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THE BIODEGRADATION OF BACTERIAL LIPOPOLYSACCHARIDES
BY MARINE INVERTEBRATES AND MARINE BACTERIA

by

Thomas Jamieson

Presented for the degree of Master of Science
in the Faculty of Science, University of Glasgow.

To Professor A.C. Wardlaw, thanks again, Thomas

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PREFACE

This thesis is the original work of the author.

Thomas Jamieson.

ACKNOWLEDGEMENTS

I should like to thank Professor A.C. Wardlaw for his guidance, encouragement and supervision throughout my period of research.

This research was made possible by the Natural Environment Research Council who provided my grant and funds for experimental materials. I express my thanks to NERC.

My special thanks go to my family and my friends for constant encouragement and support during the completion of this work.

Thanks are also due to ICI plc. for supplying me with 'Pruteen' and to Dr. J.N. Saddler for supplying me LPS-degrading bacterial strains.

In the preparation of this manuscript I should like to thank, Professor A.C. Wardlaw and Dr. J.H. Freer for reading the manuscript and for their criticisms and suggestions; Mr. I. McKie and Mr. A. Ellis for photography and Mrs. A. Mosson for fast and efficient typing.

SUMMARY

The Biodegradation of Bacterial Lipopolysaccharides by Marine Invertebrates and Marine Bacteria.

Gram-negative bacteria are abundant in marine environments and also act as a food source for filter-feeding animals such as bivalve molluscs. The question posed in this investigation was : are lipopolysaccharides (LPSs) - the cell-wall polymers unique to Gram-negative bacteria - subject to biodegradation by either molluscan gut extracts or by heterotrophic bacteria in marine sediments?

Extracts of digestive gland from 5 species of marine molluscs were prepared : Cerastoderma edule (common cockle), Modiolus modiolus (horse mussel), Mya arenaria (sand gaper), Mytilus edulis (common mussel) and Pecten maximus (great scallop). Purification was not taken beyond crude ammonium sulphate (AS) fractionation. To explore their possible hydrolytic action on LPSs, each was incubated for up to 24h with purified LPS from Salmonella minnesota, Escherichia coli O111:B4 and Methylophilus methylotrophus.

Degradation of the polysaccharide moieties of all three LPSs by all molluscan extracts was readily detected by the combined applications of descending paper chromatography, thin layer chromatography (TLC), colorimetry and gel filtration chromatography. S. minnesota LPS was degraded most by all extracts and evidence for the breakdown of the O-chain repeating unit was detected, with glucose, N-acetylglucosamine, N-acetylgalactosamine and/or galactose being released. The core-oligosaccharides of the other two LPSs were degraded : E. coli O111:B4 LPS yielded glucose and N-acetylglucosamine upon hydrolysis by the extracts, Meth. methylotrophus LPS hydrolysis released only glucose. Degradation of all three LPSs resulted in the release of other unidentified monosaccharides. Most extracts had optimum glycosidase activity at pH 4.5 against all three LPSs tested although Modiolus was most potent at pH 5.0. Of all the extracts tested, only one fraction, from Modiolus, had

demonstrable endoglycolytic activity towards LPS, releasing oligosaccharides from S. minnesota and E. coli O111:B4 LPSs.

All extracts released large quantities of inorganic phosphate from LPSs, as determined colorimetrically, but no free ethanolamine was detected by TLC.

Action on the lipid A component of the LPS molecule was investigated by the release of free fatty acids, detectable colorimetrically or by TLC. Most digestive gland preparations failed to release significant amounts of free fatty acids from LPS, despite having lipase activity towards tributyrin (the pH optimum of which was characterised for each AS fraction). However, one preparation from Cerastoderma released non-hydroxylated fatty acids from LPSs, as did Helix pomatia (common snail) gut juice, although a commercially available lipase preparation from the yeast Candida cylindracea, had very little activity against LPSs.

Marine sediments and sands from different environments were examined for both aerobic and anaerobic LPS-degrading bacteria. Numerous bacterial strains were isolated by ability to degrade tributyrin, whole Gram-negative bacterial cells, LPS or lipid A. One lipolytic bacterial isolate (P8) released fatty acids when the latter was present in nutrient culture medium. A total of nine bacterial isolates was able to grow on a medium containing LPS from Meth. methylotrophus as the sole organic nutrient and produced visible zones of degradation. These isolates were also degradative towards protein, lipids, DNA and starch. Cell-free extracts of one of these bacterial strains (M1) liberated reducing material from LPS when incubated for 24h, which suggested a saccharolytic mode of attack. In numerous tests, culture supernatant preparations from all of the other bacterial isolates (including LPS-degrading bacterial strains isolated by earlier investigators) had no detectable effect on LPS. Anaerobic LPS-degrading bacteria were sought, but not isolated, although LPS degradation was observed in anaerobic mixed liquid cultures.

In conclusion, whereas molluscan digestive gland extracts readily degraded the polysaccharide moieties of LPSs, breakdown of the lipid A component was less easily detected. Although bacterial isolates had readily demonstrable degradative activity towards LPS and other compounds on solid growth media, enzymatic breakdown of LPS was more difficult to detect and thus characterise.

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ABBREVIATIONS

| | |
|--------|---|
| AS | ammonium sulphate |
| ASW | artificial seawater |
| ASWYP | artificial seawater, yeast extract, peptone |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| EDTA | ethylenediaminetetraacetate |
| g | gram(s) |
| h | hour(s) |
| h-LPS | (partially) hydrolysed lipopolysaccharide |
| KDO | ketodeoxyoctonic acid |
| LAL | <u>Limulus</u> amoebocyte lysate |
| LPS(s) | lipopolysaccharide(s) |
| mg | milligram(s) |
| m.w. | molecular weight |
| pfu | plaque forming units |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| TBA | thiobarbituric acid |
| TLC | thin layer chromatography |

INTRODUCTION

Brief History of Endotoxin

Endotoxins are substances produced by Gram-negative bacteria and which exhibit a wide variety of toxic effects on parenteral administration to mammals. The materials can be extracted from bacterial cells, are of high molecular weight and contain carbohydrate, fatty acids and phosphorus. They are stable to boiling and are biologically extremely active, having many pathophysiological effects, including pyrogenicity and lethal toxicity.

Historical aspects of endotoxin research have been reviewed by several authors (Westphal et al., 1977; Rietschel, 1975) with Pfeiffer (1892) being cited as the originator of the term endotoxin to describe the toxin released upon bacteriolysis of Vibrio cholerae which was "closely attached to, and probably an integral part of, the bacterial body".

With the development and advancement of pharmaceutical preparations, especially drugs for injection, the problem of "injection fever" arose and was recognised by Seibert (1925). She clearly demonstrated that bacterial contamination was the source of the problem. Supporting the observations of earlier workers, it was always found that bacterial cell wall antigens were intimately associated with endotoxic and pyrogenic activities.

Endotoxic activities were only expressed by bacteria which reacted negatively by the Gram-stain. Boivin et al. (1933) were the first to devise a procedure, with trichloroacetic acid, for extracting the endotoxic complex from Gram-negative bacteria. However, protein contamination limited the purity of these preparations. Westphal et al. (1952) produced a pure protein-free lipopolysaccharide preparation by applying a hot phenol/water extraction method. They were later able to prove that it was the ubiquitous, firmly bound, lipid component (called lipid A) which was responsible for the pyrogenic and most of the endotoxic activities of the lipopolysaccharides (Galanos et al., 1972).

Extraction of LPSS

In addition to the above mentioned procedures, other solvents, including different ratios of phenol and water (Palmer and Gerlough, 1940), ethyl ether/water (Ribi et al., 1959), diethylene/glycerol (Morgan, 1937), and urea (Walker, 1940) have been used (reviewed by Nowotony et al., 1963).

Depending largely upon whether the organism displays a Smooth or Rough phenotype (Figure 6) one of two procedures are used nowadays. For Smooth-type LPS, the hot phenol/water method described by Westphal and Jann (1965) is most popular. This involves treatment of bacteria with 45% (w/w) aqueous phenol at 65-68°C followed by cooling to 10°C. The mixture separates into two phases; an upper aqueous layer of phenol-saturated water, containing LPS, soluble polysaccharides and nucleic acids and a lower layer of water-saturated phenol where the bacterial proteins are located. Since LPS exists as large aggregates in water, it can be separated from other substances by dialysis followed by ultracentrifugation. This is the most regularly used method, and gives good yields of LPS largely free of protein and nucleic acid contamination. However, Nowotony et al. (1963) suggested that phenolic treatment alters certain substituents of the LPS molecules. In addition, it should be noted that some Smooth-type LPS molecules do not partition into the aqueous phase; these include Aeromonas hydrophilia, Vibrio anguillarum (Banovb and Shaw, 1981) and Xanthomonas campestris (Hickman and Ashwell, 1966).

Rough-type (and some Smooth-type) LPS are normally obtained by the petroleum ether/chloroform/phenol method (Galanos et al., 1969). This depends upon the hydrophobicity of these glycolipids and yields are higher than when the phenol/water method is used. Due to the relative severity of previously used extraction methods (especially those involving the use of phenol), Leive and Morrison (1972) developed two milder extractions with

EDTA and aqueous butanol respectively.

Structure of LPSs

The availability of relatively protein and nucleic acid-free LPS preparations extracted by the methods of Westphal and Jann (1965) and Galanos *et al.* (1969), coupled with the biological significance of LPSs has led to numerous investigations of the structures of LPS from a wide spectrum of Gram-negative bacteria.

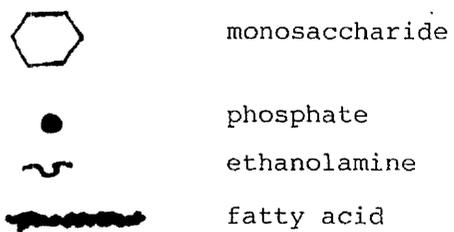
Complete LPS molecules consist of three covalently linked segments (O-chain, core-oligosaccharide and lipid A) each with distinctive composition (Figure 1) and synthesised under separate genetical control. Thus some strains produce the complete LPS structures (Smooth-type), while others produce lipid A with limited core-oligosaccharides (Rough-type). The O-chain represents a polymer of identical polysaccharide molecules and carries the antigenic determinants responsible for the serological specificity. The core-oligosaccharide carries charged substituents such as phosphate and phosphorylethanolamine and has cryptic antigenicity in Smooth strains (Brade and Galanos, 1983). Lipid A, which is the endotoxic moiety, consists of a phosphorylated hexosamine di- or monosaccharide backbone to which long chain fatty acids are bound.

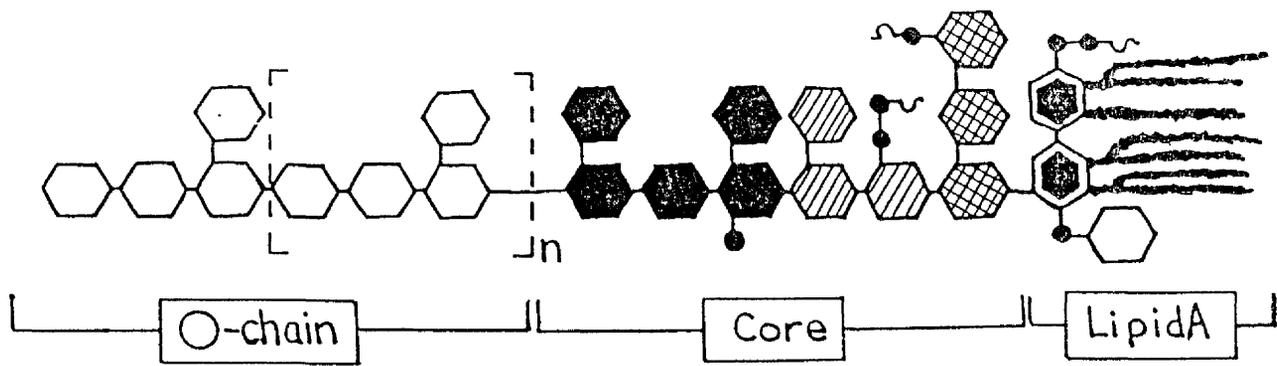
O-chain

The O-chains, which carry the main surface antigenic specificities of Gram-negative bacteria are made up of identical oligosaccharide repeating units (Robbins and Uchida, 1962). In *Salmonella* each oligosaccharide normally consists of a tetra- or pentasaccharide (Hellerqvist and Lindberg, 1971) however trisaccharides also occur. The various oligosaccharides may be differentiated immunochemically (Lüderitz *et al.*, 1966a) so that bacteria can

Figure 1 : Schematic structure of Salmonella LPS
(based on Lüderitz et al., 1982)

Key :





be grouped into "serotypes". The oligosaccharides are normally heteropolymers, although homopolymers also occur e.g. the mannan of E. coli O9 (Prehm et al., 1976). The saccharide structures in LPSs exhibit many chemical peculiarities such as the simultaneous presence of aminosugars, deoxysugars, dideoxysugars and methylsugars (Wilkinson, 1977). These constituents may be present in the main chain or as branched residues (Figure 2). Thus in Salmonella minnesota Smooth LPS, D-galactose is present in the main chain whereas N-acetyl-D-glucosamine is α -glycosidically linked as a branched residue.

Although the structure of the repeating unit is believed to be constant, certain modifying substituents, such as acetyl groups or glucosyl groups may be added in unequimolar amounts after biosynthesis or after conversion by bacteriophages. This may change the serology of the O-chain (Robbins et al., 1965; Wright and Kanegasaki, 1971).

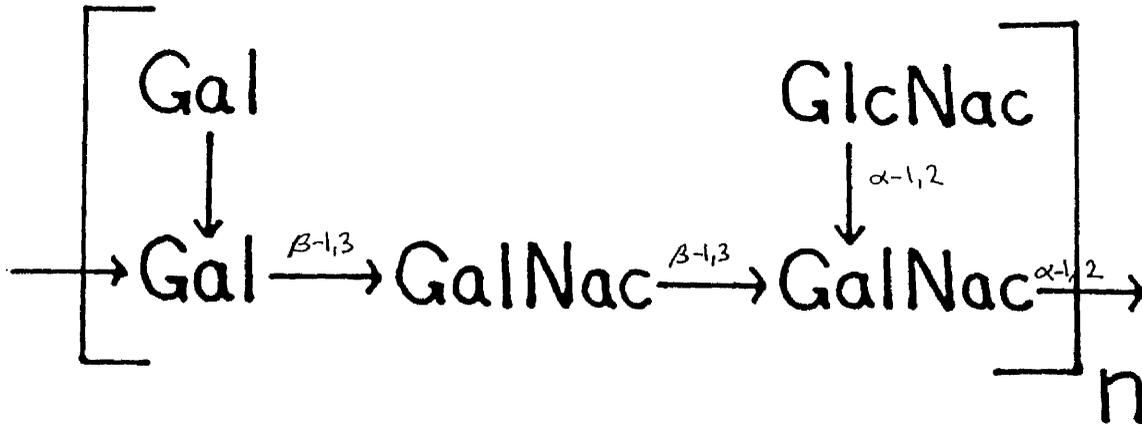
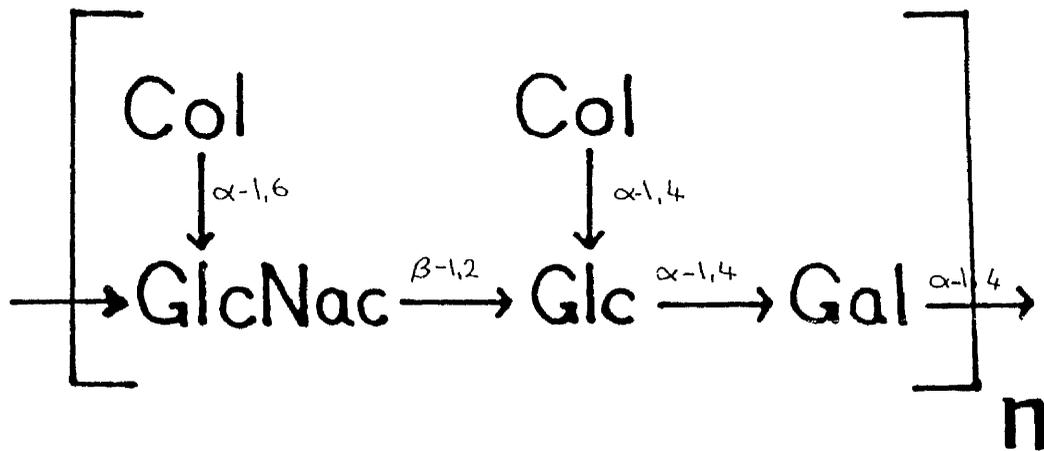
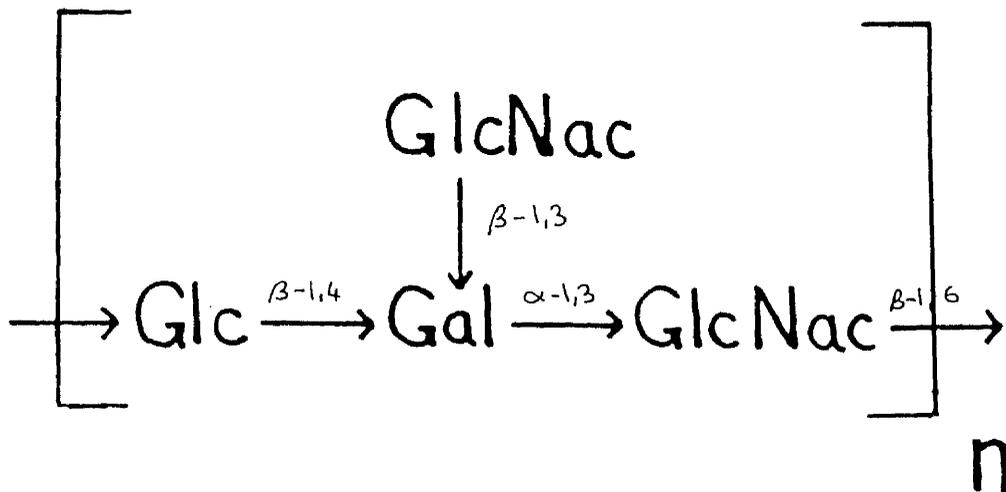
Recent advances in carbohydrate chemistry such as nuclear magnetic resonance spectrometry and fast acid bombardment spectrometry have led to O-chain structures being elucidated rapidly and completely (Lindberg, 1979; Jennings and Smith, 1980; Svenson and Lindberg, 1983). Previously it had taken up to 10 years to characterise a single O-chain structure (Lüderitz et al., 1966a). Over 100 oligosaccharide repeating unit structures have now been characterised from a wide variety of bacteria and in recent years emphasis has switched from the well studied Enterobacteriaceae (Lüderitz et al., 1982) to other Gram-negative families.

There is heterogeneity in the chain length of O-chains. With most Smooth type strains, oligomers of up to 40 repeating units are found (Tsai and Frasch, 1982; Nikaido, 1973). Bacteria appear to produce O-chains of high and low but little intermediate, degrees of polymerisation (Rottem et al., 1978; Munford et al., 1980; Carlson, 1984).

Figure 2 : Structures of the O-chain repeating units of
three enterobacterial LPSs relevant to this work
(based on (i) Lüderitz et al. (1966b), (ii) Edstrom &
Heath (1967) and (iii) Tarcsay et al. (1973)).

Key :

| | |
|--------|---------------------------------|
| Gal | galactose |
| Glc | glucose |
| GalNac | N-acetylgalactosamine |
| GlcNac | N-acetylglucosamine |
| Col | Colitose (3,6-dideoxygalactose) |

(i) Salmonella minnesota(ii) Escherichia coli O111:B4(iii) Serratia marcescens O8

Combining nuclear magnetic resonance spectrometry and computer graphics, it has been suggested that in Salmonella, O-chains form a helical coil-structure (Svenson, 1984).

Core-oligosaccharide

Whereas O-chains of LPS are highly structurally diverse, core structures are rather uniform. Thus the core structure in Figure 3(Ra) is found in all Salmonella species, and also occurs in other enterobacterial LPSs. Apart from the Salmonella type core, five different types have been found among Escherichia coli and Shigella spp. (Nikaido, 1973) and others have been reported (Lüderitz et al., 1982, see also Figure 4).

Study of the core structure of Salmonella LPS was greatly facilitated by the isolation of the various Rough-mutants which synthesised complete or incomplete core structures linked to lipid A but which were devoid of C-chains (Lüderitz et al., 1966a). These ranged from Ra mutants (lacking O-chain biosynthetic enzymes) to Re mutants containing only the linkage unit of the core to lipid A. Each Rough-mutant had LPS which sequentially lacked the specific sugar transferases for core synthesis (Figure 3).

The nature of the linkage unit has been the subject of some conjecture. In most LPSs it consists of a trisaccharide unit of 2-Keto-3-deoxy-D-manno-octonic acid (KDO), the exact arrangement of which has only recently been demonstrated (Brade et al., 1983b) and it has been further shown that α -2-4 interlinked KDO disaccharide branched residue is found in S. minnesota, S. godesburg, P. mirabilis and E. coli (Brade and Rietschel, 1984). In some LPSs, KDO is apparently absent (luminous bacteria, Kuwae and Kurata, 1983) or is present in non-molar quantities (marine bacteria, DiRienzo and MacLeod, 1978). However, it has recently been demonstrated that V. cholerae LPS, which had previously been reported to be KDO-less, contained KDO-5-phosphate which required rather more vigorous hydrolysis conditions for release. Thus it was

Figure 3 : Structures of O-chain-deficient (Ra) and
core-deficient (Rb-Re) LPSs of Salmonella
Rough mutants (based on Lüderitz et al., 1982)

Key :

| | |
|--------|----------------------------|
| KDO | 2 keto-3-deoxyoctonic acid |
| Hep | glyceromannoheptulose |
| Ⓟ | phosphate |
| Glc | glucose |
| Gal | galactose |
| GlcNac | N-acetylglucosamine |

Figure 4 : Structure of enterobacterial core types identified
thus far (based on Lüderitz et al., 1982)

Key :

| | |
|--------|----------------------------|
| KDO | 2 keto-3-deoxyoctonic acid |
| Hep | glyceromannoheptulose |
| Ⓟ | phosphate |
| EtN | ethanolamine |
| Rha | rhamnose |
| Glc | glucose |
| Gal | galactose |
| GlcNac | N-acetylglucosamine |

suggested that LPSs previously reported to contain little or no KDO may contain normal amounts arranged or substituted differently (Brade, 1985). A clear distinction should be made between the "inner core" containing KDO, mannoheptulose and non-stoichiometrically substituted by phosphate and phosphorylethanolamine, and the "outer core", which in Salmonella consists of essentially a branched pentasaccharide containing glucose, galactose and N-acetylglucosamine in the ratio 2:2:1 (Figure 3).

Lipid A

Due to the acid lability of the KDO-lipid A linkage, free lipid A can be prepared by mild acid hydrolysis and thus the structures of various lipid As have been studied.

