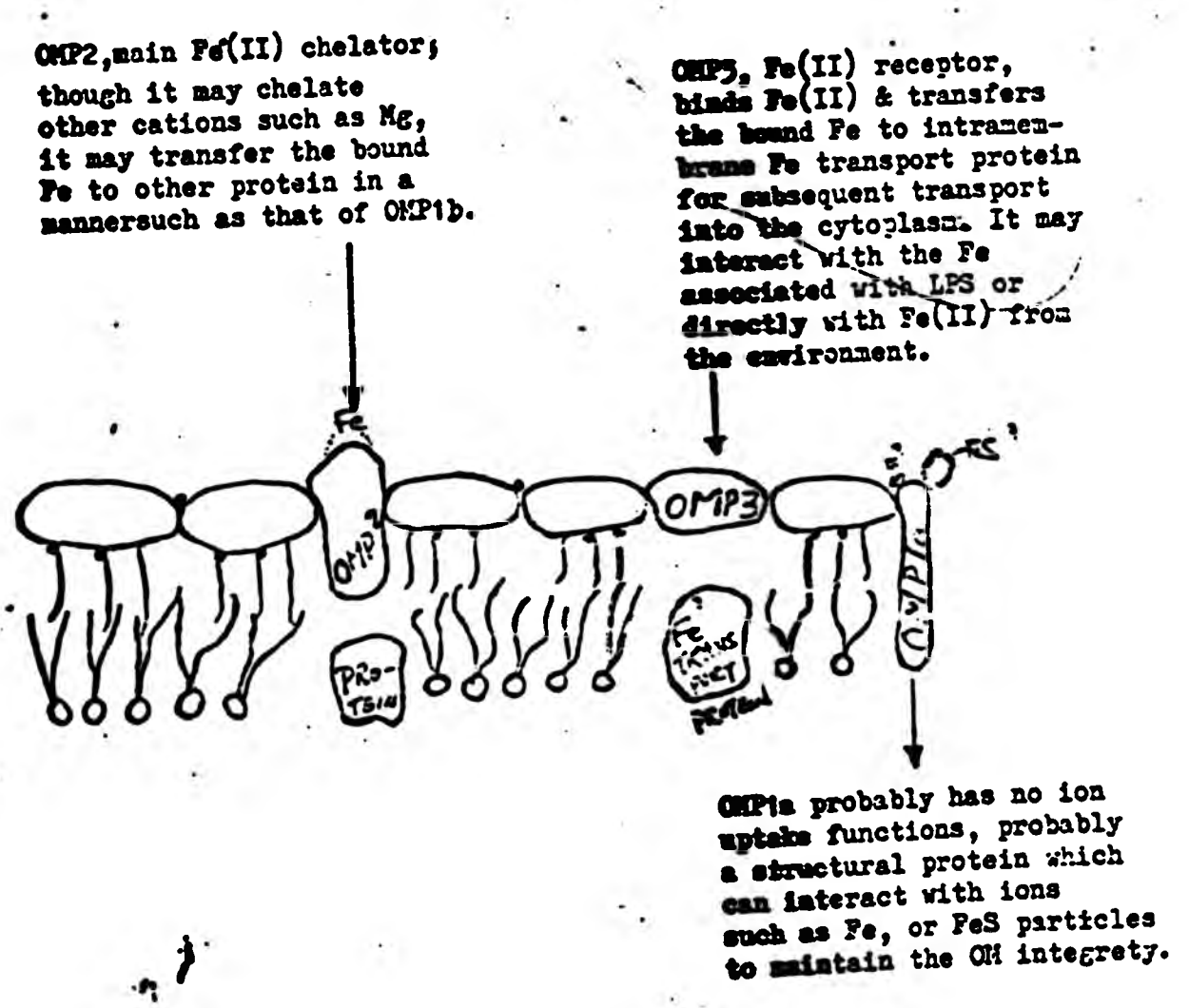
