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AN INVESTIGATION INTO THE STRUCTURE AND FUNCTIONAL ASPECTS OF THE CELL WALL OF DESULFOVIBRIO.

GRAHAM BRADLEY CBiol MIBiol.

Presented in partial fulfilment for
the degree of Doctor of Philosophy
to the
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City of London Polytechnic,
Old Castle Street, London, E1 7NT.

DEDICATION.

To my wife Susan, who provided the initial impetus for this work as well as support and encouragement throughout.

DECLARATION.

I, Graham Bradley, 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, with guidance from Mrs. C. Gaylarde and Dr. J. Johnston at the City of London Polytechnic.

Several advanced studies were undertaken in conjunction with my research programme in partial fulfilment of the degree of Doctor of Philosophy. These included a course on Microbial Chemotaxonomy at the London Hospital, Whitechapel. I also presented several research seminars to the academic staff at the City of London Polytechnic and was involved in tutorials and practical demonstrations to undergraduate students of which one demonstration was an application taken from this thesis.

I attended several meetings of the Society for General Microbiology, a FEMS symposium on Sulphate Reducing Bacteria (Freiburg, 1982), the meeting of the Royal Society on Sulphur Bacteria (February, 1982), and a meeting of the Biodeterioration Society on Microbial Corrosion of Metals (Canterbury, 1984). I have presented two poster communications together with an oral communication at the latter meeting. One paper has so far been published from the results of this investigation (Bradley *et al*, 1984).

Graham Bradley, February, 1985.

ACKNOWLEDGEMENTS.

I would like to thank Christine Gaylarde and Jean Johnston for their helpful advice and guidance on all matters. My thanks to all the staff at the City of London Polytechnic Department of Biological Sciences and to the Inner London Education Authority for their funding of this investigation.

In particular, grateful thanks to Steve Fewings for his expert advice on all aspects of technical instrumentation, to Phil Cooke and Aneal Mandal in the Department of Metallurgy and Materials, Electron Microscope Laboratory for the use of their instrument on those 'odd' biological samples, and to Patrick Foster for his help with some of the photography.

Once again my thanks are due to my wife Susan for the use of her expert biochemical knowledge in proof reading part of the text.

ABSTRACT.

AN INVESTIGATION INTO THE STRUCTURE AND FUNCTIONAL ASPECTS OF THE CELL WALL OF DESULFOVIBRIO.

GRAHAM BRADLEY

Satisfactory preparations of cell walls from Desulfovibrio vulgaris cells grown in iron rich (C+Fe) and iron poor (C-Fe) media were obtained by partial detergent solubilisation of the cell envelopes. Polyacrylamide gel electrophoresis (PAGE) has shown the presence in the outer membrane (OM) of three major proteins (OMPs 1, 2 and 3) and a relativly homogeneous lipopolysaccharide (LPS) containing no ketodeoxyoctonate (KDO). A protein of OMP 1 PAGE mobility is readily removed by the EDTA treatment of cells showing its loose association with the cell surface. Only a portion of OMP 1 is removed by digestion or acetate extraction indicating the existance of two proteins, OMP 1a and 1b which comigrate in PAGE. ^{125}I lactoperoxidase labelling of whole cells shows proteins of OMP 1 and 3 mobility to be exposed on the cell surface.

OMPs do not show hydrogenase activity and this enzyme is inactive in C-Fe cells. Numerous minor proteins are present in C+Fe OM but are absent from C-Fe OM indicating 'protection' of the cell wall by iron to detergent solubilisation.

In response to iron limitation D.vulgaris Woolwich shows an increase in the proportion of carbohydrate in the cell wall and in the yield of extractable LPS. HPLC studies have shown changes in the LPS sugars with iron limitation indicating an interaction between Fe(II) and the LPS.

Studies on the release of LPS from intact cells by an EDTA washing procedure have shown a selective interaction between Fe^{2+} and LPS in D.vulgaris. Fe(II) appears to have an important role in the stabilisation in vivo of the OM and this selective interaction may play a part in iron uptake. In vitro OM reconstitutions using extracted material have given further support to this selective interaction. The incorporation of acetate extracted OMPs into reconstituted OM has indicated a possible iron transport function for one or more of these proteins.

Calculations of Fe^{2+}:LPS molar binding ratios based on compositional data and these in vivo and in vitro studies show two forms of iron binding to the surface LPS, which may be applicable to the parameters at work in the natural environment of the bacteria.

ABBRIEVIATIONS.

ADP	adenosine diphosphate
AMP	adenosine monophosphate
APS	adenosine phosphosulphate
ATP	adenosine triphosphate
AUFS	absorbance units full scale
CM	cytoplasmic membrane
CoA	coenzyme A
EM	electron microscopy
EDTA	ethylene diamine tetraacetic acid
FAME	fatty acid methyl ester
Ferrozine	3(2-pyridyl)-5,6diphenyl 1,2,4 triazine disulphonate
GLC	gas liquid chromatography
Hase	hydrogenase
HPLC	high performance liquid chromatography
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
MBS	MOPS buffered saline
MOPS	4-morpholinopropanesulphonic acid
M.Wt.	molecular weight
n	number of experimental observations
NAD	nicotinamide adenine dinucleotide
ND	no data
NHI	non-haem iron
OD	optical density
OM	outer membrane
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
Sarkosyl	sodium lauroyl sarcosine
SD	standard deviation
SDS	sodium dodecyl sulphate
SRB	sulphate reducing bacteria
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane

The expressions Fe(II) and Fe(III) (ferrous and ferric) are used to describe the oxidation states of iron. The expression Fe^{2+} is used when it is clear that a simple ionised form of Fe(II) is involved.

CONTENTS

CHAPTER 1

INTRODUCTION.

1.1. The sulphate reducing bacteria (SRB).

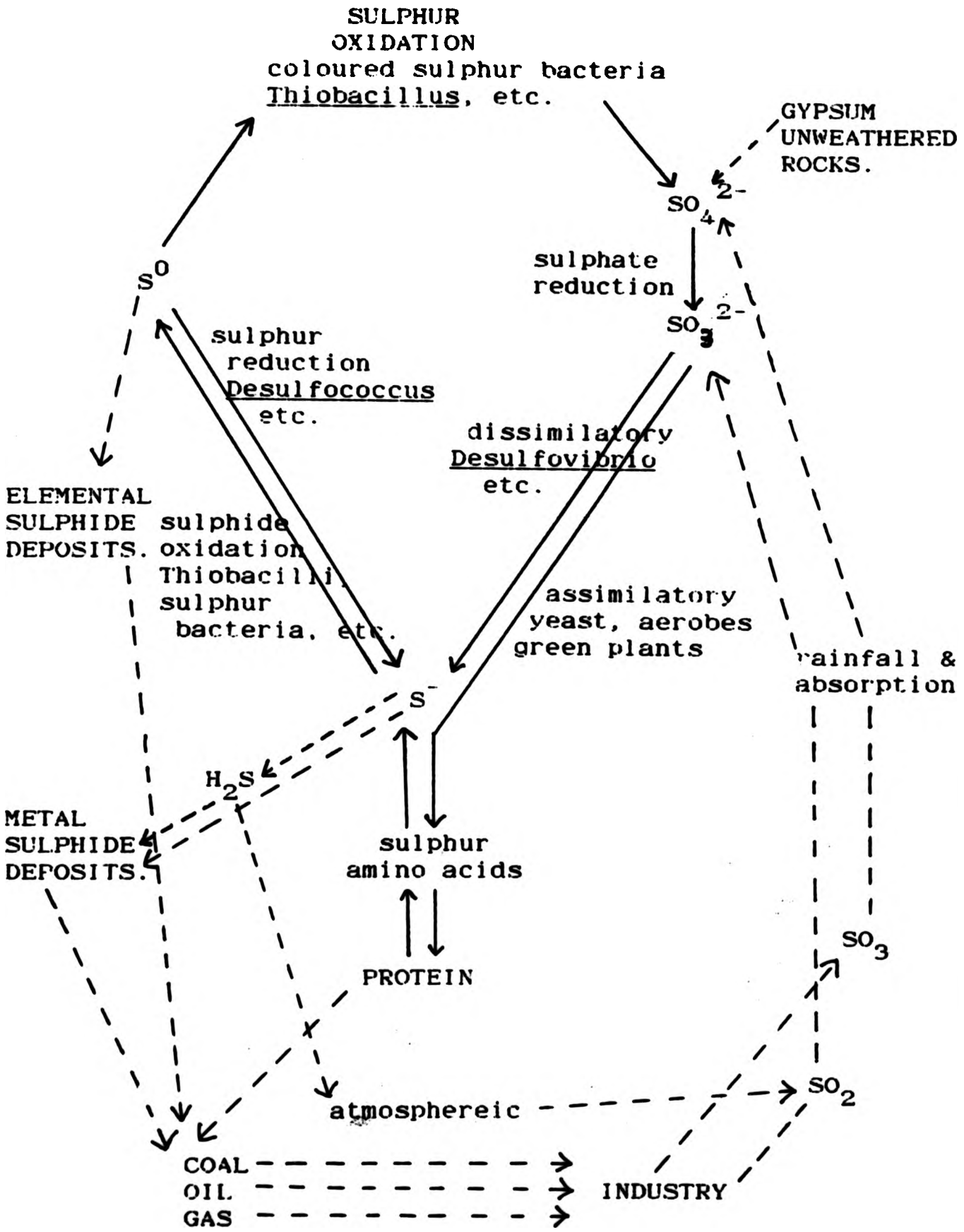
1.1.1. General description.

The name 'Sulphate reducing bacteria' is usually reserved for a group of microbes which conducts dissimilatory sulphate reduction. In this process the sulphate ion acts as an oxidising agent for the dissimilation of organic matter, as does oxygen in conventional respiration. A small amount of reduced sulphur is assimilated by the organism, but virtually all is released into the external environment as the sulphide ion, a large proportion being released as free H_2S gas. This process has been called 'sulphate respiration'.

The sulphate reducing bacteria (SRB) have been recognised for many years but comparatively little information about them has been gathered. They are all strict anaerobes, some are known to be capable of fermentative growth in the absence of sulphate, but none can grow with oxygen as the electron acceptor. They are not killed but merely inhibited by an aerobic environment and may lie dormant for long periods of time, having a remarkable capacity for survival in both terrestrial and aquatic environments. They are very widely distributed ready to become active whenever local conditions become favourable, but grow relatively slowly when compared with other common soil or water organisms.

Sulphur is transformed and translocated in the biosphere by a combination of biological and chemical agencies and the SRB play an important part in this process. This is shown in Figure 1.1. The SRB by-pass assimilatory sulphate reduction and generate hydrogen sulphide in sufficient

Figure 1.1. The global sulphur cycle showing the biological sulphur cycle (⟶) and non-biological short and long-term cycling (---→).
From Anderson (1977) and Postgate (1984).



amounts to support growth of the sulphide and sulphur oxidising bacteria. Their activities, either direct, or through the sulphur cycle, thus have a variety of ecological and economic consequences.

Pure cultures of SRB and suitable media have been available for some decades now. However the taxonomic picture is still far from satisfactory. Many of the earlier workers used impure cultures and thus some historical errors have crept into the literature. These are unravelled to some degree by the excellent review of Postgate (1984). The picture is further complicated by the pleomorphic nature of these bacteria, especially in ageing cultures and varying environmental conditions. Also in recent years several new genera have been described (Widdel, 1980; Pfennig et al. 1981), introducing a further degree of complexity.

The two longest established genera of SRB are Desulfovibrio and Desulfotomaculum which seem to be unrelated to each other apart from their ability to reduce sulphate. The genus Desulfovibrio is the best known, largely because its members are easier to isolate and purify. Both genera include terrestrial and aquatic, marine and strongly halophilic, thermophilic and psychrophilic types. Considerable adaptability is shown in terms of temperature and salinity, representatives being found in soil, fresh, marine and brackish waters, artesian waters, oil and natural gas wells, muds, sewage and rumina of sheep. They are capable of survival in vacuo or in water under considerable pressure and can tolerate pH values ranging from 5 to 9.5 together with a wide range of osmotic conditions. Morphologically desulfovibrios are curved

in shape and non-spore bearing, whereas desulfotomacula are straight and do form spores. Spore formation thus provides the absolute distinction between the two genera. Both genera are flagellated and motile, flagellar morphology being a good taxonomic criterion.

Desulfovibrio vulgaris is a non-sporulating curved rod with a single polar flagellum, most strains being highly motile in the early stages of growth. It is a mesophilic species with some 13 identified strains. Woolwich strain (NCIB 8457) was originally isolated from corrosion test specimens in the River Thames. An electron micrograph showing the morphological appearance of D.vulgaris Woolwich is given in Plate 1.1.

The SRB are adapted to almost any natural environment on this planet except the most common, an ordinary aerobic one. Their need of a low redox potential for multiplication restricts their activities to reducing environments and, though they can be isolated from almost any soil and water sample, they are not active except in anaerobic conditions. Consequently they are rarely encountered as airborne organisms, though both sporulating and non-sporulating types survive drying in soils. Once growth starts however, in a given environment, the physical and chemical nature of that environment changes, often drastically, to suit the organisms requirements and often to the costly detriment of man.

1.1.2. Metabolism.

From a biochemical viewpoint the metabolism of SRB is fascinating. Almost nothing is known of their anabolic or anaplerotic processes. The only work in this area has been

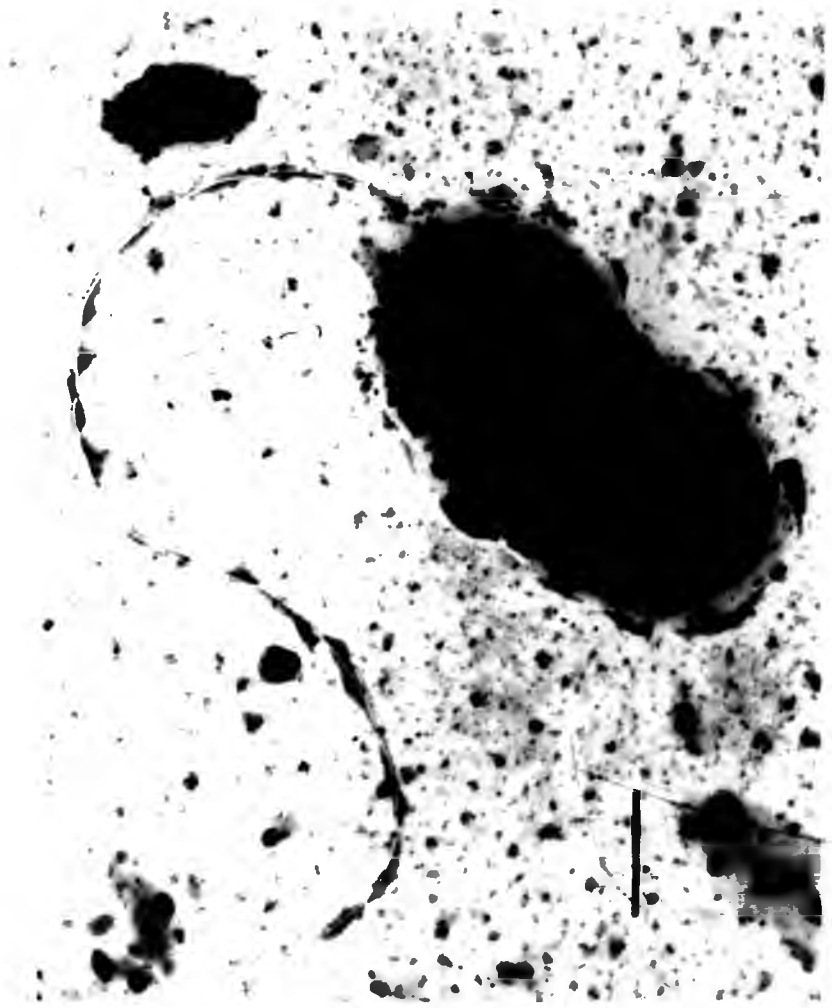
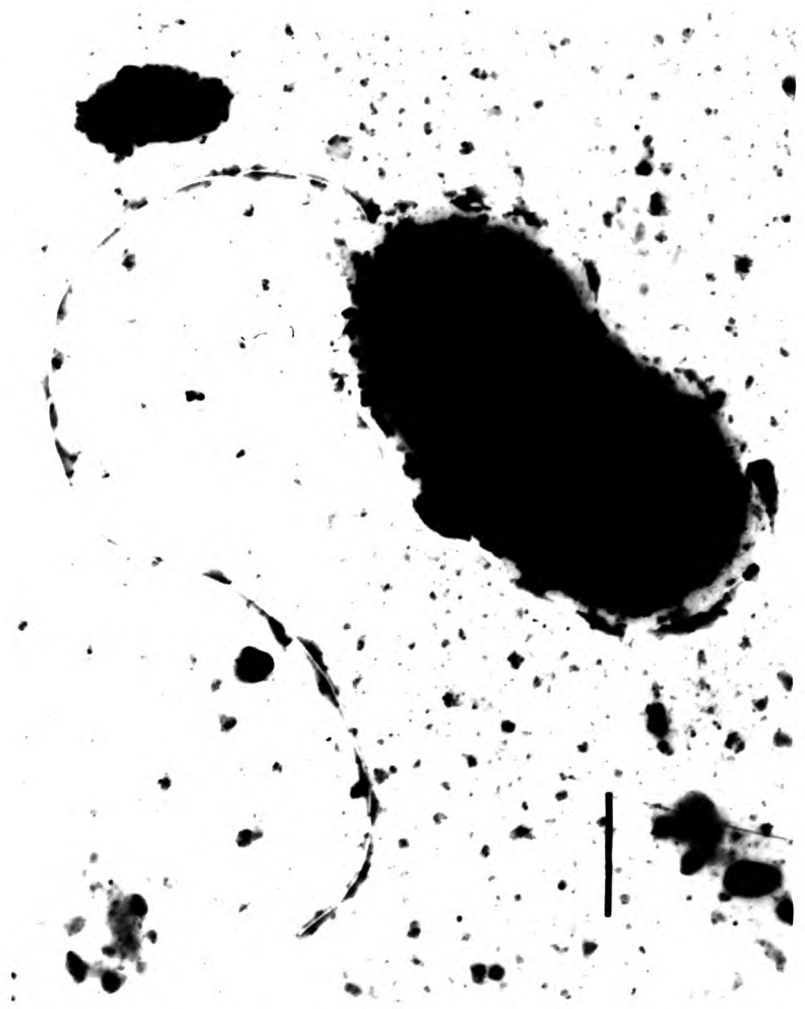


Plate 1.1. D.vulgaris Woolwich
Negative stain. x26000.
(Bar= 0.5µm).



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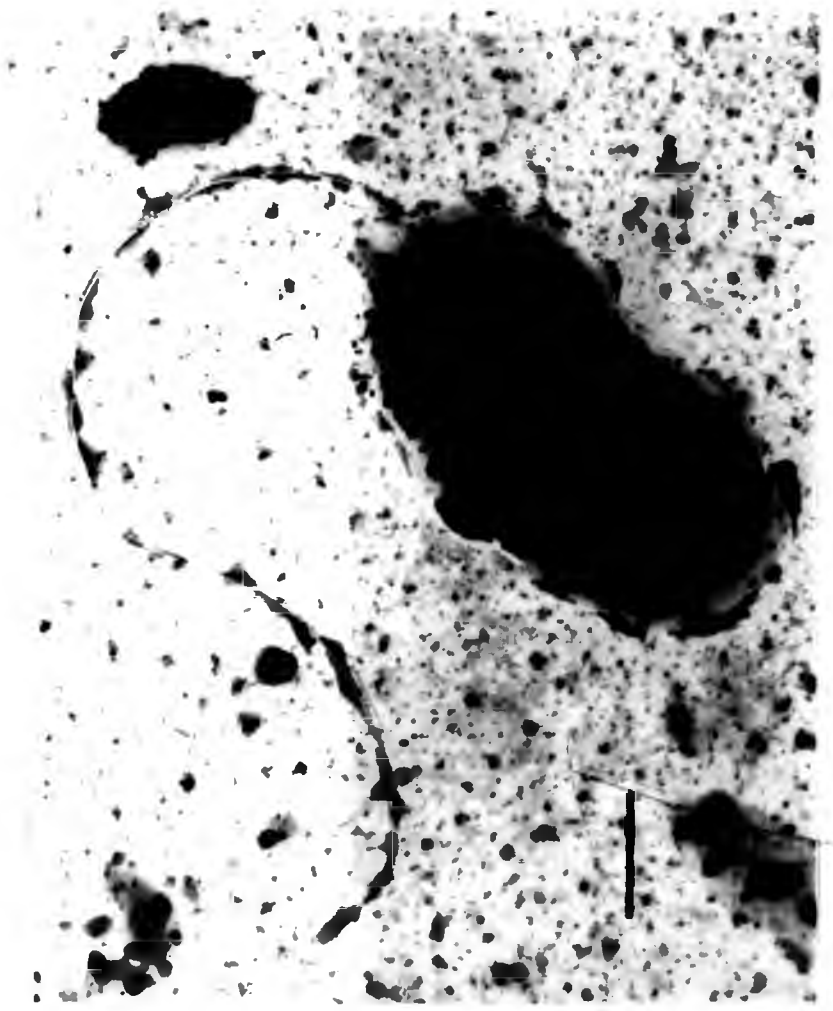

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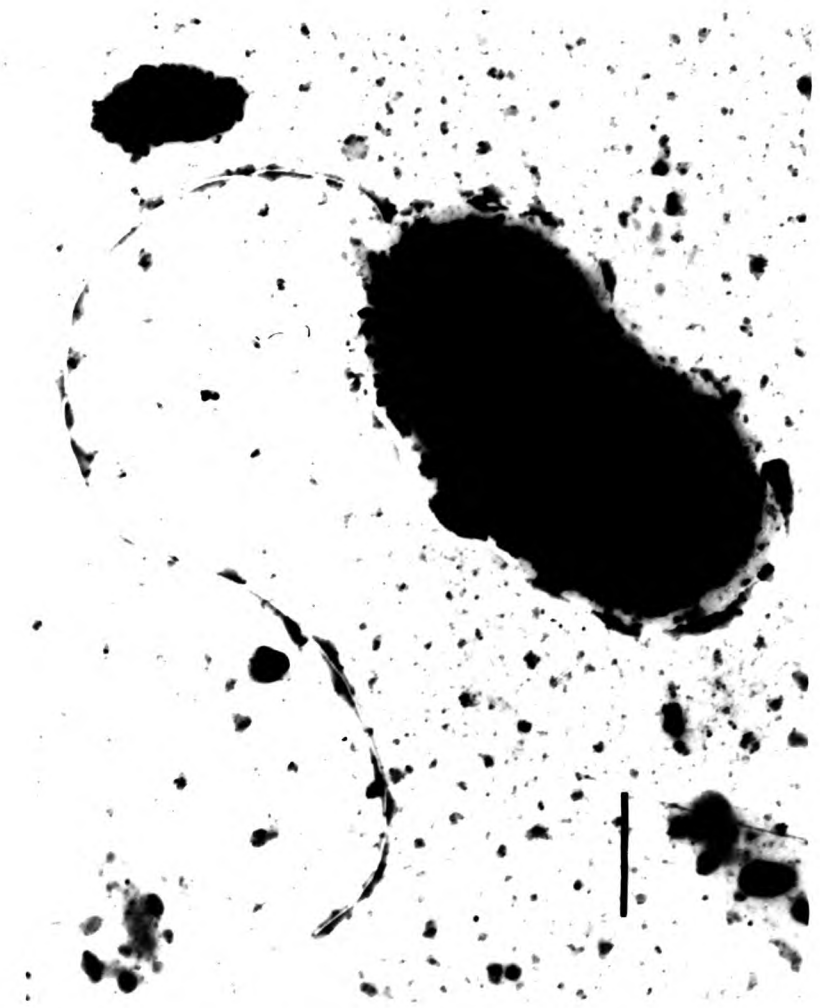
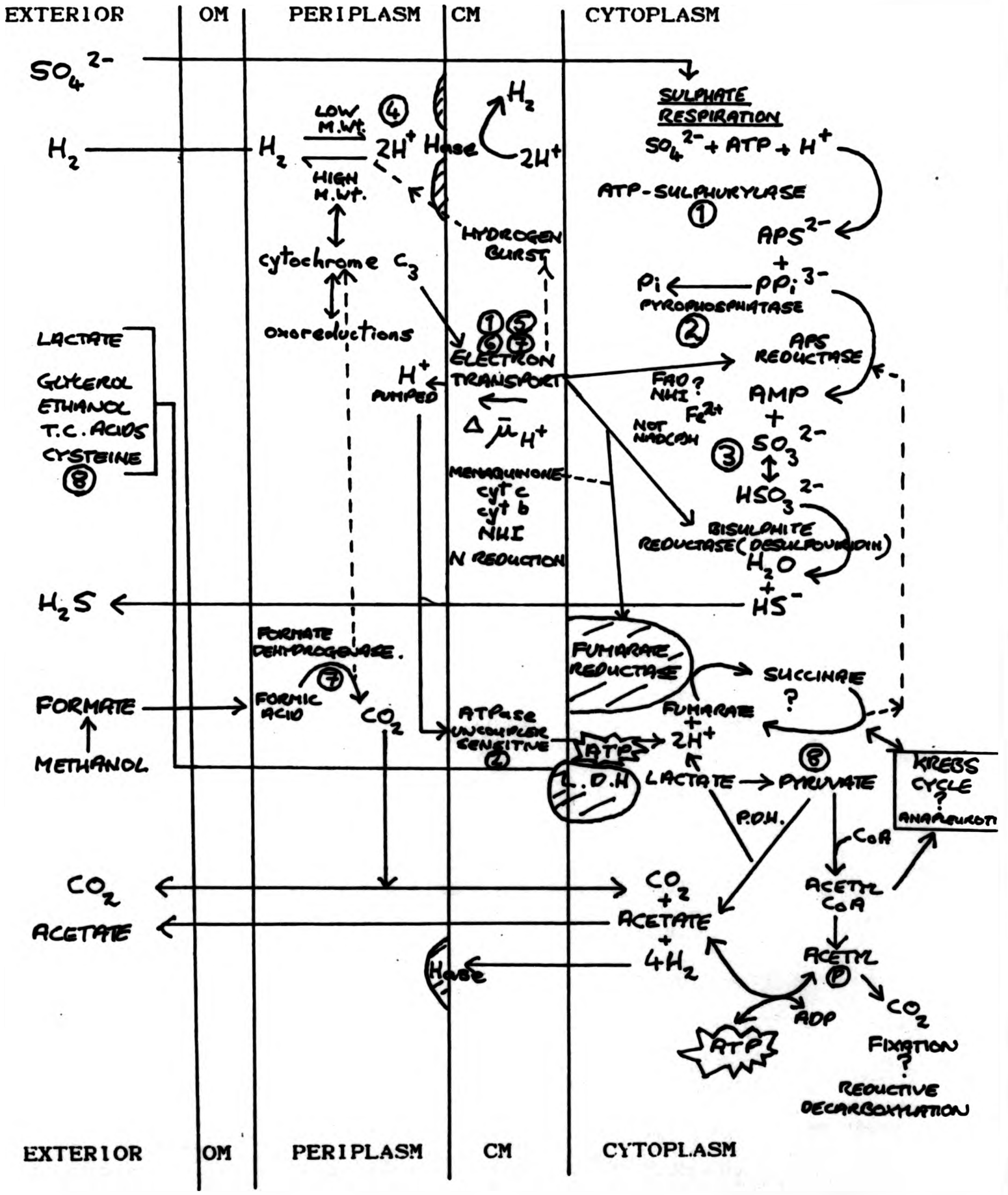


Plate 1.1. D.vulgaris Woolwich
Negative stain. x26000.
(Bar= 0.5µm).

aimed at their taxonomic identification using characteristic whole cell fatty acid compositions (Boon et al, 1977; Ueki and Suto, 1979) and their phospholipid biosynthesis (Silber et al, 1981). For obvious reasons workers have concentrated on the unique processes and enzymes of dissimilatory sulphate reduction itself. Dissimilatory sulphate reduction is essentially an oxidative form of metabolism, despite the obligate anaerobic habit of the organisms concerned, and can usually be divided into three processes. Two non-oxidative; catabolism (usually dissimilation of a carbon source) which may include substrate level phosphorylation (SLP) and electron transport coupled to ATP generation. This is followed by an oxidative process, sulphate reduction. An outline of these sequences and their topography is summarised in Figure 1.2. The organisms are capable of generating energy by the anaerobic oxidation of hydrogen gas acting as an electron donor for the electron transport chain. If the energy yield of this reaction could be coupled to the assimilation of CO_2, the organisms would be capable of growing in a purely mineral environment; i.e. they would be true autotrophs. The belief that that Desulfovibrio strains could grow autotrophically is now accepted as incorrect. However, pre-formed organic matter is required (acetate in particular) for growth and CO_2 assimilation, a reaction termed mixotrophy. The uptake of gaseous hydrogen during this type of growth is

References referred to in Figure 1.2 are: 1, Wood (1978); 2, Thauer et al (1977); 3, Siegel (1975); 4, Tsuji & Yagi (1980); 5, Vosjan (1970); 6, Badziong & Thauer (1980); 7, Peck & LeGall (1982); 8, Postgate (1984).

Figure 1.2. Schematic diagram to illustrate the topography of Desulfovibrio metabolism. The metabolic pathways are not comprehensive but indicate some of the interactions and the main catabolic sequences. Numbers refer to references listed in the text footnote.------→shows speculative electron transfer.



of importance in the removal of the protective hydrogen film during some types of metal corrosion. Most facultative anaerobes can omit the electron transport and oxidative terminal stages and rely solely on SLP for their energy budget; in this respect they resemble the fermentative obligate anaerobic bacteria, such as the Clostridia. Desulfovibrio is not capable of this fermentative type of growth, although pyruvate phosphoroclasm enables pyruvate to support sulphate-free growth in some species. The reaction is a typical phosphoroclastic split leading to the generation of acetyl phosphate and hence ATP synthesis producing acetate, CO_2 and H_2. Where pyruvate dismutation supports 'sulphate-free' growth it is analogous to the 'oxygen-free' growth of a facultative aerobe. With this ability and the oxidative nature of terminal sulphate respiration Desulfovibrio comes very close to being a facultative anaerobe whilst maintaining its strict, obligate anaerobic habit.

The popular modes of growth of Desulfovibrio are with lactate or pyruvate as reductant and carbon source, or with acetate as carbon source and hydrogen as the reductant. In this latter case there is no possibility for SLP. Glycerol, ethanol and the acids of the tricarboxylic acid cycle are all converted to acetate and CO_2 as major end-products by Desulfovibrio. During growth on lactate or pyruvate SLP is possible and sulphate reduction is simply used as an electron sink, as acetate is not metabolised further.

In Desulfovibrio there are numerous dehydrogenases and redox carriers which mediate either to sulphate reduction or through a membrane bound electron transport chain and subsequently to terminal reductases. Lactate oxidation is

mediated by a membrane bound dehydrogenase independent of NAD(P). An NADH dehydrogenase capable of linking the reduction of inorganic sulphur compounds to the oxidation of NADH has not been reported and little is known about the roles of NAD(P) in Desulfovibrio catabolism. Other dehydrogenases include; ethanol dehydrogenase, pyruvate:ferredoxin oxoreductase, formate dehydrogenase (soluble), and hydrogenase which is both soluble and membrane bound. There are a profusion of soluble redox carriers in Desulfovibrio including a periplasmic cytochrome c_3 which is the most widely studied. That electron transport is coupled chemiosmotically to phosphorylation via a series of membrane bound redox carriers (to generate the proton gradient) is now accepted (Postgate, 1984), but the actual chain involved is still obscure. Roles for both rubredoxin and menaquinone MK6 have still to be elucidated. The presence of substances characteristic of both aerobes (cytochromes, menaquinone) and of anaerobes (ferredoxins, rubredoxins and flavodoxins) once again shows the ambivalent metabolic nature of SRB.

The process of sulphate reduction carried out in the cell cytoplasm has been well studied (see references in Figure 1.2). There is initial priming of the system with the formation of adenosine phosphosulphate (APS) thereby raising the low redox potential of sulphate and enabling it to act as an oxidant. This is followed by a series of reductions through sulphite/bisulphite (and possibly trithionite and thiosulphate) to sulphide formation. This process may also be cyclic. The physiological electron donors for the various stages in sulphate reduction are still speculative. Even after more

than a decade of work on these processes the details of the sulphide-generating system are still uncertain.

Very little is known of metabolic regulation in Desulfovibrio or of its genetic regulation. The obligate anaerobic nature of these bacteria coupled with their ability to survive oxygen stress as well as their high requirement for iron makes this an interesting area for research. The plentiful supplies of iron-rich proteins in Desulfovibrio (ferredoxins, cytochrome c_3, nitrogenase, oxidases) together with the relative lack of other catalytic/control molecules (NAD(P)) shows the importance of Fe(II) levels. Iron stressed organisms may alter their metabolism by the use of flavin-centred molecules and adaptation of the component cytochromes (Postgate, 1956; Tsuji & Yagi, 1980). Exposure to oxygen leads to the modification of hydrogenase (Van der Westen et al. 1980) and the pyrophosphatase (Ware & Postgate, 1971) and both of these changes may have important regulatory functions. Phosphorylation reactions involved in metabolic control presumably also occur in Desulfovibrio.

1.2. Corrosion, adhesion and iron uptake.

The SRB are probably best known for the unpleasant odours that they generate and for the participation in the corrosion of buried and submerged iron structures. Anaerobic corrosion of this kind has certain characteristics;

i. It is restricted to anaerobic environments; clay soils, waterlogged ground, submerged structures, anaerobic areas in storage tanks, e.t.c. Iron objects subject to intermittant anaerobic conditions (e.g. tidal areas) often corrode even

faster.

ii. Corroded metals tend to show localised pitting, thereby weakening the structure.

iii. The metallic iron is removed leaving only a graphite skeleton of the structure.

iv. The bacteria adhere to the metal surface forming a black film. They also form 'tubercles' within which a restricted favourable, anaerobic environment may form.

The mechanism by which SRB may be attracted to the iron and by which adhesion occurs are unknown, although much work has been performed on the mechanisms of anaerobic metal corrosion and several theories have emerged (for reviews see Miller, 1971 and Tiller, 1983). A distinctive feature of corrosion by SRB is cathodic depolarisation. A clean ferrous surface reacts with water causing a film of hydrogen to form and iron to dissolve;

$$4Fe + 8H_2O \longrightarrow 4Fe^{2+} + 8OH^- + 8H.$$

If the hydrogen is not removed from the surface an opposing hydrogen electrode is formed and dissolution of the iron will cease. The surface is said to be polarised. However SRB can remove the hydrogen film by their hydrogenase action;

$$SO_4^{2-} + 8H \longrightarrow S^{2-} + 4H_2O.$$ (bacterial depolarisation).

This will induce further dissolution of the iron surface. The net products of the reactions themselves react to form the corrosion products iron sulphide and iron hydroxide;

$$4Fe^{2+} + 8OH^- + S^{2-} \longrightarrow FeS + Fe(OH)_2 + 2OH^-.$$

Direct attack on iron by H_2S proceeds at an appreciable rate and enhancement of iron corrosion by ferous sulphide has long been known. Thus both anodic attack by sulphide and cathodic depolarisation seem to occur. Oxidation of

sulphide to free sulphur, itself highly corrosive, is catalysed by iron and this may have an important bearing. Recent studies (Iverson & Olsen, 1983) have also implicated a highly reactive phosphorus compound, produced as a result of SRB metabolism. The extent to which types of corrosive reaction dominate in a given environment depends upon: the metal surface, presence or absence of dissolved iron and/or inorganic matter, film formation and the presence of other ions. The importance of other organisms within the microbial community and the possibility of interspecies hydrogen transfer has been stressed by Hamilton (1983).

Anaerobic corrosion by SRB is a very costly problem and its prevention is therefore an important practical problem. This may be accomplished by: the coating of metal surfaces thereby physically preventing bacterial access, aeration of the system using compressed air or a non-aggressive gravel or soil, the use of chemical biocides, or by electrochemical 'sacrificial' anodes. All of these methods are costly.

No studies exist on the adhesion of SRB to the iron surface or on the presumable concommitant uptake of Fe(II) for which Desulfovibrio has such a high requirement. Adhesion of microorganisms to surfaces in general has been studied for many years (Corpe, 1970). Many bacteria have been examined for adhesive organelles and details of cell surface structure. Cytologically demonstrable surface components such as fimbriae, capsules, slime and cements which may occur on bacterial cell surfaces largely external to the cell wall have been evaluated for adhesive properties. Capsular and slime polysaccharides have been shown to be necessary in some

genera (Fletcher & Floodgate, 1972; Obuekwe et al, 1981), and fimbriae or pili (Enterobacteriaceae) and stalks and holdfasts (Hyphomicrobium and Caulobacter) in others. No organelles of adhesion have been reported for Desulfovibrio and although a mucin-like substance has been reported in old cultures (Ochynski & Postgate, 1963) this is not present in activly metabolising cultures. However Salton (1964) recognised the overall importance of the cell wall and the external 'ionic' layer which effects overall surface charge that in turn influences attachment of the bacteria to solid surfaces. Studies on the mechanism of the events in the sorption of marine bacteria (Achromobacter and Pseudomonas) to surfaces (Marshall et al, 1971) have suggested an initial reversible ionic sorption followed by an irreversible attachment which may be accompanied by polymer production.

Presumably one of the main reasons for the association of Desulfovibrio with iron surfaces is the adsorption of Fe(II) into the cell. Other than the association of cells with the black ferrous sulphide precipitated during active metabolism no reports of molecular interaction with Fe(II) or storage within the cell have been made. Iron storage within the cell envelope has been reported in Mycobacterium (Ratledge et al. 1982) and crystalline iron is present within the cells of various magnetotactic bacteria (Blakemore, 1982). The kinetics of the uptake of metal cations into bacterial cells usually shows a biphasic form; an initial rapid binding to the surface and a secondary progressive energy dependant accumulation into the cell. Large surface binding is not usually required for the accumulation of metal cations in

the cell. The influx of a metal cation is usually accompanied by the elimination of a cation of equal charge. Some specific transmembrane influx systems exist although these may be utilised by similar 'foreign' cations. Specific uptake and accumulation of one metal by a cell is not often found. Usually a range of metals are taken up. An important consequence of the low solubility of ferric ions has been the development by microorganisms of specific ligands capable of rendering sources of iron soluble and for their facilitated uptake by the cell. These have been termed siderophores (Neilands, 1974). A mutational analysis of iron assimilation in enteric and other bacteria has revealed the presence of a multitude of methods for Fe(III) uptake (Neilands, 1982). These may be differentiated into 'low' and 'high' affinity systems. Low affinity uptake (Konisky, 1979) is operative unless iron is in short supply. Little is known of this uptake but the process can be assumed to be widely distributed in microorganisms as deletion of the high affinity uptake components is not lethal and impairs growth only under iron-restrictive conditions. The high affinity siderophore system which becomes operative, together with its membrane receptor proteins, under iron restriction has also been detected in virtually every aerobic and facultative anaerobic species critically examined for its presence (Neilands, 1984). It is also apparant that availability of iron to potentially pathogenic bacteria is of considerable importance in determining whether an infection becomes established or not (Macham, 1976). Host-iron-pathogen interactions have been held responsible for such phenomena as the fall in iron content of serum that occurs during an infection (suggested

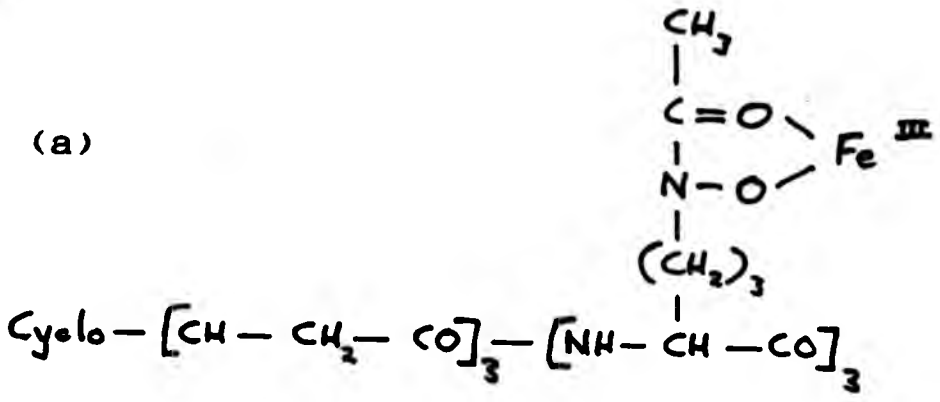
as a defence mechanism to enhance the bacteriostatic effectiveness of serum). Conversely conditions and diseases that result in greater than normal levels of serum iron (e.g. haemochromatosis and sickle cell anaemia) are frequently associated with an increased susceptibility to infection. Production of siderophores by the pathogen, amoung many other factors, is therefore important for the successful establishment of an infection, but direct *in vivo* evidence for the involvment of microbial iron binding compounds has not yet been obtained.

Siderophores have not been detected in strict anaerobes, in lactic acid bacteria, in *Legionella* spp. or in *Saccharomyces* spp. Thus although *Desulfovibrio* has not been examined for siderophores, they are unlikely to be present. Since they are low M.Wt., ferric-specific ligands designed for the solubilisation and transport of Fe(III) their afinity for all divalent cations,including Fe^{2+}, is many orders of magnitude below that for Fe^{3+} and they would be useless to *Desulfovibrio* in an environment where iron is in the reduced Fe(II) state. Examples of various siderophores are given in Figure 1.3. The ligand atoms linked to iron are predominantly oxygens, most often provided by hydroxamic acid or catechol moieties. In siderophores of the citrate family, such as schizokinen, the ß-hydroxycarboxylic acid function is linked to the iron. The affinity for Fe^{3+} is thus a consequence of pairing of the 'hard' acid (relatively small radius/charge) cation with a corresponingly hard base such as oxygen.

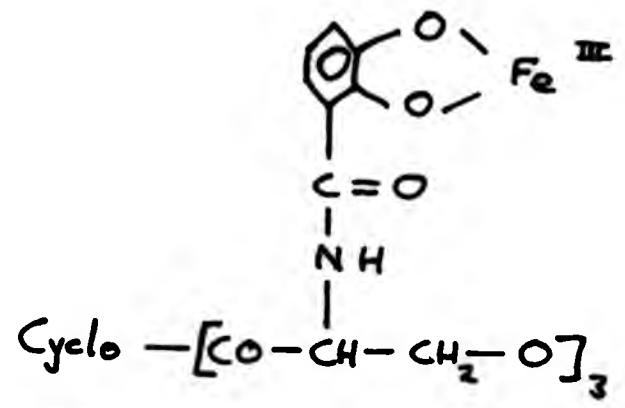
No system strictly analogous to the siderophores has been found for any other metal ion. However this should not rule out the possibility of a specific ligand system for Fe(II)

Figure 1.3. The structure of some siderophore Fe(III) ligands. (a) Ferrichrome (hydroxamate), (b) Enterobactin (catechol), (c) Schizokinin (citrate/hydroxamate). From Neilands (1984).

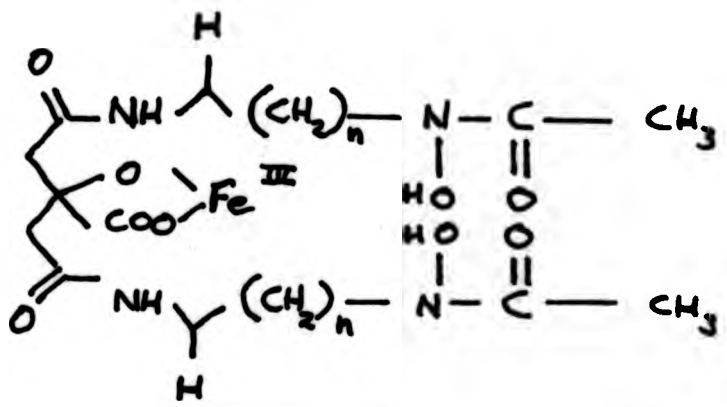
(a)



(b)



(c)



within Desulfovibrio, with its high requirement for iron, if Fe^{2+} is not readily available.

The second part of the siderophore system is the protein receptors for its uptake and utilisation (Neilands, 1982). Both parts are subject to a common regulatory device triggered by the intracellular concentration of iron. Induction of OM proteins in response to iron limitation has been reported for several Gram negative bacteria (McIntosh & Earhart, 1976; Meyer et al, 1979). Transport across the OM is presumably by facilitated diffusion whereas further transport across the CM may well be active. The membrane potential has been reported to be the driving force for siderophore iron transport in the fungus Neurospora (Huschka et al, 1983). The studies on the genetic mechanisms of siderophore regulation and receptor production/repression have provided a useful model system. The aerobactin mediated iron assimilation system has recently been successfully cloned using a plasmid vector (Bindereif & Neilands, 1983).

1.3. The Gram negative bacterial cell wall; outer membrane structure and function.

Most cells within a multicellular organism are exposed to a homeostatically controlled environment. In the case of unicellular organisms the cells are exposed to an unpredictable habitat and must be able to resist potentially toxic changes in it. In Gram negative cells such is the complexity of the outer layers of the cell that the term 'envelope' is used to describe the complete outer structures. The cell envelope is thus the interface between the organism and its habitat and hence plays an important role.

The Gram staining of bacterial cells has long been used for classification purposes and was originally based on the ability of cell envelopes to retain a dye complex. This is still one of the most valuable and widely used of staining procedures. The mechanism of the staining reaction is not fully understood but it is known that the staining response correlates not only with a difference in cell envelope structure (Rogers et al, 1980) but also with other properties. These include aminopeptidase activity (Cerny, 1980) and citrate synthetase regulation (Weitzman et al, 1975). As an aid to Gram classification a test based on the reaction of lipopolysaccharide present in the cell envelope with polymyxin B has recently been described (Weigel & Quant, 1982). Electron microscopic studies of the cell envelopes from Gram negative cells have shown them to be of a much greater complexity than those of Gram positive bacteria. Whereas Gram positive bacteria have a bounding or cytoplasmic membrane (CM) covered with a thick peptidoglycan coat (a sugar polymer with cross-linked peptides), at least four or five layers are now recognised in Gram negative cell envelopes. These can be isolated and distinguished using biochemical techniques.

Starting at the cytoplasmic aspect, a generalised Gram negative cell envelope is composed of : i.a CM, ii. a structureless 'periplasm', iii.a simple rigid peptidoglycan layer, iv.a second or outer membrane (OM), v.any exopolysaccharide or protein layer (both very variable with age and culture media). Because of the periplasmic gap and the relative ease that the OM and peptidoglycan can be stripped from cells these two outermost layers are known as the

cell wall. The actual surface of the Gram negative bacterium is therefore presented by the OM component of the cell wall and any exopolysaccharide (slime coat or capsule) that may be present. For this reason it is upon the OM that considerable attention has been focussed. The situation is further complicated by any surface organelles (flagella or pili) which must protrude through the cell wall at various points and occasional 'junction' sites between the cell wall OM and the CM (Bayer, 1981). The interaction of Gram negative bacteria with their environment, including both inert and host plant and animal tissues, will thus involve the cell wall surface components of the bacteria. Some of the more important cell wall surface activities together with their associated structure or macromolecular species are outlined in Figure 1.4.

1.3.1. Outer membrane form.

Although easily visualised with the electron microscope, the OM has only recently been isolated and studied. This is especially true of the OMs of Escherichia coli and Salmonella typhimurium and we can now claim to understand their structure and function fairly well at the molecular level (Nikaido & Nakae, 1979). In fact, together with the erythrocyte membrane, the OM of the enteric group of bacteria and those bacteria of clinical importance is one of the best understood of biological membranes. Although both CM and OM appear as typical 'unit' triple layers under the electron microscope the OM is known to differ in a number of important ways. The CM is in many ways comparable to the plasma membrane in animals; it is a phospholipid bilayer into which are embedded

Figure 1.4.
Bacterial Surface Phenomena and their Related Structure.

ACTIVITY	MACROMOLECULE/ STRUCTURE
a.) Adhesion; cell / inert surface cell / cell	slime coat pili lipopolysaccharide protein sites 'holdfasts'
b.) Ion-trapping; metal cations heavy metals	lipopolysaccharide surface charge excreted phenolics
c.) Transport; permeability	protein receptors protein pores
impermeability	lipopolysaccharide protein pore size slime coat
d.) Antigenic response;	lipopolysaccharide protein receptor
e.) Chemotaxis;	flagella proteins
f.) Periplasmic integrity;	outer membrane and peptidoglycan
g.) Cell rigidity and shape;	peptidoglycan lipoprotein

a number of different proteins (Harrison & Lunt, 1975). This membrane is semipermeable, showing active transport, carrying out energy transduction and containing some of the enzymes responsible for cellular metabolism. In contrast, the bacterial OM is dominated by a small number of major protein species (DiRienzo et al, 1978; Osborn & Wu, 1980). These proteins show little or no enzymic activity (Albright et al, 1973). The phospholipid is largely confined to the inner leaflet of the lipid bilayer whilst the outer leaflet is provided by the so-called 'lipid A' of a lipopolysaccharide (LPS) with a long sugar 'tail' extending outwards into the environment. This assymetry has been demonstrated by enzymic degradation experiments and by labelling studies with LPS-specific, ferritin-labelled antibody (Mühlradt et al, 1973). The arrangement is shown schematically in Figure 1.5.

The rigid peptidoglycan layer, thought to be anchored to the OM by specific lipoproteins (Braun & Rehn, 1969), provides support and shape to the organism. Lateral movement within the OM is somewhat slower than other membranes but whether this 'rigidity' is due to the larger amount of protein or to specific protein-protein, protein-LPS or protein-LPS-divalent cation interactions cannot be said with certainty. The matrix proteins may also be associated in some way with the lipoprotein/peptidoglycan complex. These structural properties are essential for correct functioning of the OM, and have to be borne in mind when attempting to understand its activities.

Probably the most important function of the OM is its action

Figure 1.5.
Diagrammatic representation across a Gram Negative Bacterial Outer Membrane and of a Typical Lipopolysaccharide Molecule

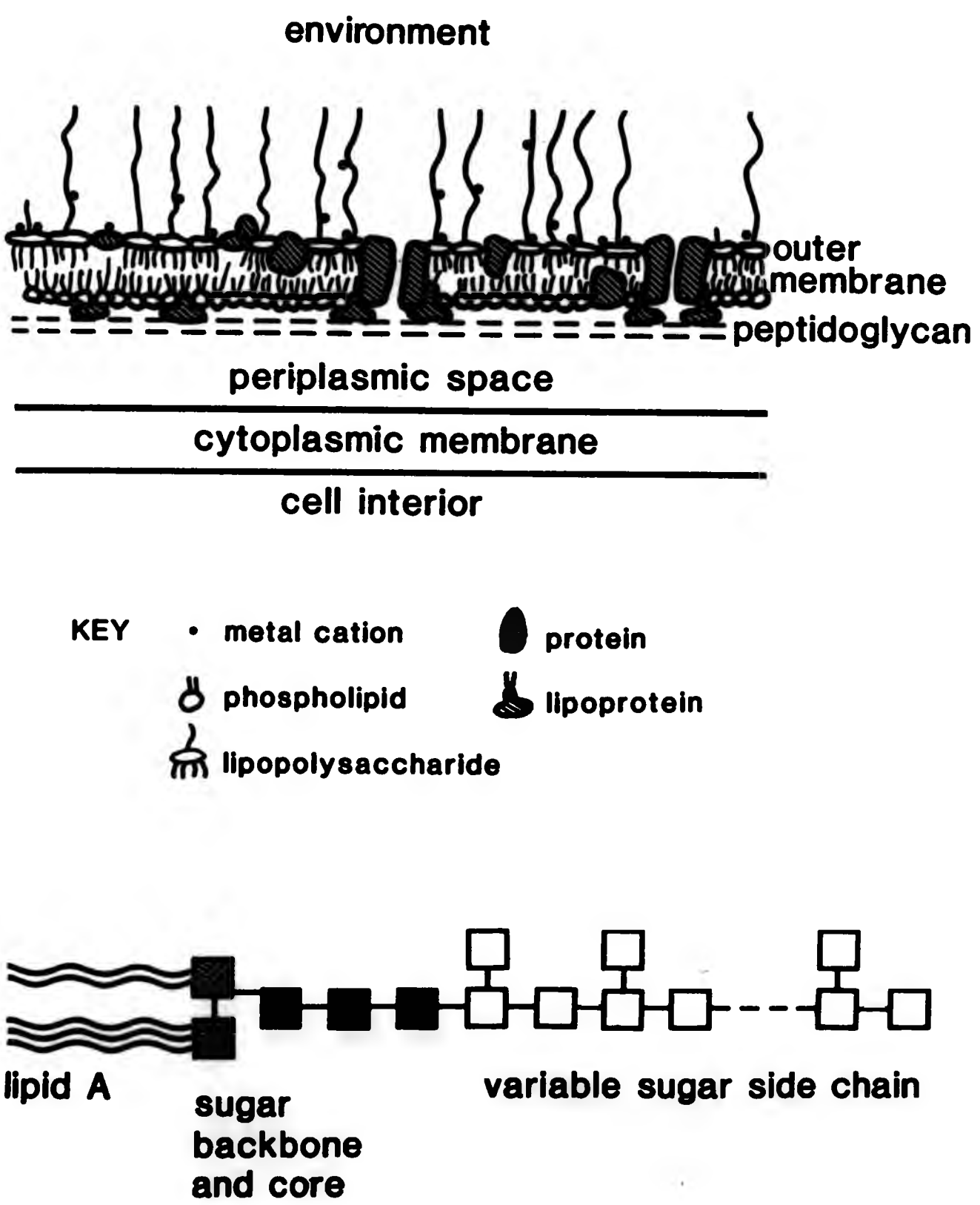
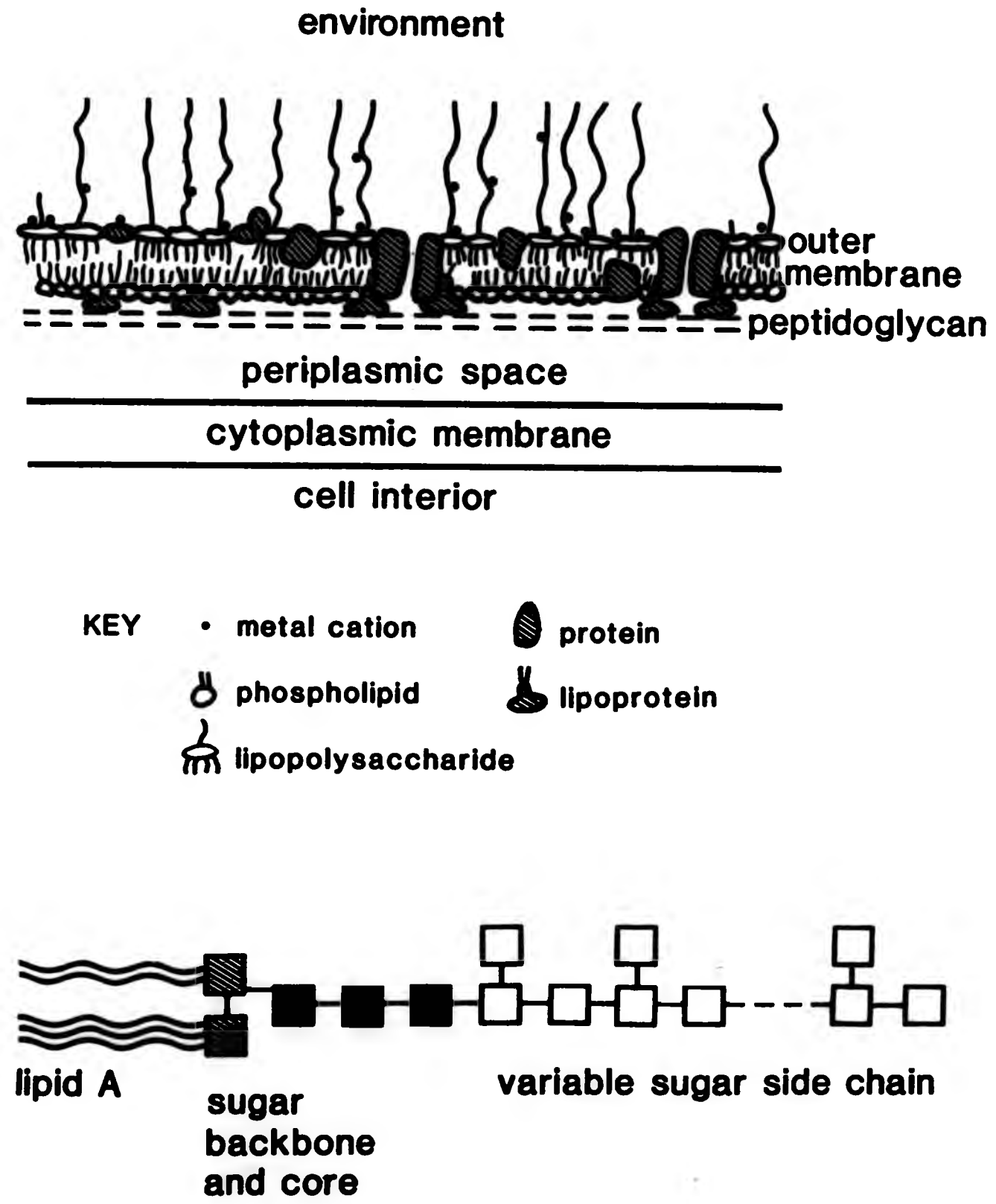


Figure 1.5.
Diagrammatic representation across a Gram Negative Bacterial Outer Membrane and of a Typical Lipopolysaccharide Molecule



as a selective permeability barrier, keeping out hydrophilic molecules above a certain size and a wider range of hydrophobic ones that would otherwise be injurious to the bacterium. It also keeps active proteins concentrated within the periplasmic space (those that are best kept away from the cytoplasmic contents). In this respect it functions in a similar manner to the lysosomal membrane of higher cells (Inouye, 1979). At the same time the OM provides specific and non-specific channels (Hancock & Nikaido, 1978) and receptor proteins (Neilands, 1982) for those nutrients and ions required for growth. It should be noted that these nutrients are transported by passive or facilitated diffusion. The active transport systems are exclusivly located in the CM. Gram negative bacteria may be more resistant to dyes, antibiotics, enzymes, e.t.c., as the OM prevents these toxic compounds from entering the cell. This is especially important for those bacteria that comprise the normal intestinal flora of animals. The OM protects the CM from direct exposure to bile salts that would lyse the cells. The most important component here is the LPS with its long polysaccharide chains, preventing penetration of certain compounds and interacting with animal tissues and substrata. Furthermore the LPS is the endotoxin, the major toxin of pathogenic enteric bacteria.

The OM also contains many receptors for phages and colicins (highly specific extracellular antibiotic proteins) and plays important roles in cell-cell interaction during conjugation as well as indirect roles in chemotaxis (Adler, 1973). The OM is thus the primary organelle in controlling the interaction of Gram negative bacteria with their

external environment.

1.3.2. Lipopolysaccharide (LPS).

Lipopolysaccharide is the unique molecular species of the OM and as such plays a part in some of its special functions. These macromolecules show a large degree of variation, especially with regard to their sugar side-chains. The classical model for a bacterial LPS is that of a smooth or 'wild' type strain of Salmonella upon which much work has been performed (Figure 1.5; Galanos et al. 1977). The use of mutant organisms abnormal in their side-chain production has proved extremely revealing, some types of LPS showing considerable shortening of this moiety. The sugar side-chain is the serologically dominant part of the molecule, responsible for the O-antigenic specificity of the bacteria. It consists of repeating units of several sugars. These may vary considerably in accord with the serological specificity of the bacteria. The side-chain may be reduced to a core region in rough strains (R-form). This core may also be sub-divided into an outer region, to which the side-chain is attached and an inner region, linked to the lipid A portion of the molecule. The lipid A is embedded within the lipid bilayer of the membrane but is responsible for the endotoxic properties of the LPS, producing the typical symptoms of bacterial infection, raised body temperature, local swelling, shock and drop in blood pressure. Both lipid A and inner core region seem to be essential for viable bacteria.

The common hexoses (D-glucose, D-galactose, D-mannose), 6-deoxyhexoses (L-rhamnose, L-fucose) and hexosamines are widely disributed in LPS. Pentoses (arabinose, ribose,

xylose) occur less frequently and their identification in LPS extracts may be indicative of contamination (Wilkinson, 1977). The core and lipid A contain some of the most distinctive components of LPS: 3-deoxy-ketoaldonic acid (KDO) and an aldopentose (both in the inner core) and glucosamine and 3-hydroxy alkanoic acids (both in the lipid A). These sugars can be useful markers for the OM and LPS during their isolation. Ion trapping by the charged sugar side-chain of LPS is important for the uptake of trace metals and in the immobilisation of growth inhibitory metal ions. Furthermore metal cations bound to the LPS seem essential for correct function (Galanos et al, 1977). One group of antibiotics appear to exert their action by replacing these cations (Hancock, 1981) and indeed some bacteria can become resistant to these antibiotics (streptomycin for example) by the synthesis of cation replacing proteins within the OM (Nicas & Hancock, 1983).

In enterobacterial LPS and presumably other KDO-containing LPS, the lipid and polysaccharide fractions are linked via this ketosidic linkage which is weak acid labile. Lipid A typically contains D-glucosamine, fatty acids, phosphate and often ethanolamine in varying proportions. The backbone of lipid A from Salmonella LPS is the β1-6 linked disaccharide of glucosamine (Wilkinson, 1977). Positions 1 and 4' of the disaccharide units are phosphorylated and position 3' is the site of KDO attachment. Fatty acids, which form the bulk of lipid A, are attached to glucosamine by both ester and amide linkages. The N-acyl residue is characteristically a 3-hydroxy alcanoic acid. An unusual feature of some lipid A is the presence of ester-linked 3-OH acids that are themselves

esterified by other fatty acids. The major fatty acids found in different lipid A fractions can be useful as taxonomic indicators.

Exactly how representative enteric bacterial LPS is of other Gram negative LPS is not certain. The absence of heptose and/or KDO from LPS has been reported for various non-enteric bacteria. Some LPS do not contain OH-acids and even glucosamine is absent from lipid A of some Rhodopseudomonas spp. (wilkinson, 1977).

1.3.3. Outer membrane proteins (OMPs).

The small number of protein species which are present within the OM in large amounts are usually designated as 'major' proteins, although this is a rather arbitrary classification. A minor protein may become a major protein when its production is fully induced. In common with other membranes these proteins may be associated with the membrane to varying degrees. 'Intrinsic' proteins are firmly embedded within the membrane, often bound hydrophobically or even covalently to other compounds. 'Extrinsic' are loosely bound to the membrane surface often by ionic forces and may be associated with metal cations. In the OM of wild type E.coli K-12 there are at least three types of 'major' OMP and several other proteins present in much smaller amounts. Figure 1.6 lists the major OMPs for four genera of bacteria which have been extensively studied. These are based on SDS-PAGE separations, and the nomenclature has by no means been standard even for investigations upon the same species. Matrix proteins (porins) are intrinsic (but non-covalent) proteins. One of these proteins (1a) of E.coli B was first

Figure 1.6. Major outer membrane proteins of four extensively studied organisms together with their probable functions. Identification is based on their mobilities in SDS-PAGE and nomenclature is not standardised.

ORGANISM	OMP Name	OMP M.Wt. (apparant)	Probable main function (if known).
E.coli	3b	—	Regulatory protein Protease?
	1a	36500	Porins
	1b	—	(small pore size).
	II (tol G ompA)	30000	Pilus conjugation Colicin receptor.
	Lipoprotein	7000	Structural.
S.typhimurium	36	36000	Porins
	35	35000	(small pore size).
	34	34000	
	33	33000	?
	Lipoprotein	7000	Structural.
P.aeruginosa	D 1/2	46000	Glucose inducible.
	E	—	
	F	41000	Major porin (large pore size).
	G	—	?
	H1	21000	LPS associated (binds phosphates),
	H2	20500	Lipoprotein (structural).
	I	—	Lipoprotein (free form).
Pr.mirabilis	C	39000	Porin.
	D	36000	Peptidoglycan-linked and porin?
	E	17000	?

Data taken from: Inouye, 1979; DiRienzo et al, 1978; Osborn & Wu, 1980; Lambert & Booth, 1982; Nixdorff et al, 1977.

isolated by Rosenbusch (1974). Its M.Wt. was calculated as 36500 and it consists of a single polypeptide of 336 amino acid residues. E.coli K-12 also contains porin protein 1b. Their relative amounts vary greatly with the growth conditions but there are about 1.5×10^5 mols. of porin protein/cell arranged as a hexagonal lattice structure covering some 60% or more of the outer surface. Evidence that the OM controlled the passage of certain molecules within a fixed size range led to the postulation of water-filled passive diffusion pores through the membrane. Nakae (1976) showed that the incorporation of porin protein into artificial LPS-phospholipid vesicles greatly enhanced their permeability to sucrose. Porins are thought to span the membrane and anchor to the underlying peptidoglycan by means of associated lipoprotein molecules. The size of the pore itself is rather variable. It appears to be smaller in the more specialised enteric bacteria while in more diverse species such as the marine pseudomonads the pore is larger in order to allow greater nutrient diffusion (Hancock et al, 1982).

The lipoprotein of the OM is one of the most thoroughly investigated of membrane proteins. This protein is the most abundant in the whole cell in terms of numbers of molecules and has many unique features (Inouye, 1979). It has a relatively small M.Wt. of 7000 and its primary structure has been established (Braun, 1975). The exact function is still unknown but a peptidoglycan-bound form certainly helps to maintain the OM structure and thus stabilise the barrier to the environment.

Protein II (tol G, ompA) also exists in large quantities in E.coli OM and is known to show anomalous mobility on SDS-PAGE (DiRienzo et al, 1978). The M.Wt. is approximatly 30000 and the protein has a high ß-structural content. (Nakamura & Mizushima, 1976). One important function of protein II is the F pilus mediated conjugation. Many of the OMPs also have secondary roles as bacteriophage or colicin receptors.

Many of the minor inducible OMPs (up to 20 in E.coli) act as specific receptors for single sugars, a cofactor, a nucleoside or a trace metal, and are often only required when the molecule is present in very low concentrations in the medium. The production of these proteins can usually be finely regulated by the organism.

Studies on the OM are important in both membrane biochemistry and other areas of molecular biology, shedding light on how transport occurs across membranes and how molecules are inserted into the membrane. The genetic regulation, biosynthesis and assembly into the membrane of OMPs forms part of the excellent review by Osborn & Wu (1980). The bacterial OM is still proving to be an invaluable model in elucidating genetic mechanisms, differentiation of membrane structures and functions, and communications with other cellular components.

1.4. Aims of the investigation.

The aim of this investigation is to establish the structure and functional relationships in the cell wall of D.vulgaris, especially those leading to an understanding of cell

adhesion to iron surfaces and iron adsorption to the cell surface with subsequent transport into the cell. The ultimate goal is to facilitate methods of preventing adhesion and therefore corrosion of iron surfaces by these SRB. In addition the taxonomy of the SRB is in a far from perfect state and any structural analysis of the cell wall may well assist with classification problems.

CHAPTER 2

METHODS.

2.1. Preparative Techniques.

2.1.1. Culture and growth.

Original freeze-dried cultures were obtained from the National Collection of Industrial Bacteria, Aberdeen. *D.vulgaris* Woolwich, NCIB 8457, was the strain used in all experiments. Woolwich strain was originally isolated from slime covering corrosion test specimens in the River Thames at Woolwich, London in 1960. Other strains were additionally utilised where useful comparisons might be made.

For the successful growth of sulphate reducing bacteria in pure culture the redox potential (E_h) of the environment must start at around -100mV (Postgate, 1984). For cultivation a modified Postgate's medium C was used routinely;

KH_2PO_4	0.5 (g/l)
NH_4Cl	1.0
Na_2SO_4	4.5
$CaCl_2 6H_2O$	0.06
$MgSO_4 7H_2O$	0.06
Sodium Lactate	6.0
Yeast Extract (Oxoid)	0.5
$FeSO_4 7H_2O$	0.004

Distilled water to one litre.

The pH was adjusted to pH 7.7 and the media autoclaved at 112^oC for 15 minutes, during which the pH fell to 7.5. Bottles were filled as full as possible to minimise air

spaces. The medium is a pale yellow transparent colour and contains only half as much yeast extract as that suggested by Postgate and contains no sodium citrate.

Iron rich (C+Fe) and iron restricted (C-Fe) media.

Sodium citrate (2.5 g/l) and one mild steel coupon (40mm x 15mm) were added to some cultures (C+Fe) to provide iron rich media and an exposed iron surface for attachment. In others the ferrous sulphate was omitted to provide an added-iron free medium (C-Fe). In the culture of D.vulgaris Venezuela, 2.5% NaCl was added to the Medium C. For maintenance of cultures 20ml. universal bottles of media were used. For cellular fractionations 500ml. bottles were inoculated and incubated at $30^{o}C$ for four days to late log phase when the cell count was approximately $5x10^{8}$ cells/ml.

Inoculations of 5ml. maintenance culture into 500mls. medium C+Fe and 15mls. maintenance culture into 500mls. medium C-Fe, were made. This gave good growth within several days. Presumably the bacteria produced sufficent H_2S from their resting metabolism to act as a redox poising agent, making added H_2S, cysteine or thioglycollate unnecessary.

Purity checks were made at inoculation by plating out onto nutrient agar and solidified medium C+Fe, and incubating at $30^{o}C$, both aerobically and anaerobically in a Gaspak (Becton-Dickinson) anaerobic jar. Plates were observed for growth over a period of seven days. Estimations of cell numbers in liquid media were made using a haemocytometer

and phase contrast microscope. Cells were observed in this manner during sonication and spher oplasting or they were heat fixed and briefly stained with Gentian Violet or more reliably by the method of Turnbull (BDH);

i. Cover smear with a mixture of one part potassium ferricyanide (2%w/v) and three parts hydrochloric acid (2%v/v) for 5-15 minutes.

ii. Transfer to 1% HCl for 5-15 minutes.

iii. Wash well with water.

iv. Counterstain with 1% aqueous neutral red.

v. Wash with water.

This procedure stains nucleic acid material red and ferrous iron a dark blue colour.

2.1.2. Sub-cellular fractionation.

1. Strategies.

The strategy adopted for isolation of membranes or cell envelope components for further analysis from a particular bacterial species is in large part dictated by the envelope structure of the organism. No one method appears to work well for all genera. Cell disruption of a Gram negative organism followed by differential centrifugation produces 'cell envelopes' containing not only the outer membrane and the peptidoglycan, but also the cytoplasmic membrane. Detailed biochemical studies of the outer membrane were thus impossible until Miura and Mizushima (1969) devised a method of isolation based on buoyant density differences. In this procedure cells of E.coli were disrupted by the osmotic lysis of lysozyme/EDTA spher oplasts (osmotically

sensitive cells in which the peptidoglycan has been digested) and the membranes were separated by equilibrium sucrose density gradient centrifugation. The OM banded at higher density due to the presence of LPS and more protein than the CM. In their classic paper Osborn et al (1972) carefully examined this procedure and minimised OM disruption due to the presence of EDTA, at least for S.typhimurium. EDTA is necessary in these procedures due to the protective nature of the OM for the underlying peptidoglycan in Gram negative species. However EDTA has been shown to release considerable amounts of LPS and protein from the cell surface (Leive, 1974) and the damage to the OM is viewed as undesirable by many workers.

Instead of lysozyme/EDTA lysis, intact cells can be disrupted by other means such as the French pressure cell or sonication and the OM complex again separated by equilibrium density gradient centrifugation. Schnaitman (1970) originally developed this method which has the advantages of the ability to handle large amounts of material and to use discontinuous gradient steps. EDTA was however still present in the gradient although a modification by Smit et al (1975) did not use this agent at any stage. Both the Osborn and the Schnaitman procedures have been successfully used for the OM isolation from such non-enteric bacteria as Neisseria gonorrhoeae, Pseudomonas spp., Caulobacter spp. and many others. In several species the OM has been purified by cell disruption, repeated washing and differential centrifugation. Thornley et al (1973) used this

method for Acinetobacter spp. and Hurlbert and Gross (1983) for Pseudomonas syringae.

In many experimental situations it is often considered complicating and unnecessary to examine the inner membrane. In instances such as these, use can often be made of techniques which allow the rapid preparation of OM material and which avoid the time-consuming step of isopycnic centrifugation.

The release of OM material by EDTA can be used to investigate protein and LPS components and this has been used in rapid procedures for preparing fractions enriched in OM proteins. Poxton and Brown (1979) have used the analysis of such OM preparations as an aid to the identification of organisms in the B.fragilis group. EDTA and mild sonication has also been used by Wolf-Watz et al (1973) for the selective removal of OM fragments from intact bacteria/spher oplasts. However, neither the Osborn method nor the EDTA release methods yield an intact OM-peptidoglycan complex (i.e. a cell wall) and as these two layers are believed to be firmly bound together (Braun, 1975) these other methods must yield fractions which are disrupted or even incomplete in some way.

Methods of OM preparation based on selective solubilisation with detergents have also been developed (Filip et al, 1973). Treatment of the cell envelope with non-ionic or weakly ionic detergents results in complete solubilisation of the CM, whereas the OM retains its morphological

integrity and bound peptidoglycan. A complete cell wall complex is thus obtained, although some lipid material may be extracted. The importance of LPS as a surface antigen has resulted in the development of selective extraction procedures for the various forms of these macromolecules. These are usually applied to isolated cell envelopes to minimise contaminating cellular material (Westphal and Jann, 1965; Galanos et al, 1969). EDTA release procedures, sucrose density gradient separations, detergent selective solubilisation and selective LPS extraction were all utilised in this investigation in an effort to determine their suitability for D.vulgaris cell envelope fractionation. The fractionation products were examined with regard to ease and rapidity of preparation, yield, their biochemical composition and suitability for the subsequent use of the fraction. The optimum method was then used in subsequent investigations.

2. EDTA washing procedure.

Cells from 500mls. culture were harvested at 3000xg for 30 minutes and washed in 50mM sodium phosphate buffer pH 7.4 containing 0.15M sodium chloride (PBS).* The pellet was resuspended in 10mls. of PBS containing 10mM EDTA and incubated at 45^{o}C for 15 minutes. The cells were then pelleted at 3500xg for 30 minutes. The supernatant was termed 'EDTA washings'. When deemed necessary the EDTA was

* In all experiments other than those on whole cells (Chapter 4) PBS was replaced by MOPS buffered saline (MBS) pH 7.2. MOPS is considered to be less biologically active than phosphate and hence a better buffering system to use.

removed by dialysis against distilled water for four to five hours.

3. Poxton/Brown outer membrane isolation.

Essentially the methods of Poxton and Brown (1979) were used. The cells were washed in EDTA as above, and then briefly vortexed, sonicated at 10μ for one minute and revortexed. They were then centrifuged at 6000xg for 30 minutes. The pellet was termed the mureinoplasts (intact cells without outer membranes), and the supernatant the outer membrane fraction plus released proteins and other molecules. The OM could be pelleted by centrifugation at 95000xg for 30 minutes, resuspended in water and stored at -70^{o}C.

This procedure was reported to standardise the conditions necessary to give optimum release of OM proteins without lysis of the bacterial (B.fragilis) cytoplasmic membrane.

4. Sucrose density gradient separations.

This method, first used by Osborn et al (1972), for preparing cytoplasmic membranes from S.typhimurium, is based on their separation by isopycnic sucrose density gradient centrifugation. Although rather prolonged, it has become the classic method for OM production for numerous enteric bacteria.

Cells from 500mls. culture were harvested at 10000xg for 5 minutes and rapidly suspended in cold 0.75M sucrose, 10mM Tris/Cl^{-} pH 7.8 for no longer than 10 minutes. Lysozyme was added to a final concentration of 100μg/ml and the suspension incubated on ice for two minutes.

Two volumes of 1.5mM EDTA pH7.5 were then slowly added.

The conditions are very important as this dilution procedure

results in the production of intact spher oplasts (osmotically sensitive cells lacking peptidoglycan). Lysis was then effected by sonication on ice for 4x one minute bursts (10μ). Any remaining intact cells were pelleted at 1200xg for 20 minutes and the supernatant was respun at 95000xg for one hour to pellet the membranes. These were then suspended in 0.25M sucrose, 3.3M Tris, 1mM EDTA and diluted to one third of the original lysate and respun. The membrane pellets were resuspended in 5mls. of 25% w/v sucrose, 5mM EDTA pH 7.5 and layered onto a 30-35% sucrose step gradient containing 5mM EDTA. This was centrifuged for 16 hours at 95000xg. Three or four bands were reported by Osborn et al for S.typhimurium. the heavier, lower band being the OM. Various modifications of the Osborn method were also attempted.

i. Cells from 500 mls. of culture were harvested at 3000xg for 30 minutes and then washed in MBS. The washed pellet was resuspended in 10mls. MBS and sonicated in an MSE Soniprep 150 for 10x one minute bursts at 10μ amplitude, with 30 second intervals. Any unbroken cells and media debris were then pelleted by centrifugation at 3500xg for 10 minutes. Complete cell envelopes were then pelleted from this supernatant by centifugation at 30000xg for 20 minutes. Envelopes were washed in MBS and resuspended in 1ml. 20%w/v sucrose, 30mM MOPS buffer pH 7.2. This suspension was layered onto a two step gradient of 60% and 70%w/v sucrose, 30mM MOPS buffer pH 7.2 and centrifuged at 90000xg for 20 hours. Bands were then removed by collecting 1 ml. fractions by suction using a peristaltic

pump. Fractions were assayed for protein (OD 280 nm) or diluted approximately ten times with water, repelleted and analysed by SDS-PAGE. This procedure was performed on both C+Fe and C-Fe cultures.

ii. Lysozyme (100µg/ml., Sigma grade 1) was added to the above procedure at either pre- or post-sonication stages.

iii. The gradient was lengthened to a three step 50%/60%/70% sucrose in MOPS buffer for cell envelope fractionation as in i.

iv. Spheroplast lysis (Osborn method) replaced the sonication procedure before separation on the modified gradients.

v. Preliminary OM enrichment by using a pre-spin of 20000xg for 20 minutes to obtain a high-speed and low-speed pellet prior to density gradient separation was also attempted.

5. Sarkosyl cell wall isolation.

It was originally reported by Filip et al (1973) that the inner (cytoplasmic) membrane of certain Gram negative bacteria could be selectivly solubilised by the ionic detergent sodium lauroyl sarkosine (Sarkosyl), leaving the cell wall (OM and peptidoglycan) intact.