The most commonly found lipid A backbone is a diphosphorylated, β -1,6-linked diglucosamine residue containing amide and ester linked long chain fatty acids (Gmeiner et al., 1969; Lüderitz et al., 1984) but diglucosamine backbones with a β 1-4 linkage have been found in some strains of E. coli and in Shigella flexneri (Adams and Singh, 1970). Lipid As of Rhodopseudomonas viridis, R. palustris and R. sulfovoridis are devoid of glucosamine and phosphate and instead have 2,3-diaminoglucose containing amide, but no ester linked long chain fatty acids (Ahamed, 1982). This type of lipid A has also been found in Pseudomonas diminuta and P. vesicularis (Wilkinson and Taylor, 1978; Mayer et al., 1984). Various models of Salmonella lipid A fine architecture have been proposed, differing in location of esterified long chain fatty acids, site of attachment to the core-oligosaccharide, or in the nature of phosphorylated substituents (Gmeiner et al., 1971; Lüderitz et al., 1973; Lüderitz et al., 1978; Westphal et al., 1983). However the model presented in Figure 5a (Galanos et al., 1984) based on high resolution mass spectrometry, two-dimensional nuclear magnetic resonance spectrometry and positive ion fast

Figure 5 Proposed chemical structures of lipid As

The primary hydroxyl group in position 6' represents the attachment site of KDO. Dotted lines represent nonstoichiometric substitutions. (based on (a) Galanos et al., 1984 and (b) Seydal et al., 1984, Imoto et al., 1985).

acid bombardment spectrometry (Imoto et al., 1983; Rietschel et al., 1984; Takayama et al., 1983) should prove to be conclusive. It consists of the β -1,6-linked glucosamine disaccharide type backbone with phosphate α -glycosidically linked to the reducing glucosamine at position C1 and another, β -esterically linked to the C4' position of the non-reducing glucosamine residue. The phosphates are non-stoichiometrically substituted by phosphorylethanolamine and 4-amino-4-deoxy-L-arabino-pyranose. The hydroxyl groups in position C3 and C3' and both amino groups are each substituted by one 3-hydroxytetradecanoyl group, three of which carry acyl residues of dodecanoic, tetradecanoic and hexadecanoic acid respectively. The hydroxyl group at position C6' represents the attachment of KDO and thus the core (and O-chain in Smooth strains).

3-hydroxy fatty acids are highly characteristic, and where present, are the only type of amide linked fatty acids found. However, some lipid As contain 2-hydroxy fatty acids and others contain very low levels of hydroxy fatty acids (Brucella abortus, Moreno et al., 1979). Except for the well studied Enterobacteriaceae, the locations of fatty acid substituents on lipid As are unknown, although their types and molar quantities have been well documented (Wilkinson, 1977). Esterification of the hydroxyl groups of 3-hydroxy fatty acids was not previously thought to commonly occur in lipid As other than Salmonella (above). However, an extensive study has recently shown that several lipid As including E. coli, V. cholerae, Fusobacterium nucleatum, Agrobacterium tumefaciens and Chromobacterium violaceum contain 3-acyloxyacyl residues which are amide linked (Wollenweber et al., 1984). The exact nature and position of these residues has been determined for E. coli and P. mirabilis and is similar to that described for S. minnesota (Seydal et al., 1984) except that in E. coli lipid A, acyloxyacyl residues are found only in the non-reducing glucosamine residue of lipid A where they are both amide and ester linked (Figure 5). Lipid As from a bacterial LPS preparation exhibit micro-heterogeneity (Nowotny, 1971; Mattsby-Baltzer et al., 1984). The phosphate

at the non-reducing end of the diglucosamine is substituted nonstoichiometrically by 4-aminoarabinose in several bacteria including Salmonella, E. coli and V. cholerae. The phosphate at the reducing end can be substituted by phosphate (E. coli), phosphorylethanolamine (V. cholerae), D-glucosamine (Chr. violaceum) or D-arabinofuranose (Rhps. tenue) (Lüderitz et al., 1982). There is also variation in the degree of esterification by long chain fatty acids. Thus lipid As prepared from a single LPS preparation can be separated by TLC and DEAE-cellulose chromatography into several fractions with different immunochemical, chemical and biological properties (Takayama et al., 1981; Mattsby-Baltzer et al., 1984).

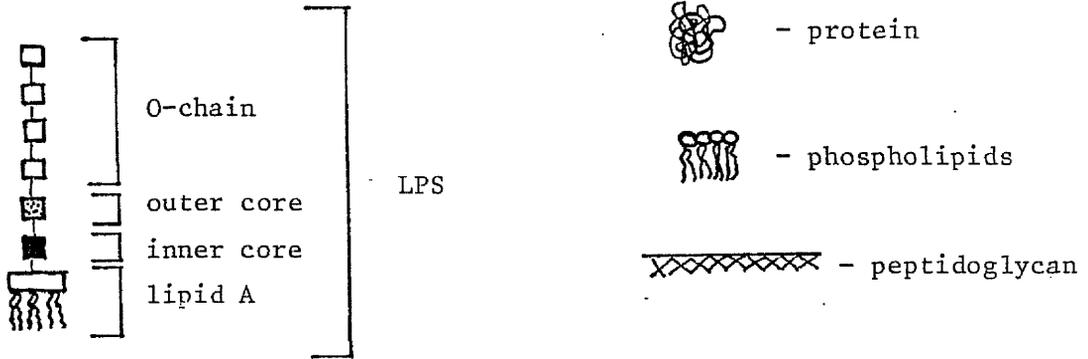
Location, Function and Biosynthesis of LPSs

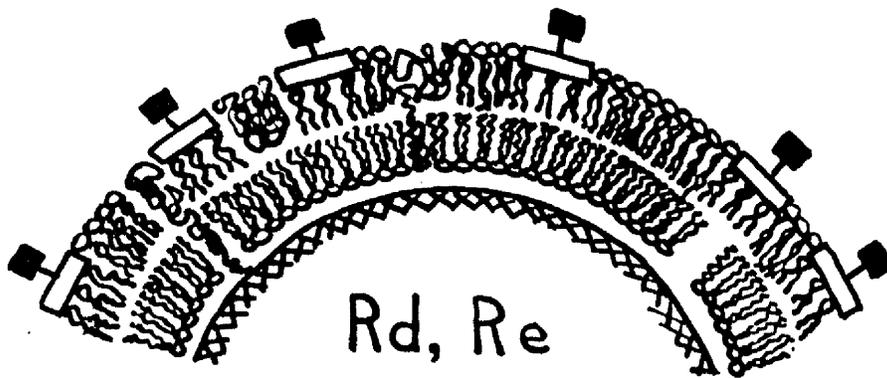
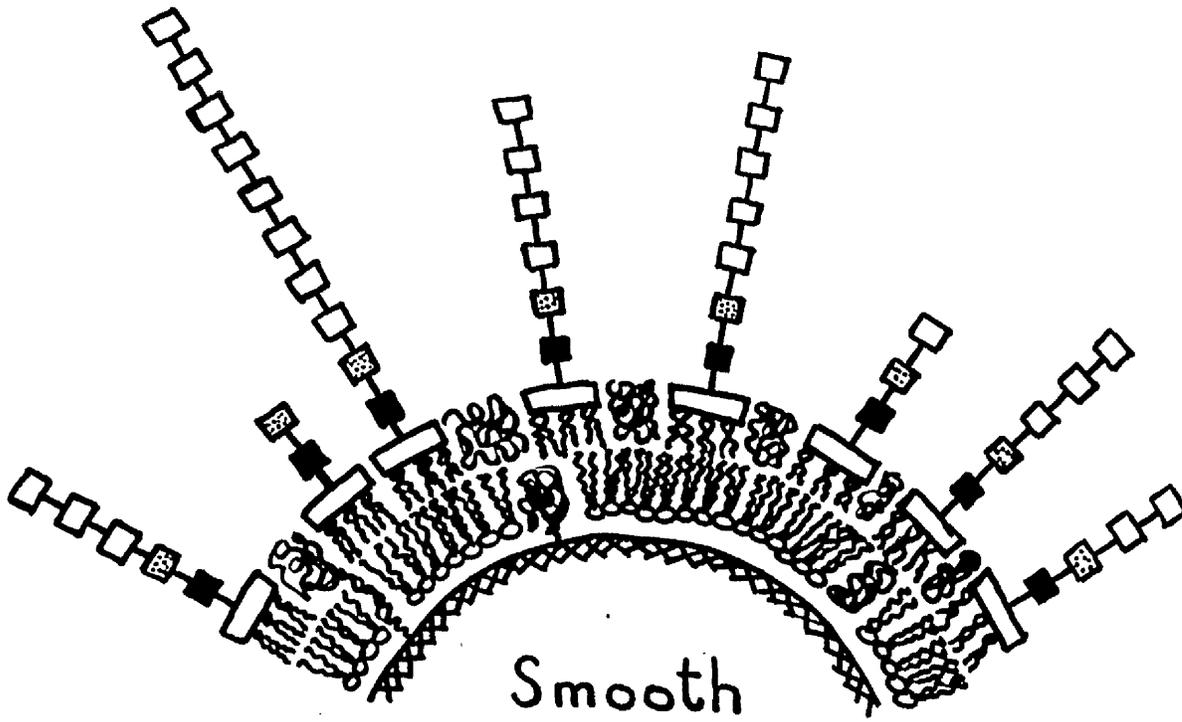
The cell envelopes of Gram-negative bacteria contain two membranes : the inner, cytoplasmic membrane and the outer membrane. The latter and the underlying layer constitute the cell wall. The outer membrane itself, consists of a bilayer containing lipopolysaccharide, proteins and phospholipids. Smit et al., (1975) could not detect phospholipid head groups in the outer leaflet of the outer membrane of Smooth strains of Salmonella typhimurium. Thus they proposed an asymmetric model in which the lipid bilayer contains LPS on one side (outer) and phospholipids on the other, with proteins occurring throughout (Figure 6). Osborn (1979), while supporting the asymmetric model, reported some phospholipids in the outer leaflet.

The function of lipopolysaccharides for bacteria that possess them have been studied by comparing Rough and Smooth strains. Rough mutants differ from their Smooth parents in having increased permeability and thus sensitivity to antibiotics (Schlecht and Westphal, 1970), penetrability by lysozyme and bile salts (Sanderson et al., 1974) and increased susceptibility to detergents and dyes (Kropinski et al., 1978). Thus the Smooth LPS represents a major permeability barrier in the cell walls of Gram-negative bacteria, as well as

Figure 6 : Proposed model for the structure of the outer membrane
of Salmonella typhimurium (based on Smit et al., 1975).

Key :





having important antigenic and pathogenic roles.

LPS core-oligosaccharide and O-chain biosynthesis have been investigated intensively in Salmonella (Nikaido, 1973; Wright and Kanegasaki, 1971). The core-oligosaccharide is made on the cytoplasmic membrane by stepwise addition of sugars to KDO-lipid A. Synthesis of the O-chain subunits occurs independently of core-lipid A synthesis, for oligosaccharide repeating units are polymerised on the cytoplasmic membrane. Transfer of the completed O-chain to the core-lipid A is followed by translocation of the completed LPS to the outer membrane.

Lipid A synthesis has been less well characterised. The enzymatic ligation of two, 2,3 diacylglucosamine nucleotide derivatives (containing amide linked 3-hydroxytetradecanoic acid), generating a tetraacyldiglucosamine, has been demonstrated in the cytosol of E. coli (Ray et al., 1984). So far, there are no data on the biosynthesis of either nucleotide precursors, although a pathway analogous to glycerophosphate biosynthesis was suggested (Raetz, 1983). Walenga and Osborn (1980) have shown that the incorporation of non-hydroxylated fatty acids are not necessary for extension of cores and O-chains, thus contributing to the heterogeneity of lipid As as described above.

It is unclear whether the KDO trimer is added as a complete unit to lipid A or whether each residue is added sequentially (Munson et al., 1978).

Endotoxic Activities of LPSS

When the biological activities of LPSS are being discussed, it is customary to refer to these substances as endotoxins. A wide variety of endotoxic activities has been reported (Milner et al., 1970; Sultzter, 1971). Parenteral administration is necessary for expressing endotoxic properties and these range from lethal toxicity and pyrogenicity, to induction of interferon production and protection against the harmful effects of radiation (Table 1).

TABLE 1Endotoxic reactions of enterobacterial LPSs or their free lipid A component(based on Westphal et al, 1983).

Adjuvant activity

Bone marrow necrosis

Complement activation

Depression of blood pressure

Embryonic bone resorption

Enhanced dermal reactivity to epinephrine

Hageman factor activation

Helper activity for Friend spleen focus-forming virus in mice

Hypothermia in mice

Induction of colony stimulating factor

Induction of early refractory state to temperature change

Induction of IgG synthesis in newborn mice

Induction of interferon synthesis

induction of mouse liver pyruvate kinase

Induction of non-specific resistance to infection

Induction of plasminogen

Induction of prostaglandin synthesis

Induction of tolerance to endotoxin

Induction of tumour-necrotizing factor

Inhibition of phosphoenol pyruvate carboxykinase

Lethal toxicity in mice

Leukocytosis

Leukopenia

Limulus lysate gelation

Local Schwartzman reaction

Table 1 (continued)

Macrophage activation

Mitogenic activity

Platelet aggregation

Pyrogenicity

Toxicity enhanced by adrenalectomy

Toxicity enhanced by BCG

Tumour necrotizing activity

Type cRNA virus release from mouse spleen cells

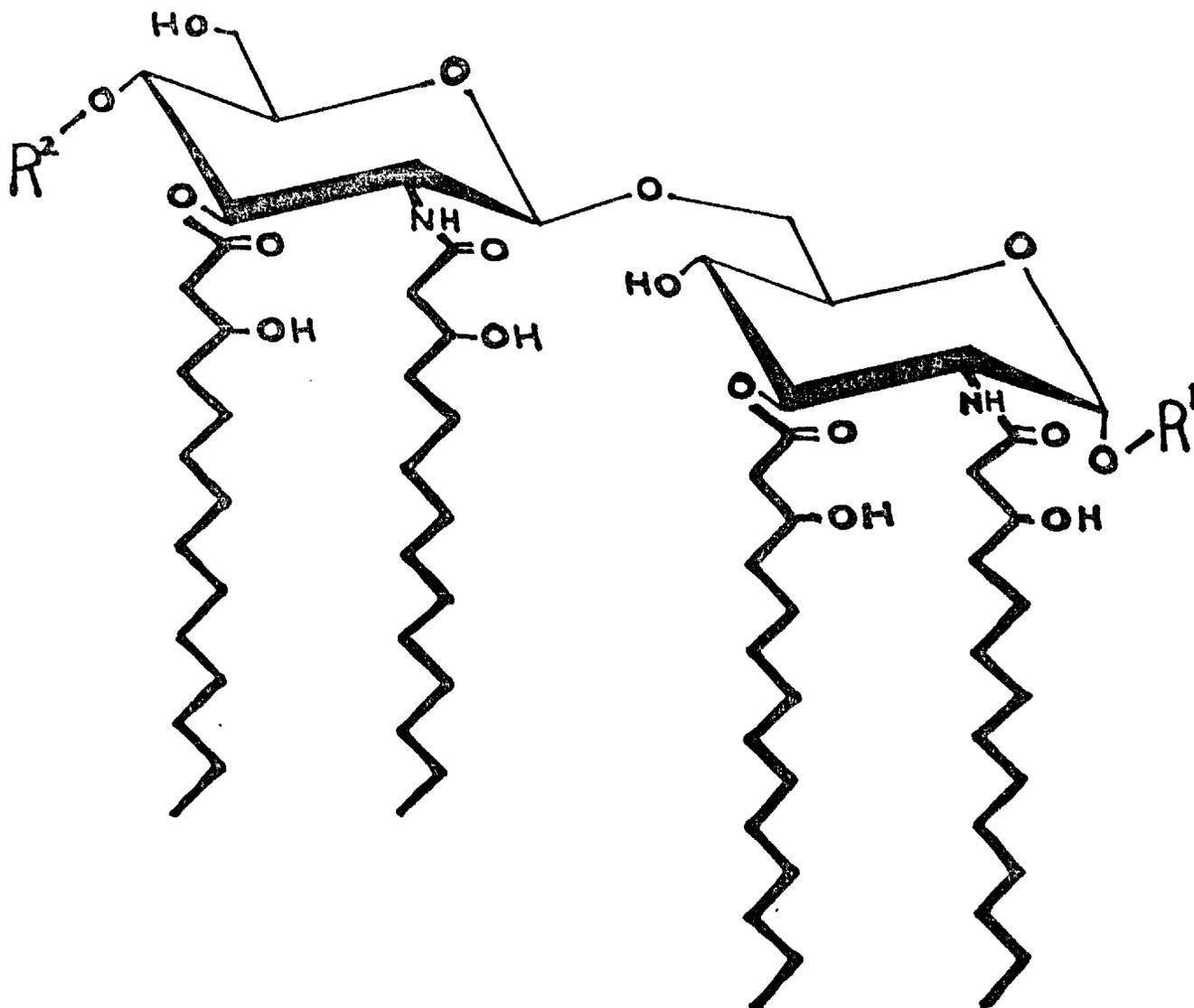
Endotoxins are composed of relatively non-toxic subunits and the formation of macromolecular aggregates is required for endotoxicity (Rudbach, 1966; Galanos and Lüderitz, 1976). McIntire et al. (1969) suggested however that the dissaggregating detergents used in these studies may block the toxic portion of the molecule. It was shown that low and high concentrations of sodium deoxycholate both caused dissociation down to the same subunit size, but that the latter complexes were non-toxic.

It is known that the polysaccharide component, although antigenic, is not responsible for endotoxicity. One effect of the polysaccharide moiety is to solubilize lipid A. Thus lipid A preparations complexed with bovine serum albumin were fully toxic (Galanos et al., 1972).

Tripodi and Nowotny (1966) hypothesised that O-acyl long chain fatty acids were responsible for the endotoxic activities of lipid A preparations. This has been supported, but not confirmed, by numerous other reports. Thus treatments which chemically or enzymatically remove O-acyl fatty acids, bring about detoxification of LPS preparations. The role of ester linked fatty acids was questioned by Kiso et al. (1981) from studies with chemically synthesised lipid A analogues, but these were based on an earlier model of lipid A structure.

Recently, Galanos et al. (1984) using synthetic analogues of the revised model of Salmonella lipid A, have shown that a synthetic β -1,6-linked glucosamine disaccharide, which carried molar equivalents of 3-hydroxytetradecanoyl residues in 2,3,2' and 3' positions and phosphoryl groups in positions 1 and 4' (406, Figure 7) exhibited lethal toxicity, β -lymphocyte mitogenicity, the capacity to engender prostaglandin formation in macrophages and to induce endotoxic tolerance as well as serological lipid A antigenicity. This represents the smallest endotoxically active lipid A substructure detected to date. The use of similar analogues strongly suggested that the 4' ester-linked phosphate groups was required for mitogenicity (404, 405 Figure 7)

Figure 7 : Structures of chemically synthesised lipid A
sub-structures (based on Galanos et al., 1984).



Nature of substituent

| Compound | R1 | R2 | Endotoxic reactions |
|----------|---------------------|---------------------|---|
| 404 | H | PO(OH) ₂ | ± pyrogenic, mitogenic, prostaglandin induction. |
| 405 | PO(OH) ₂ | H | pyrogenic, ± mitogenic, prostaglandin induction |
| 406 | PO(OH) ₂ | PO(OH) ₂ | lethally toxic, pyrogenic, mitogenic, prostaglandin induction |

and that 3-hydroxyl groups on amide-linked fatty acids required acylation for full expression of pyrogenicity and the Schwartzman reaction. This latter finding supports the observation that Ch. violaceum and Xanthomonas sinensis lipid As, which lack ester-bound, but have amide-bound 3-acyloxyacyl residues, have full endotoxicity (Wollenweber et al., 1984).

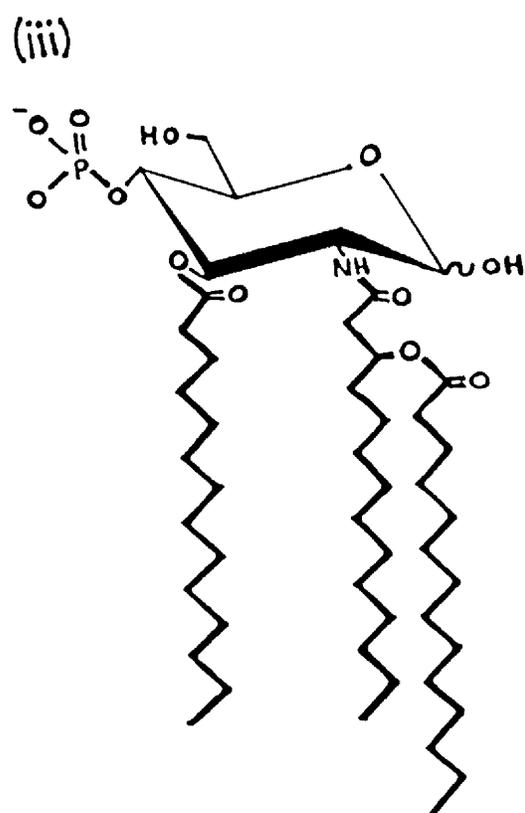
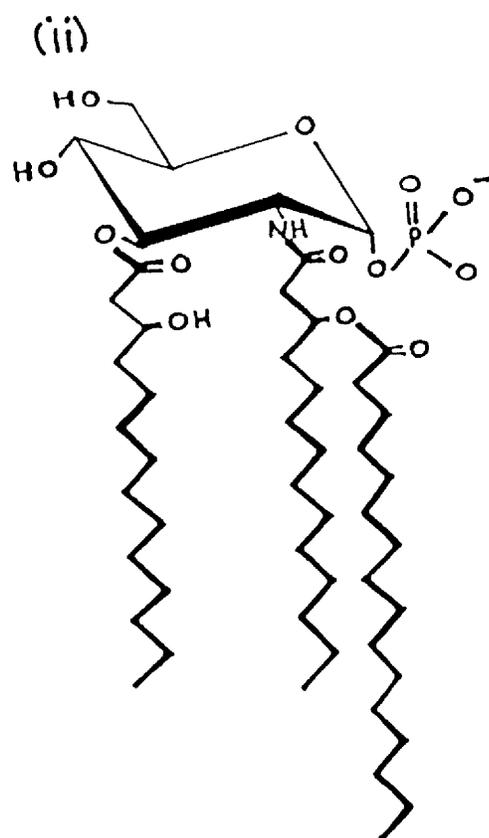
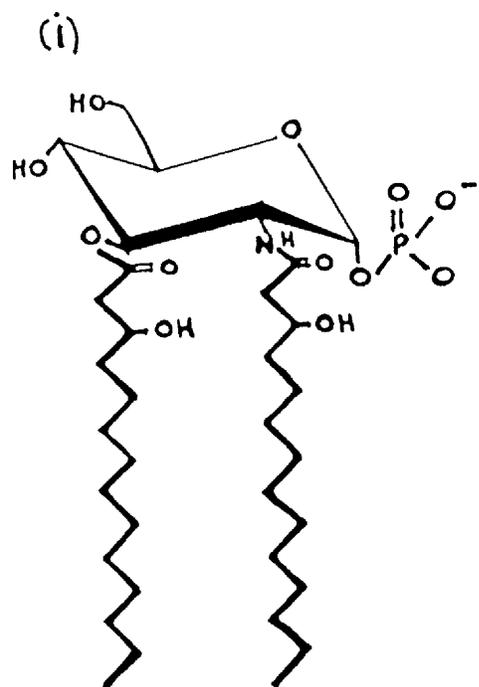
The requirement of a disaccharide unit for endotoxicity has been suggested by the non-toxicity of Rhodopseudomonas viridis, Rhps. palustris and Rhps. sulfoviridis lipid As which contain 2,3-diaminoglucose (Ahamed, 1982). Perhaps significantly in view of the above (Galanos et al., 1984), these lipid As also lack esterified long chain fatty acids. However it has recently been shown that monosaccharide lipid A sub-structures exhibit some endotoxic activities :- The E. coli lipid A precursors lipid X and lipid Y (Figure 8) induced morphological changes, prostaglandin E₂ synthesis and killing of tumour cells by peritoneal macrophages in vitro. Removal of the ester-linked 3-hydroxytetradecanoyl residue at position C3 or the phosphate at C1 abolished activity (Nishijima et al., 1985) although lipids X and Y are non-toxic (Takayama et al., 1984). Compounds have been synthesised by Kumazawa et al., (1985) which possessed N-tetradecanoyloxytetradecanoyl groups with or without a 4-O-phosphoryl group linked to glucosamine and are thus analogues of the non-reducing moiety of lipid A (Figure 8). These structures exhibited both mitogenic and polyclonal B cell activity and were non-pyrogenic.