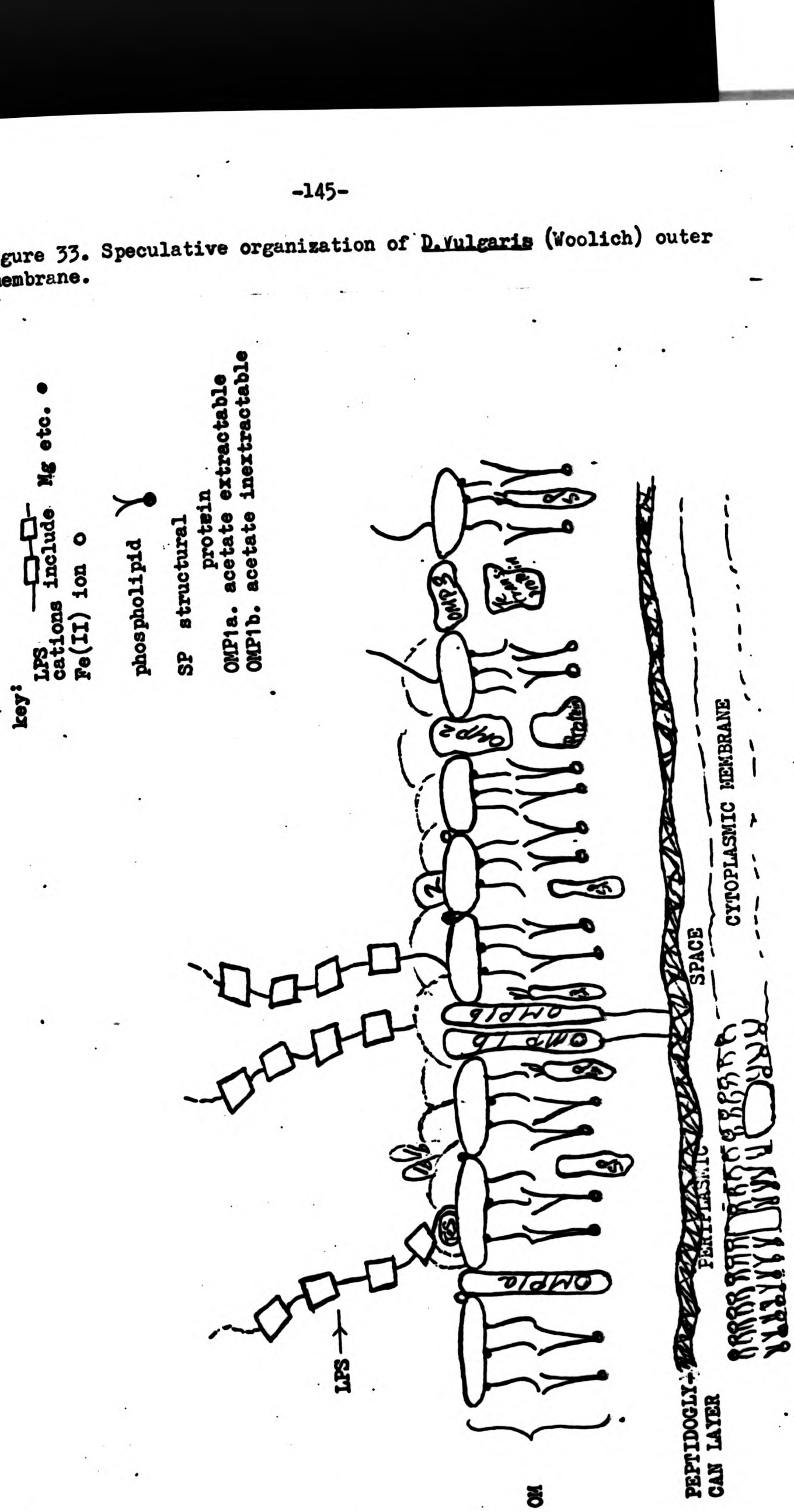