This relatively simple method of cell wall isolation has been successfully used since then (Spratt, 1977; Lambert and Booth, 1982) although no definite explanation for its effectivness has been offered. The modified method used here avoids the use of EDTA and is a relatively short, easy preparation.

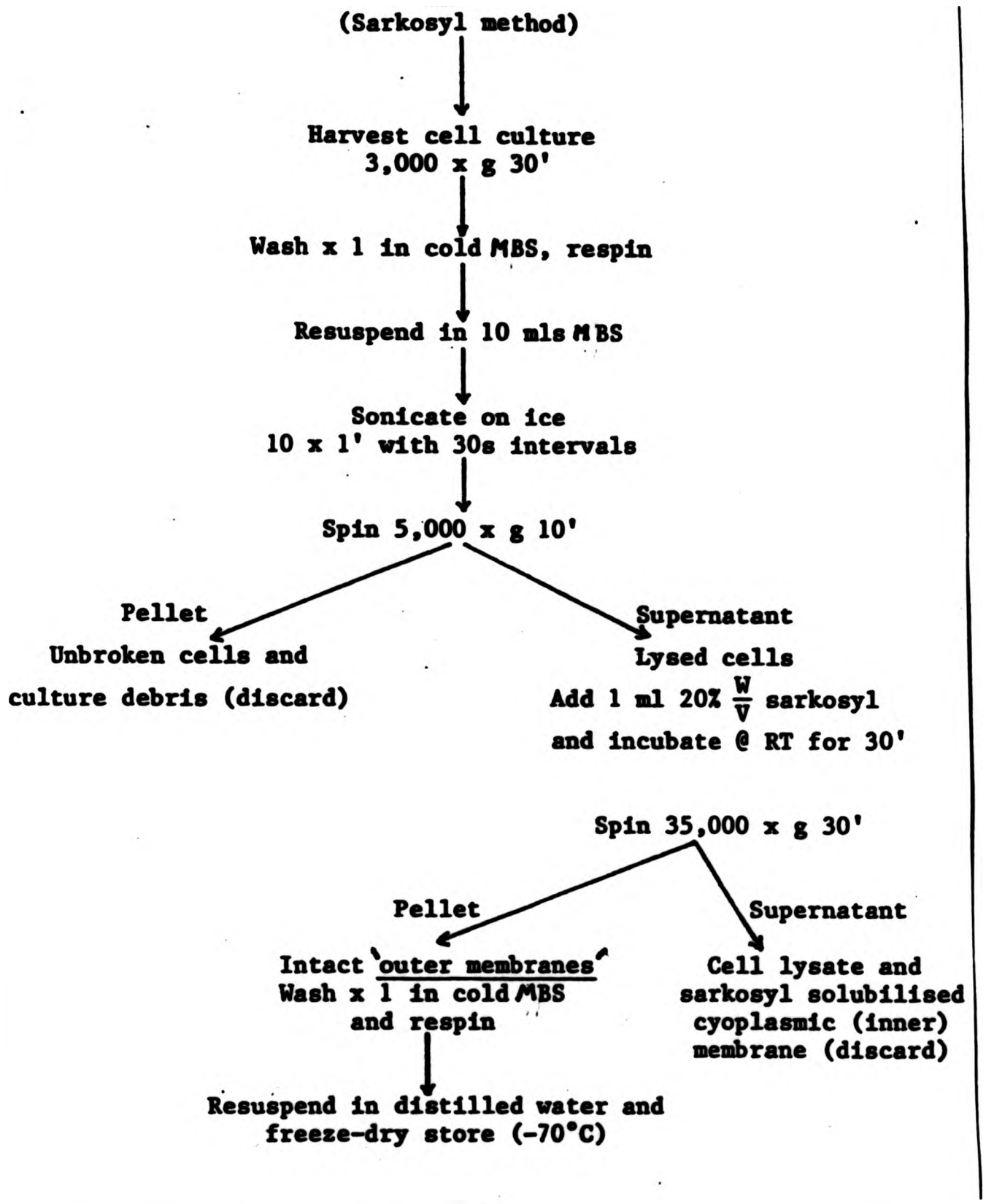
Cell envelopes were prepared as outlined above (4,i.). The supernatant containing these envelopes was taken and

to this 1ml. of 20%w/v Sarkosyl was added. This was left at room temperature for 30 minutes with occasional stirring. The unsolubilised cell walls were then pelleted by centrifugation at 35000xg for 30 minutes and washed once with MBS. This final pellet was resuspended in a small volume of water, freeze-dried and could then be stored at -70^{o}C. An outline scheme is shown in Figure 2.1.

6. Lipopolysaccharide (LPS) isolation.

Although LPS occurs in the OM as part of an insolu ble complex, it can usually be extracted in water-soluble form by various relativly mild procedures (Wilkinson, 1977). The efficiency of the extraction and the composition of the extract depend on the particular organism, the starting material (eg. whole cells or envelopes) and the methods used. Thus extracts from whole cells may be contaminated with nucleic acid and glycans from the cell interior, together with capsular polymers. For this reason it is best to extract from isolated cell envelopes thus obviating the necessity for nucleic acid removal (Johnson and Perry, 1976). Even using isolated cell envelopes dissociation of the complex may be incomplete and various LPS binding proteins have been isolated as part of the heterogeneous LPS isolates (Geyer et al. 1979). Extraction with cold aqueous TCA is the classical route to 'complete' O-antigens (protein-lipid-LPS). This complex can be split with hot aqueous phenol and this is by far the most effective general method for obtaining LPS almost free of contaminants, and has been used in many studies. After extraction the LPS is

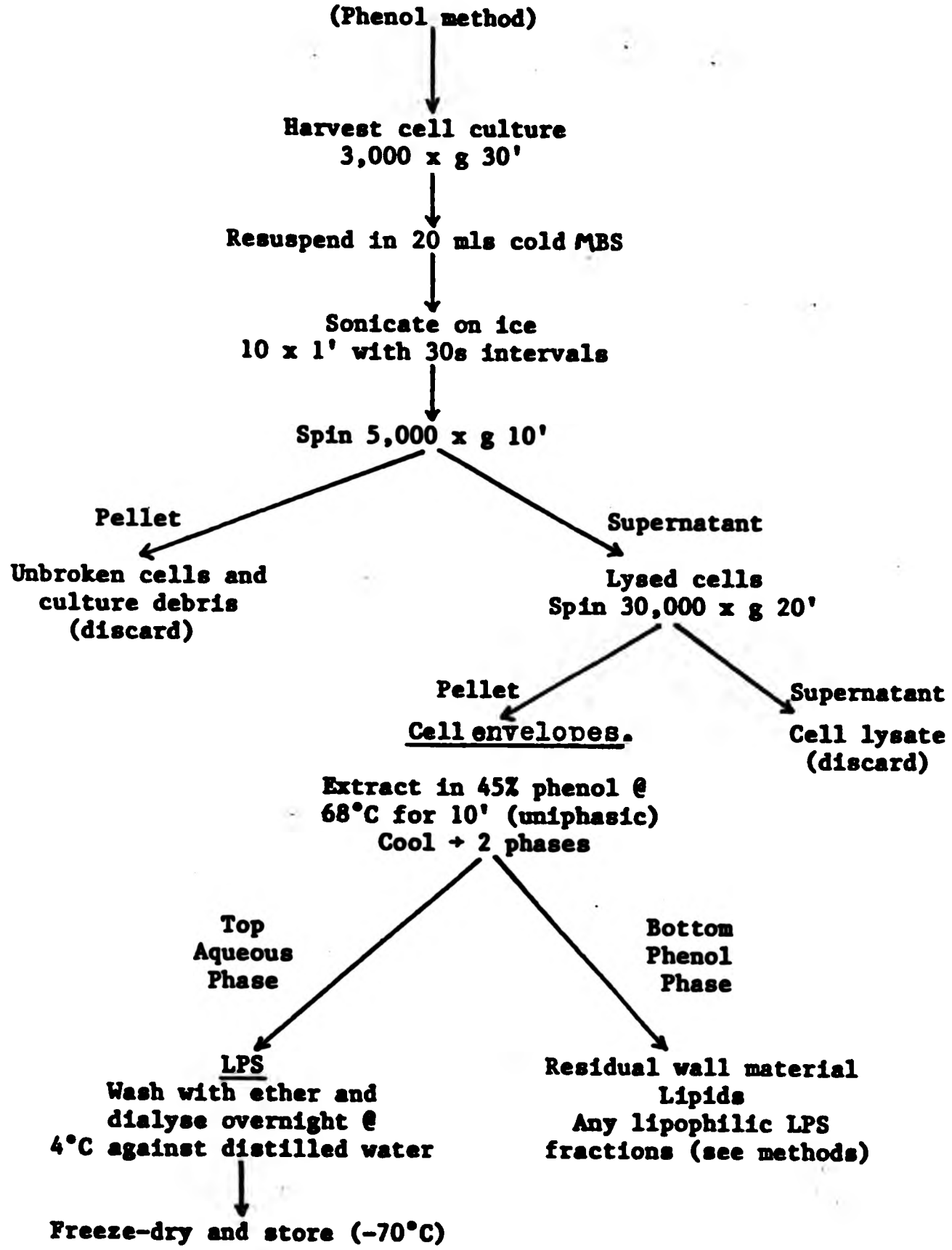
Figure 2.1. Scheme for the separation of intact Cell Walls.



usually located in the upper aqueous phase on cooling. Some relatively lipophilic LPS may however remain in the phenol layer, eg. R form LPS (with shortened carbohydrate chains) have reduced water solubility and low yields may be obtained in extractions by this aqueous phenol method. Superior yields of such products can be obtained by the mild and selective phenol-chloroform-petrol method of Galanos et al. (1969).

The method of Westphal and Jann (1965) was followed here, with some slight modifications as recommended by DePamphilis (1971). Cells from 500mls. of culture were harvested at 3000xg for 30 minutes, suspended in 20mls. of cold MBS and lysed by sonication as described above, 4i. Cell envelopes were then prepared as described (4i) and these were taken up in 50mls. of hot distilled water and added to 50mls. of hot 90%w/v aqueous phenol and incubated at 68^{o}C for 10 minutes. At this temperature the mixture was as one phase and was stirred occasionally. The mixture was then cooled on ice to 4^{o}C, whereupon two phases formed. The upper aqueous phase was removed and washed with a little diethyl ether to remove residual phenol and was then dialysed exhaustively against distilled water for 16 hours. The material was then collected by freeze-drying and stored at -70^{o}C. An outline scheme is shown in Figure 2.2.

Figure 2.2. Scheme for the separation of cell envelopes and further extraction of Lipopolysaccharide.



2.2. Analytical techniques.

1. Electron microscopy.

Transmission electron microscopy (TEM) was performed on the cell cultures and fractions. Both negative staining, using phosphotungstate, and heavy metal staining of thin sections were used. Contrast staining of acidic polysaccharides using Ruthenium Red was also utilised on the latter (Luft, 1971; Fletcher and Floodgate, 1972).

i. Negative staining (Mercer and Birbeck, 1972).

A drop of sample suspension was mixed with one drop of 2%w/v phosphotungstic acid (adjusted to pH 7.0 with NaOH). One drop of this mixture was placed on a Formvar-coated copper grid (TAAB Ltd.) and excess removed using a filter paper to give a thinner film. The residue was then allowed to dry and viewed directly.

ii. Thin sections.

Samples were pelleted wherever possible and were fixed in 2.8%v/v aqueous glutaraldehyde in 0.07M sodium cacodylate pH 6.5 (+0.5mg/ml. Ruthenium Red when used), for at least three hours at room temperature. The sample was pelleted and washed in cacodylate buffer. Postfixation was then carried out in 2%w/v osmium tetroxide in 0.07M cacodylate buffer pH6.5 (+0.5mg/ml. Ruthenium Red when used) for three hours at room temperature. The sample was pelleted and washed in cacodylate buffer.

An aliquot of the pellet was mixed, on a warm microscope slide, with 2%w/v agar solution and allowed to cool until this gelled. The agar blocks were dehydrated in methanol, (three changes each for 10 minutes) and then in propylene oxide, (two changes each for 10 minutes). They were then

placed in propylene oxide and 'Transmit EM resin' (TAAB) 1:1 for one hour (or stored overnight). They could then be placed in fresh 'Transmit' resin for at least four hours and embedded in fresh resin at 70^{o}C for 16-24 hours.
Thin sections of 20-60 nm were cut using an LKB ultra-microtome 4801A with thermal advance and glass knives. The sections were collected on acetone washed copper grids (EM scope). The sections were stained firstly with a fresh saturated solution of uranyl acetate in 75% ethanol in the dark for 30 minutes, washed in 75% ethanol and then in water. Secondly, in fresh Reynold's lead citrate (Reynold's, 1963) for 5 minutes (1.33g lead nitrate + 1.76g trisodium citrate in 30mls. water, shake for 20 minutes, 8mls 1N NaOH added and made to 50mls. with water. Used when clear. This stain may absorb CO_2 from the air, giving a precipitate of lead carbonate, and should therefore be used in a covered container with a few pellets of NaOH). The sections were rapidly washed in 0.02M NaOH and then in water. They were dried, viewed and photographed using an AEI 6B transmission electron microscope at 80kV.

iii.<u>Modifications for plasmolysis.</u>

For the electron microscopy of plasmolysed cells and that of osmotically sensitive spher oplasts the methods of Bayer (1968) were employed. Plasmolysis was achieved by suspending cells in 10mls. of Medium C + 40w/v sucrose at 30^{o}C for 2 minutes. This suspension was mixed with an equal volume of one part 12.5% glutaraldehyde in cacodylate buffer pH 6.8. one part medium C + 40% w/v sucrose.
Fixation was for two hours at room temperature. After pelleting, cells were resuspended in;

one part 0.6% OsO_4 in 0.2M cacodylate pH6.8

one part 40%w/v sucrose,

and postfixed for one hour at room temperature. This pellet was then treated as described above for thin sections, from the agar embedding stage.

iv. Uranyl acetate lipid enhancement.

An optional post-fixation/stain in 2% uranyl acetate was was used for two hours at 4^{o}C. This has been reported by Penn and Lichfield (1982) to produce enhancement of lipid material in membranes. Uranyl acetate is particularily effective for phospholipids.

v. Energy dispersive X-ray analysis.

Cell culture samples were spread thinly onto a carbon SEM plates (TAAB) and placed in an ISI-Super Mini scanning electron microscope. Energy dispersive X-ray analysis was performed using a Princeton Gamma Tech system 4 analyser.The spectra were photographed on 35mm film.

2. Polyacrylamide gel electrophoresis (PAGE).

A major breakthrough in the analysis of membrane proteins occured with the application of PAGE, particularly in detergent systems, to fractions of membrane proteins.

Polyacrylamide gels are generated by the free radical polymerisation products of acrylamide monomer (CH_2=CH-CO-NH_2) and the cross-linking co-monomer N,N'- methylene bisacrylamide (Bis, CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2). Variations in the concentrations of monomer and cross-linking agent lead to gels with a wide range of pore sizes. The range of pore sizes available may be adjusted to optimise the separation of any two components. Separating these depends on their electrophoretic mobility and molecular size.

The polymerisation reaction is initiated by a catalyst redox system which furnishes free radicals. The system used here utilises the tertiary amine N,N,N',N'-tetramethylethylene diamine (TEMED) as the catalyst and an initiator, ammonium persulphate, to generate oxygen free radicals.

Reproducibility in PAGE is critically dependant upon reproducibility in the intended polymerisation (Chrambach and Rodbard, 1971). Uniform complete polymerisation is especially dependant upon high purity monomer, co-monomer and catalyst. Variations in these may lead to drastic effects in the final resolution obtained. Impurities in the acrylamide (such as acrylic acid) can have undesirable adsorption properties which lead to abnormal and inconsistant electrophoretic behaviour. Low levels of contaminating metals can inhibit or even prevent polymerisation. It is thus advisable to use highly purified reagents or further

purify, eg. by recrystalisation of standard laboratory reagents.

Treatment of proteins with the anionic detergent sodium dodecyl sulphate (SDS) plus a reducing agent such as ß-mercaptoethanol, and its incorporation into the gel system will change their compact three-dimensional shape into rod-like structures (Reynolds and Tanford, 1970). Since the SDS molecules bind to polypeptides with a constant weight ratio (Reynolds and Tanford, 1970a) the charge per unit weight is constant and electrophoretic mobility becomes a function of molecular weight (Weber and Osborn, 1969). The technique of SDS-PAGE has been widely used to determine the molecular weights of unknown polypeptides by comparing their relative mobility (R_m) to standard polypeptides of known molecular weight (Swank et al. 1971).

The selection of the proper power during an electrophoretic run and its mode of operation depends upon a knowledge of the electrochemical properties of the electrophoresis system being used. Optimising the effects of a high potential gradient whilst minimising the effects of heat production results in the maximum obtainable resolution. Depending on the chemical makeup of the gel and anode and cathode buffers, the total resistance of the system may either increase or decrease during the course of a separation. It is often advantageous in continuous systems (the gel and tank buffer are the same) to run at a constant current, as in the tube gel system used here. However, in the slab gel system used here, a discontinuous buffer system is used and an increase in cell resistance takes place during

the run. Constant voltage is thus best used since an increase in resistance must be accompanied by a decrease in current to maintain the voltage at a constant level. This involves a decrease in total applied power while maintaining the applied voltage. Constant current operation in these circumstances would result in increased voltage (and hence damaging heat production) as the resistance increases.

The discontinuous slab system employed uses the basic principle of isotachophoresis, ie. all ions of a given sign move at the same velocity. Since at pH 6.8 glycine has a slow mobility and Cl^- a high mobility, proteins, because of their size and charge have mobilities between these. Thus in the 'stacking' gel the proteins sort themselves out between the two boundaries into zones of mobility as in isotachophoresis, and very narrow zones of protein enter the 'separating' gel. In this gel the separation is by molecular sieving, the glycinate zone can now move through the sieving proteins to form a boundary with the Cl^- ions. This boundary is seen to move down through the gel during the course of separation.

Furthermore the 'stacking' gel pH 6.8 helps to maintain the boundary between glycinate and the protein sample ions due to the low glycinate mobility. However as soon as the leading protein enters the 'separating' gel, the pH increases from 6.8 to 8.6 and this causes an enhanced mobility so that it moves rapidly away from the next stack zone. This leads to a selective destacking which results in an even more pronounced separation.

SDS/PAGE thus provides a rapid and accurate analysis of proteins from the various membrane fractions. Results are reproducible and easily comparable (especially within slab gels) and only small amounts of sample are required. There are however certain drawbacks in the interpretations which are fully discussed later.

Jann et al (1975) have also applied SDS/PAGE to the analysis of heterogenous polysaccharide chain lengths in bacterial LPS. This continuous system was utilised as a tube gel in this study.

i. Slab electrophoresis system.

Slab polyacrylamide gels were prepared from solutions shown in Appendix 1 and set in acid washed glass plates of dimensions 20cmx15cm using 1.5mm perspex spacers. Routine runs were on gels of 15% acrylamide, 0.6% Bis containing 0.37M Tris/Cl^- pH 8.6 + 0.13% SDS. Gels were polymerised overnight after which a 2-3cm 'stacking' gel of 5% acrylamide, 0.2% Bis containing 0.125M Tris/Cl^- pH 6.8 + 0.1% SDS was cast above the 'separating' gel. Sample slots were made within this gel using a perspex comb.

Samples, either freeze-dried or standards of known protein concentration were taken up in the following solutions;

Distilled water	500µl
0.5M Tris/Cl^- pH8.6 + 1mM EDTA	100µl
10% β-mercaptoethanol	100µl
20% SDS	100µl
0.001% bromophenol blue	100µl
glycerol	100µl

This was mixed well and heated at 100°C for 10 minutes, cooled and could be stored at 4°C.

Formed gels were clamped in position on the greased perspex tank. This was constructed within the Polytechnic using perspex sheets (see Appendix). The tank buffer (0.025M Tris / 0.192M glycine pH8.3 + 0.1% SDS) was placed in both reservoirs. Samples were applied to the gel using a micropipette. Up to 50µl of the prepared samples could be applied, the volume was varied according to the total amount of protein and the number of separate protein species present. The gel was then run in the constant voltage mode of a 'Vokam'(Shandon Southern) transformer, SAE 2761. 100v was used for the initial 'stacking' followed by 300v for the 'separating' gel. The run was stopped when the bromophenol blue tracking dye had reached the bottom of the gel, after a run of about 3-4 hours. During the run gels were cooled using a cold air fan over the plate.

ii. Tube electrophoresis system.

The system of Fairbanks (1971) was used for the separation of LPS, as reported by Jann et al (1975). Tube polyacrylamide gels were cast in 10cmx0.4cm (id) tubes. The gels were polymerised overnight and contained 5.8% acrylamide, 0.2% Bis with 10mM Tris pH 7.4, 20mM sodium acetate, 2mM EDTA and 1% SDS. To dry samples of extracted LPS were added the following solutions;

Distilled water	500µl
10 mM Tris/Cl^- pH8.0 + 1mM EDTA	100µl
20% SDS	100µl
10% ß-mercaptoethanol	100µl
0.001% bromophenol blue	100µl
glycerol	100µl

This was mixed well and heated at 100°C for 5 minutes, cooled and stored at 4°C if necessary.

Alternatively the LPS was dyed with Procion Blue H5R (R. Lamb). To a solution of 1.5mg LPS in 1ml water was added 1.5 mg dye in 1 ml water. After five minutes 3mg sodium chloride was added and after 30 minutes, 300µg sodium carbonate. After standing overnight the solution was added to a (2.5x 10cm) column of Sephadex G25 (Pharmacea) and eluted with distilled water. The dyed LPS was eluted with the void volume of the column. The product was then freeze-dried and treated for PAGE as above. It has been reported by Jann et al (1975) that this stained LPS can be directly observed during electrophoresis and requires no visualisation.

Gel tubes were placed in a Bio-Rad 150A electrophoresis cell with water cooling jacket and buffer placed in both reservoirs. This was 10mM Tris pH 7.4 with 20mM sodium acetate, 2mM EDTA and 1% SDS. Samples of up to 100µl were applied with a micropipette and electrophoresis carried out at 5mA/gel tube, constant current mode using a Vokam 400/100 (Shandon Southern) power pack until the tracking dye was just emerging from the gel. This was a run of about 1½ hours. Gels were then removed from tubes by 'rimming' with a long-needled syringe and stained.

iii. Staining of polyacrylamide gels.

PAGE BLUE 83, PROTEINS. PAGE Blue 83 (BDH, CI.42660) is a direct replacement for Coomassie Blue R250 (ICI), being about twice as sensitive and non-toxic. Gels were immersed in 0.05% PAGE Blue in 50% methanol, 10% acetic acid overnight. Several changes were necessary as all SDS must be removed before dye-binding can take place. Destaining was then effected in 7.5% acetic acid, 5% methanol until a clear gel

background was obtained. Protein positive bands appear bright blue.

SILVER STAIN, MACROMOLECULES. This is an extremely sensitive (x100 PAGE Blue) general stain. The procedure used here is based on that of Morrissey (1981). Gels were prefixed in 50% methanol, 10% acetic acid overnight and then soaked in water for at least two hours. They were then soaked in 5µg/ml dithiothreitol for 30 minutes, rinsed and immersed in 0.1% silver nitrate for 30 minutes. After thorough rinsing with water gels were placed in developer (50µl of 37% formaldehyde in 100ml. of 3% sodium carbonate) and then soaked in developer until the desired staining level was achieved. The reaction was stopped by adding 5ml. of 2.3M citric acid and shaking for 10 minutes. Finally gels were washed in water for 30 minutes. Gentle but thorough agitation is important during all these steps. Positive bands appear black/brown with tinges of other colours (blue and red) when freshly stained.

PERIODIC ACID/SCHIFF STAIN (PAS), CARBOHYDRATES. Gels were fixed as described previously and soaked to remove all SDS. They were then immersed in 0.5% periodic acid for two hours, 0.5% sodium arsenite in 5% acetic acid for 30 minutes and 0.1% sodium arsenite in 5% acetic acid for 20 minutes. After transfer to 5% acetic acid for 10-20 minutes, they were stained in Schiff's reagent (BDH) overnight. Carbohydrate bands appeared light pink.

Gels were sketched and photographed in order to record the separations achieved.

Densitometer scans were made of PAGE Blue stained gels (Cello 3 densitometer, Shandon Southern Products).

3. Protein Estimation.

The Lowry method (Lowry et al. 1951) is probably the most suitable method for the estimation of membrane proteins. Direct measurement at 280nm can be impaired by traces of insoluble material scattering light, and the Biuret method is rather insensitve. The major disadvantage of the Lowry method is that colour development varies between proteins and a calibration curve based on a standard protein such as bovine serum albumen (BSA) is not altogether satisfactory. As significant differences occur between fractions, especially with glycoproteins, ideally such a pure protein fraction should be calibrated against itself. Carbohydrate reduces the estimation coeffecient by a larger fraction than can be attributed to the dry weight of the carbohydrate compound itself. It should be noted that, as the original publication reported, the calibration curve is not fully linear. The method used here is modified by the addition of sodium deoxycholate (DOC) to facilitate the dissolution of the proteins (Maddy and Spooner, 1970).
Lipids and EDTA do not interfere with the colour development.

Reagents used;

i. Copper-alkali solution; 100mls.of 0.1N sodium hydroxide and 100mls. of 4% w/v anhydrous sodium carbonate were mixed and 2mls. of a 2%w/v sodium potassium tartrate added followed by 2mls. of a 1%w/v copper sulphate solution. The mixture was prepared fresh each day of use.

ii. Folin-Ciocalteau reagent; the commercial reagent was diluted 1:1 with water, ie, 1M with respect to acid.

iii. Protein sample; the protein or membrane suspension was

made 0.25M with respect to sodium hydroxide and 2%w/v with respect to DOC.

5mls. of the copper-alkali reagent were added to 1ml. of protein sample containing up to 400µg of protein, mixed, and allowed to stand at room temperature for 10 minutes. 0.5ml. of the diluted Folin reagent was added as rapidly as possible and mixed. Colour was then developed for 30 minutes and read against a reagent blank at 750nm. The colour is stable. BSA was used as a protein standard.

4. Carbohydrate Estimation.

i. Anthrone method; this is a general test for hexoses. They are dehydrated by strong sulphuric acid to give furfural and similar products, which react with anthrone to give a blue/green complex. The extinction depends on the compound used&is constant for a particular sugar.
4mls. of 0.2%w/v anthrone in concentrated H_2SO_4 (fresh) was added to 1ml. of carbohydrate sample and rapidly mixed. Tubes were covered and placed in a boiling water bath for 10 minutes. After cooling , the absorbance was read at 620nm. Glycogen (Sigma) was used as a standard sugar.
ii. Phenol/sulphuric acid method; the Anthrone method was replaced in later estimations by the more reliable phenol/ sulphuric acid method (Dubois et al. 1956). To 1ml. of carbohydrate solution was added 0.5ml. 5% aqueous phenol and 2.5ml. concentrated H_2SO_4. The tube was mixed well and boiled for 15 minutes covering tube tops with foil. The tube was then cooled rapidly and read at 490nm against a reagent

blank. Glycogen was again used as a standard. The phenol/ sulphuric acid method does not detect sugar alcohols or amino sugars.

5. Lipid assay and gas-liquid chromatography (GLC).

Ester bound carboxylic acids may be analysed qualitatively by GLC or quantitatively by the hydroxylamine procedure. The alkaline hydroxylamine procedure of Snyder and Stephens (1959) may be applied directly to some classes of lipids for the quantitative determination of ester groups. However, in the case of more complex lipids, eg. bacterial phospholipids and LPS, the liberation of carboxylic acids or their transesterification into methyl esters is necessary. This reaction may then be followed by quantitative determination of the methyl esters or by their GLC.

i. Acid methanolysis; Acid methanolysis of isolated cell envelopes, cell walls or LPS was carried out by the method of Minnikin et al (1975). To a dry sample was added; 2.5mls. methanol, 2.5mls. toluene and 0.1ml. concentrated sulphuric acid , this was left overnight at 50^{o}C. The methyl esters were then extracted into hexane (about 1.5mls.), and were then dried using a little anhydrous sodium sulphate if they were to be used for GLC.

ii. Alkaline hydroxylamine procedure; For the alkaline hydroxylamine reagent 2g hydroxylamine hydrochloride were dissolved in 2.5mls. water and made up to 50mls. with ethanol. This was termed solution A. 4g of sodium hydroxide dissolved

in 2.5mls. water and made up to 50mls. with ethanol were termed solution B. A and B were mixed, producing a white precipitate and after 10 minutes the mixture was filtered and the filtrate used fresh as the reagent.

For the ferric perchlorate reagent 5g non-yellow ferric perchlorate were added to 10mls. 70% perchloric acid and 10mls. water. A stock solution was produced by making this up to 100mls. with ethanol. The stock may be stored at $4^{o}C$. For use 4ml. stock was mixed with 70% perchloric acid made up to 100mls. with ethanol and used immediately.

Aliquots of methyl esters in hexane were taken and made up to 2mls. with hexane. 2mls. of the alkaline hydroxylamine reagent were added and this left at room temperature for 15 minutes. The tubes were then briefly immersed in a water bath at $67^{o}C$ to evaporate the hexane and then reimmersed for 15 minutes. Tubes were removed, cooled and 5mls. of ferric perchlorate reagent added. Colour developed after standing at room temperature for 30 minutes. The absorbance was then read at 520nm. Blanks of hexane were run, together with ester standards dissolved in hexane.

iii. Gas liquid chromatography; For the analysis of fatty acids in isolated cell envelopes, cell walls and LPS, the acid methanolysis procedure was utilised as described above. Samples were then taken up in a small volume of hexane and injected into a Perkin Elmer model F30 machine. Separation was on an OV225 stainless steel column of length 2 metres packed with 3% SP1000 on Chromosorb W (100-200 mesh).

A flame ionisation detector and chart recorder (Perkin Elmer

56) were attached. Both injector and detector temperature were set at 250°C. Nitrogen was the carrier gas at 90 pounds/ square inch pressure. The initial oven temperature was 170°C for 15 minutes and this was increased to 240°C at a rate of 10°C/minute. Separation was deemed complete after 30 minutes. Standard bacterial fatty acid methyl ester mixture (FAME, Supelco) was run using identical conditions to establish tentative identification , together with known straight chain FAME s.

6. Polar lipid extraction and thin layer chromatography (TLC).

The method of Bligh and Dyer (1959) as modified by Minnikin et al (1979) was used for this extraction. Up to 50mg. of dry cell envelopes or cell wall were placed in a universal bottle and 2.75mls. methanol added, this was stirred for two minutes. After cooling to 4°C 1.25 mls. chloroform and 0.75ml. 0.3% aqueous sodium chloride was added and the mixture stirred for two hours. The material was pelleted on a bench centrifuge and re-extracted with 2mls. of chloroform/ methanol/0.3% saline (50:100:40:) for 15 minutes. The material was again pelleted and both supernatant extracts bulked. 1.75mls. chloroform and 1.75mls. 0.3% saline were added, the mixture was shaken and centrifuged to separate the two phases. The lower chloroform layer contained the polar lipids. This was removed using a Pasteur pipette and evaporated to dryness under a stream of nitrogen.

If the lipids were to be stored for any length of time, a few mg. of BHT antioxidant was added to the extraction solvents.

For analysis by TLC extracts were resuspended in a small volume of chloroform/methanol (2:1) and applied to a silica

gel H plate presprayed with 5mM borate solution and redried. The plate was then developed in chloroform/methanol/water (65:25:4). To visualise the lipid classes plates were sprayed with the following reagents;

General lipid spray: 10%w/v solution of molybdophophoric acid in ethanol. Plates were sprayed until wet and heated at 150°C for 15 minutes. Lipids appear as blue spots on a yellow background.

Lipids with alpha amino groups: 0.2%w/v ninhydrin in water-saturated butan-1-ol. Plates were sprayed lightly and heated to 100°C for 5 minutes. Lipids which contain alpha amino groups appeared as red/violet spots on a white background.

Lipids containing sugar residues: 15%w/v alpha naphthol in 95% ethanol was prepared. To 10.5mls. of this solution was added 6.5mls. concentrated sulphuric acid, 4.5mls. ethanol and 4mls. water. The plates were sprayed lightly and heated at 120°C for 10 minutes. Sugar-containing lipids (glycolipids) gave purple/red spots on a pink background.

7. <u>Isoprenoid quinone extraction.</u>

The method of Collins and Jones (1981) was followed using dry cell envelopes or cell walls. Up to 10mg. of material was extracted with 10 mls. of chloroform/methanol (2:1) for two hours in a dark room with constant stirring. The suspension was then passed through a Whatman #1 filter paper and the filtrate evaporated to dryness under a stream of nitrogen. The extract was then taken up in a small volume of chloroform/methanol and applied to a silica gel G_{60} F_{254} TLC plate. This was developed in petrol/ether (85:15) and examined

under U/V light (254nm). Menaquinone bands were removed by scraping the plate and eluting with a little acetone, they were evaporated to dryness and re-dissolved in absolute ethanol. A U/V spectrum was recorded using a scanning spectrophotometer.

8. Lipopolysaccharide dye assay.

The hypochromic shift that occurs on the addition of LPS to a cationic carbocyanine dye forms the basis of a useful assay. Janda and Work (1971) reported a sensitive, specific colourimetric assay for LPS that was technically easy to perform. Furthermore , LPS detection was not limited to that in a purified state but worked well with culture filtrates. Detection and quantitation of LPS had usually involved extraction and purification followed by chemical analysis for key components or biological tests for endotoxic activity. Zey and Jackson (1973) further studied this assay describing the optimum assay conditions. The analysis is based on the ability of a cationic carbocyanine dye, 1-ethyl-2[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidine)-2-methylpropenyl] naphtho[1,2d]-thiazolium bromide (Eastman Kodak, 'LPS dye') to form aggregates which have different absorption maxima at different molar concentrations and in different solvents. Janda and Work (1971) showed that the dye alone had an absorption maximum at 510nm whereas in combination with LPS this shifted to a shorter wavelength between 468 and 478nm. Samples of LPS-dye mixtures were scanned against blanks of dye reagent to avoid excess dye masking any shift to the

shorter wavelength. New reagent blanks were also prepared for each sample due to the pronounced temperature effect on dye solutions.

Optimal conditions for the assay may vary with the source of LPS due to its inherent heterogeneity, reflecting differences in the structure of the LPS or a difference in the ability of each type of LPS to aggregate. In a dim room and using a foil-covered container the following solutions were mixed; 10mg. LPS dye, 10ml. 0.2M sodium acetate buffer pH 4.05, 10ml. 1,4-dioxane. After 30' mixing, 80mls. of buffer was added and this stock solution was stored at 4°C for up to eight hours before use.

To 25mls. of the above mixture, 0.5ml. 0.01M ascorbate was added and this mixture used within one hour. For the assay 1ml.of sample or water blank, 0.4ml. acetate buffer and 0.6ml. of the dye reagent were mixed in the given order. This was incubated at room temperature for 5 minutes and read at 460nm or scanned over this wavelength, against fresh reagent blanks. Up to 50µg of LPS may be used per assay.

9. <u>2-keto-3-deoxyoctonic acid (KDO) determination.</u>

The method used is highly sensitive and depends upon the oxidation of free KDO by periodate to yield β-formylpyruvic acid. This is condensed with thiobarbituric acid (TBA) to obtain a pink chromogen which can be estimated colourimetrically The original method is that of Wiessbach and Hurwitz (1959). Several other carbohydrates with similar structure of the first four carbon atoms will yield a pink colour with TBA; these include 2-deoxyribose and sialic acid.

KDO is liberated by very mild acid hydrolysis. 1-2mg. of

sample (LPS) was suspended in 0.7ml. of 0.018N sulphuric acid and hydrolysed at 100°C for 20 minutes. 0.75ml. of 0.25N periodic acid in 0.125N sulphuric acid was then added directly to the hydrolysate and incubated at room temperature for 30 minutes. 1.5ml. of 2%w/v sodium arsenite in 0.5N HCl was added whilst mixing, to terminate the oxidation. Iodine may be evolved at this stage. 3mls. 0.6%w/v TBA (adjusted to pH2) was then added with mixing, followed by heating at 100°C for 20 minutes. The sample was slowly (important) cooled to room temperature and the absorbance read at 548nm. The colour was stable at room temperature for at least 30 minutes.

This method gives sensitivity to the µg range. To permit assay down to the ng range a further extraction of the chromogen mey be performed by adding 1ml. of iso-amyl alcohol: 12N HCl (1:1) or acid butanol (butan-1-ol + 12%v/v 12N HCl) and shaking for 20 seconds. After a short centrifugation the upper, pink, organic phase can be removed and read in a micro-cuvette.

10. LPS sugar hydrolysis, paper chromatography and High Performance Liquid Chromatography (HPLC).

Strong hydrolysis; The yield from 3 or 4 separate extractions of LPS (5mg) or commercial S.typhimurium LPS was dissolved in 2mls. of 0.5N HCl and sealed in an ampoule. This was placed in an oven at 100°C for 4 hours.

Weak hydrolysis; was performed similarly but using 1%v/v acetic acid at 100°C for 90 minutes.

The ampoules were then chilled on ice, opened and the contents

sample (LPS) was suspended in 0.7ml. of 0.018N sulphuric acid and hydrolysed at 100°C for 20 minutes. 0.75ml. of 0.25N periodic acid in 0.125N sulphuric acid was then added directly to the hydrolysate and incubated at room temperature for 30 minutes. 1.5ml. of 2%w/v sodium arsenite in 0.5N HCl was added whilst mixing, to terminate the oxidation. Iodine may be evolved at this stage. 3mls. 0.6%w/v TBA (adjusted to pH2) was then added with mixing, followed by heating at 100°C for 20 minutes. The sample was slowly (important) cooled to room temperature and the absorbance read at 548nm. The colour was stable at room temperature for at least 30 minutes.

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Weak hydrolysis; was performed similarly but using 1%v/v acetic acid at 100°C for 90 minutes.

The ampoules were then chilled on ice, opened and the contents

centrifuged at 3000xg, 15 minutes to pellet the Lipid A portion of the hydrolysate. Released sugar could be monitored at this point by the phenol/sulphuric acid method (2.2.4). Hydrolysis was also followed by SDS/PAGE silver staining of both fractions (2.2.2). The sugars contained in the supernatant were subjected to both paper chromatography and HPLC.

Paper chromatography was performed as described by Plummer (1978). Small aliquots were spotted onto Whatman filter paper and developed using isopropanol/water (4:1) overnight. Spots were visualised by spraying with aniline/diphenylamine reagent (1% aniline, 1% diphenylamine in acetone + 10ml. 85% phosphoric acid) for sugars and heating to 100°C until various coloured spots appeared.

HPLC; For HPLC hydrolysed sugar samples were passed through a SEP-PAK C_{18} cartridge (Waters). The cartridge was first activated with 2mls. methanol followed by 4mls. water. Sugars were eluted immediatly from the cartridge leaving any remaining lipid and/or hydrophobic contaminants on the cartridge. Sugar samples were then freeze-dried and taken up in a small aliquot of the column mobile phase.
A Waters HPLC system was utilised for the separation, incorporating a UK6 injector, 6000A solvent delivery system, 401 refractive index detector, an Enica 10 (Delsi) integrator/ printer for run monitoring, and a chart recorder (JJ) for continuous (baseline) monitoring. The column was a Waters µBondapak 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 between 1.5 and 2.0 mls/minute. Acetonitrile was Fisons HPLC grade and double distilled water was further purified before use by passage through a Millipore 'Norganic' (trace organic removal) cartridge and filter membrane. All solvents were degassed before use by nitrogen bubbling or evacuation. A general scheme for HPLC sample preparation is given in Figure 2.3.

11. Assay of iron (II).

The spectrophotometric estimation of iron (II) as described by Carter (1971) was used. This makes use of the colour complex formed by reduced iron with the complexing agent 'Ferrozine' (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid) -1,2,4-triazine. Sigma.). The reagents used were;

i. 10% ammonium acetate buffer

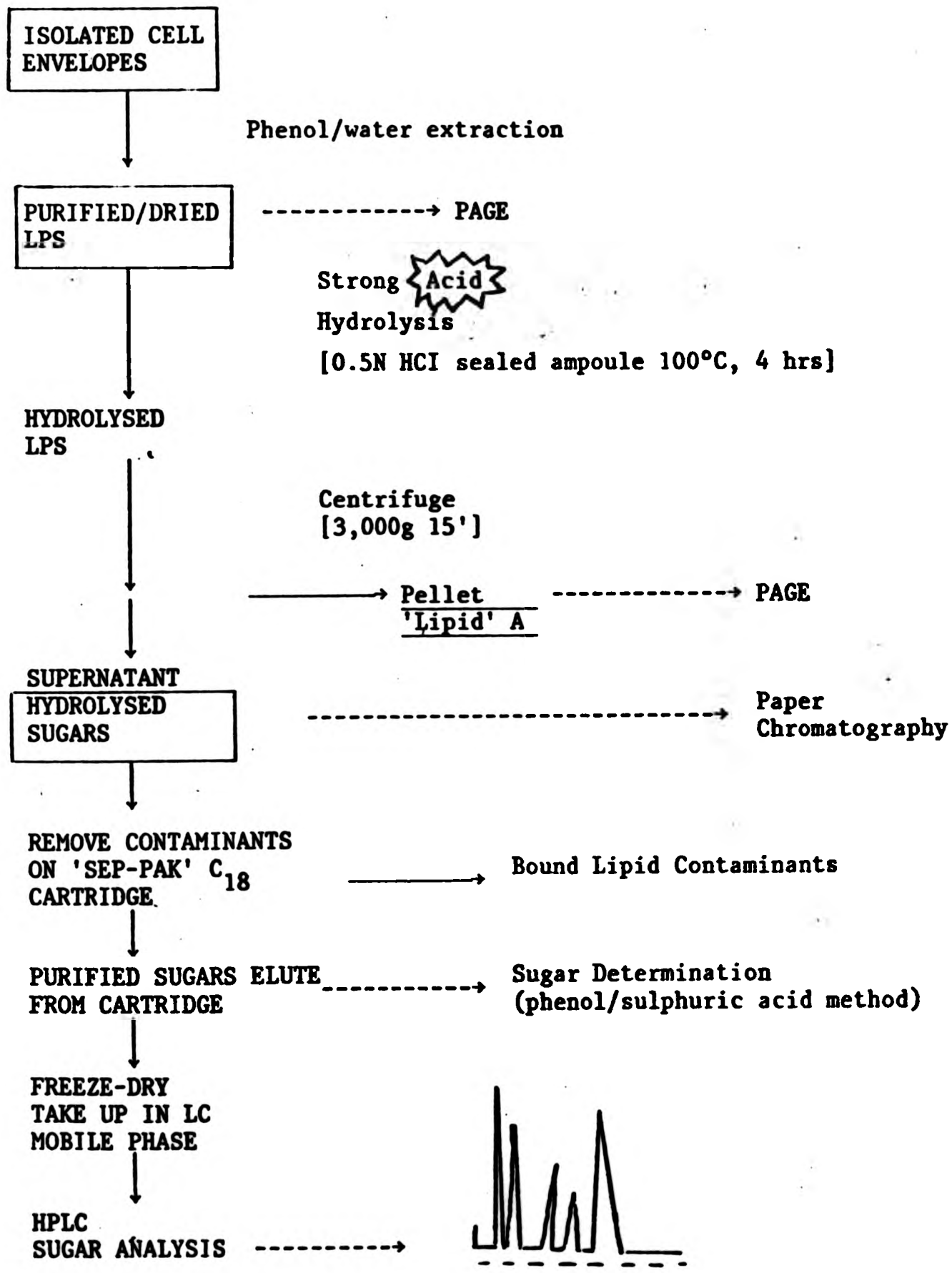
ii. 0.02% ascorbate in 0.2N HCl.

iii. Colour reagent- 75mg. Ferrozine in 25mls. water + one drop of concentrated HCl.

iv. Standard iron- transfer 50mg. $FeSO_4.7H_2O$ to a one litre flask, add 0.5ml. concentrated sulphuric acid and dilute to volume. This stock was diluted 1:100 for the working standard (1µg/ml.).

To 1ml. of sample 1ml. of reducing agent was added and the mixture allowed to stand for 5 minutes. 0.1ml. colour reagent and 0.8ml. of buffer were added, mixed and the absorbance read at 562nm after 5 minutes. In cases of high protein content of the sample it was necessary to remove this protein to avoid a precipitate in the final solution. This was done

Figure 2.3. General scheme for the preparation of LPS sugar hydrolysate for HPLC.



using 1ml. 10% TCA precipitation and centrifugation prior to addition of buffer, using 2mls. of clear supernatant and appropriate correction factors. This method is extremely sensitive for iron (II), measuring ng amounts.
In this investigation the reactions were carried out directly in new, disposable, 3ml. plastic cuvettes thus avoiding the preparation of iron-free glassware.

2.3. Structural and functional analysis.

2.3.1. Whole cell studies.

1. EDTA LPS release.

The cultures of D.vulgaris Woolwich in Medium C, C-Fe and C+Fe were as previously described. Bacteria were harvested in late exponential phase by centrifugation (3000xg, 30minutes) washed in PBS and resuspended in PBS to a density of approximately 10^{10} cells/ml. This suspension (2mls.) was added to tubes containing 0.5ml. 10mM ascorbate, the required volume of 200mM metal cation salt solution and distilled water to a final volume of 4mls. Final concentrations of cations varied between 0.01mM and 17mM. Distilled water replaced cations in control treatments. Salts used were; $FeSO_4.7H_2O$, $CaCl_2.6H_2O$, $MgSO_4.7H_2O$ and $ZnSO_4.7H_2O$. Tubes were incubated at 0°C for 15 minutes. Cells were then pelleted and resuspended in 2mls. PBS containing 10mM EDTA. After incubation at 45°C for 15 minutes the suspension was centrifuged and the supernatant was assayed for protein by the Lowry method (2.2.3.) and LPS by the carbocyanine dye method (2.2.8.). LPS released was calibrated against standards of LPS extracted from D.vulgaris cultures by the

phenol/water method (2.1.6). The change in LPS release was calculated by subtracting the control values from those obtained after cation treatment. SDS-PAGE was also performed as previously described (2.2.2).

2. ^{55}Fe whole cell labelling.

Cultures of D.vulgaris Woolwich (C-Fe) were harvested by centrifugation, washed once in MBS and repelleted as previously described. This pellet was taken up in 3mls. MBS and 0.5ml. 100mM ascorbate. 150µl of ^{55}Fe (127.5µCi, 32.1mg Fe, Amersham International) was added. This suspension was incubated for either;

i. 10 minutes on ice,

ii. one hour at room temperature,

iii. two hours at room temperature.

The suspension was then spun on a bench centrifuge and the resultant pellet washed three times to remove unbound isotope. The final pellet was analysed by SDS-PAGE and gels were dried onto filter paper using a vacuum pump and slight warming together with a porous support. To minimise cracking of the 15% polyacrylamide gels these were cut into individual lanes before drying. Autoradiographs were exposed at room temperature using Kodak X-Omat Rapid X-ray film, for at least 14 days before development.

3. ^{125}I/lactoperoxidase labelling of surface proteins.

Cultures of D.vulgaris Woolwich were grown in Medium C-Fe and C+Fe as previously described. Cells were harvested by centrifugation, washed once in MBS and spun. The pellet was

taken up in 10mls. MBS. To 1ml. of this suspension at room temperature was added 0.5ml. lactoperoxidase (10IU*/ml. fresh in MBS, Sigma 85 IU/mg.) and 1µl. of $Na^{125}I$ (100mCi/ml. Amersham International). Four 100µl aliquots of 10mM H_2O_2 at 1½ minute intervals were added and the iodination stopped after 10 minutes by the addition of 20mM cysteine. The cells were then pelleted on a bench centrifuge and washed four times in 0.3M MOPS buffer pH 7.2 before resuspension in 0.5ml. distilled water. These cells were then analysed by SDS-PAGE and autoradiographs prepared of the separations as described above (2.3.1.2).

2.3.2. Cell fraction studies.

1. Protection, digestion and OMP extraction of cell walls.

The preparation of Sarkosyl cell walls from D.vulgaris (C-Fe) Woolwich was performed as described under preparative techniques (2.1.2.). Cell wall which was termed 'protected' was prepared in the same manner except for the addition of 100µg/ml. Fe(II) and 100µg/ml. ascorbate and an incubation of 10 minutes on ice prior to sonication. Incubations of up to two hours both pre- and post-sonication were also investigated, as was the inclusion of a short burst of H_2S gas prior to Sarkosylisation.

Further solubilisations of C-Fe cell walls were achieved using 2% SDS at 30°C for 15 minutes and at 60°C for 30

* 1 IU lactoperoxidase is the amount that will form 1mg. purpurogallin from pyrogallol in 20 seconds at 20°C, pH6.0.

minutes. Protein digestion was performed on cell walls using protease (Sigma crude pancreatic type 1) 100µg/ml. at 30°C for 15 minutes at pH7.2.

In all the above experiments residual material was pelleted by high speed centrifugation and subjected to SDS-PAGE analysis.

The removal of OMP's from Sarkosyl-prepared cell walls was attempted using acetate extraction (Nixdorff et al. 1977). Samples of C-Fe cell wall or pre-digested cell walls were extracted with 50% acetic acid for 30 minutes on ice. The suspension was centrifuged at 95000xg for 30 minutes and the supernatant material dialysed overnight against distilled water to remove acetic acid. The material could then be concentrated and analysed for protein, SDS-PAGE or utilised in further experiments. The residual pellet was also analysed.

2. Hydrogenase studies.

Cells of D.vulgaris Woolwich and Hildenborough were grown to late exponential phase in medium C+Fe and C-Fe, as previously described. The cells were harvested, washed and fractionated into;

i. Crude cell lysate (sonicate with whole cells removed).

ii. Cell envelope (particulate fraction).

iii. Cytoplasm (soluble fraction).

iv. Sarkosyl-prepared cell wall (OM + peptidoglycan).

v. Sarkosyl-solubilised CM.

Fractionations were performed as described under preparative techniques (2.1.2.).

Early work on the mechanism of hydrogenase action was performed by Peck et al (1955, 1956) using various manometric techniques

and suitable electron acceptors and donors. The enzyme may be assayed by either the evolution or the consumption of hydrogen gas provided a suitable substrate and conditions are given. Recent assay procedures include an 'enzymic electric cell' method (Yagi et al. 1973) and an isotope exchange and conversion method (Yagi et al. 1973). The classic manometric assay was utilised in this study, using the more convenient evolution of hydrogen as a measure of enzymic activity. The method of van der Western et al (1978) was followed with some modifications.

The reaction was carried out in a Warburg apparatus at 30°C under nitrogen. Tris/Cl^- buffer pH7.5 was used and hydrogen evolution from 90mM dithionite with 6mM methyl viologen as electron carrier was measured. 0.1mg. BSA was added to minimise adsorption of the enzyme to the glass during dilution. The center well contained 0.15ml. 20%w/v KOH. The final volume of the reaction mixture was 3mls. The reaction was started, after preincubation of the flasks to a steady reading, by tipping the enzyme from the side-arm. Determinations were performed in duplicate, and readings were taken at about 2½ minute intervals. Readings were corrected using a thermobarometer run concurrently. Warburg flasks were calibrated by the calculation and use of a flask constant (KH_2) using the 'ferricyanide-hydrazine' method (Umbreit et al. 1972). Protein was estimated by the Lowry method (2.2.3). One unit (U) of hydrogenase activity was defined as the amount to catalyse the production of 1µmol. H_2/minute. Results were calculated and expressed as U/mg protein.

3. Reconstituted vesicle studies.

A. Vesicle formation.

0.5mg of lipid was placed in a series of bijou bottles and dried down to a thin film using a stream of nitrogen gas. Up to 18 bottles were used per experiment. The bottles could be sealed and stored at -20°C for several days in this form. The lipid used was either the product of a polar lipid extraction on D.vulgaris C+Fe cell envelopes (2.2.6) ie. a natural lipid extract, or a synthetic mixture of 0.45mg. phophatidylethanolamine and 0.05mg. diphosphatidylglycerol (both Sigma). Double distilled water, D.vulgaris LPS or commercial LPS (up tp 500µg) and/or various protein extracts (up to 150µg) were then added to a final volume of 1.6ml. The lipid was hydrated by a brief vortex mix and vesicles were formed by two brief 30 second bursts from a Soniprep ultrasonic disintegrator at low amplitude (2µm) using a microtip probe. These procedures were all performed at 4°C.

B. ^{55}Fe binding.

0.4ml. of 100mM ascorbate in 300mM MOPS buffer pH 7.2 was added to the formed vesicles. In blocking experiments 20mM calcium was also included here. 5µl of ^{55}Fe ($5x10^5$ cpm, 127µg Fe, 1.1mM; Amersham International) was added, the bottles mixed and left at room temperature for 30 minutes. The separation of the vesicles from unbound ^{55}Fe was then attempted by several methods;

i. Aliquots were removed to polypropylene microfuge tubes and spun in a Beckman microfuge for 10 minutes (9000xg). 500µl of the unbound ^{55}Fe supernatant was then removed for analysis. The samples were mixed with 10mls. aqueous liquid scintillation cocktail (Beckman Ready-Solv E.P.) and counted

in a Beckman LS 7500 liquid scintillation counter. Bound ^{55}Fe was calculated by subtracting these results from parallel water/isotope controls.

ii. 1ml. of Chelex 100 cation chelating resin (Biorad, 1g/10ml. presoaked in MOPS buffer pH7.2) was added to the incubation bottles and, after standing at room temperature for 15 minutes, 500µl of the clear upper solution was removed for liquid scintillation counting. This enabled direct readings of bound ^{55}Fe to be made. Water blanks were also run.

iii. Aliquots of the vesicle/^{55}Fe mixture were filtered through a Milipore (0.45µm) filter. The filter was allowed to dry, placed in a vial of scintillation cocktail and counted after standing for at least 30 minutes. This method also enabled direct readings of ^{55}Fe binding to be taken. Water blanks were also run.

C. <u>Fe(II) release.</u>

Vesicles were also formed in the presence of 100mM $FeSO_4$ to trap Fe(II) within the formed vesicles. Non-entrapped Fe(II) was removed by the addition of a few beads of the cation exchange resin Chelex 100 previously equilibrated with buffer. Aliquots of the Fe(II) loaded vesicles were then added to the 'Ferrozine' assay system for Fe(II) (2.2.11.) and changes in the absorbance at 562nm due to the formation of the Fe(II)-ferrozine complex in the extra-vesicular solution were monitored.

RESULTS.

CHAPTER 3

CULTURE, GROWTH CHARACTERISTICS & MORPHOLOGY OF D.VULGARIS IN IRON RESTRICTED (C-Fe) & IRON RICH (C+Fe) MEDIA.

RESULTS

Because of their strict anaerobic habit, relatively slow rate of growth and rather capricious nature, the culture of Desulfovibrio is not a simple task. However reproducible batch cultures which could be harvested at late log phase after a period of about four days were obtained by growth in 500mls. of medium C+Fe. The cell count reached a maximum of approximately $5x10^8$ cells/ml. Occasionaly, for no apparent reason, the growth was observed to follow a linear course maturing at the same time. These curves are shown in Figure 3.1. Cells harvested from this medium were contaminated with large amounts of ferrous sulphide precipitate; however this was acceptable and probably represents the condition of cells found around exposed iron surfaces in nature. Most cells were seen to be very motile when examined under the light microscope.

A similar growth was observed in medium C when a slightly larger inoculum was made. Less ferrous sulphide was produced, the culture having a dark grey turbidity rather than black. Growth in medium C-Fe was erratic, even with a higher inoculum, and often did not occur at all. An example is shown in Figure 3.2.

It was noted that the previous experience of the cells influenced the growth observed in subsequent cultures (Figure 3.3). Bacteria which had once managed to grow in medium C-Fe had no difficulty on reinoculation. Thus once established in medium C-Fe the cells were easily grown using the usual precautions against contamination. Medium C-Fe grown cultures were a pale buff colour and tended to be less smelly than iron grown cultures. Cells were still very motile. The

Figure 3.1. Sample growth curves for D.vulgaris in 500mls. batch culture medium C+Fe, showing exponential and linear growth.

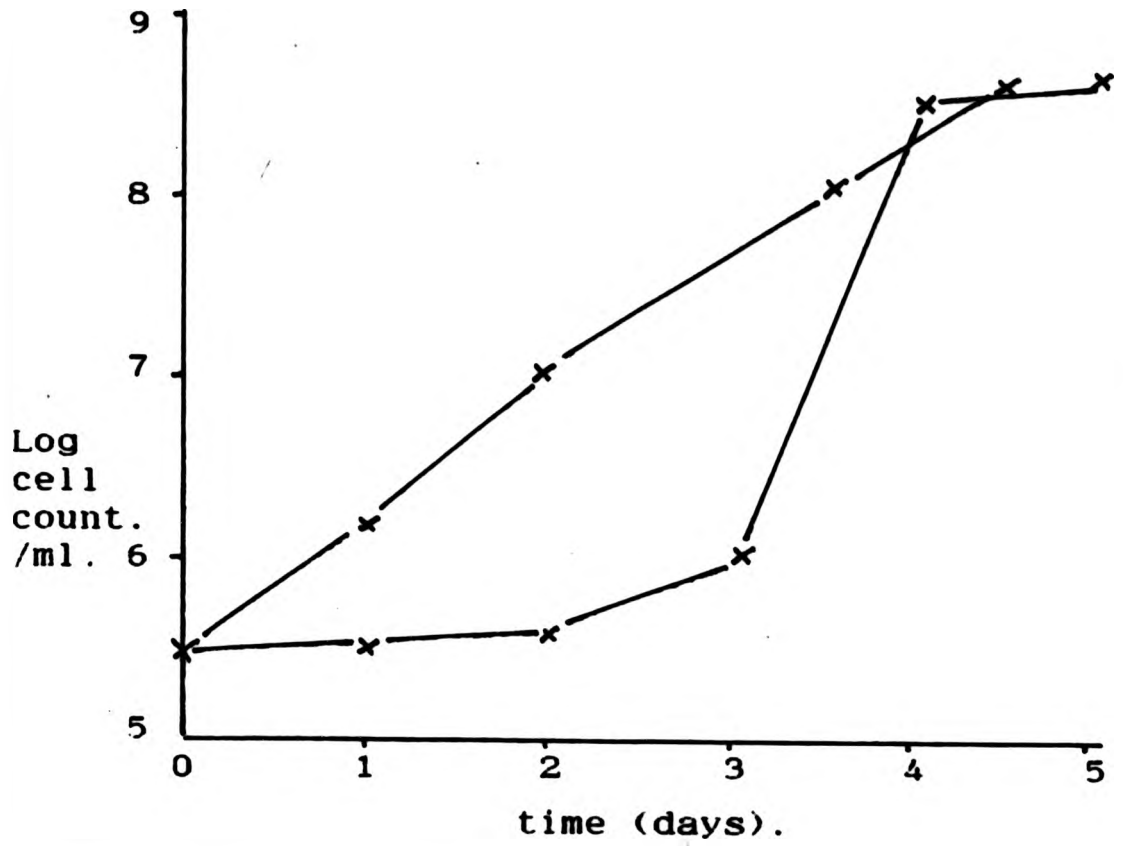


Figure 3.2. Sample growth curves for D.vulgaris in 500mls. batch culture medium C (×) and C-Fe (⊙), demonstrating occasionally observed failure of growth in medium C-Fe.

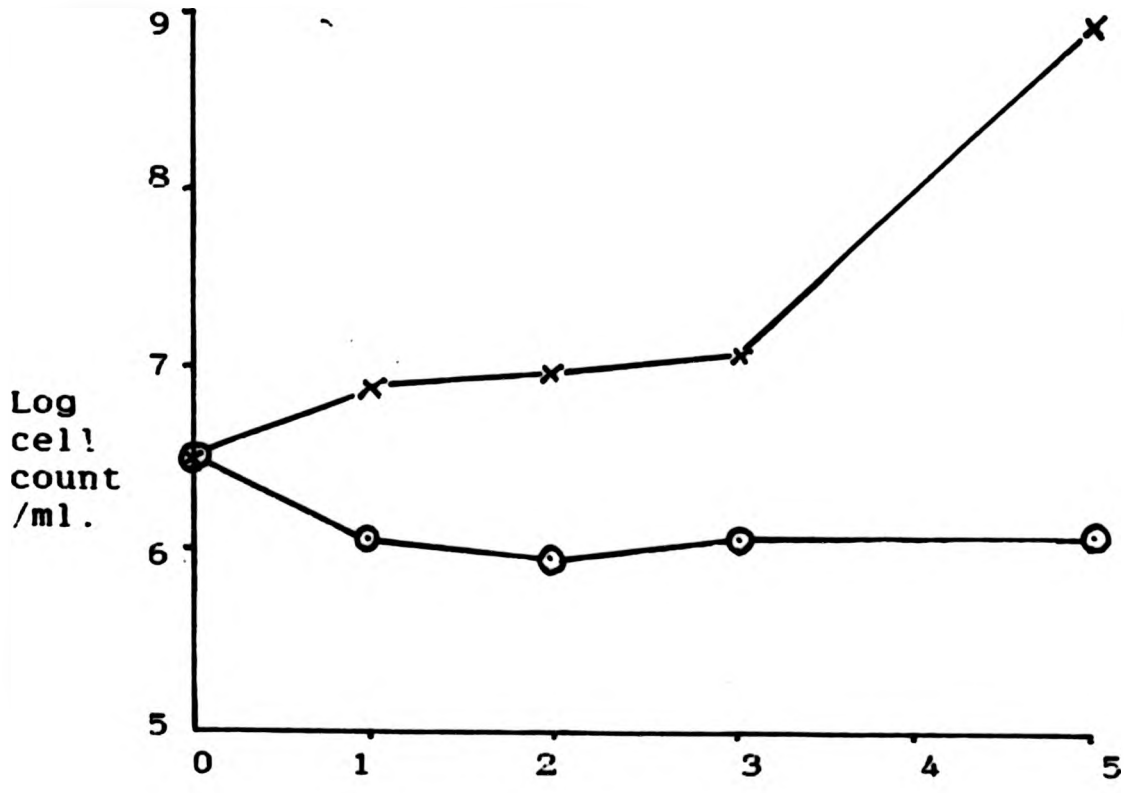
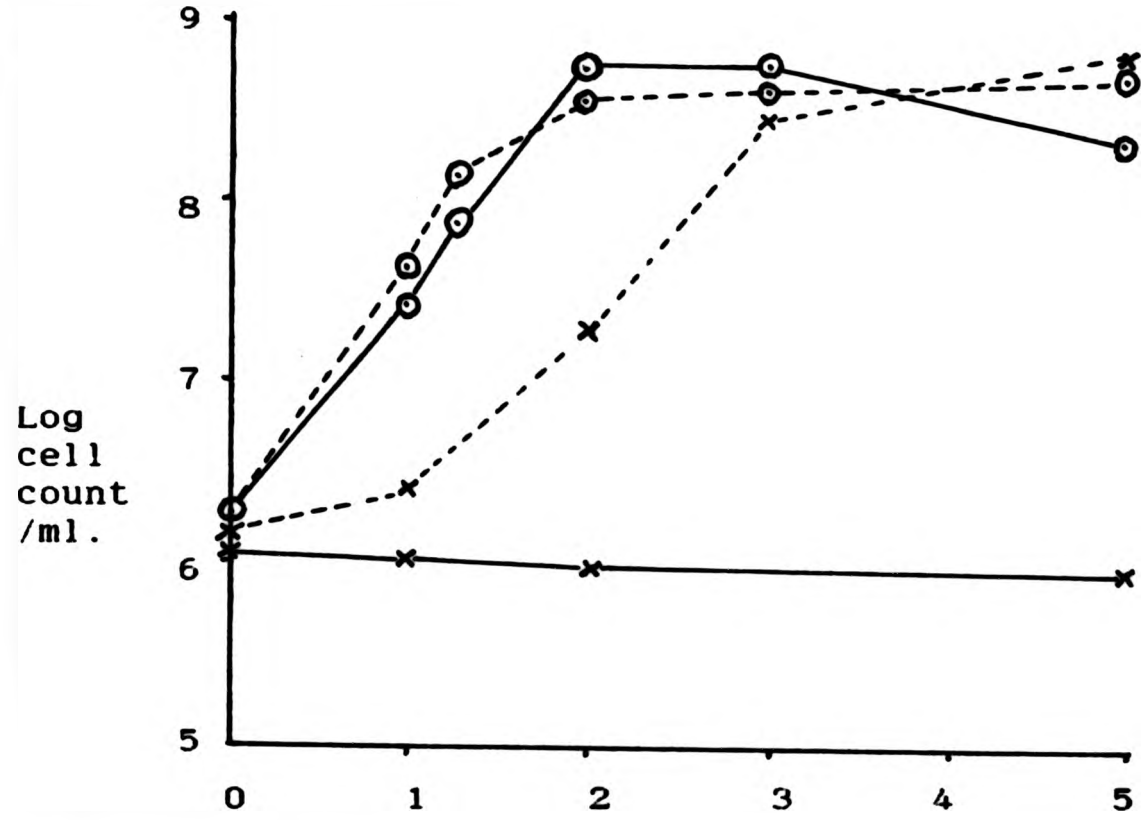


Figure 3.3. Sample growth curves for <u>D.vulgaris</u> in 500mls. batch culture medium C+Fe (⟵⟶) and C-Fe (⟵⟶) when inoculated from C+Fe (×) or C-Fe (⊙) cultures.



various media were assayed for iron (II) using the Ferrozine method and the results are shown in Figure 3.4.

Cells examined in the electron microscope after negative staining (Plates 3.1 & 3.2) showed no discernable surface structures other than the single, polar flagellum and, in many cases, adsorbed ferrous sulphide particles. The surface itself appeared as a rather fuzzy area showing little contrast. When grown in medium C-Fe cells were seen to be of a more pronounced vibrioid shape (Plate 3.3) having a smoother surface and less bound, dark particulate matter than their C+Fe counterparts. Some dark particles of ferrous sulphide were still visible however. A comparison of the two surfaces is shown in Plates 3.4 & 3.5.

Thin sections of intact cells showed a typical Gram negative cell envelope (Plates 3.6 & 3.7), consisting of cytoplasmic membrane, a variable periplasmic space and an outer membrane with a fuzzy peptidoglycan layer. No unusual features were seen and no internal membrane systems were discernable.

The sections of C-Fe grown cells showed them to be of a more pleiomorphic character, many containing electron transparant areas (Plates 3.8 & 3.9). The cell envelope of these cells was less defined than that of C+Fe grown cells, the OM being rather wavy, no gross differences were seen however.

Post-fixation of cells with 2% uranyl acetate to enhance the lipid material revealed stained areas on the outer surface layers (Plate 3.10).

Ruthenium Red contrast staining of intact cells (Plate 3.11) for polysaccharide material revealed a considerable amount of densely staining material surrounding some cells.

Figure 3.4. Iron present in the growth media.

Medium	Iron concentration. ng/ml.	µM
C+Fe including steel plate after.....		
1 hour	1.8×10^3	31.3
3 days	19.8×10^3	353.0
C+Fe no steel plate.	1.0×10^3	18.1
Medium C.	963 (890)*	17.2 (15.9)*
Medium C-Fe.	81	1.45

Estimated by the Ferrozine method (mean values of three estimations).

* calculated value from amount added.

Plate 3.1. <u>D.vulgaris</u> Woolwich C+Fe grown intact cell. Negative stain showing adsorbed FeS colloidal material. x60000 (bar=0.1µm).

Plate 3.1. D.vulgaris Woolwich C+Fe grown intact cell. Negative stain showing adsorbed FeS colloidal material. x60000 (bar=0.1µm).

Plate 3.2. <u>D.vulgaris</u> Woolwich C+Fe grown intact cell. Negative stain showing close-up of adsorbed FeS particle. x80000 (bar=0.1µm).

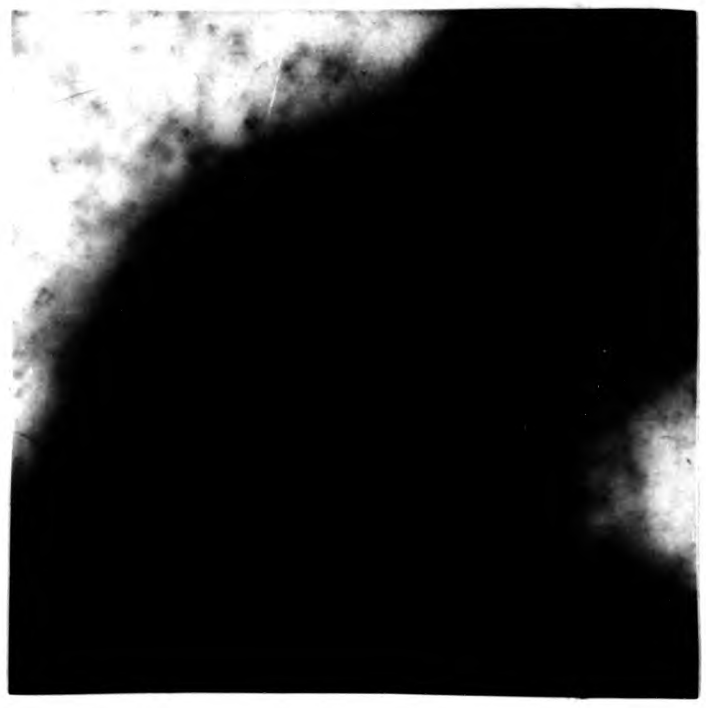


Plate 3.3. <u>D.vulgaris</u> Woolwich C-Fe grown intact cells. Negative stain. x40000 (bar=0.1µm).

Plate 3.2. D.vulgaris Woolwich C+Fe grown intact cell. Negative stain showing close-up of adsorbed FeS particle. x80000 (bar=0.1µm).

Plate 3.3. D.vulgaris Woolwich C-Fe grown intact cells. Negative stain. x40000 (bar=0.1µm).

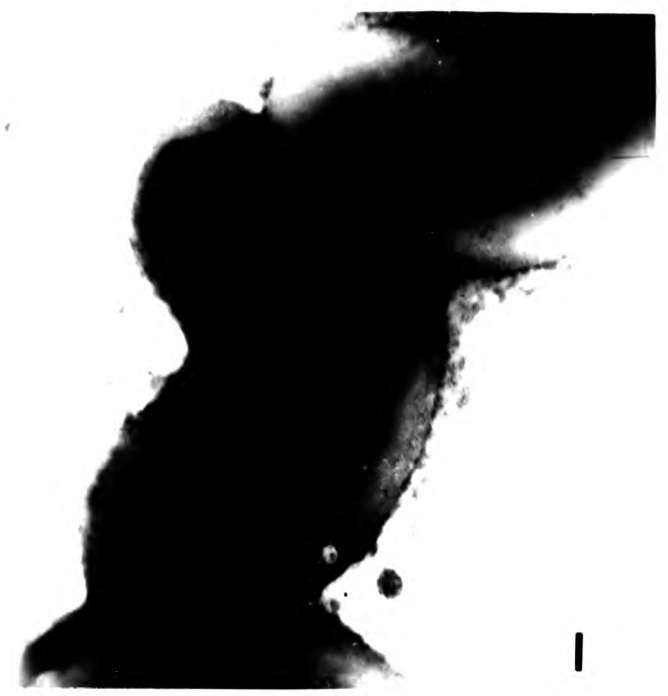


Plate 3.4. Portion of D.vulgaris Woolwich C+Fe grown cell surface. Negative stain x120000 (bar=0.1µm).

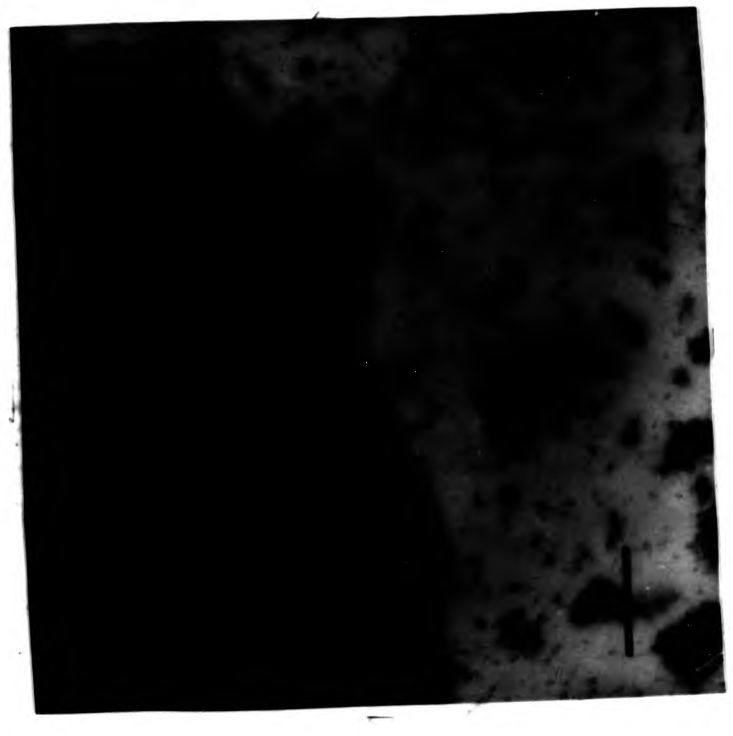


Plate 3.5. Portion of D.vulgaris Woolwich C-Fe grown cell surface. Negative stain x120000 (bar=0.1µm).

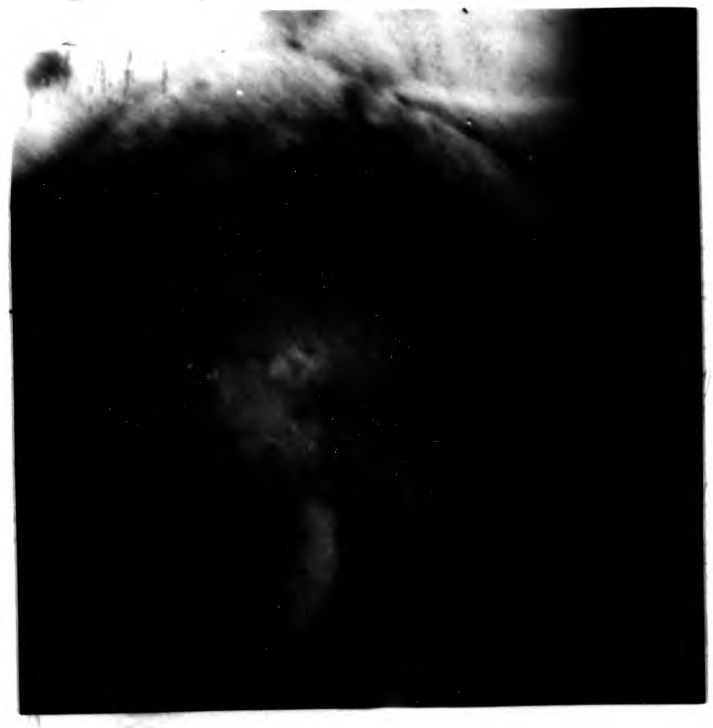


Plate 3.4. Portion of D.vulgaris Woolwich C+Fe grown cell surface. Negative stain x120000 (bar=0.1µm).

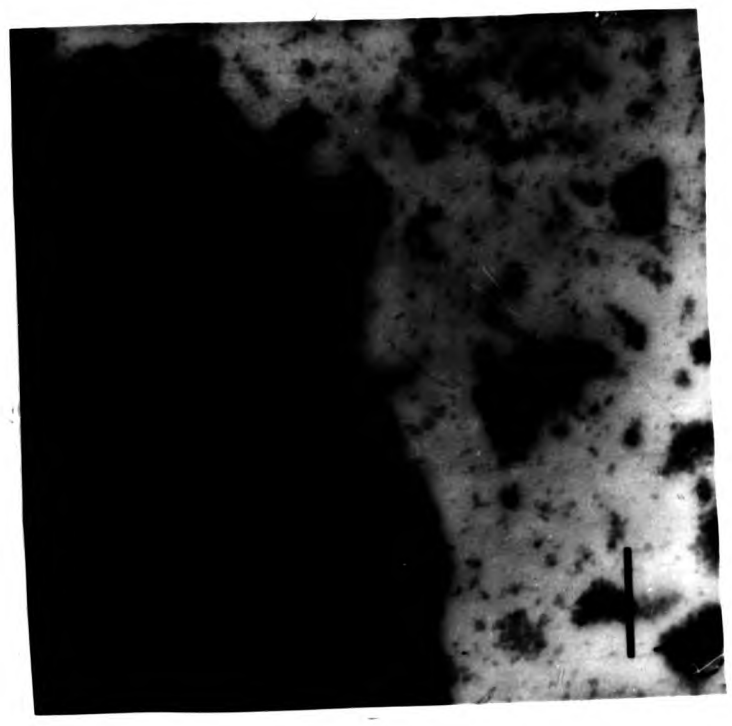


Plate 3.5. Portion of D.vulgaris Woolwich C-Fe grown cell surface. Negative stain x120000 (bar=0.1µm).

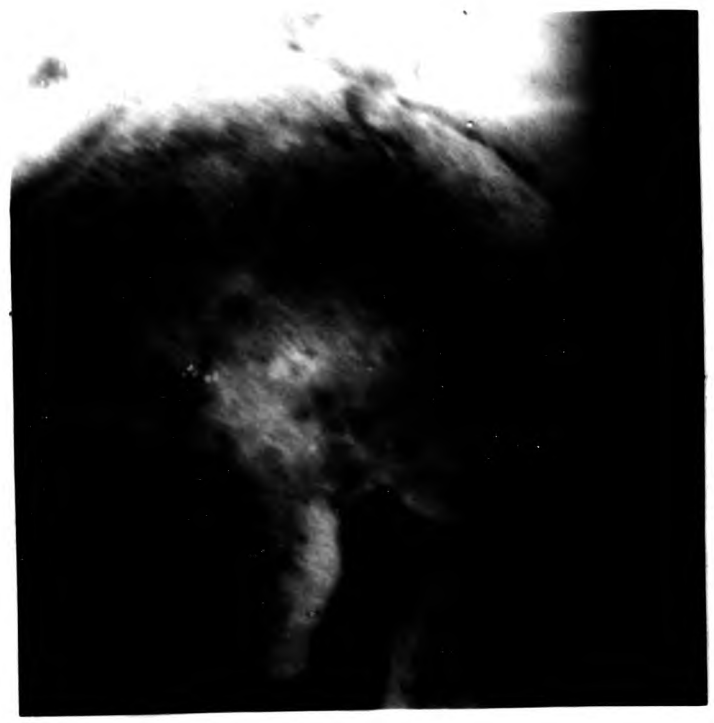


Plate 3.6. D.vulgaris Woolwich C+Fe grown intact cell.
Thin section through cell envelope.
x400000 (bar=25nm).