The non-toxicity of monophosphoryl lipid A, prepared by Takayama et al., (1981, below), was explained by its insolubility or by its altered chemical configuration compared to biphosphoryl lipid A (Galanos et al., 1984; Kanegasaki et al., 1984). The phosphate group at the reducing C1 was not considered the "toxophore" of the molecule. However, these latter groups based their criticisms on chemically synthesised lipid A analogues which lacked non-hydroxylated fatty acids, thus the proposal by Amano et al., (1982) that, since this non-toxic lipid A retained anti-tumour-activity, it

Figure 8 : Chemical Structures of Lipid A Monosaccharide

Sub-structures

- (i) Lipid X (based on Takayama et al., 1984)
- (ii) Lipid Y
- (iii) analogue of non-reducing sugar moiety of lipid A
(based on Kumazawa et al., 1985)



could have potentially beneficial applications in immunotherapy, has not been disproved. Relationships between structures and functions of endotoxins will be most clearly elucidated by the study of chemically synthesised structures. The complete synthesis of E. coli lipid A has recently been achieved (Imoto et al. 1985) and thus potentially useful sub-structures may be similarly synthesised.

Methods of Detecting LPSs

Most assays for detecting LPSs involve measuring particular biological activities of the molecules. Complement activation is not used as a method for detecting LPS in pharmaceuticals or serum samples for it has been shown that mitogenic and pyrogenic activities are expressed by different chemical configurations than those that activate complement (Elin et al., 1976; Galanos et al., 1984). Other biopolymers including pneumococcal cell walls and water-insoluble glucans of Streptococci also activate complement (Winkelstein and Tomasz, 1977; Inai et al., 1976). Rough LPSs activate complement via the classical pathway, whereas Smooth LPSs activate complement via the alternative pathway and the activation of the alternative pathway is sensitive to slight variations in the chemical structure, but not to large variations in the chain length of the O-chains of LPS (Grossman and Leive, 1984). It is known that high anti-complementary activity is shown by LPS preparations having high degrees of aggregation and relatively low levels of phosphate (Galanos and Lüderitz, 1976; Galanos et al., 1984).

The most sensitive, rapid and convenient bio-assay for LPS is the Limulus Amoebocyte Lysate (LAL) assay (Levin and Bang, 1964). Minute quantities of endotoxin (pg-ng) activate a cascading coagulation mechanism in the amoebocyte lysate of the horseshoe crab Limulus polyphemus, resulting in the formation of a gel. The LAL has been developed as a useful diagnostic aid for coliform bacteria (Scheifle et al., 1981) and for detecting endotoxin in cerebrospinal

fluid (Munford *et al.*, 1984), water samples (Jorgensen *et al.*, 1976), sterile surgical equipment (Kundsinn and Walter, 1980), vaccines (Geier *et al.*, 1978), antibiotic solutions (Case *et al.*, 1983) and others. Since the mechanism of LAL gelation was elucidated (Takagi *et al.*, 1979), synthetic, chromogenic substrate LAL assays have been developed (Tsuji *et al.*, 1984). However, the LAL assay has been shown to be unspecific for certain bacterial products (Wildfleuer *et al.*, 1974) and when applied to serum samples, inhibitors of gelation must first be removed by heating and dilution, thus diminishing sensitivity (Scheifle *et al.*, 1981). Recently, however, Paulssen *et al.*, (1985) have reported that ultrafiltration using an Amicon filter unit removed inhibitors such as sodium and calcium salts and solutions containing antibiotics, thus allowing detection of endotoxin. There is good correlation between the LAL and the United States Pharmacopeia (USP) pyrogen test (1975), which measures the rise in temperature in rabbits injected with endotoxins. This latter test is inconvenient, expensive and variable, although it is highly sensitive and can detect down to 100pg/ml of endotoxin.

Other bioassays are the histamine hypersensitisation test (Bergman *et al.*, 1977) and the chick embryo cytotoxicity test (Finkelstein, 1964), both of which are time consuming and may be unspecific. The lethal toxicity to mice sensitised by injection with D-galactosamine, however provides a useful assay of the endotoxic properties of an unknown sample (Galanos *et al.*, 1979).

Enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of LPSs. Some of these are highly specific for a typical group of LPSs however (Rigby, 1984; Dahlén and Mattsby-Baltzer, 1983), although a system to detect anti-lipid A antibodies in serum was developed for diagnostic purposes by Fink and Galanos (1981) which had broad specificity, since this structure is highly conserved.

Radioimmunoassays are also limited due to their specificity for particular O-antigens (Gutowski and Jacobs, 1979). However, Nolan et al. (1982) developed a method with broad specificity which detected lipid A obtained from various endotoxins of various origins after acid hydrolysis of LPSs, although the method is to date slightly less sensitive than the LAL. Another method of detecting LPSs is the measurement of chemically released hydroxy fatty acids. This has been applied to endotoxins in serum (Maitra et al., 1978) and to endotoxins found in marine sediments (Parker et al., 1982). Finally there are methods depending on bioluminescence (Ulitzur et al., 1979), the colorimetric estimation in response of LPS to a carbocyanine dye (Janda and Work, 1971) and haemagglutination inhibition (Neter, 1956).

Chemical Detoxification of Endotoxic Activities

A comprehensive study of the effect of alkaline hydrolysis (which liberates esterified fatty acids from LPS) upon various biological activities of endotoxins was presented by Neter et al. (1956). The rates of both saponification and detoxification were markedly increased by carrying out the reaction in ethyl alcohol or dimethylsulphoxide (Niwa et al., 1968).

Boivin et al. (1933) used dilute acetic acid to degrade endotoxins with subsequent loss of toxicity. This was confirmed by Tal and Goebel (1950). In kinetic studies, Ribi et al. (1964) found that the rate at which the biological activities of endotoxins were abolished paralleled the dissociation of the complex into particles which were 1% of the size of the original endotoxin.

Takayama et al. (1981) reported the preparation of a non-toxic lipid A fraction : LPSs from Re strains of S. minnesota and S. typhimurium treated with dilute acetic acid (pH 4.5) released KDO. Free lipid A was further treated with dilute hydrochloric acid, selectively removing the phosphate

group at the reducing end of the glucosamine disaccharide (Figure 5(a), C1). Finally, monophosphoryl lipid A was separated from the reaction mixture by TLC and DEAE-cellulose chromatography. The chick embryo lethal dose (CELD) of monophosphoryl lipid A was 10,000 fold greater than diphosphoryl lipid A and it was less pyrogenic. However monophosphoryl lipid A retained full antitumour activity (Amano et al., 1982).

Detoxification without loss of pyrogenicity and antigenicity may also be achieved by treating LPS or heat-killed Gram-negative bacteria with ionizing radiation (Previte et al., 1967). However, Czako et al. (1983) reported that ionizing radiation brought about (i) loss of polysaccharide moieties and (ii) the alteration of lipid A, both of which might have been expected to affect the activities reported by the previous authors.

The following treatments produced materials (termed "endotoxinoids" by Nowotny, 1963) with reduced pyrogenicity or lethal toxicity; lithium aluminium hydride, transesterification with boron trifluoride, O-acyl cleavage with potassium methylate and treatment with pyridine-formic acid.

Prigal et al. (1973) showed that LPSs from six bacterial strains could be rendered non-toxic by treatment with ferric chloride as measured by the mouse lethality test. Since chromic chloride gave the same result, it was suggested that the trivalent metal ions in some way altered the chemical configuration of the endotoxin.

Acetylation of LPS reduced pyrogenicity at least one hundred fold (Martin and Marcus, 1966); upon deacetylation activity was recovered.

Schenck et al. (1969) achieved a very high level of detoxification by succinylation and bromoacetylation. Chedid (1976) found that LPSs thus detoxified retained their capacity to stimulate murine lymphocytes, to protect mice against lethal radiation and to induce abortion.

Non-Destructive Detoxification of Endotoxic Activities

Polymyxin B binds to LPS at its lipid A-KDO region (Bader and Teuber, 1973) blocking many of its biological effects in vitro (Neter et al., 1958; Bannatyne et al., 1977) and its toxic effects in vivo (Corrigan and Bell, 1971; From et al., 1979). Cooperstock and Riegler (1981) markedly decreased the endotoxic activity in suspensions of Bordetella pertussis, E. coli, H. influenzae and P. aeruginosa by treatment with polymyxin B. A role in reducing the inflammatory reactivity of LPS in vaccines of Gram-negative bacteria was suggested.

Bishop and Desnick (1981) used affinity chromatography to remove endotoxins from preparations of human α -galactosidase A which is parenterally administered to patients suffering from deficiency in the enzyme. Sephacryl gel filtration was included in the purification of human arylsulphatase A by Sarafian et al. (1982) in order to remove endotoxins. The complete removal of endotoxins from human placental granulocyte-macrophage colony stimulating factor (HP-CSF) was achieved by its adsorption to LAL proteins either directly, or following coupling to Sepharose beads (Wollman et al., 1982). Activated charcoal and Dowex 1 - X 2 were the most efficient in the removal of endotoxins in vitro in a study of several resins by Nolan et al. (1975). The level of endotoxin in influenza virus vaccine lots was reduced 10-20 fold after barium sulphate adsorption-elution. However, despite these numerous reports of methods of removing endotoxins from various materials, it is known that endotoxins are "sticky" and bind to some protein preparations for human injection such as human growth hormone (Axelsson, 1985).

Ditter et al. (1983) showed that bentonite, surgically implanted in the stomach of mice, prevented subsequent orally induced endotoxemia. It was speculated that similar methods may be applied to remove circulating endotoxins in vivo. However, Maitra et al. (1981) found that in vitro binding of endo-

toxins by various matrices was greatly reduced in the presence of serum. Thus the use of these materials in removing circulating endotoxins in vivo may be limited.

Biodegradation of LPSs

Slime Moulds

Amoebae of the phagotrophic slime mould Dictyostelium discoideum cultivated on the Gram-negative bacteria Salmonella london or Escherichia coli B as the sole organic nutrient source, released into the medium partially degraded bacterial LPSs (Malchow et al., 1967) lacking both hydroxy and non-hydroxy fatty acids. It was later shown that the diglucosamine backbone of lipid A was present, thus ruling out the possibility that the entire lipid A component with attached fatty acids was split off (Malchow et al., 1969). However, the same investigators later reported little release of 3-hydroxytetradecanoic acid (Nigam et al., 1970). The degraded material was 50 fold less toxic for mice than untreated LPS (Malchow et al., 1969).

Two fatty acyl amidases from D. discoideum were shown to act sequentially in vitro on E. coli K-12 LPS which had been partially degraded beforehand by alkali and acid treatment. Thus amidase-I cleaved 3-hydroxytetradecanoic acid from the amino group on the reducing end glucosamine residue of phosphorylated, diglucosamine backbone containing only amide-linked 3-hydroxytetradecanoic acid. Amidase-II, removed the remaining 3-hydroxytetradecanoic acid from the amino group in the distal glucosamine residue (Rosner et al., 1979a). Both enzymes were partially purified (Verret et al., 1982a) and some of their properties characterised (Verret et al., 1982b). Both were specific for long chain fatty acids, although the 3-hydroxyl group was not required for hydrolysis. The substrate of amidase-I was resistant to amidase-II. It was also reported that phosphatases and fatty acyl esterases acting on LPS or LPS derivatives were major contaminants of the amidases prior to purification,

although these were not further characterised. Phosphatase was largely removed by heating at 60°C for 5 min, while fatty acyl esterase activity was reduced by gel filtration. N-acetylglucosaminidase activity towards p-nitro-phenyl-N-acetylglucosamine glucoside was also detected in crude extracts, but the effect of this enzyme on LPS or its derivatives was not reported.

Another slime mould, Physarum polycephalum also degraded LPSs from various Gram-negative bacteria both in vivo and in vitro (Saddler et al., 1979a). As with D. discoideum, the polysaccharide portion of LPS was scarcely effected, but long chain fatty acids were released from lipid A. No detectable 3-hydroxytetradecanoic acid was released, which is the same as found by Nigam et al., with D. discoideum.

Helix pomatia

Degradation of LPSs from several Gram-negative bacteria by gut juice of the snail Helix pomatia was reported by Saddler et al. (1979b). Little effect was detected on the immunodominant sugar content of LPS O-chains, but there was extensive loss of anticomplementary activity and changes in the electrophoretic pattern of LPSs in polyacrylamide gels. It was concluded that lipid A was the main site of attack, with fatty acids being removed, thus resembling the degradation of LPSs by slime moulds.

Bacterial Isolates

The Gram-positive bacterium, Bacillus firmus was isolated from soil by Voets and Beyaert (1970) and was capable of growing on solid medium with LPS from Salmonella minnesota as the sole organic nutrient source. They later found that Bacillus macerans, also isolated from soil, grown in liquid mineral media containing different bacterial LPSs, hydrolysed LPS to lipid A and a polysaccharide fraction. The latter component was not further degraded but

free fatty acids and phosphate were liberated from lipid A (Voets et al., 1973).

A microbial enzyme preparation was reported by Vincent and Cameron (1965) to be able to degrade various LPSs. Culture supernates of Streptomyces griseus caused a drop in turbidity of 1% (w/v) LPS solutions. An enzymatic mode of action was suggested by the sensitivity of the activity to heating to 100°C for 1 min and its defined pH optimum of pH 7.2. However, there was poor reproducibility of these findings.

Cortez (1976) isolated three unidentified bacteria from soil which degraded LPSs extracted from two Gram-negative bacteria also found in soil. Whole-cell extracts of the degrading bacteria exhibited glycolytic activity towards LPS, producing a series of oligosaccharides when analysed by gel filtration chromatography, although these were of unidentified molecular weight.

LPS-degrading bacteria were isolated from marine sediments by Saddler and Wardlaw (1980) by serial enrichment in sea water with added LPS. Each of three isolates varied in their ability to degrade either lipid or polysaccharide components of LPSs. Cell-free extracts were not prepared from these organisms.

Sonic extracts of Neisseria gonorrhoeae and N. meningitidis degraded the purified LPS from the former bacterium, with the complete loss of antigenicity and immunogenicity (Apicella et al., 1978). Due to the chemical complexity of the O-chain of the organism, the involvement of more than one glycolytic enzyme was suggested. The extracts had no effect on LPSs from other Gram-negative organisms, and similarly prepared sonic extracts from other bacteria had no effect on gonococcal LPS. It was suggested that the ability to degrade major antigenic components of the cell wall should impair the recognition of the organism by the host's immune system. In addition, the haptenic O-chain

subunits thereby released would be available to neutralise protective antibodies.

Sediments and Soils

LPSs from two soil Gram-negative bacteria were degraded by Mediterranean red soil (Cortez, 1977a). Release of carbon dioxide from soil samples incubated with LPSs was interpreted as being indicative of degradation of the added substrate by the microbial flora of the soils. The polysaccharide portion of LPS was identified as the site of attack (Cortez, 1977b). One of the LPSs was less readily degraded which was explained as being due to LPS forming complexes with clay particles and metal ions present in the soil and thus being protected from hydrolysis. Degradation of both of the LPSs was greater in montmorillonite clay than in kaolinite and in the presence of Fe^{3+} ions rather than Ca^{2+} ions. It was proposed that degradative enzymes were also adsorbed onto clay particles, occupying similar sites to LPS molecules, and thus "masking" their own active sites (Cortez, 1976).

Evidence for rather extensive degradation of LPSs in marine sediments was provided by Saddler and Wardlaw (1980). Marine sediments at different depths and sites were extracted with phenol/water and/or trichloroacetic acid and assayed for various indicators of LPS. KDO was not detected in any of the samples taken below a depth of 4cm ; anticomplementary activity decreased with increasing depth. When killed enterobacteria were incubated with marine sediments, degradation of LPS was observed. Purified LPSs were also degraded when added to sediments, with the polysaccharide component degraded faster (as demonstrated by the loss of KDO and serological specificity) than the lipid moiety (demonstrated by loss of anticomplementary activity and 3-hydroxytetradecanoic acid). Both amide- and ester-linked fatty acids of the lipid A were lost.

Phages

Bacteriophages of several species of Enterobacteriaceae possess structural proteins which enzymatically degrade the LPS of their host cell wall, which is their site of attachment. Most have been found to cause depolymerisation of the O-chain to units of di and tri-repeating units. Iwashita and Kanegasaki (1973) showed that the tail protein of ϕ P22 was an endoglycolytic enzyme that depolymerised the O-chain of Salmonella typhimurium LPS. It was specific for α -rhamnosyl-1,3-galactose linkages. Phages from five strains of Enterobacteriaceae degraded LPS in situ in their host cell wall (Lindberg, 1973). Rieger-Hug et al. (1977) isolated six Smooth-specific phages from sewage, all of which had characteristic endoglycolytic activity upon their respective purified LPS. Among other phages that have been shown to degrade O-chains are ϕ^{15} (Kanegasaki and Wright, 1973), ϕ^{34} (Iwashita and Kanegasaki, 1975), $\phi 8$ (Reske et al., 1973) and 1(40) of Salmonella johannesberg (Chaby and Girard, 1980).

Salmonella phage c341, although being Smooth-specific, hydrolysed the O-acetyl substituents of LPS O-chains, while having no effect upon the glycosidic linkages (Iwashita and Kanegasaki, 1975).

There have been no reports of phage enzymes that are active against the core-oligosaccharide or lipid A.

Mammalian Systems

There is relatively little information about the metabolism of LPS in mammals, except various reports of interactions between LPS and serum or hepatic components; few of these have given clear demonstrations of enzymatic degradation. The action of serum in lowering the pyrogenicity of endotoxins (Lüderitz et al., 1958) was suggested by Keene et al. (1961) to involve enzymatic degradation. Two serum fractions were implicated in enzymatic detoxification by Skarnes (1966; 1968). One of these (α_1 -lipoprotein esterase) degraded endotoxi

to some extent but did not inactivate it, while the other (α_1 globulin-esterase) destroyed toxicity but not serological activity. Degradation of LPSs from E. coli, S. marcescens and Sh. flexneri were reported by Schultz and Becker (1967) to be brought about by a detoxifying lipoprotein lipase. Rutenberg et al. (1967) showed that esterase activity in denervated reticuloendothelial cells of the spleen rapidly detoxified endotoxins. An esterase-rich fraction from rabbit polymorphonuclear leukocytes detoxified endotoxins (Skarnes, 1970). This was shown by Gimber and Rafter (1969) to be due to a lipase.

Recently, Hall and Mumford (1983) reported that the granule fraction of human neutrophils contained one or more enzymes that partially deacylated S. typhimurium lipid A in vitro by removing non-hydroxylated fatty acids. It was suggested that similar degradation of LPSs occurs in vivo. Freudenberg et al. (1984) found that partially deacylated LPS could be recovered from the liver and other organs such as the spleen, of rats that had been parenterally administered LPS from Salmonella abortus-equi. The material was less toxic than untreated LPS by a factor of 30.

Contrary to the above reports of LPS biodegradation by mammalian systems, an extensive study of Lehrer and Nowotny (1972) failed to identify any enzyme of mammalian origin with significant biochemical or biological effects on LPS. They concluded that such an enzyme for which they coined the title "endotoxinase" would probably be highly specific for LPS.

Occurrence of LPS in Marine Environments

Gram-negative bacteria are known to be abundant in soils, fresh-water, mammalian digestive tracts, and in marine sediments among other environments. Use of the LAL test in recent years had added to the wealth of documentation of the ubiquity of LPS in Nature.

Gram-negative bacteria account for 80-95% of the prokaryotes found in marine environments (Zobell and Upham, 1944; Rheinheimer, 1974). Grimes et al. (1984) reported that the predominant bacterial type in marine environments (Pseudomonas sp.) was superceded by Vibrio sp. when samples were taken near the dump-sites of pharmaceuticals.

The bacterial biomass of marine mud-flats was estimated as 1.1g bacterial dry weight/cubic foot of surface layer based on viable counts, which were assumed to recover only 10% of the organisms present (Zobell and Feltham, 1942). It was further estimated that in the course of 24h, 11g of bacterial dry weight/cubic foot should be produced.

Sullivan et al. (1976) estimated bacterial biomass in the sea by comparing the LAL test and ATP measurements. They concluded that over 50% of the total biomass, in the marine environment consisted of bacteria. Assuming that Gram-negative bacteria contain, on average, 1% LPS by dry weight, they calculated that LPS accounted for around 0.5% of the total biomass in the marine environment. However, this surprisingly large estimation of LPS content may be unreliable. The LAL test is influenced by a variety of factors such as salts and pH and variations in results have been obtained between operators with the same samples from marine environments (Watson et al., 1977). Microorganisms attached to sediments must also be quantitatively recovered from sediments prior to the LAL test (Parker et al., 1982).

Saddler and Wardlaw (1980) estimated that in the top 1cm layer of sediments close to a sewage outfall there was either 4g or 300mg of bacterial dry weight/kg dry weight of marine sediment depending on whether the calculations were based on anticomplementary activity or 3-hydroxytetradecanoic acid estimation respectively. From these figures, estimations of 40mg or 3mg of LPS/kg dry weight of marine sediment can be made. LPS was readily extractable from the surface layer of marine sediments by sequential application of the phenol/water

and trichloroacetic acid extraction methods. Sewage-contaminated sediments were richer in LPS than natural sediments and the concentration of LPS dropped off steeply with depth of burial.

Parker et al. (1982) recovered LPS added to sediments by extraction with a single-phase chloroform/ methanol mixture. This method was simpler, and the subsequent estimation of LPS (by analysis of hydroxy fatty acids), was reported to be about five-fold more sensitive than the phenol/water or trichloroacetic acid procedure. From their data it can be estimated that in estuarine sediments, bacteria constitute about 750mg dry weight/kg dry weight of sediment, of which LPS should contribute on average 7.5mg/kg dry weight. Thus, taking into account the increased efficiency of their experimental system these estimates were of the same order as those of Saddler and Wardlaw (1980).

Nutrition of Filter-Feeding Marine Bivalves

The abundance of Gram-negative bacteria in the marine environment suggests that these organisms may constitute an important food source for filter-feeding marine animals. Marine bivalve molluscs in general, feed by drawing a continuous current of water into the mantle cavity and sieving particles of suitable size for subsequent degradation by enzymes from the digestive system (Reid, 1966).