Figure 33. Speculative organization of D.Vulgaris (Woolich) outer membrane.



Further work suggested by these results could include:

1. PYMS analyses of isolated lipid from the LPS to enable one fuller interpretion of the PYMS data.

2. Omp specific antibody labelling of whole cells. The use of gold-labelled antibody could provide a better indication of the location of Omps in the OM.

3. Isolation & purification of Omps - for use in liposome formation to analyse the possibility of their being pore porteins.

4. Analysis of the ionogenic properties of anti-LPS Fab treated and untreated cells using cell electrophoresis.
A change in migration pattern will indicate the different ionogenic properties of the cell surfaces, therefore giving a better indication of whether LPS is the sole factor involved in the adhesion process.

5. The identification of LPS on PAGE gels could be improved by the use of proteinase K and periodate in the silver stain method of Tsai and Frasch cited by Hitchcock and Brown,1983.

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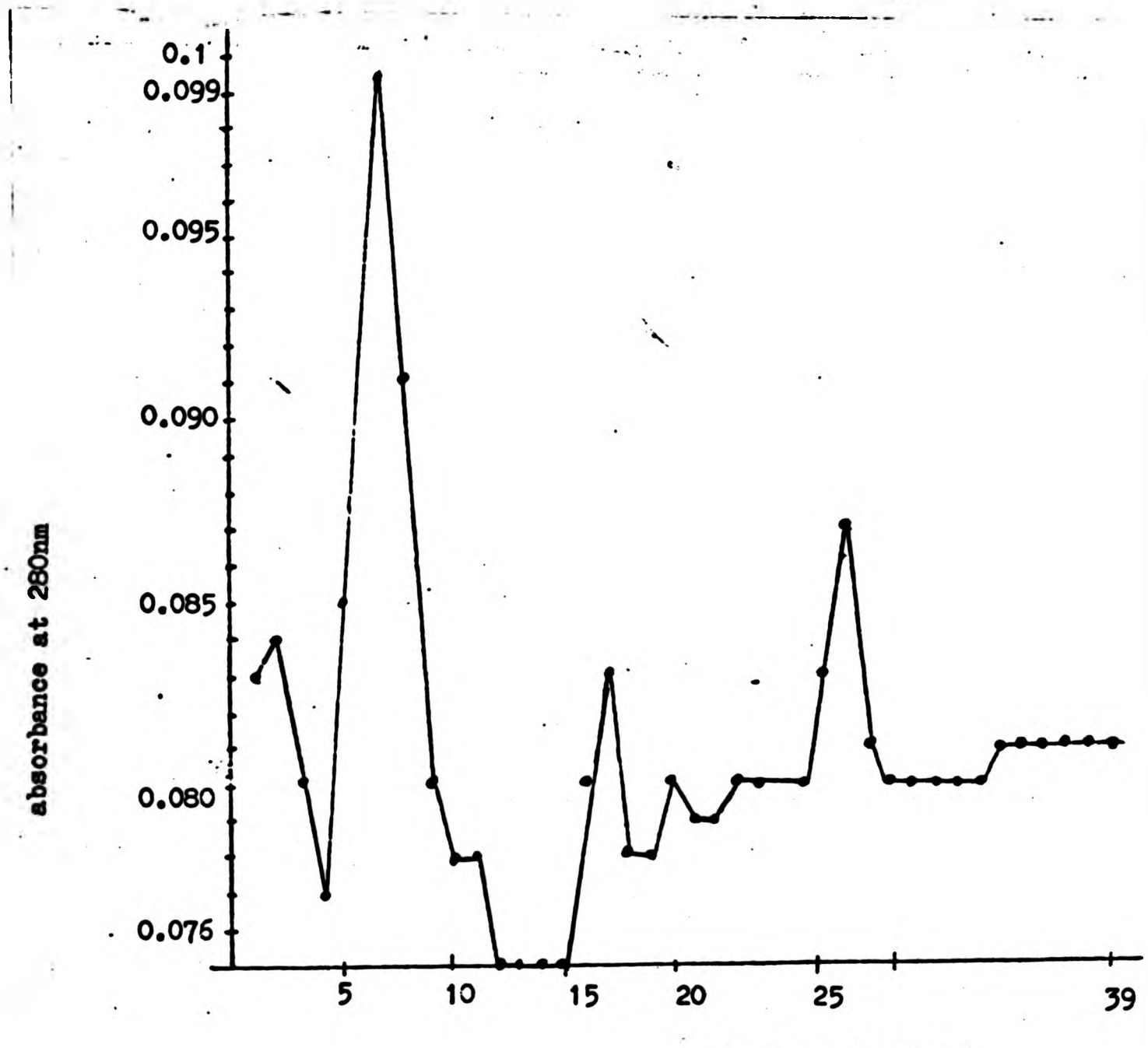
Appendix
Elution profile of IgG fragments through carboxymethyl cellulose ion exchange column.