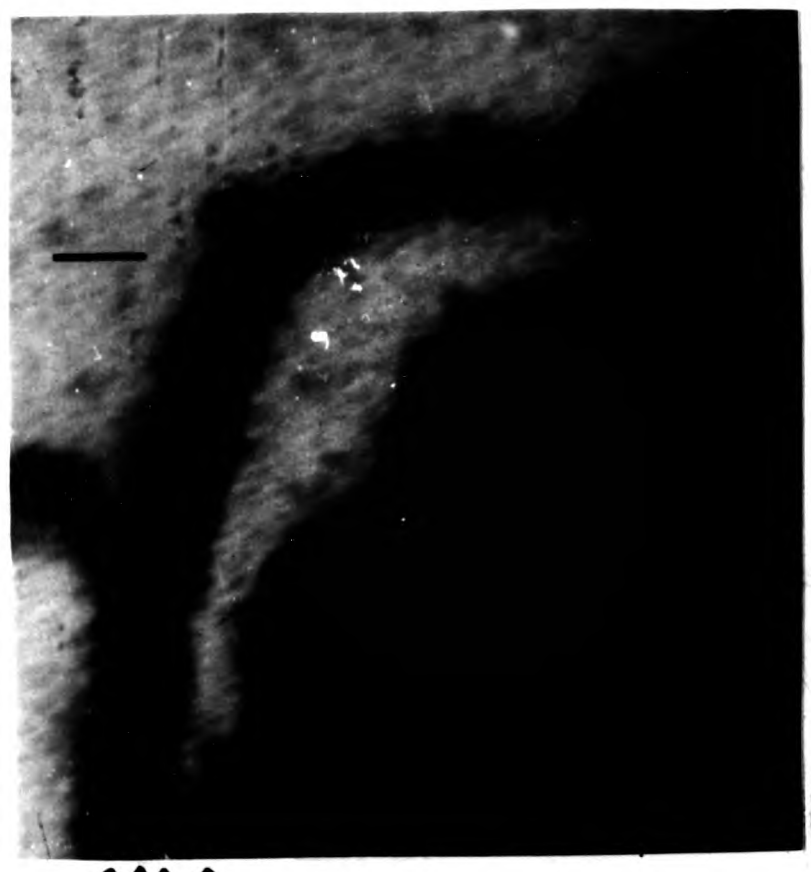


Plate 3.6. D.vulgaris Woolwich C+Fe grown intact cell.
Thin section through cell envelope.
x400000 (bar=25nm).

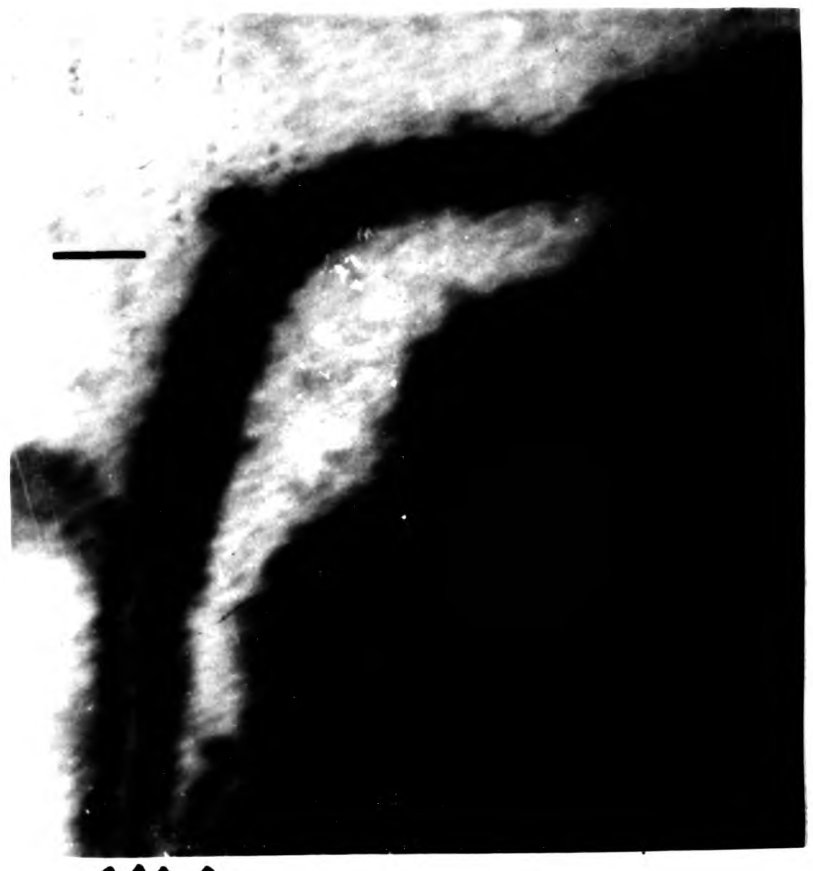


Plate 3.7. D.vulgaris
Woolwich C+Fe grown
intact cell. Thin
section X40000 (bar=0.1µm).

Plate 3.8. D.vulgaris
Woolwich C-Fe grown intact
cell. Thin section.
x40000 (bar=0.1µm).

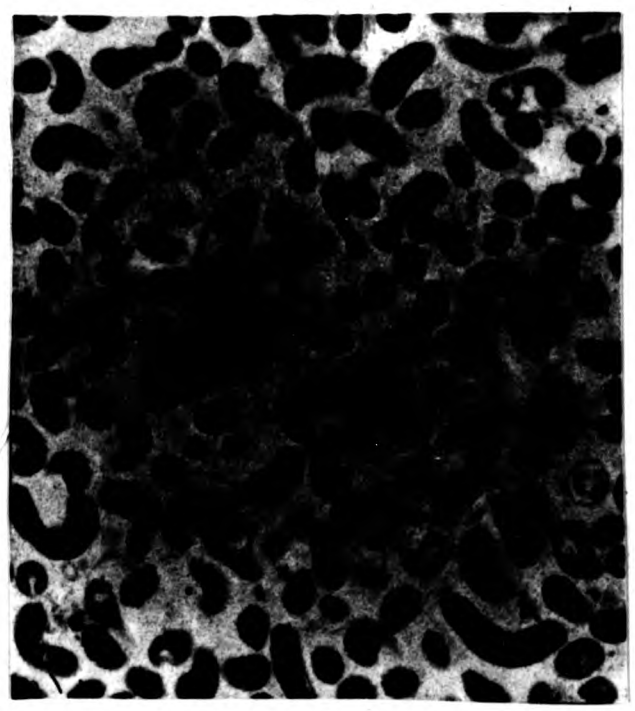


Plate 3.9. D.vulgaris
Woolwich C-Fe grown intact
cells. Thin section.
x6000 (bar=0.5µm).

Plate 3.7. D.vulgaris Woolwich C+Fe grown intact cell. Thin section X40000 (bar=0.1µm).

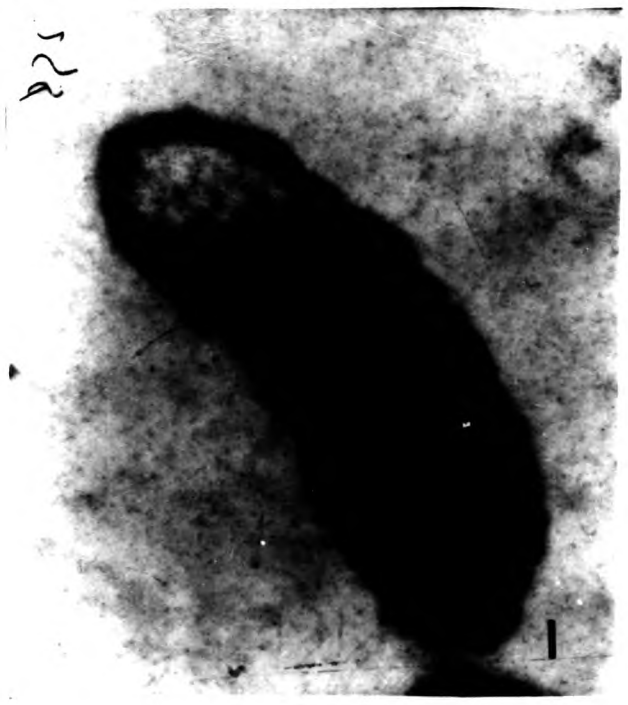


Plate 3.8. D.vulgaris Woolwich C-Fe grown intact cell. Thin section. X40000 (bar=0.1µm).

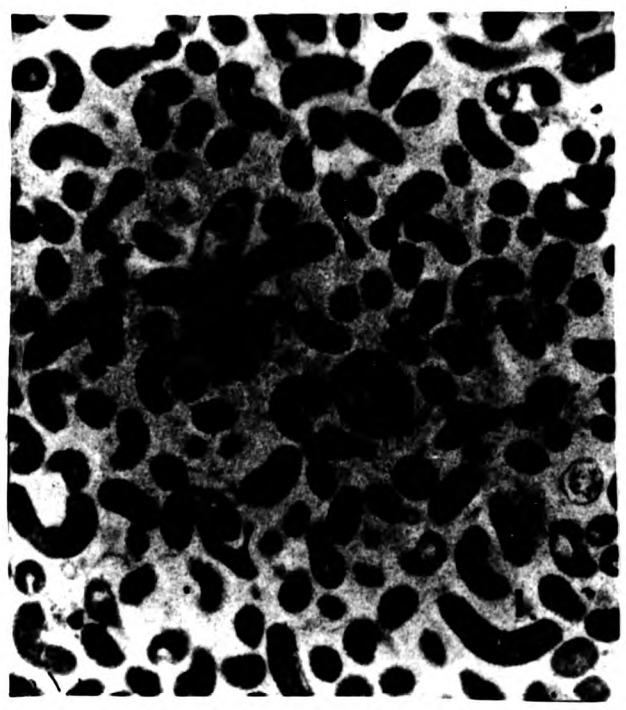


Plate 3.9. D.vulgaris Woolwich C-Fe grown intact cells. Thin section. X6000 (bar=0.5µm).

Plate 3.10. D.vulgaris Woolwich C+Fe intact cells.
Thin section contrast stained to show lipid enhancement in cell wall (arrowed). x50000 (bar=0.1µm).

Plate 3.11. D.vulgaris Woolwich C+Fe intact cells.
Thin section contrast stained with Ruthenium Red to enhance polysaccharide material. x50000 (bar=0.1µm).

Plate 3.10. D.vulgaris Woolwich C+Fe intact cells.
Thin section contrast stained to show lipid enhancement in cell wall (arrowed). x50000 (bar=0.1µm).

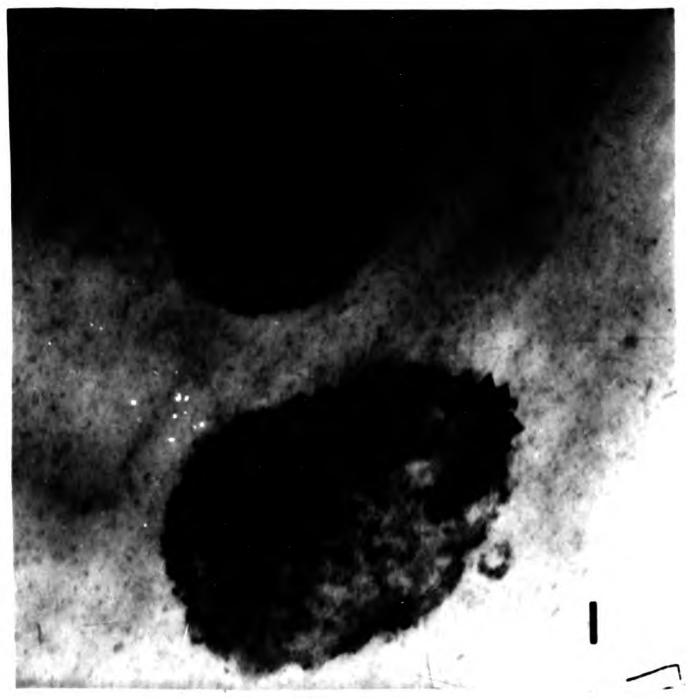
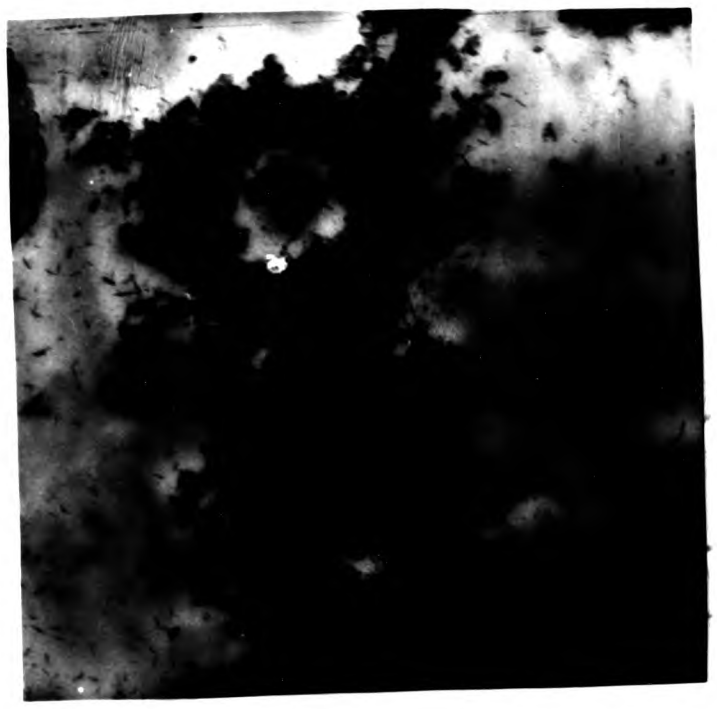


Plate 3.11. D.vulgaris Woolwich C+Fe intact cells.
Thin section contrast stained with Ruthenium Red to enhance polysaccharide material. x50000 (bar=0.1µm).



However, although seen in the majority this was not present in all cells observed.

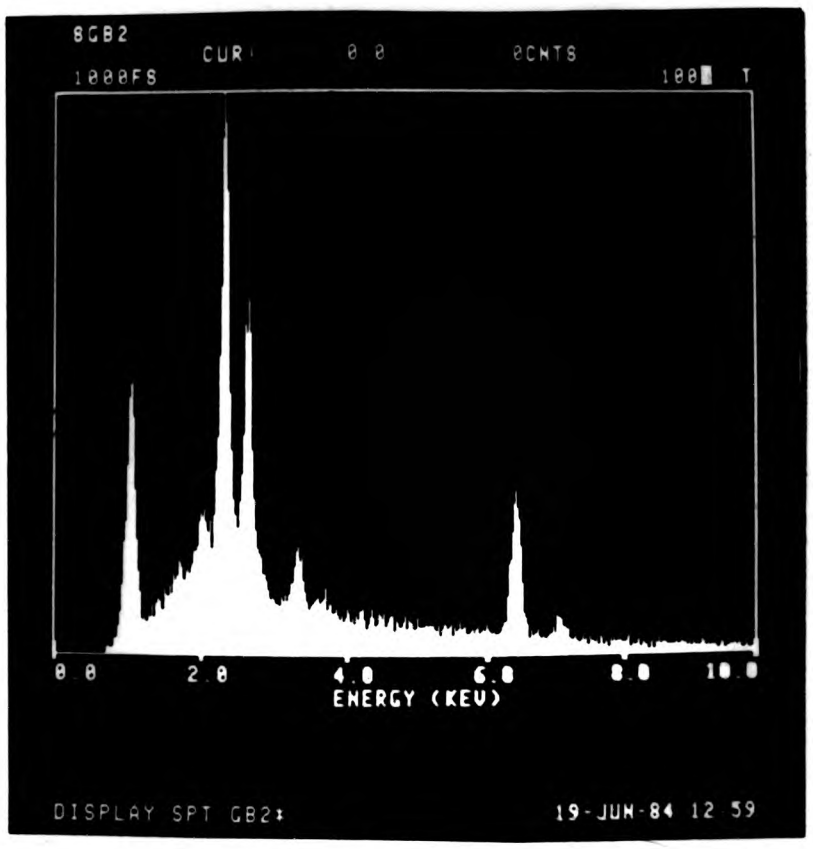
The cells grown in medium C+Fe, including a large proportion of the black precipitate from this medium, when dried and examined by X-ray dispersive analysis gave the spectra shown in Plate 3.12 a-d. Iron and sulphur are seen to be two of the major components, together with large amounts pf sodium and chlorine. Both potassium and phosphorus are present in smaller amounts. This type of chemical analysis does not detect elements below fluorine in the periodic table and the bulk of the organic material is therefore ignored (C, H, O, N).

DISCUSSION

The successful culture of D.vulgaris Woolwich in iron restricted C-Fe medium represents a significant step in providing cellular material from organisms under iron stress for further experimental work and for direct comparison with cells under optimum iron availability. Neilands (1984) suggests that iron levels in growth media of 10μM and 0.1μM may be regarded as 'high' and 'low' respectively, for aerobes or facultative anaerobes. The adventitious contamination of minimal media provides iron at a concentration of about micromolar, which is borderline for the expression of the twin elements of the siderophore system in these organisms. Indeed the ion-exchange resin scrubbed 'iron-free' medium used by M^{C}Intosh and Earhart (1977) still contained 22.4ng/ml. (0.4μM) iron. However Postgate (1984) states that sulphate

Plate 3.12. X-ray energy dispersive analysis of D.vulgaris Woolwich C+Fe grown cells including black precipitate from this medium.

a) Energy spectrum.



b) Identification of all peaks.

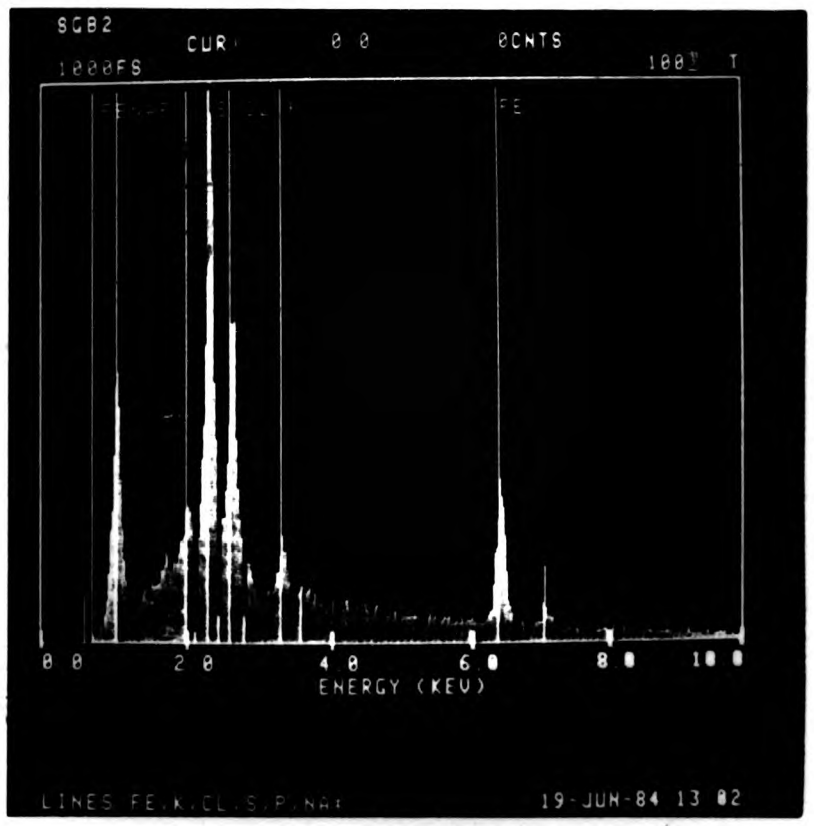
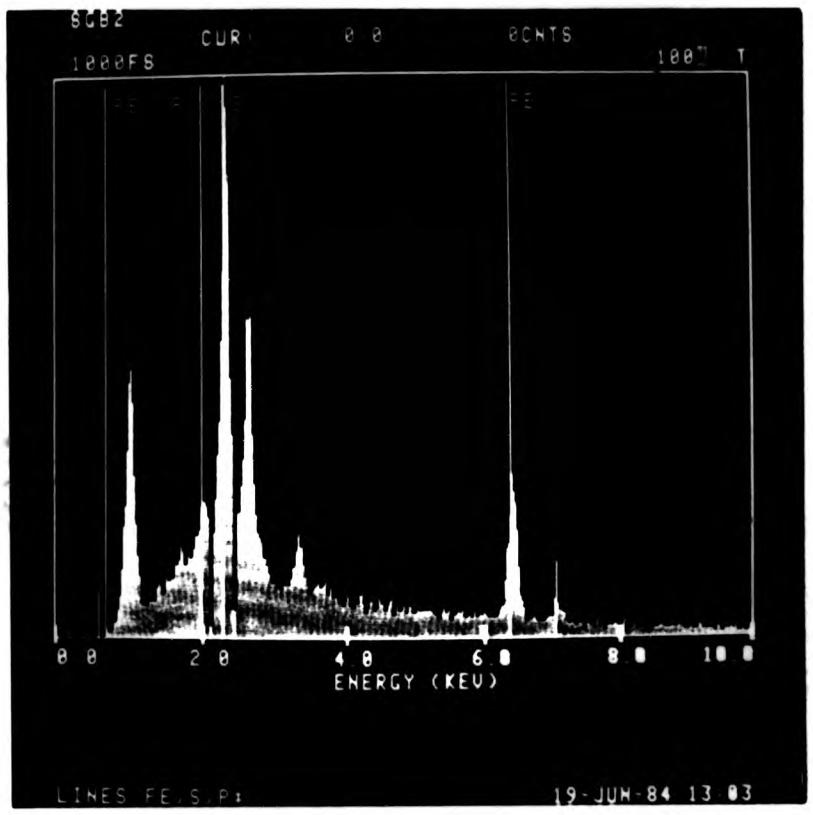
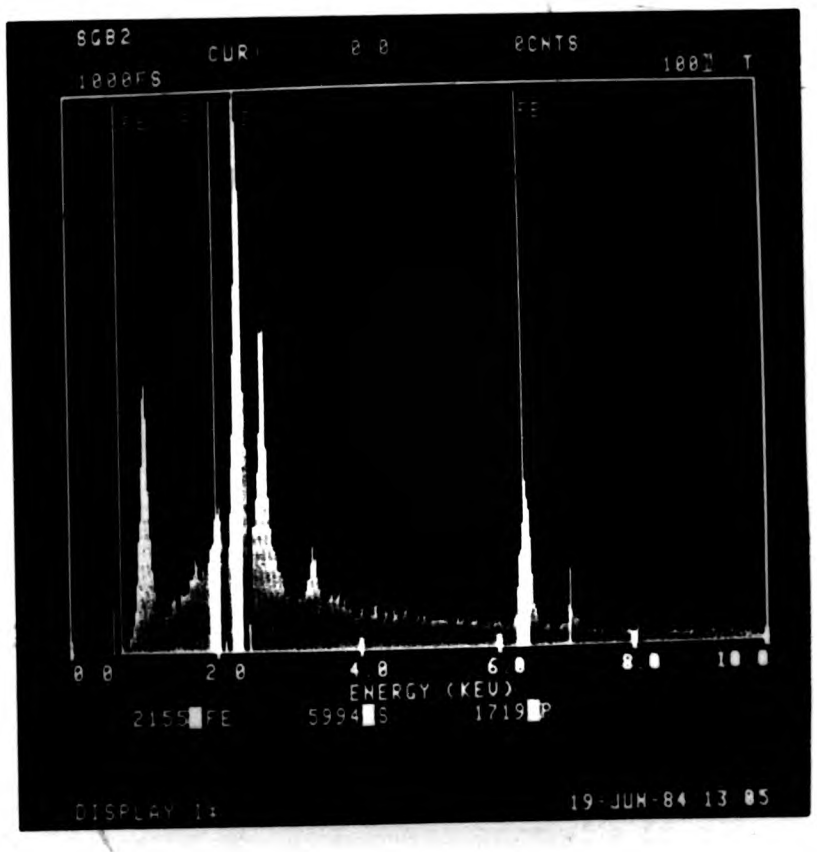


Plate 3.12. continued.

c) Chosen elements selected.



d) Integrated peak intensities for chosen element windows.



reducing bacteria in general have a high requirement for iron and his studies (1956) demonstrated a high requirement of at least 10μM iron for the optimal growth of D.desulfuricans in batch culture. The arbitrary 'high' and 'low' iron levels for Desulfovibrio can be regarded as ten-fold higher than those of other bacteria; 100μM and 1.0μM respectively, 10μM being a borderline level. The iron levels in the media used in these present studies confirm these figures. The iron limited C-Fe medium, producing iron-stressed cells, had a 'low' level of 1.45μM iron. Medium C provides a level of around borderline of 17.2μM and the iron rich medium provides 'high' levels of iron at at least 353μM with non-limiting iron availability from the mild steel plate. The need for slightly larger inoculations into medium C and especially into medium C-Fe probably reflects the need for a low redox potential (2.1.1) before growth can commence, as no redox poising agent was included in the media the lowering of the initial E_h relies on the transfer of sufficient hydrogen sulphide from the stock culture. The linear growth curve observed in some C+Fe cultures has been attributed to the supposed unavailability of iron as it precipitates as the sulphide (Postgate, 1984). The inclusion of citrate in medium C is meant to aid this iron availability. However the logic in this action is based on a knowledge of iron transport in D.vulgaris which does not exist, and which is one of the main objectives of these investigations.

The 'training' of organisms for growth in medium C-Fe can be explained by the intracellular regulation of various factors

in response to iron limitation (eg. extra-cellular iron chelators, iron transport proteins, or the synthesis of alternative enzyme pathways and redox pigments) and is more appropriately discussed later in the light of the results from further investigations.

The electron microscopy of intact cells was,on the whole, rather disappointing, a 'clasical' Gram negative cell envelope being revealed with no apparant, previously unseen, structures or appendages. Contrast staining for lipid enhancement and polysaccharide material showed some possible locations of these macromolecules on the outer surface, but this was far from conclusive (especially from a structural and functional viewpoint). C-Fe cells were clearly seen to be under stress from their variable morphology and the inclusion of electron transparent areas, which may well be polyhydroxybutyrate storage granules. These are known to be produced under certain stress conditions (Dawes and Senior, 1973).
Negative staining to show the cell surface proved to be even more unyielding showing a fuzzy indistinct surface even around bound ferrous sulphide particles. These dark particles were still present in C-Fe grown cells although in less abundance and the surface of the cells was generally smoother and cleaner. This however gave no clue as to the nature of the association of iron with the bacterial surface. Examination of these dark particles by the X-ray energy dispersive analysis on the abundant ferrous sulphide precipitate produced in C+Fe grown cultures indicated, as well as iron and sulphur, substantial amounts of sodium and chlorine and smaller amounts of potassium and phosphorus. These are not unexpected

because of their presence in all growth media; however the presence of phosphorus should not pass unnoticed since it has a potential involvement in the processes of iron corrosion as outlined in Chapter 1.

These initial studies have therefore succeeded in determining the growth characteristics of D.vulgaris in iron rich (C+Fe) and iron restricted (C-Fe) media and have proved useful in confirmimg the general morphology of these cells.

CHAPTER 4

WHOLE CELL STUDIES.

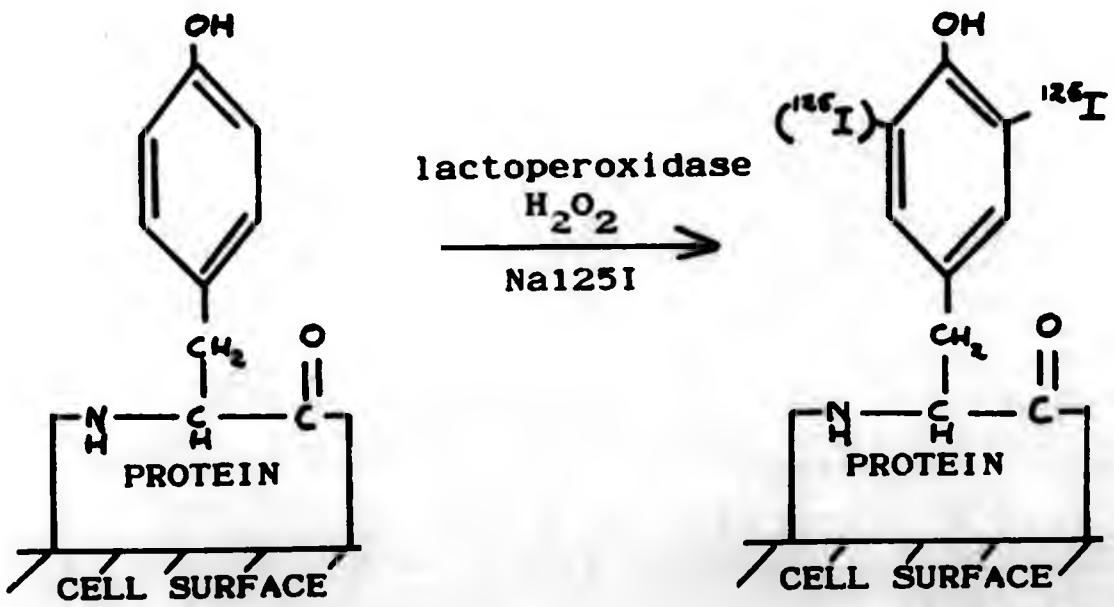
The studies on whole cells are intended to demonstrate the molecular species which are exposed on the surface of the bacterium by release or binding studies, without prior disruption of the organism.

It is well established that the LPS located in the external leaflet of the OM can interact with various metal cations (Galanos et al, 1977) This is important for in vivo membrane assembly and for the barrier function of the OM (Leive, 1974). Leive et al (1968) have demonstrated that the metal cation chelator EDTA releases LPS and protein material from the surface of some Gram negative bacteria. The divalent cation availability in the growth medium, together with the affinity of the LPS for these cations, determine in part the amount of LPS held in place in the OM by ionic binding. This, in turn, influences the proportion of LPS that can be released by EDTA treatment of whole cells. LPS of enteric bacteria contain a number of potential cation binding sites (Schindler and Osborn, 1979) having a high affinity for calcium and magnesium ions. In these present studies the material released from whole D.vulgaris cells by EDTA treatment was examined and comparison made between the effects of preincubation with different divalent metal cations on the release of LPS by EDTA treatment using cultures grown in the media of varying iron availability. It was hoped that such studies might indicate the degree of interaction between LPS and various metal cations in vivo.

The exposure of the OM proteins on the cell surface of D.vulgaris C-Fe and C+Fe grown cells was investigated by treating whole cells with 125I/lactoperoxidase. Membrane

proteins are asymmetrically distributed across the bilayer and for some membrane proteins this can be investigated directly because they have endogenous markers, eg. a haem binding region or a glycosylated region. For proteins with no marker, reagents which label the protein but do not traverse the bilayer have been developed. The radio-iodination of tyrosine and histidine residues using lactoperoxidase and hydrogen peroxide to generate the necessary oxidising conditions (Morrison, 1974) has been applied to a number of Gram negative bacteria (Lambert and Booth, 1982). Lactoperoxidase is too large to penetrate the OM of intact cells (M.Wt. 77500) and is therefore assumed to label only those proteins which have such residues exposed on the surface of the cell (Figure 4.1). In addition to this protein marker, radioactive ^{55}Fe (II) was also used as a tracer in an effort to determine directly surface material concerned with iron binding.

Figure 4.1. Schematic representation of surface protein labelling by 125I/lactoperoxidase.



RESULTS

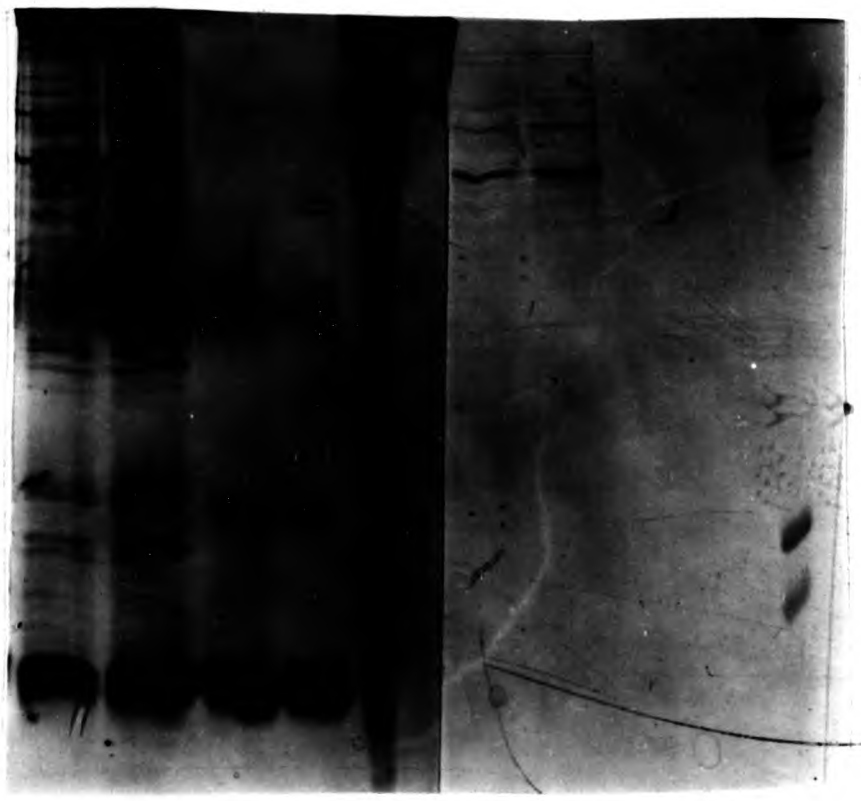
4.1. EDTA washes and release studies.

The washing of cells by the EDTA method removed between 3% and 8% of the total estimated cell protein. When examined by SDS-PAGE (Plate 4.1) the 'EDTA' washings showed one predominant polypeptide band of apparent M.Wt. 54000 and several minor bands. A plot of log M.Wt against relative mobility is shown in Figure 4.2. Staining by the silver method revealed further bands comparable to those bands obtained with LPS extracted by the phenol/water method.

Examination of EDTA washed cells by electron microscopy revealed certain differences from intact cells (Plates 4.2 and 4.3). Negative staining showed clearly the release of cellular material. The cell had a much more defined outline. The surface itself took on a more granular appearance although no regular arrays noted by Slytr (1978) or striations/ring structures reported by Every et al (1983) for certain other Gram negative organisms, were visible. Some cells did show 'bulges' in the cell wall (Plate 4.4), presumably indicating a weakening of the cell wall structure prior to eventual lysis if contact with EDTA was prolonged. Thin sections revealed no distinct differences, the complete cell envelope still being visible. Ruthenium Red stained polysaccharide material did however seem somewhat more diffuse after EDTA treatment, (Plate 4.3).

The preincubation of cells in ferrous salt solutions caused a marked increase in the EDTA-induced release of LPS above

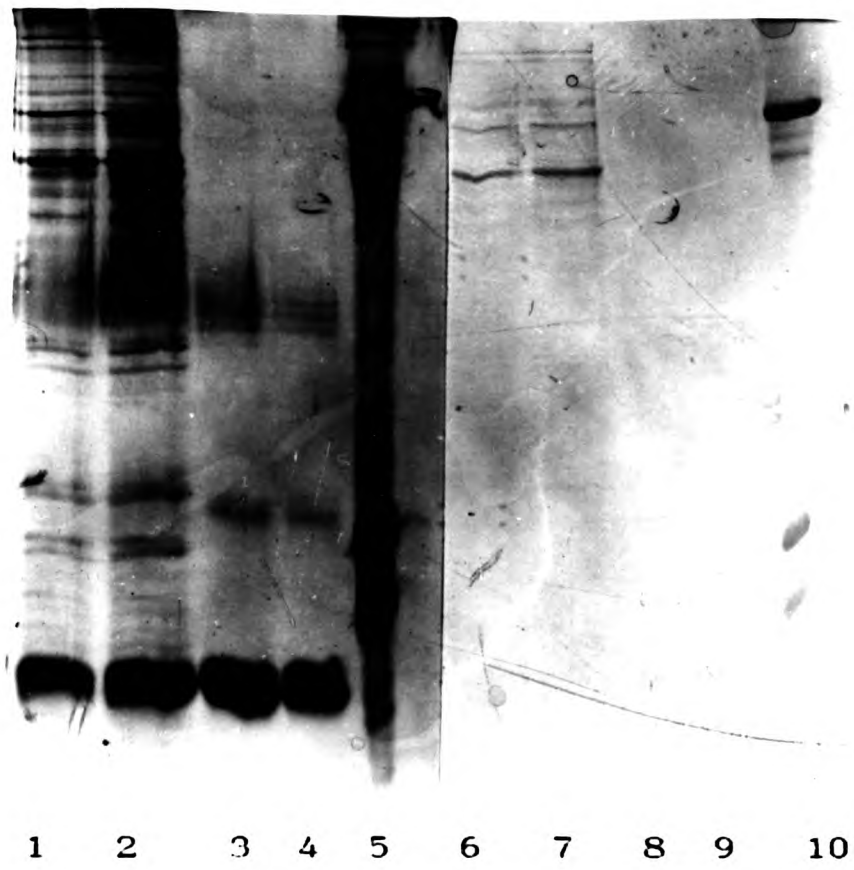
Plate 4.1. SDS-PAGE analysis of <u>D.vulgaris</u> Woolwich 'EDTA washings' of whole cells (C-Fe) and after preincubation in 10mM ferrous ions.



1 2 3 4 5 6 7 8 9 10

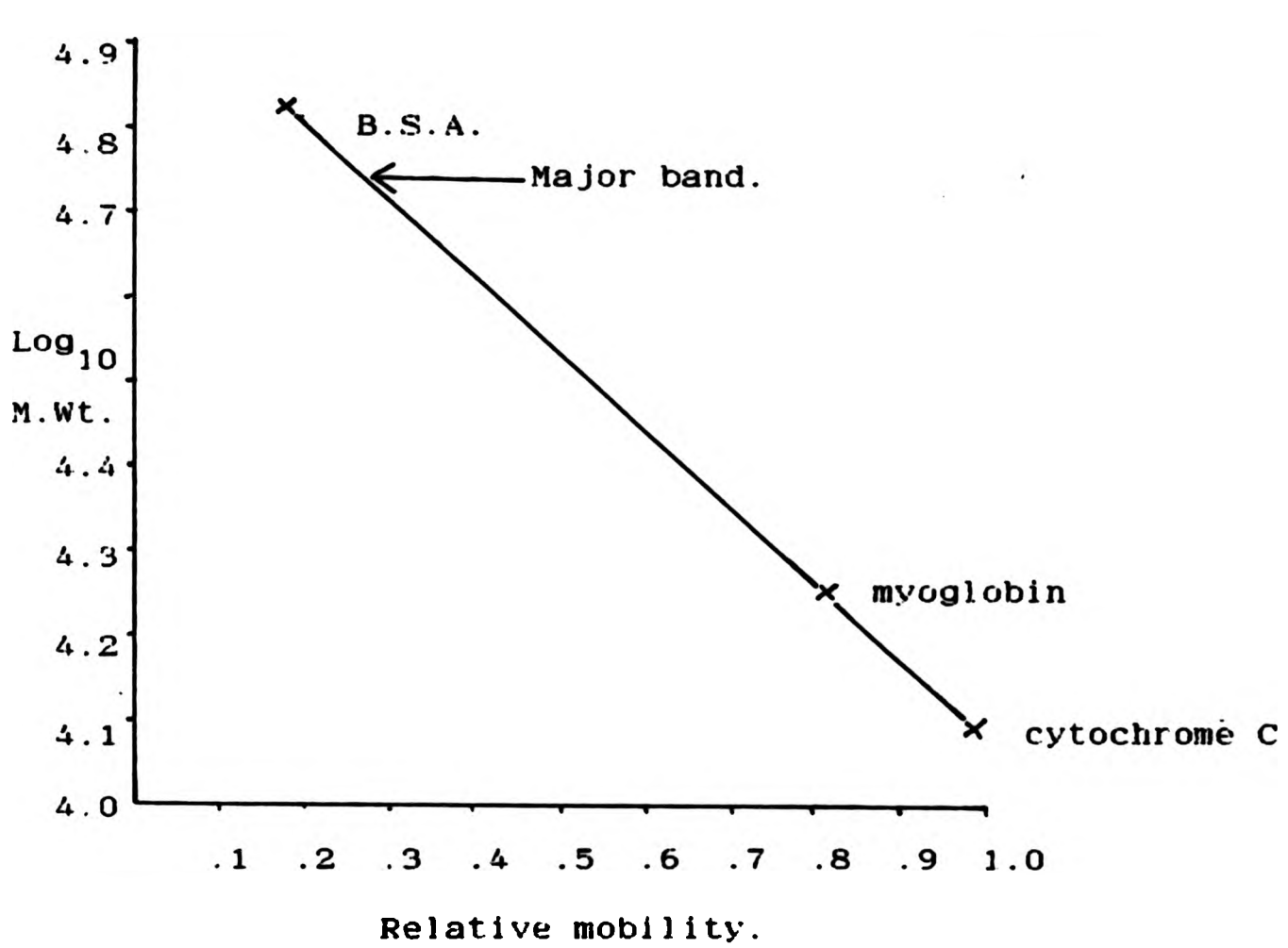
Gels were stained with silver (lanes 1-5)and PAGE Blue 83 (lanes 6-10). Lanes 1&6, EDTA whole cell 'washings'; lanes 2&7, EDTA 'washings' after preincubation of cells in 10mM Fe^{2+}; lanes 3,4,8&9 phenol/water extracted <u>D.vulgaris</u> LPS; lanes 5&10, standard marker proteins .

Plate 4.1. SDS-PAGE analysis of D.vulgaris Woolwich 'EDTA washings' of whole cells (C-Fe) and after preincubation in 10mM ferrous ions.



Gels were stained with silver (lanes 1-5)and PAGE Blue 83 (lanes 6-10). Lanes 1&6, EDTA whole cell 'washings'; lanes 2&7, EDTA 'washings' after preincubation of cells in 10mM Fe^{2+}; lanes 3,4,8&9 phenol/water extracted D.vulgaris LPS; lanes 5&10, standard marker proteins .

Figure 4.2. Determination of M.Wt. for major polypeptide band found in D.vulgaris EDTA 'washings' of whole cells, using plot of $\log_{10}$ M.Wt. standard proteins against their relative mobility.



Results of a single typical gel separation were used.

Major band M.Wt. = 54000.

Plate 4.2. D.vulgaris C+Fe intact cell. Negative stain during EDTA 'washing'. Showing removal of surface material. x50000 (bar=0.1µm).

Plate 4.3. D.vulgaris C+Fe intact cell. Thin section and Ruthenium Red contrast stain during EDTA 'washing'. Note intact outer membrane. x50000 (bar= 0.1µm).

Plate 4.4. D.vulgaris C+Fe intact cell. Negative stain after EDTA washing. Showing more defined outline granular surface and bulge (arrowed). x50000 (bar= 0.1µm)

Plate 4.2. D.vulgaris C+Fe intact cell. Negative stain during EDTA 'washing'. Showing removal of surface material. x50000 (bar=0.1µm).

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Plate 4.4. D.vulgaris C+Fe intact cell. Negative stain after EDTA washing. Showing more defined outline granular surface and bulge (arrowed). x50000 (bar= 0.1µm)

that of the controls (Figure 4.4a and b). The control levels of LPS and protein release for the three media types are shown in Figure 4.3 . The increase in EDTA-induced release occured for cells grown either in medium C or in medium C-Fe. Other divalent cations used (Zn^{2+}, Ca^{2+}, Mg^{2+}) were unable to mimic this effect, although there was a slight increase in LPS release from medium C-grown cells preincubated with calcium at levels above 1.8mM (Figure 4.4a). Cells grown in medium C+Fe (Figure 4.4c) and all cells preincubated with Fe^{2+} concentrations greater than 10mM showed a gradual decrease in this response. Although up to 150µg protein/ml. mixture was removed from C-Fe grown cells by EDTA treatment, no correlation was observed between this and cation incubation concentration (Figure 4.5). Protein release followed a very variable course for all growth media.

The SDS-PAGE separation shows both protein and LPS material released by EDTA treatment after cation incubation. The LPS dense staining band and the 54000 M.Wt. protein are prominent (Figure 4.1). No changes in the number of bands were noted with different ion concentrations, nor was there any difference in band-pattern between cells grown in the different media.

4.2. 125I/lactoperoxidase labelling.

Whole cell labelling by the 125I/lactoperoxidase method proved successful in labelling several proteins. Plate 4.5 shows a contact print of an autoradiograph prepared from an SDS-PAGE separation of labelled whole C-Fe-grown cells. This clearly shows there to be four labelled bands; one heavily labelled, two equally well labelled and one faint

Figure 4.3. Control levels (no cation incubation) of LPS and protein released by EDTA treatment of cultures from different growth media.

Medium	µg LPS released /2ml. mixture.	Corrected* ratio of LPS released.	µg protein released/2ml. mixture.
C+Fe	620±90	20.4	209±62
C	270±58	2.2	252±94
C-Fe	218±72	1.0	143±42

Values are means of at least three separate experiments ± S.D.

* The corrected ratio was calculated by dividing the mean LPS release for each medium by the estimated carbohydrate value (Chapter 5) for the corresponding cell wall fraction.

Figure 4.4. Change in release of LPS by EDTA treatment of D.vulgaris after preincubation in metal cation solutions. Points represent means of at least three separate experiments.

a) Cells grown in medium C. ○ iron □ calcium △ magnesium.

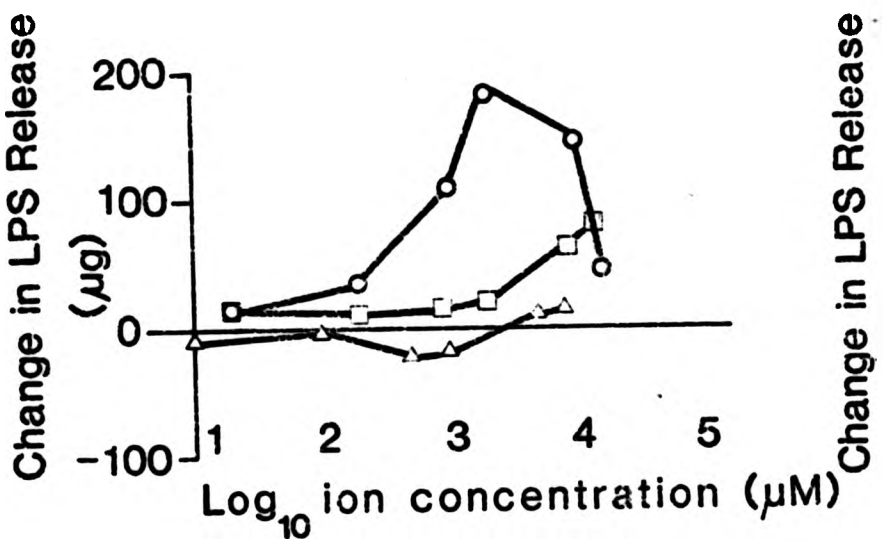


Figure 4.4 continued;

b) Cells grown in medium C-Fe. ○ iron □ calcium ▽ zinc.

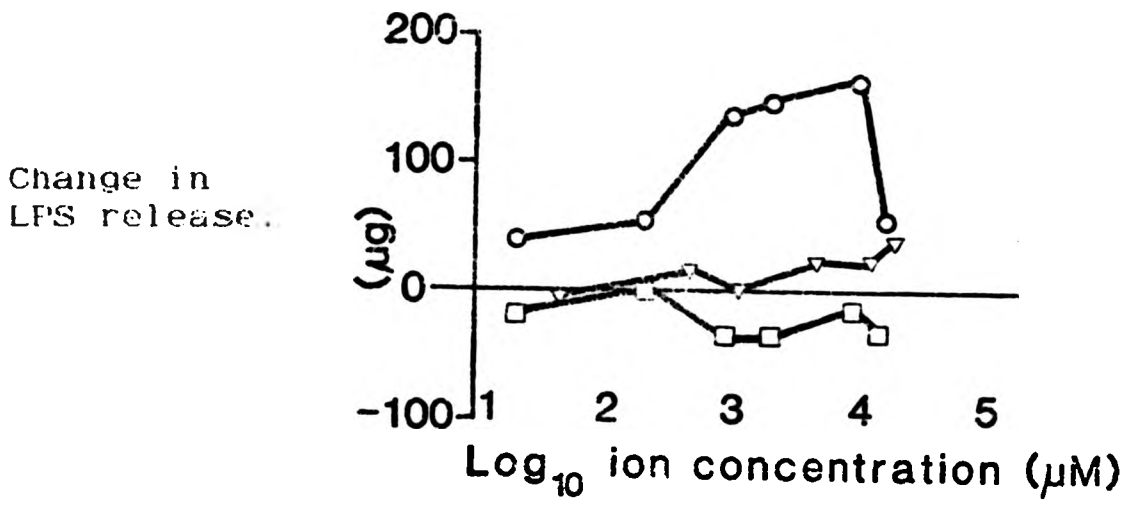


Figure 4.4 continued;

c) Cells grown in medium C+Fe. O iron □ calcium.

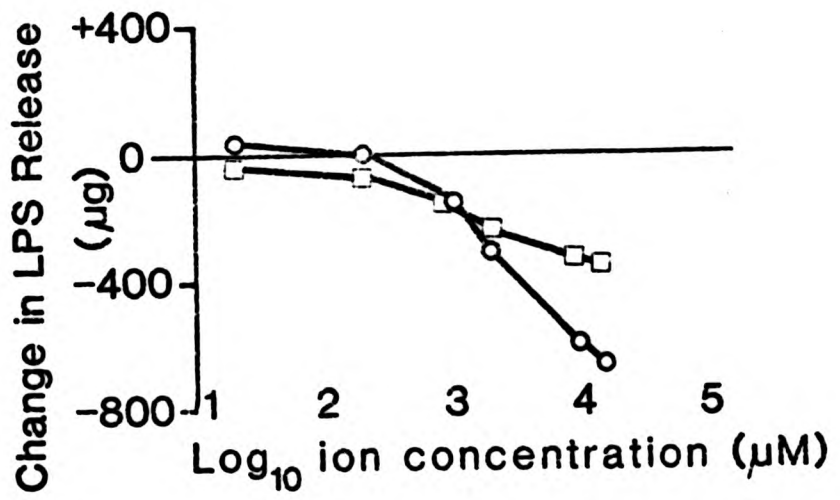
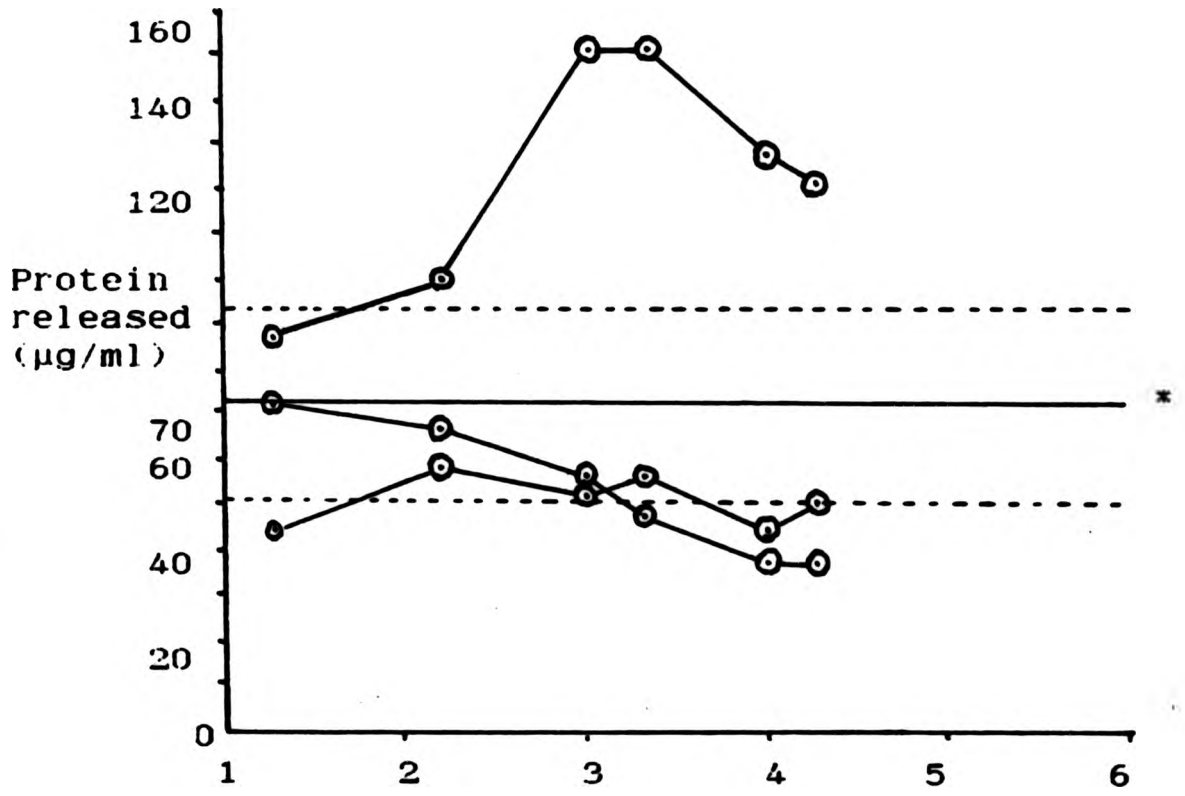


Figure 4.5. Protein released by EDTA treatment of D.vulgaris C-Fe cells after preincubation in ferrous ion solutions. Curves represent three separate experiments.



* represents mean control (no ion incubation) value ± S.D. for three experiments.

Plate 4.5. Contact print of an autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase labelled D.vulgaris Woolwich C-Fe grown cells.

Two lanes are shown from duplicate separations.

Plate 4.5. Contact print of an autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase labelled D.vulgaris Woolwich C-Fe grown cells.

Two lanes are shown from duplicate separations.

Plate 4.6. Contact print of an autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase labelled D.vulgaris Woolwich C+Fe grown cells.

Three tracks are shown, the central one is inverted.

Plate 4.6. Contact print of an autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase labelled D.vulgaris Woolwich C-Fe grown cells.

Three tracks are shown, the central one is inverted.

band. It must be emphasised at this point that the density of labelling is only indicative of the number of exposed susceptible residues and non-labelling does not necessarily mean non-exposure at the surface, only a lack of favourable binding sites. An autoradiograph of similarily labelled C+Fe grown cells (Plate 4.6) shows only very faint labelling of the C-Fe dense band and no other bands are visible.

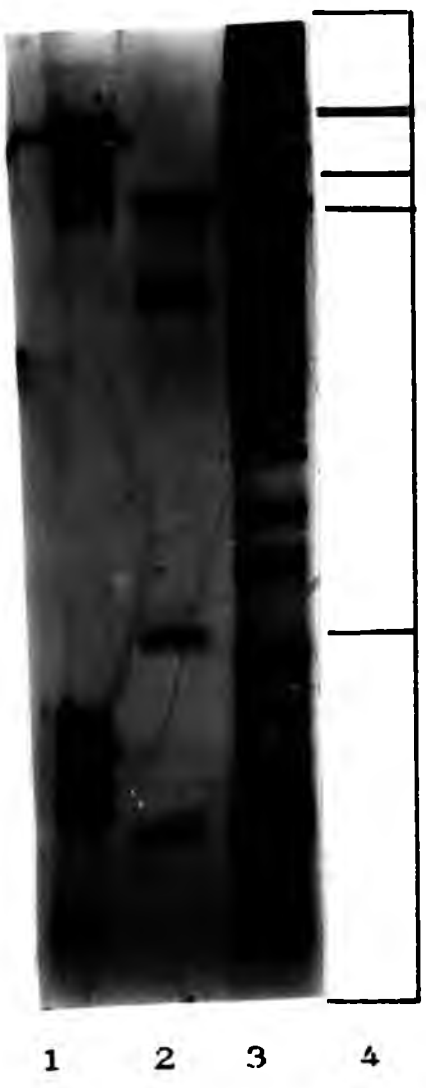
Plate 4.7 shows a sketch of the labelled D.vulgaris C-Fe whole cell autoradiograph aligned adjacently to SDS-PAGE separations of both C-Fe whole cells and Sarkosyl prepared cell walls, both stained by the silver method. The whole cell profile is predictably very complex, and two of the lactoperoxidase labelled proteins can be seen to align, having similar electrophoretic mobilities, with two of the bands found in the C-Fe cell wall fraction. The analysis of these cell wall fractions is discussed in detail in Chapter 5.

4.3. 55Fe whole cell labelling.

Whole cell labelling by incubation with ^{55}Fe as the ferrous ion proved to be only of limited use. Plate 4.8 shows contact prints of autoradiographs prepared from SDS-PAGE separations of labelled whole D.vulgaris C-Fe grown cells incubated for the indicated lengths of time.

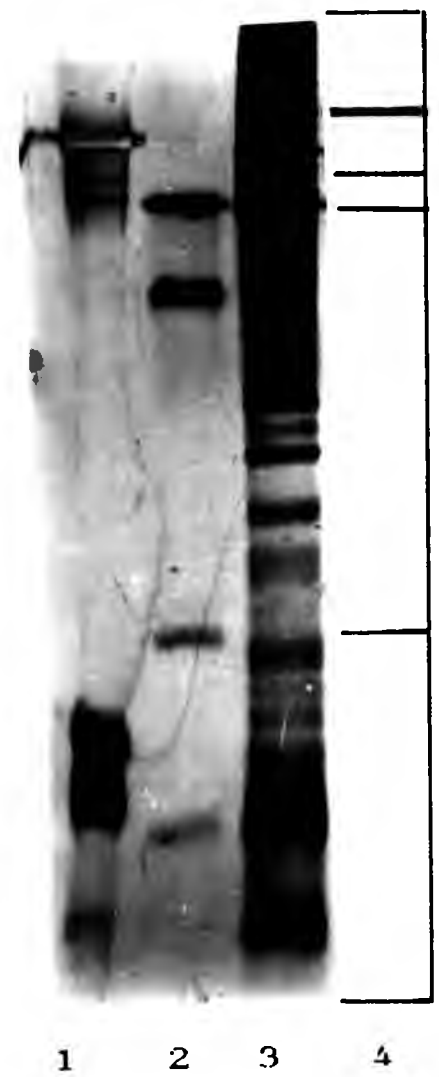
Incubations of 10 minutes at 0°C and one hour at room temperature revealed bands at the ends of the gel runs. An incubation of two hours at room temperature showed a similar single band but this was much more defined as a definite gel band.

Plate 4.7. Sketch of autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase-labelled D.vulgaris Woolwich C-Fe grown cells aligned adjacently to an SDS-PAGE separation of C-Fe whole cells and Sarkosyl prepared C-Fe cell walls, both stained by the silver method.



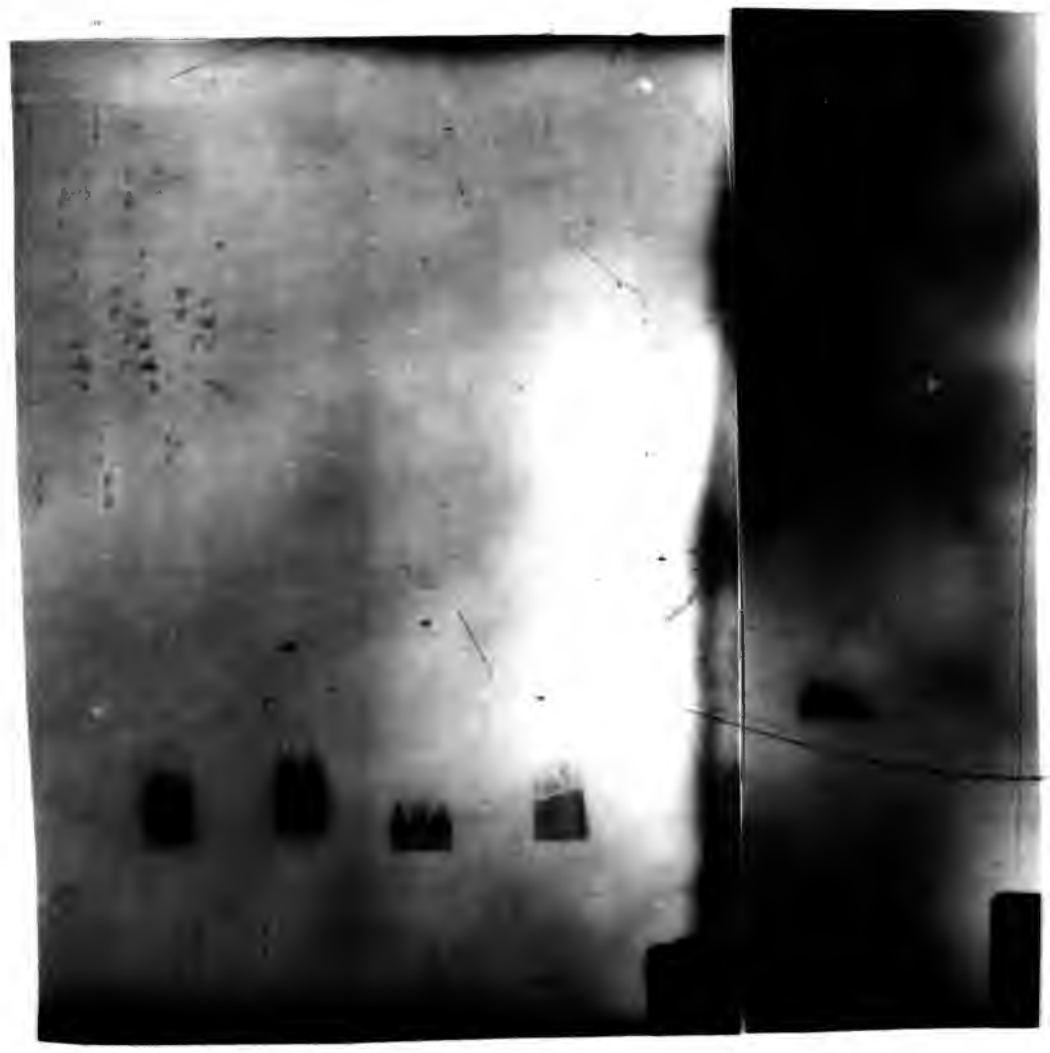
Lane 1, protein markers; lane 2, Sarkosyl prepared C-Fe cell walls; lane 3, C-Fe whole cells (iodinated); lane 4, sketch of autoradiograph from an SDS-PAGE separation of ^{125}I/ lactoperoxidase labelled C-Fe whole cells (aligned using marker proteins on original gel).

Plate 4.7. Sketch of autoradiograph prepared from an SDS-PAGE separation of ^{125}I/lactoperoxidase-labelled D.vulgaris Woolwich C-Fe grown cells aligned adjacently to an SDS-PAGE separation of C-Fe whole cells and Sarkosyl prepared C-Fe cell walls, both stained by the silver method.



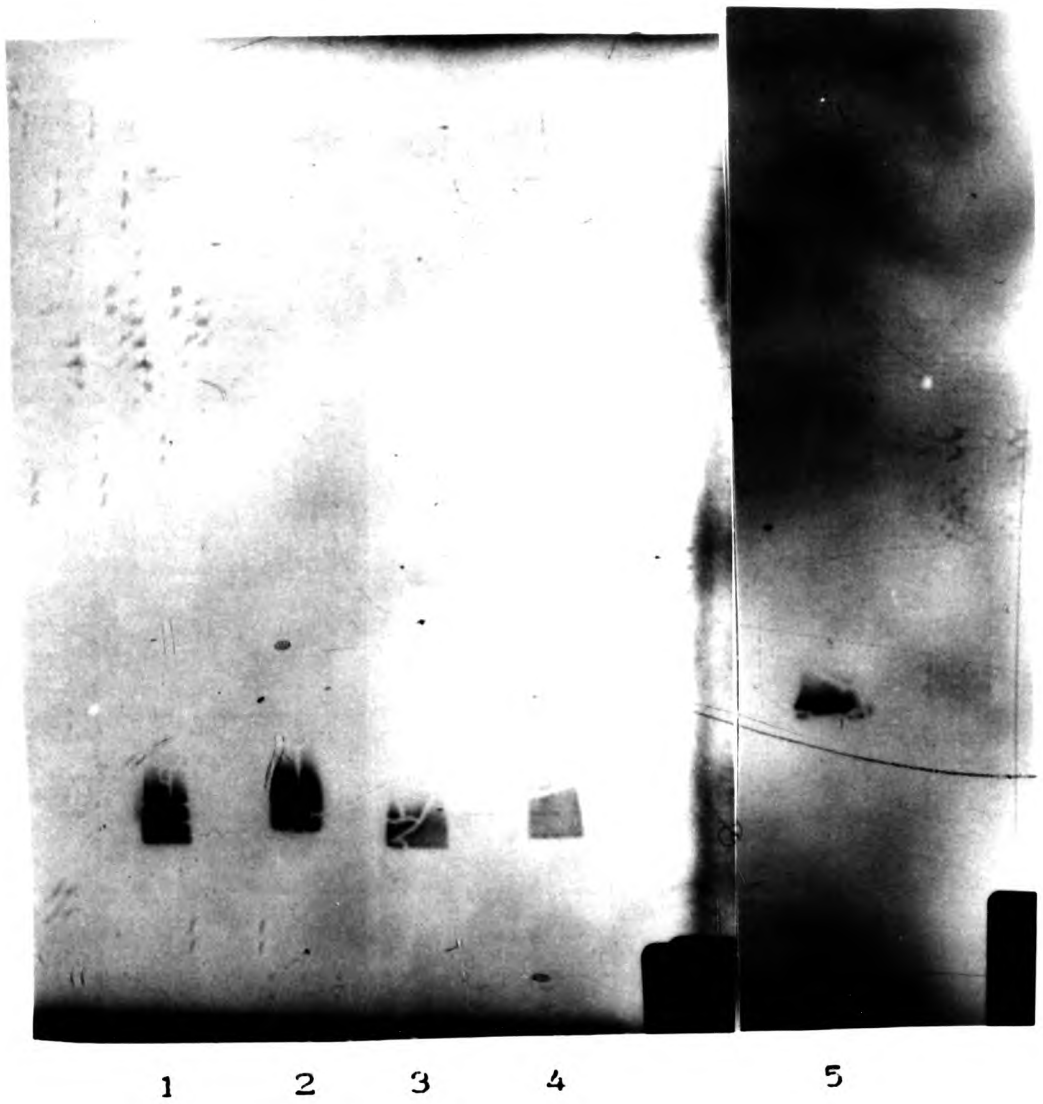
Lane 1, protein markers; lane 2, Sarkosyl prepared C-Fe cell walls; lane 3, C-Fe whole cells (iodinated); lane 4, sketch of autoradiograph from an SDS-PAGE separation of ^{125}I/ lactoperoxidase labelled C-Fe whole cells (aligned using marker proteins on original gel).

Plate 4.8. Contact prints of autoradiographs prepared from SDS-PAGE separations of ^{55}Fe labelled whole D.vulgaris Woolwich C-Fe grown cells.



Lane 1&2, two hour incubations; lanes 3&4, one hour incubations; lane 5, 10 minute incubation (0°C).

Plate 4.8. Contact prints of autoradiographs prepared from SDS-PAGE separations of ^{55}Fe labelled whole D.vulgaris Woolwich C-Fe grown cells.



Lane 1&2, two hour incubations; lanes 3&4, one hour incubations; lane 5, 10 minute incubation (0°C).

DISCUSSION.

The release of surface material from whole cells demonstrates several molecular species which present this surface to the environment, in D.vulgaris. Lipopolysaccharide and protein material are relesed from both iron rich and iron limited cell cultures, and this material must be relatively weakly held at the cell surface by ionic interactions.

The lactoperoxidase labelling indicates at least four proteins are exposed at the cell surface in C-Fe grown cells. The excess iron in C+Fe grown cells appears to interfere with the labelling due to iron hindrance or protection of the surface. Two of the labelled proteins are of similar M.Wt. (54000 and 18400) to two of the proteins found in Sarkosyl prepared C-Fe cell walls. The possibility that these are the same proteins, as is the 54000 M.Wt. protein released by EDTA treatment, cannot be proven at this stage.

The identities of the other two lactoperoxidase labelled proteins (M.Wt. 78000 and 56000) are unknown. They did not appear as stained bands in the Sarkosyl cell wall separation and were not therefore major OM proteins. They may be surface components retained by the cells during harvesting and washing prior to iodination or possibly flagellar components. One could also be lactoperoxidase itself (M.Wt. 77500) which has been retained in the cell pellet. This is a disadvantage of this labelling method and other iodination technques can be used, such as the use of Chloramine T as the oxidising agent, this method has the disadvantage of being rather harsh however. It has also been reported that this type of study may label inner membrane proteins in some organisms (Loeb et al, 1983; Cushley et al, 1983).

The single bands obtained with ^{55}Fe whole cell labelling probably reflect the passage of free ferrous ions through the polyacrylamide gel. Any ferrous iron ionically bound during the incubation periods may well become dissociated during the solubilisation of the intact cells with the strongly ionic detergent used. Two hour incubations did reveal a definite band but this did not correspond to any macromolecular band stained by the silver or PAGE Blue 83 methods and its identity remains obscure.

The EDTA release study demonstrates an important interaction between ferrous ions and the LPS of D.vulgaris in vivo. A short incubation with ferrous ions increases the amount of EDTA-releasable LPS in D.vulgaris grown in iron limited media. The inability of other cations, in particular Zn^{2+} which has a higher affinity for EDTA than ferrous ions (log_{10} stability constants 16.7 and 14.3 respectively), to mimic this effect indicates the interaction is specific. This does not, however, imply that other sites do not exist for other divalent cations (or further sites for Fe^{2+}).

The decrease in LPS release shown in iron rich cultures could be due to the binding of ferrous ions to molecules other than LPS or to simple precipitation of ions once LPS saturation is reached during preincubation. Both of these would effectively reduce the amount of EDTA available for reaction with cation-bound LPS. The decrease in LPS release at ferrous ion preincubation concentrations above 10mM in C-Fe cultures is probably due to EDTA limitation (this point is reached sooner in medium C grown cells due to some previously bound iron) rather than iron saturation. One would

expect the decrease in LPS release in C+Fe cells to tail-off above 10mM iron incubation. The 10mM curve turning point in C-Fe grown cells indicates a 1:1 Fe^{2+}:EDTA binding. It is also interesting to note that, although this is not the maximal removal of LPS if EDTA concentrations were increased, an incubation with 10mM Fe^{2+} is capable of releasing 170µg of LPS (Figure 4.4a). This is in 2mls. of mixture thus; 10µmoles of bound Fe^{2+} allows the release of 85µg of LPS or 6.6µg Fe^{2+} allows the release of 1µg LPS. 1µg of C-Fe D.vulgaris LPS in vivo appears to bind 6.6µg ferrous iron which needs to be removed before the LPS is released from the OM.

CHAPTER 5

SUB-CELLULAR FRACTION STUDIES.

The whole cell studies described in the previous chapter have indicated the presence of LPS and several protein species at the surface of the bacterium. They have also provided evidence of a selective interaction between this LPS and Fe^{2+}. To further analyse the cell surface of D.vulgaris C+Fe and C-Fe grown cells sub-cellular fractions of the bacteria were prepared to isolate the cell envelope and further purify the OM from this in an intact state. The strategies for these fractionations have been described in 2.1.2.1. It was hoped that these fractions would provide important structural knowledge together with an insight into the relative localisations of the various macromolecules present.

The analysis of the cell envelope and OM involved total protein, total carbohydrate and polar lipid analysis coupled to analysis of the fatty acid composition (a major taxonomic criterion). Various strains of D.vulgaris were compared and contrasted to aid classification and note common features. The various iron containing media were again used in an attempt to assign iron associated structural changes to functions.

When examining the effectiveness of any cellular subfractionation procedure various criteria and techniques may be used to estimate the purity of a given fraction. Probably the most convenient and widely accepted approach is to measure the variety of biochemical markers whose enzymatic activity may be determined. The most widely used are the enzymes and pigments associated with the respiratory chain and oxidative

phosphorylation in the CM. However this does not prove practical in many anaerobes for reasons which are discussed later. Chemical determinations are also used in the analysis of structural and other components which are not amenable to detection by enzymatic assay. Their value is probably greatest when considering the purity of cellular compartments such as the cell wall and OM, which are low in metabolic activity. The best marker for the OM in this respect is the LPS which possesses several unique chemical constituents which are amenable to assay. SDS-PAGE can easily be applied to the problem of determining membrane purity especially for estimating cross-contamination of OM/CM. Furthermore LPS present can be monitored by SDS-PAGE. Electron microscopy is often the most convenient and rapid method of estimating the degree of contamination caused by morphologically recognisable structures such as flagella, mesosomes and various membranes with associated peptidoglycan.

In this study the respiratory pigment menaquinone was assessed as a potential CM marker to check for OM purity. Menaquinones (vitamin K) are respiratory isoprenoid quinone pigments present in many bacterial membranes and menaquinone 6 has been reported to be present in Desulfovibrio in relatively large amounts (Maroc et al. 1970).

The various fractions prepared were also assayed for the enzyme hydrogenase in an attempt to localise this corrosion-related protein within the cell envelope. The biological activation of molecular hydrogen to serve as an electron or hydrogen donor is catalysed by the enzyme hydrogenase

(hydrogen: ferricytochrome c_3 oxoreductase, EC1.12.2.1).

$$H_2 \rightleftharpoons 2H^+ + 2e$$

The hydrogen metabolism of Desulfovibrio is mediated by the reversible iron-sulphur containing enzyme present in most strains (Huynh et al, 1984). In addition to carbon sources, gaseous hydrogen can sometimes act as electron donor to the electron transport chain (see 1.1.2). Other functions of the hydrogenase have also been postulated, such as an 'escape valve' to dispose of excess reducing power (Grey & Gest, 1965). Desulfovibrio hydrogenases have been reported to be both soluble and membrane bound (Yagi, 1970; Yagi et al. 1978), and to be present as two or more different proteins (Tsuji & Yagi, 1980). The latter authors postulate a high and low M.Wt. enzyme; the high M.Wt. being constitutive and catalysing an initial 'burst' of H_2 during substrate level phosphorylation of lactate to acetate, whereas the low M.Wt. enzyme is induced by this initial H_2 burst to feed subsequent electron transport phophorylation once the sulphate reducing system is 'primed'. Important features of the Desulfovibrio hydrogenase are its reported presence in the periplasm of the cell for D.gigas (Bell et al. 1974; Hatchikian et al. 1978) and the fact that in D.vulgaris Hildenborough it can be easily released into soluble form by EDTA/alkaline washing of whole cells (Van der Westen et al. 1978). This location on the periphery of the cell and M.Wt. of about 58000 in the Hildenboruogh strain, make it an attractive candidate for an OM protein, all the more so when one considers the advantages to the cell of a hydrogenase located on the cell surface and the possible removal of a hydrogen film as being implicated in iron corrosion (cathodic depolarisation; 1.2 & Khosrovi et al, 1971).

RESULTS

5.1. Cell envelope and Sarkosyl OM analysis.

5.1.1. Electron microscopy.

The fractions obtained during the fractionation of whole D.vulgaris Woolwich C+Fe and C-Fe grown cells were examined by electron microscopy using both thin sections and negative staining. The cell envelope fraction (Plates 5.1, 5.2 & 5.3) shows this to be composed of irregular shaped envelopes free of any intact cells. The OM and CM are clearly visible in thin sections. The removal of the cell cytoplasm did not render the cell surface more amenable to negative staining. The cell surface was still granular with no discernable structures, arrays or striations.

After solubilisation with Sarkosyl both C+Fe and C-Fe cells yielded a residual pellet on centrifugation. The C-Fe pellet was of a translucent nature and pale yellow in colour, the C+Fe pellet was still apparantly rich in ferrous sulphide, being black coloured. These fractions were examined under the electron microscope and are shown in Plates 5.4, 5.5 & 5.6. The Sarkosyl C+Fe cell wall fraction shows single membrane vesicles, sometimes broken, of various sizes. In thin section these are seen to consist of a single membrane bilayer together with a fuzzy, internal, electron-dense layer, the whole being similar in structure to the outer layers of the complete cell envelope. Preparations from C+Fe cultures contained large amounts of dense granular material often obscuring the envelopes (Plate 5.4). C-Fe preparations produced similar

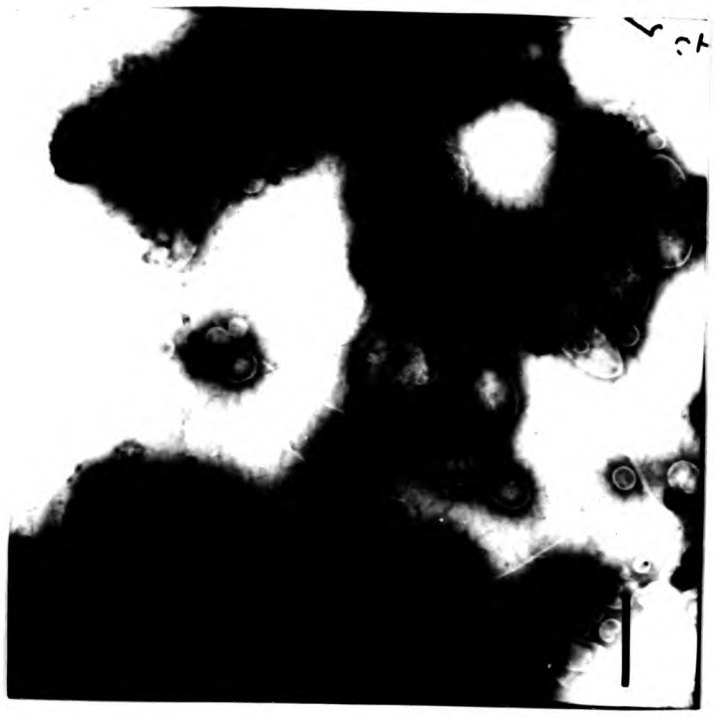


Plate 5.1. D.vulgaris C+Fe cell envelopes. Negative stain. x20000 (bar=0.5µm).