Since Zobell and Feltham (1938) showed that the mussel Mytilus californianus could be nourished by a diet of bacteria alone, it has generally been accepted by subsequent investigators that bacteria do serve as a source of nutrition for filter-feeding marine bivalves (reviewed by Birkbeck and McHenry, 1982). Conflictingly however, Ukeles and Sweeney (1969) found no evidence for utilization of bacteria, and even considered that bacteria merely interfered with the digestion of phytoplankton by packing the alimentary tract.

The degradation of bacteria by marine bivalves has been poorly studied.

McHenery *et al.* (1979) showed with radioactively labelled bacteria that those with cell walls which were sensitive to Mytilus edulis lysozyme were rapidly degraded in vivo. Bacterial polymers were apparently degraded and reassembled into mussel biopolymers with the exception of bacterial DNA which was degraded and excreted. Extracts of the digestive gland degraded rapidly whole cells of lysozyme-sensitive, but not insensitive, bacteria in vitro (Birkbeck and McHenery, 1982).

Although numerous enzyme activities have been reported in marine bivalves (Reid, 1968), such activity against bacterial constituents have not been generally considered, apart from the lysozyme studies of McHenery *et al.* (1979). Thus Hylleberg Kristensen (1972) conducted an extensive study of the carbohydrases of marine invertebrates (including filter-feeding bivalves) in relation to their natural substrates. But perhaps due to the conflicting evidence upon the nutrition of marine bivalves (above), ^{she} studied only the breakdown of simple saccharides and plant and algal polysaccharides.

Stark and Walker (1983) found that di and trisaccharides along with starch, glycogen and laminaran (a β -1, 3-glucan, characteristic of algal cell walls) were rapidly degraded by digestive diverticula extracts of the deep-sea scallop (Pecten maximus). Seiderer *et al.* (1982) found little difference between the enzyme spectrum of two species of mussel which had different modes of nutrition (one fed on kelp detritus, the other on plankton). Laminarinase and α -amylase were the strongest activities among those assayed in extracts of the crystalline style of these animals.

The occurrence of several different glycosidases in marine invertebrates ~~has~~ been reported. Wojtowicz (1972) detected α -glucosidase, β -glucosidase, β -galactosidase, laminarinase and chitobiase in the digestive gland of the scallop Placopecten magellanicus. The crystalline style contained α -amylase and laminarinase activity. β -N-acetylglucosaminidase was partially purified

from the digestive glands of three clams and was found to contain several other glycosidase activities (Santoro and Dain, 1981). While purifying β -glucuronidase from the periwinkle Littoriana littorea, Diez and Cabezas (1979) reported ten other glycolytic enzymes in hepatopancreas extracts. However, in the above studies, activities were detected against p-nitrophenylglycosides. Thus although the extracts contained enzymes capable of hydrolysing saccharide linkages known to be present in LPSs, there is no evidence as to whether they are active against this substrate in its polymerised state. In this context, the β -glucuronidase purified by Diez and Cabezas (1979) was inactive against macromolecules containing β -glucuronic linkages.

The pH optima of carbohydrases from the digestive glands of filter-feeding marine bivalves range from pH 3.6-5.5 (Wojtowicz, 1972; Santoro and Dain, 1981). McHenery and Birkbeck (1982) reported that the lysozyme of M. edulis had a pH optimum of pH 4.6 at high ionic strength, but had greatest activity in low ionic strength, pH 7.1. M. edulis chitinase was also purified and found to have optimum enzyme activity at pH 4.8 (Birkbeck and McHenery, 1984).

Molluscan lipases in general, have not been well studied. George (1952) showed that in marine bivalves, lipid digestion took place in conjunction with the dissolution of the crystalline style. The crystalline style lipase from the surf clam Spisula solidissima was shown to have low substrate specificity and to have a specific activity of only 2% of that shown by standard hog pancreatic lipase (Patton and Quinn, 1973). Lipases from various tissues of some marine filter-feeding bivalves were reported by Reid (1968). Pecten had weak lipases in the stomach fluid while the cockle Cardium (= Cerastoderma), and especially the clam Mya, had high activity. In all the bivalves tested, lipases of the crystalline style were weaker than stomach fluid lipases.

In conclusion, it is generally accepted that phytoplanktonic algae and non-living detritus, along with bacteria, are the principal food sources of

filter-feeding marine bivalves (Bayne, 1976) although their relative importance remains to be determined (Birkbeck and McHenry, 1982).

OBJECT OF RESEARCH

While there is a vast literature on the chemistry, immunology and pharmacology of bacterial lipopolysaccharides (LPSs), comparatively little is known about the fate of these substances in the natural environments where Gram-negative are abundant. This thesis considers aspects of the fate of LPS in marine environments, where Gram-negative bacteria are the predominant prokaryotes.

The primary aims of this work were (a) to examine the digestive systems of a variety of marine filter-feeding bivalves for LPS-degrading activity (b) to investigate the occurrence of LPS-degrading bacteria in marine coastal sediments and (c) to attempt some characterisation of the enzymes involved from both molluscan and bacterial sources.

MATERIALS AND METHODS

LPSs, Bacteria and Marine Invertebrates

Lipopolysaccharides (LPSs) from Salmonella typhosa O901, Salmonella minnesota and Escherichia coli O111:B4 extracted by the phenol/water method of Westphal et al. (1952) were purchased from Sigma. LPS from Methylophilus methylotrophus was extracted from 'Pruteen', a single-cell-protein product of I.C.I. Plc., routinely by the method of Westphal et al. (1952), although occasionally by other methods.

Escherichia coli NRE 600, was provided by Dr. K.Y. Lee. Two strains of LPS-degrading marine bacteria were provided by Saddler and Wardlaw (1980). Strain A was a Bacillus spp. and Strain B was a Coryneform spp. Marine invertebrates were obtained from the Specimen Supply Department of the University Marine Biology Station, Millport, Isle of Cumbrae. Tissues were either dissected at the Marine Station or the animals were transported live to this department and dissected immediately on arrival. They were the filter-feeding bivalves; Cerastoderma edule (common cockle); Modiolus modiolus (horse mussel); Mya arenaria (sand gaper); Mytilus edulis (common mussel) and Pecten maximus (great scallop).

Preparations and Isolations

(a) LPS from Methylophilus methylotrophus

Westphal Method

Whole, dried cells of the methanol-utilizing Gram-negative organism Methylophilus methylotrophus were supplied by I.C.I. in a granular form as 'Pruteen'. The following procedure was found to be the most convenient adaptation of the Westphal method (Westphal et al., 1952) :

'Pruteen' granules (100g) were ground to a fine powder in a mortar and added to a 5 litre conical flask containing 1 litre of distilled water in a steamer and allowed to stand with occasional mixing for 20 min. The temper-

ature was then adjusted to 68°C by placing the flask in a water bath at that temperature, after which, 1 litre of 90% (w/v) aqueous phenol, pre-heated to 68°C, was added and the mixture shaken vigorously for 30 min. The mixture was cooled to around 10°C, centrifuged at 8,000 r.p.m. in a Sorval Centrifuge for 45 min and the upper aqueous layer collected. To the lower phenol layer was added another 1 litre of distilled water and the extraction repeated. The combined aqueous layers were dialysed against running water for three days and distilled water for one day. After centrifugation at 10,000 r.p.m. for 15 min to remove particulate material, the supernate was concentrated from around 2 litres to around 250ml in an Amicon ultrafiltration unit with a 100,000 D exclusion limit. The retentate was centrifuged at 40,000 r.p.m. for 4h in an MSE Superspeed 65 centrifuge. The sedimented pellet was resuspended in distilled water and ultracentrifugation repeated. The resuspended pellet was then treated with DNase (from bovine spleen, type V, Sigma) RNase A (from bovine pancreas, type 1-A, Sigma), protease (type V, from Streptomyces griseus) and finally ultracentrifuged and lyophilised.

Galanos Method

The procedure described by Galanos et al. (1969) was followed. Granular 'Pruteen' (20g) was ground in a mortar until a fine powder was obtained and was added to 100ml of extraction mixture (90% w/v aqueous phenol:chloroform:petroleum ether, 2:5:8) and the procedure was completed as described by Galanos et al. (1969) but without the primary homogenisation step.

EDTA Method

LPS was released from ground 'Pruteen' by a scaled up modification of the method of Leive and Morrison (1972). 'Pruteen' (100g) was washed three times in 3ℓ of 0.12M Tris-HCl, pH 8.0 by centrifugation at room temperature. The temperature was then brought to 37°C and EDTA was added in the same buffer to

give a final volume of 500ml and 0.01M EDTA. After 5 min gentle agitation at 37°C, MgCl₂ was added (0.05M final concentration) and after centrifugation at 10,000 r.p.m. for 20 min in a Sorval centrifuge, the supernate was dialysed for 24h against several changes of buffer, beginning with buffer of high ionic strength, to ensure that nucleotides and other charged molecules passed the dialysis barrier, followed by distilled water. After protease treatment, Amicon ultrafiltration and ultracentrifugation with repeat wash, the material was lyophilised.

(b) Aqueous Extracts of Marine Invertebrate Digestive Glands

A method based on that of Diez and Cabezas (1979) to extract enzymes from the hepatopancreas of the periwinkle (Littorina littorea) was used. All steps were carried out in a 4°C cold room.

Digestive glands from 10 animals were homogenised in distilled water (1:6, w/v) in a mortar with washed sand. The suspension was allowed to settle for 2h and the crude extract was centrifuged for 20 min at 10,000 r.p.m. in a Sorval centrifuge. Solid (NH₄)SO₄ was added with stirring to the supernate to 50% saturation. After allowing to settle for 12h the resulting precipitate was removed by centrifugation (10,000 r.p.m., 30 min, Sorval), resuspended in a minimum volume of distilled water, against which it was dialysed overnight, centrifuged to remove particulate material and lyophilised (this was known as AS fraction 1).

Solid (NH₄)SO₄ was added to 70% saturation to the supernate obtained after removal of the precipitate formed at Step 1. This was allowed to settle for 12h and treated as above (this gave AS fraction 2).

Solid (NH₄)SO₄ was added to 100% saturation to the supernate obtained after removal of the precipitate formed at Step 2. This was allowed to settle

and treated as above (this was AS fraction 3).

(c) Culture Supernates of LPS-Degrading Bacteria

(i) ASWYP (Artificial Seawater, Yeast extract, Peptone) alone or containing LPS from Meth. methylotrophus (0.01%, w/v) in 500ml flasks was inoculated with 10ml of an overnight broth culture of the organisms in ASWYP and shaken in an orbital shaker (150 r.p.m.) at 25°C until growth passed the peak of optical density as estimated at 595nm. Bacteria were removed by centrifugation at 10,000 r.p.m. in a Sorval centrifuge. Solid $(\text{NH}_4)\text{SO}_4$ was added to the supernate to 70% or 80% saturation and the precipitate formed at 18h at 4°C was collected by centrifugation at 10,000 r.p.m. for 30 min, suspended in a minimum volume of ASW (Artificial seawater) and dialysed against a large volume of ASW. If any precipitate formed after dialysis, this was removed by centrifugation.

(ii) LPS from Meth methylotrophus (0.1%, w/v) in 50ml of ASW was inoculated with 1ml of an overnight bacterial culture in the same medium and shaken in an orbital shaker (150 r.p.m., 25°C). Growth could not be monitored easily in this medium by optical density because of its initial opacity. Also, because bacterial growth was clumped and adhered to the sides of the flask, viable counts were erratic. Hence, at various times up to four days after inoculation, bacteria were removed by centrifugation and the supernate treated as above.

(d) Isolation of LPS-Degrading Bacteria.

Marine sediment samples (0-4cm from the surface) were collected from various sites on the Isle of Cumbrae, at Helensburgh and at the Dunstaffnage Marine Laboratory, Oban. Bacteria capable of LPS degradation were isolated by two similar methods :-

(i) Direct Inoculation onto Selective Medium

Sediment samples (one loopful) were inoculated into a double-layered solid medium with a basal layer of ASWYP agar and an upper layer of ASWLPS agar (Table 2). After incubation at 25°C, colonies showing zones of clearing of LPS were sub-cultured to a solid medium containing a basal layer of ASW agar and an upper layer of ASWLPS agar. After incubation, isolated colonies surrounded by zones of clearing, were selected as having LPS-degrading capability.

(ii) Enrichment, Followed by Inoculation onto Selective Medium

Sediment samples (5g) were incubated at 25°C in 20ml of ASW containing 1mg/ml LPS on an orbital shaker at 150 r.p.m. for two days. A loopful was then sub-cultured and incubated on solid medium with ASW agar base and ASWLPS agar overlay and isolated colonies selected as above.

To isolate anaerobic LPS-degrading bacteria, marine sediments were used as inocula for methods (a) and (b) above, but plates were incubated in anaerobic jars at 25°C for up to 4 days.

(e) Agar and Silica Gel, LPS-Containing Discs

(i) Molten agar (1.5%, w/v) containing 5mg/ml LPS from Meth. methylotrophus was sonicated until opaque. After autoclaving for 30 min at 5 p.s.i., 7.5ml was allowed to solidify in 4.5cm petri dishes. Using a 5mm cork borer, discs of the opaque LPS agar were aseptically cut and removed to universal containers of Robertson's Cooked Meat medium.

(ii) Silica gel discs were prepared from the medium of Thatcher and Weaver (1974) with LPS from Meth. methylotrophus (5mg or 500µg/ml) being added with sonication immediately after neutralisation of the sodium silicate solution. After sterilisation by filtration through 0.45µm filters the gel was allowed to

TABLE 2 MEDIAArtificial Sea Water Yeast Extract, Peptone (ASWYP)

| | |
|-----------------------|----------|
| Yeast Extract (Difco) | 3g |
| Peptone (Bacto) | 5g |
| ASW | 1,000 ml |

When used as solid medium, 1.5% (w/v) agar (Difco) was added.

Artificial Seawater, LPS (ASWLPS) agar

| | |
|-------------------------------------|--------|
| LPS (<u>Meth. methylotrophus</u>) | 0.4g |
| agar | 1.5g |
| ASW | 100 ml |

Autoclaved for 30 min. at 5 p.s.i.

set and the procedure was as above for agar discs.

(f) Agar Diffusion Plates, for the Detection and pH Optimisation of Lipase Activity

Pre-heated 0.1M sodium citrate/phosphate buffer was added with rapid mixing to an equal volume of 2% (w/v) molten agar containing 0.4% (w/v) tributyrin at 60°C (final solution - 0.05M sodium citrate/phosphate, 1% (w/v) agar and 0.2% (w/v) tributyrin). After brief sonication of the molten solution, 4.5ml was pipetted onto 5 x 5cm clean glass plates. Once set, 2mm wells were cut with a stainless steel cork-borer, and plugs removed by suction. By starting with 0.1M sodium citrate/phosphate buffer over a pH range from 4-10, the pH of the solidified plates could be varied. The pH of the buffer/agar mixture was measured using a pH meter with a temperature probe prior to pouring.

(g) Partially De-acylated LPS (h-LPS)

S. typhosa LPS was partially de-acylated by the method of Warren et al. (1977) :- 20mg of LPS was treated with 5ml of 0.25N NaOH for 60 min at 37°C followed by dialysis against phosphate buffered saline, final pH, 7.0.

(h) Heat-Killed Whole Bacteria

E. coli NRE 600 was grown to stationary phase in nutrient broth, washed in phosphate buffered saline and killed by heating at 80°C for 30 min.

Colorimetric Assays

All colorimetric readings were made on a Pye-Unicam SP8-100 spectrophotometer with 1cm path-length cells of glass or silica.

(a) Protein

The method of Lowry et al. (1951) was used, with bovine serum albumin

(Sigma) as standard.

(b) Carbohydrate

The total sugar content of samples was determined by the method of Dubois et al. (1956) with glucose as standard.

(c) Reducing Sugar Equivalentents

The method of Park and Johnson (1949) was used with glucose as standard. The test sample (5 or 10 μ l) was added to a tube containing 600 μ l of : distilled water - ferricyanide solution (0.05% (w/v) aqueous potassium-ferricyanide, stored in a brown bottle) - carbonate/cyanide solution (0.53% (w/v) aqueous sodium carbonate, 0.065% (w/v) potassium cyanide (1:1:1, (v/v/v))). Tubes were heated in a boiling water bath for 15 min, cooled, and 1ml of ferric iron reagent (0.15% (w/v) ferric ammonium sulphate, 0.4% sodium dodecyl sulphate in 0.05N sulphuric acid) was added. After shaking, tubes were allowed to stand for 15 min after which the resulting blue colour was read at 620nm.

(d) Nucleic Acid

LPS preparations (1mg or 250 μ g/ml) in distilled water were scanned for absorption over a range from 400nm to 200nm with a chart recorder speed of 0.2cm/sec and wavelength speed of 5nm/sec. A peak at 260nm was assumed to indicate nucleic acid contamination of LPS. Salmon testes DNA type III (Sigma) was used as standard.

(e) Phosphorus

Free phosphorus (as phosphate) was estimated by the ammonium molybdate method (Ames, 1966) with 1% ascorbic acid as the reducing agent but without prior acidic digestion of the samples. KH_2PO_4 was used as standard, and extinctions were read at 820nm (Ames, 1966).

(f) KDO

Free KDO was estimated by the method of Karkhanis et al. (1978) but without the primary sulphuric acid release step, KDO or KDO lactone (Sigma) were used as standard.

(g) Fatty Acids

Fatty acids were estimated by the Duncombe Method (1963) with myristic acid as standard. To 500 μ l of test-sample in a stoppered test-tube was added 1ml of copper reagent (9 volumes of aqueous 1M triethanolamine, 1 volume of 1N acetic acid and 10 volumes of 6.45% (w/v) $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$). After addition of 3ml of chloroform the tubes were mixed by inversion for $1\frac{1}{2}$ min. After allowing to settle for 15 min, the upper aqueous layer was removed by suction with a pasteur pipette, the last remnants were filtered from the chloroform layer with Whatman No.1 filter paper. Finally, 0.25ml of diethyldithiocarbamate reagent (0.1% (w/v) sodium diethyldithiocarbamate in n-butanol) was added to the chloroform solution and the yellow colour that developed was read at 440nm.

Detection of Degradation Products of LPS by Chromatography(a) Thin Layer Chromatography (TLC)

TLC plastic or glass sheets of silica gel 60F₂₅₄ (20 x 20cm, 0.22mm thickness, Merck) were used.

Monosaccharides

After application of test-samples (25-50 μ l) directly to TLC plates and development twice with methylethyl ketone:acetic acid:methanol (6:2:2, (v/v/v)), plates were dried and sprayed with 1% (w/v) orcinol in 50% (v/v) H_2SO_4 and heated at 100°C for 10-20 min. Authentic sugar standards were also applied to aid in identification (1 μ l of 1% (w/v) solution in distilled water. Sugars gave varying shades of brown.

Oligosaccharides

Test-samples (50-100 μ l) were applied directly to TLC plates with a wick consisting of a 20 x 20cm piece of Whatman 3MM chromatography paper folded into a 20 x 1cm pad and clamped to the top 1cm of the plate. Plates were developed for 12-18h in a freshly prepared solution of n-propanol:acetic acid:water (3:2:2, (v/v/v)). (Holmes and O'Brien, 1979). After drying, the carbohydrate-containing compounds were detected by spraying with the orcinol reagent described above.

Ethanolamine

Test-samples (50-100 μ l) were applied directly to TLC plates and developed in the solvent system described above (monosaccharides). After allowing to dry the plates were sprayed with 0.5g benzoquinone in 10ml pyridine + 40ml n-butanol. Standard ethanolamine (10 μ l of 1% (w/v) aqueous solution) was applied and gave a red spot at room temperature.

Fatty acids

After acidification of test-samples (500 μ l) by adding 50 μ l of 1N HCl, 500 μ l of diethylether was added. After whirli-mixing for 1 min and centrifuging at 4,000 r.p.m. for 1 min, 150 μ l of the supernatant ether extract was applied to TLC plates with capillary tubes. Plates were developed in a solution of petroleum ether:diethylether:acetic acid (80:20:1, (v/v/v)) in chromatography tanks lined with filter paper to saturate the atmosphere (Legakis and Papavassiliou, 1974). Plates were allowed to dry and were sprayed with 3% (w/v) cupric acetate solution in 8% (v/v) aqueous H₃PO₄ until transparent. The plates were then heated at 180^oC until fatty acids appeared as charred spots. (Fewster et al., 1969). Myristic acid and β -hydroxymyristic acid were applied (25 μ l of 1% (w/v)) in chloroform as standards.

(b) Paper Chromatography

Test-samples (25-100 μ l) were applied to Whatman No. 1 chromatography paper (21 x 56cm) and developed in the descending direction in a solvent consisting of n-butanol:pyridine:water (6:4:1, (v/v/v)) for 18h. After drying, sugars were visualised by the alkaline silver nitrate method of Trevalyan et al. (1950). Authentic sugars (1 μ l of 1% (w/v) aqueous solution) were applied and appeared as brown spots when stained.

(c) Gel Filtration Chromatography

After application of test-samples (100 μ l) to the top of a 50 x 1cm column of Biogel P4 (exclusion limit 4,000, Bio-Rad) with a flow rate of 20ml/h and a void volume of 11ml which had previously been equilibrated with 0.05M sodium acetate buffer, pH 7.0, fractions (1ml) were collected and assayed for total sugar by the method of Dubois et al. (1956) (Cortez, 1976).

Detection of Lipolytic Activities of Crude Extracts from Marine Invertebrates

Homogenate supernates of digestive gland and crystalline style, were incubated with LPS, partially de-acylated LPS (h-LPS, prepared as described above) or whole heat-killed bacteria, after which the free fatty acid content was estimated colorimetrically.

Detection and pH Optimisation of Lipase Activity

Lyophilised enzyme preparations (5 μ l of 10mg/ml in distilled water) were applied to wells cut in tributyrin agar diffusion plates and incubated at 25°C for 18h in a humid atmosphere, followed by measurement of the resultant zones of clearance, due to lypolysis, with the aid of a plate microscope.

Degradation of LPS by AS precipitated Aqueous Extracts from Marine Invertebrates.

AS fractions were incubated with LPS and subsequently assayed for degradation of the sugar component (manifested by an increase in reducing sugar

equivalents), or free phosphorus and free KDO by the respective colorimetical assays. Liberated monosaccharides were analysed by TLC and paper chromatography. Oligosaccharides were sought by both TLC and gel filtration chromatography. Free ethanolamine was tested for TLC.

In experiments to detect release of free fatty acids, the AS fraction from each marine invertebrate with strongest lipase activity against tributyrin was incubated with LPS at the pH of optimum activity, and liberated free-fatty acids estimated colorimetrically and by TLC.

Degradation of Substrates other than LPS, by LPS-Degrading Bacteria

Bacterial isolates were inoculated onto double-layered solid media in which the base was ASWYP agar or ASW agar (Table 2) with substrate (see below) suspended in the upper layer. All incubations were at 25°C, for up to four days when degradation was read.

The substrates were :

Lipids

Tributyrin and tripalmitin (1%,w/v) which had been emulsified by sonication. Lipolytic bacteria produced a clear zone around regions of growth.

DNA

DNA from salmon testes (1%,w/v) was mixed in molten agar by brief sonication with a sonic probe. After inoculation and incubation, DNA degradation was detected by flooding the plates with 0.5N HCl which precipitated undegraded DNA, producing clear zones around degradative colonies.