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P21 ***A Comparison of Lipopolysaccharides (LPS) from Iron-rich and Iron-free Cultures of Desulfovibrio vulgaris***

L.K. SIEW and C.C. GAYLARDE (Department of Biological Sciences, City of London Polytechnic)

LPS from *D. vulgaris* (Woolwich) has been shown to bind Fe^{2+} ions selectively. It may be involved in iron-uptake by the cells. The extracted LPS from iron-rich or iron-poor cultures was analysed by HPLC and immunological techniques. Initial results indicate that changes occur in the LPS on iron-depletion. The possible significance of these changes to iron-uptake by the cells is discussed.

P22 ***Stability of Immobilized Phosphatases in Soil***

R.G. BURNS and J.N. LADD* (Biological Laboratory, University of Kent, and *CSIRO, Division of Soils, Glen Osmond, South Australia)

Polyphenolic-phosphatase complexes were prepared as analogues of naturally occuring humic-enzyme complexes. When added to soil, the immobilized enzymes were remarkably stable, e.g. acid phosphatase, 43% activity at 50 days (soluble, 100% inactivated at 50 days); alkaline phosphatase, 79% activity at 50 days (15 days). The possible application of immobilized phosphatases to phosphate solubilization in soil will be discussed.

ABSTRACTS
TRADE EXHIBITION

Microbe 86

XIV INTERNATIONAL CONGRESS OF MICROBIOLOGY

7-13 September 1986 Manchester, England

P.B22-3 The Specific Transport and Regulation of Manganese in the Yeast, Candida utilis.

D.J. PARKIN and I.S. ROSS: Department of Biological Sciences, University of Keele, Keele, Staffordshire, U.K.

Uptake of ^{54}Mn from 10 nM Mn^{2+} was shown to be both energy-dependent and pH-dependent. Analysis of the uptake kinetics revealed an apparent half-saturation constant, K_t, of 16.4 nmol Mn^{2+} (g dry wt)$^{-1}$ min^{-1}. Mn^{2+} uptake was highly specific, being unaffected by 100-fold molar excess of Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} but inhibited 30 to 40% by 1000-fold molar excess of Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} and Ni^{2+}. Zn^{2+} competitively inhibited Mn^{2+} uptake having a K_i value some 3-fold greater than the K_t for Mn^{2+}. This system is only operated at submicromolar concentrations and is independent of the non-specific Mg^{2+}-transporter. The Mn^{2+} transport system observed appears to be analagous to the specific metal transporter systems reported in the bacteria. Efflux studies indicated that a low level metabolic exchange of Mn occurs. Cellular Mn^{2+} levels remain relatively constant during growth in batch culture. Cells grown in varying Mn^{2+} concentrations in continuous culture exhibited markedly different ^{54}Mn uptake curves; Mn^{2+} uptake was greatly repressed in cells grown in 100 μM Mn^{2+} as compared to cells grown in 0.45 μM Mn^{2+}.

P.B22-4 Cell characteristics of Desulfovibrio vulgaris in iron-replete and iron-deficient conditions

L.K.SIEW and C.C. GAYLARDE: Department of Biological Sciences, City of London Polytechnic, London U.K.

Desulfovibrio has a high requirement for iron. Nevertheless, cells can be induced to grow in low-iron media, although their morphology is altered under these conditions. Minor variations between iron-rich and iron-poor cells are seen on analysis by pyrolysis -mass spectrometry. Gross differences between the outer membranes of the two cell types are apparent in SDS-PAGE gels. The depletion of many outer membrane proteins and increase in one major protein in iron-deficient cells may indicate that the latter protein is involved in iron-uptake. These differences do not appear to be due to changes in plasmid content of the cells.

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