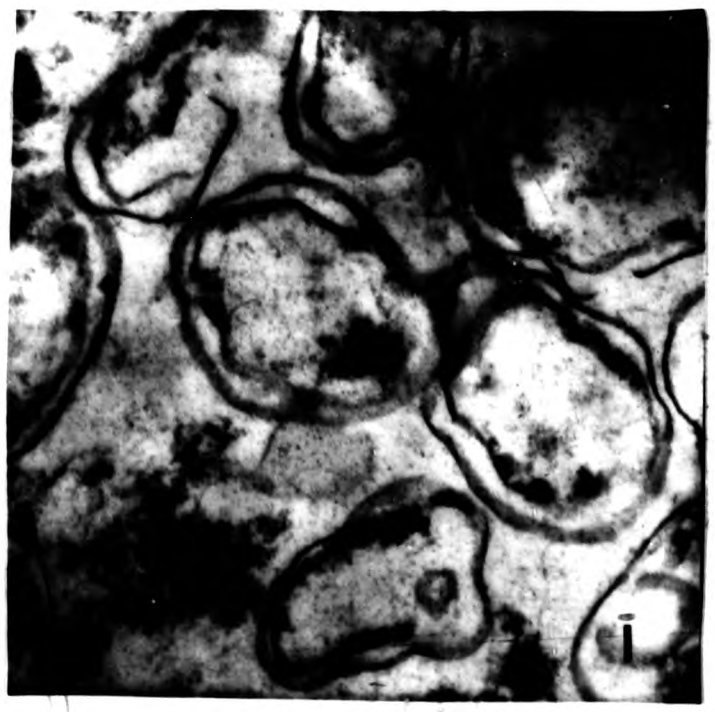
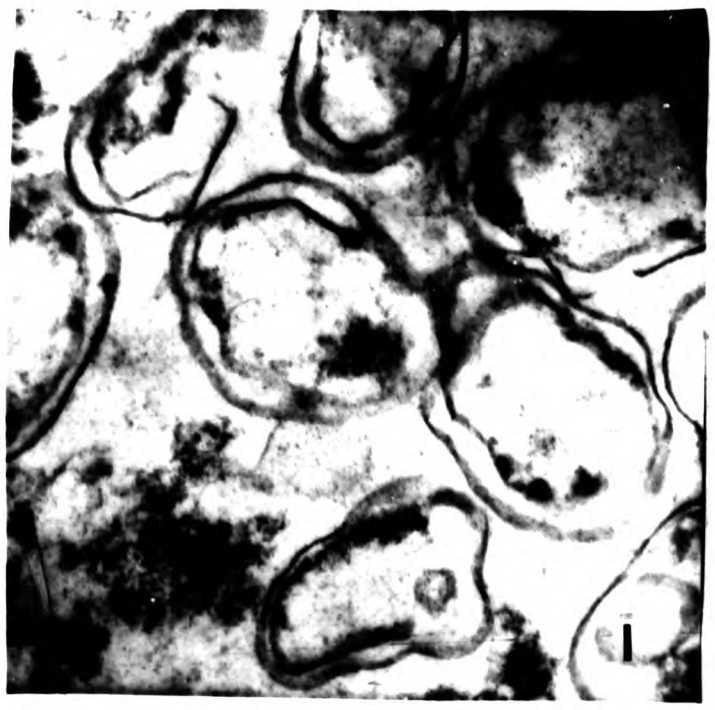


Plate 5.2. D.vulgaris C+Fe cell envelopes showing double membrane system. Thin section. x40000 (bar=0.1µm).

Plate 5.3. D.vulgaris C+Fe cell envelope. High magnification, negative stain. x100000 (bar=0.1µm).

Plate 5.1. D.vulgaris C+Fe cell envelopes. Negative stain. x20000 (bar=0.5µm).



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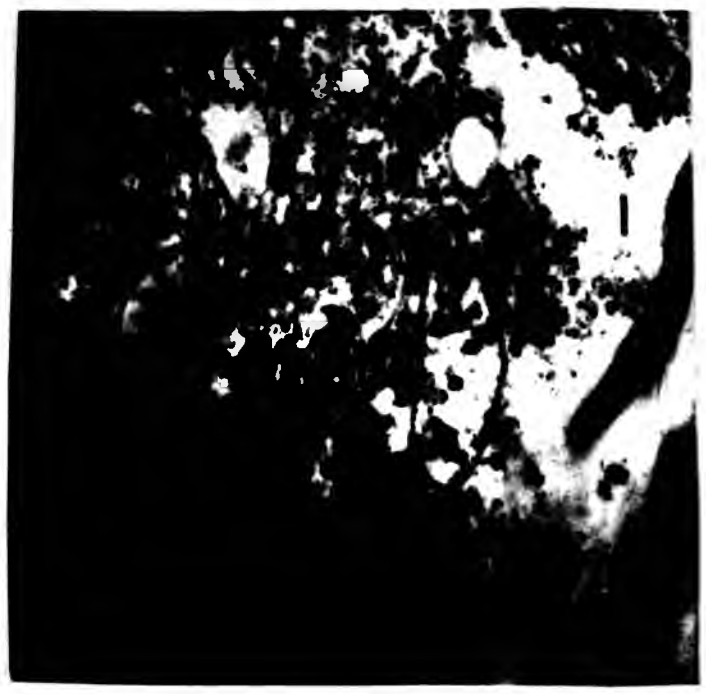


Plate 5.4. D.vulgaris C+Fe Sarkosyl cell wall. Thin section showing single membrane and FeS material. x40000 (bar=0.1µm).

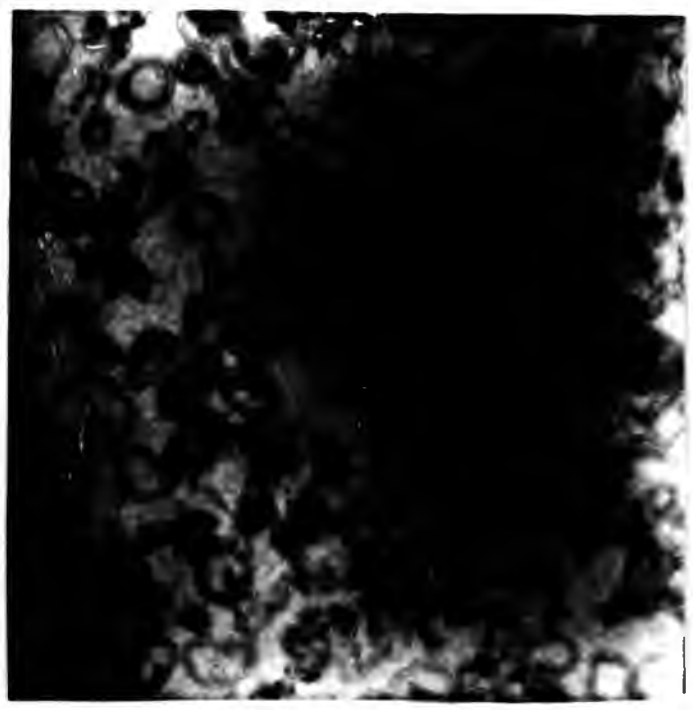


Plate 5.5. D.vulgaris C-Fe Sarkosyl cell wall. Thin section showing single membrane. x60000 (bar=0.1µm).

Plate 5.4. D.vulgaris C+Fe Sarkosyl cell wall. Thin section showing single membrane and FeS material. x40000 (bar=0.1µm).

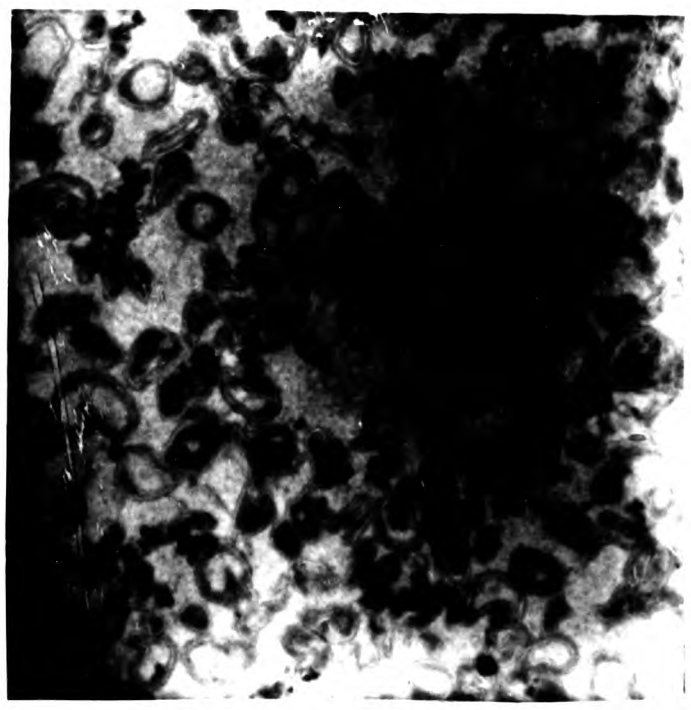


Plate 5.5. D.vulgaris C-Fe Sarkosyl cell wall. Thin section showing single membrane. x60000 (bar=0.1µm).

Plate 5.6. <u>D.vulgaris</u> Woolwich C+Fe Sarkosyl cell wall. High magnification, thin section showing single membrane and fuzzy internal layer. x200000 (bar=50nm).

Plate 5.6. D.vulgaris Woolwich C+Fe Sarkosyl cell wall. High magnification, thin section showing single membrane and fuzzy internal layer. x200000 (bar=50nm).

single membrane vesicles but of cleaner nature with much less granular material. Negative staining of Sarkosyl prepared cell walls (Plates 5.7 & 5.8) was disappointing, revealing little detail, although small transparant spheres on the dark staining vesicles were sometimes visible.

5.1.2. Yields and composition.

The yields obtained by the fractionation of the cell envelope and Sarkosyl preparation of the cell wall are given in Figure 5.1. The C+Fe Sarkosyl cell wall was analysed for total protein, carbohydrate, lipid, LPS, Fe^{2+}, and KDO. The results are shown in Figure 5.2. Comparisons of the C+Fe Sarkosyl cell wall protein and carbohydrate content were made for three strains of D.vulgaris and these are shown in Figure 5.3. Comparisons of the protein and carbohydrate content in the Sarkosyl cell walls were also made for Woolwich preparations from the three types of media and these are given in Figure 5.4. Fe^{2+} was assayed in C-Fe Sarkosyl cell walls but was below the limit of detection (using sample levels which did not result in turbidity in the assay system).

5.1.3. SDS-PAGE separations.

SDS-PAGE analysis of Woolwich C+Fe Sarkosyl cell walls (Plate 5.9) showed a complex pattern of polypeptides with three major bands and up to 20 minor bands. The pattern obtained was highly reproducible. Quantitative staining was best obtained by using PAGE Blue 83 but this stain was unable to resolve LPS material. Silver staining was more sensitive and was able to visualise LPS bands. Densitometer scans are given in Figure 5.5, showing their numerical designation. The M.Wt s. of the three major proteins, designated OMP s 1, 2 & 3, were estimated from a plot of $\log_{10}$ M.Wt. (standards)

Plate 5.7. D.vulgaris C-Fe Sarkosyl cell walls. Negative stain x20000 (bar=0.5µm).

Plate 5.8. D.vulgaris C-Fe Sarkosyl cell walls. High magnification, negative stain . x80000 (bar= 0.1µm).

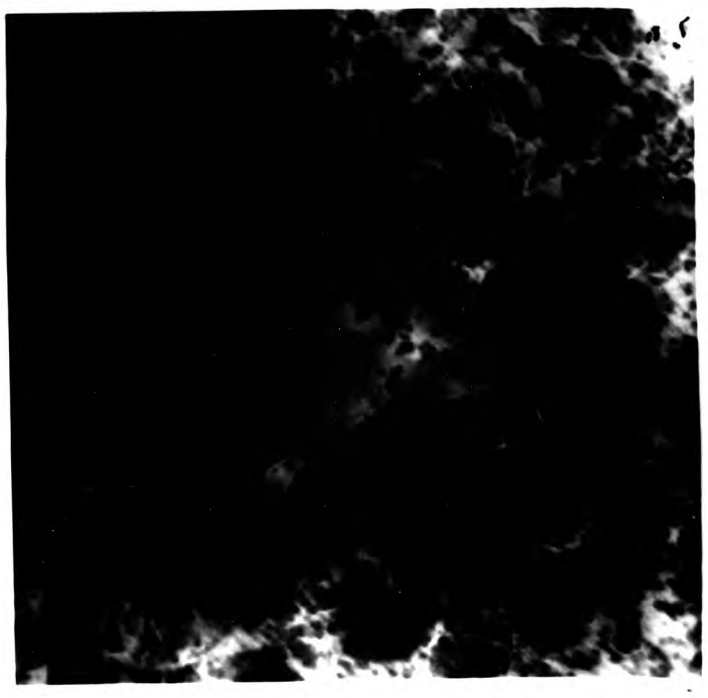


Plate 5.7. D.vulgaris C-Fe Sarkosyl cell walls. Negative stain x20000 (bar=0.5µm).

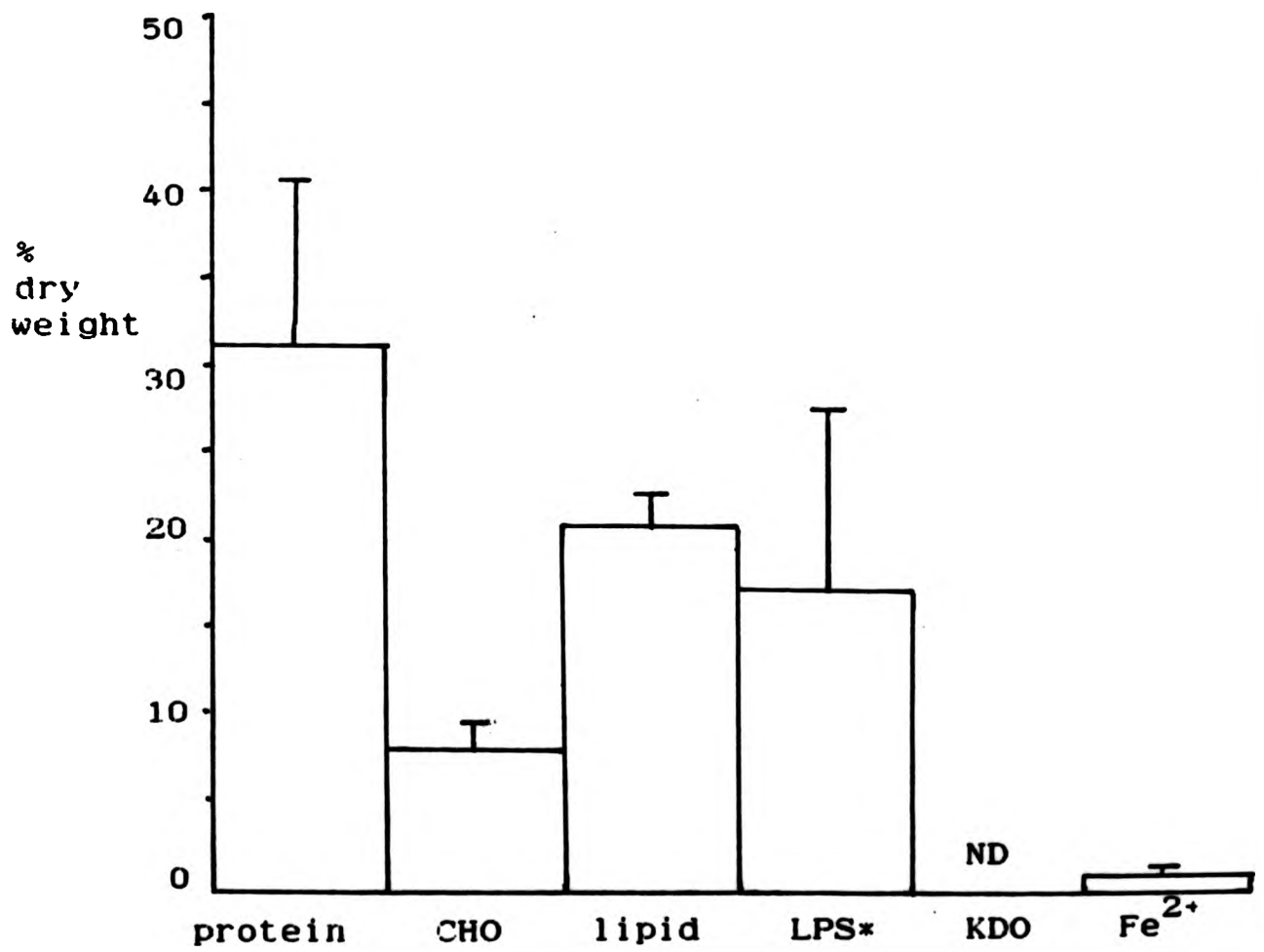
Plate 5.8. D.vulgaris C-Fe Sarkosyl cell walls. High magnification, negative stain . x80000 (bar= 0.1µm).

Figure 5.1. Fraction yields for cell envelope and for Sarkosyl cell wall preparations.

D.vulgaris preparation.	Mean dry weight extracted (mg) /500ml. culture	Mean dry weight extracted (mg) /10^8 cells.
Woolwich.		
cell wall C+Fe	4.0	0.76 (n=13)
cell wall C	3.3	0.67 (n=7)
cell wall C-Fe	1.8	0.67 (n=5)
cell envelope C+Fe	9.8	2.10 (n=9)
Hildenborough		
cell wall C+Fe	4.1	1.00 (n=3)

n= number of separate fractionations.

Figure 5.2. *D.vulgaris* Woolwich C+Fe Sarkosyl cell wall analysis expressed as per cent total dry weight.



**estimated as LPS using extracted *D.vulgaris* material.

Figure 5.3. Comparison of total protein and total carbohydrate content in C+Fe Sarkosyl cell walls for three strains of D.vulgaris.

(hatched=protein).

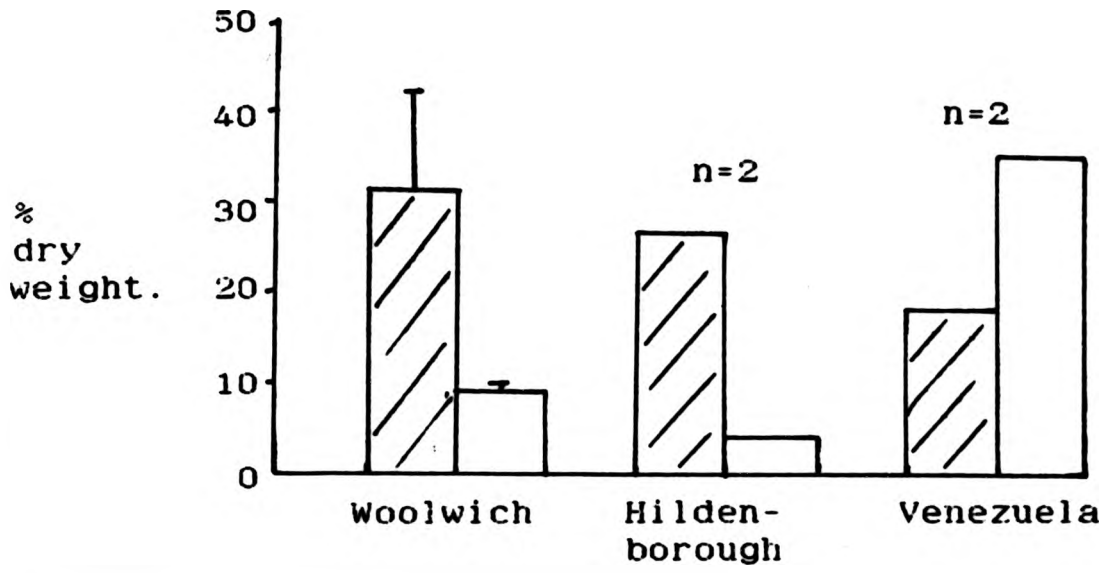


Figure 5.4. Comparison of total protein and carbohydrate content in Sarkosyl cell walls of D.vulgaris Woolwich from three growth media.

(hatched=protein).

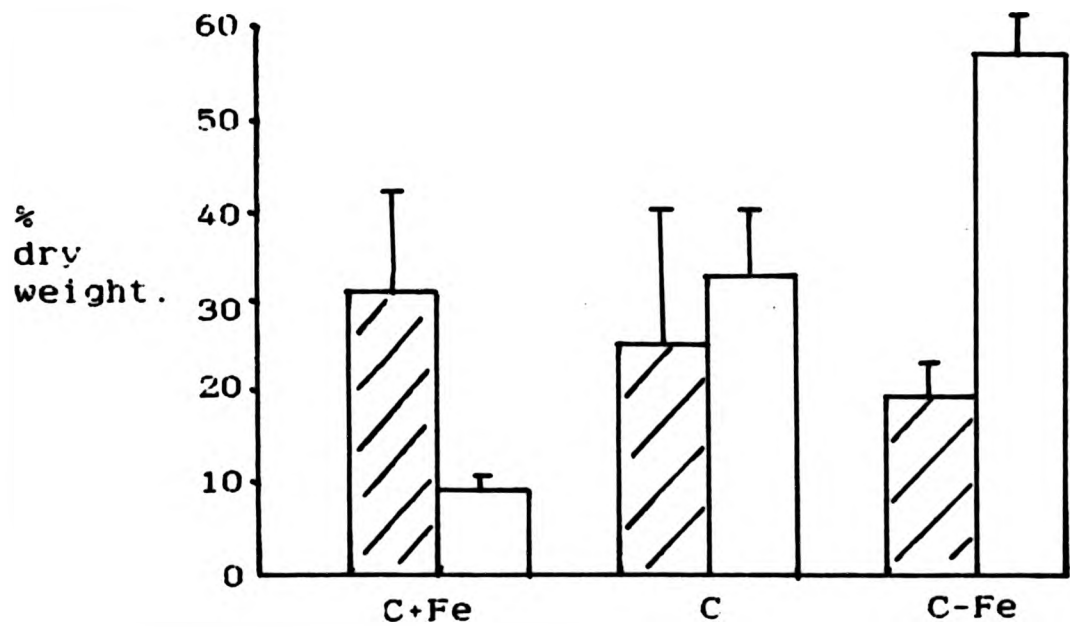
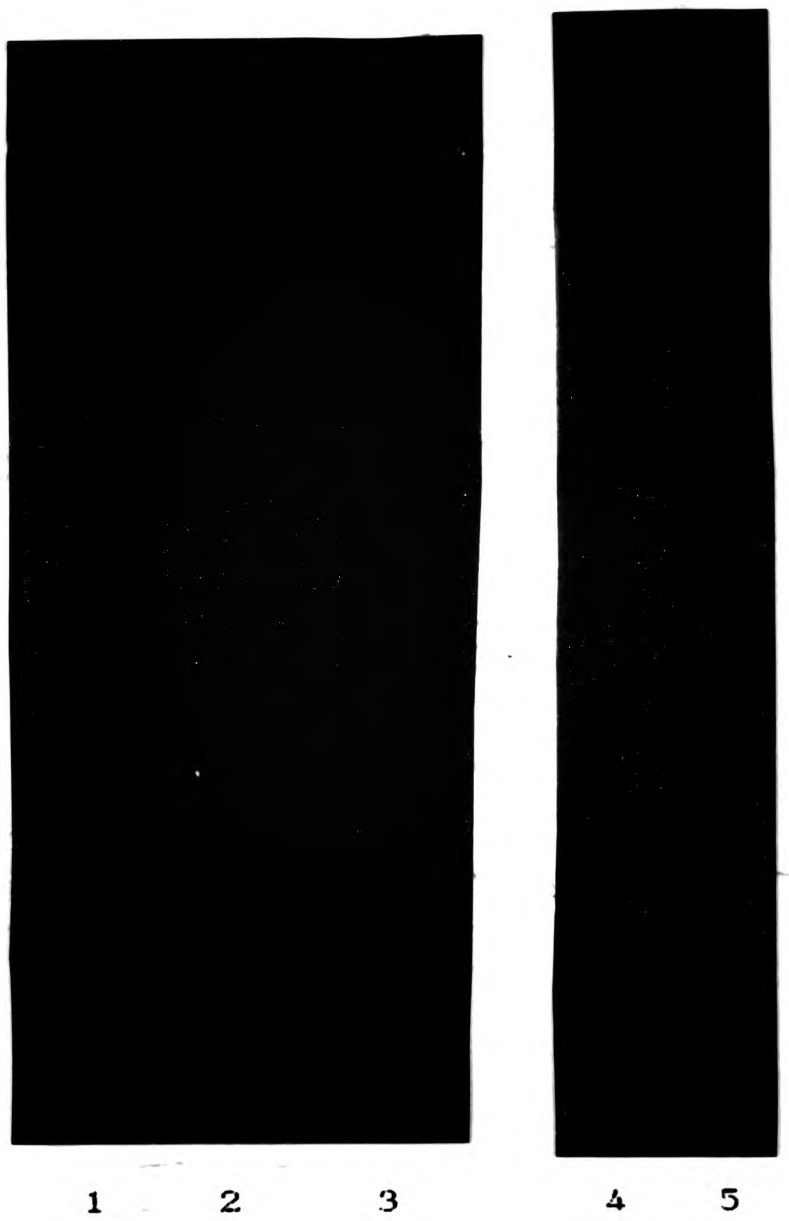


Plate 5.9. SDS-PAGE analysis of Sarkosyl cell walls from D.vulgaris Woolwich grown in medium C+Fe and C-Fe.

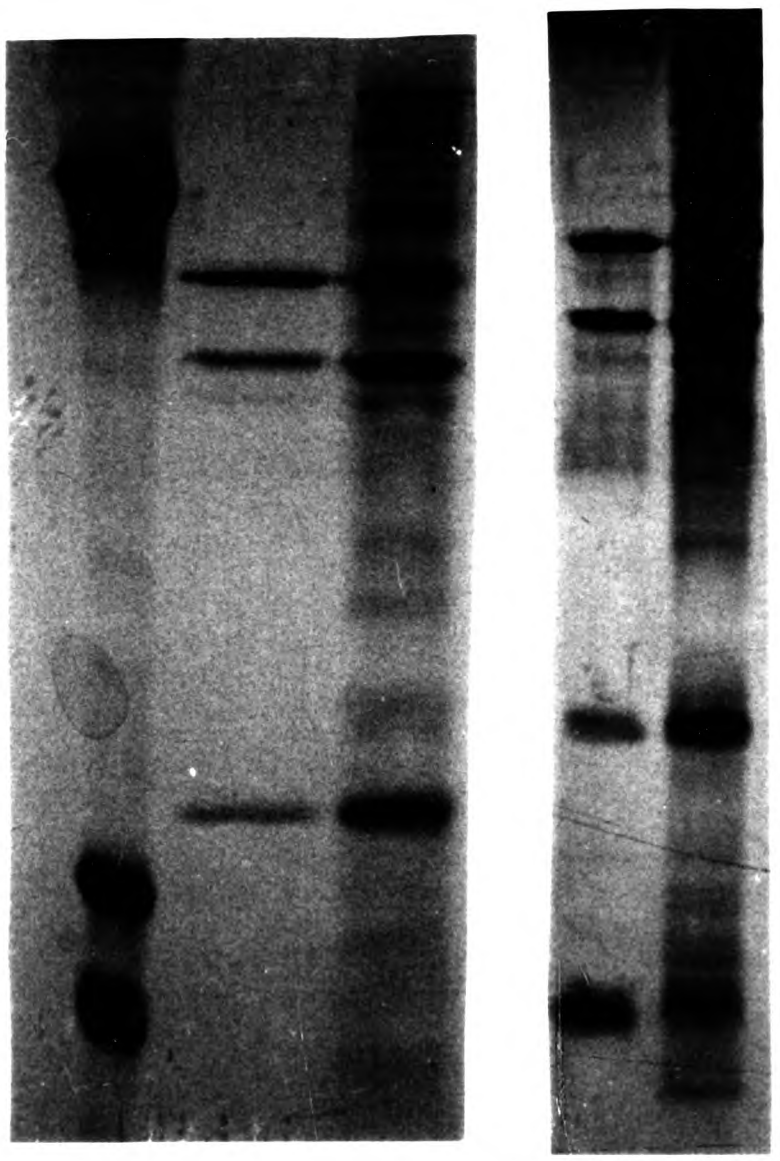


Lanes 1-3 are stained using PAGE Blue 83, lanes 4 & 5 using Silver method. Lane 1, marker proteins; lanes 2 & 4, C-Fe cell walls; lanes 3 & 5, C+Fe cell walls.

Plate 5.9. SDS-PAGE analysis of Sarkosyl cell walls from D.vulgaris Woolwich grown in medium C+Fe and C-Fe.

Lanes 1-3 are stained using PAGE Blue 83, lanes 4 & 5 using Silver method. Lane 1, marker proteins; lanes 2 & 4, C-Fe cell walls; lanes 3 & 5, C+Fe cell walls.

Plate 5.9. SDS-PAGE analysis of Sarkosyl cell walls from D.vulgaris Woolwich grown in medium C+Fe and C-Fe.

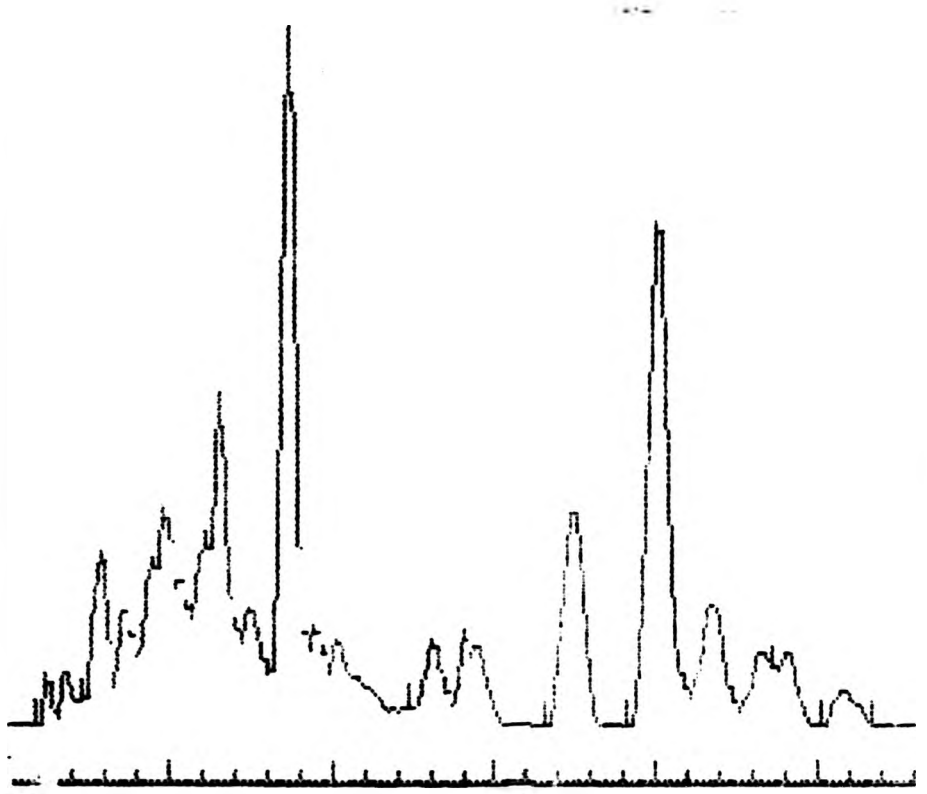


1 2 3 4 5

Lanes 1-3 are stained using PAGE Blue 83, lanes 4 & 5 using Silver method. Lane 1, marker proteins; lanes 2 & 4, C-Fe cell walls; lanes 3 & 5, C+Fe cell walls.

Figure 5.5. Densitometer scan of a PAGE Blue 83 stained SDS-PAGE analysis of a) C+Fe Sarkosyl cell walls from D.vulgaris Woolwich b) C-Fe cell walls c) EDTA washings from whole cells.

a).



#	% Prot.	OMP	M.Wt.
21	1.3	3d	
20	2.1	3c	
19	2.6	3b	
18	4.7	3a	
17	17.4	OMP 3	18300
16	7.4	2g	
15	2.2	2f	
14	1.7	2e	
13	2.4	2d	
12	4.5	2b	
11	2.2	2a	
10	16.5	OMP 2	46000
9	3.9	1a	
8	8.1	OMP 1	54000
7	4.1	g	
6	2.9	f	
5	4.3	e	
4	3.5	d	
3	2.5	c	
2	3.9	b	
1	1.8	a	

Figure 5.5. b).

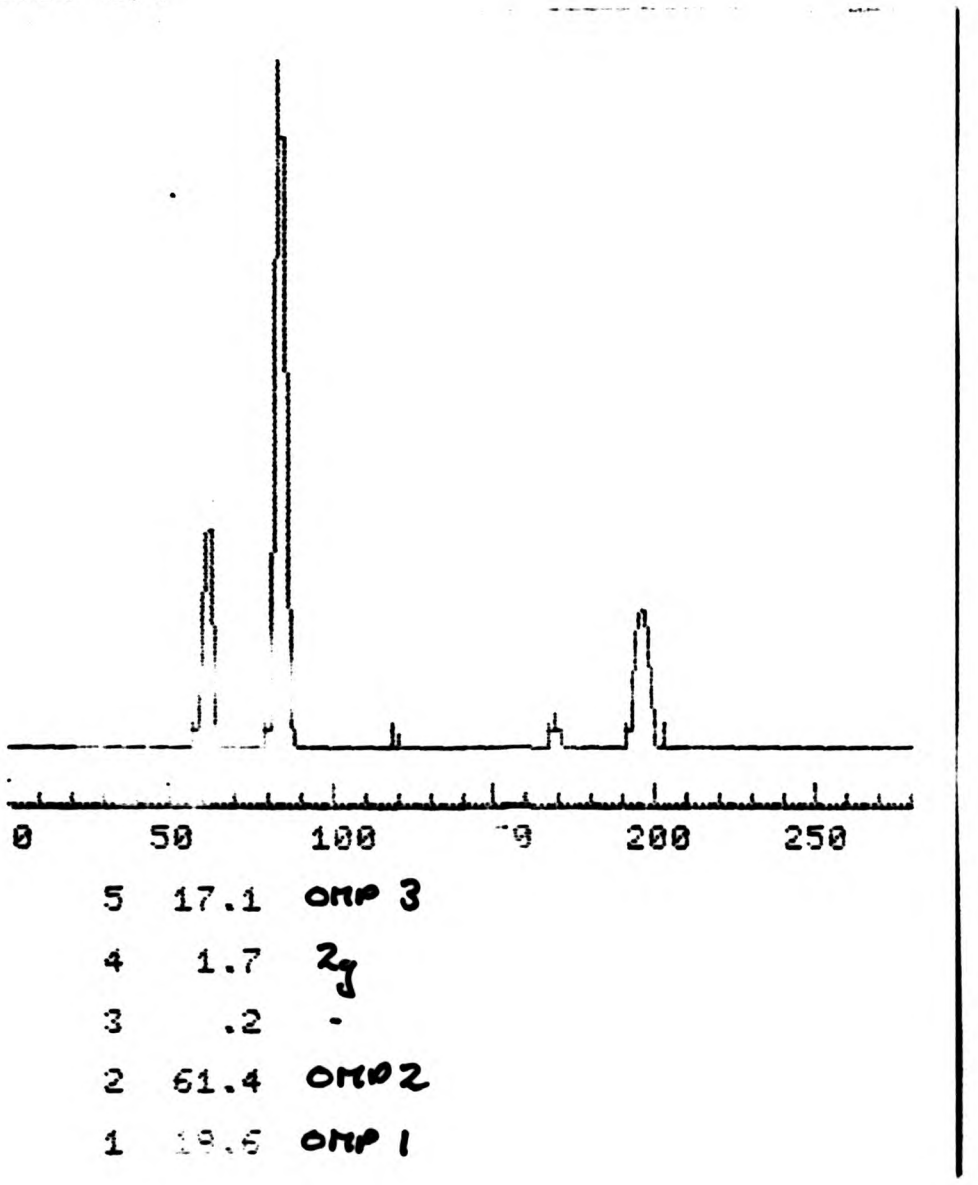
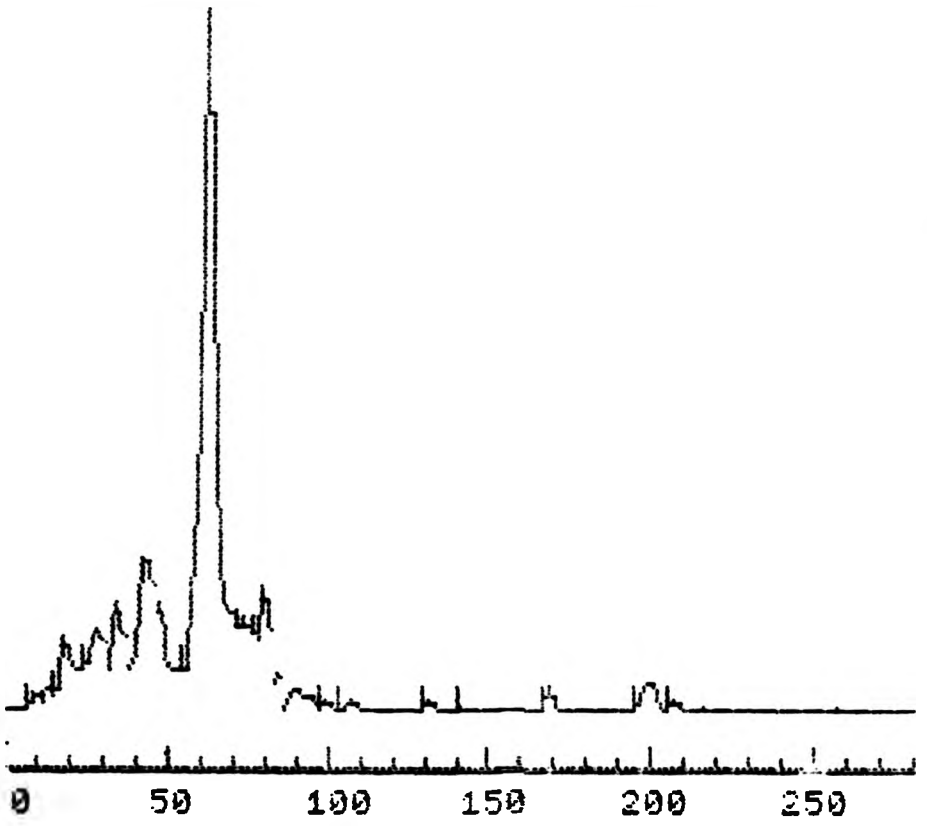


Figure 5.5. c).



17	1.5	OMP 3
16	.6	2_g
15	.0	-
14	.2	-
13	.3	-
12	.2	-
11	1.7	2c
10	.8	-
9	5.5	OMP 2
8	5.0	la
7	50.9	OMP 1
6	6.5	f
5	10.6	e
4	5.2	d
3	4.6	c
2	5.1	b
1	1.3	a

against relative mobility in the gel and were found to be; 54000, 45000 and 18300 respectively (figure 5.6). Polypeptide band 2g was observed only with PAGE Blue 83 being seen as a 'negative' band in silver stained gels.

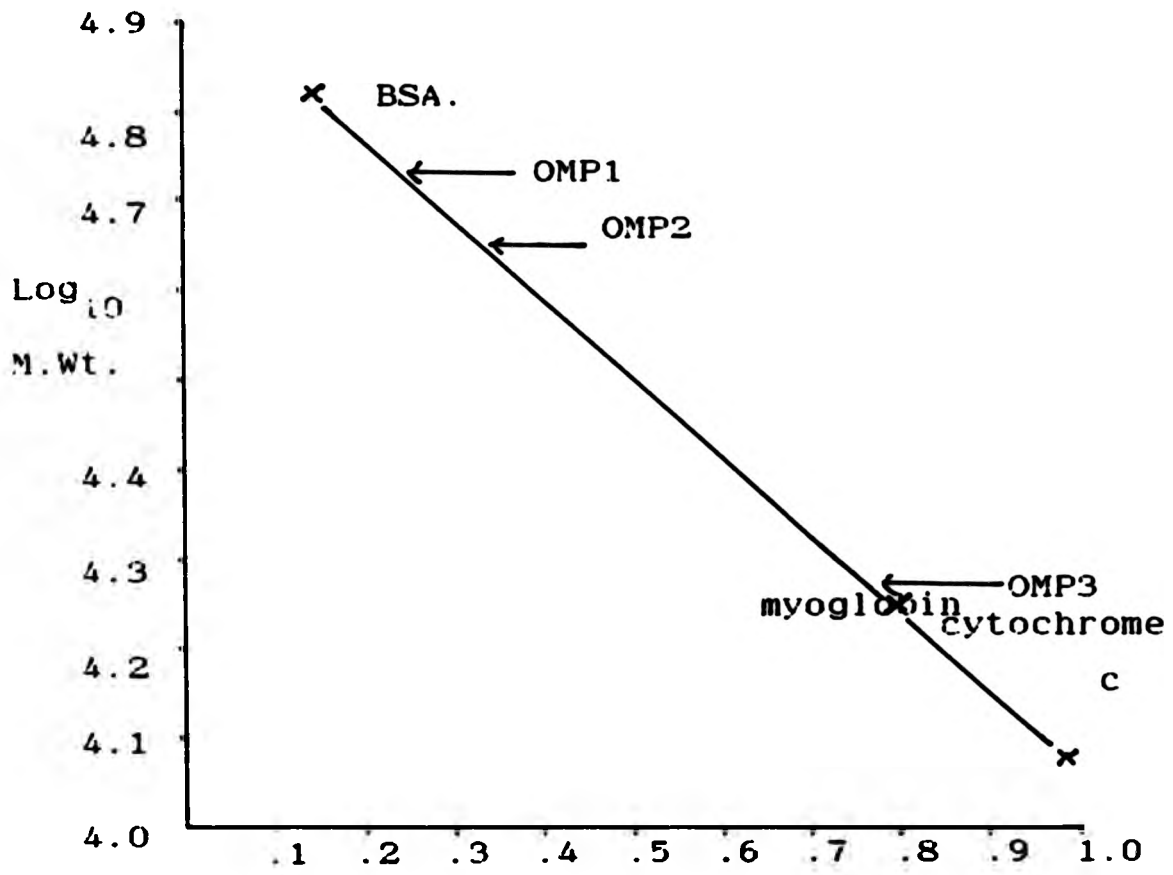
The analysis of Woolwich C-Fe Sarkosyl cell walls by SDS-PAGE showed a considerable simplification of the polypeptide pattern when compared with C+Fe cell walls. This was shown well in PAGE Blue 83 stained gels but is also seen with the enhanced sensitivity of silver staining. C-Fe Sarkosyl cell walls retained only the major OMP s 1, 2 & 3, LPS bands and faint minor bands b, 2a and 3b. although these latter minor bands and LPS are only visible in silver stained gels. SDS-PAGE analysis was also performed on several other strains of D.vulgaris C+Fe Sarkosyl prepared cell walls. These separations are shown in Plate 5.10.

5.1.4. Protection, digestion and OMP extraction.

Plate 5.11. shows the SDS-PAGE analysis of Woolwich C-Fe Sarkosyl cell walls after 'protection', protease digestion or further solubilisation with detergent. Only LPS material was removed after further solubilisation with 2% SDS at 30°c, whereas only OMP 1 was partially retained after a similar treatment at 60°c. OMP 1 was also the only polypeptide band retained after treatment with protease although several small digest fragments are seen on the gel. LPS material is of course also resistant to protease action.

A preincubation or 'protection' of C-Fe cells with Fe^{2+} prior to Sarkosyl cell wall isolation gave an enhanced retention

Figure 5.6. Determination of M.Wt. for major OMP s found in C-Fe Sarkosyl cell walls of D.vulgaris Woolwich, using a plot of $\log_{10}$ M.Wt. standard proteins against their relative mobilities in SDS-PAGE.



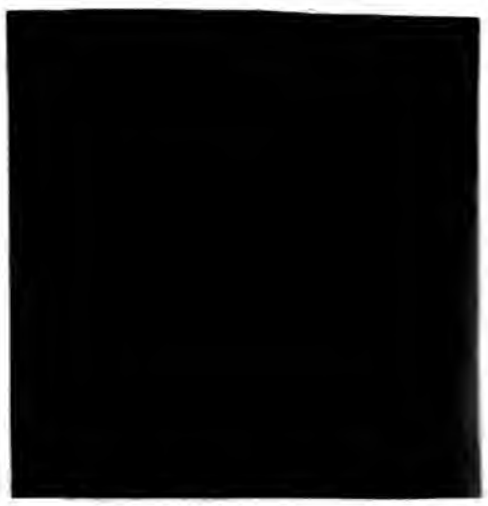
Results of a single typical gel separation were used.

M.Wt's.; OMP1= 54000
OMP2= 45000
OMP3= 18300.

Plate 5.10. SDS-PAGE analysis of Sarkosyl cell walls from several species and strains of Desulfovibrio.

1 2 3 4 5 6 7 8 9 10

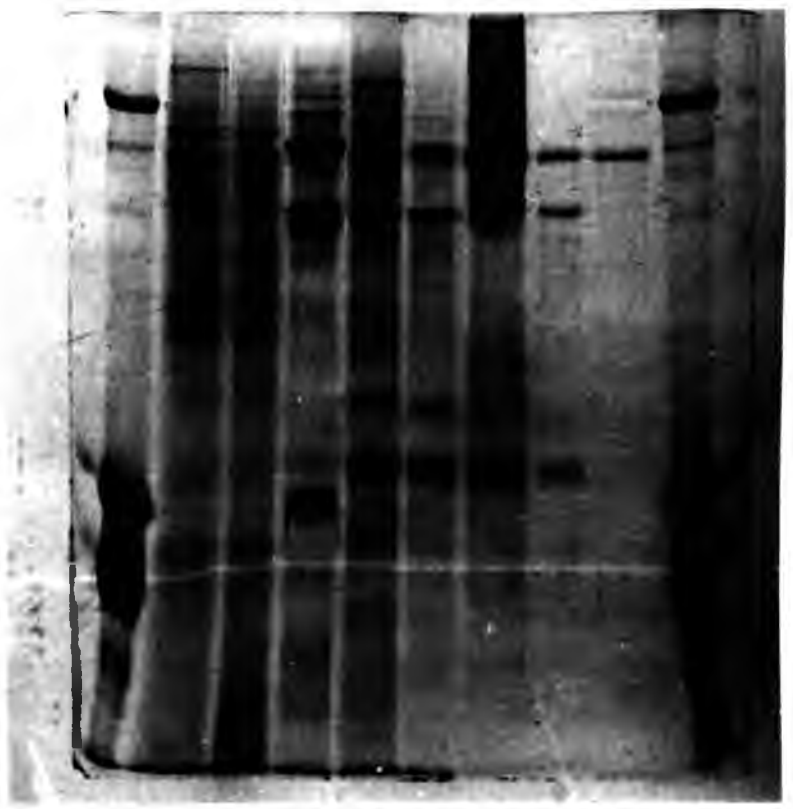
Gel stained using PAGE Blue 83. All cultures except lane 8 are grown in medium C+Fe. Lanes 1 & 10 protein markers. Lane 2, D.desulfuricans; lane 3, D.africanus; lane 4, D.vulgaris (Venezuela); lane 5, D.vulgaris (Holland); lane 6, D.vulgaris (Hildenborough); lane 7, D.vulgaris (Woolwich); lane 8, D.vulgaris (Woolwich) C-Fe; lane 9, D.vulgaris (Woolwich) EDTA washings.



1 2 3 4

Inset showing close-up of OMPs 1 & 2 stained using the Silver method. Note slight band variation and colour. Lane 1, Woolwich C-Fe; lane 2, Woolwich C+Fe; lane 3, Hildenborough; lane 4, Venezuela.

Plate 5.10. SDS-PAGE analysis of Sarkosyl cell walls from several species and strains of Desulfovibrio.



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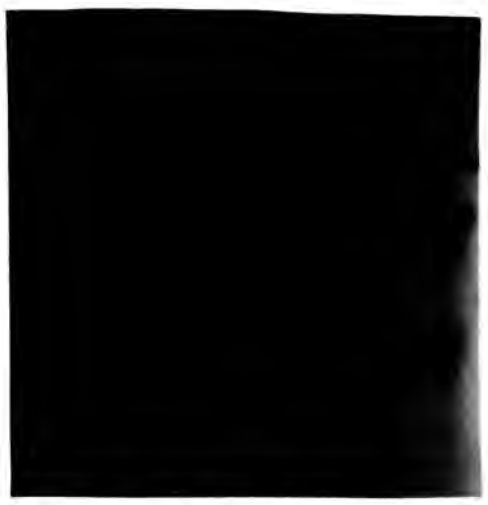

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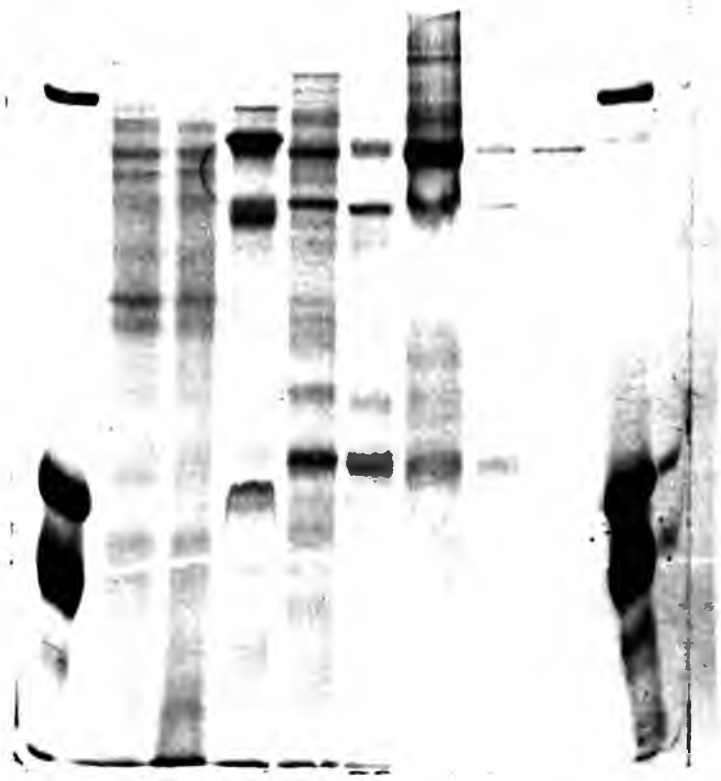
Plate 5.10. SDS-PAGE analysis of Sarkosyl cell walls from several species and strains of Desulfovibrio.



1 2 3 4 5 6 7 8 9 10

Gel stained using PAGE Blue 83. All cultures except lane 8 are grown in medium C+Fe. Lanes 1 & 10 protein markers. Lane 2, D.desulfuricans; lane 3, D.africanus; lane 4, D.vulgaris (Venezuela); lane 5, D.vulgaris (Holland); lane 6, D.vulgaris (Hildenborough); lane 7, D.vulgaris (Woolwich); lane 8, D.vulgaris (Woolwich) C-Fe; lane 9, D.vulgaris (Woolwich) EDTA washings.

1 2 3 4

Inset showing close-up of OMPs 1 & 2 stained using the Silver method. Note slight band variation and colour. Lane 1, Woolwich C-Fe; lane 2, Woolwich C-Fe; lane 3, Hildenborough; lane 4, Venezuela.

Plate 5.11. SDS-PAGE analysis of D.vulgaris Woolwich C-Fe Sarkosyl cell walls after 'protection', protease digestion or further solubilisation with SDS.

1 2 3 4 5 6 7 8 9 10

Lanes 1-5 stained using PAGE Blue 83, lanes 6-10 using Silver method. Lane 1, protein markers; lane 2, cell wall after 2% SDS at 60°c for 30 minutes; lane 3, Sarkosyl C-Fe cell wall; lane 4, protease digested cell walls; lane 5, 'protected' cell wall with 100µg Fe^{2+} prior to Sarkosylisation; lane 6, Sarkosyl C-Fe cell wall; lane 7, cell wall after 2% SDS at 30°c for 15 minutes; lane 8, as lane 4; lane9, as lane 5; lane 10, Sarkosyl C+Fe cell walls.

Plate 5.11. SDS-PAGE analysis of D.vulgaris Woolwich C-Fe Sarkosyl cell walls after 'protection', protease digestion or further solubilisation with SDS.

1 2 3 4 5 6 7 8 9 10

Lanes 1-5 stained using PAGE Blue 83, lanes 6-10 using Silver method. Lane 1, protein markers; lane 2, cell wall after 2% SDS at 60°c for 30 minutes; lane 3, Sarkosyl C-Fe cell wall; lane 4, protease digested cell walls; lane 5, 'protected' cell wall with 100µg Fe^{2+} prior to Sarkosylisation; lane 6, Sarkosyl C-Fe cell wall; lane 7, cell wall after 2% SDS at 30°c for 15 minutes; lane 8, as lane 4; lane9, as lane 5; lane 10, Sarkosyl C+Fe cell walls.

Plate 5.11. SDS-PAGE analysis of D.vulgaris Woolwich C-Fe Sarkosyl cell walls after 'protection', protease digestion or further solubilisation with SDS.

1 2 3 4 5 6 7 8 9 10

Lanes 1-5 stained using PAGE Blue 83, lanes 6-10 using Silver method. Lane 1, protein markers; lane 2, cell wall after 2% SDS at 60°c for 30 minutes; lane 3, Sarkosyl C-Fe cell wall; lane 4, protease digested cell walls; lane 5, 'protected' cell wall with 100µg Fe^{2+} prior to Sarkosylisation; lane 6, Sarkosyl C Fe cell wall; lane 7, cell wall after 2% SDS at 30°c for 15 minutes; lane 8, as lane 4; lane9, as lane 5; lane 10, Sarkosyl C+Fe cell walls.

of OMP's 2 and 3 (especially evident in PAGE Blue 83 stained gels), together with a slight increase in the higher M.Wt. bands otherwise only seen in C+Fe cell walls. Pre- or post-sonication incubation with Fe^{2+} or the use of a short burst of hydrogen sulphide gas prior to Sarkosylisation gave no change in the result and furthermore the 'protections' were never able to completely restore the SDS-PAGE band pattern of C+Fe cell walls.

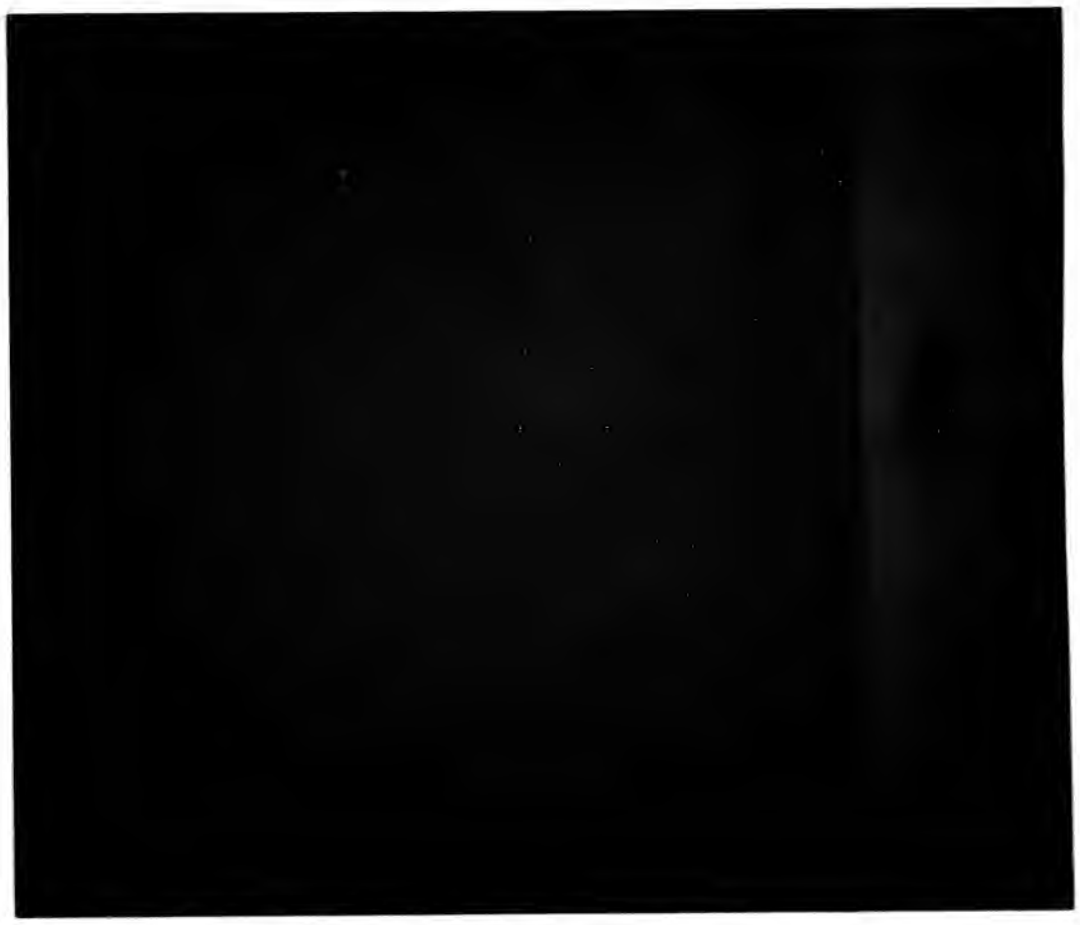
The extraction of C-Fe Sarkosyl cell walls with 50% acetic acid caused the removal of OMP's 2 & 3 together with a portion of OMP 1 and some LPS material. On dialysis against distilled water to remove acetic acid the extracted proteins were seen to form a milky suspension. Prior 'EDTA washing' of the whole cells reduced the amounts of extractable LPS and OMP 1 (subsequently called OMP 1a) yielding mainly OMP's 2 & 3 in the acetate extract.

Protease resistant OMP 1 could not be acetate extracted and was subsequently called OMP 1b. This protein was retained in the residual acetate-extracted pellet.

5.1.5 Polar lipid extraction and Thin Layer Chromatography (TLC).

Polar lipids were successfully extracted from both D.vulgaris Woolwich C+Fe cell envelopes and Sarkosyl cell walls. The material was of a faint yellow colour when dried under nitrogen. A typical TLC separation is shown in Plate 5.12. Cell envelope lipid extract gave three phosphomolybdic acid stained spots of mobility; 13.5, 11.4 and 8.2 cms. with a faint spot at 10.1cms. The lipids from Sarkosyl cell walls gave a similar separation but with much fainter visualisation

Plate 5.12. Typical separation of C+Fe D.vulgaris Woolwich cell envelope polar lipid extract by TLC.
Lanes 1, 6 & 7 envelope lipids; lane 2, phosphatidyl ethanolamine; lane 3, phosphatidyl glycerol; lane 4, cardiolipin; lane 5, phosphatidyl serine.



α-napthol spray | phosphomolybdic acid spray | ninhydrin spray

Sample	Mobility of spot(s), cms.
Lipid extract	13.5, 11.4, (10.1), 8.2.
P.ethanolamine	8.2
P.glycerol	8.6
Cardiolipin	10.6
P.serine	2.5

Plate 5.12. Typical separation of C-Fe D.vulgaris Woolwich cell envelope polar lipid extract by TLC.
Lanes 1, 6 & 7 envelope lipids; lane 2, phosphatidyl ethanolamine; lane 3, phosphatidyl glycerol; lane 4, cardiolipin; lane 5, phosphatidyl serine.

α-napthol spray | phosphomolybdic acid spray | ninhydrin spray

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P.glycerol	8.6
Cardiolipin	10.6
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Plate 5.12. Typical separation of C-Fe D.vulgaris Woolwich cell envelope polar lipid extract by TLC.
Lanes 1, 6 & 7 envelope lipids; lane 2, phosphatidyl ethanolamine; lane 3, phosphatidyl glycerol; lane 4, cardiolipin; lane 5, phosphatidyl serine.

α-napthol spray | phosphomolybdic acid spray | ninhydrin spray

Sample	Mobility of spot(s), cms.
Lipid extract	13.5, 11.4, (10.1), 8.2.
P.ethanolamine	8.2
P.glycerol	8.6
Cardiolipin	10.6
P.serine	2.5

of the latter three spots. The 8.2cm. spot had a mobility the same as phosphatidylethanolamine standard and gave its characteristic red spot with ninhydrin spray reagent. The faster moving 13.5cms. spot gave a positive rose-pink reaction with α-napthol glycolipid spray. The two fainter spots were not identified although both had a mobility close to that of cardiolipin standard.

5.1.6. Gas liquid chromatography (GLC).

Successful GLC separations were obtained for standard bacterial fatty acid methyl esters (FAME s) and with FAME s prepared from Woolwich C+Fe cell envelopes and Sarkosyl C+Fe cell walls, by the acid methanolysis procedure. These are shown in Figures 5.7, 5.8 and 5.9. Relative peak areas were calculated and expressed as a percentage of the total peak area. The area of each peak was calculated as;

Area = Peak height × width at ½ height.

The results are shown in Figures 5.10 and 5.11. It is seen that the unknown 'U2' is the most abundant fatty acid component in both fractions together with large amounts of 18:0, ai17:0 and 16:0. The C18 fatty acids show a different ratio in the two fractions.

5.1.7. Isprenoid quinone extraction.

Material was extracted from Woolwich C+Fe cell envelopes and the extract analysed by TLC. The separation shown in Figure 5.12 was achieved. The faster moving band corresponding to Menadione standard, was eluted from the plate and its U/V spectrum recorded. This is shown in Figure 5.13. This appeared to be characteristic of a menaquinone in exhibiting several

Figure 5.7. Typical GLC separation achieved using commercial standard bacterial FAMEs (Supelco).

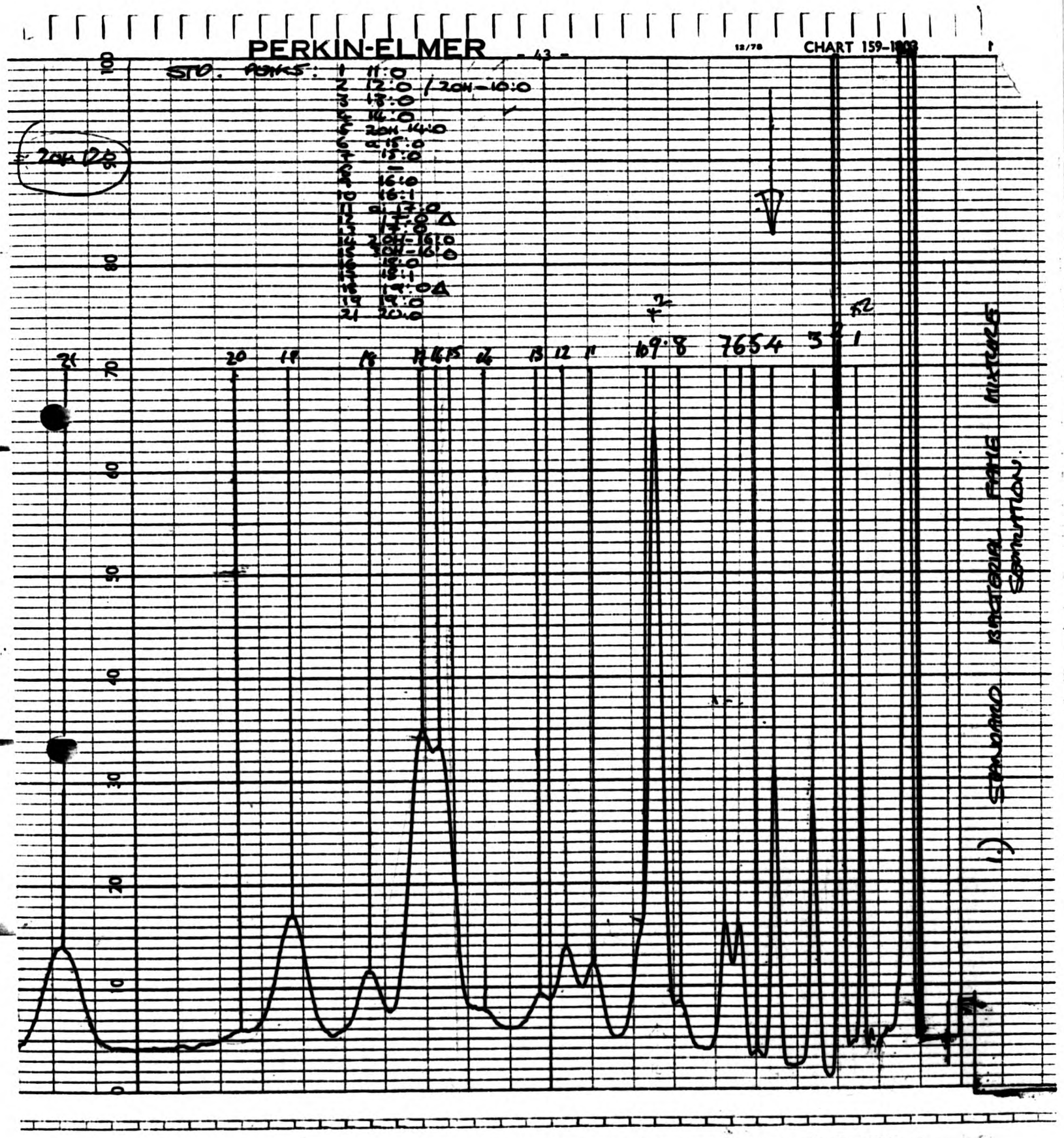


Figure 5.8. Typical GLC separation achieved using FAMEs prepared from <u>D.vulgaris</u> Woolwich C+Fe cell envelopes.

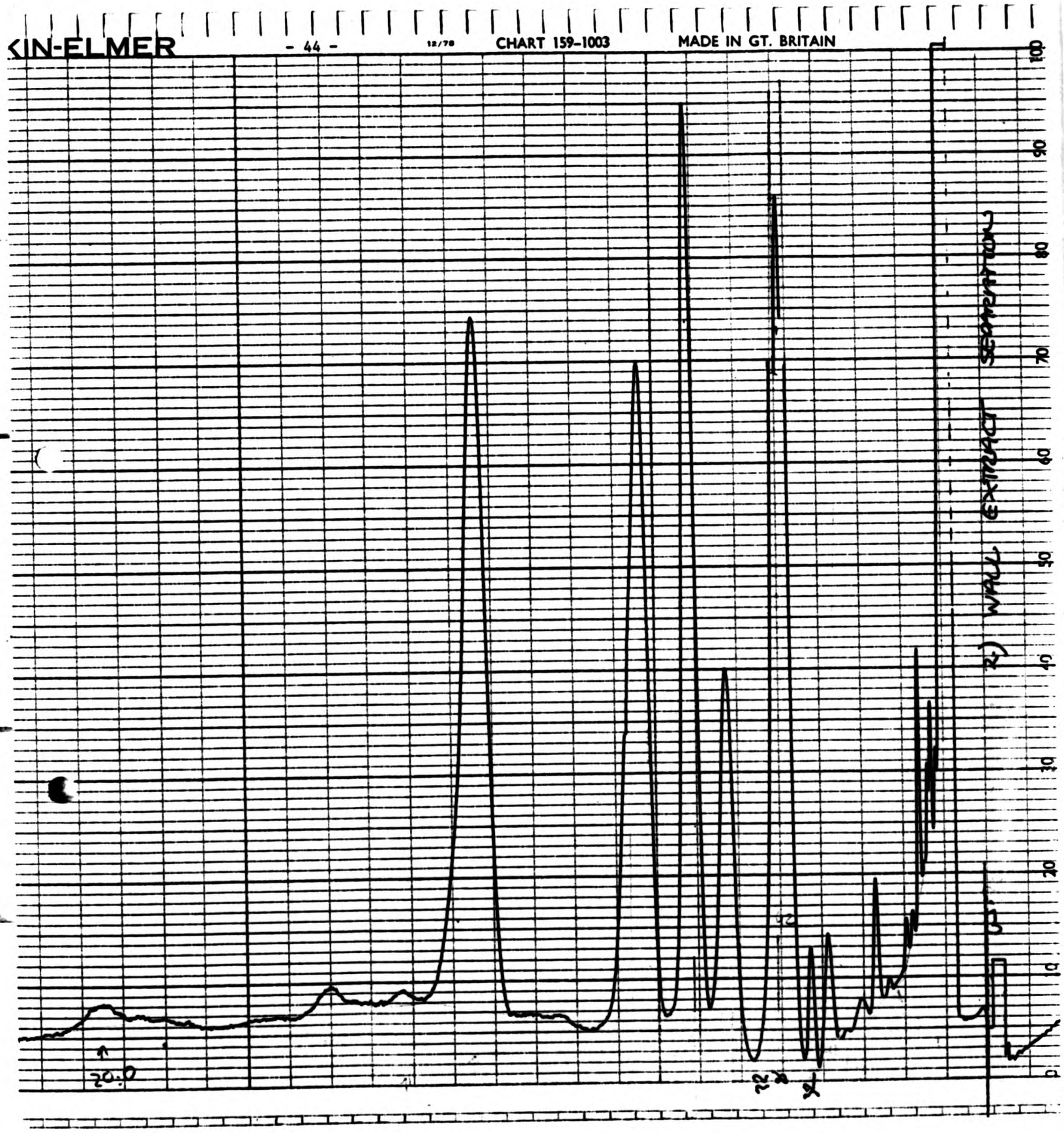


Figure 5.9. Typical GLC separation achieved using FAMEs prepared from <u>D.vulgaris</u> Woolwich C+Fe Sarkosyl prepared cell walls.

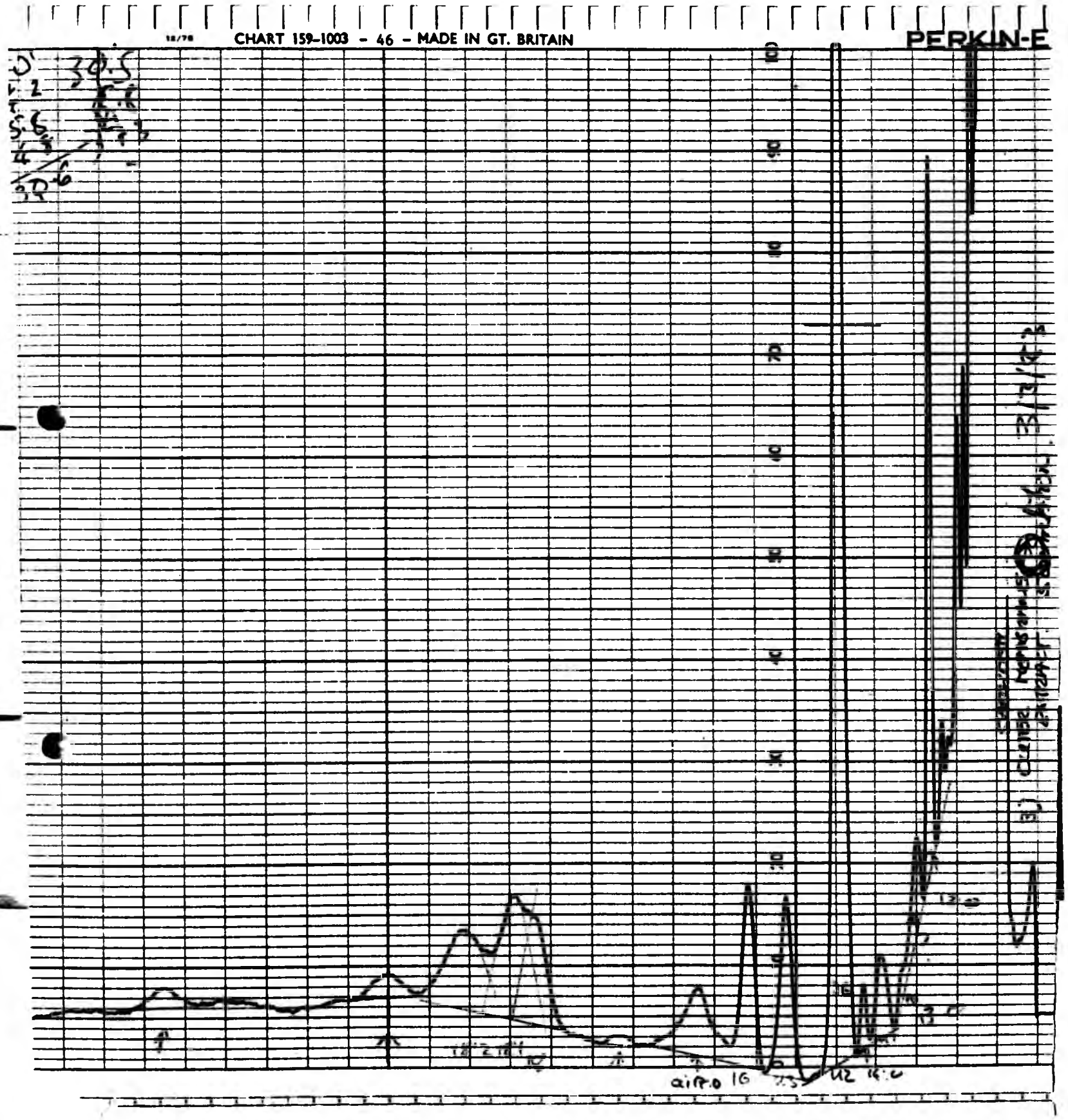


Figure 5.10. GLC separation of FAME's from D.vulgaris C+Fe cell envelope shown as percentage of total peak area against retention time.

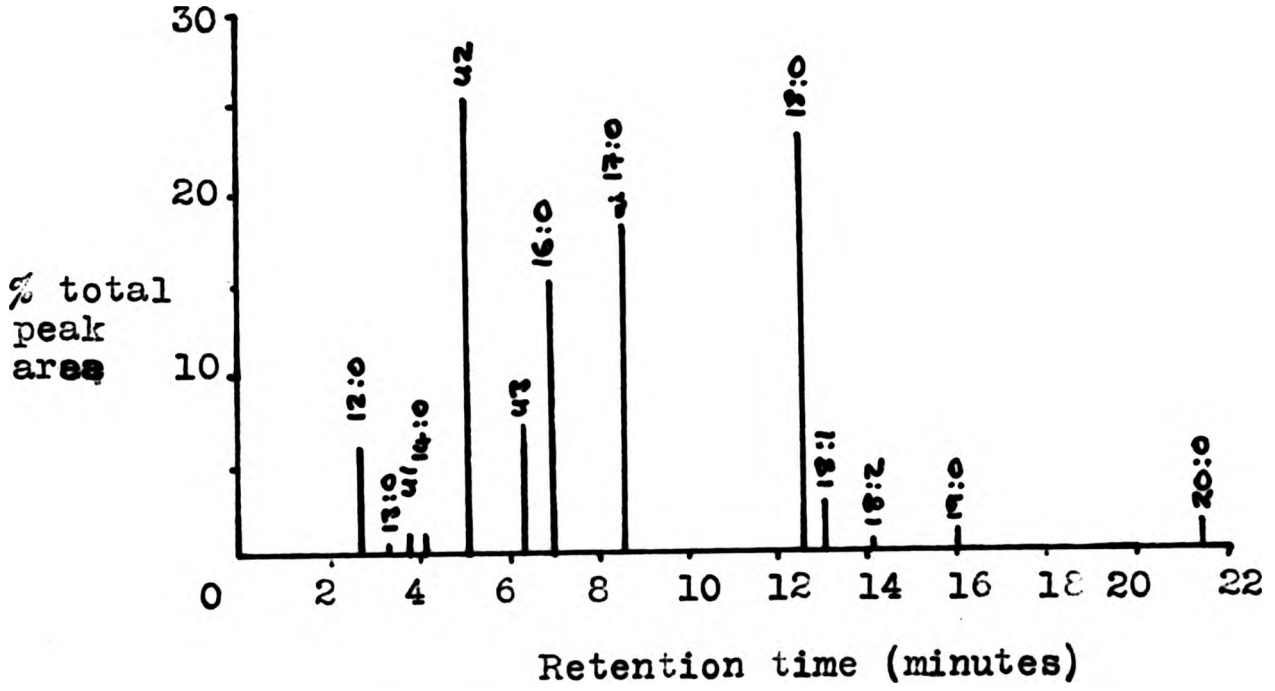


Figure 5.11. GLC separation of FAME's from D.vulgaris C+Fe Sarkosyl cell walls shown as percentage total peak area against column retention time.