Protein

Casein (Difco) (1%,w/v) emulsified by sonication. Proteolytic bacteria produced a clear zone around areas of growth.

Starch

Soluble starch (Difco) (1%,w/v) was mixed by shaking. After incubation plates were flooded with Grams Iodine, staining the medium blue/black except for clear zones around colonies where there had been complete hydrolysis of starch, or reddish-brown zones indicating partial hydrolysis.

Anaerobic Degradation of LPS

Universal containers containing Robertson's Cooked Meat medium and agar discs with incorporated LPS were inoculated with marine sediments collected from the Isle of Cumbrae (Table 8) and incubated at 25°C. Discs were removed at various times up to 7 days and inspected for clearing of opacity. Silica gel discs were treated similarly, but LPS was visualised by the silver stain of Tsai and Frasch (1982) which stained LPS-containing discs brown, while discs without LPS remained almost colourless.

RESULTS

Section 1-Degradation of LPSs by Marine Invertebrates

Degradation of Polysaccharide Moieties

Detection

In order to test whether filter-feeding marine bivalves were capable of enzymatically breaking down the polysaccharide moieties of LPSs to monosaccharides, which might then act as a source of nutrition, it was decided to test their digestive gland as the most likely source of such enzymes. In a previous study by Saddler *et al.* (1979b) using another mollusc, the snail Helix pomatia, no evidence for polysaccharide breakdown was detected. Nevertheless, in the present studies, snail gut juice was used as a positive control owing to its well documented broad spectrum of carbohydrase activities (Holden and Tracey, 1950).

Crude extracts from the digestive glands of five species of bivalves were prepared as in Table 3 but prior to AS precipitation, were tested for their ability to degrade LPSs from different bacteria. However, these extracts themselves contained very large amounts of carbohydrate which caused undesirably high background values in the assays for total carbohydrate and reducing sugars and the appearance of very heavy sugar spots on analysis by descending paper chromatography. This presented a problem in the detection of LPS-derived monosaccharides. However, despite this high level of background sugar, the results suggested that LPS was degraded by these extracts to the monosaccharide level. This could have been more unequivocally demonstrated if radioactively labelled LPS had been available.

It was clearly necessary to remove the contaminating sugars from the experimental system. Therefore, the crude extracts of digestive glands were subjected to AS precipitation (Table 3) which successfully removed almost all of the low molecular weight sugars, although there was still some high molecular weight carbohydrate which co-precipitated with the proteins. This

TABLE 3

Yield of high molecular weight material precipitated by AS from the digestive gland homogenates of 5 species of marine invertebrates.

| marine invertebrate | wet weight of digestive gland tissue (g) | Dry weight of material precipitated by AS (mg) | | | TOTAL |
|---------------------|--|--|--------------------------------|---------------------------------|-------|
| | | 0-50% saturation (fraction 1) | 50-70% saturation (fraction 2) | 70-100% saturation (fraction 3) | |
| <u>Mya</u> | 10.1 | 32 | 60 | 27.5 | 119.5 |
| <u>Modiolus</u> | 11.3 | 25 | 40 | 39.5 | 104.5 |
| <u>Cerastoderma</u> | 5.0 | 33 | 21.5 | 3.2 | 57.7 |
| <u>Mytilus</u> | 4.9 | 40 | 15.5 | 4.1 | 59.6 |
| <u>Pecten</u> | 33.4 | 486 | 236.5 | 176 | 898.5 |

(The digestive glands of 10 specimens of each species were pooled. The figures here represent one batch of marine invertebrates. Four such batches were used during the course of this work).

polysaccharide contamination had low reducing sugar equivalent per mg, and no monosaccharides could be detected by descending paper chromatography and thus was not a major problem to further experiments.

The three AS fractions were labelled fraction 1 (0-50% AS saturation), fraction 2 (50-70% AS saturation) and fraction 3 (70-100% AS saturation). When they were incubated (1mg/ml lyophilised material in 0.05M sodium acetate buffer pH 4.0) with LPSs (from E. coli O111:B4, S. minnesota, Meth. methylo-trophus, 4mg/ml), it was found that fractions 1 and 2 from all five bivalves produced heavy spots corresponding to glucose when the digests were analysed by descending paper chromatography (Figure 9). This was found also, when papers were rechromatographed after the initial solvent development. Fraction 3 from all bivalves however, produced either a very faint spot or no spot at all. Enzyme/LPS mixtures, enzymes alone and LPSs alone analysed prior to incubation produced no spots, although Modiolus fractions 1 and 2 produced faint spots corresponding to glucose after the enzyme extracts alone had been incubated and subsequently analysed. No other enzyme extracts or LPSs alone produced any spots on analysis following incubation. There was thus good evidence that fractions 1 and 2 from all bivalves had the ability to release monosaccharide from LPS after incubation.

Since the principle of the staining method used to develop the chromatograms is based on the reaction of silver nitrate with reducing sugars, it was possible that non-reducing sugars were also being liberated from LPS by the extracts but were not being detected. However, it was noted, with authentic reducing and non-reducing sugars on the chromatograms, that whereas the former were the first to appear, the latter developed slightly more slowly but attained the same final intensity as reducing sugars. Thus fractions 1 and 2 from all five bivalves released glucose from all three LPSs tested and it was unlikely that non-reducing sugars were undetected.

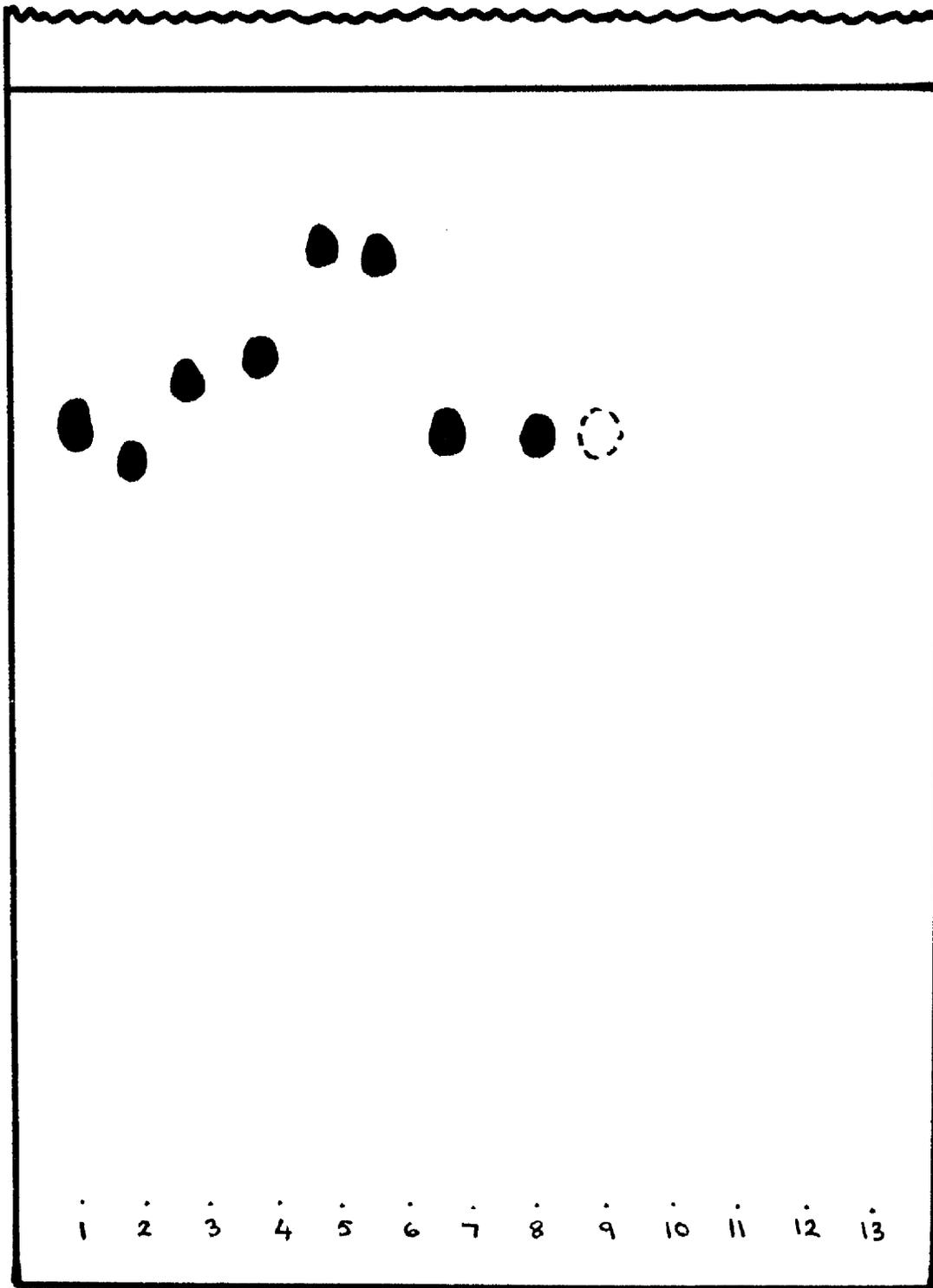
Figure 9 : Schematic representation of paper chromatogram of monosaccharides released from LPSs by marine invertebrate AS extracts

Key :

- | | |
|--------------------------|--|
| 1. glucose | 7. marine invertebrate fraction 1 + LPS |
| 2. galactose | 8. marine invertebrate fraction 2 + LPS |
| 3. mannose | 9. marine invertebrate fraction 3 + LPS |
| 4. fructose | 10. marine invertebrate fraction 1 alone |
| 5. N-acetylglucosamine | 11. marine invertebrate fraction 2 alone |
| 6. N-acetylgalactosamine | |
| | 12. marine invertebrate fraction 3 alone |
| | 13. LPS alone |

enzyme/LPS mixtures were incubated at 25°C for 24h and 30µl were applied to chromatograms.

LPS (4mg/ml (w/v)) was from S. minnesota, E. coli O111:B4 or Meth. methylotrophus.



Once it had been established that attack on LPS was taking place, quantitation was based on the methods used to study LPS depolymerisation by bacteriophage (Reiger-Hug et al., 1976). Tables 4-6 show that all bivalve fractions tested and H. pomatia gut juice had readily detectable ability to release reducing sugar equivalents from LPS. It can be seen that LPSs had very low reducing power prior to incubation with the extracts, which is consistent with their high molecular weights.

Comparing enzyme/LPS mixtures, enzymes alone and LPSs alone assayed prior to and after incubation, apart from the differences indicated above in enzyme/LPS mixtures, enzymes increased in reducing sugar content to some extent upon incubation. Most notably, Modiolus fraction 1 and especially fraction 2 produced a large increase in reducing power. LPS preparations alone did not increase in reducing power on incubation.

Comparing the relative degradation of each LPS, Figure 1\ shows that when net release of low molecular weight sugars (reducing sugar equivalents) are compared, S. minnesota LPS was degraded most by all enzyme preparations tested. Apart from Modiolus and Mytilus the other enzyme extracts degraded Meth. methylotrophus LPS second in rank. E. coli O111:B4 showed the least evidence of degradation by the enzyme extracts apart from those exceptions mentioned above. This was in good correlation with the relative sugar composition of each LPS (Table 7).

Comparing the ability of each molluscan enzyme extract to degrade all three LPSs, it can be seen that, in decreasing order Mya > Cerastoderma > Mytilus > Pecten > Modiolus. However, there were exceptions, e.g. Pecten degraded Meth. methylotrophus LPS more than did Mytilus, but degraded S. minnesota LPS and E. coli O111:B4 LPS to an equal or less degree than did Mytilus. H. pomatia was second only to Mya in degradation of LPS.

When each fraction was analysed for protein content, it was found that

TABLE 4

Formation of reducing sugar equivalents from E. coli 0111 : B4 LPS
by molluscan extracts

| Mollusc | AS fraction | μg reducing sugar equivalents/ml | |
|----------------------|-------------|---|--------------|
| | | enzyme + LPS | enzyme alone |
| <u>Mya</u> | 1 | 380 | 90 |
| | 2 | 390 | 80 |
| <u>Modiolus</u> | 1 | 150 | 75 |
| | 2 | 365 | 265 |
| <u>Cerastoderma</u> | 1 | 300 | 75 |
| | 2 | 360 | 80 |
| <u>Mytilus</u> | 1 | 340 | 100 |
| | 2 | 270 | 100 |
| <u>Pecten</u> | 1 | 265 | 100 |
| | 2 | 230 | 80 |
| <u>Helix pomatia</u> | gut juice | 385 | 170 |

after incubation at 25°C for 24h, 10 μl assayed. LPS alone contained 40 μg reducing sugar equivalent/ml. The figures in the table are the average of three individual experiments carried out in duplicate and represent the amount of reducing sugar equivalent/ml after incubation, after subtraction of the readings given prior to incubation.

TABLE 5

Formation of reducing sugar equivalents from Meth. methylotrophus

LPS by molluscan extracts .

| Mollusc | AS fraction | µg reducing sugar equivalents/ml | |
|----------------------|----------------|----------------------------------|-----------------|
| | | enzyme + LPS | enzyme alone |
| <u>Mya</u> | 2 | 480 | 80 |
| <u>Modiolus</u> | 2 | 330 | 265 |
| <u>Cerastoderma</u> | 2 | 380 | 80 |
| <u>Mytilus</u> | 1 | 265 | 100 |
| <u>Pecten</u> | 1 | 365 | 100 |
| <u>Helix Pomatia</u> | gut juice | 505 | 170 |

after incubation at 25°C for 24h, 10 µl assayed. LPS alone contained 30 µg reducing sugar equivalents/ml. The figures in the table are the average of three individual experiments carried out in duplicate and represent the amount of reducing sugar equivalents/ml after incubation, after subtraction of the readings given prior to incubation.

TABLE 6

Formation of reducing sugar equivalents from S. minnesota LPS by molluscan extracts.

| µg reducing sugar equivalents/ml | | | |
|----------------------------------|-------------|--------------|--------------|
| Mollusc | AS fraction | enzyme + LPS | enzyme alone |
| <u>Mya</u> | 1 | 585 | 90 |
| | 2 | 615 | 80 |
| <u>Modiolus</u> | 1 | 325 | 75 |
| | 2 | 490 | 265 |
| <u>Cerastoderma</u> | 1 | 535 | 75 |
| | 2 | 475 | 80 |
| <u>Mytilus</u> | 1 | 425 | 100 |
| | 2 | 520 | 100 |
| <u>Pecten</u> | 1 | 460 | 100 |
| | 2 | 485 | 80 |
| <u>Helix pomatia</u> | gut juice | 675 | 170 |

after incubation at 25°C for 24h, 10 µl assayed. LPS alone contained 35 µg reducing sugar equivalents/ml. The figures in the table are the average of three individual experiments carried out in duplicate and represent the amount of reducing sugar equivalents/ml after incubation, after subtraction of the readings given prior to incubation.

TABLE 7 Comparison of total sugar content of LPSs

| lipopolysaccharide | % sugar (w/w) as glucose) |
|----------------------------------|------------------------------|
| <u>E. coli</u> O111:B4 | 34 |
| <u>Meth. methylotrophus</u> | 61 |
| <u>S. minnesota</u> [*] | 100 |

estimated by the method of Dubois et al. (1956)

* this LPS contains lipid A, but due to the presence of some sugars which react more strongly than the glucose used as standard, it has an apparent higher content of sugar.

whereas some fractions consisted of ~90% protein (e.g. Cerastoderma fraction 1) others consisted of < 50% protein (e.g. Cerastoderma fraction 2). Comparing relative degradative potencies of each molluscan extract per mg of protein (Figure 10) it can be seen that at least one fraction from each marine invertebrate was more potent than H. pomatia with the exception of Modiolus. Pecten, which was considered least active when comparison was made on the basis of activity/per mg of lyophilised material (Figure 11), was among the most degradative when activity is expressed per mg of protein.

Identification of Released Products

Since the results (above) suggested that rather extensive degradation of all three LPSs was taking place, it was surprising to find that glucose was apparently the only monosaccharide product. As can be seen from Figures 2 and 3, in S. minnesota LPS glucose is present only in the main core region, in E. coli O111:B4 LPS it is present in the main chain of both the O-chain and the core region (Figures 2, 4(R3)). Hence it was expected that some other main-chain monosaccharides should be released along with glucose. Preliminary experiments with TLC to detect monosaccharide products in comparison with descending paper chromatography, showed that this method was more sensitive than the latter, since glucose spots could be detected by TLC using 10% of the sample required for detection by paper chromatography.

Fractions 1 and 2 were incubated as previously, with each LPS and 30µl of each sample was analysed by TLC. Figure 12a shows the pattern obtained when representative fractions were incubated with S. minnesota LPS. Both fractions from each bivalve gave similar spot patterns. In the photograph shown, some bands that were visible on the TLC plate to the naked eye are not visible. Since in other experiments these were clearly visible, a schematic interpretation was constructed of spots that were routinely found in over five individual

Figure 10 : Comparison of net formation of reducing sugar
equivalents from LPS by molluscan extracts per
mg of protein

Key :

-  E. coli O111:B4 LPS
-  Meth. methylotrophus LPS
-  S. minnesota LPS

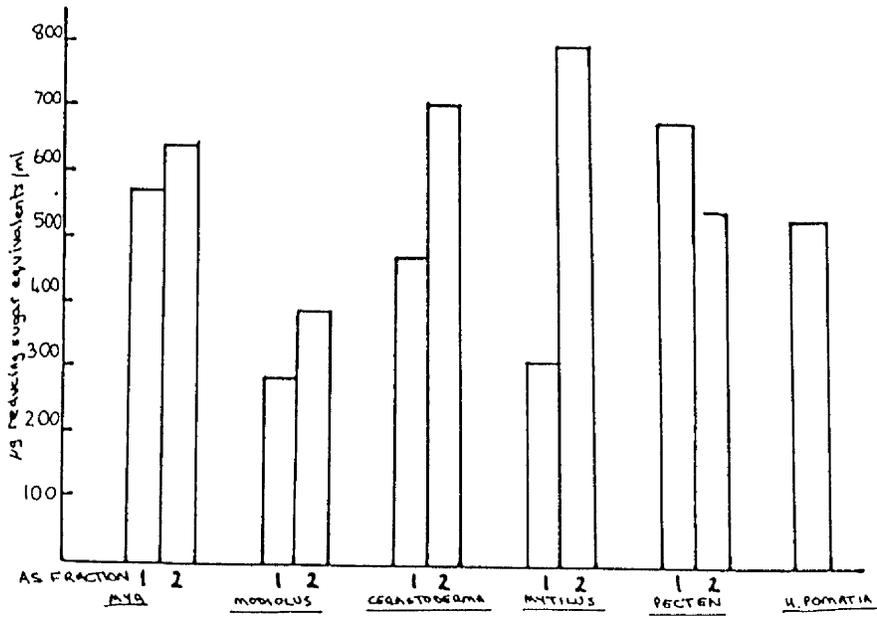
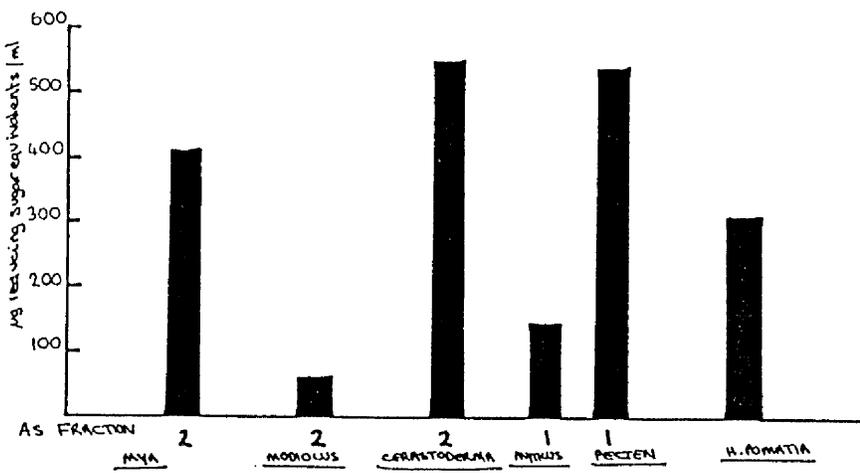
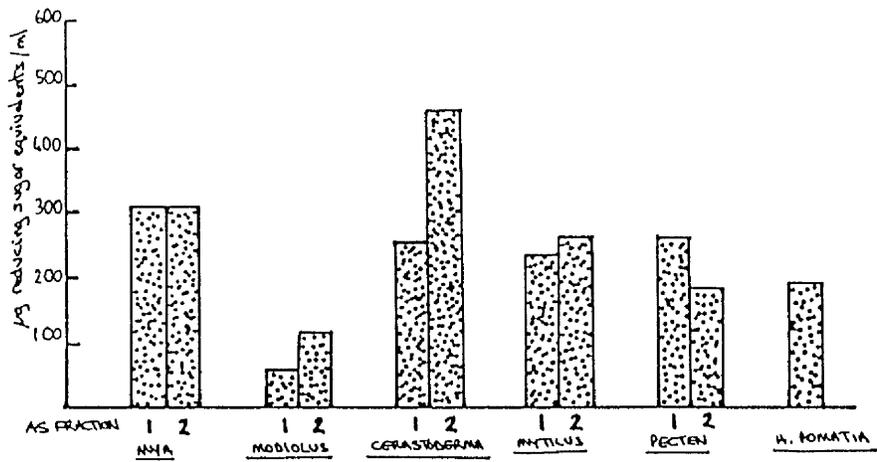


Figure 11 : Comparison of net formation of reducing sugar
equivalents from LPS by molluscan extracts per
mg of lyophilised material

Key :

- S. minnesota LPS
- E. coli O111:B4 LPS
- Meth. methylotrophus LPS

Figure 12 : TLC analysis of monosaccharide products of S. minnesota LPS
degradation by marine invertebrate extract fractions

A - photograph of TLC plate from an individual experiment

B - schematic diagram, showing spots obtained consistently
over several experiments.