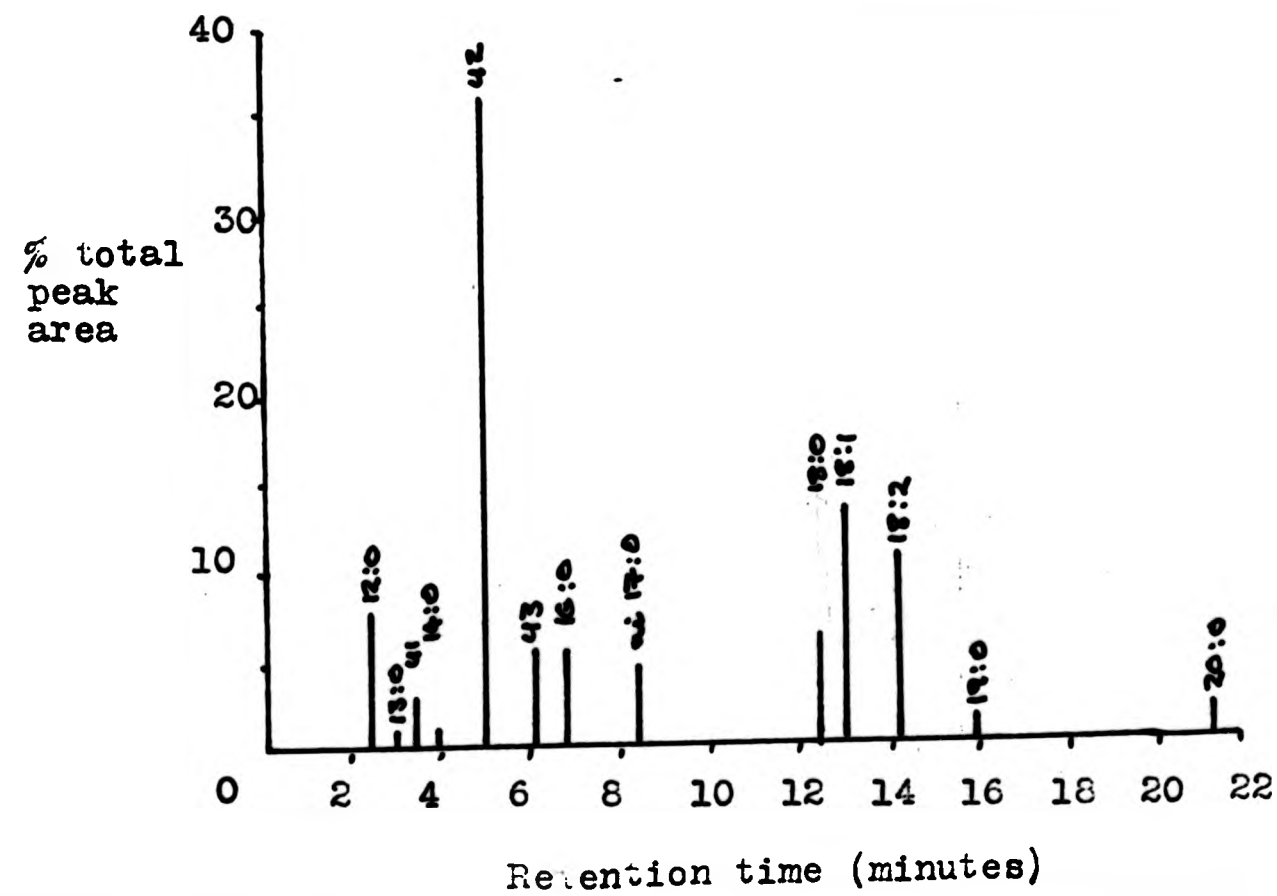
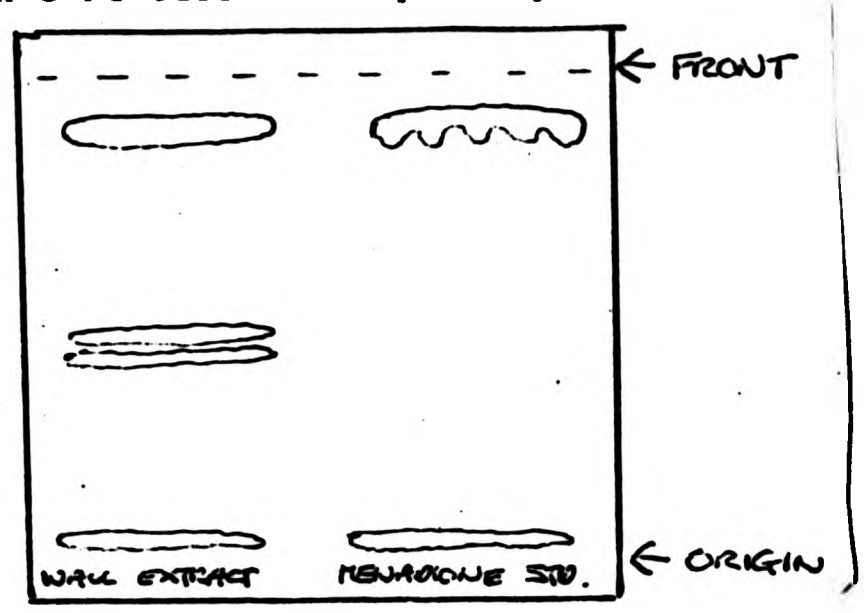


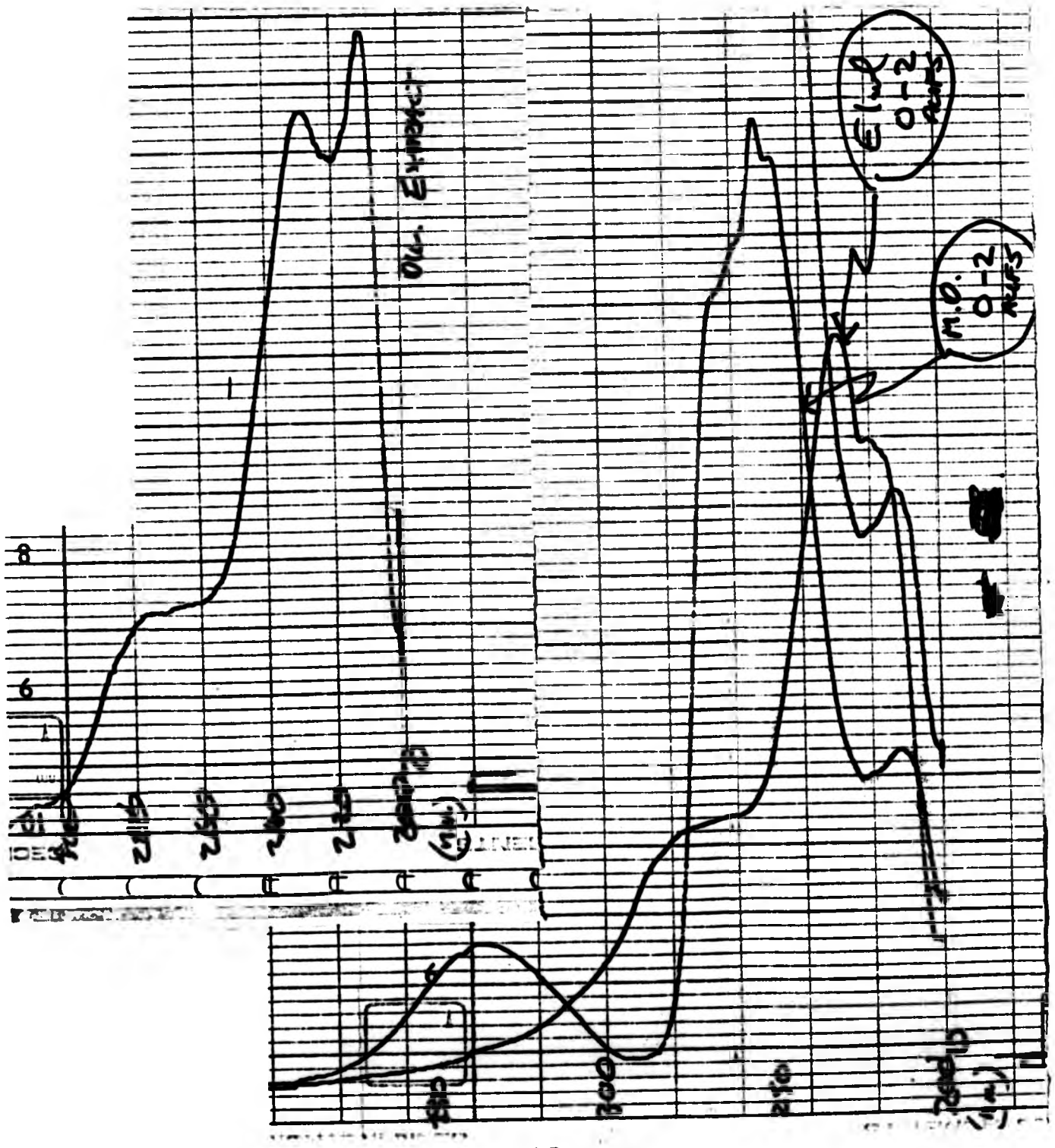
Figure 5.12. Diagrammatic representation of a separation of a D.vulgaris Woolwich C+Fe cell envelope isoprenoid quinone extract by TLC.



Menaquinones
R_f=0.8

Ubiquinones
R_f=0.4

Figure 5.13. Ultraviolet spectra of material prepared by TLC separation of isoprenoid quinone extract of D.vulgaris Woolwich C+Fe cell envelopes.



peaks. Menaquinones exhibit adsorption maxima at 242, 248, 260, 269 and 369nm. Ubiquinones show only a single peak usually at about 270nm.

Much smaller amounts of menaquinone material were isolated from Sarkosyl C+Fe cell walls for an equivalent dry weight of material, but relatively large amounts of starting material were needed in order to extract a reasonable amount of menaquinone for quantitative comparisons between the two fractions to be made. Further quantitative extractions were therefore not attempted.

5.2. Other isolation methods.

5.2.1. Poxton/Brown isolation.

Fractions obtained using D.vulgaris Woolwich C+Fe cells were analysed for protein content and by SDS-PAGE. The results are shown in Figure 5.14 and Plate 5.13. It should be remembered that the initial EDTA 'wash' would remove 3-8% of the total cell protein. Up to 28% of the cell protein is removable by the Poxton/Brown method, substantially more than by mere EDTA 'washing'. Only a small amount of this is pelletable by high-speed centrifugation.

SDS-PAGE of the pellet fraction shows a complex band pattern. A band corresponding to the mobility of OMP1 in Sarkosyl cell walls is prominent together with fainter bands for OMPs 2 and 3. The supernatant fraction is reminiscent of an EDTA 'wash' separation (which is shown for comparison in Plate 5.13) including the prominent 54000 M.Wt. band.

Electron microscopy of a thin section of the pellet fraction (Plate 5.14) revealed single membrane vesicles together with some granular material.

Figure 5.14. Protein content of fractions obtained from D.vulgaris Woolwich C+Fe by the Poxton/Brown method.

Fraction		% total cell protein.
Residual mureinoplast pellet.		70-85%
OM fraction.	supernatant	1.5-28%
	pellet	0.5-3.4%

Ranges are from four separate experiments.

Plate 5.13. SDS-PAGE analysis of Poxton/Brown OM fractions from D.vulgaris Woolwich C+Fe stained using PAGE Blue 83.

Lane 1, protein markers; lane 2, EDTA washings; lane 3, Poxton/Brown pellet fraction; lane 4. Poxton/Brown supernatant fraction.

Figure 5.14. Protein content of fractions obtained from D.vulgaris Woolwich C+Fe by the Poxton/Brown method.

Fraction		% total cell protein.
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Lane 1, protein markers; lane 2, EDTA washings; lane 3, Poxton/Brown pellet fraction; lane 4, Poxton/Brown supernatant fraction.

Plate 5.14. Poxton/Brown OM pellet fraction from D.vulgaris Woolwich C+Fe cells. Thin section x100000. (bar=0.1µm).

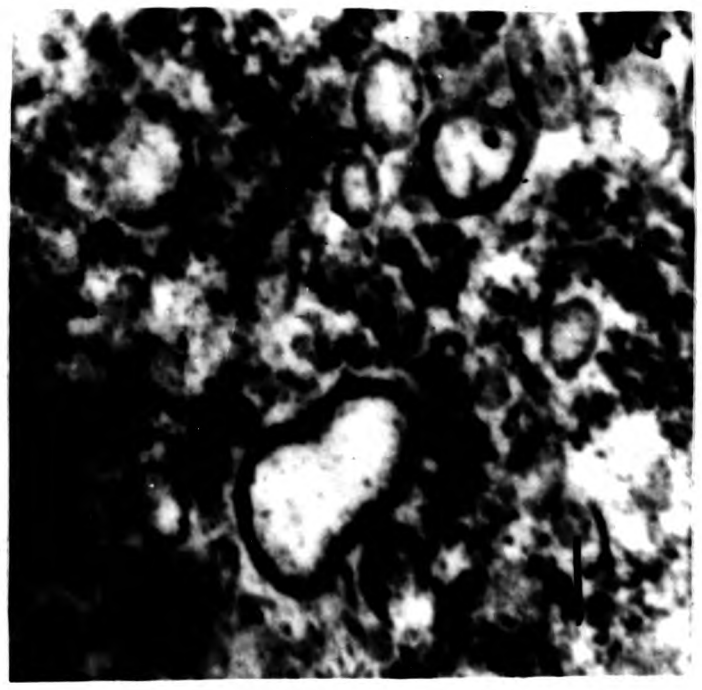


Plate 5.15. Pellet obtained by sucrose density gradient centrifugation (Osborn method) of lysed spheroplasts from D.vulgaris Woolwich C+Fe cells. Thin section x100000. (bar=0.1µm)

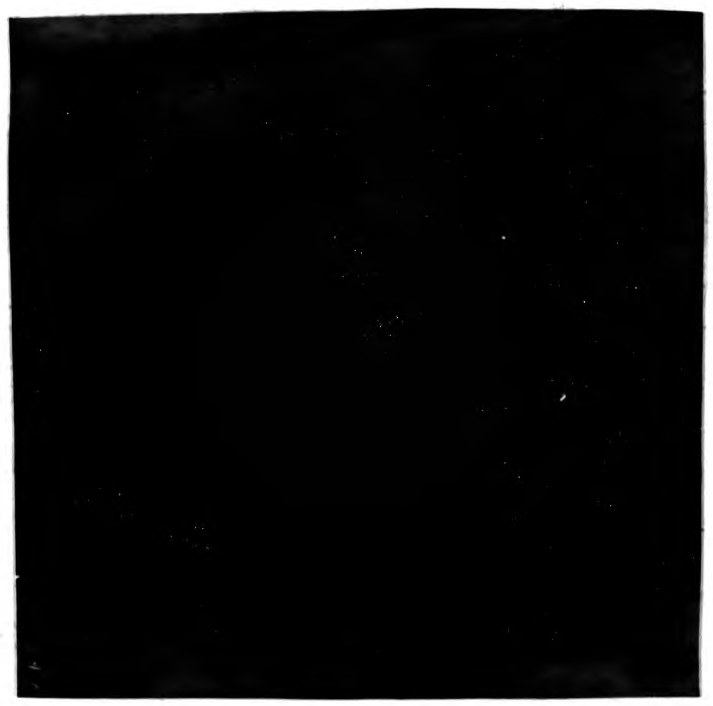
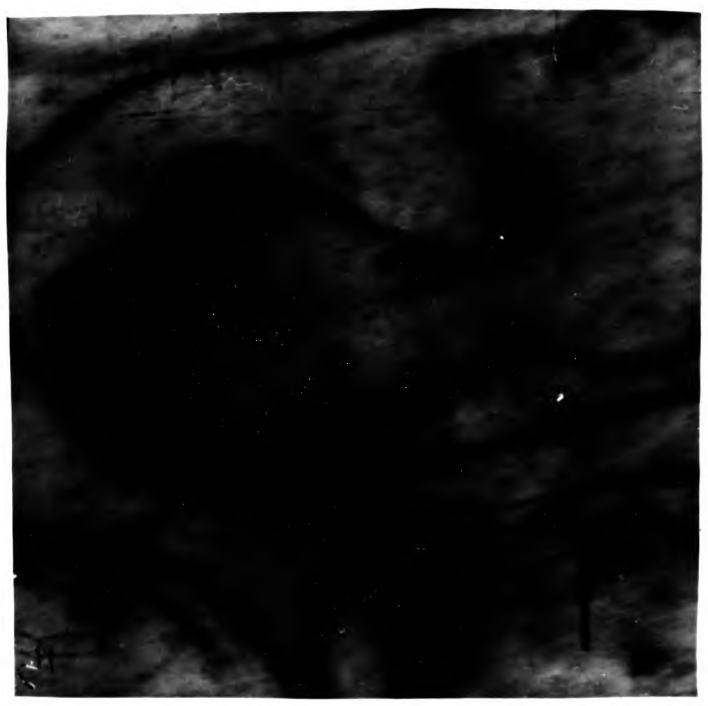


Plate 5.14. Poxton/Brown OM pellet fraction from D.vulgaris Woolwich C+Fe cells. Thin section x100000. (bar=0.1µm).

Plate 5.15. Pellet obtained by sucrose density gradient centrifugation (Osborn method) of lysed spheroplasts from D.vulgaris Woolwich C+Fe cells. Thin section x100000. (bar=0.1µm)



The use of EDTA in this method and the release of LPS material by this reagent makes the use of LPS as an OM marker in this system unreliable.

5.2.2. Sucrose density gradient separations.

The attempted separation using the 'classical' method of Osborn et al (1972), did not achieve a resolution of the lysed spher oplasts on the sucrose gradient. A single pellet was obtained at the bottom of the centrifuge tube. An increase in the sucrose cushion concentration to 60%w/v yielded no better separation. Examination of a thin section of this pellet by electron microscopy (Plate 5.15) showed what appeared to be plasmolysed or lysed cells lacking in cytoplasmic contents but with both CM and OM present. The OM did seem to be lacking in the fuzzy internal layer seen to be present in Sarkosyl cell walls (Plate 5.6) and in cell walls of intact cells (Plate 3.6). The spheroplasting and osmotic lysis was therefore deemed to be successful.

The separation of both sonically prepared Woolwich C-Fe cell envelopes (Figure 5.15) and C+Fe cell envelopes (Figure 5.16) on the modified (two step, 60-70%w/v) sucrose gradient did achieve a resolution into three distinct bandings. These were easily removed and analysed for protein and by SDS-PAGE. Although small differences were noted, the three fractions for both types of cell envelope appeared to be identical in their major band patterns. The LPS marker for OM was present in all of the fractions and the major OMP's seen in Sarkosyl cell walls were also present in all fractions.

The addition of lysozyme, 'spheroplast lysis,' or lengthening

Figure 5.15. Separation of D.vulgaris Woolwich C-Fe sonically prepared cell envelopes on two-step sucrose density gradient showing a) separation, b)SDS-PAGE analysis, c) profile of bands.

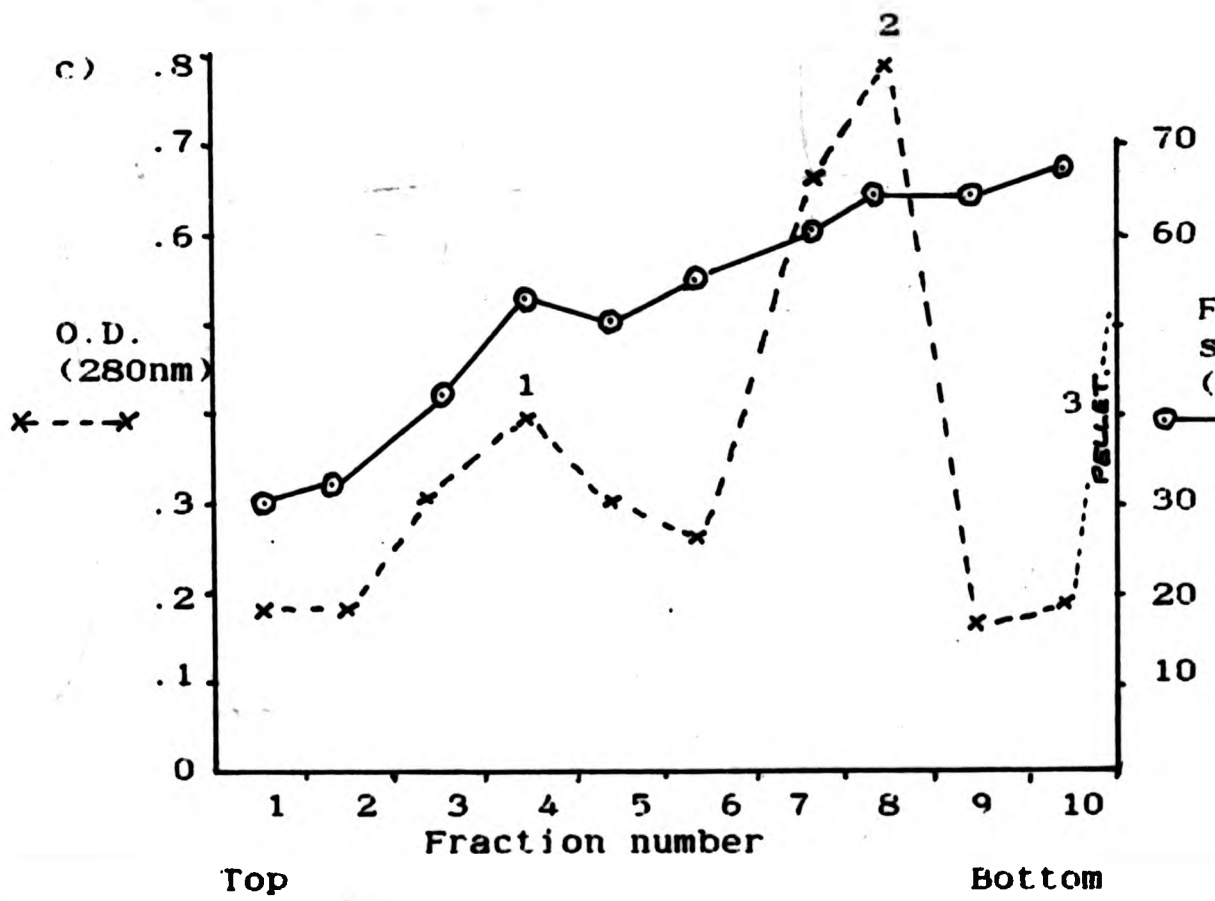


Figure 5.15. Separation of D.vulgaris Woolwich C-Fe sonically prepared cell envelopes on two-step sucrose density gradient showing a) separation, b)SDS-PAGE analysis, c) profile of bands.

a)

Band.

1

2

3

Pellet

b)

Lane 1; Band 1
2; Band 2
3; Band 3
4; Sarkosyl C+Fe OM
5; Sarkosyl C-Fe OM.

c)

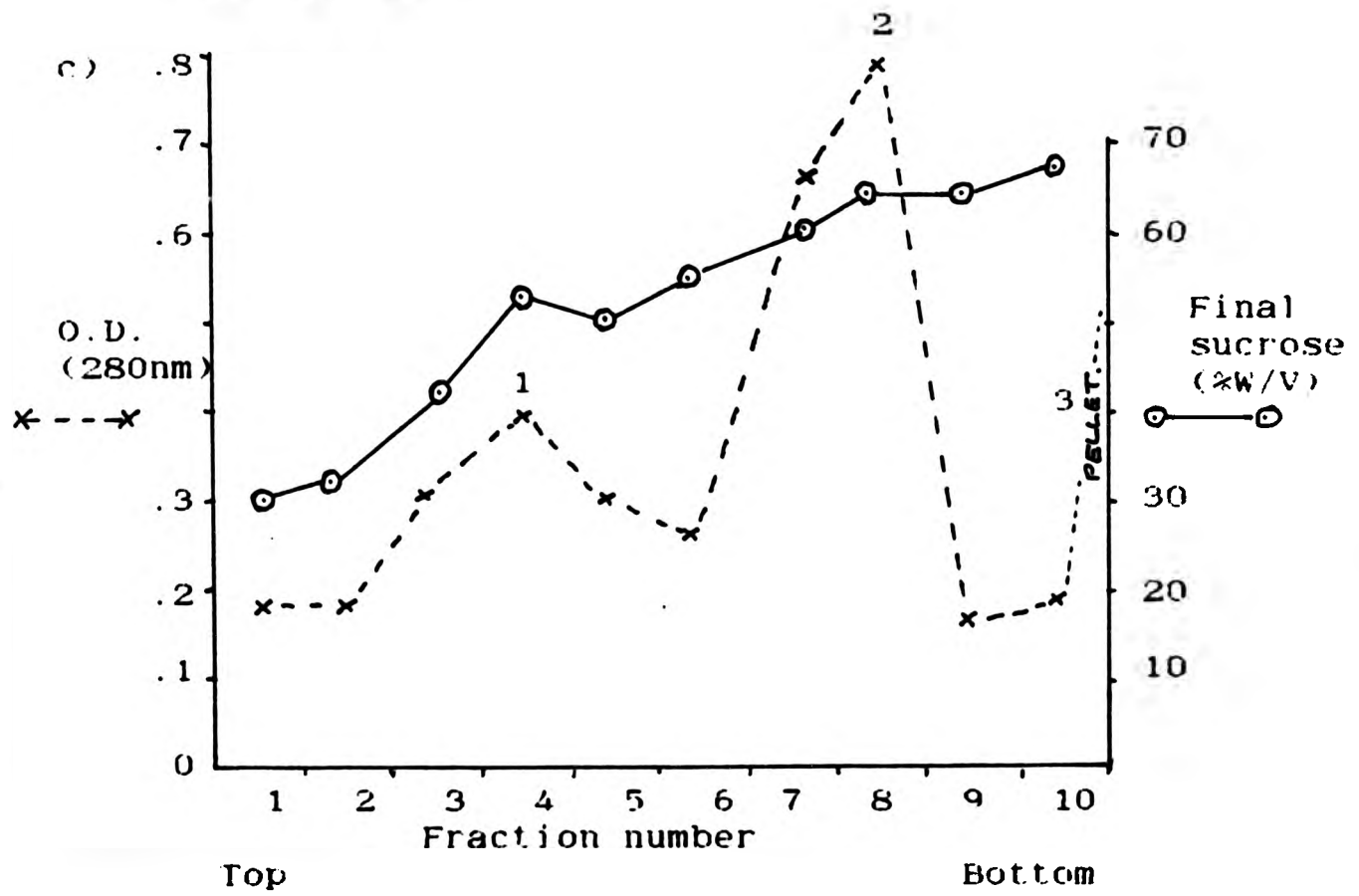


Figure 5.15. Separation of D.vulgaris Woolwich C-Fe sonically prepared cell envelopes on two-step sucrose density gradient showing a) separation, b)SDS-PAGE analysis, c) profile of bands.

a)

b)

c)

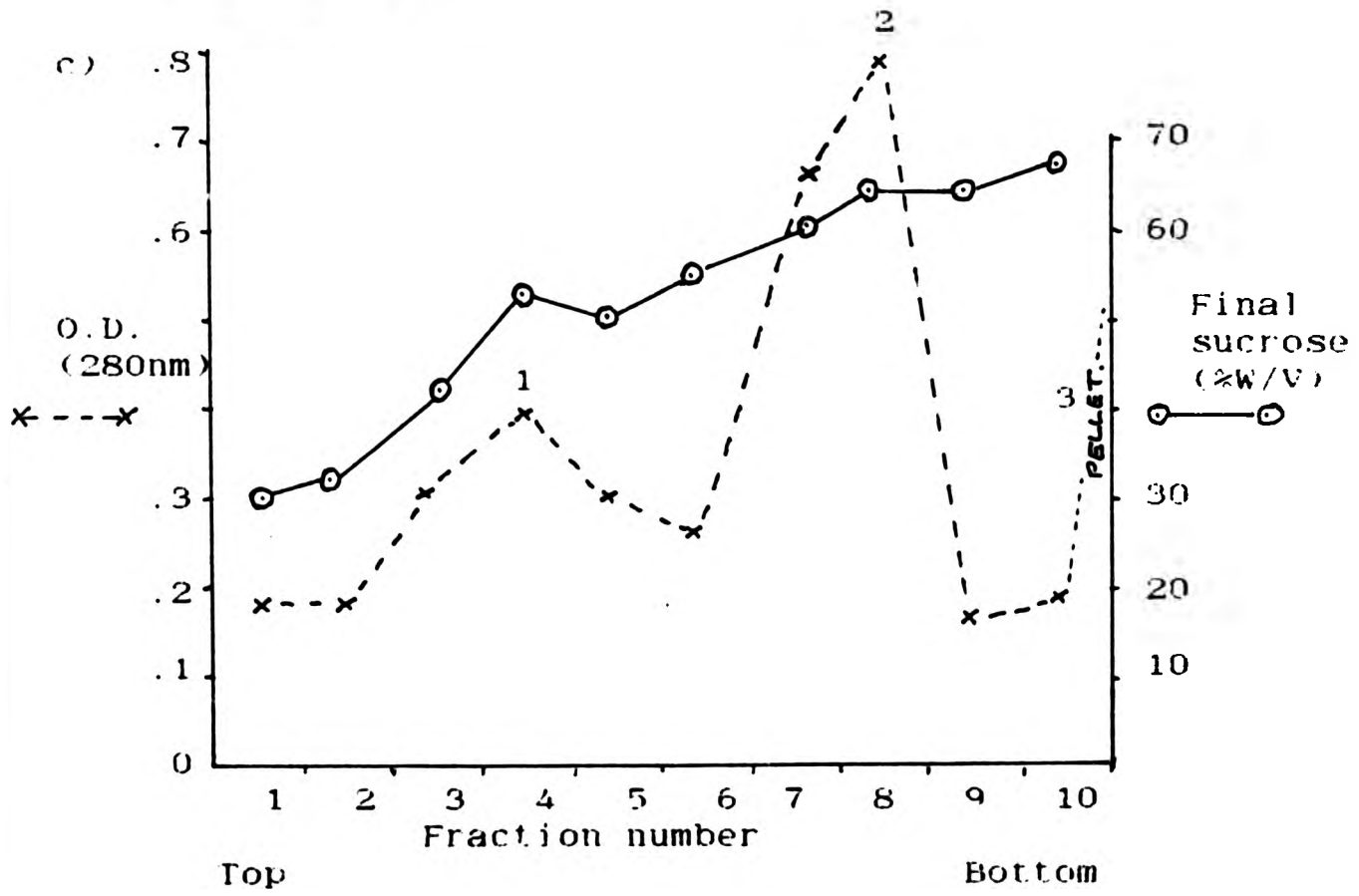


Figure 5.16. Separation of D.vulgaris Woolwich C+Fe sonically prepared cell envelopes on two-step sucrose density gradient (60-70%W/V) showing a) separation and b) SDS-PAGE analysis.

a)

Band.

‹ 1

‹ 2

‹ 3 (pellet)

b)

Lane; 1, Sarkosyl C+FE OM
2, Sarkosyl C-Fe
3, Band 1
4, Band 2
5, Band 3 pellet

Figure 5.16. Separation of D.vulgaris Woolwich C+Fe sonically prepared cell envelopes on two-step sucrose density gradient (60-70%W/V) showing a) separation and b) SDS-PAGE analysis.

a)

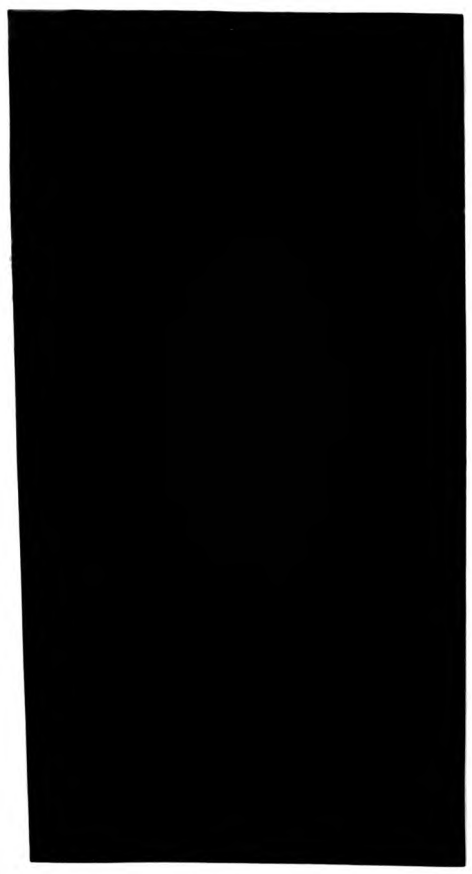
Band.

< 1

< 2

< 3 (pellet)

b)



Lane; 1, Sarkosyl C+FE OM
2, Sarkosyl C-Fe
3, Band 1
4, Band 2
5, Band 3 pellet

Figure 5.16. Separation of D.vulgaris Woolwich C+Fe sonically prepared cell envelopes on two-step sucrose density gradient (60-70%W/V) showing a) separation and b) SDS-PAGE analysis.

a)

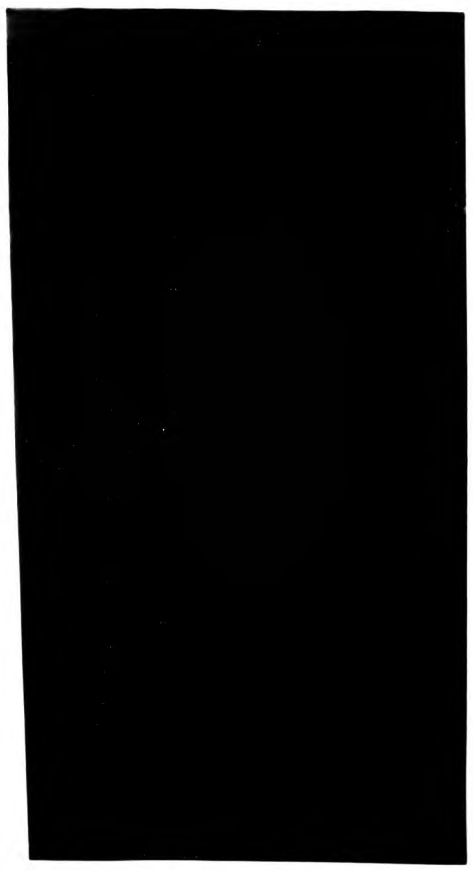
Band.

< 1

< 2

< 3 (pellet)

b)



Lane; 1, Sarkosyl C+FE OM
2, Sarkosyl C-Fe
3, Band 1
4, Band 2
5, Band 3 pellet

Figure 5.16. Separation of D.vulgaris Woolwich C+Fe sonically prepared cell envelopes on two-step sucrose density gradient (60-70%W/V) showing a) separation and b) SDS-PAGE analysis.

a)

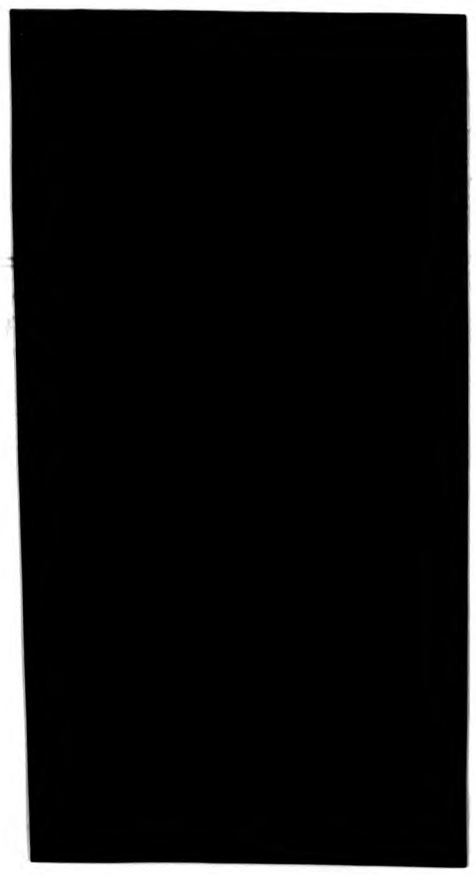
Band.

‹ 1

‹ 2

‹ 3 (pellet)

b)



Lane; 1, Sarkosyl C+FE OM
2, Sarkosyl C-Fe
3, Band 1
4, Band 2
5, Band 3 pellet

Figure 5.16. Separation of D.vulgaris Woolwich C+Fe sonically prepared cell envelopes on two-step sucrose density gradient (60-70%W/V) showing a) separation and b) SDS-PAGE analysis.

a)

Band.

< 1

< 2

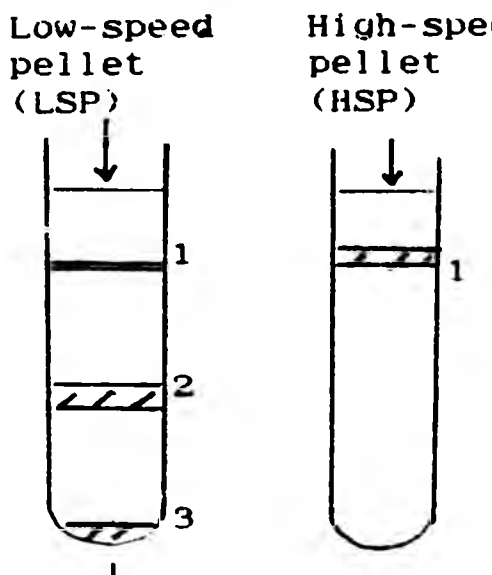
< 3 (pellet)

b)

Lane; 1, Sarkosyl C+FE OM
2, Sarkosyl C-Fe
3, Band 1
4, Band 2
5, Band 3 pellet

Figure 5.17. Separation of D.vulgaris Woolwich C-Fe cell walls on two-step sucrose density gradient after preliminary low-speed spin (20000xg) and high-speed spin (90000xg), showing a) sketch of separations, b) SDS-PAGE analysis, c) profile of bands. The position of Sarkosyl C-Fe OM on the two-step gradient is also shown.

a)



b)

Lanes; 1=Sarkosyl OM.C-Fe
2=HSP1
3=LSP1
4=LSP2
5=LSP3 pellet

c)

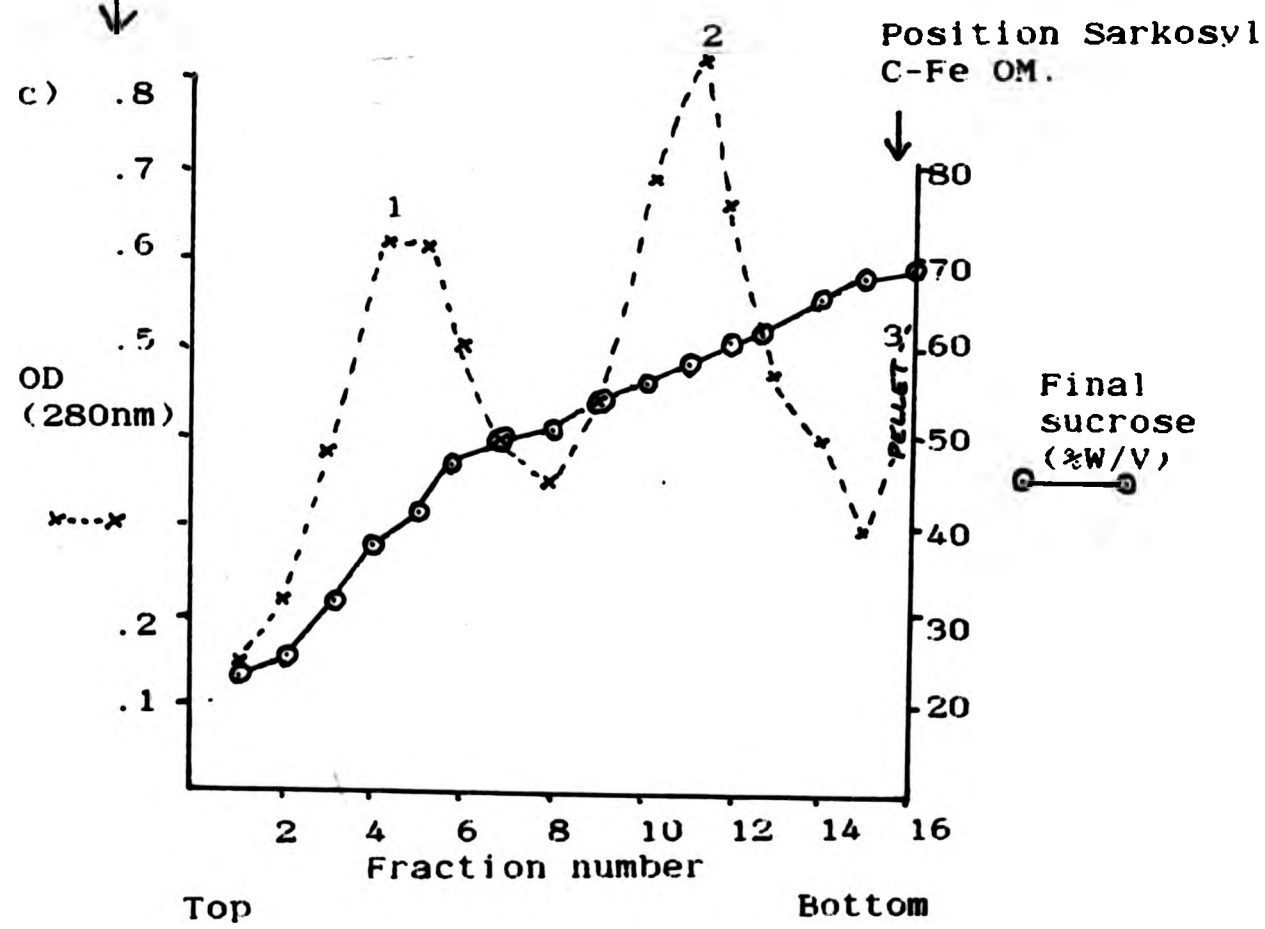
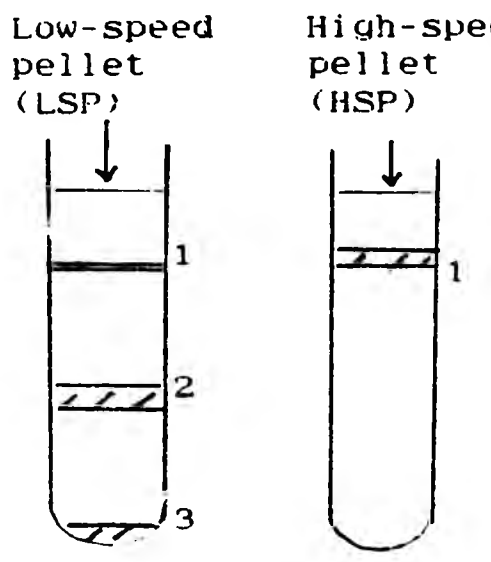


Figure 5.17. Separation of D.vulgaris Woolwich C-Fe cell walls on two-step sucrose density gradient after preliminary low-speed spin (20000xg) and high-speed spin (90000xg), showing a) sketch of separations, b) SDS-PAGE analysis, c) profile of bands. The position of Sarkosyl C-Fe OM on the two-step gradient is also shown.

a)



b)

Lanes; 1=Sarkosyl OM.C-Fe
2=HSP1
3=LSP1
4=LSP2
5=LSP3 pellet

c)

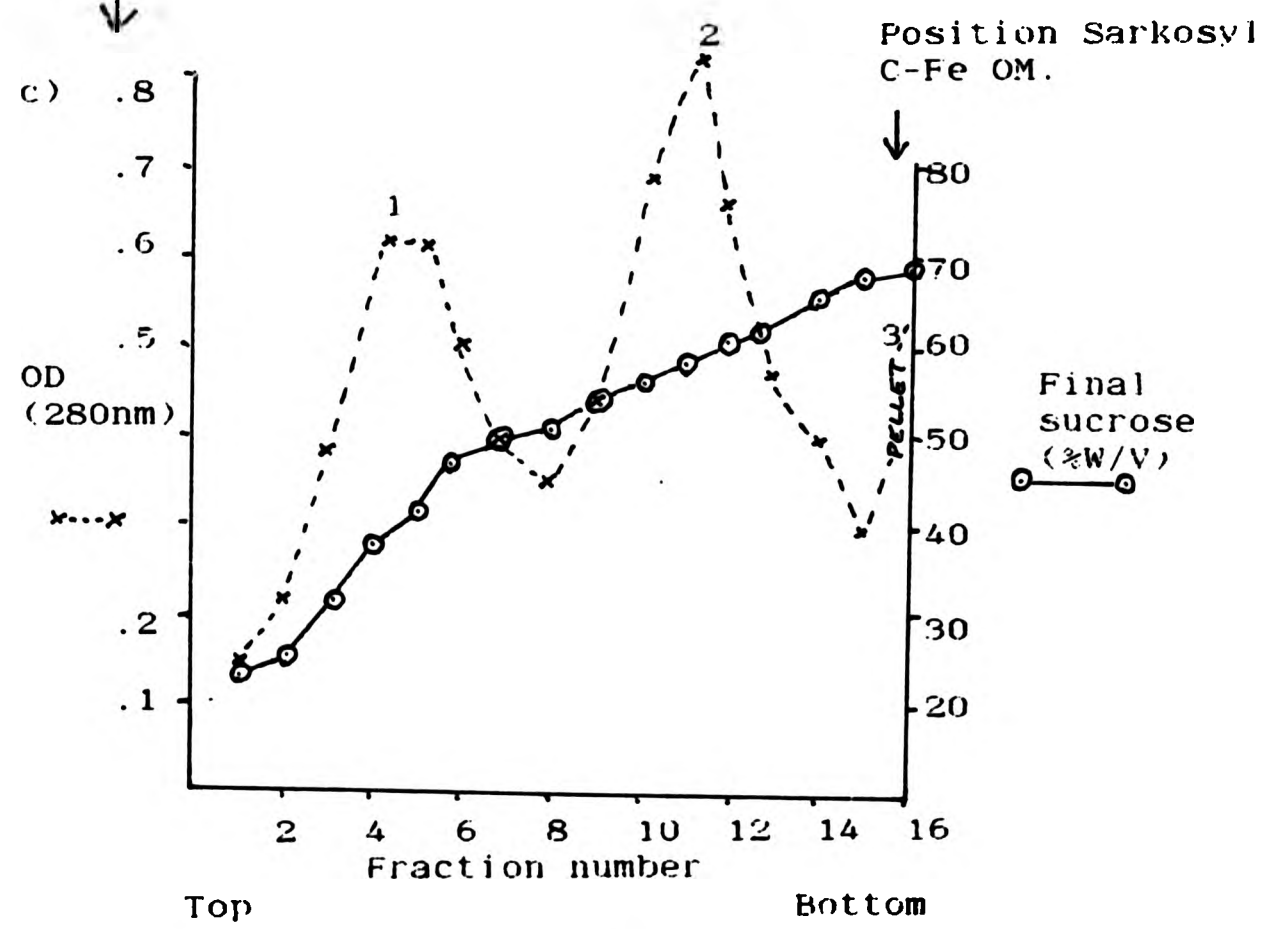
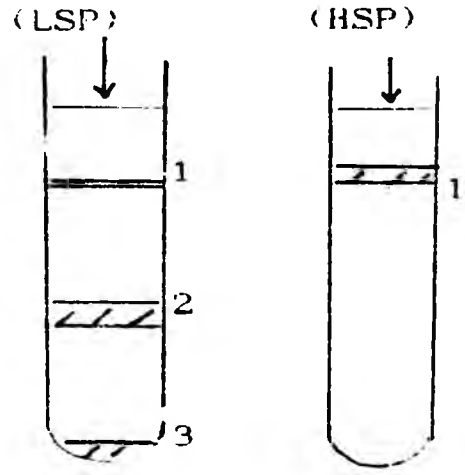


Figure 5.17. Separation of D.vulgaris Woolwich C-Fe cell walls on two-step sucrose density gradient after preliminary low-speed spin (20000xg) and high-speed spin (90000xg), showing a) sketch of separations, b) SDS-PAGE analysis, c) profile of bands. The position of Sarkosyl C-Fe OM on the two-step gradient is also shown.

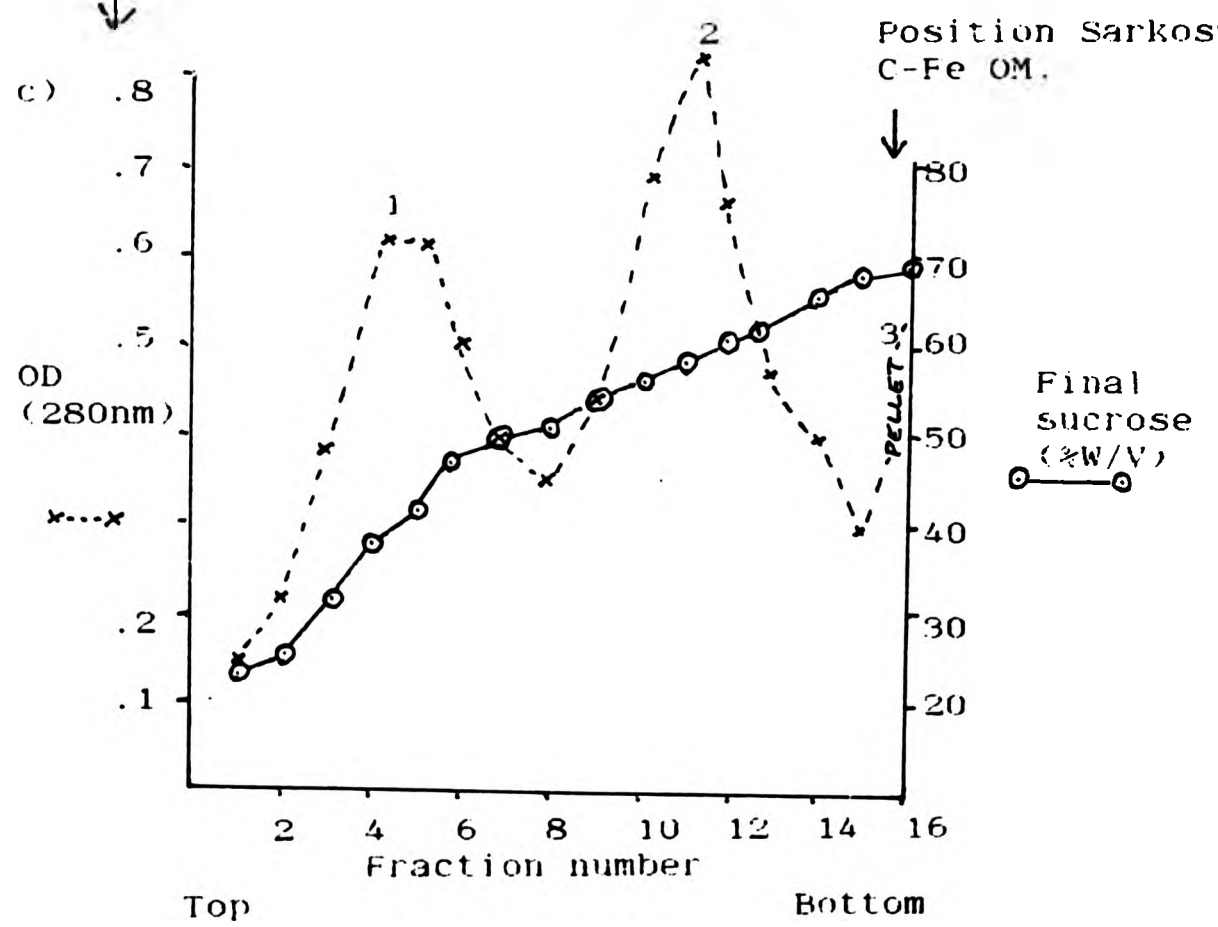
a)



b)

Lanes; 1=Sarkosyl OM.C-Fe
2=HSP1
3=LSP1
4=LSP2
5=LSP3 pellet

c)



of the sucrose gradient (three step, 50-60-70%w/v) all had little or no effect on the separations. A preliminary spin to enrich a pellet in OM appeared only to remove the fraction of lighter density from the gradient (Figure 5.17), otherwise no differences were seen in the fractions obtained. The result of loading a Sarkosyl C-Fe cell wall onto a two step gradient is also shown in Figure 5.17.

5.3. Hydrogenase studies.

D.vulgaris Woolwich C+Fe crude cell lysate (sonicate with whole cells removed) was seen to contain 1.05 U/mg hydrogenase. The evolution of hydrogen gas is seen from Figure 5.18 to be linear with time over the first 5-10 minutes under the conditions used, facilitating the estimation of reaction velocities. Woolwich C-Fe cell lysate contained no detectable activity. However the restoration of approximatly 13% original (C+Fe) hydrogenase activity to C-Fe lysates was possible by either adding Fe^{2+}, prior to cell disruption or a 15 minute incubation at 0°c after disruption. These results are shown in tabular form in Figure 5.19.

The hydrogenase assayed here (Woolwich strain) was very stable to air (Figure 5.20). At least 25% activity still remained after storage for two weeks at 4°c in aqueous solution.

Kinetic parameters for the crude enzyme were calculated from Figures 5.21 and 5.22. From a plot of reaction velocity against substrate concentration, the k_m value for methyl viologen with sodium dithionite was 0.31mM and the V_{max} was 1.1U/mg. (0.2mM and 4600U/mg. are comparable values for the highly purified Hildenborough strain enzyme of

Figure 5.18. Evolution of hydrogen gas against time for D.vulgaris Woolwich cell lysates from C+Fe, C-Fe and 'restored' cultures.

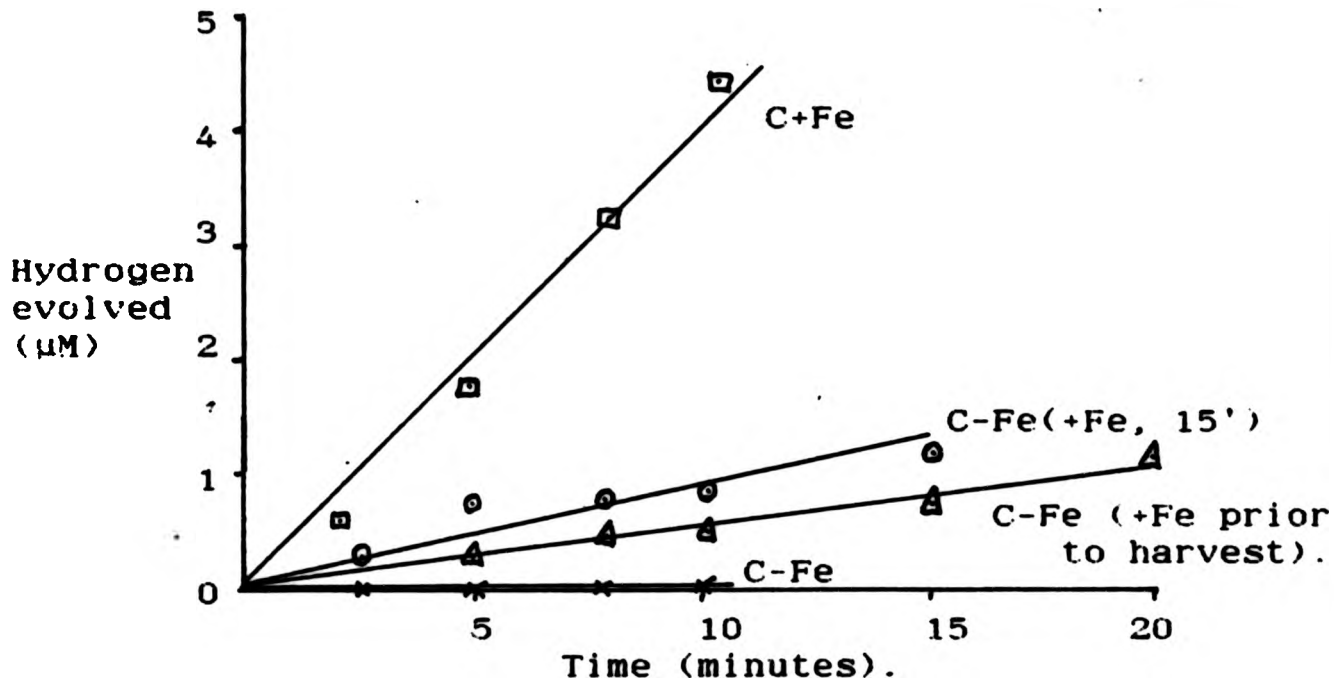


Figure 5.19. Hydrogenase activities in D.vulgaris Woolwich cell lysates from C+Fe, C-Fe and 'restored' cultures.

Cell lysate	Specific activity (Units/mg protein)	% maximal activity.
C+Fe	1.05	100
C-Fe	0	0
C-Fe + 15 minute incubation with Fe^{2+} at 0°c	0.152	13.0
C-Fe + Fe^{2+} added prior to sonication.	0.158	13.5

Figure 5.20. The effect of storage (4°c, aqueous solution in air) upon hydrogenase activity in the cell lysate from D.vulgaris Woolwich C+Fe.

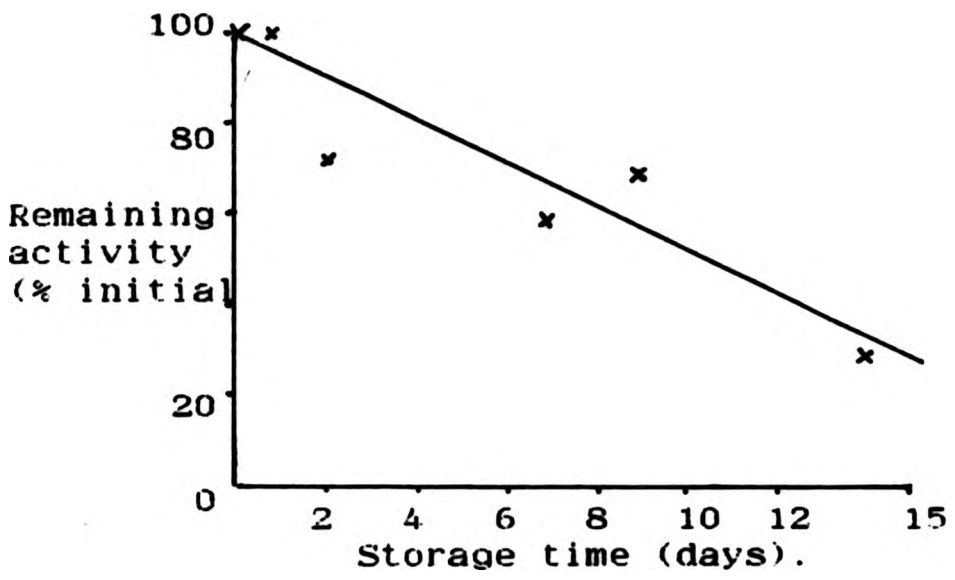


Figure 5.21. Michaelis/Menten plot of reaction rate against substrate concentration (methyl viologen) for D.vulgaris Woolwich C+Fe cell lysate.

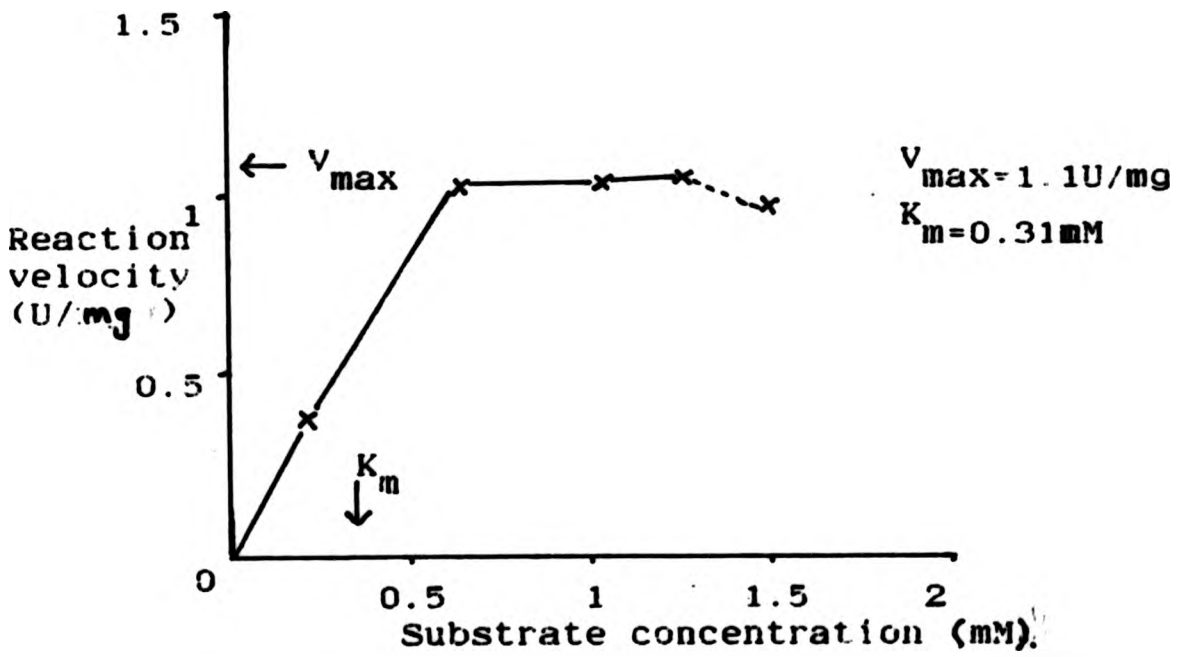


Figure 5.22. Double reciprocal plot (Lineweaver/Burk) of reaction velocity against subtrate concentration (methyl viologen) for D.vulgaris Woolwich C+Fe cell lysate.

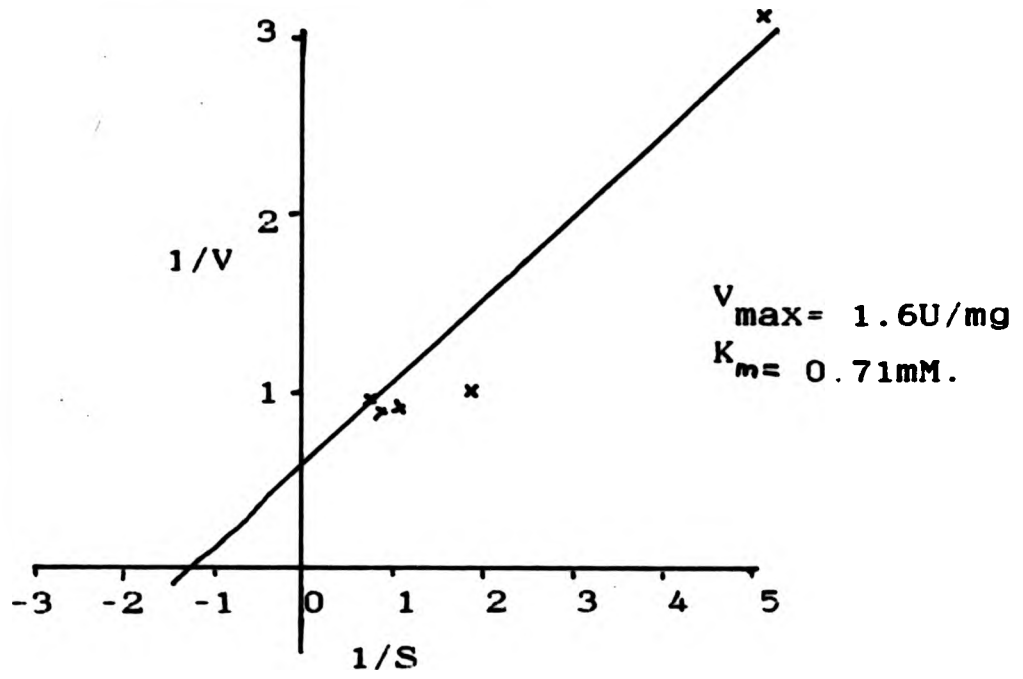


Figure 5.23. Localisation of hydrogenase activity within D.vulgaris Woolwich C+Fe and Hildenborough C+Fe cell fractions.

Cell fraction	Woolwich		Hildenborough	
	% initial activity	specific activity (U/mg)	% initial activity	specific activity (U/mg)
cell lysate	100	3.9	100	2.6
cell envelope (particulate)	13	1.1	19	0.92
cell cytoplasm (soluble)	39	1.8	60	2.1
cell wall (Sarkosyl)	0	0	0	0
cytoplasmic membrane (Sarkosyl soluble)	5.2	0.6	-	-

van der Westen et al, 1978). The available data was not adequate for the potentially more accurate double reciprocal plot to be of use, although Figure 5.22 shows the values so obtained.

On further fractionation of the crude cell-free lysate in both Woolwich and Hildenborough strains only one third of the residual activity remained in the particulate fraction, (Figure 5.23). Upon Sarkosylisation no activity was found to be associated with the cell wall and only 5.2% initial activity in the CM (Woolwich). Attempts to activate the enzyme in cell walls by addition of c ysteine or a crude cytochrome c_3 (C-Fe cytoplasmic fraction) proved fruitless.

DISCUSSION.

The Sarkosyl fractionation method has proved a reproducible procedure for the production of a cell wall fraction from D.vulgaris cells grown in both medium C+Fe and C-Fe. The fraction contains broken vesicles of various sizes composed of a single membrane bilayer (the OM) and an internal electron dense structure (peptidoglycan), which are both identical to the cell wall when seen in the thin section of an intact cell. The lack of EDTA in this procedure is a distinct advantage in view of the results shown in Chapter 4. Its use would most certainly make the isolation of an intact cell wall unlikely. However the possibility of obtaining a 'pure' isolated cell wall is doubtful due to the presence of junction sites (Bayer, 1981) with the CM and flagellar penetration structures. The cell wall (especially OM) should not be regarded as a discrete organelle but as a dynamic structure

in common with other membrane systems. With these points in mind the Sarkosyl method may be regarded as a rapid and reproducible method for the production of a cell wall fraction.

The Poxton/Brown method also appeared to yield a fraction containing cell wall fragments. However the small amount of material produced by this method and the loss of LPS and protein material released by the use of EDTA make this an unsuitable method for routine analytical use.

Sucrose density gradient separations in the form of the 'classic' method (Osborn et al, 1972) were totally unsuccessful. Even after several modifications of this system, D.vulgaris cell envelopes were unamenable to sucrose density gradient OM/CM separation. The fractions obtained probably reflect morphological rather than structural differences. They reflect the various sizes of OM and CM (or both) particles. Thus copurification may be due to; i. certain domains of CM having the same density as OM, ii. membrane fragments of one type becoming entrapped within vesicles of the other membrane, or loosely associated with the other membrane, iii. the existance of adhesion sites. In particular the presence of divalent cations is widely thought to adversely affect the separation of OM and CM in sucrose gradients (Nikaido & Nakae, 1979). This could well be the case for D.vulgaris cultures where Fe^{2+} abounds around the cell wall even in iron restricted media. Divalent cations may cause the annealing of OM and CM (Owen et al. 1982). Difficulties are also experienced where the LPS in the cell wall is lacking in long 'O' side-chains, although this can be offset by retaining

an intact peptidoglycan layer, ie. by not using lysozyme digestion. Ultrasound is not usually used for cell disruption prior to gradient fractionation. French pressure cell lysis is the most common method and this was not possible in these investigations. Lysozyme/ spheroplast lysis however resulted in little improvement in these separations. Extreme use of ultrasound may indeed randomise membrane preparations, but the EM studies suggest that this has not occurred in these studies. The most promising resolution occurred after the incorporation of a pre-spin into the method. The pellet of this separation was slightly enriched in LPS and contained the major OMPs. Subsequent washing of this fraction may finally remove CM contaminants. Sarkosyl prepared cell walls also pelleted on this gradient.

Sucrose density gradients are thus ineffective for the fractionation of D.vulgaris OM/CM, using these methods. They are exremely long-winded and were an unsuitable method for routine use.

Sarkosyl cell wall yields from the various Woolwich cultures of the three media (Figure 5.1) show very small differences. The loss of iron clearly visible from the colour of C-Fe extracts (being buff rather than black) must therefore be compensated for in some way, possibly by an increase in the production of LPS.

The Sarkosyl C+Fe cell wall composition (31% protein, 8% carbohydrate, 21% lipid, 0.5% Fe^{2+} and containing 17.5% LPS with no detectable KDO) left 40% of the dry weight unaccounted for. This may have been sulphide or precipitates of sulphur other than FeS notably those seen in the X-ray dispersion

analysis (Plate 3.12). The peptidoglycan layer present is likely to be composed of amino-sugars and non-aromatic amino acids neither of which would give values in the assays used for carbohydrate or protein. Any amino-sugars in the LPS would also fail to give values.

A significant increase is seen in the percentage dry weight of carbohydrate within the cell walls of C-Fe grown cells whilst the protein shows a very slight decrease (Figure 5.4). This may demonstrate an increase in 'O' side-chain length in response to iron limitation. Hildenborough and Woolwich Sarkosyl cell walls show similar carbohydrate and protein compositions (Figure 5.3) whilst Venezuela shows a reversal in the amount of protein to carbohydrate, this may also show a higher LPS content or longer 'O' side-chain in Venezuela cell walls.

SDS-PAGE analysis of Sarkosyl C+Fe cell walls showed a complex pattern of polypeptide and LPS bands. Whether the simplification of this pattern to just OMP's 1, 2, 3 and LPS is related to structural changes or to increased solubilisation is a matter of speculation. However the partial return/ increased retention of some proteins (Plate 5.11) in 'protected' cell wall points to iron binding as a hindrance to Sarkosyl solubilisation rather than a metabolic consequence of iron deficiency, possibly demonstrating the protective nature of iron surface binding to Desulfovibrio in their natural environments. Although induction as well as repression of certain Gram negative OM proteins in response to iron limitation has been reported (McIntosh & Earhart, 1976; Meyer et al. 1979) this was not on the scale observed here. The Sarkosyl method was therefore not suitable to demonstrate

such changes in Desulfovibrio due to this 'protection' by iron binding.

Differences are observed in the SDS-PAGE cell envelope pattern of C+Fe and C-Fe cells but this would be expected due to the change in many enzymes and cofactors of respiration, present within the CM, as a consequence of iron limitation.

The importance of OMP 1 is emphasised by its presence in all the strains analysed (Plate 5.10). The identical M.Wt's. of OMP 1, the major polypeptide released by EDTA washes of whole cells and one of the proteins labelled by lactoperoxidase surface protein studies (Plate 4.7), leads one to consider them as one and the same. This is far from conclusive however. Further purification and separation on a different electrophoresis or chromatography system may provide proof, but this is not a simple task with this partially hydrophilic membrane protein.

OMP 1 is also relatively insensitive to protease digestion and to 2%SDS solubilisation at 60°c. The former property may indicate protection of the molecule by being embedded within the OM, whereas the latter property has been implicated with peptidoglycan association (Nixdorff et al, 1977).

All three major OMP s are resistant to 2%SDS solubilisation at 30°c, when LPS is removed. Proteins having this property and an apparant M.Wt. of 21000 (similar to OMP 3) have been reported present in nine species of Gram negative bacteria and identified as lipoproteins (Mizuno, 1979). The ability to extract OMP 1 only partially with acetate, its partial release by EDTA washes (some OMP 1 always remains in Sarkosyl cell walls prepared from EDTA washed cells) and partial resistance to protease digestion (protease resistant OMP 1

cannot be acetate extracted) indicates OMP1 may be two proteins which comigrate in the SDS-PAGE system used here. These were therefore labelled OMP1a (removable) and OMP1b (bound). A table summarising the major OMP properties is given in Figure 5.24. These properties and acetate extraction may provide useful fractions for later experiments.

A menaquinone was extracted from D.vulgaris cell envelopes and cell walls but due to the relatively large amounts of starting material needed and difficulty of quantification it did not prove to be a good marker for cell wall purity.

Polar lipid extractions showed that a large proportion of the cell envelope and cell wall lipid was present as phosphatidylethanolamine. This was in accordance with previous work (Makula & Finnerty, 1974) were considerable differences among Desulfovibrio species were shown. One of the detected phospholipids may have been cardiolipin (diphosphatidylglycerol) which was also previously reported present by Makula & Finnerty. Phosphatidyl serine reported by these authors, was not found in this study. The necessary enzymes for phosphatidylglycerol and cardiolipin synthesis have been demonstrated in Desulfovibrio by Silber et al, 1981. They also report a lack of plasmalogens (O-alk-1-enyl substituent at the C1 atom of glycerol), often common in anaerobes, in D.vulgaris. The unidentified lipid (Plate 5.12), possibly a glycolipid, ran equally well in cell envelope and wall extract separations on TLC, and should be noted here. The results therefore confirm previous taxonomic studies and this extract of polar lipids, in reasonable quantities, should be useful in further work upon membrane reconstitutions.

Figure 5.24. Table showing susceptibility of the D.vulgaris Woolwich major OM proteins to various treatments.

Major OMP	EDTA release	Protease action	Acetate extraction	Lacto-peroxidase labelling.
1a	+	+	+	+
1b	-	-	-	+
2	-	+	+	-
3	-	+	+	+

A reasonable separation of FAME's from cell envelope and cell wall fractions was achieved by GLC, although many peaks remained unidentified. The ratios of 18:0, 18:1 and 18:2 are interesting and characteristic of the two different fractions. Previous work in this area has only been performed using whole cell FAME's for taxonomic purposes (Boon et al. 1977); Ueki & Suto, 1979; Taylor & Parkes, 1983). In all these studies monoenoic C_{15} and C_{17} and ß-hydroxy fatty acids were shown to be common. In this present analysis on intact cell envelopes the relative amounts of fatty acids were; U2>18:0>17:0>16:0>U3>12:0>18:1>18:2>traces.
U2 was very abundant in both fractions and may have been OH-12:0 or i/ai 15:0. The examination of purified major OMP's for FAME s ie. identification as lipoproteins, may be a useful extension of this work.

The lack of hydrogenase activity in D.vulgaris Woolwich C-Fe cells has been reported for other strains (Postgate, 1956; Tsuji & Yagi, 1980) although no explanation was offered for this. The latter authors state that cytochrome c_3 levels still remain constant. Whether iron is needed for hydrogenase synthesis, is a necessary cofactor, confers resistance to inhibitors (eg. atmospheric oxygen) or whether alternative metabolic pathways make the enzyme unnecessary during iron starvation, is a matter of speculation. Of interest is the restoration of approximately 13% hydrogenase activity to C-Fe lysates (Figure 5.19) by Fe^{2+} incubation either prior to or after cell disruption. Previous reports on the necessity of iron for the activity of the enzyme are conflicting (Riklis & Rittenberg, 1961) as indeed are reports of reactivation

by SH-group reagents, enhanced activity using cytochrome c_3, oxygen sensitivity and M.Wt. (Postgate, 1984). These variations can, in part, be explained by the various forms of hydrogenase within a single species and considerable variation with growth conditions. D.vulgaris strains previously noted appear to have two hydrogenases (Tsuji & Yagi, 1980). These high and low M.Wt. species may catalyse the evolution and consumption of hydrogen, respectively, under physiological conditions. The isolated enzyme can catalyse the reaction to equilibrium. The air stability of the enzyme assayed here (Figure 6.20) was in common with the well characterised enzyme of van der Westen (1978) from Hildenborough strain which was extremely air stable, 44% activity remaining after a similar storage to that performed here. The low level of activity associated with the particulate fraction in these studies is in marked contrast to van der Westen et al (1978) where the 'major part' of the activity was found to be associated with the particulate material after mechanical disruption, and to Yagi et al (1978) where hydrogenase activity was mainly particulate, only a 'minor fraction' liberated upon sonic disruption of D.vulgaris Miyazaki. van der Westen et al (1980) have reported that exposure of intact cells to oxygen may affect subsequent extracted hydrogenase oxygen sensitivity. The present studies show that hydrogenase activity is not associated with the OM in Woolwich and Hildenborough C+Fe strains. Complete inactivation of the enzyme by Sarkosyl detergent is unlikely as some residual activity is located in the Sarkosyl solubilised Woolwich CM. Oxygen sensitivity of the particulate hydrogenase only (not soluble enzyme) cannot be ruled out.

To summarise, the results provide much useful background data supporting many of the taxonomic studies by other workers. Possible methods for easy strain identification, especially the Sarkosyl cell wall SDS-PAGE patterns (Plate 5.10 inset) are envisaged. Many of the fractionation procedures will be of value in further studies.

CHAPTER 6

LIPOPOLYSACCHARIDE STUDIES.

Results from the previous chapters have demonstrated the presence of LPS in the cell wall of D.vulgaris, its possible involvement in a selective interaction with Fe^{2+} and possible composition changes within the LPS in response to iron limitation. The silver stain has proved an excellent method for the visualisation of LPS material after separation on SDS-PAGE. It was therefore proposed to investigate the chemical nature of isolated LPS from various D.vulgaris strains using the methods tried on isolated cell walls in the previous chapter, and to investigate the sugars of the 'O' side-chain by hydrolysis and subsequent chromatography. Especially useful would be a comparison of the 'O' side-chains from the LPS of C+Fe and C-Fe grown cells. The results of this type of analysis may enable speculation upon the type(s) of chemical interaction between D.vulgaris LPS and ferrous ions and possibly to its transport into the cell.

RESULTS

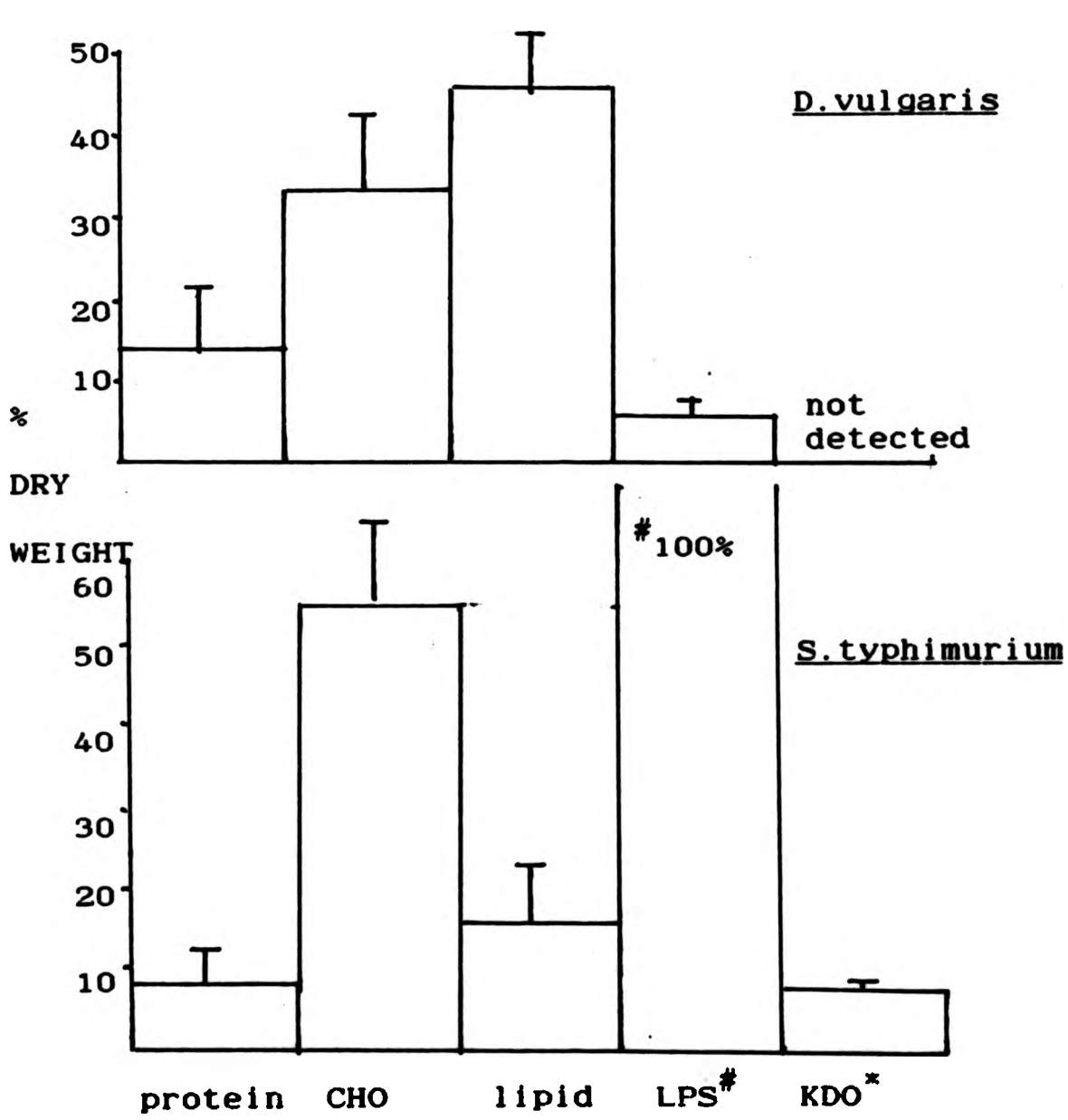
6.1. Yields and composition.