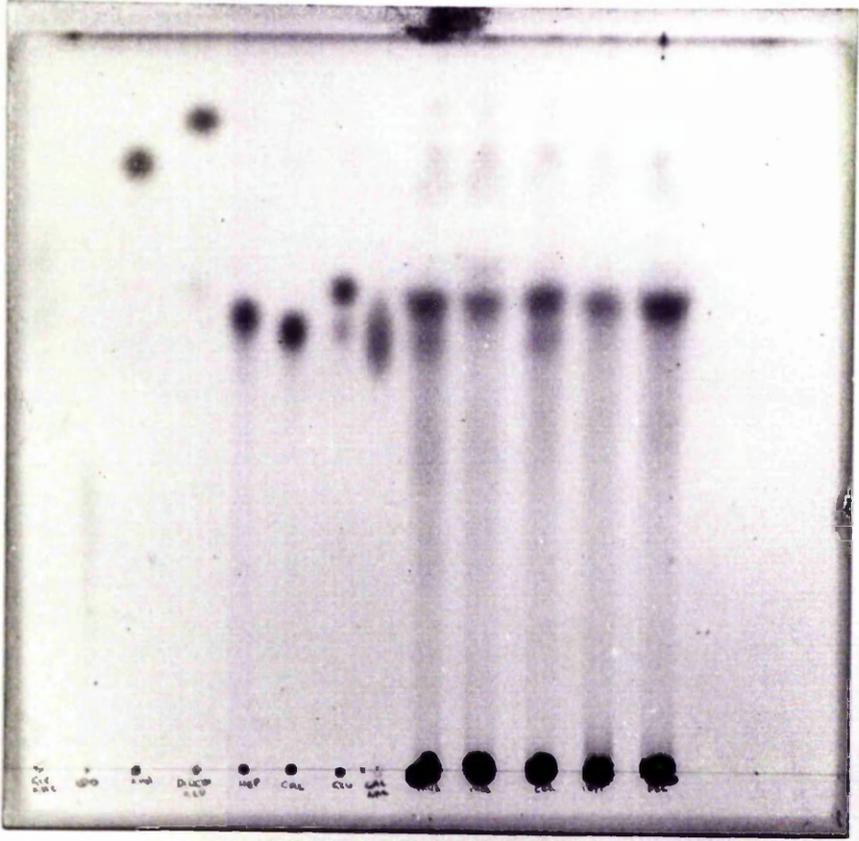
Key :

A + B

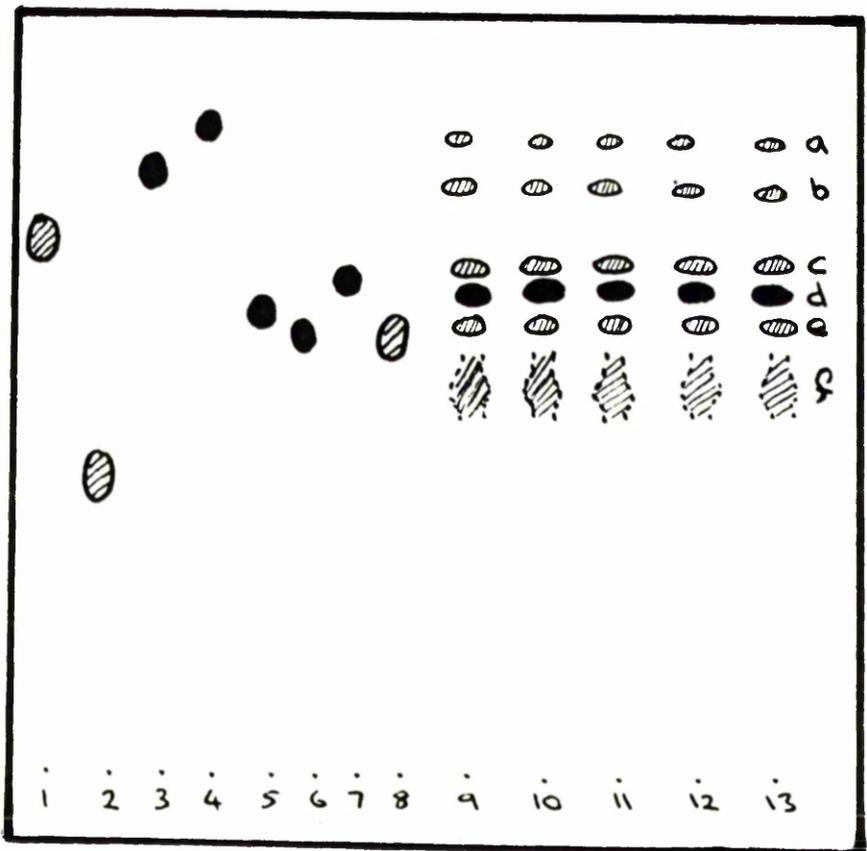
1. N-acetylglucosamine
2. KDO
3. rhamnose
4. dideoxyglucose
5. mannoheptulose
6. galactose
7. glucose
8. N-acetylgalactosamine
9. Mya fraction 2 incubated with LPS
10. Modiolus fraction 2 incubated with LPS
11. Cerastoderma fraction 2 incubated with LPS
12. Mytilus fraction 1 incubated with LPS
13. Pecten fraction 1 incubated with LPS

C

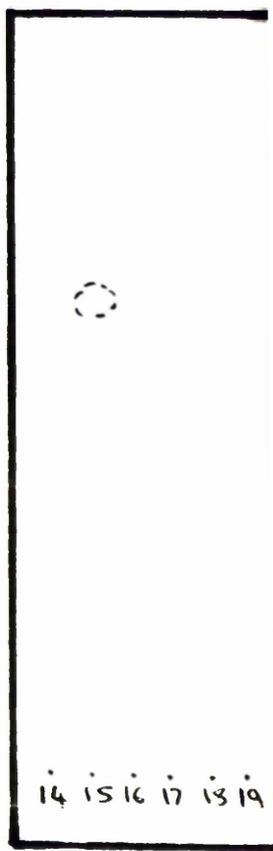
14. Mya fraction 2 incubated alone
15. Modiolus fraction 2 " "
16. Cerastoderma fraction 2 "
17. Mytilus fraction 1 "
18. Pecten fraction 1 "
19. S. minnesota LPS "



B



C



experiments (Figure 12b).

Spot d was always the most intense spot and had a migration position identical to glucose. Spot c had a similar migration position and staining appearance to N-acetylglucosamine. Spot e migrated similarly to both galactose and N-acetylgalactosamine. The very diffuse spot, f, could not be identified even when compared to sugars not known to be present in this LPS (Figures 2,3). Similarly spots a and b were always found but did not correspond to any monosaccharides known to be present in S. minnesota LPS. However, by comparing with several monosaccharides it was found that they migrated along with dideoxyglucose and rhamnose respectively. Although not shown here, it was found that H. pomatia gut juice produced bands b, d and f only, when incubated with this LPS.

Figure 13 shows that when extracts were incubated with E. coli O111:B4 LPS, glucose was the major monosaccharide product (identified as spot c) N-acetylglucosamine was identified as spot b. Unfortunately, no authentic colitose (3,6-dideoxygalactose) standard was available in order to detect its release from this LPS, of which it is a major component (Figure (Figure 2,4(R3))). However it would have been expected to travel just behind dideoxyglucose. The monosaccharide product with most similar migratory pattern (spot a) consistently migrated with rhamnose. H. pomatia released spots a and c only.

Although Meth. methylotrophus LPS contains several deoxysugars (Collins, 1984), Figure 14 shows that none were detected after incubation of extracts with LPS. Glucose (spot a) was again the major product detected. Spots b and c could not be identified. It was noted that the only difference between the sugars released by fractions 1 and 2 from any bivalve from any particular LPS, were spots b and c from Meth. methylotrophus LPS : from each bivalve, one fraction produced b and c, the other produced b or c. H. pomatia was not tested against this LPS.

All enzyme/LPS mixtures, enzymes alone and LPS alone produced no spots

Figure 13 : TLC analysis of monosaccharide products of E. coli O111:B4
LPS degradation by marine invertebrate extract fractions

A - photograph of TLC plate from an individual experiment

B - schematic diagram, showing spots obtained consistently over
several experiments

Key :

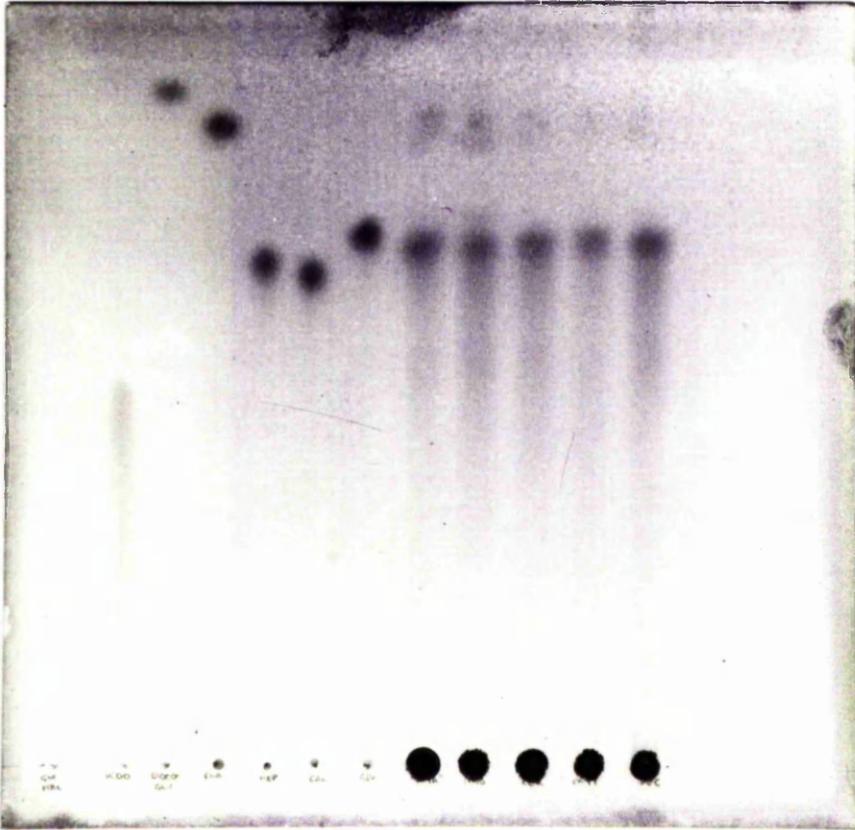
A + B

1. N-acetylglucosamine
2. KDO
3. dideoxyglucose
4. rhamnose
5. mannoheptulose
6. galactose
7. glucose
8. Mya fraction 2 incubated with LPS
9. Modiolus fraction 2 incubated with LPS
10. Cerastoderma fraction 2 incubated with LPS
11. Mytilus fraction 1 incubated with LPS
12. Pecten fraction 1 incubated with LPS

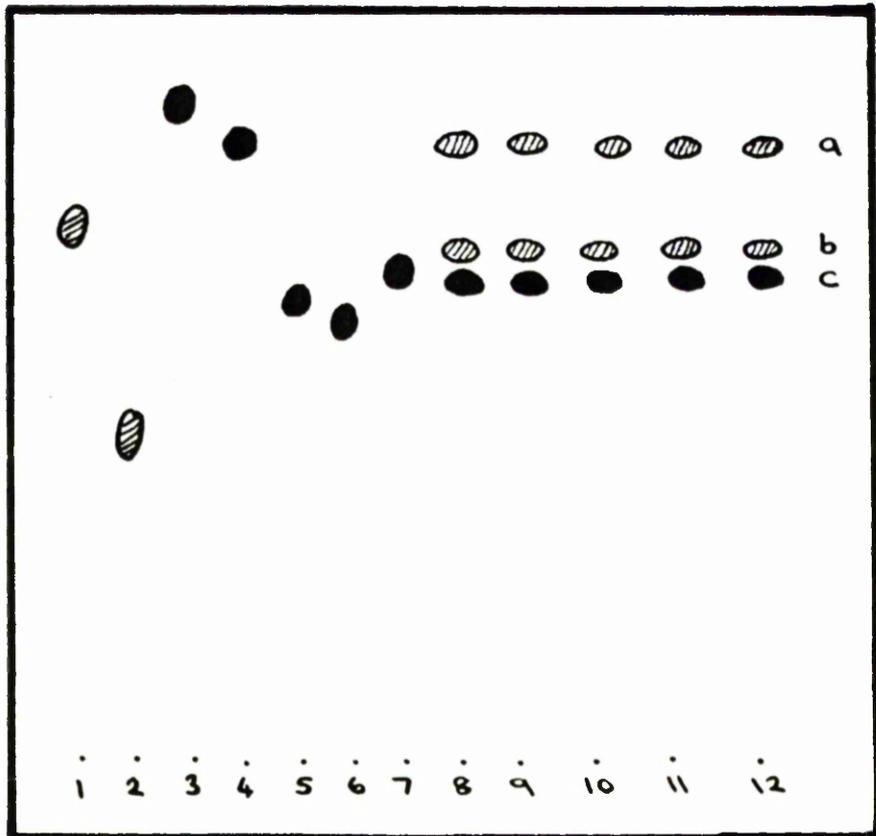
C

13. Mya fraction 2 incubated alone
14. Modiolus fraction 2 " "
15. Cerastoderma fraction 2 " "
16. Mytilus fraction 1 " "
17. Pecten fraction 1 " "
18. E. coli O111:B4 LPS " "

A



B



C

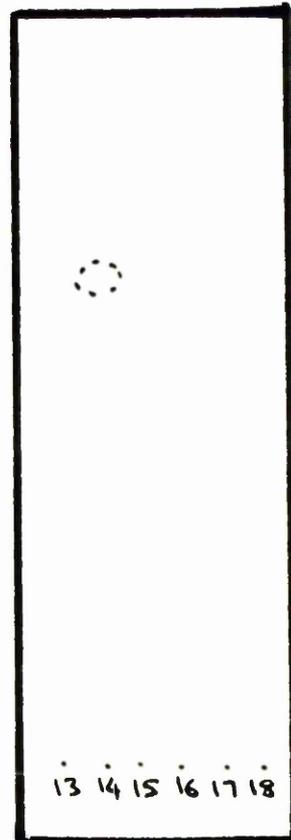


Figure 14 : TLC analysis of monosaccharide products of Meth. methylotrophu
LPS degradation by marine invertebrate extract fractions

A - photograph of TLC plate from an individual experiment

B - schematic diagram, showing spots obtained consistently,
over several experiments.

Key :

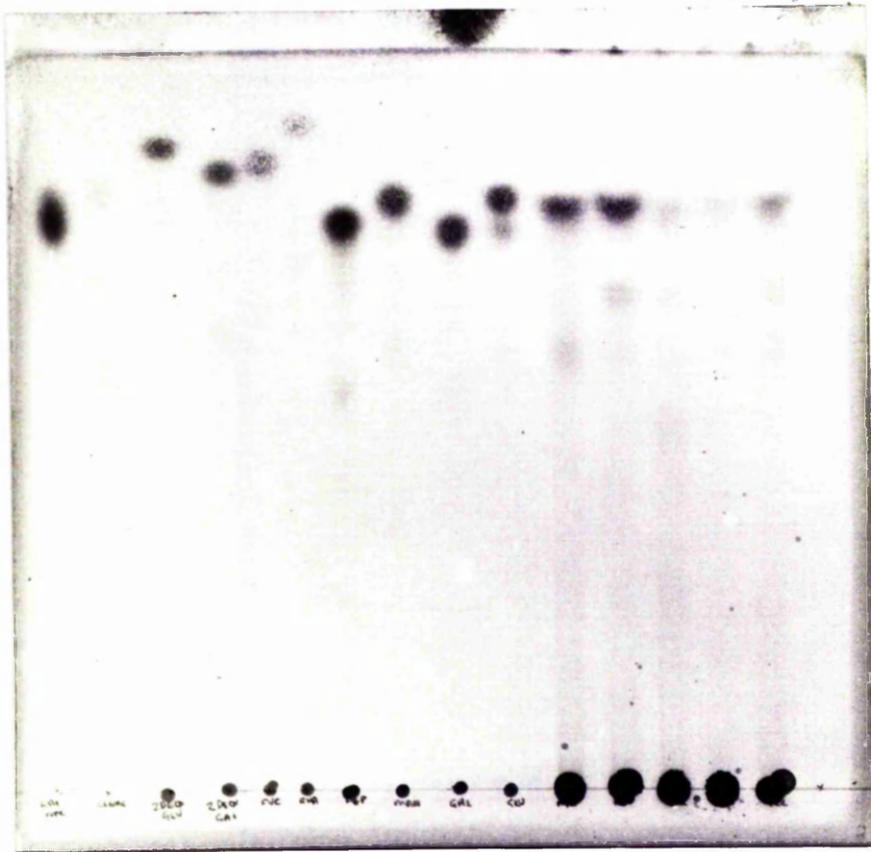
A + B

1. N-acetylgalactosamine
2. N-acetylglucosamine
3. 2-deoxyglucose
4. 2-deoxygalactose
5. fucose
6. rhamnose
7. mannoheptulose
8. mannose
9. galactose
10. glucose
11. Mya fraction 2 incubated with LPS
12. Modiolus fraction 2 incubated with LPS
13. Cerastoderma fraction 2 incubated with LPS
14. Mytilus fraction 1 incubated with LPS
15. Pecten fraction 1 incubated with LPS

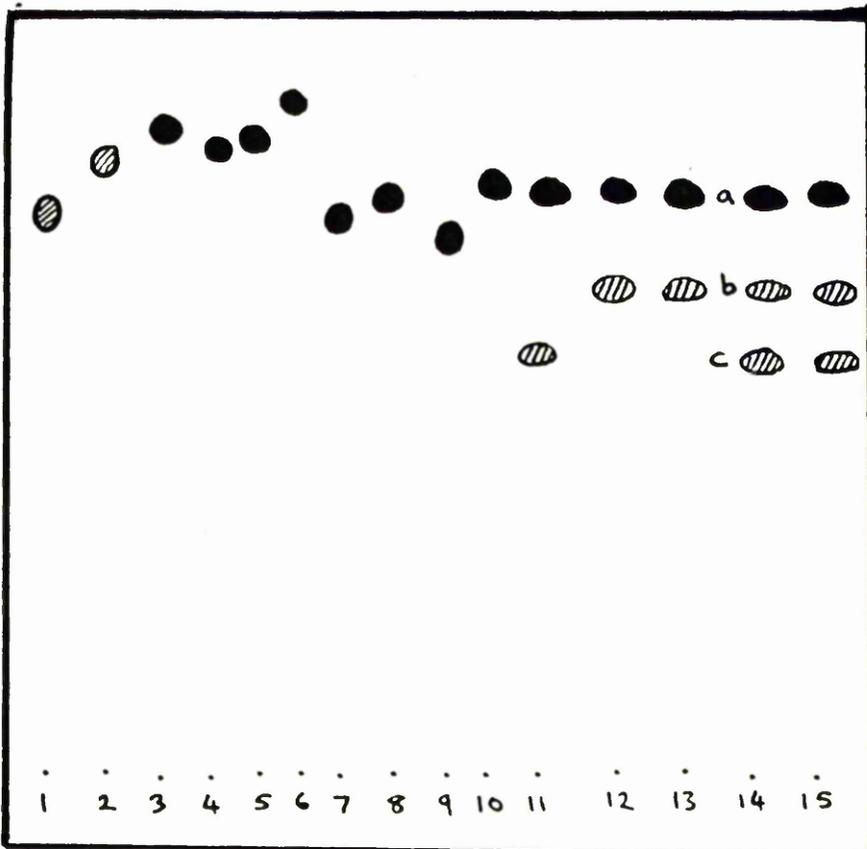
C

16. Mya fraction 2 incubated alone
17. Modiolus fraction 2 " "
18. Cerastoderma fraction 2 "
19. Mytilus fraction 1 "
20. Pecten fraction 1 "
21. Meth. methylotrophus LPS "

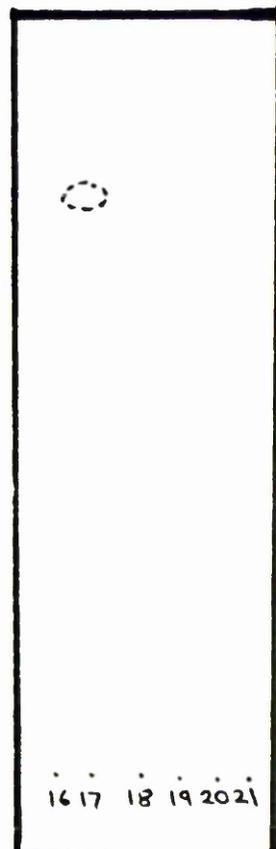
A



B



C



when analysed prior to incubation. However, Modiolus fractions 1 < 2, as expected produced spots corresponding to glucose, when analysed after incubation.

Relative Release of Glucose

Since there was a relationship between the amount of reducing sugars released from LPSs and their total sugar content (S. minnesota > Meth.methylotrophus > E. coli O111:B4) (Figures 10, 11), and the major monosaccharide product was glucose, it was expected that when examined by TLC, the intensity of glucose spots should follow this trend. However when the amount of sample applied was decreased (10µl) so that only glucose spots were visible after staining the plates, the most intensely staining glucose spots were formed in the order :- S. minnesota > E. coli O111:B4 > Meth. methylotrophus (Figure 15). This suggests that unidentified spots b and c (Figure 14) from degraded Meth. methylotrophus (or other sugars that escaped detection by TLC) contributed significantly to the reducing power of the LPS hydrolysis products. Conversely, in S. minnesota and E. coli O111:B4 LPS enzyme hydrolysates, glucose may have been the major contributor to the reducing power.

Despite finding evidence for monosaccharides other than glucose being released some particular monosaccharides were also expected to be detected, especially galactose which is a major constituent of all LPSs tested. Some fractions were tested for the ability to convert galactose to glucose, to test whether this could explain the ubiquity of glucose in TLC analyses. After incubation of galactose with extracts it was found that in all experiments, subsequent TLC produced a major galactose spot and a fainter spot corresponding to glucose. However, the results were inconclusive and the commercially supplied sugars were of dubious purity, since in occasional experiments, galactose alone produced this double-spot profile. It is possible that, supplied with appropriate co-factors, the enzyme extracts may have this ability.

Figure 15 : TLC estimation of the relative release of glucose from LPS
by marine invertebrate extract fractions

A - photograph of TLC plate from an individual experiment

B - schematic diagram, showing spots obtained consistently
over several experiments

Key :

A + B

1. Modiolus fraction 2 incubated with E. coli O111:B4 LPS
2. " " " " S. minnesota LPS
3. " " " " Meth. methylotrophus LPS
4. Cerastoderma fraction 2 " " E. coli O111:B4 LPS
5. " " " " S. minnesota LPS
6. " " " " Meth.methylotrophus LPS

C

7. Modiolus fraction 2 incubated alone
8. Cerastoderma fraction 2 " "
9. E. coli O111:B4 LPS " "
10. S. minnesota LPS " "
11. Meth. methylotrophus LPS " "

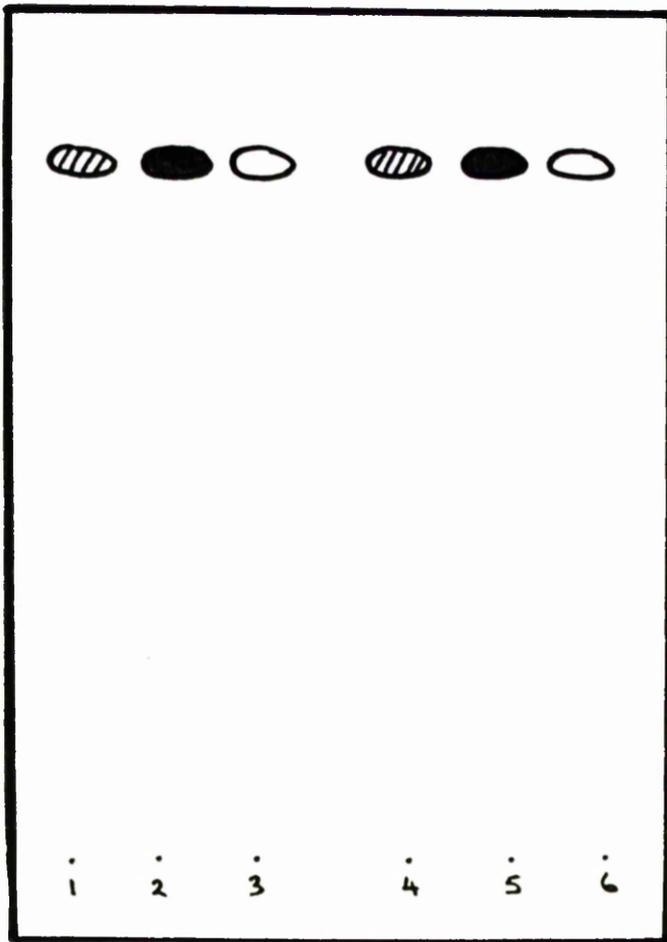
10µl of sample applied

shading indicates intensity of spot.

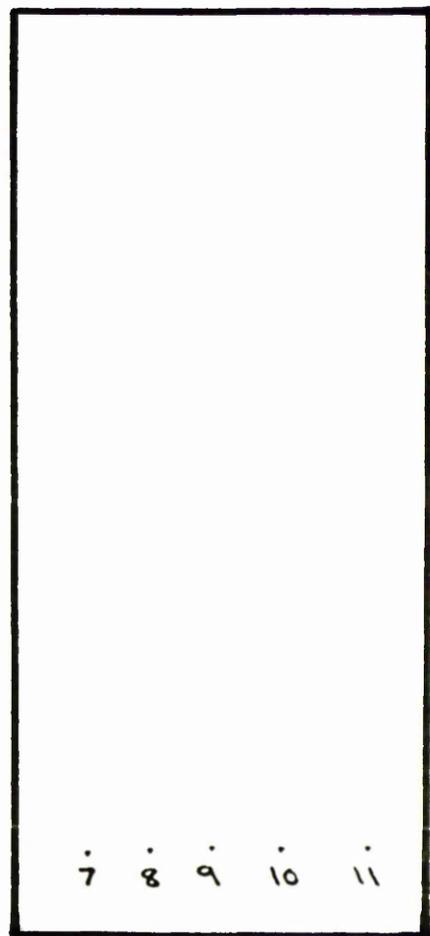
A



B



C



pH Optimisation

Other investigators have suggested that the pH optima of marine invertebrate glycolytic enzymes are in the acidic range. In order to define the pH optima of the bivalve glycolytic activity detected (above), one fraction from each was chosen on the basis of strongest previously determined activity and also availability. Enzymes were incubated with the three previously tested LPSs in 0.05M sodium acetate buffer over a range from pH 4.0 to pH 5.5, following which reducing sugar equivalents were estimated.

It is evident from Figure 16 that whereas all previous experiments had been arbitrarily incubated at pH 4.0, the pH optimum for each fraction tested did not have much greater activity. However, Modiolus, which had the least active fractions at pH 4.0, was found to be > 50% more active at pH 5.0, at least against E. coli O111:B4 and Meth. methylotrophus LPSs. S. minnesota LPS was degraded more efficiently at pH 4.5. Apart from Modiolus, all other fractions had pH optima between pH 4.0 and pH 4.5, for all three LPSs. Cerastoderma activity rose at pH 5.5, however unfortunately, activities were not tested beyond pH 5.5, hence it is not known whether the actual optimum activity for Cerastoderma is found at pH values > 5.5.

Rate

All previous experiments had been incubated for 24h. Hence it was decided to determine whether this length of incubation was needed for this level of degradation. Since Mya fraction 2 produced most sugar release (per mg of lyophilised material) it was used to determine the rapidity of degradation of S. minnesota LPS (which was degraded most). Degradation was determined as previously, but incubation was in pH 4.5 buffer with 5µl aliquots being removed at various times, and the reaction stopped by the 15 min boiling step in the reducing sugar assay.

Figure 16 : Effect of pH on the formation of reducing sugar
equivalents from LPS by marine invertebrate extract
fractions

Key :

△-----△ E. coli O111:B4 LPS
●-----● Meth. methylotrophus LPS
⊙-----⊙ S. minnesota LPS

- A Modiolus fraction 2
- B Cerastoderma fraction 2
- C Mytilus fraction 1
- D Pecten fraction 1
- E Mya fraction 2

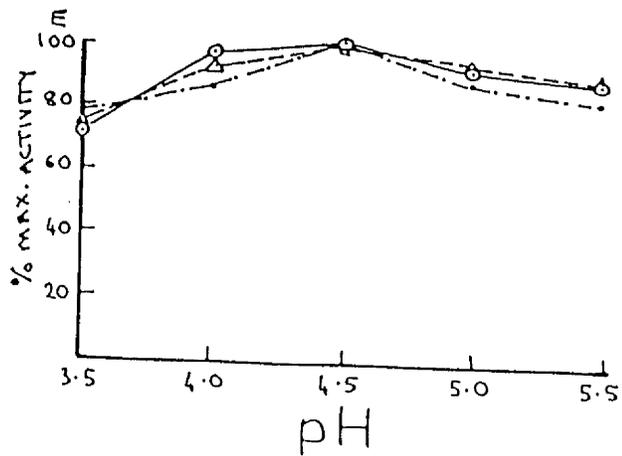
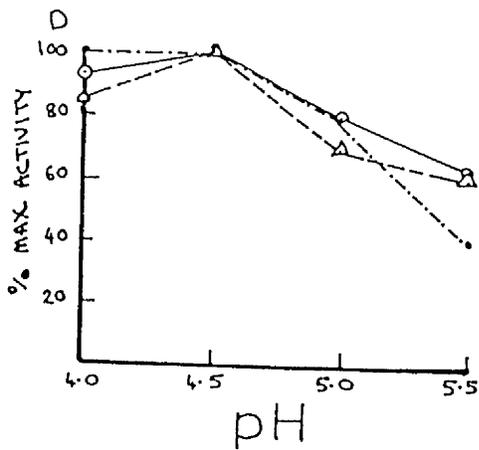
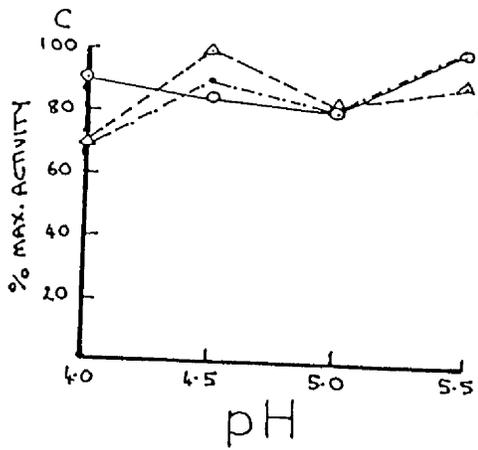
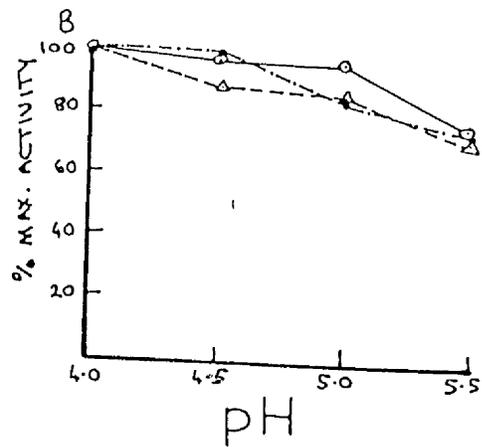
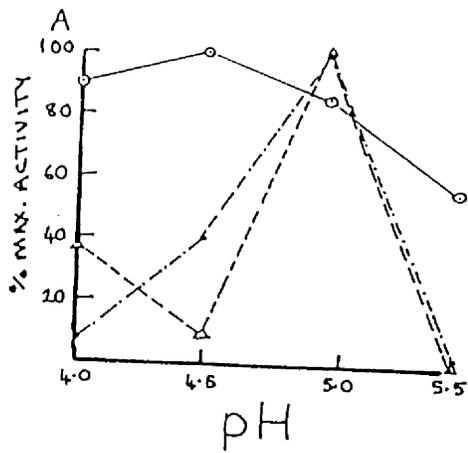


Figure 17 shows that degradation was relatively more rapid than expected; 80% of the activity expressed after 24h could be detected after 3h and 50% took place in 1h. Prolonged incubation (beyond 24h) would not bring about total breakdown of the polysaccharide to monomers, since although 600µg/ml reducing sugar equivalents were released, LPS was present as 4mg/ml.

Release of Oligosaccharides

LPS derived oligosaccharides may have potential application in vaccine production against Gram-negative bacterial infections (Svenson and Lindberg, 1983). With this in mind and in order to detect LPS degradation products that were previously undetected (above), it was decided to test bivalve enzyme extracts for the ability to release oligosaccharides from LPS.

Both fractions 1 and 2 from all five bivalves, along with H. pomatia gut juice were incubated with LPS from S. minnesota, E. coli O111:B4 and Meth. methylotrophus in conditions as previously described. After TLC analysis (up to 100µl applied) by a method which separates oligosaccharides with between 4 and 10 monosaccharide units, it was found that only Modiolus fraction 1 produced an oligosaccharide spot, which migrated along with the tetrasaccharide, stachyose (Figure 18). A tetrasaccharide product was identified from all three LPSs, perhaps suggesting that different endoglycosidases were responsible for each product, since each LPS has a distinctive sequence of monosaccharides.

As before, the relevant controls were made and all were negative, including Modiolus fraction 1 incubated alone, which had previously been found to release monosaccharide.

H. pomatia produced very faint 'tetrasaccharide' spots when incubated with S. minnesota or E. coli O111:B4 LPS but was not tested against Meth. methylotrophus LPS.

Figure 17 : Rate of formation of reducing sugar equivalents
from S. minnesota LPS by Mya fraction 2

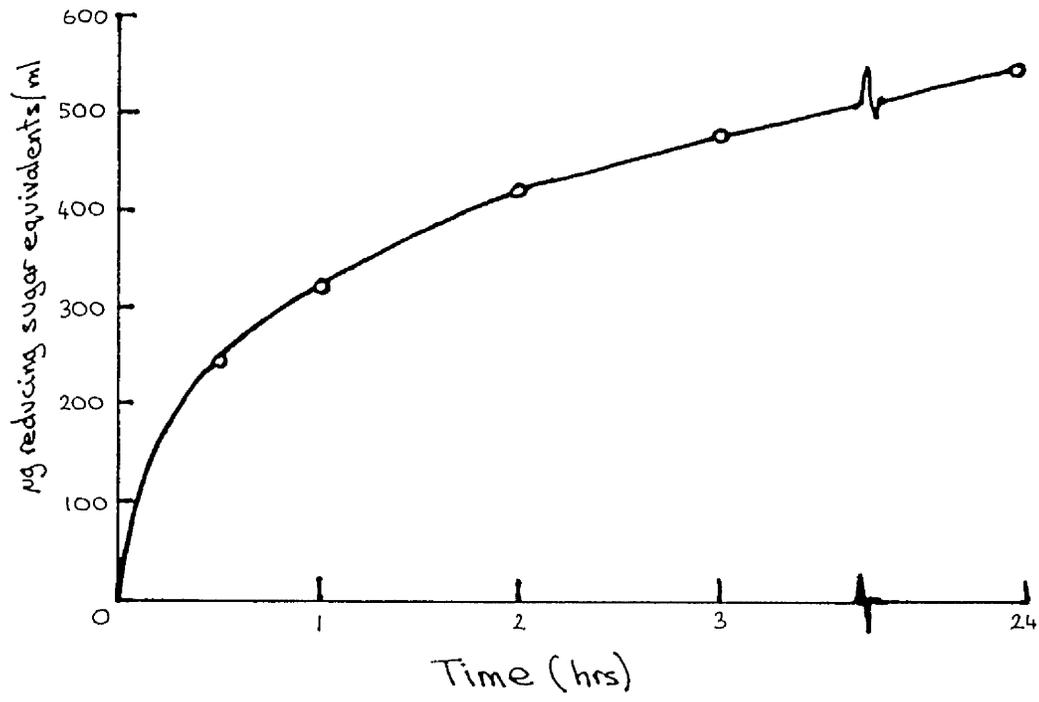
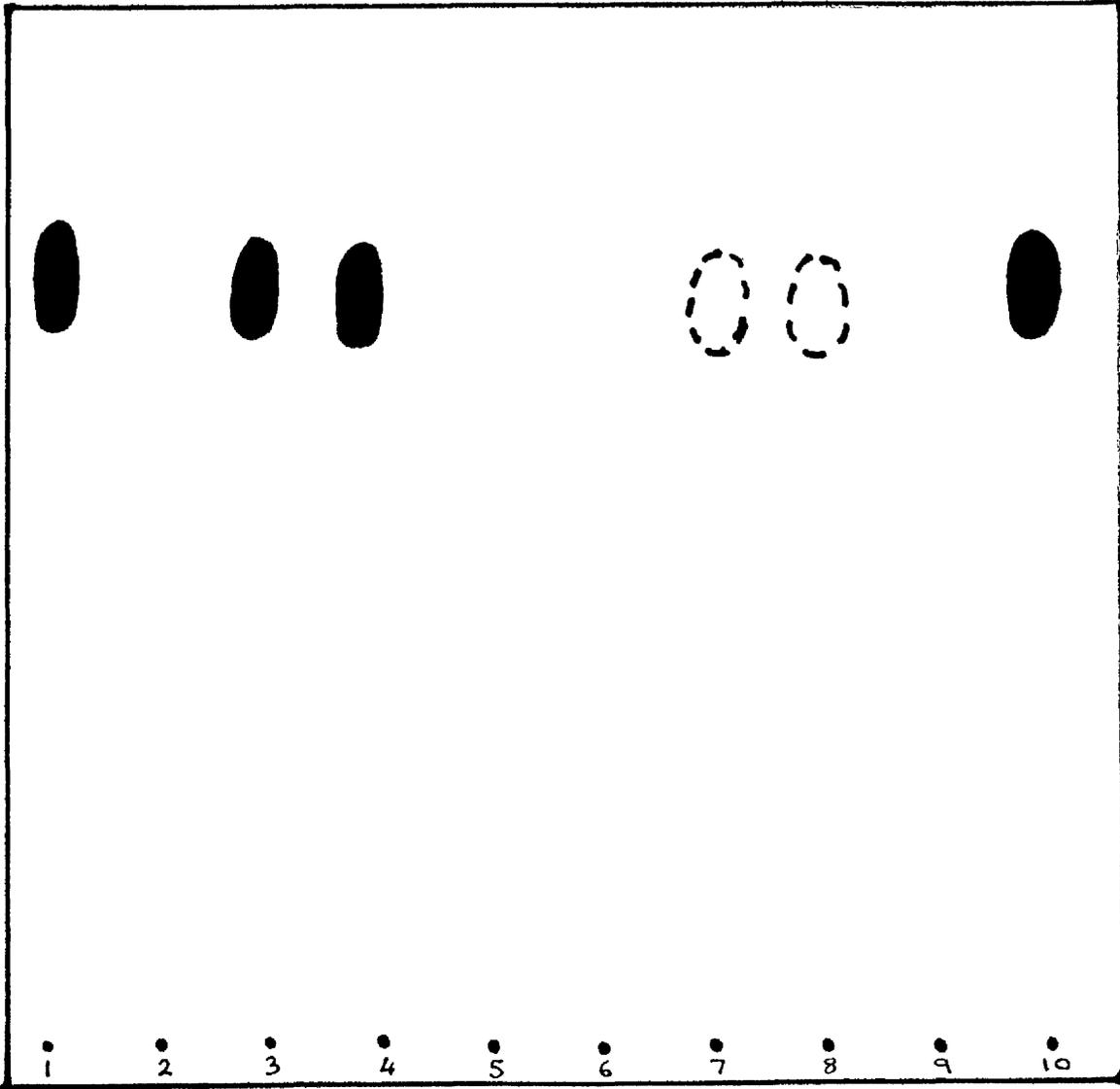


Figure 18 : Release of oligosaccharides from LPS by molluscan
extracts as detected by TLC

Key :

1. stachyose
2. Modiolus fraction 1
3. Modiolus fraction 1 incubated with S. minnesota LPS
4. Modiolus fraction 1 incubated with E. coli O111:B4 LPS
5. S. minnesota LPS
6. E. coli O111:B4 LPS
7. H. pomatia gut juice incubated with S. minnesota LPS
8. H. pomatia gut juice incubated with E. coli O111:B4 LPS
9. H. pomatia gut juice
10. stachyose

after incubation at 25°C for 24h, pH 4.0 sodium acetate 0.05M, 75µl applied.



It was possible that those enzyme preparations that did not produce detectable oligosaccharide after 24h (above) may have liberated oligosaccharides at an earlier stage in the degradation and that these were subsequently degraded to di or monosaccharides. However, all enzyme preparations were tested after 4h, 12h and 18h, and no oligosaccharides were detected.

Mya fraction 2 was studied in more detail and after incubation with S. minnesota LPS for 30 min, 1h, 2h and 3h no oligosaccharides were detected.

A study was made of the activity of Modiolus fraction 1 over a period of 24h with samples being analysed after 4h, 8h, 16h, 18h and 24h. It was found in two experiments, but inconsistently, that 'tetrasaccharide' was detectable after 4h and throughout the rest of the incubation. After 8h a larger oligosaccharide appeared which had an apparent drop in m.wt. when sampled after 16h. The same spot was present after 18h but had decreased in intensity. At 24h only the tetrasaccharide spot remained.

With the solvent system and TLC apparatus used in these experiments, tri, di and monosaccharides migrate off the top of the TLC plate as the solvent front progresses. To test whether extracts liberated such products from LPS, the solvent was allowed to migrate only as far as the top of the plate, at which point plates were dried and spots detected. It was found that stachyose standard migrated 5cm and that whereas standard tri, di and monosaccharides could be differentiated, only monosaccharide spots (as noted above) were detected. By this method, the 'tetrasaccharide' produced by Modiolus fraction 1 from LPSs was not resolved, appearing as a heavy smear.

It was assumed that the 'tetrasaccharide' spot seen in TLC represented a particular oligosaccharide species from each LPS. In order to attempt to identify the monosaccharides present, preparative TLC was carried out by applying 500 μ l of incubation mixtures of Modiolus fraction 1 with S. minnesota and E. coli O111:B4 LPS to plates and developing as before. Then, without staining, silicate was removed from the plates in areas where the tetra-

saccharides were expected to be located and the powder was eluted as described by Holmes and O'Brien (1978). It was planned to identify the monosaccharide products of acid hydrolysis of the dried eluates, however it was found that insufficient material was recovered for analysis.

Gel filtration chromatography was used as an alternative method to prepare liberated oligosaccharides for analysis. It was also thought that oligosaccharides possibly released by other bivalve extracts, not detected by TLC, may be detected by this method (Cortez, 1976).

Thus Mya fraction 2 incubated with S. minnesota LPS was tested for release of oligosaccharides by this method along with Modiolus fraction 1. When column fractions were analysed however, even Modiolus, which had previously been shown by TLC to release oligosaccharide from LPS, did not produce a corresponding peak (Figure 19) even when 500µl of sample were applied to the column. LPS breakdown products eluted as two peaks (tubes 29-31; 34-36). Filtration was on a molecular size basis, hence it is surprising that a species smaller than the tentatively identified hexose was observed. Mya fraction 2 produced a similar elution profile (Figure 20). The later eluted peak presumably represents spots a and (or) b detected by TLC examination of monosaccharide products (Figure 12). It is not known whether they are pentoses, or whether various deoxysugars migrate more slowly through the gel matrix. Stachyose and glucose eluted in peaks 27-28 and 29-31 respectively. Enzyme fractions, analysed alone produced a small amount of 'background' in the void area (tubes 14-16) which was unaltered by prior incubation.

Unexpectedly, there was not a major difference in the area of the peak corresponding to undegraded LPS between Modiolus and Mya incubation mixtures. However, Mya incubation mixtures produced larger peaks of LPS degradation products.

Figure 19 : Analysis of oligosaccharide products of S. minnesota
LPS degradation by Modiolus fraction 1, detected by
gel filtration chromatography.

Key :

- A. S. minnesota LPS (4mg/ml)
- B. S. minnesota LPS (4mg/ml) incubated with Modiolus
fraction 1 (1mg/ml)
- C. stachyose and glucose standards (see text)
- D. Modiolus fraction 1 (1mg/ml) incubated alone

after incubation at 25^oC for 24h, pH 4.0 sodium citrate 0.05M,
100µl applied.