The yields obtained by the Phenol/water extraction of D.vulgaris Woolwich cell envelopes are given in Figure 6.1. The extracted C+Fe LPS and that of commercial (Phenol/water extracted) S.typhimurium LPS were analysed for total protein, carbohydrate, lipid, KDO and comparative LPS values given by the carbocyanine dye assay. The results are shown in Figure 6.2. Comparisons of the protein and carbohydrate content of the LPS were also made for C-Fe Woolwich and for C+Fe Hildenborough strain; these are given in Figure 6.3.

Figure 6.1. Fraction yields for hot phenol/water LPS extraction of D.vulgaris Woolwich cell envelopes.

Preparation	Mean dry weight extracted mg/500ml culture	Mean dry weight extracted mg/10^8 cells	
LPS (C+Fe)	1.7	0.37	(n=8)
LPS (C-Fe)	2,9	0.79	(n=4).

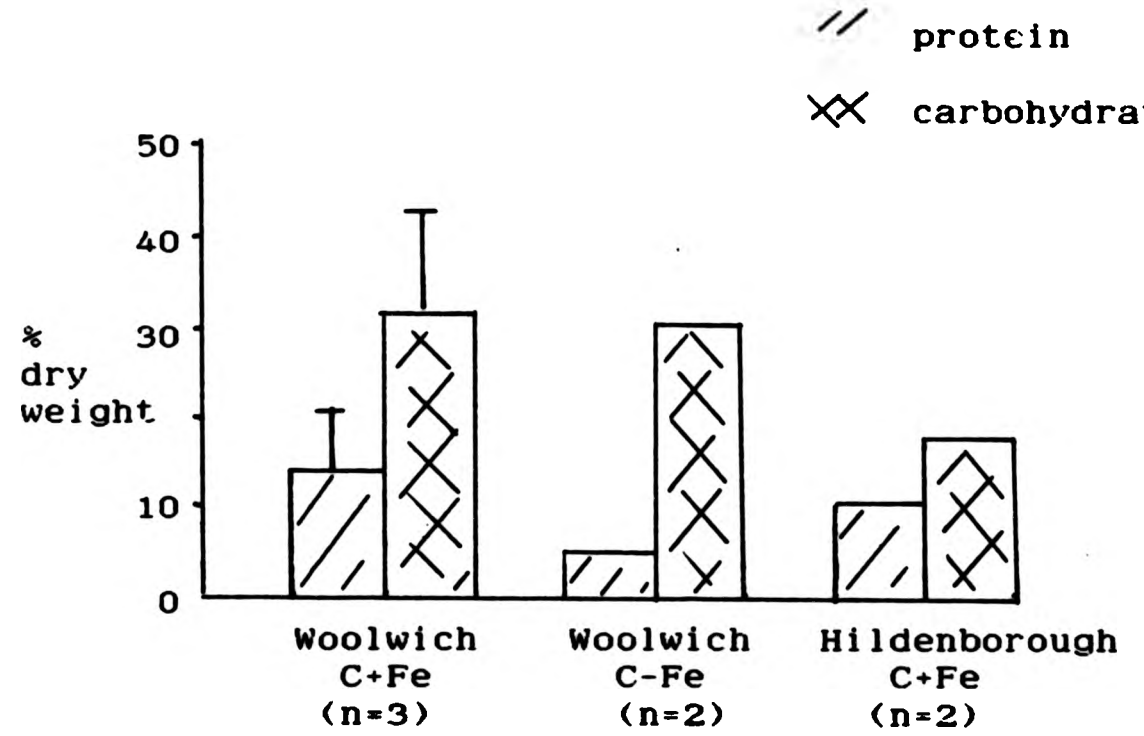
Figure 6.2. Comparison of D.vulgaris Woolwich C+Fe extracted LPS with commercial S.typhimurium LPS by chemical analysis expressed as % total dry weight.



Estimated using S.typhimurium LPS as standard.

* Expressed as O.D. units x 10^2/100µg dry weight LPS.

Figure 6.3. Comparison of total protein and carbohydrate content of <u>D.vulgaris</u> LPS from two strains and two growth media.



6.2. SDS-PAGE separations.

6.2.1. Tubes.

Separations on SDS-PAGE tube gels after Jann et al (1975), and staining using the PAS stain for carbohydrate were successful for both D.vulgaris extract and commercial S.typhimurium LPS. The S.typhimurium LPS gave a more complex band-pattern as shown in Plate 6.1. The D.vulgaris extract was also stained using Procion blue H5R and excess dye removed by passage through a Sephadex G25 column (Plate 6.2.).
It was not possible to load sufficient of this stained material onto the gel to allow visual monitoring during separation and this method was deemed of no advantage.

6.2.2. Slabs.

The excellent resolution obtained using slab SDS-PAGE with cell wall separations (Chapter 5) including LPS material within them, together with silver staining for visualisation gave an excellent opportunity for the analysis of extracted LPS by this method. Results of separations using LPS from the various media and strains of Desulfovibrio and that obtained with S.typhimurium LPS are shown in Plate 6.3. To determine the possible nature of the bands shown by silver staining a parallel separation of D.vulgaris Woolwich C-Fe LPS was stained using the PAS method. All three bands were seen to give positive reactions for carbohydrate when the amount loaded permits.

6.3. Gas liquid chromatography (GLC).

Successful GLC separations were obtained with FAME's prepared from Phenol/water extracted D.vulgaris Woolwich C+Fe LPS. These were compared with those of the standard bacterial FAME's and other separations shown in Chapter 5 (Figures

Plate 6.1. Tube SDS-PAGE analysis of D.vulgaris Woolwich C+Fe extracted LPS and commercial S.typhimurium LPS stained by the PAS method.

1 2

Lane 1, D.vulgaris Woolwich C+Fe LPS; lane 2, commercial S.typhimurium LPS.

Plate 6.2. Removal of excess Procion Blue H5R from dyed D.vulgaris LPS by passage through a Sephadex G25 column. Dyed LPS is shown arrowed.

Plate 6.1. Tube SDS-PAGE analysis of D.vulgaris Woolwich C+Fe extracted LPS and commercial S.typhimurium LPS stained by the PAS method.

1 2

Lane 1, D.vulgaris Woolwich C+Fe LPS; lane 2, commercial S.typhimurium LPS.

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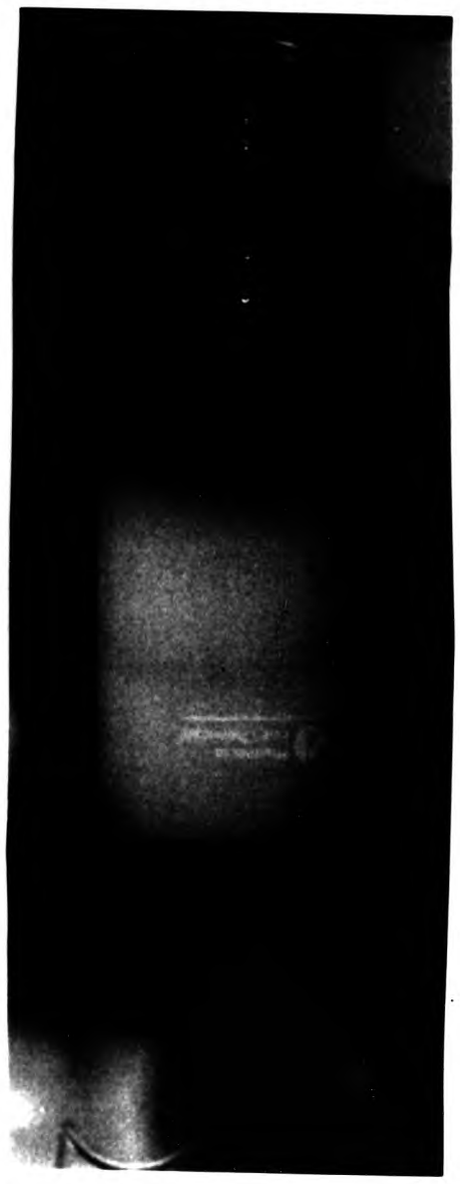


Plate 6.1. Tube SDS-PAGE analysis of D.vulgaris Woolwich C+Fe extracted LPS and commercial S.typhimurium LPS stained by the PAS method.

1 2

Lane 1, D.vulgaris Woolwich C+Fe LPS; lane 2, commercial S.typhimurium LPS.

Plate 6.2. Removal of excess Procion Blue H5R from dyed D.vulgaris LPS by passage through a Sephadex G25 column. Dyed LPS is shown arrowed.

Plate 6.3. Slab SDS-PAGE analysis of Desulfovibrio LPS from various strains and media together with S.typhimurium LPS.

1 2 3 4 5 6 7 8

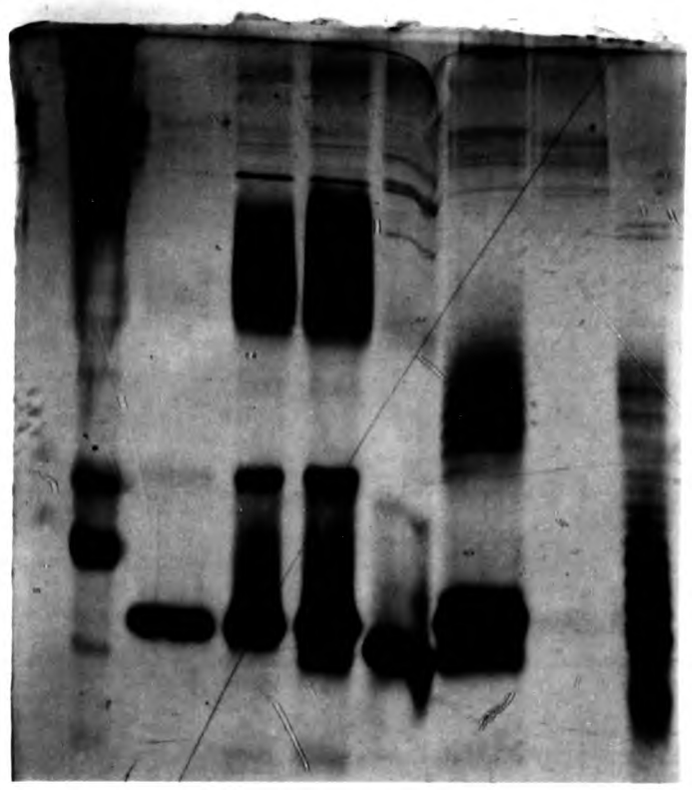
Lane 1, protein markers; lane 2,3, D.vulgaris Woolwich C-Fe LPS; lane 4, D.vulgaris Woolwich C+Fe LPS; lane 5, D.vulgaris Hildenborough C+Fe LPS; lane 6, D.vulgaris Holland C+Fe LPS; lane 7, D.gigas C+Fe LPS; lane 8, commercial S.typhimurium LPS. All loadings are of 30µg except lane 2 (15µg) and lane 7 (10µg).

Plate 6.3. Slab SDS-PAGE analysis of Desulfovibrio LPS from various strains and media together with S.typhimurium LPS.

1 2 3 4 5 6 7 8

Lane 1, protein markers; lane 2,3, D.vulgaris Woolwich C-Fe LPS; lane 4, D.vulgaris Woolwich C+Fe LPS; lane 5, D.vulgaris Hildenborough C+Fe LPS; lane 6, D.vulgaris Holland C+Fe LPS; lane 7, D.gigas C+Fe LPS; lane 8, commercial S.typhimurium LPS. All loadings are of 30µg except lane 2 (15µg) and lane 7 (10µg).

Plate 6.3 Slab SDS-PAGE analysis of Desulfovibrio LPS from various strains and media together with S.typhimurium LPS.



1 2 3 4 5 6 7 8

Lane 1, protein markers; lane 2,3, D.vulgaris Woolwich C-Fe LPS; lane 4, D.vulgaris Woolwich C+Fe LPS; lane 5, D.vulgaris Hildenborough C+Fe LPS; lane 6, D.vulgaris Holland C-Fe LPS; lane 7, D.gigas C+Fe LPS; lane 8, commercial S.typhimurium LPS. All loadings are of 30µg except lane 2 (15µg) and lane 7 (10µg).

5.7 - 5.11). Similar calculations were made and these are shown in Figures 6.5 and 6.6. Large amounts of U2 are again seen and the ratios of 18:0, 18:1 and 18:2 should be noted.

6.4. LPS sugar analysis.

6.4.1. Hydrolysis.

The weak and strong acid hydrolysis of D.vulgaris Woolwich hot phenol/water extracted LPS was monitored by several methods for its efficacy. Estimation of sugar using the colourimetric phenol/sulphuric acid method showed that no detectable sugar was released by weak acid hydrolysis, but 128µg/mg LPS of sugar was released by the strong hydrolysis procedure. Furthermore an insoluble lipid pellicle was only produced after strong acid hydrolysis. SDS-PAGE analysis of hydrolysis fractions is shown in Plate 6.4. Weak hydrolysis shows the appearance of a slightly faster moving band but definite retention of the dense staining band. Strong acid hydrolysis shows the complete removal of the dense band and the appearance of a very faint band in the soluble fraction. Only strong acid hydrolysis was used for the subsequent analysis of LPS sugars by chromatography.

6.4.2. Paper chromatography.

Paper chromatography did not give particularily good resolution of hydrolysed LPS. Separations took long periods of time, up to 20 hours, and a single faint spot was obtained. This did not exactly correspond to any of the standard sugars. A typical separation is shown in Plate 6.5.

6.4.3. High performance liquid chromatography (HPLC).

Excellent separations of standard sugars and LPS hydrolysates were obtained within 12 minutes using a flow rate of

Figure 6.5. Typical GLC separation achieved using FAMEs prepared from <u>D.vulgaris</u> Woolwich C+Fe LPS.

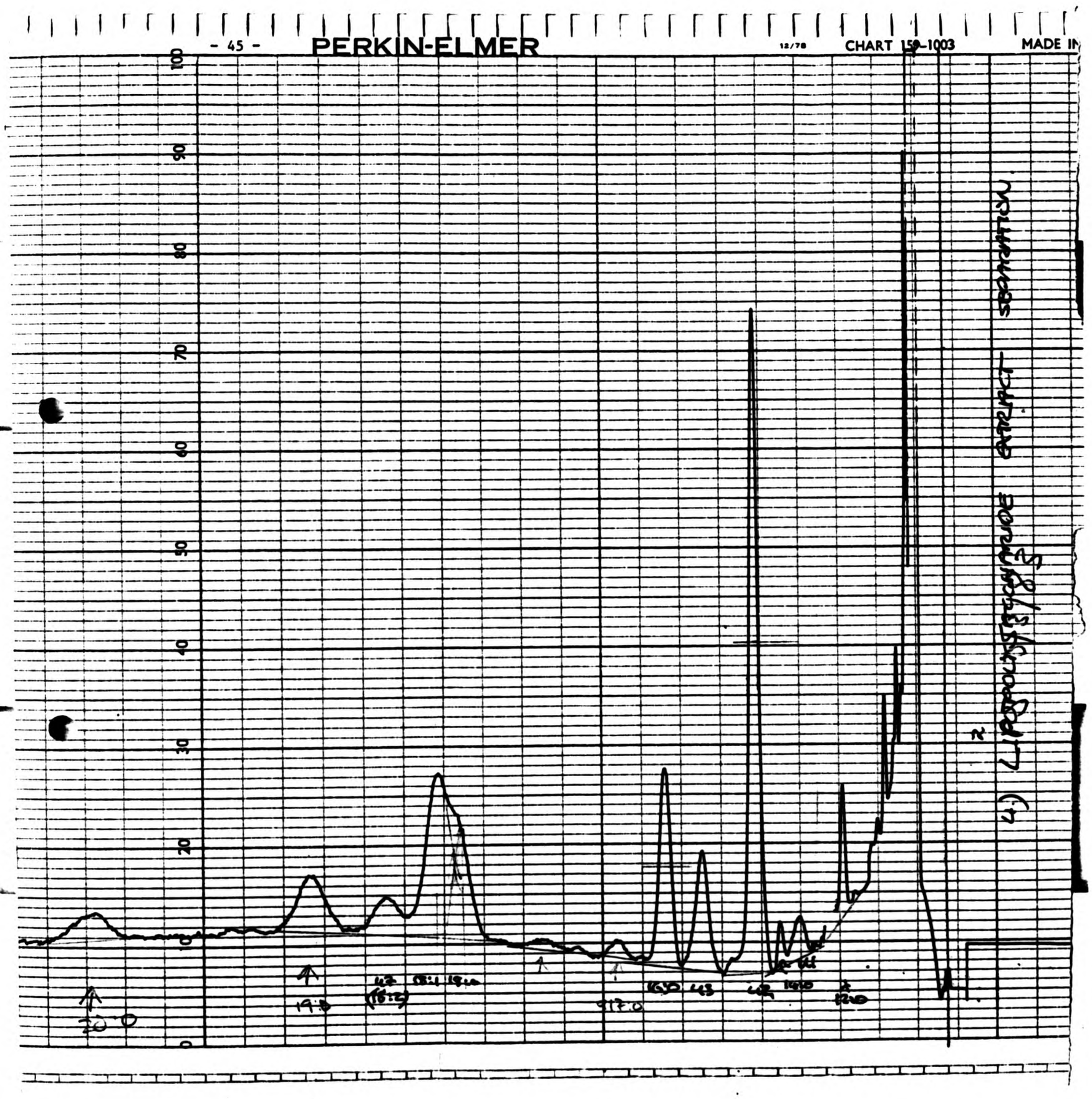


Figure 6.6. GLC separation of FAME's from D.vulgaris C-Fe extracted LPS shown as percentage total peak area against retention time.

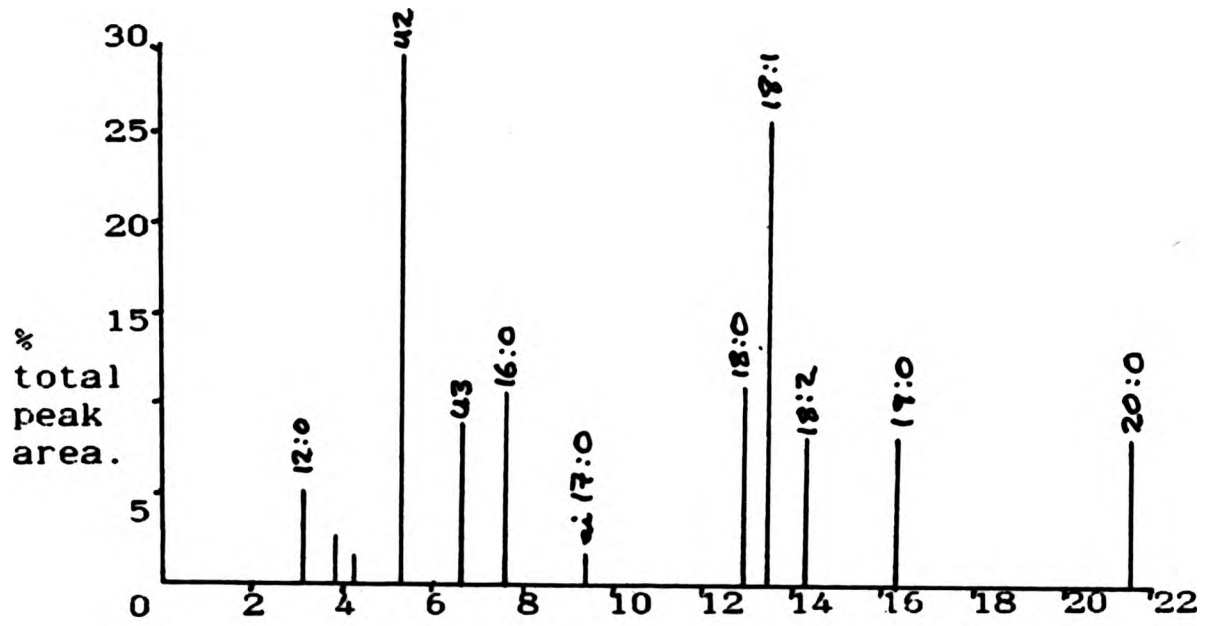


Plate 6.4. SDS-PAGE analysis of hydrolysis fractions from both weak and strong acid hydrolysis of D.vulgaris Woolwich C+Fe LPS. Gel is silver stained.

1 2 3 4 5

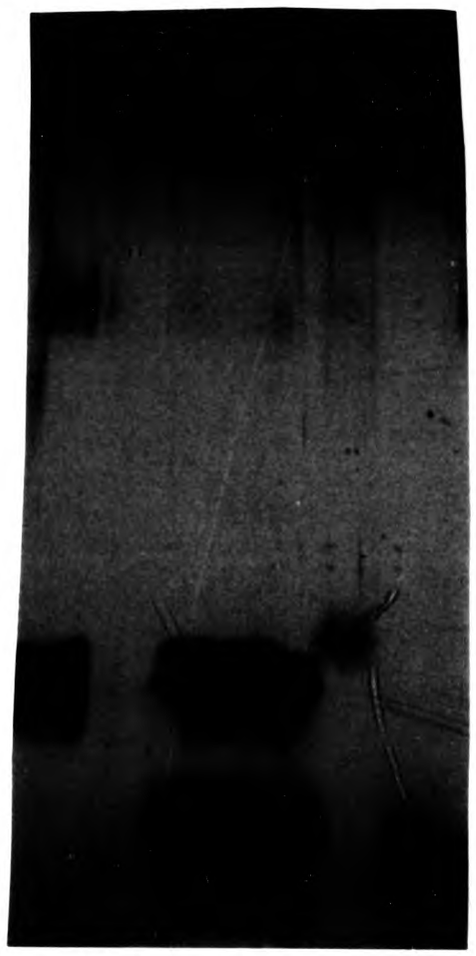
Lanes; 1= Unhydrolysed LPS.

2= Soluble (sugar) fraction
3= Pelletable LPS.
} Weak acid hydrolysis.

4= Soluble (sugar) fraction.
5= Lipid pellicle.
} Strong acid hydrolysis

Plate 6.4. SDS-PAGE analysis of hydrolysis fractions from both weak and strong acid hydrolysis of D.vulgaris Woolwich C+Fe LPS. Gel is silver stained.

1 2 3 4 5

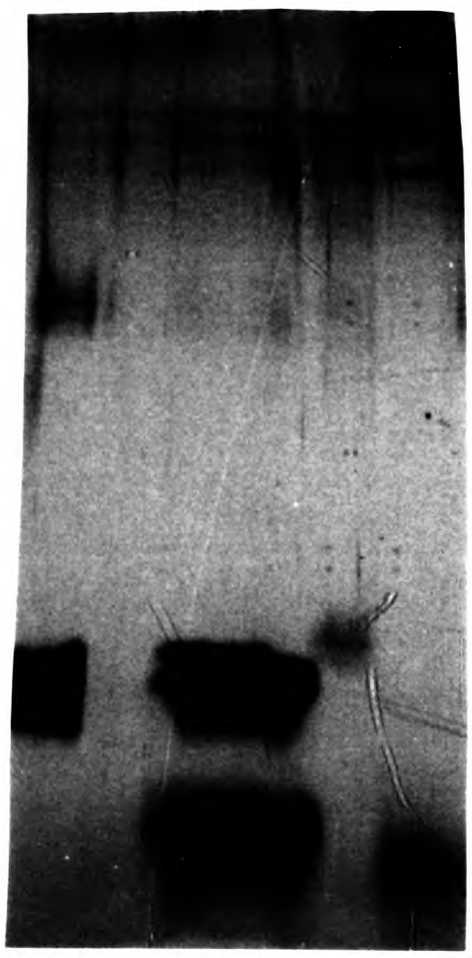


Lanes; 1= Unhydrolysed LPS.

2= Soluble (sugar) fraction ┐
3= Pelletable LPS. ┘ Weak acid hydrolysis.

4= Soluble (sugar) fraction. ┐
5= Lipid pellicle. ┘ Strong acid hydrolysis

Plate 6.4. SDS-PAGE analysis of hydrolysis fractions from both weak and strong acid hydrolysis of D.vulgaris Woolwich C+Fe LPS. Gel is silver stained.



Lanes; 1= Unhydrolysed LPS.

2= Soluble (sugar) fraction
3= Pelletable LPS.
} Weak acid hydrolysis.

4= Soluble (sugar) fraction.
5= Lipid pellicle.
} Strong acid hydrolysis

Plate 6.5. Typical sugar hydrolysate separation by paper chromatography and table of migration distances and R_{glu} values.

Sample	Spot colour	Migration# distance (cms.)	R_{glu}
Sugar hydrolysate	Faint brown	22.2	1.04
Glucose	black	21.4	1.00
Mannose	brown	24.4	1.14
Xylose	black/grey	24.8	1.16
Fructose	orange	23.4	1.09
Rhamnose	yellow	27.3	1.28
Ribose	grey/blue	24.7	1.15
Galactose	black	19.6	0.92

values obtained are from more than one run.

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Ribose	grey/blue	24.7	1.15
Galactose	black	19.6	0.92

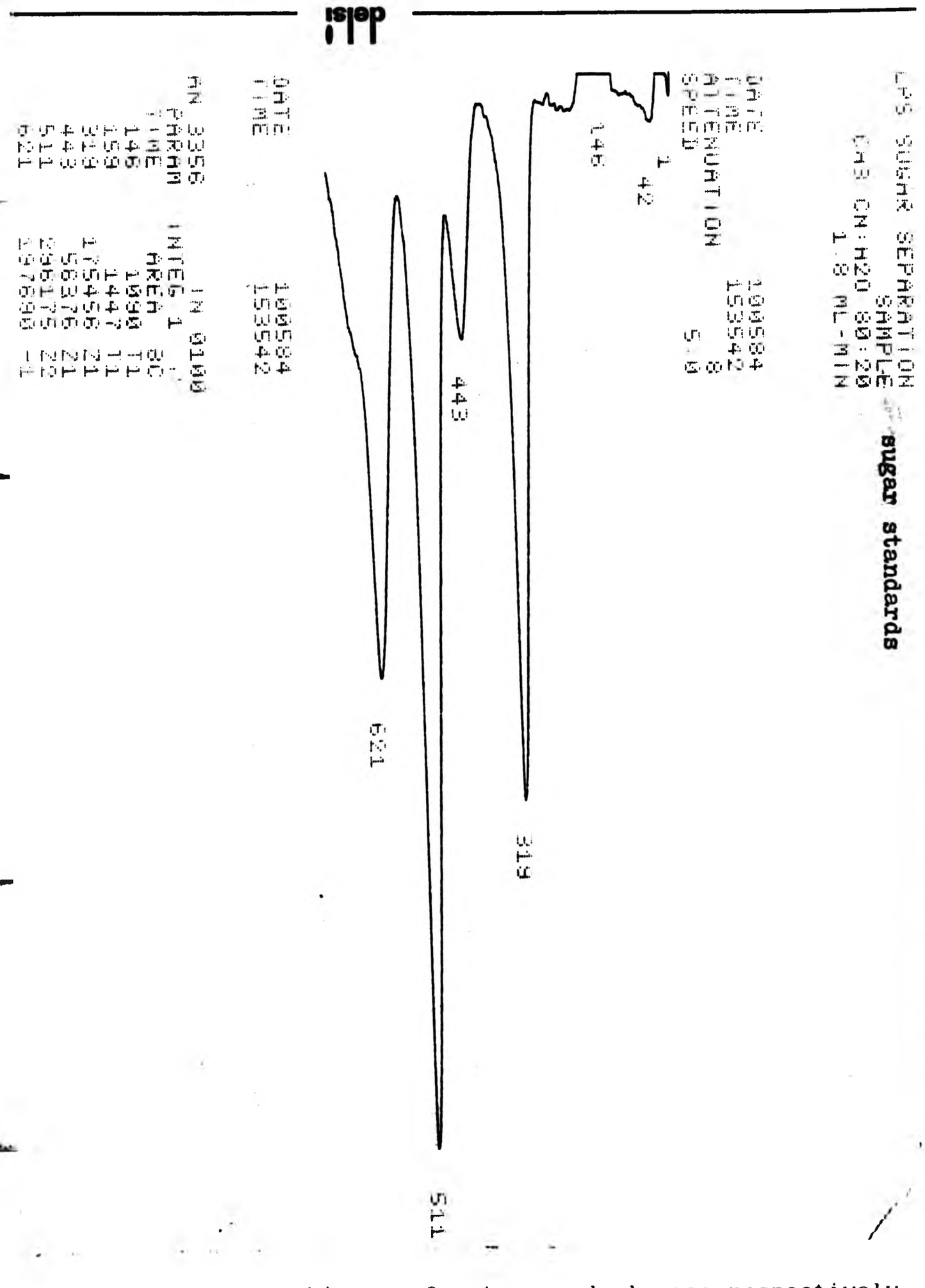
values obtained are from more than one run.

1.8ml./minute. Separations of sugar standards are shown in Figure 6.7 and 6.8. Hydrolysates of S.typhimurium LPS gave complex separations which were highly reproducible. Figures 6.9 and 6.10 show these hydrolysates from two separate preparations. An inset of the sugars reported present in this type of LPS is also given. Separations of two separate hydrolysates are shown for LPS sugars from both D.vulgaris Woolwich C+Fe and C-Fe cells in Figures 6.11 to 6.14. The hydrolysates are the products of three or four separate LPS extracts bulked prior to hydrolysis. Several peaks are observed but the pattern is simpler than S.typhimurium separations. Distinct differences are noted between C+Fe and C-Fe sugar hydrolysates. Especially notable is an increase in the peaks of about 350 seconds retention time and a diminution in the trailing peak (about 600 seconds retention time) in C+Fe separations. Unfortunately an injection trough was usually seen at about 110 seconds and this could not be eliminated.

DISCUSSION.

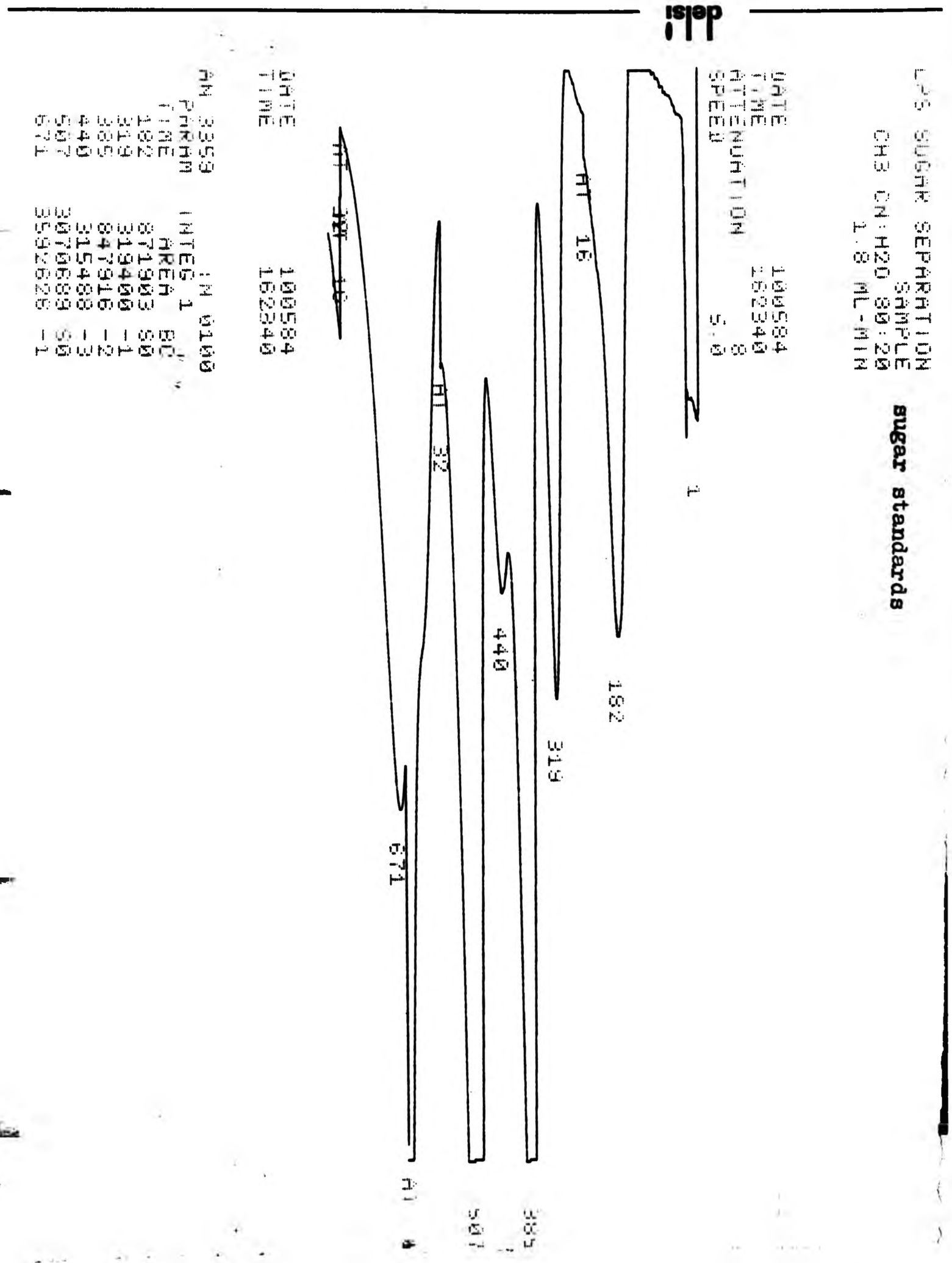
The yield of LPS from C-Fe cultures is seen to be 2.1 times that extracted from C+Fe cultures (Figure 6.1), this may be due to poor extraction in C+Fe cultures because of the protective bound iron or may point to an actual increase in the LPS content of the cell wall in C-Fe cultures. This latter possibilty would complement the seven-fold increase in carbohydrate content of the cell wall seen in Chapter 5 (Figure 5.4), in response to iron limitation. No corresponding increase in carbohydrate content of the extracted LPS is seen for C-Fe grown extracts (Figure 6.3) and this would

Figure 6.7. Typical HPLC separation achieved using commercial sugar standards.



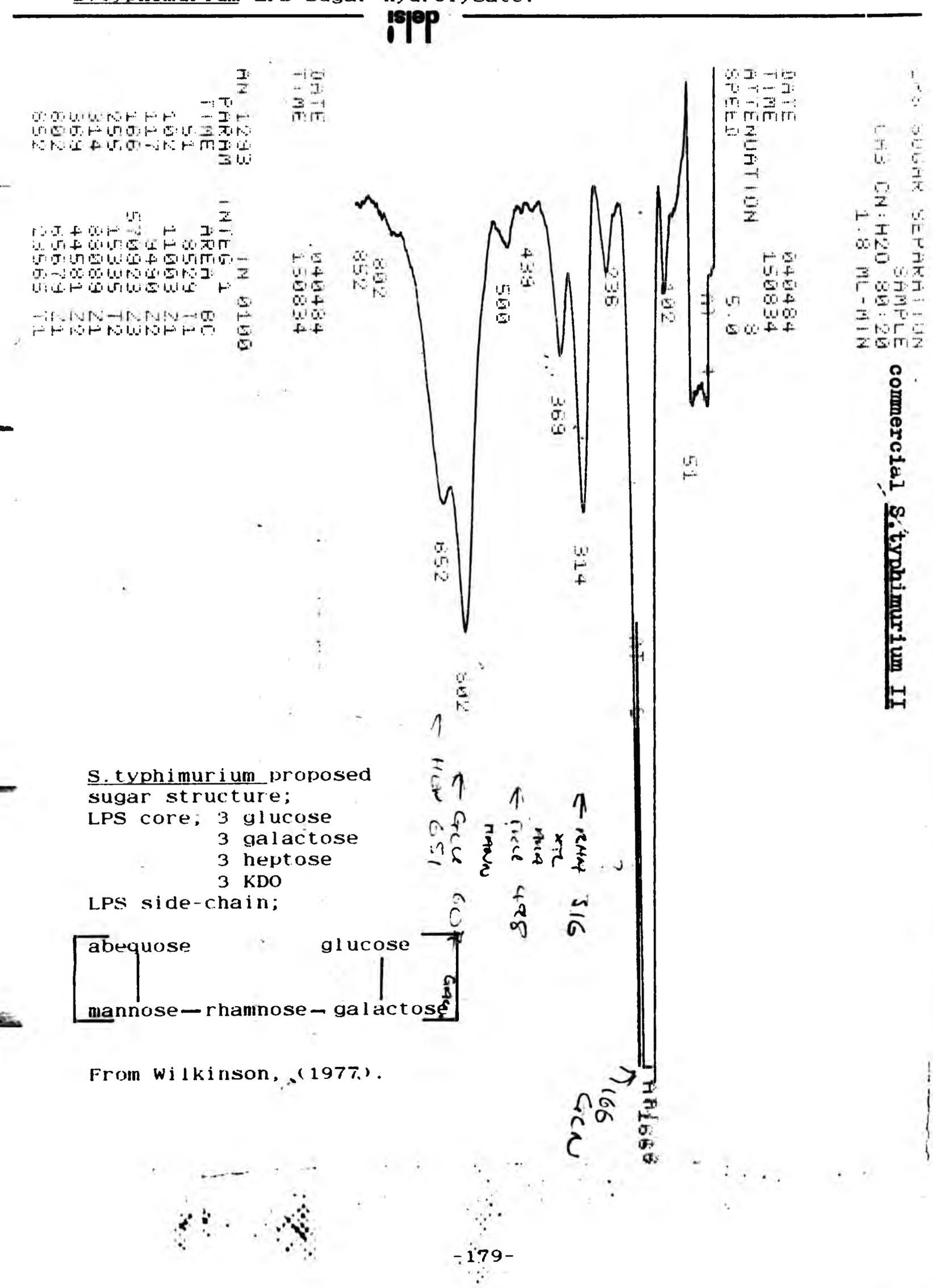
Peaks are rhamnose, arabinose, fructose and glucose respectively.

Figure 6.8. Typical HPLC separation achieved using commercial sugar standards.



Peaks are glucosamine, rhamnose, xylose, arabinose, fructose, glucose and heptose respectively.

Figure 6.9. Typical HPLC separation achieved using commercial S.typhimurium LPS sugar hydrolysate.



S.typhimurium proposed sugar structure;
LPS core; 3 glucose
3 galactose
3 heptose
3 KDO
LPS side-chain;

From Wilkinson, (1977).

Figure 6.10. Typical HPLC separation achieved using commercial S.typhimurium LPS sugar hydrolysate. Separate hydrolysate to Figure 6.9., showing reproducibility of methods.

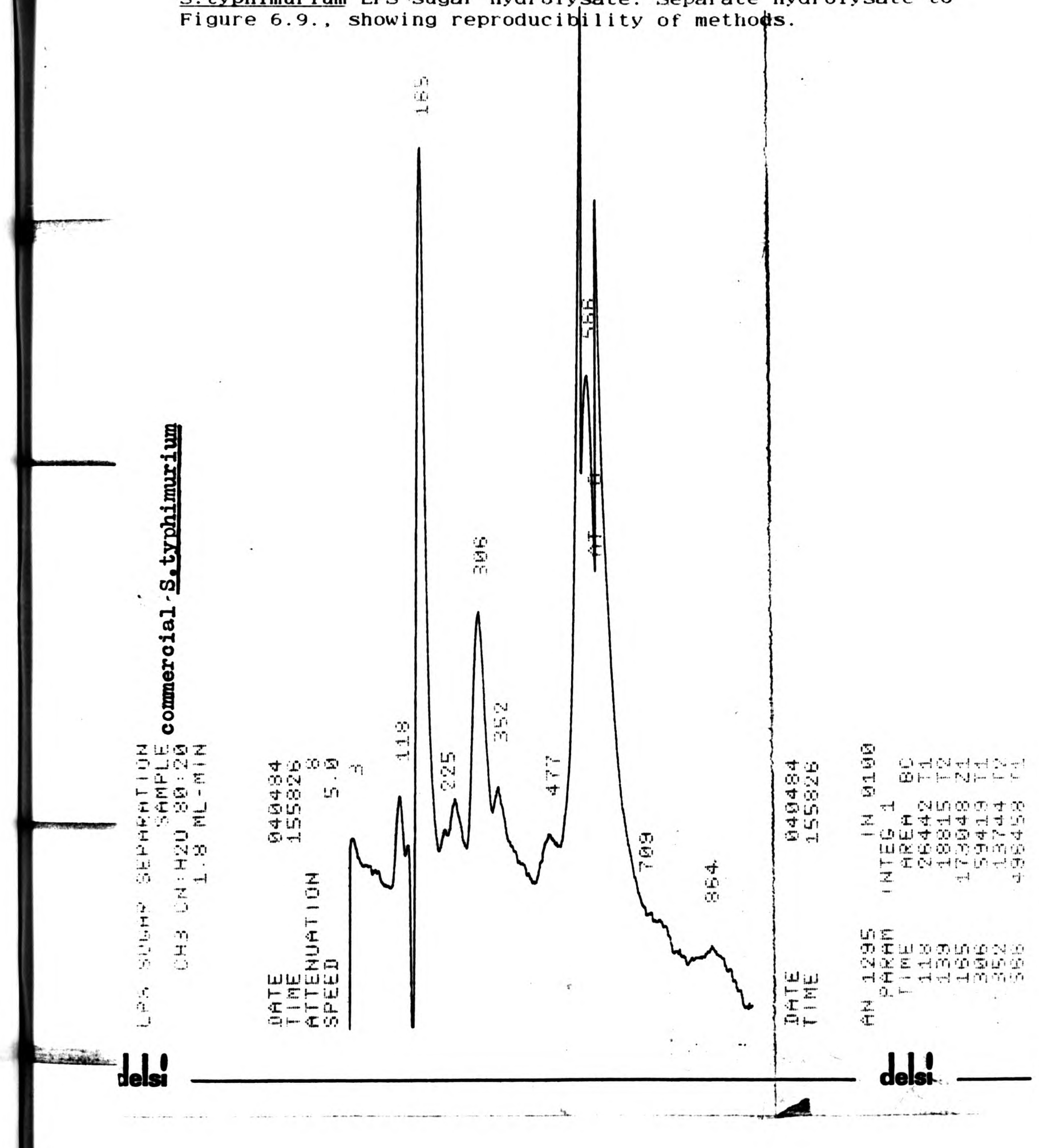


Figure 6.11. Typical HPLC separation achieved using <u>D.vulgaris</u> C+Fe LPS sugar hydrolysate.

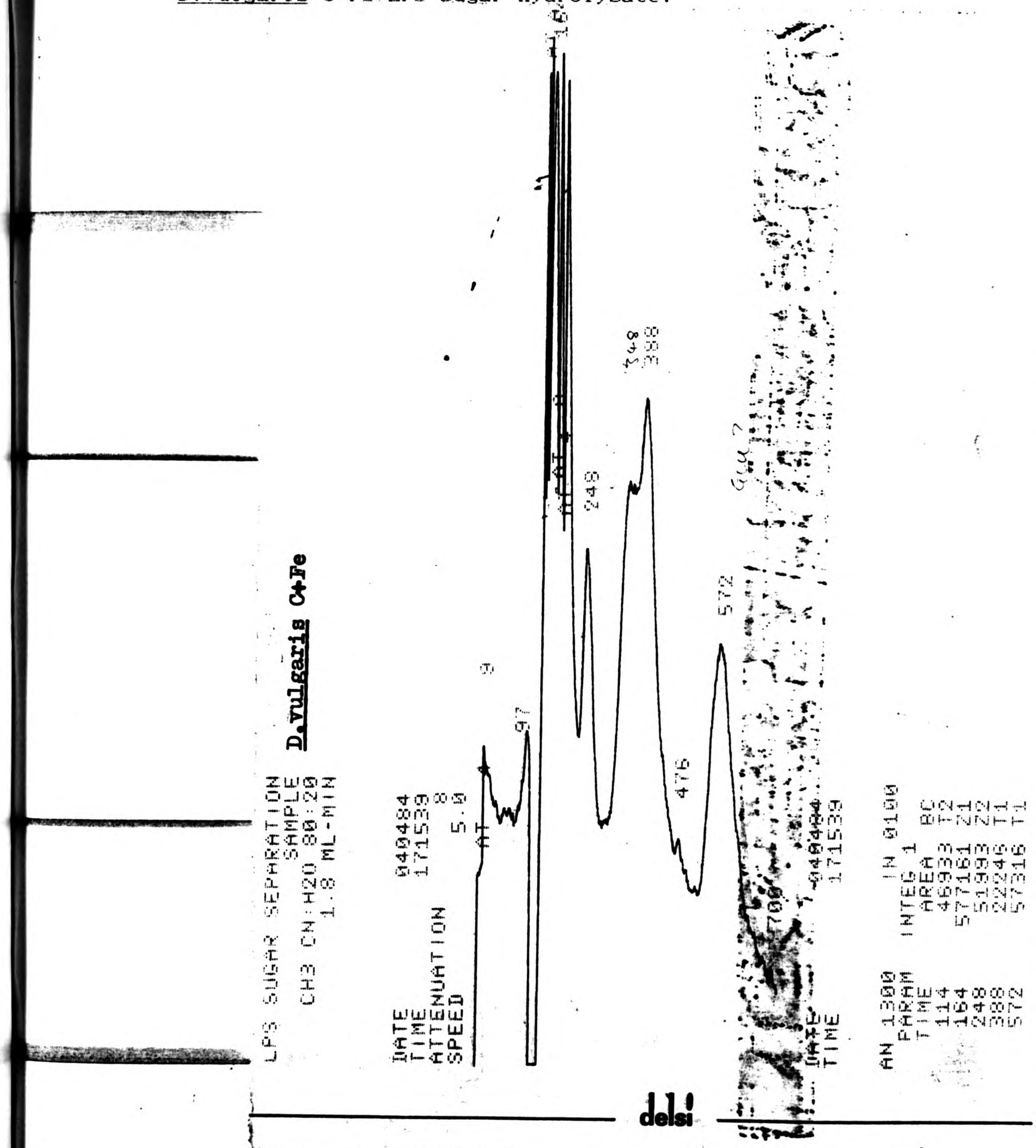


Figure 6.12. Typical HPLC separation achieved using D.vulgaris Woolwich C+Fe LPS sugar hydrolysate.

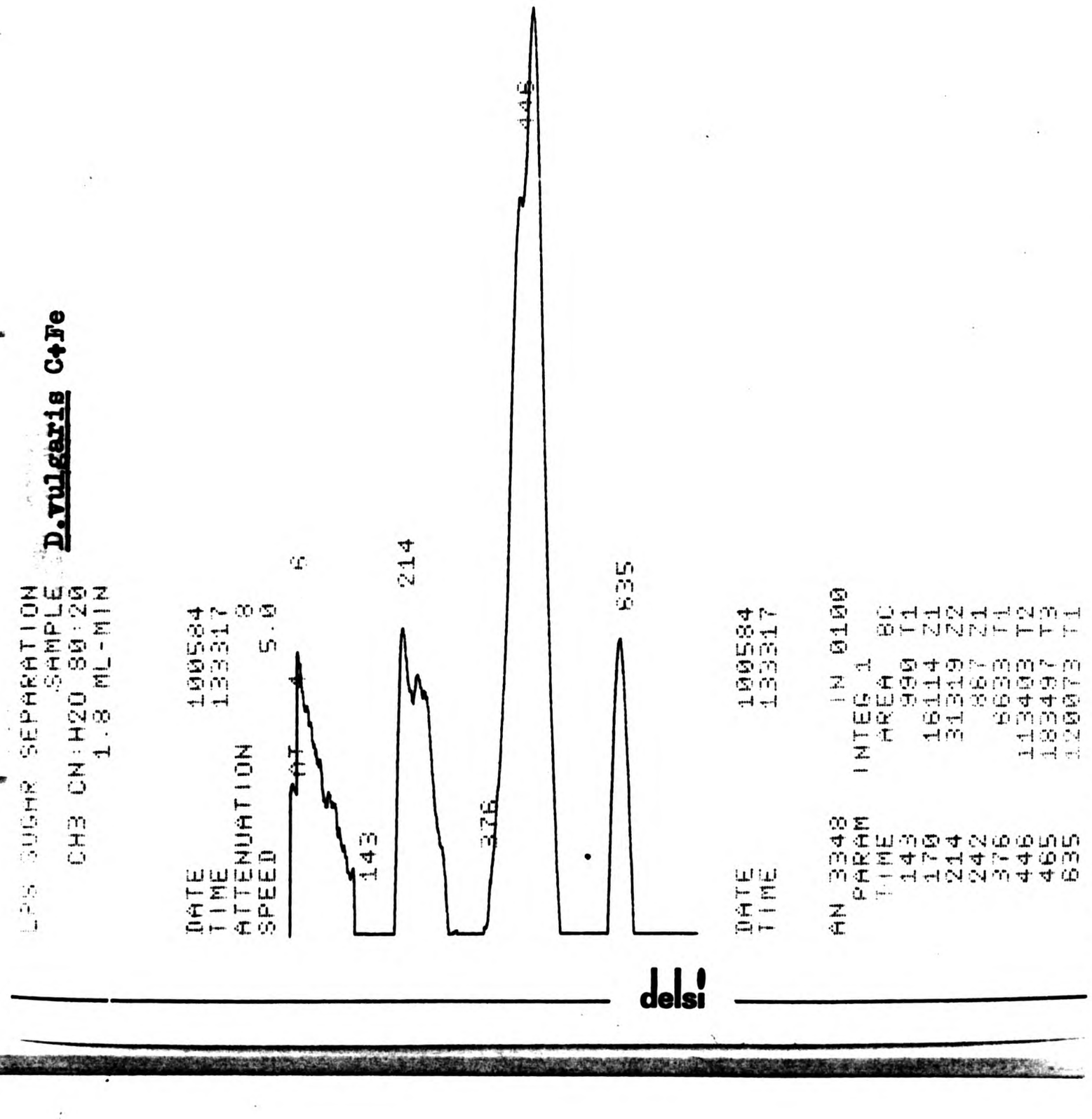


Figure 6.13. Typical HPLC separation achieved using <u>D.vulgaris</u> Woolwich C-Fe LPS sugar hydrolysate. Note especially peaks marked ' ** '.

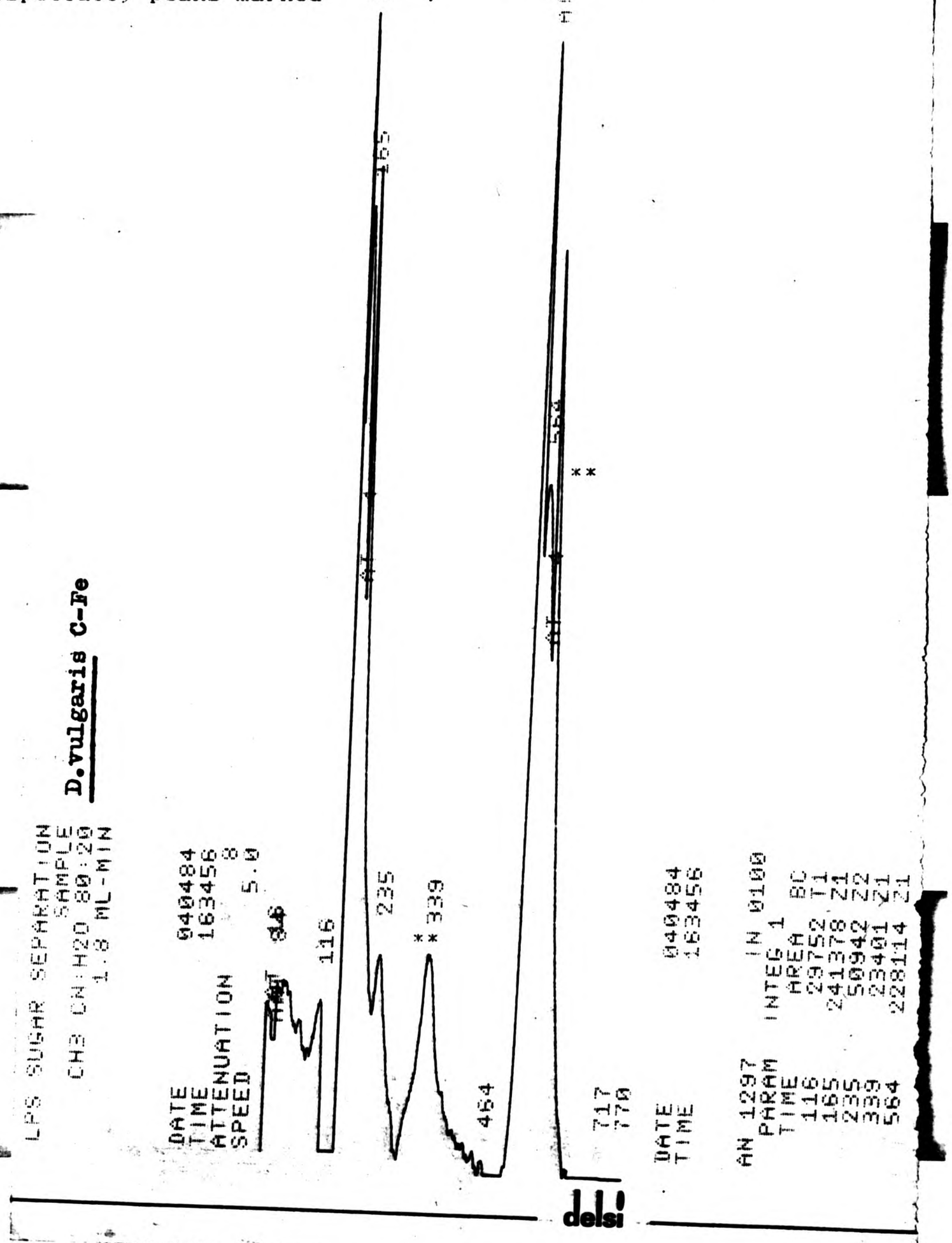
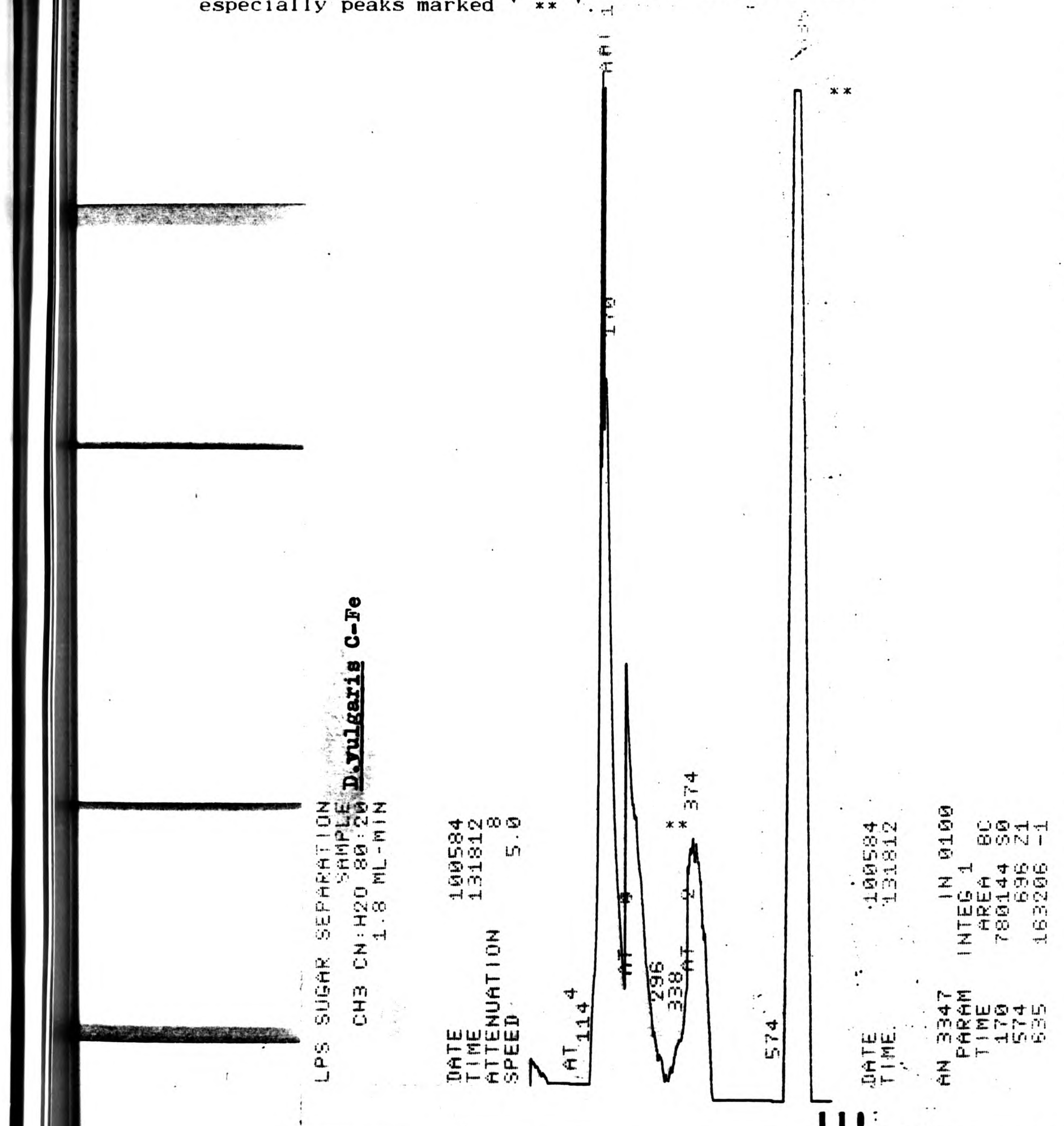


Figure 6.14. Typical HPLC separation achieved using D.vulgaris Woolwich C-Fe LPS sugar hydrolysate. Note especially peaks marked ' ** '.



suggest an increase in the number of LPS molecules rather than any lengthening of the 'O' side-chain in response to iron limitation. This is further supported by the lack of any change in the band pattern on SDS-PAGE analysis of C-Fe and C+Fe LPS (Plate 6.3), although the chemical nature of these observed bands is as yet uncertain.

The simpler band patterns seen in both tube and slab gels for D.vulgaris LPS when compared with S.typhimurium LPS, together with a lower carbohydrate content but increased lipid, point to a shorter and less variable 'O' side-chain. The lack of KDO also shows a difference in basic structure between the two types of LPS; this is in common with other genera including Bacteroides sp. and Anabaena variabilis (Galanos et al, 1977). SDS-PAGE analysis of D.vulgaris Woolwich C-Fe or C+Fe LPS (Plate 6.3) when silver stained, shows a fast moving, dense black band and a slower cluster of golden bands. A second black band is seen on excess loading. The nature of these bands is not at all certain although a similar cluster of trailing bands has been thought to be the various lengths of 'O' side-chain present within the LPS population in P.aeruginosa (Kropinski et al, 1982). Three LPS SDS-PAGE fractions have also been analysed in Alteromonas haloplanktis (DiRienzo & McLoed, 1978) with rather uncertain findings. More recently the numerous bands obtained by silver staining of S.anatum LPS on SDS-PAGE have been reported to represent the differing number of 'O'-polysaccharide repeating units within that LPS molecule (McConnell & Schoelz, 1983). These bands have further been shown to contain O-serogroup-

specific antigenic determinants in E.coli using immunoblotting techniques (Karch et al. 1984). D.vulgaris Woolwich would appear to be very homogeneous with respect to the types of 'O' side-chains within a LPS population, especially when compared to the heterogeneity of S.typhimurium. Carbohydrate is present in all the bands observed with D.vulgaris Woolwich as revealed by PAS staining and more especially within the dense black band which is seen to disappear upon strong acid hydrolysis of the LPS (Plate 6.4). This homogeneity is also shown in Hildenborough strain but not in Holland or Venezuela which show a more complex system. This observation complements the higher % dry weight of carbohydrate seen in D.vulgaris cell walls reported in Chapter 5.

D.vulgaris Woolwich LPS gave much lower values in the carbocyanine dye assay (Figure 6.2) than LPS from S.typhimurium again reflecting the differences in composition. The length of 'O' side-chain and charge upon it reflects the ability to form aggregates with the cationic dye. However, excellent standard curves were constructed using D.vulgaris LPS (see appendix). It should also be noted that although the dye assay method gave very reliable results for released and extracted LPS, peak shifts to other wavelengths were often observed with cell wall or envelope fractions containing bound LPS which resulted in some low readings for LPS content with these fractions (such as Figure 5.2).

The lack of any hydrolysis when using weak acid is not surprising due to the lack of KDO in D.vulgaris LPS. This 'weak link' between the core and 'O' side-chain in the LPS

of enteric bacteria enables their hydrolysis to be achieved using weak acid. Strong acid was however successful in D.vulgaris and yielded, in addition to the sugar hydrolysate, a lipid pellicle (the lipid A) which may be useful in further studies. The paper chromatography of the hydrolysates was not sufficiently sensitive due to the heteropolymeric nature of the sugar side-chain. A much larger loading of the paper would be required, which would hinder the separation aswell as using up valuable material. HPLC coupled to refractive index detection proved to be much more informative. Several sugars were tentatively identified in the reproducible separations achieved with S.typhimurium LPS, some corresponding with the known composition of this LPS (Wilkinson, 1977). Several peaks were not identified although abequose standard was not available to run. Ribose presence may be due to some nucleic acid contamination. It should be emphasised that the composition of S.typhimurium LPS is very batch variable. The identification of sugars by this method in a complex separation of this type is very tentative and although this may have been possible for the D.vulgaris separations this was not the prime objective of the investigation. The most important findings are the differences in peaks obtained using C+Fe and C-Fe LPS. Whether these peak changes represent changes in the LPS sugars or different separations on the HPLC column (which is based on both ion-exchange and reverse phase techniques, Waters Associates) due to charge differences brought about by interaction with ferrous ions is at this stage uncertain. However the previous data indicating no change in the length of the 'O' side-chain or change in the % dry weight of carbohydrate tends to favour the latter

proposal and this would support the demonstration of some form of selective interaction between D.vulgaris LPS and ferrous ions (Chapter 4). HPLC has thus proved a very useful tool in the analysis of LPS sugars.

CHAPTER 7

RECONSTITUTED OUTER MEMBRANE VESICLES.

The previous studies have enabled the isolation of various macromolecules which are present in the cell wall of D.vulgaris and it is proposed to utilise these in an attempt to reconstitute OM vesicles which are structurally and functionally similar to the OM in its natural state. By the manipulation of the vesicle components it should be possible to further investigate their individual properties. Previous in vivo studies (Chapter 4) have indicated a selective interaction between the LPS and ferrous ions in D.vulgaris and this in vitro study seeks to extend this investigation. It is further hoped to investigate the possible passage of metal cations through the reconstituted OM by initial vesicle entrapment of Fe^{2+} and subsequent leakage.

Dry films of most naturally occuring phospholipids swell spontaneously when in contact with aqueous solutions to form multilayered structures, or liposomes, composed of concentric bimolecular leaflets of phospholipid separated by small aqueous spaces. The water content and temperature of the dispersion is important but such vesicles can be produced simply by shaking phosphatidylcholine in water (Harrison & Lunt, 1975). More stable homogeneous liposome preparations can be obtained by sonication, which promotes formation of single-bilayer-bounded vesicles which have been shown to be completely sealed. The superficial resemblance of liposomes to biological membranes is obvious and they have been used as simple model membrane systems in many studies (Sessa & Weissmann, 1968) particularly for studying the the properties of bio-membranes (Singer, 1973). However, liposomes more closely approach bio-membranes with the addition of proteins

and various methods have been developed for the incorporation of functioning protein molecules into liposomes (Eytan, 1982):-

i. Detergent dialysis; involving co-solubilisation of proteins and phospholipids in detergent with the subsequent removal of the detergent by dialysis.

ii. Detergent dilution; co-solubilisation followed by dilution of small aliquots forming tight reconstituted proteo-liposomes.

iii. Sonication; Sonication of a mixture of phospholipid and protein. The liposomes can be preformed but this may require longer sonication periods and hence possible protein denaturation.

iv. Freeze-thaw sonication; Preformed liposomes mixed with protein aliquots are rapidly frozen and slowly thawed. Reconstitution is completed by a brief sonication.

v. Direct incorporation; By simple incubation of suitable proteins.

vi. Fusion; Proteo-liposomes can often be induced to fuse with other vesicles, planar membranes or cells.

The choice of method is determined simply by determining that suitable for the efficient functioning of the protein to be incorporated. No one method is suitable for every protein. For effective reconstitution mixtures of lipids are however recommended and various extracts have been used. Where synthetic mixtures are used it is best to remove neutral lipids. The lipid:protein ratio is also of importance. In natural systems this is between 0.25 and 1.0 . Functional reconstitution usually requires an increased ratio of 20 for most transmembraneous functions. The possible orientation

of proteins within the liposomes should also be borne in mind when interpreting results of reconstitution experiments.

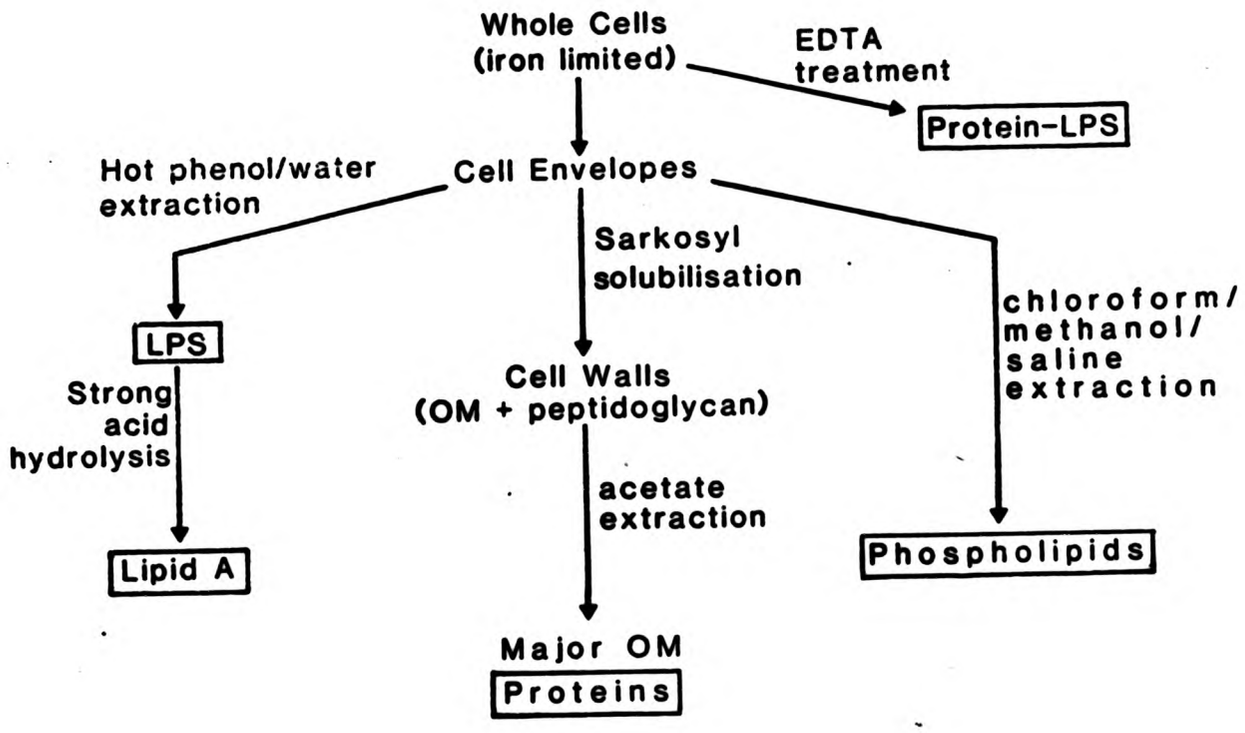
Reconstituted membrane systems utilising bacterial OM components have been reported in the pioneering studies of Nikaido & Nakae and their co-workers in their work on trans-membrane hydrophilic pores in Pseudomonas aeruginosa (Nakae, 1975, 1976a,1976b; Hancock & Nikaido, 1978). A simpler system, not containing LPS, has also been reported for Proteus mirabilis by Nixdorff et al (1977). Also of note are liposomal studies of mobile carrier ionophores for Fe(II) (Young & Gomperts, 1977).

The workers on reconstituted OM vesicles have favoured the use of natural, extracted phospholipids for their reconstitutions. However, the ratio of this material to other components is variable. All workers have utilised the sonication method for their reconstitutions using either a bath or microtip probe.

For the reconstitutions performed in these studies it was necessary to make use of the various assumptions and calculations made in previous chapters regarding the composition of extracts and the approximate molecular weights of certain molecules in order to determine the optimum proportions of constituents. A scheme summarising the component fractions used is given in Figure 7.1.

Figure 7.1.

FRACTIONATION OF COMPONENTS



RESULTS

7.1. Vesicle formation.

The variability of component ratios and the use of natural extracts by other workers did not aid the decision as to which ratios to use with D.vulgaris extracts. The formation of vesicles was monitored by the use of negative staining and thin sections for electron microscopy. The use of up to 500µg of extracted D.vulgaris LPS appeared to result in vesicles incorporating LPS (Plates 7.1, 7.2 and 7.4) and the use of excess LPS showed the appearance of LPS aggregates outside the formed vesicles (Plate 7.3). Thin sections of lipid/LPS vesicles showed the presence of membrane bilayers (Plate 7.5), these were however much thinner in cross-section than those of isolated cell walls seen in previous work (Plate 5.5). Results of previous work (Chapter 6) and calculations made from these (Chapter 8) suggest an average M.Wt. for D.vulgaris LPS of about 4000, and 734 for that of phospholipid. Thus the maximum amounts of LPS and phospholipid used here (500µg of each) correspond to a molar ratio of 5.5:1 (lipid:LPS).

7.2. 55Fe (II) binding.

Vesicles incorporating lipid only, lipid and D.vulgaris LPS, and lipid and S.typhimurium LPS, were utilised for iron binding experiments. After incubation the free iron was removed by one of three methods; centrifugation, Chelex 100 (chelating resin) or filtration. The results were corrected using water blanks and are shown in Figure 7.2. Filtration removal gave the highest apparent binding of iron whereas centrifugation gave lower readings, but which were still reproducible. Chelex 100 gave comparable values to the above methods for lipid only vesicles but the inclusion of LPS gave values below the water

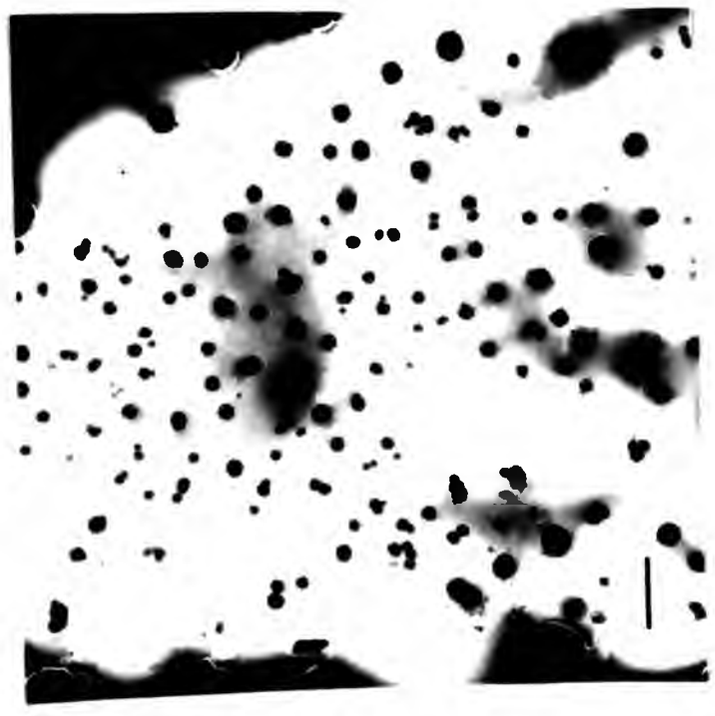


Plate 7.1. Vesicles formed from extracted D.vulgaris polar lipid only. Negative stain. x4000 (bar=1.0µm).

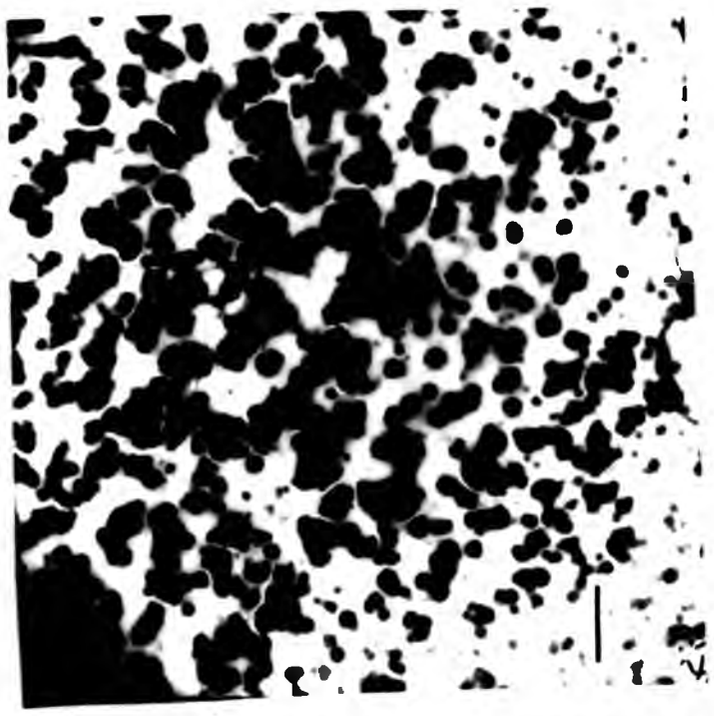


Plate 7.2. Vesicles formed from lipid and D.vulgaris LPS (5.5:1). Negative stain. x4000 (bar=1.0µm). See alsp Plate 7.4.

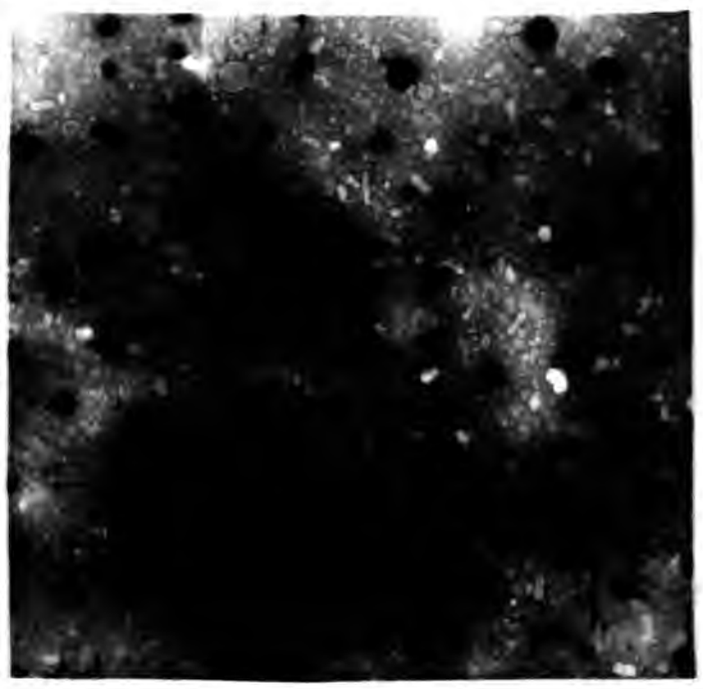


Plate 7.3. Vesicles formed from lipid and excess LPS (shown as aggregates). Negative stain. x16000 (bar=1.0µm).

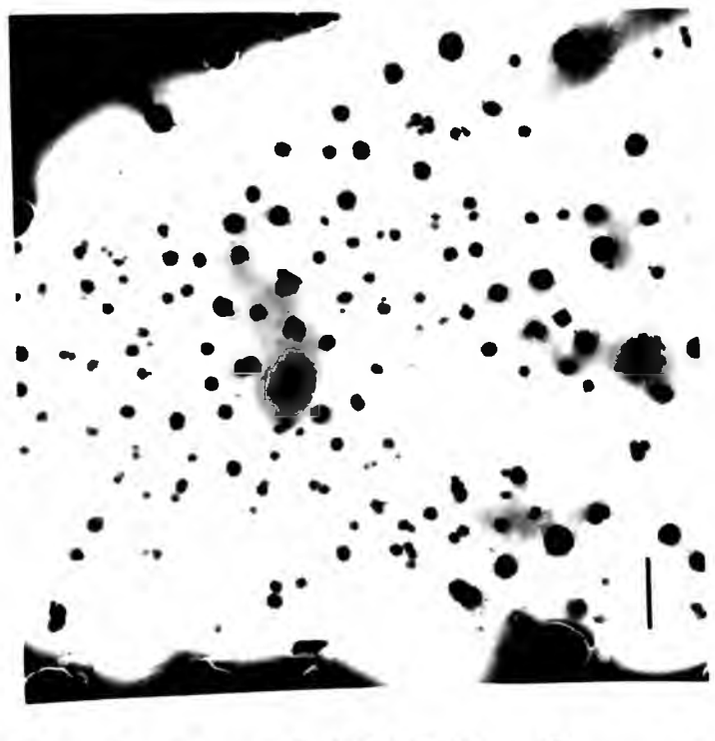


Plate 7.1. Vesicles formed from extracted D.vulgaris polar lipid only. Negative stain. x4000 (bar=1.0µm).

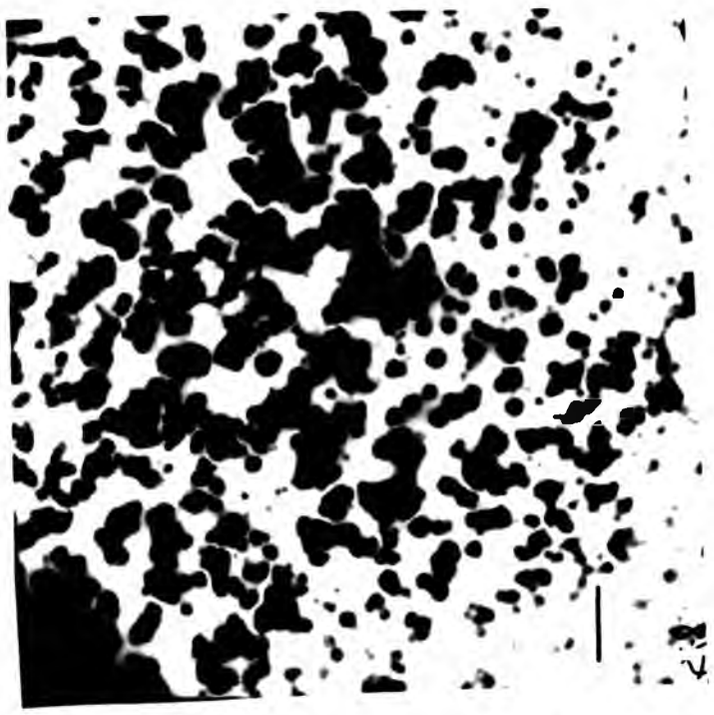


Plate 7.2. Vesicles formed from lipid and D.vulgaris LPS (5.5:1). Negative stain. x4000 (bar=1.0µm). See alsp Plate 7.4.

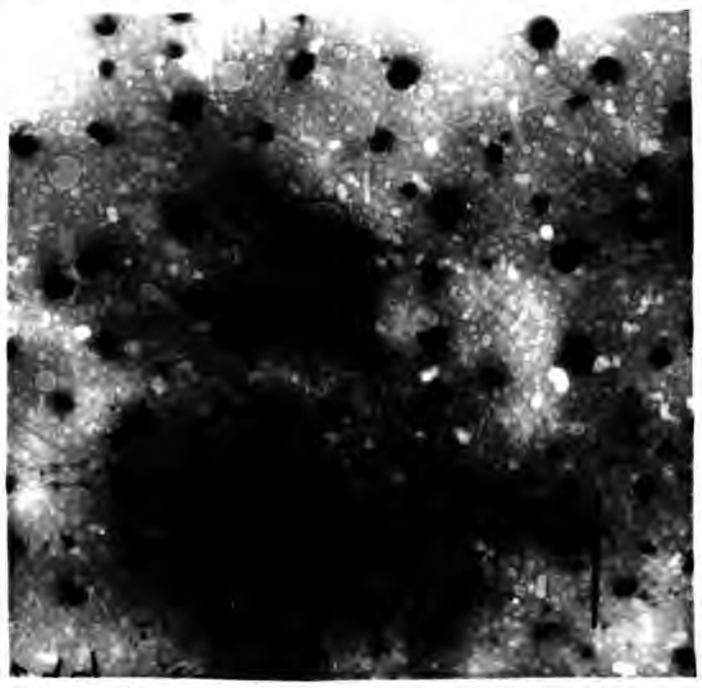


Plate 7.3. Vesicles formed from lipid and excess LPS (shown as aggregates). Negative stain. x16000 (bar=1.0µm).

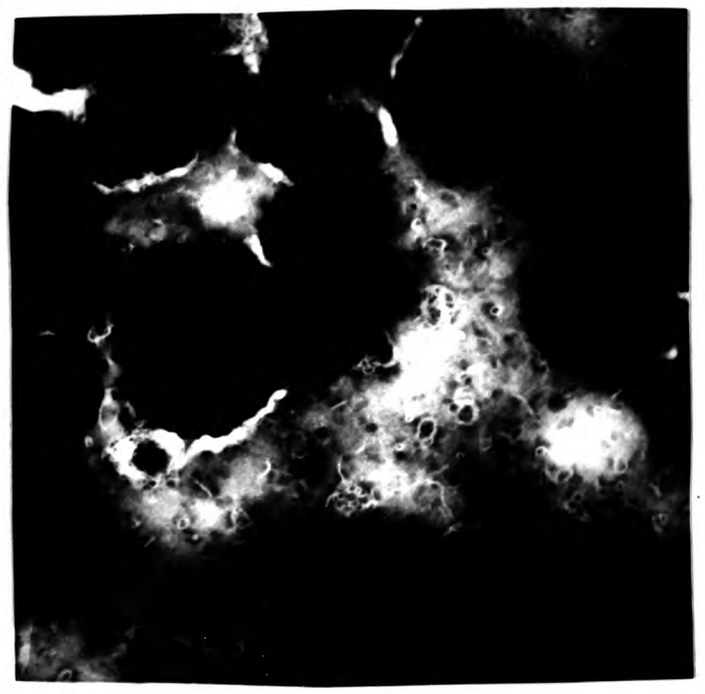


Plate 7.4. Vesicles formed from D.vulgaris lipid and LPS (5.5:1). High magnification, negative stain. x60000 (bar=0.2µm).

Plate 7.5. Vesicles formed from D.vulgaris lipid and LPS (5.5:1). Thin section. x40000 (bar=0.2µm). c.f. Plate 5.5.

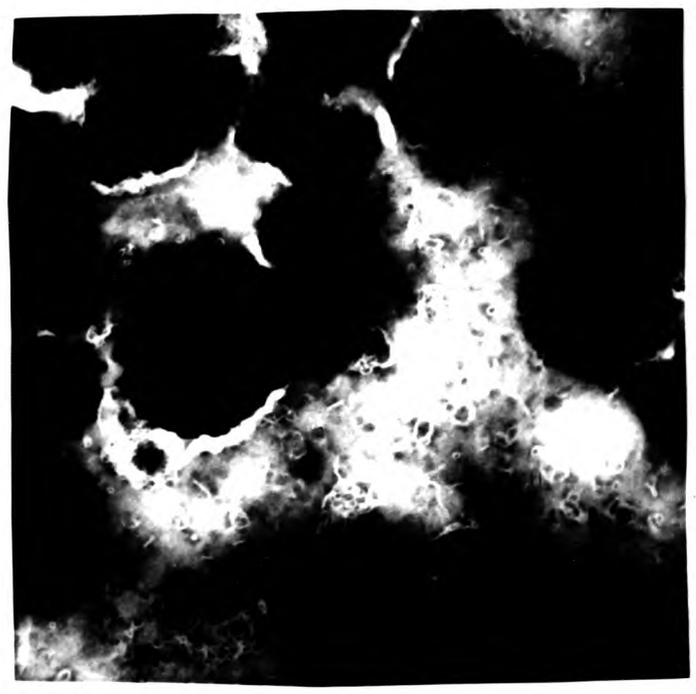


Plate 7.4. Vesicles formed from D.vulgaris lipid and LPS (5.5:1). High magnification, negative stain. x60000 (bar=0.2µm).

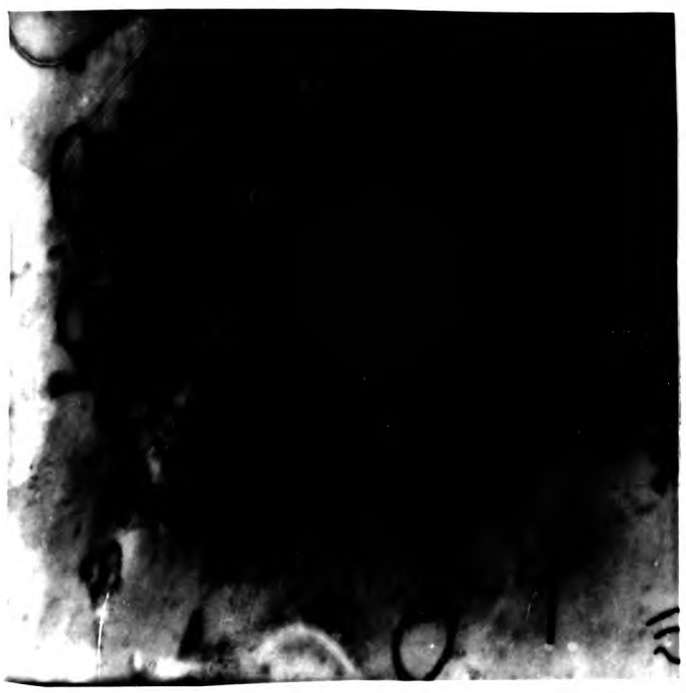


Plate 7.5. Vesicles formed from D.vulgaris lipid and LPS (5.5:1). Thin section. x40000 (bar=0.2µm). c.f. Plate 5.5.

Figure 7.2. Iron bound (µg) to various reconstituted vesicles measured after three separate techniques for the removal of free iron.

Separation method.	Vesicle composition.		
	Lipid only	Lipid + *D.vulgaris* LPS (500µg)	Lipid + *S.typhimurium* LPS(500µg).
i.Centrifugation	6.0±0.5	19.0±4.0	39.4±12.4
ii. Chelex 100 (cation chelating resin).	15.2±0.3	-52.0±1.0	-46.5±0.9
iii. Filtration	83.5±14.5	100.9±16.0	ND

N.B. All values are corrected using water blanks. n≥3.

blanks. Because of the reproducibility of conditions and the ease of the method the centrifugation removal technique was used for further binding studies.

Lipid/LPS vesicles utilising amounts of D.vulgaris and S.typhimurium LPS up to 500µg were assayed for iron binding and the curves produced are shown in Figure 7.3. These experiments were repeated in the presence of 20mM calcium in an attempt to block iron binding to the two types of LPS and the results are also given in Figure 7.3. Figures 7.4 and 7.5 show the relative ability of calcium to block iron binding in the two types of LPS-containing vesicles. The binding of iron to LPS vesicles is demonstrated as a histogram in Figure 7.6, together with the results from vesicles incorporating lipid A fractions, OMPs and EDTA washings. Binding to synthetic phospholipid vesicles is also shown.

7.3. Fe(II) release.

The leakage of vesicle entrapped Fe^{2+} was investigated and the effect of subsequent additions of cell wall extracts were monit ored. the results are shown as original traces in Figure 7.7 (experiments 1-4). The ionophore A23187 (Sigma) produced a small increase in Fe(II) release above the basal level. OMPs 2/3 (the product of acetate-extracted cell walls prepared from EDTA washed cells) was the only fraction tested which produced a result. Addition of this fraction saw an inibition of Fe(II) release even in combination with other fractions and the ionophore. EDTA washings and OMP 1b (the residue after acetate extraction for OMPs 2/3) gave no response.

The effect of cell wall extracts directly (no vesicles present) on the Ferrozine assay system was also monitored

Figure 7.3.

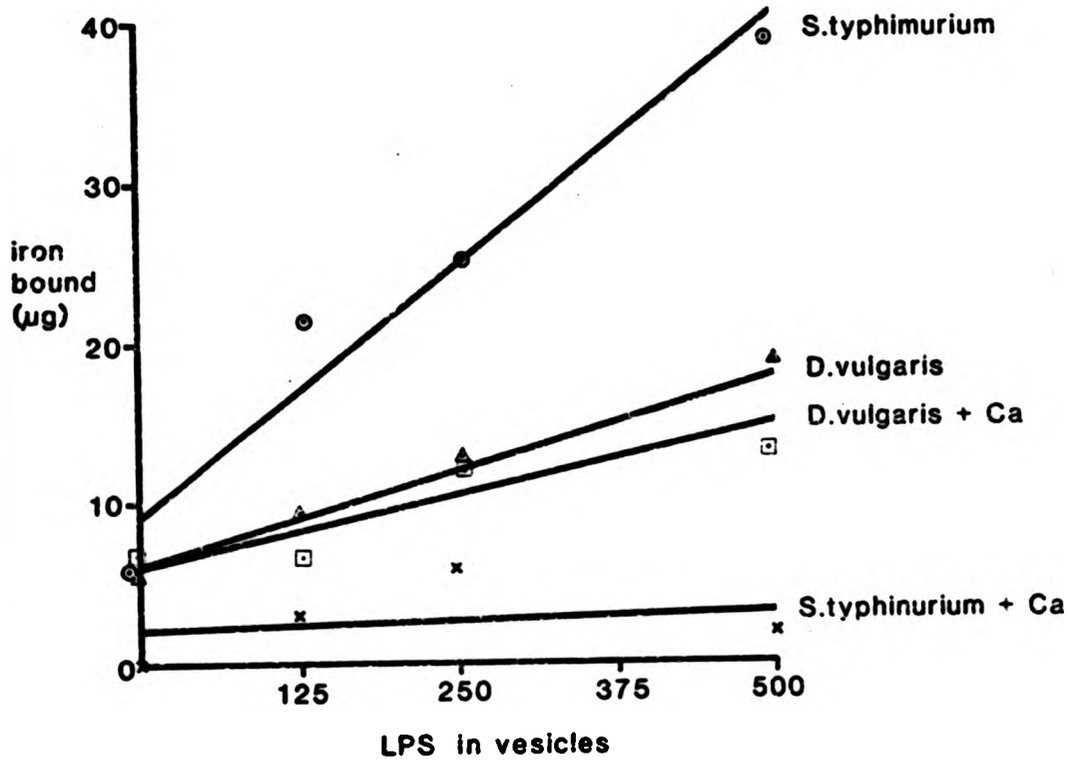
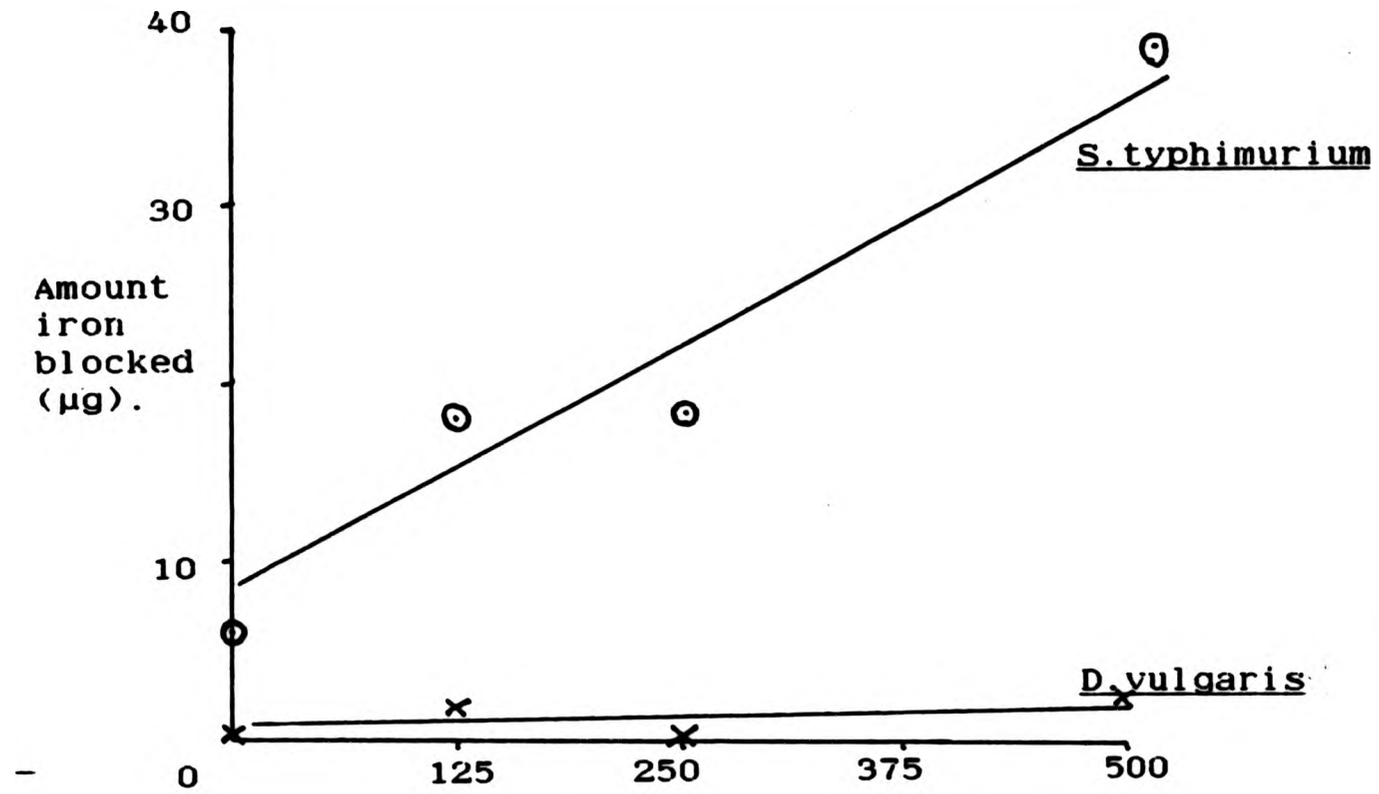

^{55}Fe BOUND TO LIPID / LPS VESICLES
OF D.VULGARIS AND S.TYPHIMURIUM &
IN THE PRESENCE OF CALCIUM (20mM)
40
30
20
10
0
iron bound (µg)
S.typhimurium
D.vulgaris
D.vulgaris + Ca
S.typhinurium + Ca
125
250
375
500
LPS in vesicles
Values shown are means of three experiments
curves represent calculated regression slopes

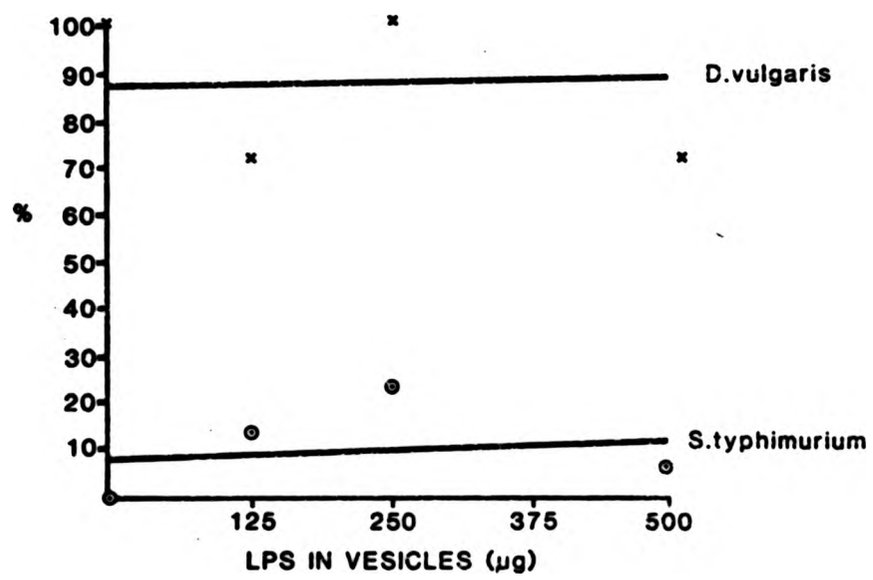
Figure 7.4. The amount of iron (^{55}Fe) (II) normally binding to lipid/LPS vesicles of D.vulgaris and S.typhimurium extracts that is blocked by the presence of calcium (20mM).



Values shown are means of three experiments and represent calculated regression slopes.

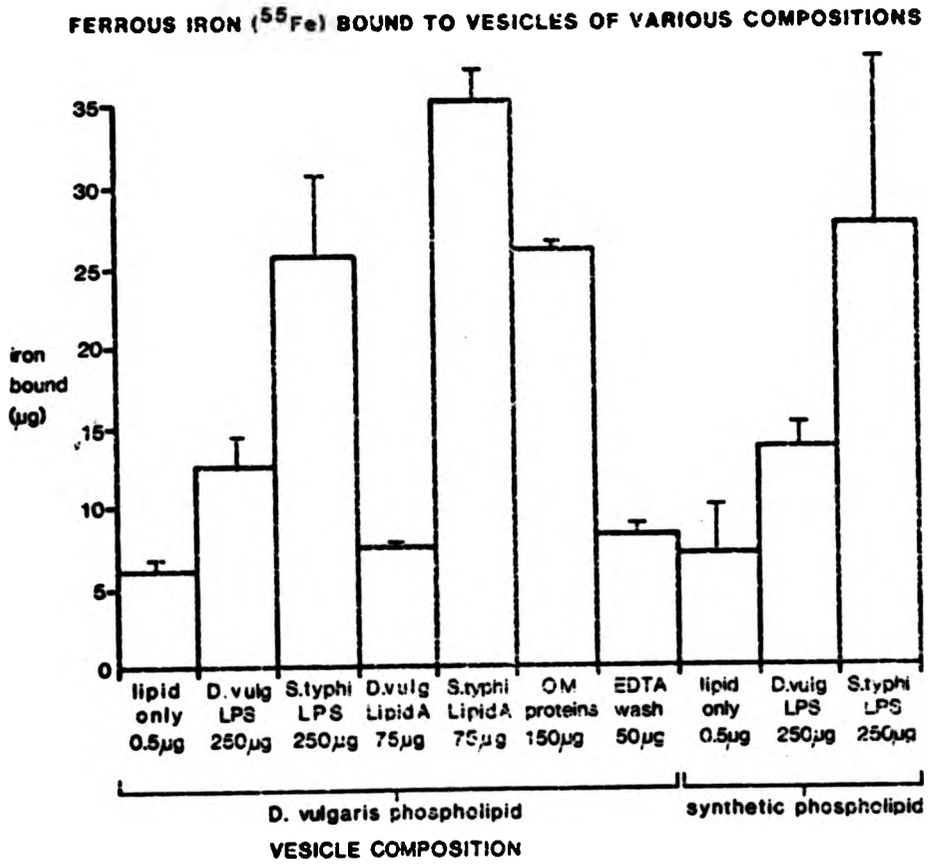
Figure 7.5.

^{55}Fe BINDING TO LIPID/LPS VESICLES OF D.VULGARIS AND S.TYPHIMURIUM IN THE PRESENCE OF Ca (20mM), AS A %AGE OF THAT BINDING IN THE ABSENCE OF Ca.



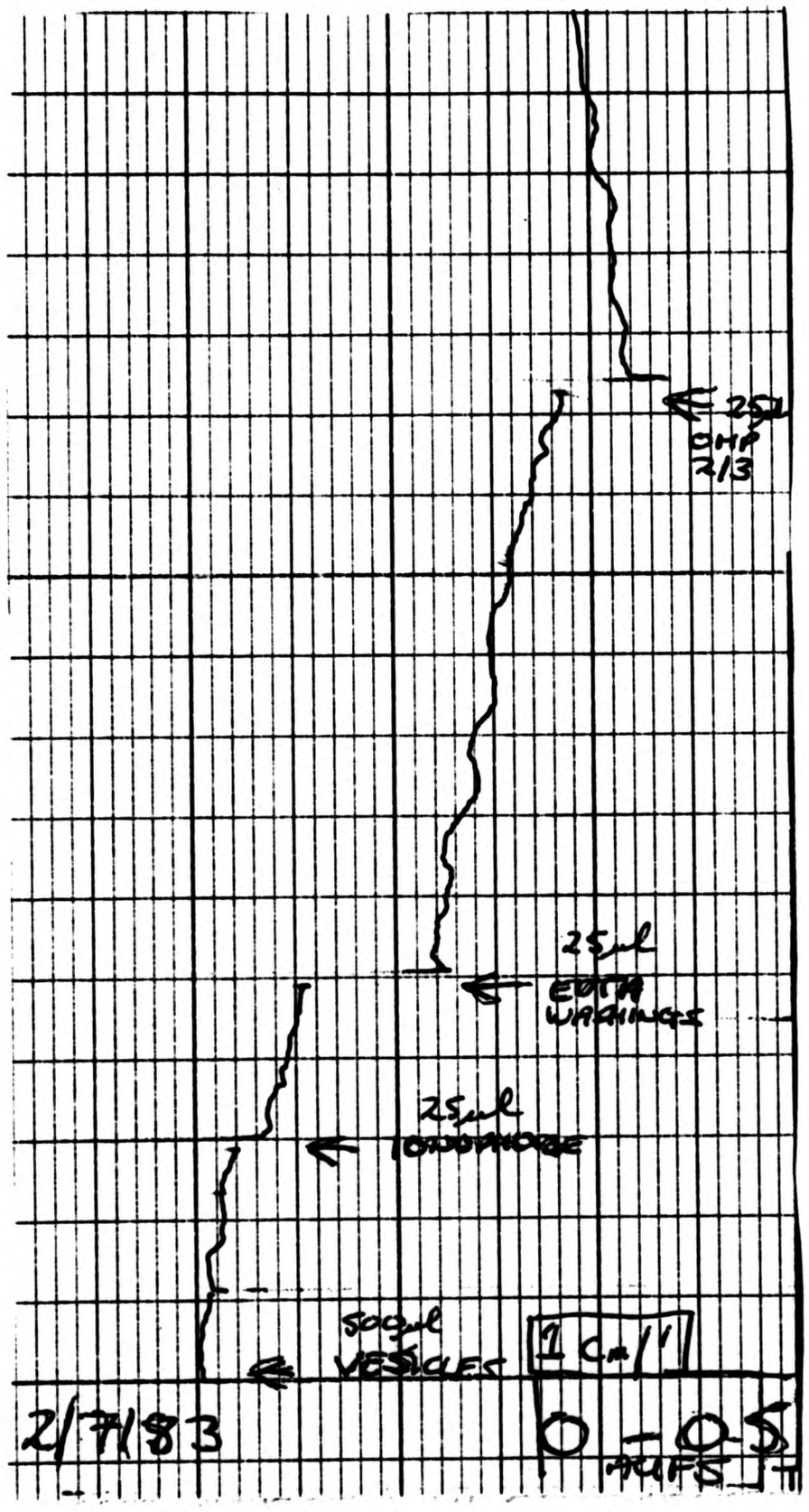
Values shown are means of three experiments
curves represent calculated regression slopes

Figure 7.6.

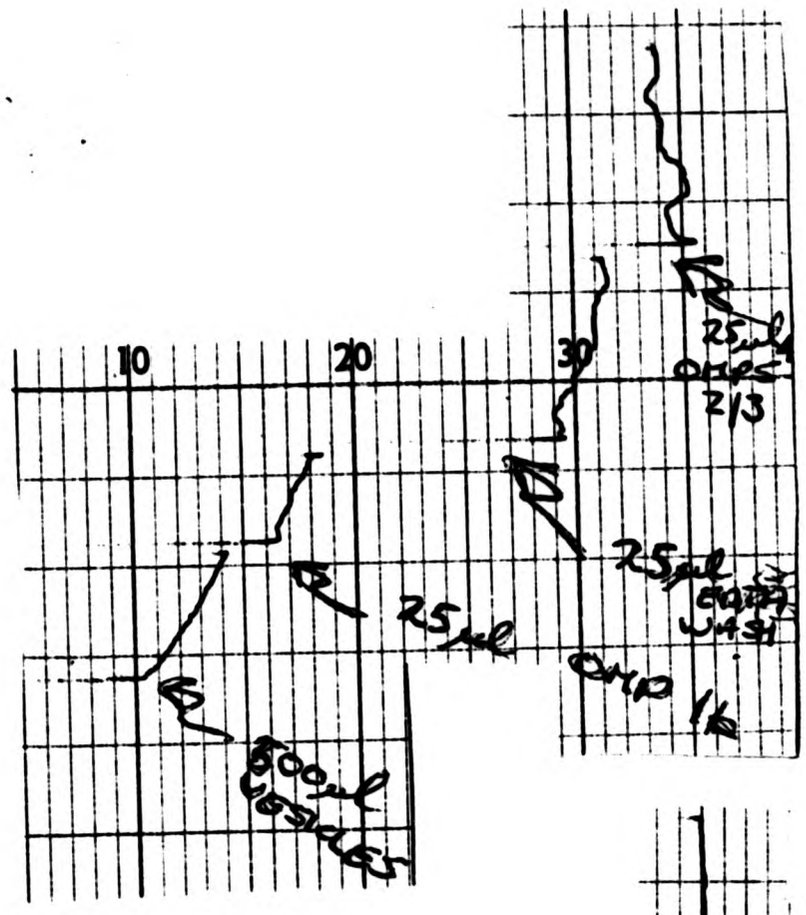


Values are means of at least three experiments (Bar=S.D)

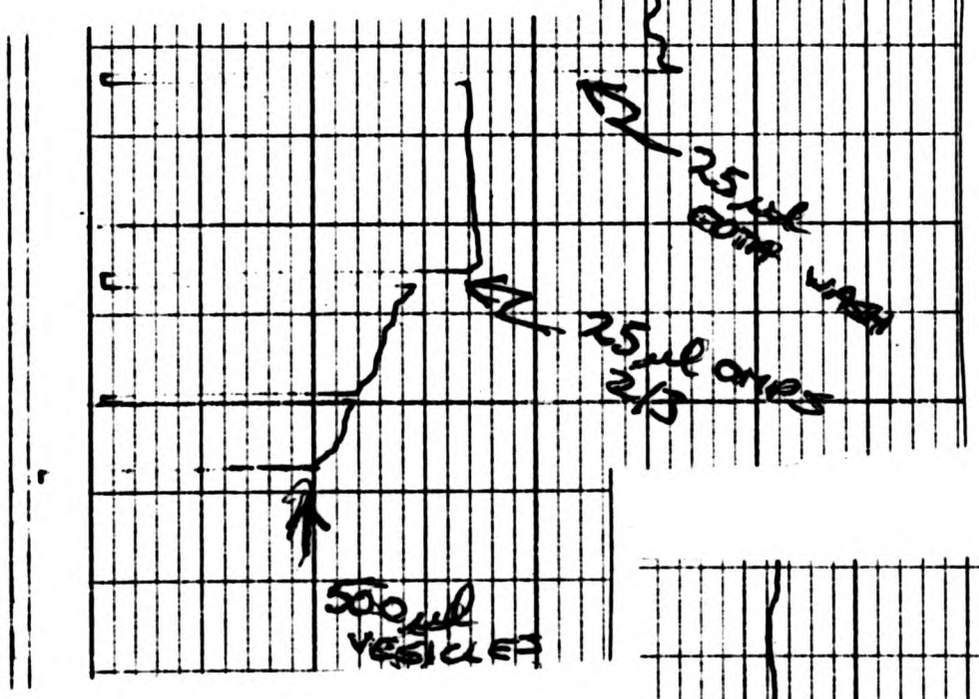
Figure 7.7. Traces showing change in optical density with time for Ferrozine system containing D.vulgaris lipid vesicles with entrapped Fe^{2+}, and after subsequent addition of cell wall fractions or ionophore.



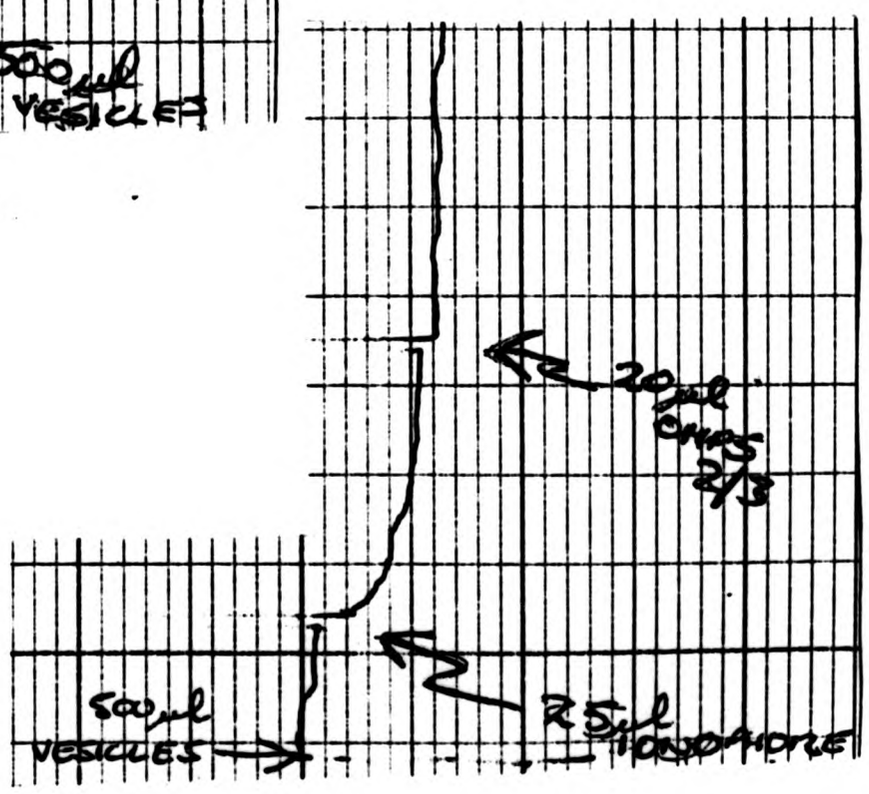
a) EXPERIMENT 1.



d) EXPERIMENT 4.



c) EXPERIMENT 3.



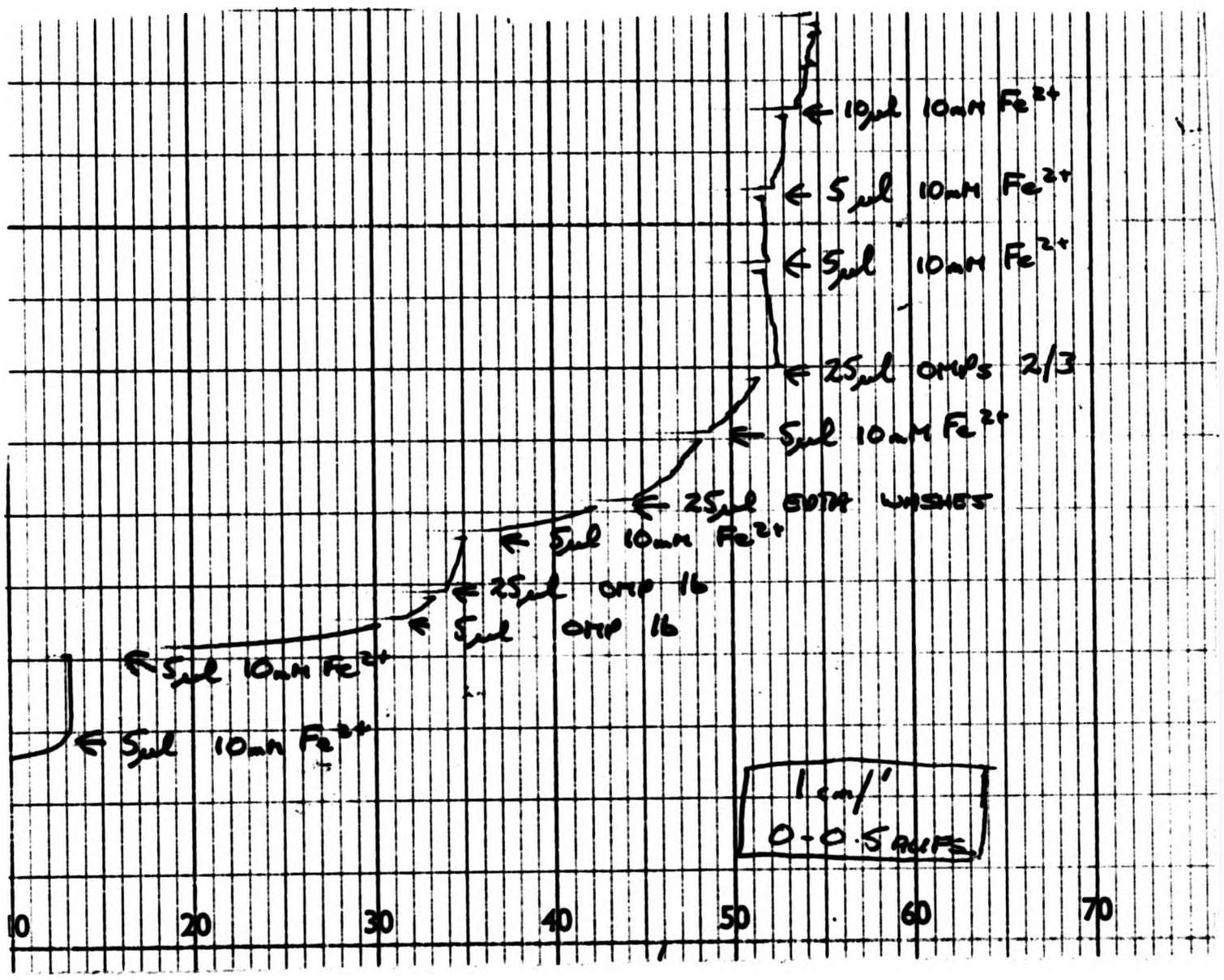
b) EXPERIMENT 2.

as a control to aid the interpretation of results. This is shown as original traces in Figure 7.8 (experiments 1-4). Only the OMPs 2/3 are seen to inhibit the Ferrozine assay system, the other cell fractions show no response. EDTA solution is capable of inhibiting the assay system.

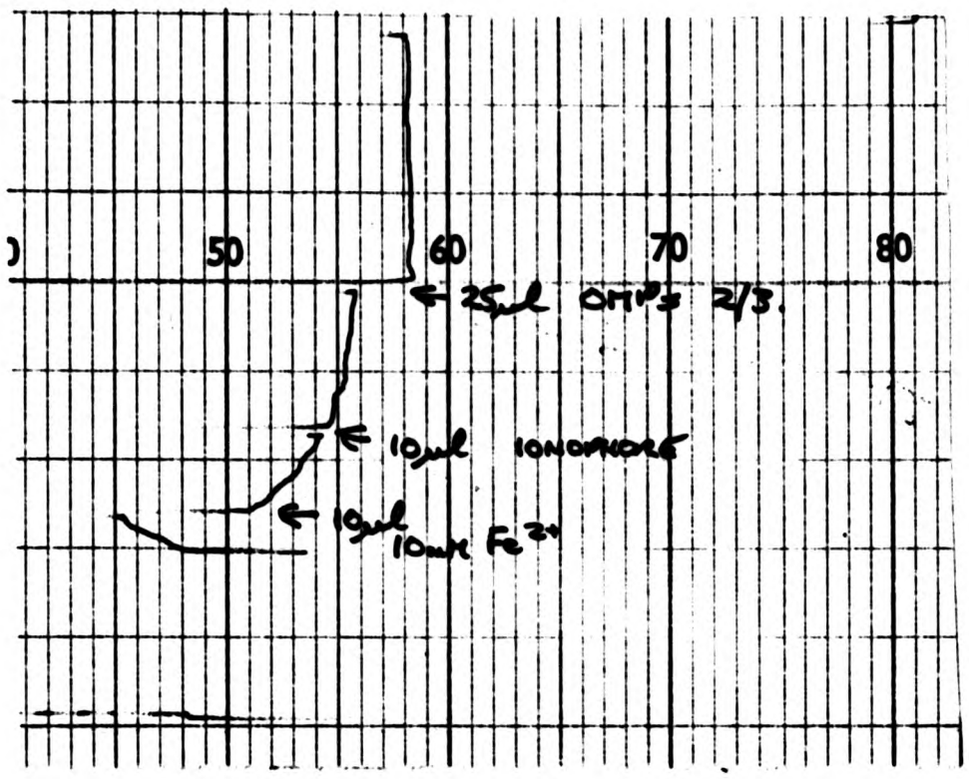
DISCUSSION.

The formation of D.vulgaris lipid/LPS (5.5:1) vesicles was successful as judged by electron microscopy. However, the demonstration of pure lipid and lipid/LPS vesicles by electron microscopy is not especially easy (Muller, 1980). The use of phosphotungstate for negative staining produces flattened vesicles although natural lipid vesicles are more stable. For thin sectioning, fixation by glutaraldehyde cross-links proteins and is therefore of little use, whereas osmium tetroxide primarily fixes across unsaturated fatty acids and therefore offers some stabilisation especially in bacterial fatty acid containing systems. The LPS appears to be incorporated into the vesicles at this ratio (5.5:1, Plate 7.2) although how the LPS molecules are orientated with regard to the lipid bilayer is not known and this may have an important bearing on the subsequent binding of Fe^{2+} to the vesicles. One may well expect molecules to be randomly inserted into the lipid vesicle bilayer upon reconstitution, however, surprisingly this has not been found to be the case with many protein incorporation systems where the protein is vectorally orientated. This may well be the case with LPS, a molecule which is amphipathic, where the more hydrophobic moiety is inserted first and ends up exposed to the inner surface of the vesicles. The 'O' side-chain would then be

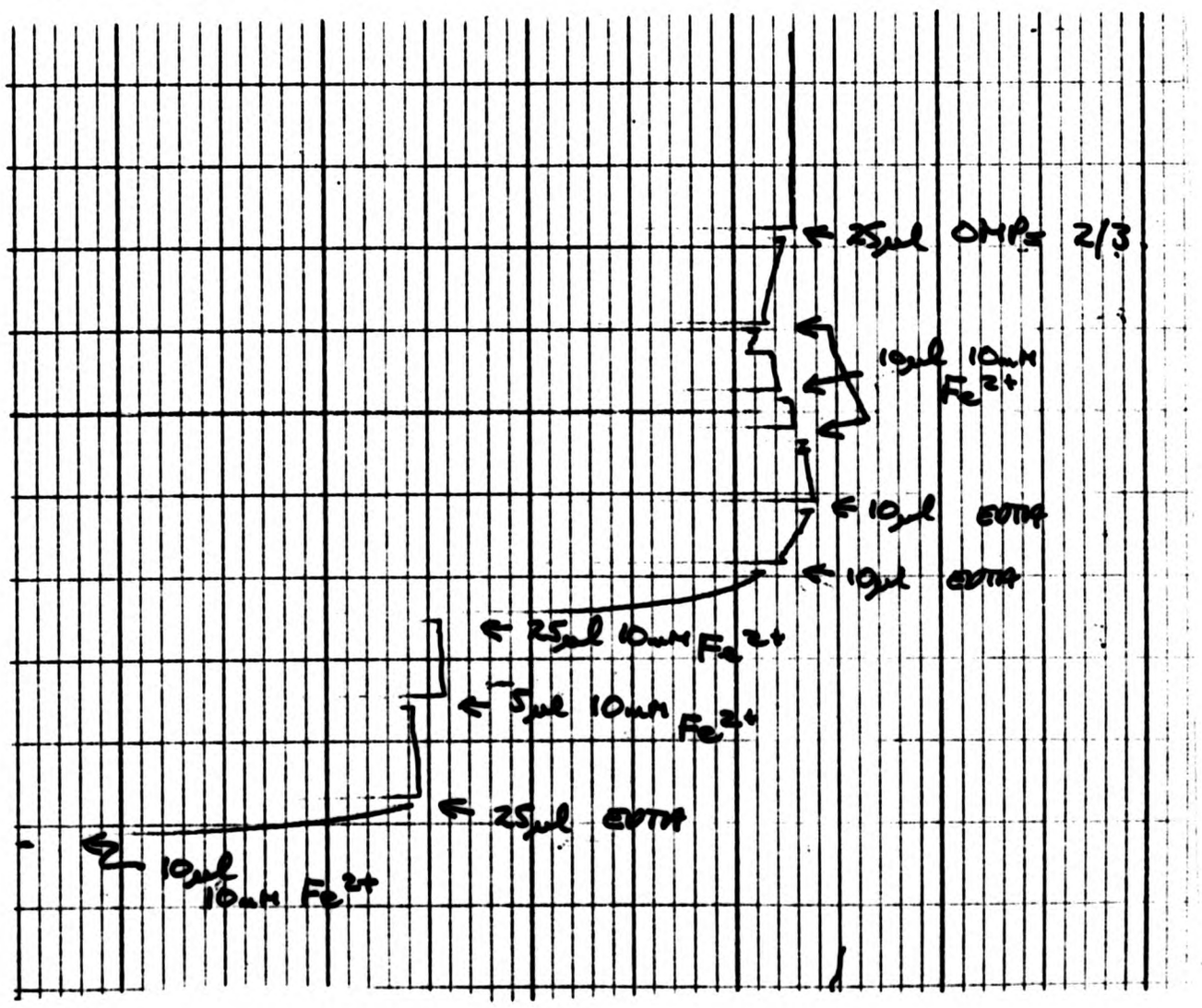
Figure 7.8. Traces showing change in optical density with time for Ferrozine system after addition of Fe^{2+} solution, D.vulgaris cell wall extracts and EDTA solution.



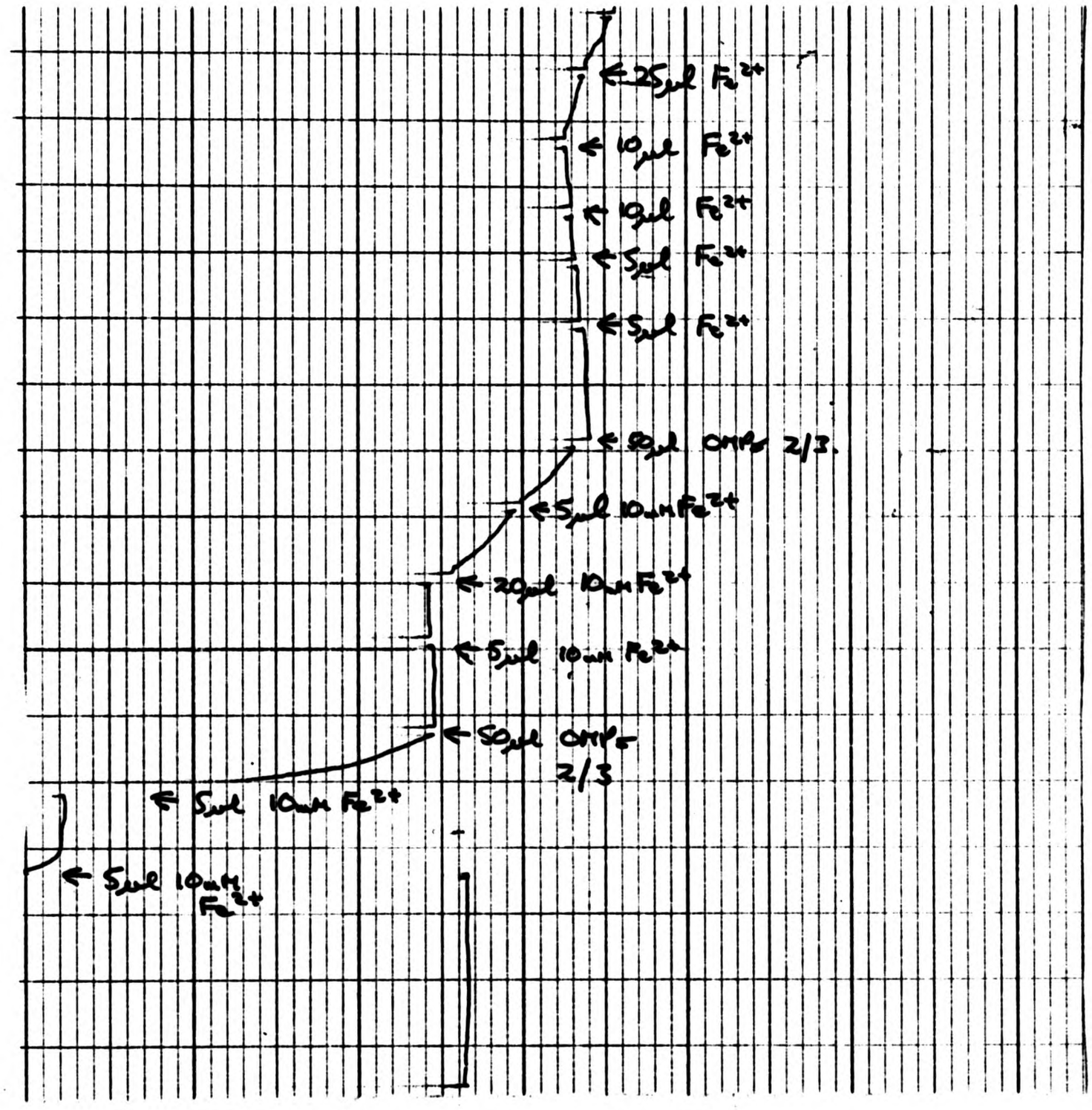
a) EXPERIMENT 1.



c) EXPERIMENT 3.



b) EXPERIMENT 2.



d) EXPERIMENT 4.

exposed to the outer mileau as in the natural membrane.

The vesicles bind $^{55}Fe(II)$ effectively as demonstrated by the three types of separation techniques (Figure 7.1). Filtration and centrifugation are both good methods for free iron removal. The use of Chelex 100 for this purpose however does not give expected results with the inclusion of LPS into the vesicles. This can be attributed to the possible stripping of LPS from the vesicles (along with any bound iron) as is seen in the *in vivo* chelator experiments, hence giving a very low 'iron bound' reading. Indeed the chelating ability of Chelex 100 is improved by the presence of *D.vulgaris* or *S.typhimurium* LPS. Both *D.vulgaris* lipid and synthetic lipid vesicles show Fe^{2+} binding to a similar degree (Figure 7.6) and the nature of this binding is unknown. Both types of LPSwhen incorporated into the vesicles are able to show Fe^{2+} binding, this being more pronounced in *S.typhimurium* LPS. These results do not necessarily show a greater mole:mole binding of Fe^{2+} however as the two types of LPS differ in molecular size and the previous cationic history of the commercial LPS is not known. The reconstitution of electro-dialysed (Galanos *et al*, 1975) LPS may enable a more direct comparison to be made.

The inclusion of Ca^{2+} (20 fold excess over Fe^{2+}) caused a marked drop in the linear iron binding curve for lipid/ *S.typhimurium* LPS vesicles, but little interference was seen with *D.vulgaris* LPS-containing vesicles (Figure 7.3). This is further emphasised in the plots shown in Figures 7.4 and 7.5. In *S.typhimurium* lipid/LPS reconstitutions Ca^{2+} effectively competed with the Fe^{2+}, only 15% of the Ca^{2+}-

free binding remaining in 250µg LPS/lipid vesicles when Ca^{2+} was present. These results support the previous in vivo findings reported in Chapter 4, using the EDTA washing techniques, and further point to a selective interaction between Fe^{2+} and the LPS in D.vulgaris.

Figure 7.6 further shows that iron binds to S.typhimurium. but not to D.vulgaris lipid A. This points to a seemingly totally different site of cation binding in the two types of LPS. When one considers the differences in the LPS demonstrated in the previous results and the possible selective nature of D.vulgaris cation binding this does not seem unlikely. The possible nature of these binding sites and the significance of this interaction with regard to transport/metabolism by the cell and cell/iron adhesion will be fully discussed in Chapter 8.

The pronounced iron binding to vesicles containing OMPs (Figure 7.6) is noticable and prompted the further fractionation into OMPs 2/3 and 1b by the use of EDTA washed cells for cell wall preparation and acetate extraction, together with their use in studies on Fe(II) release from vesicles (Figures 7.7 and 7.8). The only noticable effect of the various extracts was the ability of OMPs 2/3 to inhibit the apparant release of iron even after ionophore treatment. This result prompted the testing of this fraction on just the Ferrozine assay mixture, which it was able to inhibit in a similar manner to the EDTA cation chelator. It thus seems that OMPs 2/3 have a chelator function of their own, rather than a direct effect on the leakage of iron from

reconstituted OM vesicles. This initial study together with the binding ability of isolated OMPs 1/2/3 may well repay further investigations.

reconstituted OM vesicles. This initial study together with the binding ability of isolated OMPs 1/2/3 may well repay further investigations.

CHAPTER 8

GENERAL DISCUSSION.

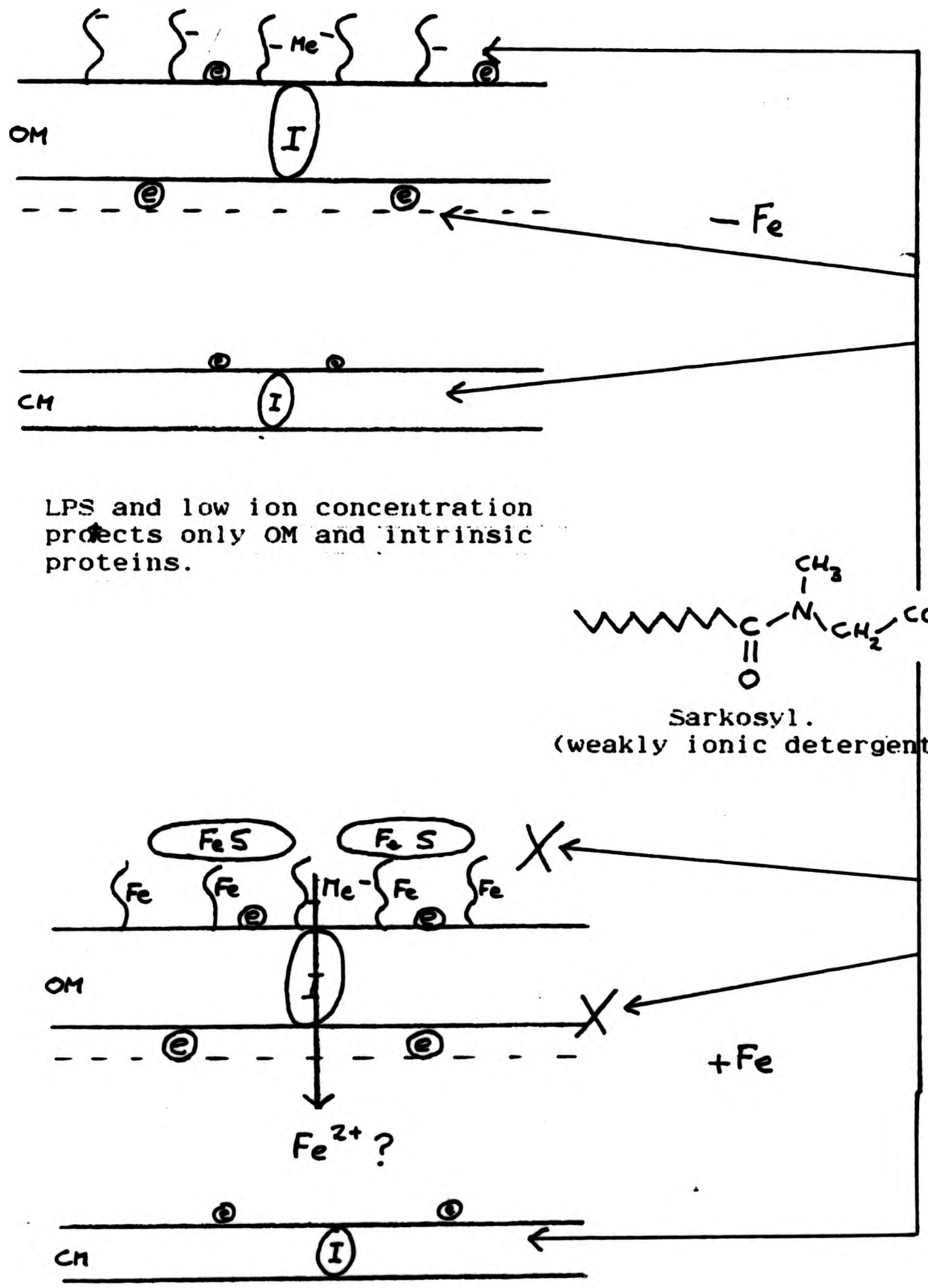
The Sarkosyl solubilisation technique (Filip et al, 1973) has proved extremely useful for the investigation of the cell wall structure of D.vulgaris grown in iron rich (C+Fe) and iron poor (C-Fe) media. Although some studies suggest that the material obtained by this technique is not complete OM + peptidoglycan in its native state (Owen et al, 1982; Chopra & Shales, 1980) and Sarkosyl had been shown to penetrate the OM in some instances (Anwar et al, 1983), the result is a loss of some lipid material and OMPs rather than any contamination by CM material. The Sarkosyl method is frequently used for the localisation of protein within the cell envelope. In these studies on D.vulgaris, broken vesicles of OM + peptidoglycan material which are morphologically similar to the intact cell wall structure were obtained. This material is rich in proteins and contains both lipid and LPS material. The most interesting feature of Sarkosyl cell wall preparation from D.vulgaris is the gross difference in the protein composition of C+Fe and C-Fe grown cells. The scale of these changes points to their being of an artifactual nature i.e. that the ferrous sulphide precipitate in C+Fe cultures acts in a protective manner rendering the OM insoluble to Sarkosyl. The ability of iron prior to Sarkosylisation to restore some of these proteins to C-Fe grown cells supports this view, although some proteins may well be iron regulated. Sarkosylisation does not therefore provide a good method of detecting protein induction or repression caused by iron limitation per se in D.vulgaris OM. C-Fe cell walls prepared by Sarkosylisation yielded material from which all of the minor (C+Fe) protein bands had been removed. It is considered that these proteins were either

extrinsic membrane proteins, located at one face of the membrane, or adventitious proteins bound during extraction. These proteins are presumably protected from solubilisation by the ionic binding of Fe^{2+} or FeS particles in C+Fe cultures. In spite of this protein loss Sarkosylisation of C-Fe cell envelopes provides a convenient means of obtaining material rich in only the major cell wall proteins, LPS and lipid. These are certainly the major macromolecules involved in the OM structure and in its principal functions. The diagrammatic representation of Sarkosyl C+Fe and C-Fe cell envelope solubilisation is shown in Figure 8.1.

The 'classical' sucrose density gradient separation did not prove effective in fractionating cell wall material. The high concentration of Fe^{2+} and other metal cations in D.vulgaris cell envelopes, even in iron restricted cultures, may have been the cause of this, along with the lack of extensive 'O' side-chains in D.vulgaris LPS. The best hope for a second, comparative, cell wall fractionation by density differences appears to be from the pelleting of a low-speed deposit and subsequent extensive washing procedures. Further hopes for cell wall isolation may also lie with the emerging technique of 'membrane monolayers' (Nermut, 1982), in which cells are adsorbed to a suitable support material prior to lysis and treatments to remove the CM.

PAGE has shown the presence in the OM of three major proteins (OMPs 1, 2 and 3). The orientation of these proteins within the OM is not completely clear. 125I/lactoperoxidase labelling studies have shown that both OMP1 and OMP3 appear

Figure 8.1. Diagrammatic representation of the Sarkosyl solubilisation of D.vulgaris Woolwich C+Fe and C-Fe cell envelopes.



exposed on the surface in intact cells. Only OMP1 is retained upon protease treatment of isolated cell walls, indicating some form of protection within the membrane. Furthermore only OMP1 is resistant to 2% SDS treatment at 60°c (all three OMPs are resistant at 30°c whereupon LPS is removed).
This seems to suggest a complex role for OMP1, borne out by its presence in all strains examined, in that it appears to span the membrane, being exposed at the cell surface and bound to the petidoglycan. An apparant contradiction is the removal of a protein of the same M.Wt. as OMP1 by the EDTA washing of cells. If these proteins are identical, this could be explained by the postulation of several molecular forms of OMP1 or the presence of two proteins which comigrate on SDS-PAGE. This is further supported by the partial extraction only of OMP1 by acetate. OMP1 is, then, considered to be composed of OMP1a (removable, extrinsic) and OMP1b (bound, intrinsic). The exact structural differences and functions await further studies. OMP1b does not appear to be a porin, since it does not facilitate Fe^{2+} release from reconstituted vesicles, but it is likely that it is necessary for the structural integrity of the OM.
OMP2 is intrinsic, may not be exposed to the surface, but is not apparently bound to the peptidoglycan. OMP3 is also intrinsic but is exposed to the surface and may have some form of receptor function.

Hydrogenase studies failed to show any activity within the OM, ruling out this function for OMPs 1, 2 and 3. Hydrogenase activity is only present in C+Fe cultures. The enzyme is, apparently, periplasmic and could be loosely associated with.

the OM or CM, although most of the activity in these studies was found in the soluble fraction. This enzyme has recently been shown to contain non-haem iron at 12 atoms/molecule (Huynh et al. 1984) and this high iron content may explain its absence in C-Fe cultures.

The presence of superoxide dismutase has been reported in several obligate anaerobes including D.desulfuricans and Desulfotomaculum nigrificans (Hewitt & Morris, 1975) and the presence of this type of enzymic activity within the cell wall may provide substantial protection to the organism against oxygen toxicity. Prokaryotes (and mitochondria) have iron (or manganese) associated superoxide dismutases while the cytoplasm of eukaryotes contains the copper enzyme (Williams,1982). The difference between the two enzymes is in their stability, copper being bound to the enzyme with a high stability unlike iron (or manganese) which is bound much more weakly. Thus an iron containing superoxide dismutase is only stable in environments where there is a reasonably high concentration of available ferrous ions. Further searches for the functions of the major OMPs of D.vulgaris cell walls could therefore centre on superoxide dismutase assays. Several genera of SRB have been reported by Hewitt & Morris (1975) to contain this enzyme. The inability of these organisms to grow in air does not mean that the enzyme renders them no significant service. The reversibly bacteriostatic rather than bactericidal oxygen sensitivity of Desulfovibrio may well be due to the presence of superoxide dismutase activity. Many other oxygenase enzymes are associated with iron. Indeed studies on the mechanism of oxygenase action have shown that most, if not

all, dioxygenase reactions involve iron and that iron-bound oxygen appears to be the activated form before reaction with the substrate (Hayaishi & Nozaki, 1969).

The possibility that one or more of the OMPs are iron receptors or transporters is an attractive proposition and is made more likely by the iron chelating ability of crude preparations of OMPs 2 and 3 demonstrated by the binding of Fe^{2+} to reconstituted vesicles containing these proteins and their inhibition of the Ferrozine assay system for Ferrous iron. D.vulgaris differs in many ways from other Gram negative bacteria with regard to iron availability. Iron is necessarily already present in relativly large amounts within the outer limits, at least, of the cell envelope and present as the Fe(II), rather than the Fe(III) form. Many other bacteria have up to three high affinity siderophore iron chelating systems together wih cell envelope protein receptors (Chapter 1) aswell as a low affinity uptake system. D.vulgaris is unlikely to synthesise the usual Fe(III) ion siderophores together with their receptors. These highly specific Fe(III) ligands, being phenolates and hydroxamates, have a low affinity for Fe(II) and are unlikely candidates in Desulfovibrio. Specific Fe^{2+} transport systems through the OM may well be unnecessary if sufficient Fe^{2+} permeates to the periplasm through OM pores as, presumably, do many small hydrophilic compounds (lactate and sulphate inwards, H_2S outwards). This is especially true if concentrations of Fe^{2+} in the outer cell layers are high, as they must be in this bacterium. D.vulgaris may well be expected to have relatively large OM pores. This has been demonstrated in other non-enteric bacteria

(Hancock & Nikaido, 1978).

The problems of free Fe^{2+} diffusion into the periplasm, when high H_2S concentrations are diffusing out from the cell cytoplasm are obvious. Although iron is more soluble in its reduced state (expected maximum concentrations from solubility products at pH 7 are 10^{-1}M for Fe^{2+}, 10^{-18}M for Fe^{3+}), its precipitation as ferrous sulphides by the H_2S produced during bacterial metabolism will lower its availability to the cells. Thus although the redox potential provides a control of Fe(II) availability, the increase in H_2S provides another restriction. The solubility product of FeS is approximatly 10^{-17} when H_2S is at a concentration of 10mM; this gives 10^{-10}M S^{2-} at pH7 and the concentration of Fe^{2+} is limited to 10^{-7}M (Williams, 1982). Keeping this calculation in consideration it would not be unreasonable to expect some form of specific Fe(II) binding, giving an ease of availability, and an uptake system in Desulfovibrio.

The interactions of the various OMPs with each other, with LPS, with the lipid or with other extrinsic proteins is still obscure but may play an important part in their functioning. Kropinsky et al (1982) have postulated that the number of open functional protein pores in P.aeruginosa and hence OM permeability to small hydrophilic molecules, is directly influenced by the state of the LPS. In D.vulgaris OMP 1a is a good candidate for LPS association, being removable using EDTA (together with LPS) but retained by Sarkosyl prepared C-Fe cell walls. It is the lipid material within normal membrane systems that provides the fluidity for protein-protein interactions to occur, and although phospholipid

only forms the inner leaflet of the OM it is still of importance for these interactions and for the production of certain 'domains' of protein and other components within the membrane system. Polar lipid extraction and TLC of D.vulgaris cell envelope fractions has revealed the presence of phospholipids in keeping with those of other Gram negative anaerobic bacteria (mainly phosphatidylethanolamine). GLC FAME separations from whole cells have proved useful taxonomic pointers in other studies (refer to Chapter 5). The results from this present study using FAME analysis by GLC of D.vulgaris cell envelope, cell wall and LPS, give a useful comparison of fatty acid distribution within the cell envelope if one assumes all of the 18:1 to reside in the LPS (Figures 8.2 and 8.3). This is not an unreasonable assumption from the results given in Figures 5.10, 5.11 and 6.6. From Figure 8.3 the lipid distribution of the OM is seen to be 60% LPS and 40% other (phospholipid) highlighting the importance of the LPS contribution to the lipid bilayer in D.vulgaris.

The LPS analysis in this study has enabled an idea of the nature of this macromolecule in D.vulgaris to be formed and to contrast it with S.typhimurium LPS, as well as giving useful taxonomic pointers. The banding of LPS on silver stained SDS-PAGE appears to be a useful and rapid additional criterion for classification within Desulfovibrio. Above all however, the in vivo release studies and in vitro binding studies have shown a selective interaction between ferrous ions and LPS in D.vulgaris. In order to obtain an estimate of the molar binding ratio of ferrous ions and LPS of

Figure 8.2. Relative % fatty acids found in fractions of D.vulgaris Woolwich C+Fe cell envelope based on GLC separations of FAMEs using 18:1 as a 100% marker for LPS.

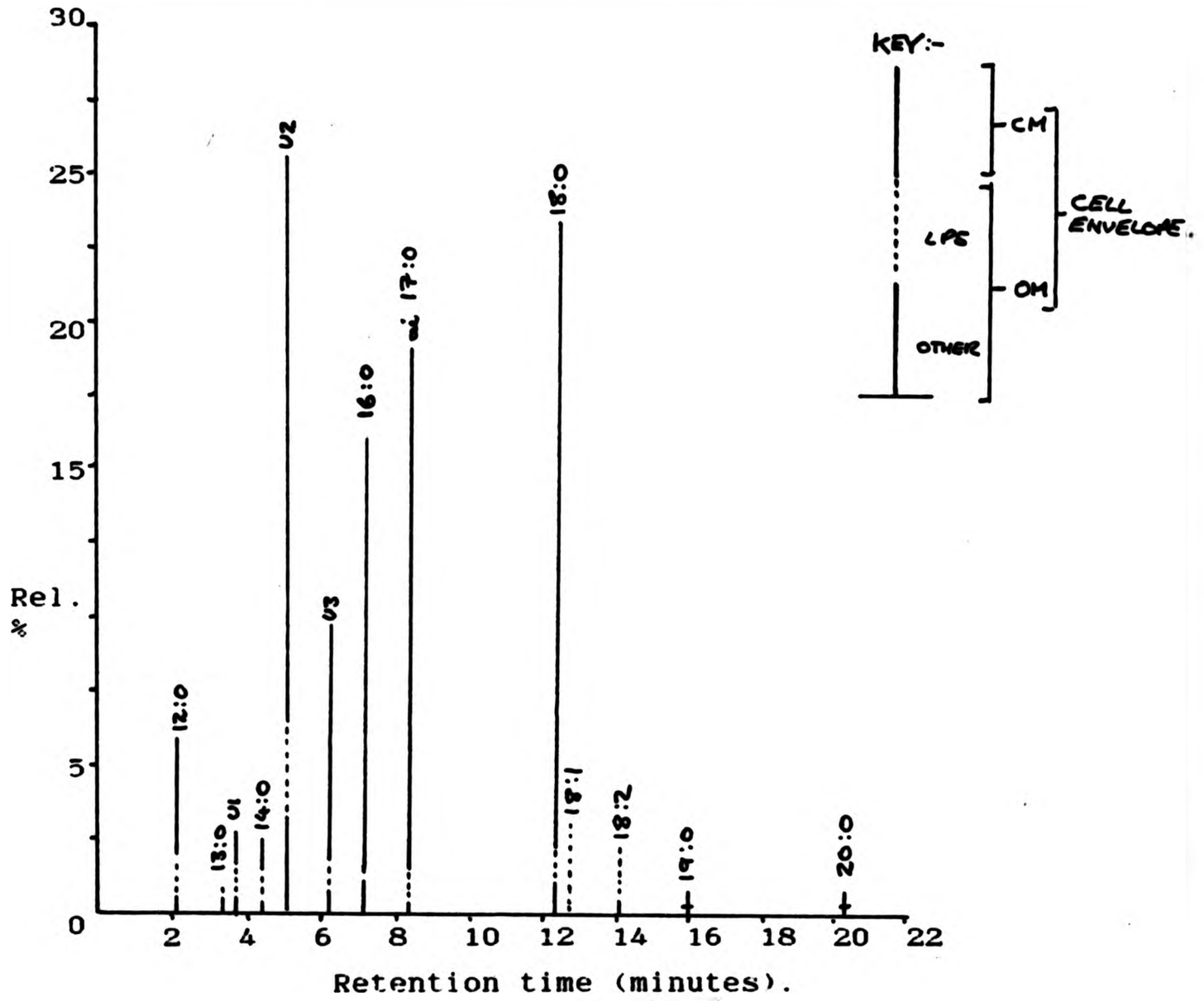
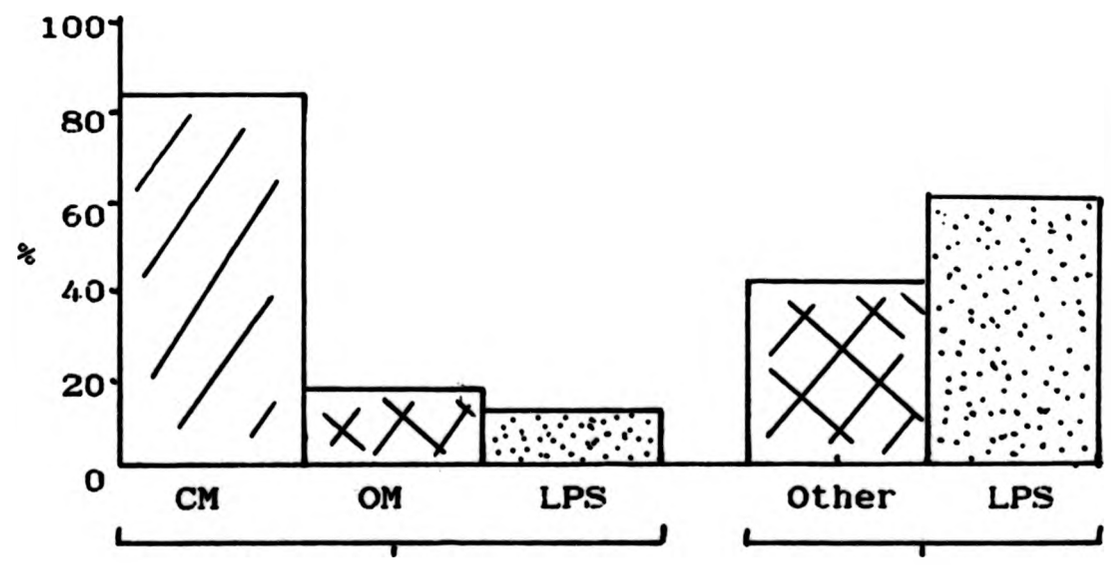


Figure 8.3. % total lipid (as fatty acid) found in cell envelope fractions from D.vulgaris Woolwich C+Fe, based on GLC separations.



D.vulgaris. one needs first to estimate the M.Wt. of the LPS. In the case of Salmonella LPS this value means very little due to the extreme variability and heterogeneity of the molecule. However in D.vulgaris Woolwich estimation of the M.Wt. may be more meaningful as the molecule is simpler and more homogeneous (Chapter 6). From the compositional data of Chapter 6 the dry weight of Woolwich LPS is 46% lipid. It is not unreasonable to assume there to be six fatty acid residues since this number is found in many other species and this region tends to be conserved (Wilkinson, 1977; Rogers et al, 1980). If the length of each residue is 18 C atoms then the M.Wt. of this moiety is 1800 and that of the whole LPS approximately 3913. A similar calculation for S.typhimurium LPS arrives at an average estimated M.Wt. of 12000. These figures are of little value in themselves but serve to contrast the two types of LPS.

Similarily using these figures, the compositional data for carbohydrate (Chapter 6) and 180 as the average monosaccharide M.Wt., one arrives at a figure of seven monosaccharide units per LPS for D.vulgaris Woolwich. This is in contrast to 38 monosaccharide units per LPS for S.typhimurium. These figures would not include amino-sugars and would assume an equal dispersion of heptoses and pentoses. This latter figure compares well with that of approximately 50 units (excluding amino-sugars) proposed for the 'O' side-chain and core region of S.typhimurium ('O' antigen factors 4, 5 and 12) by Galanos et al (1977). From the in vivo release studies of Chapter 4, 1µg of LPS appeared to bing 6.6µg of Fe^{2+}. Using the above M.Wt. estimation for LPS one arrives at a ratio of one mole LPS binding 461 moles Fe^{2+}, a seemingly large number of

ferrous ions for one molecule of LPS. From the *in vitro* studies of binding to reconstituted vesicles and allowing for the adsorption of ferrous ions to the lipid only, one is able to calculate a mean binding of 12.4µg of Fe^{2+} to vesicles containing 250µg of *D.vulgaris* LPS. Using the apparent M.Wt. of 3913 calculated above, this provides a molar binding ratio of 1.8 moles Fe^{2+} per mole *D.vulgaris* LPS for these *in vitro* studies. The apparent incompatability of these results may point to two types of interaction which are at work under *in vivo* and *in vitro* conditions.

In response to iron limitation *D.vulgaris* Woolwich appears to: increase carbohydrate within the cell wall; increase the amount of extractable LPS but show no lengthening of the 'O' sugar side-chain in this molecule; and either replace LPS sugars (or sugar moieties) or form a complex interaction between LPS and Fe^{2+}. These results together with the selective interaction demonstrated between LPS and Fe^{2+} indicate that LPS plays a very important role in the adsorption of iron to the cell surface and consequently adsorption of the cell to exposed iron surfaces in *D.vulgaris* Woolwich. The OMPs are probably more concerned with subsequent transport of iron into the cell, structural and other functions previously mentioned.

The peptidoglycan has been reported to bind metal ions in *E.coli*, (Hoyle & Beveridge, 1984). However when the intact cell wall is considered it would appear these ions are trapped by the fabric of the OM before contact with the peptidoglycan monolayer. Furthermore the peptidoglycan is a thin, simple layer in Gram negative organisms and iron deposition in the

cell wall of A.laidlawii is known to occur even when this organism is lacking in any peptidoglycan material (Hirsch, 1984). It seems unlikely therefore that peptidoglycan is of great importance in iron binding in D.vulgaris.

The, what must be, comparatively small 'O' side-chain in D.vulgaris Woolwich LPS emphasises that there is no need for the protection afforded by long and variable sugar side-chains but highlights the importance of the core and lipid A regions of the LPS. These regions contain a very high intensity of charge groups in the enteric bacteria (Nikaido & Nakae, 1979) including phosphate, ethanolamine phosphate, ethanolamine pyrophosphate and the carbonyl group of KDO. However it appears both KDO and heptose are absent from D.vulgaris Woolwich LPS. This has been found to be the case in other non-enteric bacteria, Bacteroides spp. and Anabaena variabilis (Galanos et al. 1977). The core region appears of paramount importance for metal ion binding in enteric bacteria, and the 'O' side-chain takes on a more protective function, but in D.vulgaris Woolwich the shortened, simpler sugar side-chain takes on the prime function of cation binding whilst having little protective function (or presumably antigenic nature). The bound FeS probably affords an alternative method of protection. These differences in metal cation binding are shown in the binding of Fe^{2+} to lipid A fractions reconstituted in OM vesicles, where Salmonella lipid A retains a high degree of binding, unlike that of D.vulgaris where once the side-chain sugars are removed by hydrolysis binding falls to the basal level. The binding in these two types of LPS is thus very different.

cell wall of A.laidlawii is known to occur even when this organism is lacking in any peptidoglycan material (Hirsch, 1984). It seems unlikely therefore that peptidoglycan is of great importance in iron binding in D.vulgaris.

The, what must be, comparatively small 'O' side-chain in D.vulgaris Woolwich LPS emphasises that there is no need for the protection afforded by long and variable sugar side-chains but highlights the importance of the core and lipid A regions of the LPS. These regions contain a very high intensity of charge groups in the enteric bacteria (Nikaido & Nakae, 1979) including phosphate, ethanolamine phosphate, ethanolamine pyrophosphate and the carbonyl group of KDO. However it appears both KDO and heptose are absent from D.vulgaris Woolwich LPS. This has been found to be the case in other non-enteric bacteria, Bacteroides spp. and Anabaena variabilis (Galanos et al. 1977). The core region appears of paramount importance for metal ion binding in enteric bacteria, and the 'O' side-chain takes on a more protective function, but in D.vulgaris Woolwich the shortened, simpler sugar side-chain takes on the prime function of cation binding whilst having little protective function (or presumably antigenic nature). The bound FeS probably affords an alternative method of protection. These differences in metal cation binding are shown in the binding of Fe^{2+} to lipid A fractions reconstituted in OM vesicles, where Salmonella lipid A retains a high degree of binding, unlike that of D.vulgaris where once the side-chain sugars are removed by hydrolysis binding falls to the basal level. The binding in these two types of LPS is thus very different.

This is not unexpected when one considers the selective nature of D.vulgaris Woolwich LPS for Fe^{2+}.

The chemical nature of this selective iron binding in D.vulgaris is unknown. Muhlradt et al (1977) concluded from a ^{31}P NMR study of the phosphate groups in LPS and lipid A of Salmonella that the bacterial surface charge can be modified in order to adapt to the ionic environment. This occurs by the addition or omission of substituents neutralising the overall negative charge of KDO and phosphate groups in the core. In Salmonella this was by substitution of the lipid A with 4-aminoarabinose. The study emphasised the core and lipid A importance in surface charge regulation in this enteric organism but gives little clue to the type of chemical interactions at work in D.vulgaris. Schindler & Osborn (1979) have also studied the binding of divalent cations to the core and lipid A regions using fluorescent probes and highlighted these regions in the binding of Ca^{2+} and Mg^{2+} in S.typhimurium. Their results suggest two sites of differing affinity: phosphate groups forming a low affinity site and a 'cage' of carboxylate clusters from the highly negative branched KDO region forming a high affinity site. All their LPS tested showed a binding ratio of 1 mole Ca^{2+} per mole LPS monomer, slightly lower than that observed in the D.vulgaris in vitro reconstition binding studies and much lower than that observed in the in vivo release studies. The absence of KDO in D.vulgaris Woolwich again, of course dictates a different structure and results imply not only a high affinity binding of Fe^{2+} but also a selective interaction. In looking for a potential binding site for D.vulgaris LPS one has to examine which type of ligands bind to the first

transition series of divalent cations and how these ligands may be arranged to select for one particular metal cation i.e. Fe^{2+}. Figure 8.4 shows the binding stability constants of these metal ions to various ligand groups common in biological molecules. Looking at Figure 8.4 one can see that Zn^{2+}, Cu^{2+} and Ni^{2+} all have high binding strengths and are predicted to bind to two or more N- or S- donors with perhaps carboxylate donors. Ca^{2+} and Mg^{2+} have low binding strengths and bind to only O- donor ligands rarely assisted perhaps by N- donor imidazole (Mg^{2+}). These predictions have been confirmed by X-ray structural studies on coordination sites for these metal ions in various protein ligands (Williams, 1982). There remains from Figure 8.4 the group of metal ions Fe^{2+}, Mn^{2+} and Co^{2+} which are of an intermediate character in their ligand forming nature. Many iron and cobalt containing enzymes however are based on the binding of small organic ring chelates i.e. haem and corrin. They provide the advantage of not exchanging metal ions and thereby maintaining the integrity of these iron and cobalt containing macromolecules. There are many iron/protein complexes which show these intermediate exchange rates and some of these are shown in Figure 8.5. The binding constants for these enzymes are known to be approximately 10^8 and they therefore need a standing iron concentration of approximatly 10^{-8}M to prevent dissociation, one possible reason for the high iron requirement by Desulfovibrio. The liganding groups are indeed intermediate in character between those which bind Ca(II) and those which bind Zn(II). An example is seen in the binding of Mn(II) in Concanavalin A (Reeke et al. 1978), here one imidazole group and several oxygen donors bind Mn(II) but

Figure 8.4. The stability constants of complexes of the first transition series of divalent cations with various ligands of biological importance. Effective constants at pH7 are reduced by competition from H^+. Mg(II) and Ca(II) complexes are all weaker than Mn(II) complexes. (From Williams, 1982).

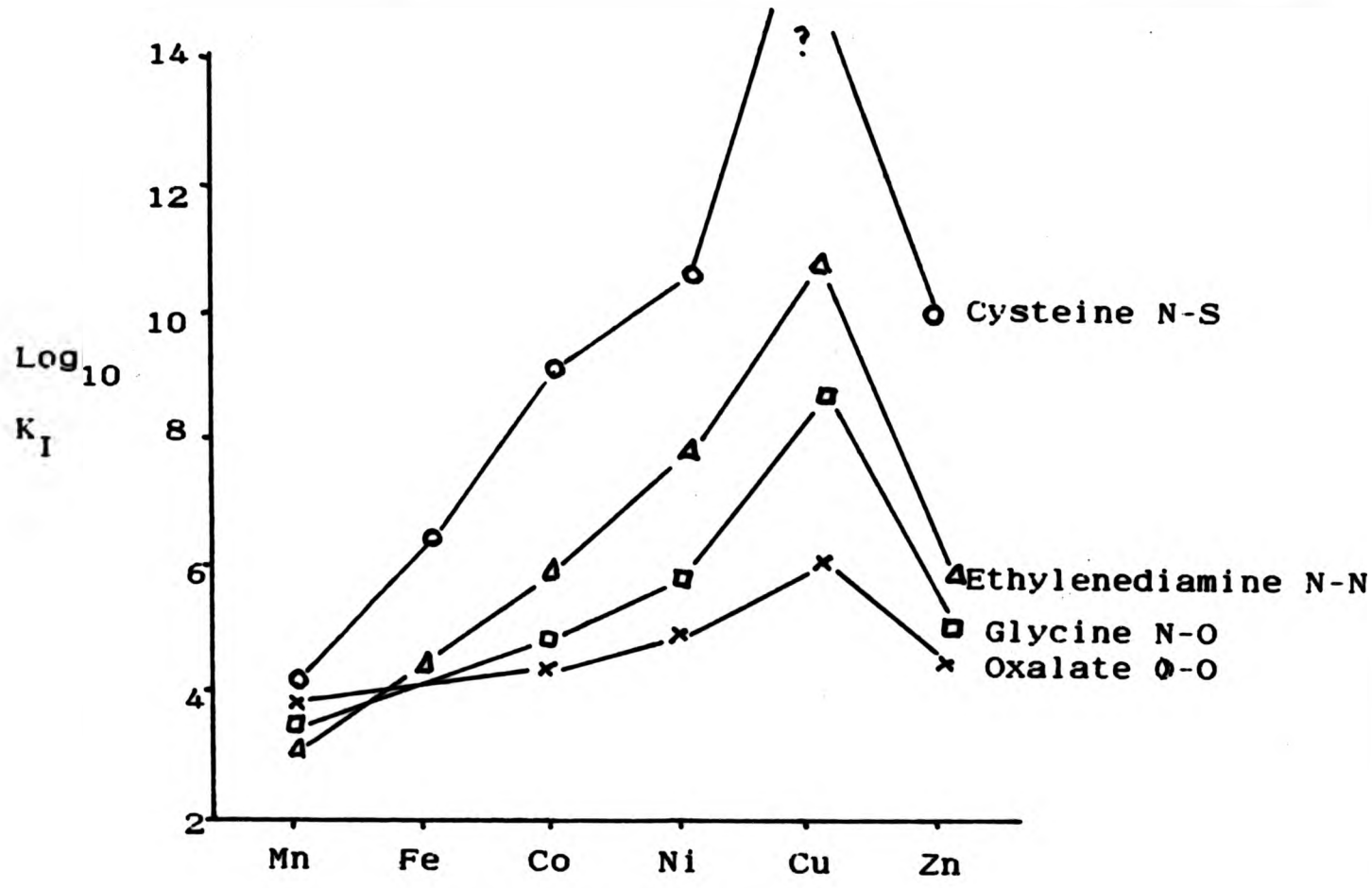


Figure 8.5. Some readily dissociable (intermediate exchange rate) Fe(II) proteins and their locations.

PROTEIN	LOCATION
Acotinase	Mitochondria
Superoxide dismutase	Prokaryote cytoplasm (cell wall)?
Ferredoxin II	SRB cytoplasm
Oxygenases	Prokaryote cytoplasm (cell wall)?
Lysine/proline oxidases	Cytoplasm
Chelatase for porphyrin synthesis	Mitochondria
Hydrogenase	SRB periplasm?

such a site is insufficiently electronegative to bind metals such as Cu(II), Zn(II) and Ni(II) and the presence of one nitrogen donor discriminates heavily against Mg(II) and Ca(II). A second potential way of discriminating against the heavier transition metal ions and yet maintaining selectivity for iron (II) versus Mg(II) and Ca(II) is to increase the bond lengths of the nitrogen (especially) and oxygen donor ligands or by increasing the coordination number i.e. the size of the metal binding cavity. This could be accomplished in two ways; ligand atoms can be constrained by the ligand (polysaccharide or protein) framework so that the donor atoms cannot collapse to their optimal bond distances with the metal ion, or mutual repulsion between the donor ligand atoms will limit their ability to make close contact with the smaller cations at the end of the transition series. Thus a constrained large cavity rather than a flexible one will favour to some degree Fe(II) rather than Zn(II). Discrimination in favour of Fe(II) over Mn(II) can be achieved by larger numbers of nitrogen bases within complexes of high coordination number. A prospective binding site for Fe(II) on the surface of D.vulgaris can thus be envisaged to require; i. a relativly low electronegativity consisting of a combination of O- and several N- donor ligands, ii. a high coordination number resulting in mutual repulsion of donor ligands to give a large cavity binding site. Thus more than one LPS monomer may be involved in the binding site (together possibly with protein) and this may explain why the binding ratio (from in vitro studies) is not a whole integer.

A further consequence of the intermediate binding nature

of iron (II) may lie in its possible role in intracellular catalytic and control functions; strongly bound metal ions tend to have truley catalytic functions (Zn, Ni) whereas truley control functions reside in metal-activated molecules (group IIa cations). Perhaps Fe(II) may perform in Desulfovibrio the functions that NAD undertakes in other organisms, having catalytic and control functions and dissociating readily but slowly from enzymes; another reason for the high requirement of iron in Desulfovibrio. A dissociable Fe_4S_4 unit has been reported present in a control electron transport protein of the sulphur bacteria and the functional value of the dissociation ;

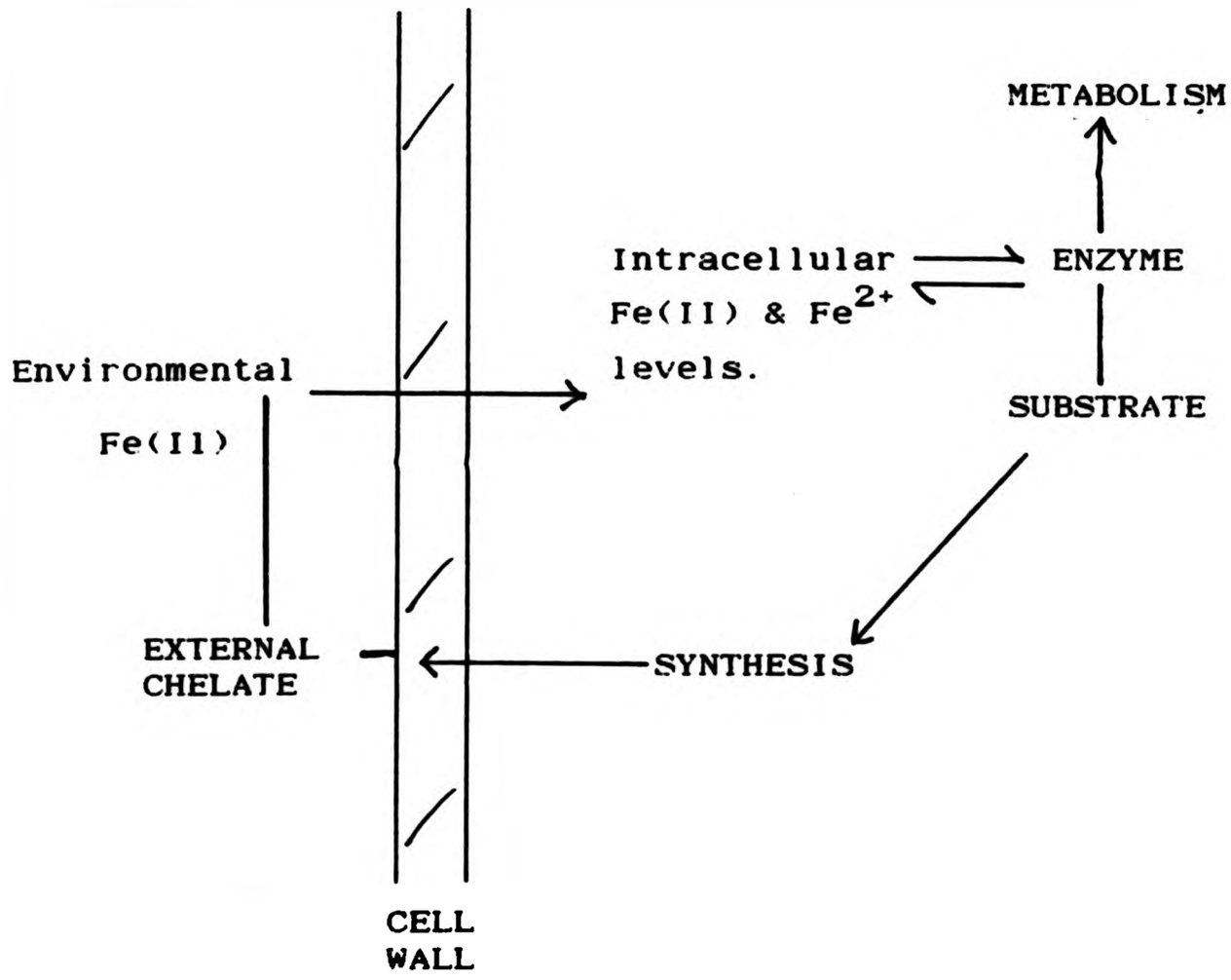
$Fe_4S_4 = Fe_3S_3 + Fe^{2+} + S^{2-}$

has been discussed by Xavier et al (1981).

If one considers the dissociable iron-containing enzymes in general; their substrates are related to the precursor molecules required for the synthesis of iron-uptake components (Williams, 1982). In its simplest form this is shown where citrate is either involved directly or is the precursor of an iron chelating agent e.g. schizokinen (Chapter 1). A major pathway of citrate metabolism is via acotinase which is an enzyme that requires free Fe^{2+}. This link can be demonstrated for other enzymes and is shown diagramatically in Figure 8.6. The link may also be useful in further investigations on Desulfovibrio. where a knowledge of the chelator/uptake system can lead to speculation on the intracellular functions or vice versa.

When one considers the selective binding of iron(II) to LPS in D.vulgaris Woolwich one must keep in mind the nature

Figure 8.6. The link between prokaryote cytoplasmic free iron (Fe^{2+}), Fe(II), and Fe(II) in the environment is controlled by the synthesis of chelates (either scavenging or attached to the cell wall. This synthesis is regulated by the free Fe^{2+} itself by the switching on or off of dissociable enzymes which are dependant upon Fe(II).
(Adapted from Williams, 1982).



of the prefered natural environment where much of the iron is present as colloidal FeS. Thus although the initial binding of Fe(II) as Fe^{2+} may be at the selective sites just described and display the binding ratio of 1.8:1 (Fe^{2+}:LPS), this bound metal would act as nucleation sites for the deposition of more metal as Fe(II), possibly by colloidal interactions. The end result would be an accumulation of precipitated metal above stoichiometric amounts as demonstrated in the binding ratio of 461:1 from in vivo release studies. Non-selective secondary binding of Fe(II) as FeS once the primary LPS sites are saturated is also supported by the decrease in LPS release at high iron concentrations seen in these studies (Figure 4.4c). The large amount of iron bound and its importance in D.vulgaris OM stabilisation is further emphasised when one examines the basal levels of LPS release in the three types of growth media (Figure 4.3). When corrected for varying carbohydrate composition these are 20.4:2.2:1 for C+Fe:C:C-Fe. This represents the relative amounts of LPS held in place in the OM by cationic binding. This binding of divalent cations has already been proposed as a major force holding the LPS in the OM (Leive, 1974) but is not the only bond, as not all LPS is released by cation chelator treatment. Cationic bound LPS is probably in equilibrium with other, non-EDTA releasable LPS, emphasising the dynamic nature of the OM. A supply of metal cations would shift the equilibrium , thereby releasing more LPS on cation chelation using EDTA.

The primary selective binding site of Fe^{2+} to the LPS is more likely to be concerned with the direct uptake of Fe(II) into the cell, either by low affinity simple diffusion

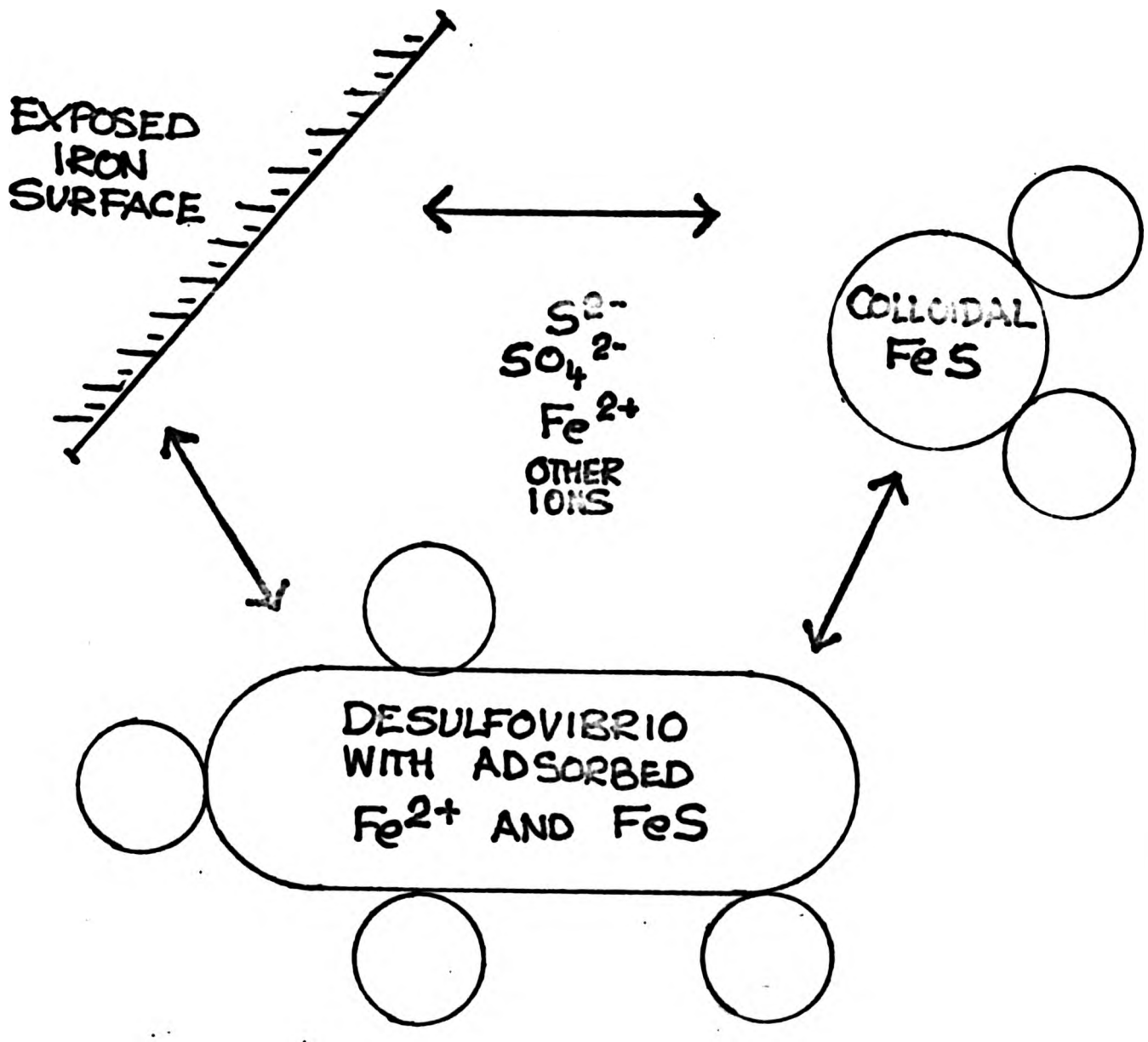
through protein pores or by an OMP mediated (facilitated diffusion) system into the periplasm and thence by active transport through the CM. The secondary colloidal binding of FeS to the surface of the bacterium in metabolising iron-rich cultures is of more importance when one considers the relationship of the bacterium to its particular environment, and the adhesion to exposed metal surfaces. Both the bacterial cells and FeS are in the size range 1nm to 1μm i.e. large molecules to small particles, and may be regarded as colloidal systems (Shaw, 1970). In considering such systems factors to be taken into account are: the particle size, particle shape and flexibility, particle-particle interactions, particle-solvent interactions and surface (including electrical) properties. The latter also applies to any exposed iron surface. Owing to the immense complexity of colloidal systems the subject can not always be treated with the exactness associated with certain branches of physical chemistry. This is especially true of a microbial culture or a natural environment (with many micro-habitats) which is in a state of biochemical flux, and in which very little, if anything, is known of the factors listed above. However, the binding of FeS to the surface of Desulfovibrio or its adsorption to iron surfaces must have a profound effect on the immediate environment of the bacterium and cannot be overlooked. Undoubtably the increased density would tend to cause the bacterium to sink, in its aqueous environment, to the bottom of a pond/sea where growth conditions would be more suitable (organic matter, low oxygen levels). Indeed the protection afforded by covering the surface with FeS could be a great selective advantage,

Coflocculation and sedimentation would modify behaviour affecting for example: predator/prey interactions, immediate pH, nutrient input, metabolite output and the penetration of inhibitory substances (as is suggested by the Sarkosyl treatments in this study) such as U/V light, X-rays and oxygen. The protection against atmospheric oxygen, especially once all nutrients are utilised, when normal metabolism ceases and the environment returns to an aerobic one, may be invaluable to the cells' survival.

A most important property of colloidal dispersions is the tendancy of the particles to aggregate. Encounters between particles dispersed in liquid media occur frequently as a result of Brownian motion and the stability, i.e.adsorption, of a dispersion is determined by the interaction between the particles during these encounters. In this case of course, the bacteria are themselves mobile and such encounters within a culture, including those between cells and metal or other 'inert' surfaces, would be expected to occur more frequently. The principal cause of aggregation is the Van der Waals attractive forces between particles (dispersion or London forces), whereas stability against aggregation is a consequence of repulsive interactions between similarily charged electrical double layers and particle-solvent affinity. The nature of the charge on surfaces and the presence of an electrical double layer surrounding such surfaces is impossible to predict in these circumstances. Experiments upon the surface charge of the various components of this system would be extremely interesting (e.g. cell micro-electrophoresis upon D.vulgaris under various conditions)

and would be a useful extension of this present study. It is thus seen that numerous interactions are possible and these will depend upon certain ionic species being present in excess within the culture. This is summarised diagramatically in Figure 8.7. However one can state certain generalisations. Sols of metals, sulphur and metallic sulphides usually carry negative charges. This may depend on how the colloid was first formed (Shaw, 1970). Also, sulphides, as a general rule, preferentially adsorb ions which are common to them i.e. a colloidal FeS particle or an FeS covered SRB will tend to adsorb either Fe^{2+} or S^{2-}. Thus the net charge at the surface would depend upon which ion is in excess i.e. whether the bacteria were actively metabolising or not. This behaviour is not unreasonable for it indicates a natural tendency of the ionic crystal (of the FeS particle) to extend its lattice. Thus the stability of negatively charged sols (including sulphide-covered SRB) in the presence of H_2S (i.e. during active metabolism) is due to the adsorption of sulphide ions $(FeS)S^{2-}$. Cessation of bacterial metabolism would cause a drop in H_2S levels, removal of this electrostatic repulsion and colloidal flocculation. However, this is obviously a very simplistic approach and does not account for interactions at an exposed iron surface or for numerous variations in the overall environment due to localised nutrient concentrations. Studies upon the surface charge of the cells and other colloidal material present would be of great value. It has been reported by other workers (Busch & Stumm, 1968; Marshall et al, 1971) that the pH and electrolyte concentration of the medium can affect adhesion of other bacterial species.

Figure 8.7. Diagram showing a summary of some of the numerous colloidal interactions and ionic species possible within the natural environment of Desulfovibrio.



Firm adhesion to a metal surface is presumably only a selective advantage when shear forces exist, which cannot always be the case in SRB habitats. The bacterial sorption to inert surfaces has been shown to occur in two phases (Fletcher & Floodgate, 1973); i. an indirect effect which arises by the bacterial modification of the physico-chemical environment of the metal i.e. a reversible phase during which the bacteria (and associated colloidal matter) are held at the surface by a balencing of Van der Waals attractive forces and the electrical repulsive energies of the two surfaces, ii. a time-dependent irreversible phase, in which bacteria are firmly attached to the surface. The importance of colloidal interactions within the SRB environment and the results of this study lead one to conclude that the former effect is the most important or indeed only one necessary in Desulfovibrio adhesion to metal surfaces. The relationship between SRB attachment to iron surfaces and the corrosion of such structures has not been the direct object of this study but presumably for an organism to play a significant role in the corrosive processes it should be closely associated with the corroding surface (Obuekwe et al. 1981). This has been shown to be the case for D.vulgaris (Gaylarde & Johnston, 1980), and the first phase of attachment would provide an intimate colloidal covering enabling the corrosive processes to occur. In a related phenomenon, ore leaching by bacteria, attachment of the bacteria to the ore particles is common but direct attachment is not a prerequisite for leaching of ore material (Berry & Murr, 1978).

The logical extension of this work from an applications point of view would be the search for an analogue or disruptive factor which could block the selective nature of D.vulgaris cell walls towards Fe^{2+}, allowing the binding of other metal cations and the destabilisation of the FeS colloidal lattice or more effectively its initial build-up from the primary binding sites. Such a chemical would need to fulfill certain requirements in line with currently used biocides i.e. be inexpensive, relatively non-polluting or biodegradable and easily applied to the site of potential danger. Such commercial requirements are by no means easily met even if such a substance could be found.

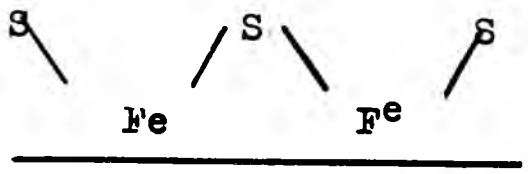
Diagramatic representations of D.vulgaris LPS with speculations on the mechanism of Fe^{2+} binding are given in Figure 8.8. Further work on the chemical and immunological nature of D.vulgaris Woolwich LPS would be most useful. The potential of this molecule as a selective Fe^{2+} chelator, with apparantly little antigenic activity may be of some use, and would warrant further study. Much of the information gained from this investigation is summarised in Figure 8.9, which shows the current state of knowledge on the D.vulgaris OM.

Figure 8.8. Speculative diagrammatic representation of a D.vulgaris Woolwich lipopolysaccharide molecule showing selective Fe^{2+} binding site.

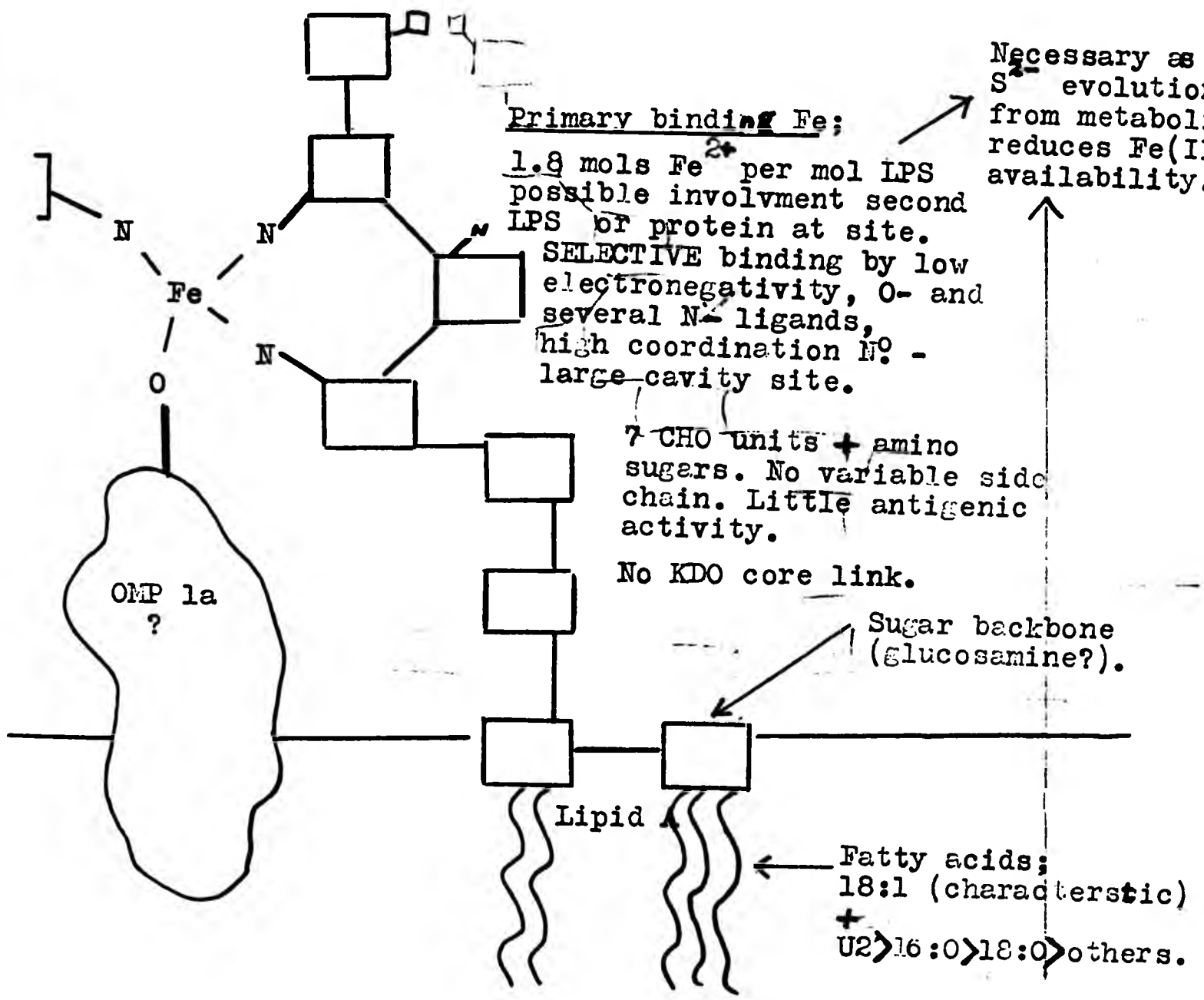
M.Wt 3913
46% lipid
34% CHO
14% associated protein.

C/F. Salmonella;
M.Wt. 12000
>50 sugars
very variable
'O' antigenic.

Iron limitation →
113% increase in LPS
no change in side-chain length.

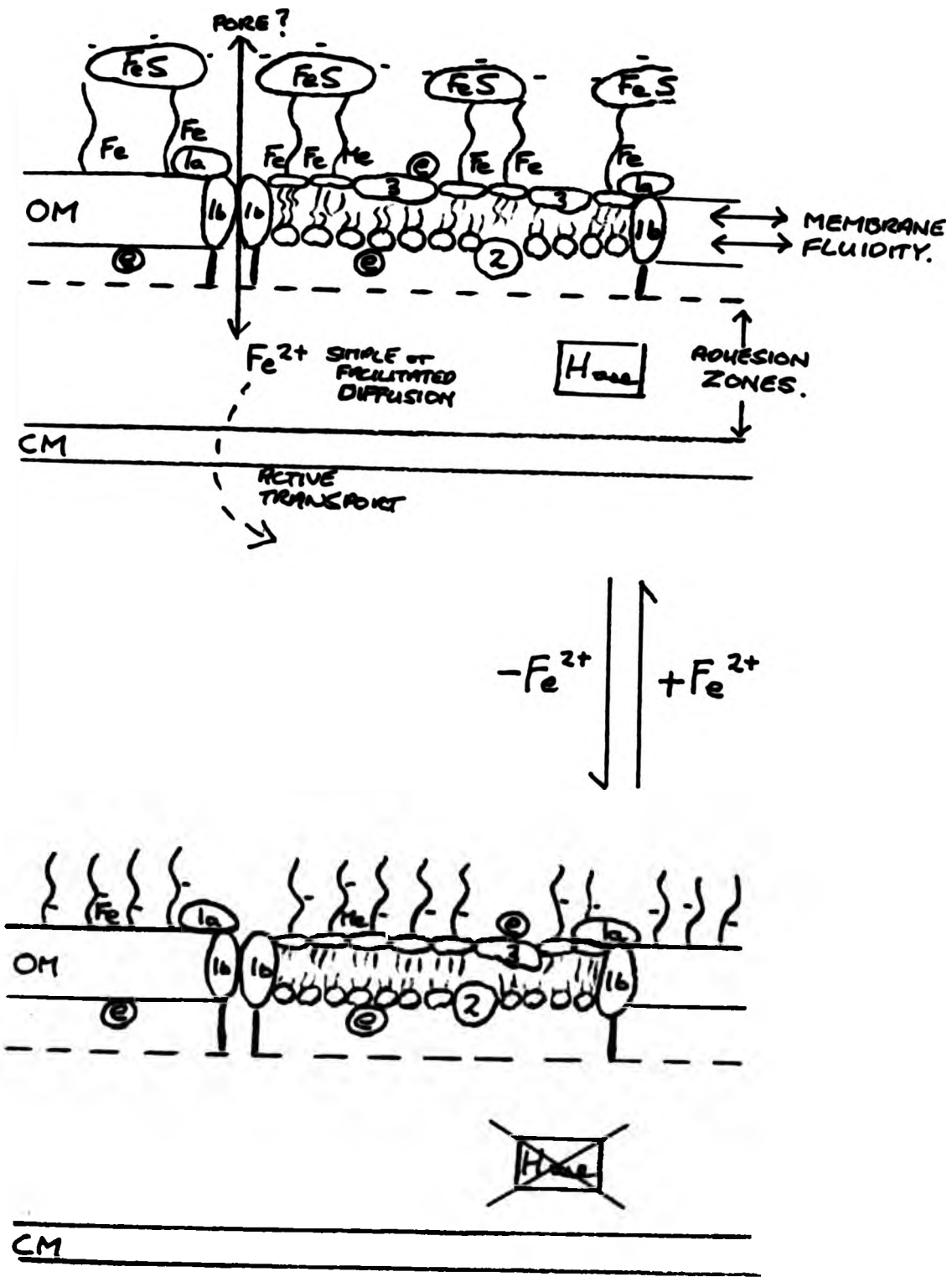


Secondary binding Fe;
Non-stoichiometric FeS colloidal interaction using 1° site as nucleus.



OUTER MEMBRANE BILAYER.

Figure 8.9. Diagram of the cell wall of <u>D.vulgaris</u> Woolwich incorporating some of the results from this current investigation.



KEY:-

- PHOSPHOLIPID (MAINLY P.E.).
- LPS.
- MAJOR OMP.
- EXTRINSIC PROTEIN.
- --- PEPTIDOGLYCAN.
- Me METAL.

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APPENDIX.

Materials.

All chemicals were of AnalaR grade where obtainable and used without further purification. These were purchased from BDH Chemicals PLC.

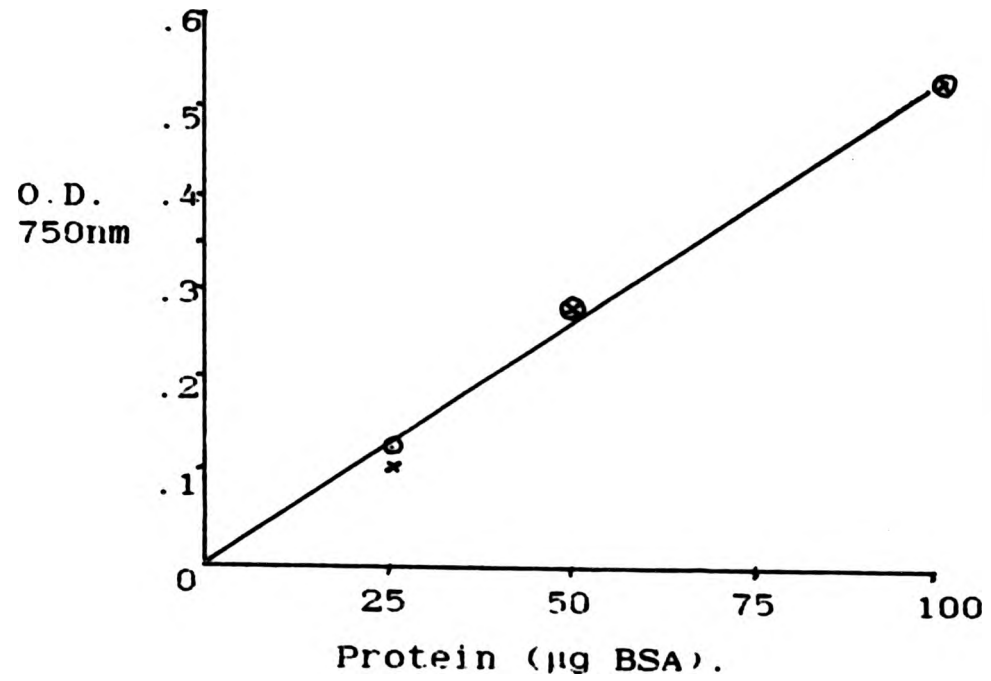
Fine chemicals were purchased from Sigma Chemicals PLC. Further specifications are listed below:
Acrylamide, NN'-methylenebisacrylamide (Bis) and sodium dodecyl sulphate (SDS) were BDH 'specially purified for electrophoresis ' (Electran) grade.
Ethylenediamine tetraacetic acid (EDTA) was the disodium salt, Sigma grade.
N-Lauroylsarcosine (Sarkosyl) was the sodium salt, Sigma grade.
The standard lipopolysaccharide from S.typhimurium was purchased from Sigma but had been extracted using the phenol/water procedure.
'EM' grade chemicals were used for all electron microscopy where possible.

All 'low-speed' centrifugation was performed on an MSE HS18 ultracentrifuge using a 8x50 or 6x250mls. head.
All 'high-speed' centrifugation was performed on an MSE 'Superspeed 65' or 'Europa 65M' ultracentrifuge using a 3x25mls. swing-out head and 10mls. tube inserts.

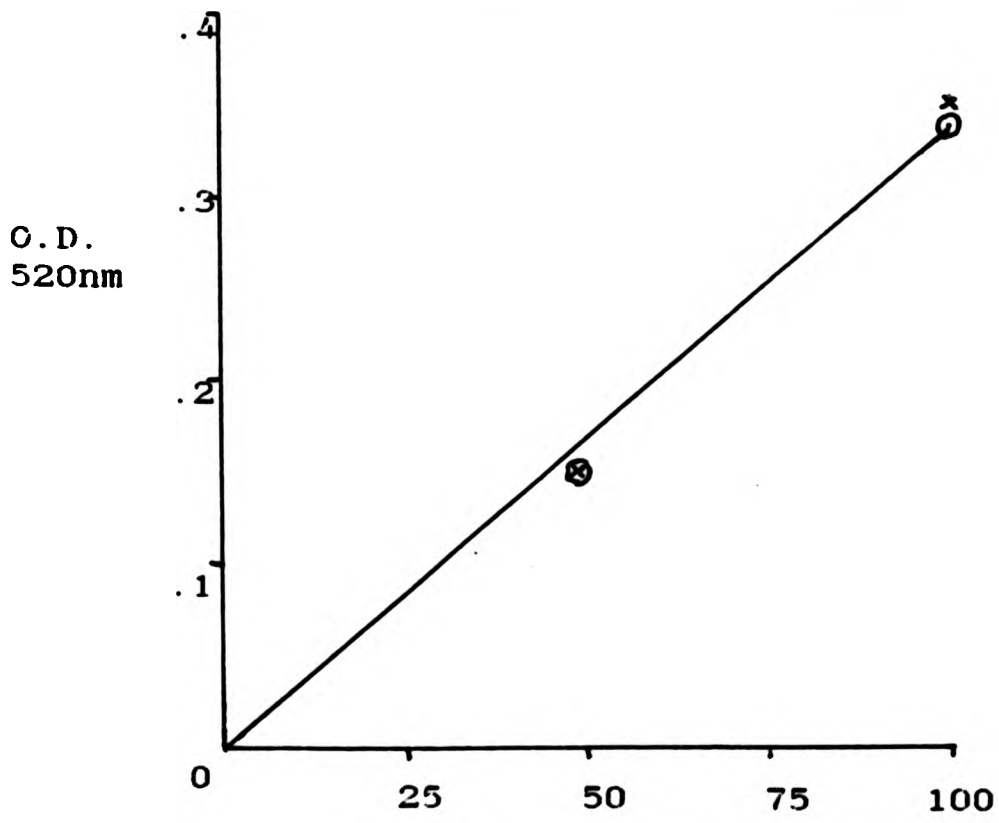
Optical densities were read using 1cm. lightpath cuvettes on a Cecil CE292 Digital spectrophotometer or were scanned using a Perkin-Elmer 552 UV-VIS spectrophotometer.

STANDARD CURVES.

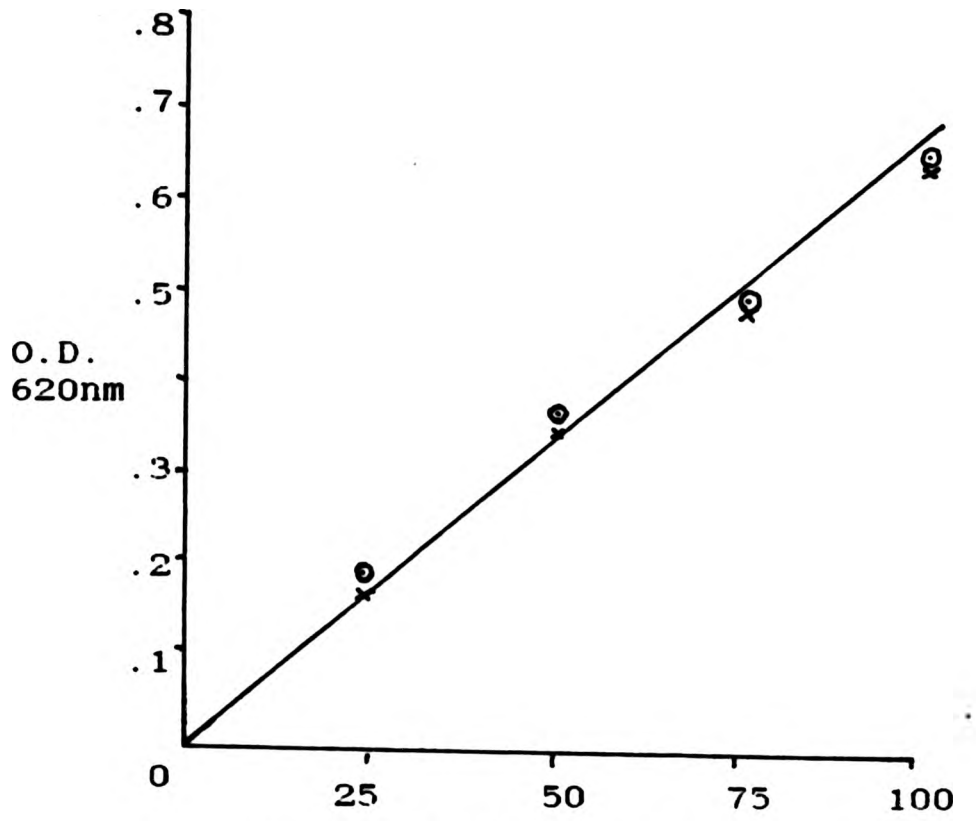
Estimation of protein using the Lowry method (2.2.3).



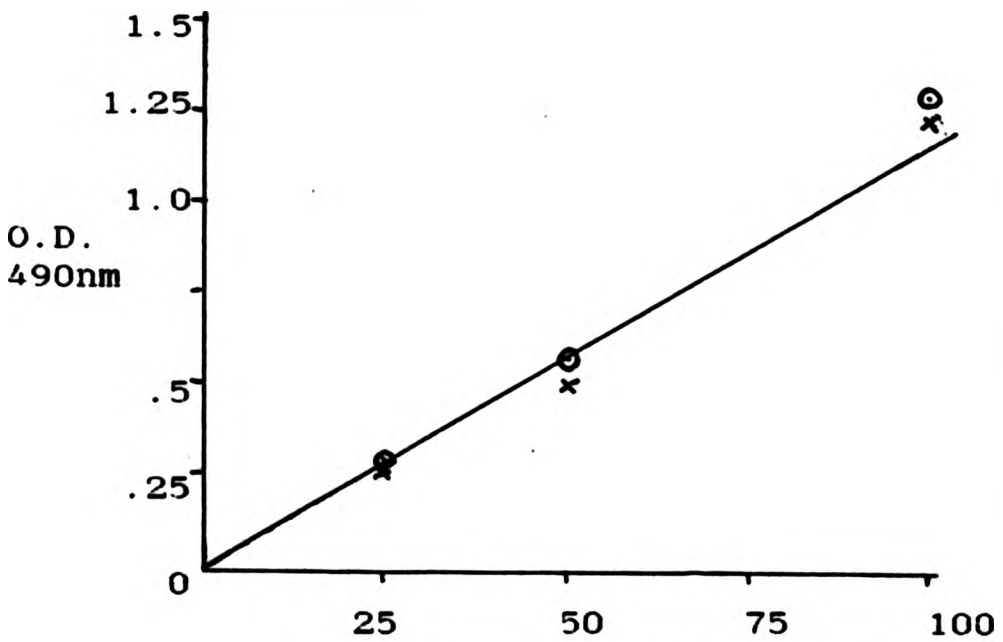
Estimation of lipid using the alkaline/hydroxylamine procedure, (2.2.5).



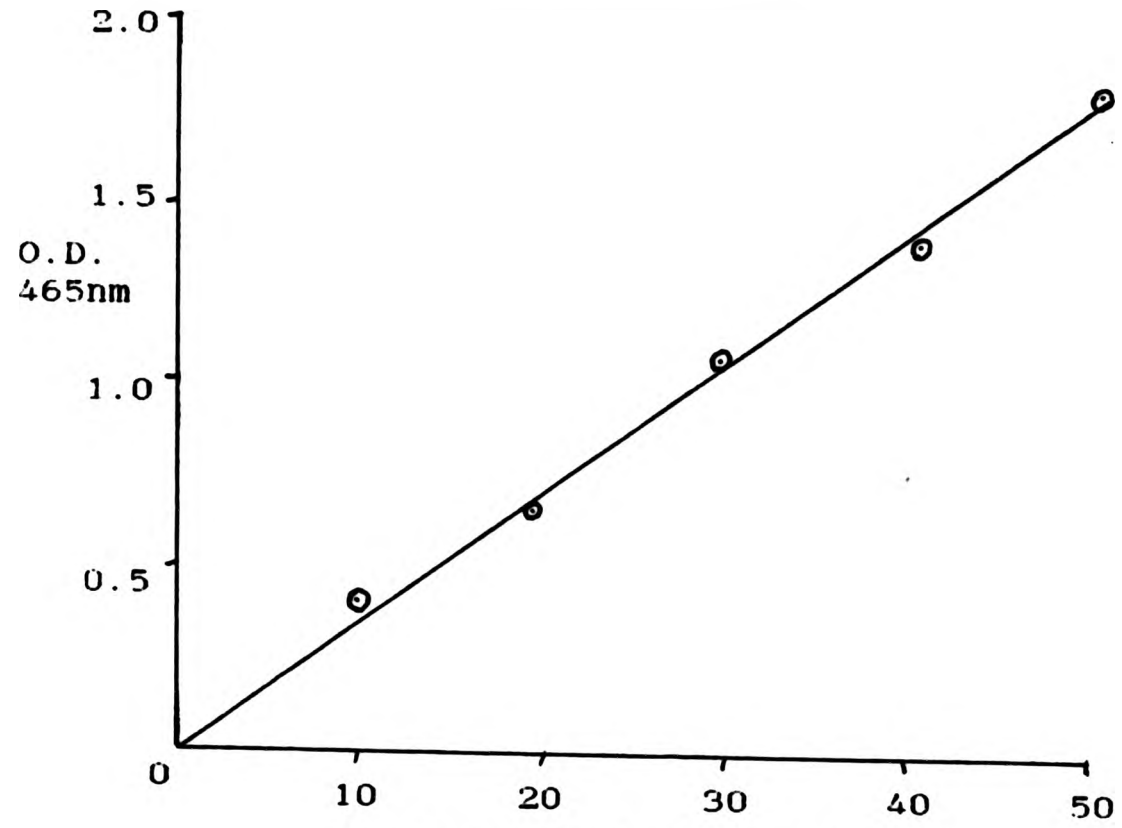
Estimation of carbohydrate using the Anthrone method (2.2.4).



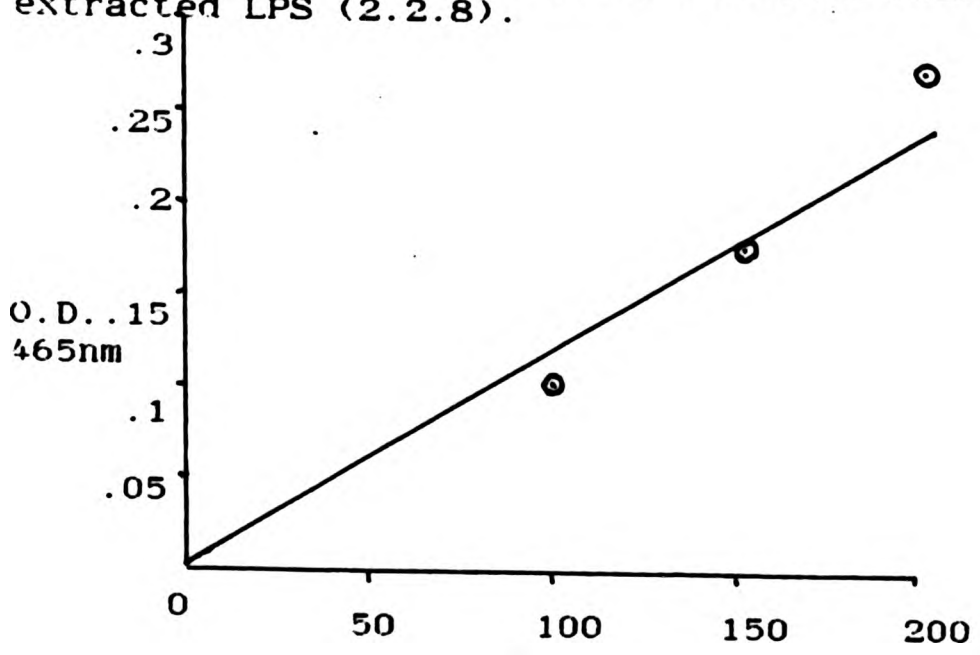
Estimation of carbohydrate using the phenol/sulphuric acid method (2.2.4).



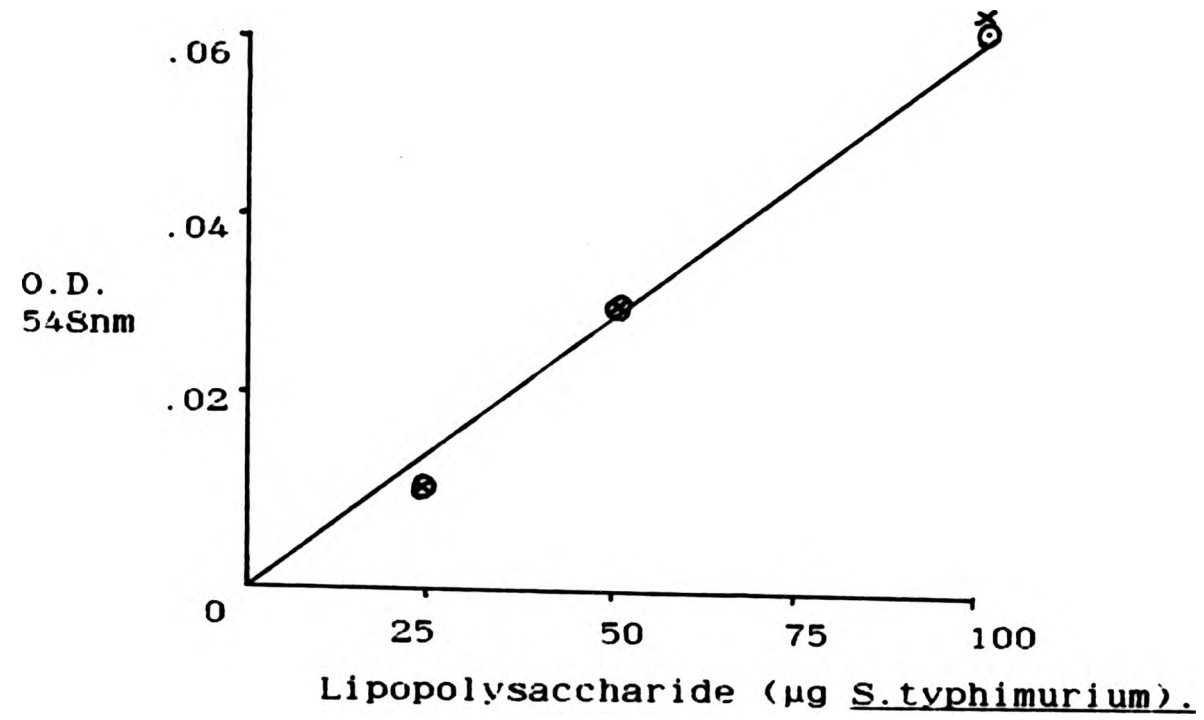
Lipopolysaccharide dye assay using S.typhimurium commercial LPS (2.2.8).



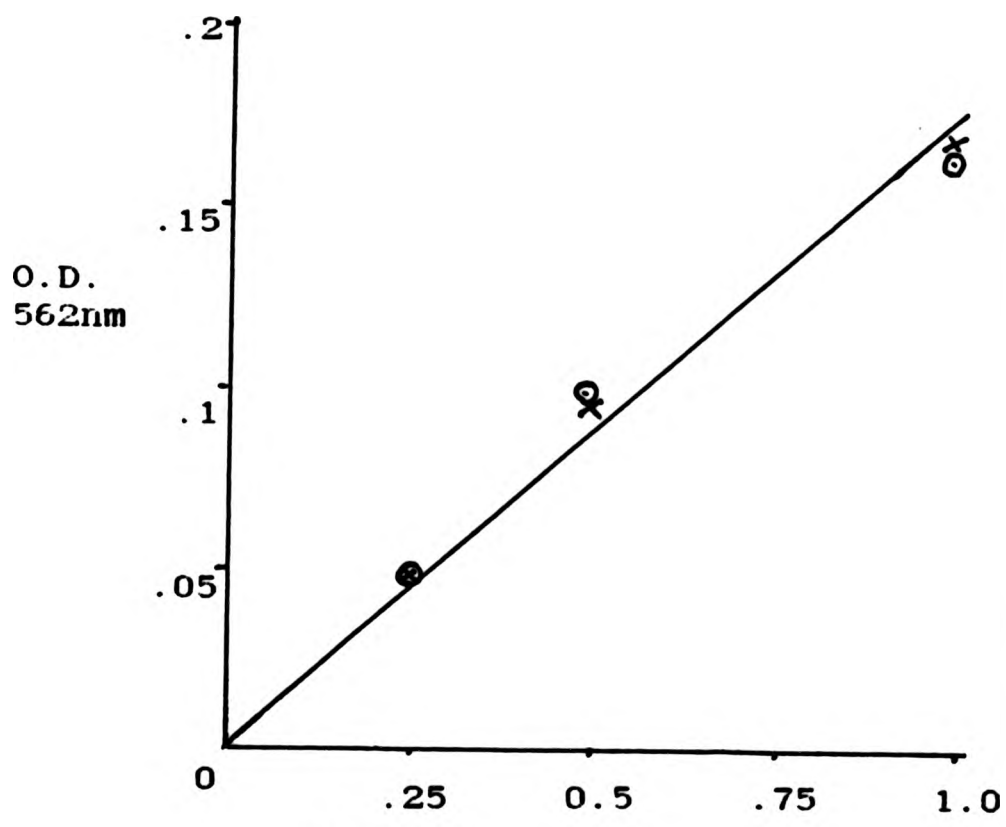
Lipopolysaccharide dye assay using D.vulgaris Woolwich C+Fe extracted LPS (2.2.8).



Estimation of KDO using the thiobarbituric acid method (2.2.9).



Estimation of iron (II) using the Ferrozine method (2.2.11).

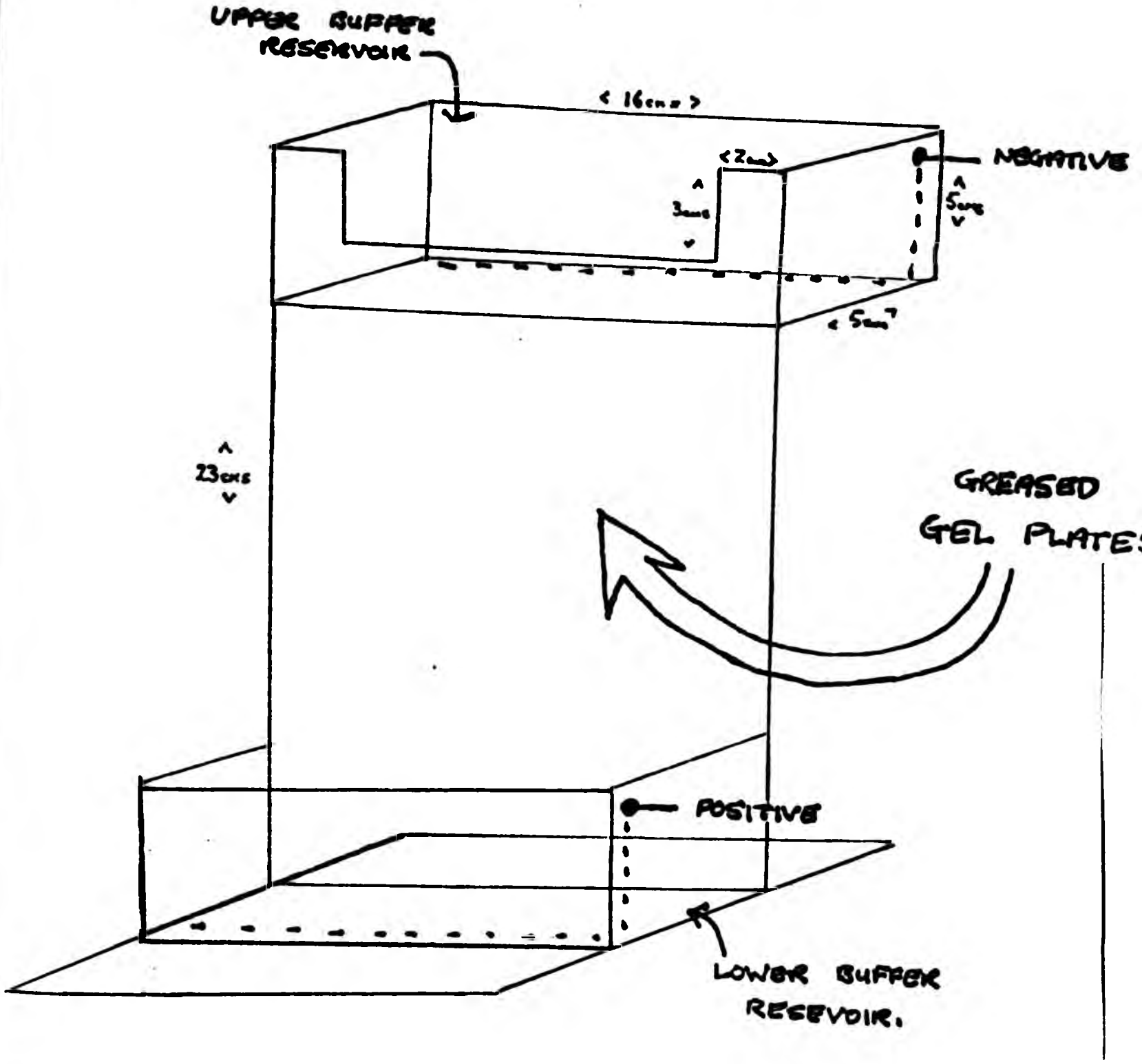


Solutions required for the production of polyacrylamide gels.

Solutions	15% separating slab gel	5% stacking slab gel	5.8% tube gel
50% acrylamide 2% BIS solution	9.0	1.0	2.32
2.2 M TRIS/Cl^- pH 8.6	4.95	-	-
1.25 M TRIS/Cl^- pH 6.8	-	1.0	-
100 mM TRIS pH 7.4 Sodium acetate 200 mM EDTA 20 mM + 10% SDS	-	-	2.0
20% SDS	0.2	0.05	-
0.5% TEMED	1.5	0.5	1.0
1.5% ammonium persulphate	1.5	0.5	1.0
Water	12.85	6.95	13.68
FINAL VOLUME	30	10	20

All volumes are in mls.

Sketch of polyacrylamide electrophoresis tank constructed from perspex sheets and platinum electrodes and utilised for all slab PAGE in this study.



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A Selective Interaction Between Ferrous Ions and Lipopolysaccharide in *Desulfovibrio vulgaris*

By G. BRADLEY, C. C. GAYLARDE* AND J. M. JOHNSTON

Department of Biological Sciences, City of London Polytechnic, Old Castle Street, London E1 7NT, UK

(*Received 1 August 1983; revised 29 September 1983*)

Lipopolysaccharide and protein material were released from cells of *Desulfovibrio vulgaris* on treatment with 10 mM-EDTA. Preincubation with up to 15 mM-ferrous ions caused increased removal of lipopolysaccharide from bacteria grown in iron-limited medium, but preincubation with calcium, magnesium, or zinc ions was unable to induce this response. Ferrous ions appear to have an important role in the stabilization *in vivo* of the outer membrane of *D. vulgaris*. The selective interaction between ferrous ions and *D. vulgaris* lipopolysaccharide may play a part in iron uptake.

INTRODUCTION

Desulfovibrio vulgaris is a Gram-negative, anaerobic sulphate-reducing bacterium, which has a high requirement for inorganic iron (Postgate, 1981). The cells associate with exposed iron surfaces and may cause costly corrosion problems in metal structures wherever anaerobic conditions arise. Much work has been performed on the chemical and electrochemical processes of anaerobic iron corrosion and several theories have been proposed to explain this action of *Desulfovibrio* (for a review, see Miller, 1971; Iverson, 1974; Iverson & Olson, 1983), but little or no work has been carried out on the interactions between iron and the *Desulfovibrio* surface at the molecular level.

It is well established that the lipopolysaccharide (LPS) located in the external leaflet of the outer membrane (OM) of Gram-negative bacteria can interact with various metal cations. This is important for membrane assembly (Galanos *et al.*, 1977) and for the barrier function of the OM (Leive, 1974). Leive *et al.* (1968) have demonstrated that the metal cation chelator EDTA releases LPS and protein material from the surface of some Gram-negative bacteria. The divalent cation availability in the growth medium, together with the affinity of the LPS for these cations, determines in part the amount of LPS held in place in the OM by cationic binding. This, in turn, influences the fraction of LPS that can be released by EDTA treatment of whole cells. Lipopolysaccharides of enteric bacteria contain a number of potential cation-binding sites (Schindler & Osborn, 1979) having a high affinity for calcium and magnesium ions. The replacing of these metal cations is the proposed mode of action for various cationic antibiotics (Hancock, 1981) and for the development of resistance to these drugs by the synthesis of cation-replacing proteins (Nicas & Hancock, 1983).

In the studies reported here, we have compared the effects of preincubation with different divalent metal cations on the release of LPS by EDTA treatment, using cultures of *D. vulgaris* (Woolwich) grown in media of varying iron availability.

METHODS

Growth media and conditions. Desulfovibrio vulgaris Woolwich (NCIB 8457) was grown as 500 ml batch cultures at 30 °C. Media used were: Medium C (Postgate, 1981) modified by the omission of sodium citrate (C); Medium C

Abbreviation: OM, outer membrane.

0022-1287/84/0001-1417 $02.00

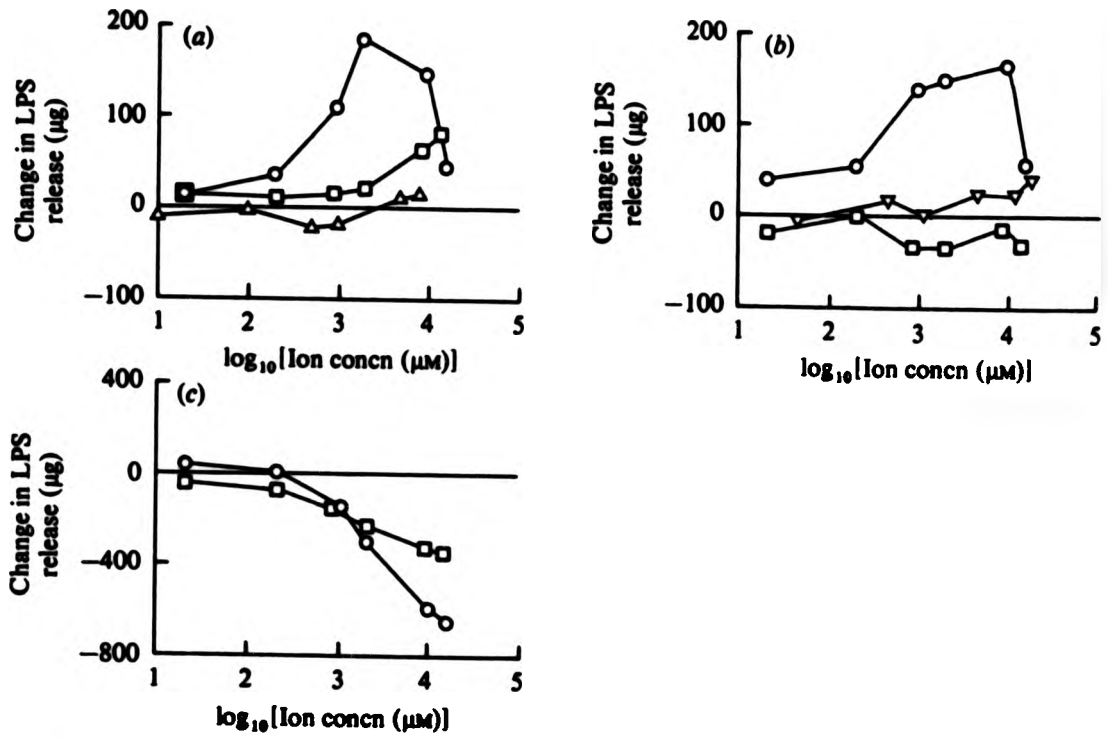


Fig. 1. Change in release of LPS by EDTA treatment of *D. vulgaris* after preincubation in metal cation solutions. Points represent mean values of at least three separate experiments. Cells grown in (*a*) iron-limited (C) medium, (*b*) iron-free (C − Fe) medium, and (*c*) iron-rich (C + Fe) medium. ○, Iron; □, calcium; △, magnesium; ▽, zinc.

including 0·25% (w/v) sodium citrate with a mild steel coupon (40 × 15 mm) added to provide a relatively iron-rich medium together with an exposed iron surface (C + Fe); Medium C modified by the omission of ferrous sulphate and sodium citrate providing a medium free of added iron (C − Fe). All media were adjusted to pH 7·5.

Release of LPS. Bacteria were harvested in late-exponential phase by centrifuging (3000 *g*, 30 min), washed in PBS (0·15 M-NaCl; 0·05 M-PO_4; pH 7·4) and resuspended in PBS to a density of approximately 10^{10} cells ml^{-1}. This suspension (2 ml) was added to tubes containing 0·5 ml 10 mM-ascorbate, the required volume of 200 mM-metal cation salt solution and distilled water to a final volume of 4 ml. Final concentrations of cations varied between 0·01 mM and 17 mM. Distilled water replaced cations in the control treatments. Salts used were: $FeSO_4.7H_2O$, $CaCl_2.6H_2O$, $MgSO_4.7H_2O$ and $ZnSO_4.7H_2O$. Tubes were incubated at 0 °C for 15 min. Cells were then pelleted and resuspended in 2 ml PBS containing 10 mM-EDTA. After incubation at 45 °C for 15 min, the suspension was centrifuged and the supernatant was assayed for protein by the Lowry method using bovine serum albumin as a standard, and for LPS using the carbocyanine dye assay of Zey & Jackson (1973). LPS released was calibrated against standards of LPS extracted from *D. vulgaris* cultures. The change in LPS release was calculated by subtracting the control values from those obtained after ion treatment.

LPS extraction. Envelopes of *D. vulgaris* were isolated by resuspending washed cells in 20 ml ice-cold PBS and lysing by sonication (MSE Soniprep 150). Unbroken bacteria and debris were removed by centrifuging (5000 *g*, 10 min) and the supernatant was recentrifuged (30000 *g*, 20 min) to pellet the envelopes. LPS was extracted from this fresh preparation by the hot aqueous phenol procedure of Westphal & Jann (1965).

Analytical PAGE. SDS-PAGE was performed on slab gels containing 15% (w/v) acrylamide pH 8·6. Gels were stained using Page blue 83 (BDH, Coomassie blue equivalent) or the silver method of Morrissey (1981), modified by the use of 15% (w/v) acetic acid plus 10% (w/v) methanol as the fixative.

RESULTS

Release of LPS by EDTA treatment

Preincubation of cells in ferrous salt solutions caused a marked increase in the EDTA-induced release of LPS above that of the control (Fig. 1). This occurred for cells grown either in iron-limited (C) or in added iron-free (C − Fe) medium. Other divalent cations used (Zn^{2+}, Ca^{2+} and Mg^{2+}) were unable to mimic this effect, although there was a slight increase in LPS release from medium C-grown cells preincubated with Ca^{2+} at levels above 1·8 mM (Fig. 1*a*). Cells grown in iron-rich (C + Fe) medium (Fig. 1*c*) and all cells preincubated with Fe^{2+}

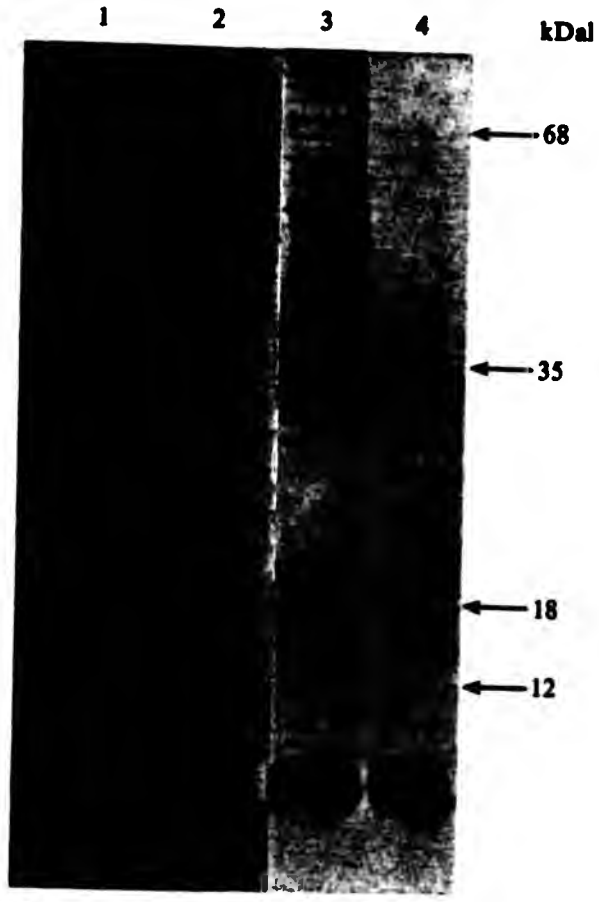


Fig. 2. SDS-PAGE analysis of samples from *D. vulgaris* iron-limited cultures. Gels were stained with Page blue 83 (lanes 1 and 2) and silver (lanes 3 and 4). Lanes 1 and 3, EDTA-removed material after preincubation in 10 mM-ferrous ions; lanes 2 and 4, phenol/water-extracted LPS.

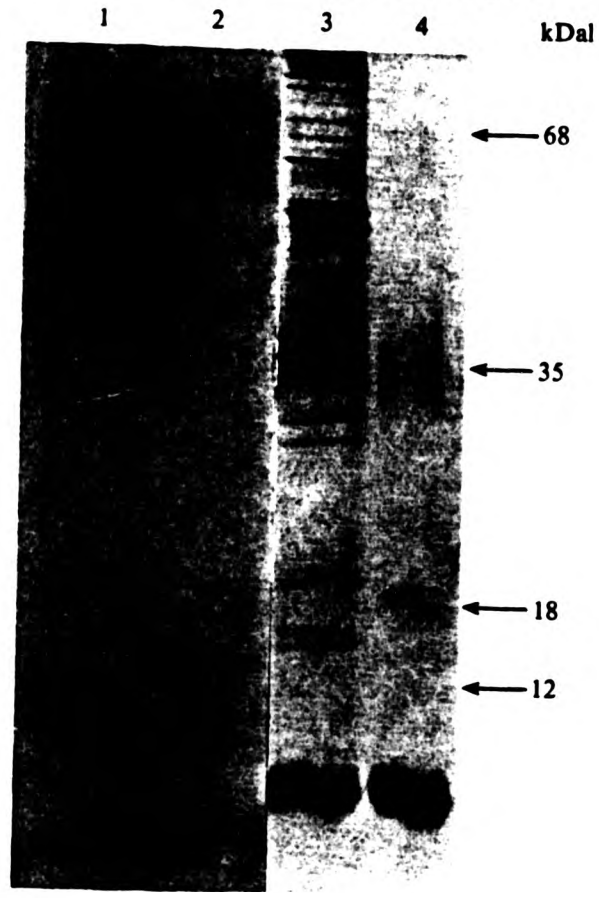
concentrations greater than 10 mM showed a gradual decrease in this response. Although up to 150 μg protein ml^{-1} was removed from the cells by EDTA treatment, no correlation was observed between this and the cation concentration (data not shown).

SDS-PAGE analysis

LPS extracted from cells by the aqueous phenol method could not be visualized by Page blue 83. However, the silver stain allowed the detection of three broad bands, the fastest being a dense black colour (Fig. 2). Identical bands were found in EDTA-released material, where again the fast dense black band was the most heavily stained. Numerous polypeptide bands were also revealed, both by silver and Page blue 83 staining, the major protein having an approximate molecular weight of 54000. No changes in the number of bands were noted with different ion concentrations, nor was there any difference between LPS extracted from cells grown in the various media (results not shown).

DISCUSSION

This study demonstrates an important interaction between ferrous ions and the LPS of *D. vulgaris in vivo*. A short incubation with ferrous ions increases the amount of EDTA-releasable LPS in *D. vulgaris* grown in iron-limited media. The inability of other cations, in particular Zn^{2+} which has a higher affinity for EDTA than ferrous ions (log_{10} stability constants 16·7 and 14·3, respectively), to mimic this effect indicates that the interaction is specific. The decrease in LPS release shown in iron-rich cultures and with ferrous ion preincubation concentrations above 10 mM in other cultures could be due to the binding of ferrous ions to molecules other than LPS, or to the precipitation of ions once LPS saturation is reached during preincubation. This would effectively reduce the amount of EDTA available for reaction with cation-bound LPS.



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SDS-PAGE analysis using the silver stain shows that some of the material released by EDTA treatment is identical to LPS extracted from cell envelopes by the aqueous phenol method. The nature of the bands seen in the latter extract is unknown, but the slower running cluster (position 35 kDal, Fig. 2) bears some resemblance to that reported in *Pseudomonas aeruginosa* by Kropinski *et al.* (1982), who suggested that these bands correspond to the O antigen polysaccharide side chains. In addition to these LPS-associated bands, EDTA causes the release of other material which may also be visualized by Page blue 83. These bands correspond to some of those found in the purified OM of *D. vulgaris* (authors' unpublished observations) and are, presumably, components released on partial breakdown of the membrane.

The possession of ferrous ion-selective sites in the LPS of *D. vulgaris* may aid the bacteria in the uptake of iron from their anaerobic environment. Although iron is more soluble in its reduced state (expected maximum concentrations from solubility products at pH 7 are 10^{-1} M for ferrous, 10^{-18} M for ferric ions), its precipitation as ferrous sulphides by the hydrogen sulphide produced during bacterial metabolism will lower its availability to the cells. Since *Desulfovibrio* has a high requirement for iron, the ability to bind ferrous ions selectively is obviously desirable. Alternative methods of iron uptake have been reported in other bacterial genera. Siderophores (Neilands, 1974) or other low-affinity uptake systems (Konisky, 1979) have been found in a number of cells and the possibility of their presence in *Desulfovibrio* should not be excluded. Induction, as well as repression, of certain Gram-negative OM proteins in response to iron limitation has been reported (McIntosh & Earhart, 1976; Meyer *et al.*, 1979). Such iron-specific compounds have, as yet, not been detected in *Desulfovibrio* and this report is the first evidence of any molecular interaction between these bacteria and ferrous ions. Further work will be required to elucidate more fully the nature of the ion binding and its significance in *Desulfovibrio*.

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Society for General Microbiology

SGM—97th ORDINARY MEETING
University of Warwick, 11–15 April
(Last date for booking—11 March)

Published with the *Society for General Microbiology Quarterly*, Volume 10, Part 1

OFFERED PAPERS: GENERAL (POSTER)

Wednesday, 13 April
Concourse and Adjoining Rooms

1115–1245: Authors will be in attendance. Posters will remain on view until 1700.

P28 *Preparation and Preliminary Analysis of the Outer Membrane of Desulfovibrio vulgaris*
G. BRADLEY, C. GAYLARDE and J. JOHNSTON (Department of Biological Sciences, City of London Polytechnic)

Outer membranes of *D. vulgaris* (Woolwich) have been prepared using differential solubilization of cell envelopes by the detergent *N*-lauryl-sarcosine. SDS-PAGE analysis of the membranes shows the presence of three major polypeptides, one of which is readily removed from intact cells by EDTA treatment. Preliminary results indicate the presence of a single molecular species of lipopolysaccharide containing no ketodeoxyoctonate. The possible functions of the membrane components in the cells in their natural environment will be discussed.

Society for General Microbiology

SGM – 101st ORDINARY MEETING
Sheffield, 18 – 20 September

(Last date for booking – 20 August)

Published with the *Society for General Microbiology Quarterly*, Volume 11, Part 3

OFFERED PAPERS: GENERAL (POSTER)

Wednesday, 19 September
Arts Tower Foyer

1130–1230: Authors in attendance.

P27 *The Binding of Ferrous Ions to Reconstituted Outer Membrane Vesicles of Desulfovibrio vulgaris*
G. BRADLEY and C.C. GAYLARDE (Department of Biological Sciences, City of London Polytechnic)

Model membrane vesicles were reconstituted from combinations of phospholipid, lipopolysaccharide and outer membrane proteins of *D. vulgaris* Woolwich. The binding of ^{55}Fe to these vesicles and to vesicles composed of commercial phospholipid and lipopolysaccharide from *Salmonella typhimurium* was measured. The influence of Ca^{2+} on ^{55}Fe binding for the two types of lipopolysaccharide will be shown.

PROGRAMME

UNIVERSITY OF KENT AT CANTERBURY

BIODETERIORATION SOCIETY

MICROBIAL CORROSION OF METALS

SUMMER MEETING

JULY 5th & 6th. 1984

JULY 5th

12.30	Registration
1 pm	Lunch
2 - 2.40	Principles of Electrochemical Corrosion. M.J. Robinson, Cranfield Institute of Biotechnology
2.40 - 3.30	The Mechanisms of Corrosion of Ferrous Metals by Sulphate-reducing Bacteria R.A. King & J.D. Stott, Corrosion & Protection Centre, UMIST
3.30 - 4.00	Tea
4.00 - 4.40	The Importance of Cell-Iron Interactions in Desulfovibrio-related Corrosion. G. Bradley & C. Caylarde, City of London Polytechnic.
7 - 15 pm	Conference Dinner

July 6th

9.30 - 10.00	Sulphate-reducing Bacteria and the Oil Exploration Industry P.F. Sanders & W.A. Hamilton, Aberdeen University
10.00- 10.40	Pitting Corrosion of Mild Steel by Sulphate-reducing Bacteria J.A. Hardy, BP, Sunbury
10.40 - 11.10	Coffee
11.10 - 11.50	Sulphide Generation in Metal-working Fluids E.C. Hill, Cardiff
11.50 - 12.30	Corrosion Produced by Microorganisms within Fuel Systems J.T. Gisborne, T. Cartledge & J. McEvoy, ARE Dockyard Laboratory, Portsmouth & Trent Polytechnic
12.30 - 2.00	Lunch
2.00 - 2.40	The Role of Dissolved Organics in Corrosion A. Chamberlain, Surrey University
2.40 - 3.10	Corrosion Due to Differential Aeration Induced by Microorganisms A.K. Tiller, National Corrosion Centre, NPL, Teddington