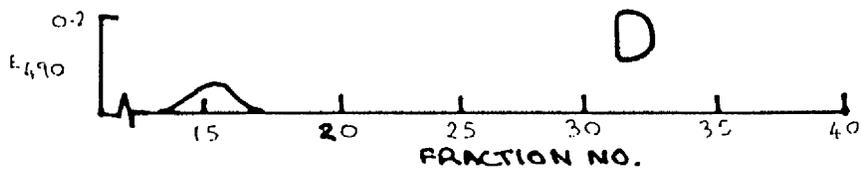
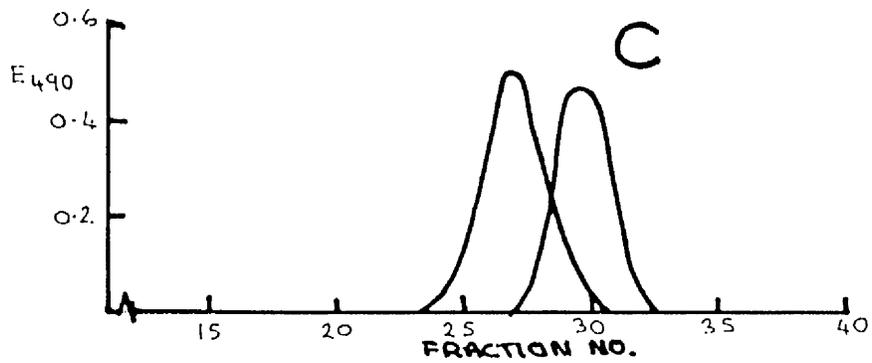
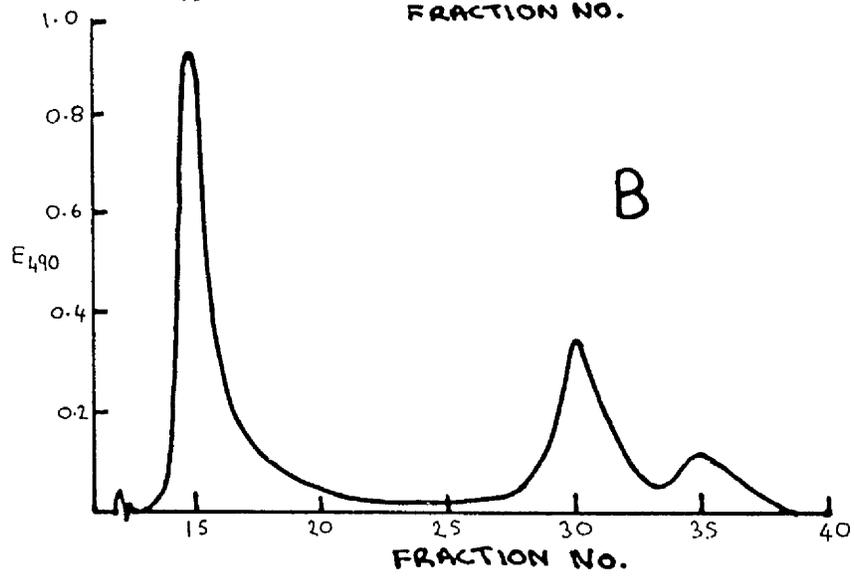
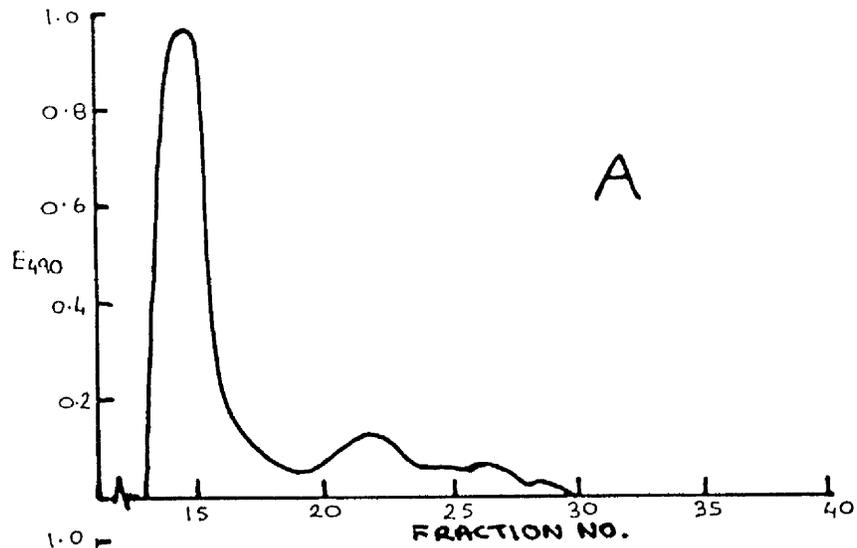
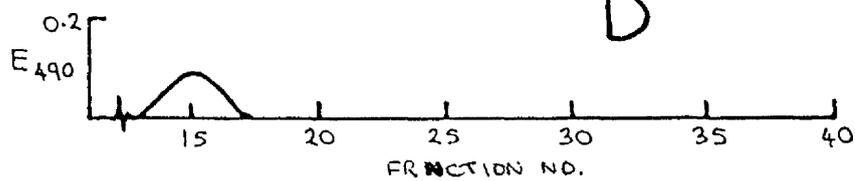
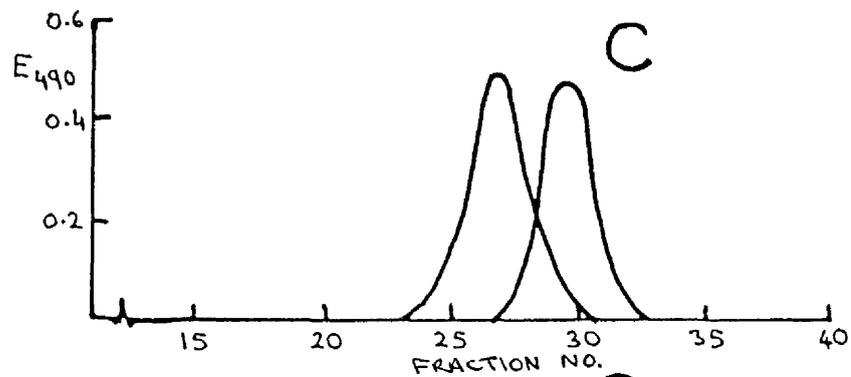
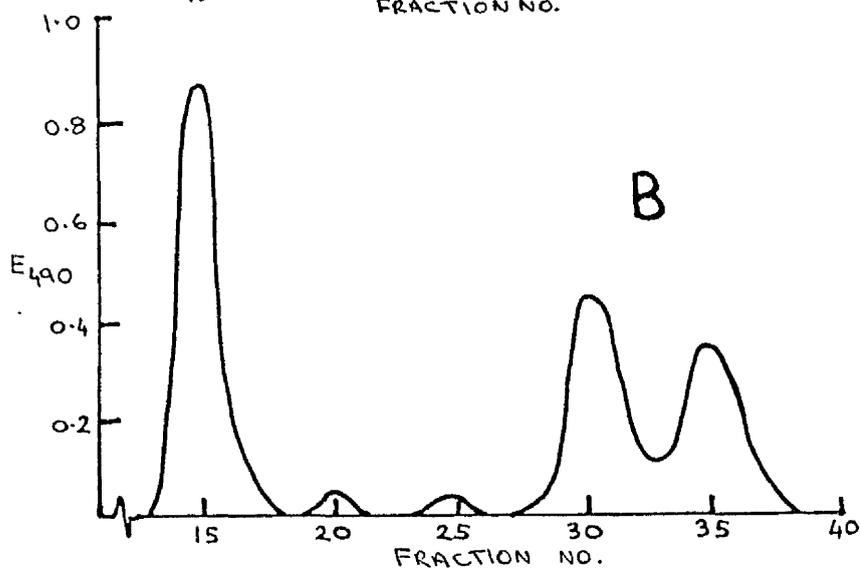
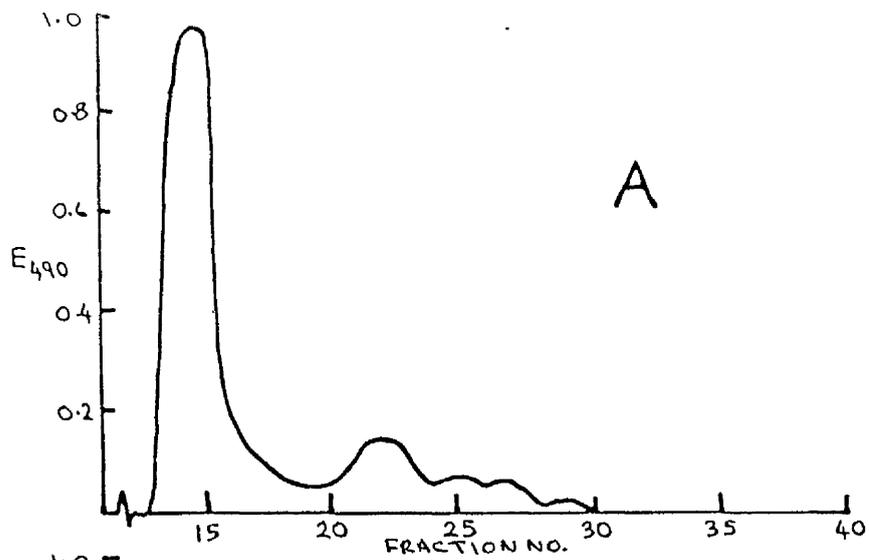


Figure 20 : Analysis of oligosaccharide products of S. minnesota
LPS degradation by Mya fraction 2, detected by gel
filtration chromatography

Key :

- A. S. minnesota LPS (4mg/ml)
- B. S. minnesota LPS (4mg/ml) incubated with Mya fraction 2
(1mg/ml)
- C. stachyose and glucose standards (see text)
- D. Mya fraction 2 (1mg/ml) incubated alone

after incubation at 25^oC for 24h, pH 4.0 sodium acetate 0.05M,
100µl applied.



Release of Non-saccharide Components

Ethanolamine

Ethanolamine is a characteristic component of the inner core-lipid A region of LPS (Figures 1,3,4). Its release from LPS would, therefore, indicate a particular degree of LPS breakdown and, since it is linked to LPS via phosphoryl linkages, it may be involved directly or indirectly in the removal of phosphate from LPS.

Bivalve extracts were incubated with LPSs and the digests tested for ethanolamine by TLC. However, although authentic ethanolamine standard could readily be detected, none was seen in enzyme/LPS mixtures. However more than 5µg of ethanolamine must be present in order to be detected by this method, and it is possible that small quantities were released but were undetected. Since ethanolamine constitutes 1.5% (w/w) of rough LPSs there should have been detectable ethanolamine present if there had been significant enzymatic release.

Phosphate

It was reported in the Introduction that phosphate linked to lipid A may play a role in endotoxicity of LPS. It was thus decided to test bivalve extracts for the ability to dephosphorylate LPS, as a preliminary step in perhaps preparing an enzyme extract with the ability to lower the endotoxicity of LPS preparations.

Fractions were tested for their ability to release free inorganic phosphate from LPS. Table 8 shows that all enzyme extracts tested released phosphate from LPSs (4mg/ml), with Mya having the highest phosphatase activity. More was released from S. minnesota LPS by all extracts than from E. coli O111:B4 LPS, which is in agreement with the phosphate content of the respective LPSs. All incubations were at pH 4.0, so it is possible that activities may be considerably greater, as it is known that phosphatases fall into two broad

TABLE 8 Release of inorganic phosphate from LPSs by
marine invertebrate extracts

| Marine invertebrate | µg inorganic phosphorus/ml | |
|---------------------|------------------------------|--------------------------------|
| | extract | extract |
| | + <u>S. minnesota</u> LPS | + <u>E.coli</u> O111:B4 LPS |
| <u>Mya</u> | 95 | 68 |
| <u>Modiolus</u> | 39 | 28 |
| <u>Cerastoderma</u> | 73 | 68 |
| <u>Mytilus</u> | 72 | 68 |
| <u>Pecten</u> | 72 | 70 |

LPS contained < 1µg inorganic phosphorus/ml.

Only fraction 1 from each marine invertebrate was used and contained no detectable inorganic phosphorus.

Extracts and LPS incubation mixtures were tested for inorganic phosphorus after 24h at 25°C. Prior to incubation, they contained <1µg inorganic phosphorus/ml.

categories :- alkaline and acid. These results would justify further work on solubilised lipid A preparations.

KDO

In experiments to determine whether free KDO was released from LPS, as an indicator of inner core region degradation, bivalve extracts were incubated with LPSs at pH 4.0 as previously and assayed for release of KDO.

However, the red chromophore that developed upon assay due to degradation products of LPS had an absorption maximum at 530nm instead of the authentic 550nm. All enzyme extracts gave a greater release of this product from S. minnesota LPS than E. coli O111:B4 LPS (Table 9).

It was suspected that the period of time in the boiling step in the assay, in which the red chromophore develops may be critical. To test this, authentic KDO was assayed by the documented procedure but the normal boiling step (15 min) was varied from between 5 and 45 min. Figure 21 and Table 10 show that the red colour which formed changed in both intensity and absorption maximum, with boiling time and had an absorption maximum of 530nm if the boiling was for 30 min. This was seen with large amounts of KDO (25µg) it is therefore possible that smaller amounts might have given an absorption peak at 530nm after much less than 30 min, but this length of boiling time was not used.

As an alternative source of the apparent shift in the absorption spectrum of KDO, other sugars were tested to see if they cross-reacted in the assay. Only N-acetylgalactosamine gave a notable reading at 550nm; but had an absorption peak at 440nm (Table 11) and is, in any case, not present in the LPS of E. Coli O111:B4.

When attempts were made to detect release of KDO by TLC, none was found in any of the enzyme/LPS incubation mixtures. Hence it appears most likely

TABLE 9 Reaction of LPS degradation products in the TBA assay(i) S. minnesota LPS

| Marine invertebrate | absorption at | |
|---------------------|---------------|-------|
| | 550 | 530 |
| <u>Mya</u> | 0.300 | 0.500 |
| <u>Modiolus</u> | 0.228 | 0.378 |
| <u>Cerastoderma</u> | 0.210 | 0.356 |
| <u>Mytilus</u> | 0.218 | 0.358 |
| <u>Pecten</u> | 0.208 | 0.348 |
| (LPS) | 0.092 | 0.034 |

(ii) E. coli O111:B4 LPS

| Marine invertebrate | absorption at 550nm |
|---------------------|------------------------|
| <u>Mya</u> | 0.133 |
| <u>Modiolus</u> | 0.114 |
| <u>Cerastoderma</u> | 0.129 |
| <u>Mytilus</u> | 0.116 |
| <u>Pecten</u> | 0.144 |
| (LPS) | 0.077 |

Incubation mixtures were tested after 24h at 25°C.

Extracts alone had zero absorption reading in the range 500nm - 600nm.

Figure 21 : Variation in the absorption maxima in the TBA assay
by authentic KDO with time of boiling.

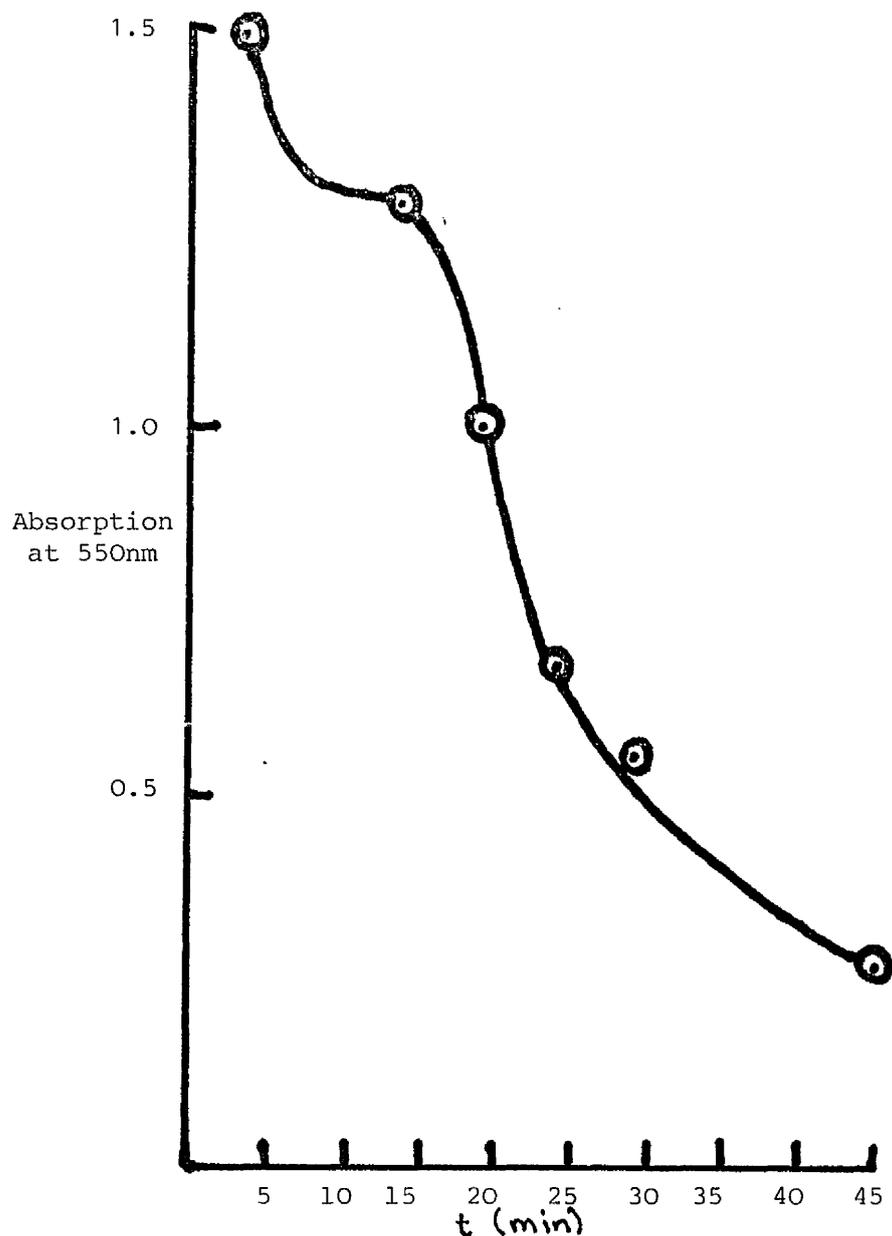


Table 10 : Variation in the wavelength giving maximum absorption in
the TBA assay by authentic KDO, with time of boiling

| time of boiling | absorption peak other than 550nm (reading) |
|-----------------|---|
| 5 | None |
| 10 | " |
| 15 | " |
| 20 | " |
| 25 | 533nm (0.704) |
| 30 | 530nm (0.693) |
| 45 | 526nm (0.465) |

TABLE 11Cross-reacting sugars in the TBA assay

| sugar | absorption at 550 nm | other absorption peak (reading) |
|-----------------------|-------------------------|------------------------------------|
| KDO | 0.315 | NONE |
| KDO lactone | 0.300 | 530 nm (0.350) |
| N-acetylgalactosamine | 0.179 | 440 nm (0.195) |
| fructose | 0.000 | NONE |
| galactose | 0.000 | NONE |
| glucose | 0.000 | NONE |
| mannoheptulose | 0.023 | NONE |
| mannose | 0.000 | NONE |
| N-acetylglucosamine | 0.035 | 432 nm (0.050) |

All sugars were tested as 1 $\mu\text{g/ml}$ in distilled water.

that free KDO is not released from LPS by the enzyme extracts tested, but that they do release a sugar (or sugars) which cross-react in its colorimetric assay.

Release of Fatty Acids

In order to estimate the prospects of the purification, from marine filter-feeding bivalve tissue, of an enzyme extract capable of diminishing the endotoxicity of LPS preparations by the release of free fatty acids, it was decided, initially, to test crude digestive gland homogenates for the ability to release free fatty acids from LPS. This would also provide information on the degradation of lipid A as possible source of nutrition by the bivalves.

Before setting up the biodegradation assays, the amount of total fatty acids was measured colorimetrically after treating LPS with alkali. From these tests it was estimated that an incubation mixture with 2mg/ml LPS should give detectable free fatty acids if as little as 1% of the total was released.

(a) Crude Extracts

Bivalve digestive glands (25%, w/v) or crystalline style (1%, w/v) were homogenised in 0.2M citrate/phosphate buffer pH 6.0 or in 3% (w/v) NaCl/0.02M sodium acetate buffer, pH 4.6 in a glass grinder with a Teflon ball (Birkbeck and McHenry, 1982) followed by centrifugation at 10,000 r.p.m. for 10 min in a Sorval centrifuge at 4°C.

LPS

To 400µl of the above supernate was added 100µl of 10mg/ml LPS from either S. minnesota or S. typhosa 0901 and the mixture incubated at 22°C for up to 90 min, after which the free fatty acid content was assayed colorimetrically.

There was no obvious release of free fatty acids from LPS by most extracts. However, Table 12 shows Cerastoderma and H. pomatia gave a net release of fatty acids. Free fatty acids present in extracts alone and the problem of interpretation of

TABLE 12

Release of fatty acids from LPS by Molluscan extracts

| Mollusc | incubation time (min.) | µg free fatty acids/ml | | |
|---------------------|------------------------------|------------------------|------------------|--------------|
| | | Extract + LPS | Extract alone | LPS alone |
| <u>Mya</u> | 0 | 31.5 | 24 | <2.5 |
| | 90 | 30 | 18 | " |
| <u>Modiolus</u> | 0 | 60 | 39 | " |
| | 60 | 81 | 59 | " |
| <u>Cerastoderma</u> | 0 | 30 | 30 | " |
| | 90 | 42 | 30 | " |
| <u>Mytilus</u> | 0 | 70 | - | " |
| | 90 | 85 | 92 | " |
| <u>Pecten</u> | 0 | 90 | 84 | " |
| | 90 | 90 | 81 | " |
| <u>H. pomatia</u> | 0 | 30 | 29 | " |
| | 90 | 60 | 34 | " |

marine invertebrate digestive gland homogenate supernates were incubated in 0.2M citrate/phosphate buffer at 22°C.

H. pomatia gut juice was incubated in the same buffer at 30°C.

figures represented mean readings from experiments done in duplicate and repeated up to 4 times.

the zero time increment for each sample however made firm conclusions difficult. The buffer used in these experiments (phosphate buffered saline, 0.02M, pH 6.2) was chosen since lipolytic activity towards LPS was reported by Saddler et al. (1979) in the gut juice of another mollusc, H. pomatia in these conditions. Birkbeck and McHenry (1982) reported that homogenisation and subsequent incubation in 3% NaCl/0.02M sodium acetate buffer, pH 4.6 to be an effective method for the detection of bacteria-degrading activity from Mytilus edulis digestive glands. However, when these conditions were used with Cerastoderma digestive glands, and the homogenate supernate subsequently incubated with LPS, the net release was less than that yielded by the previously used procedure (Table 13). After many repeats of these experiments with variations in the test conditions, it was concluded that release of fatty acids from LPS by digestive gland extracts other than Cerastoderma's was insignificant.

Whole Bacteria

To test whether the 3% NaCl/0.02M sodium acetate buffer extracts (above) released fatty acids from whole bacteria, heat-killed E. coli were used to replace LPS in experiments similar to those above.

Table 14 shows that extracts of Cerastoderma digestive gland and crystalline style released fatty acids from whole bacteria, but not as effectively as did H. pomatia gut juice. Thus the extraction procedure used here yielded a product with lipase activity but not towards free LPS.

h-LPS

One reason why fatty acids were not readily released from LPS might be steric hindrance i.e. enzymes could not get access to the lipid moieties of the LPS molecules because of their probable location inside a micelle. However, it was found that the addition of the disaggregating agents triethylamine, sodium deoxycholate or Triton X-100 (1%, w/v) did not enhance

TABLE 13

Release of fatty acids from LPS by Cerastoderma digestive gland extract.

| | Extract + LPS | | Extract alone | | LPS alone | |
|--|---------------|-----|---------------|------|-----------|------|
| incubation time (min.) | 0 | 180 | 0 | 180 | 0 | 180 |
| free fatty acids ($\mu\text{g/ml}$) | 20 | 24 | 15 | 17.5 | <2.5 | <2,5 |

incubated in 3% NaCl/0.02M sodium acetate buffer pH 4.0, 22°C

figures represent mean of 3 experiments done in duplicate

TABLE 14

Release of fatty acids from whole bacteria by Molluscan extracts

| Molluscan tissue extract | incubation time (min.) | μg free fatty acids/ml | | |
|--|---------------------------|-----------------------------------|------------------|-------------------|
| | | Extract + bacteria | Extract Alone | Bacteria Alone |
| <u>H. pomatia</u> gut juice | 0 180 | 90 280 | 7 17.5 | 62.5 50 |
| <u>Cerastoderma</u> digestive gland | 0 180 | 28 74 | 16 15 | 25 29 |
| <u>Cerastoderma</u> style | 0 180 | 30 53 | 0 0 | 33 45 |

marine invertebrate extracts were incubated in 3% NaCl/0.02 M sodium acetate buffer pH 4.6, 22°C.

H. pomatia gut juice incubated in 0.2M citrate/phosphate, pH 6.0, 30°C.

N.B. Apparent high fatty acid content of bacteria alone in H. pomatia experiments is due to volumetric differences in the protocol.

release of fatty acids from LPS by Cerastoderma extracts.

Mild-alkaline treatment, which releases some fatty acids from LPS, leaves a structure which should be more accessible to lipases. Cerastoderma extracts were marginally more active against this substrate than towards whole LPS (Table 15).

(b) AS Extracts

pH Optimisation

Prior to testing ammonium sulphate (AS) extracts from bivalve digestive glands for the ability to release fatty acids from LPS, the pH of optimal lipase activity was estimated against tributyrin. This was done by measuring the diameter of clear zones produced by enzyme preparations upon incubation in wells cut in tributyrin agar diffusion plates (Figure 22). Cerastoderma and Modiolus fraction 1 contained most (or all, in the latter case) of the lipase activity, with optimum activity being detected at pH 8 for Mya and Cerastoderma and pH 7 for Modiolus. Mytilus also had an optimum at pH 7 but was strongest in fraction 2. No lipase could be detected in either of the Pecten fractions, even after prolonged incubation.

In control experiments, H. pomatia gut juice had an optimum of pH 7 at 37°C. Lipase from Candida cylindracea (Sigma) was used both as a control in LPS degradation studies and also to test the efficacy of the assay method. The experimentally determined optimum was pH 8 at 37°C for C. cylindracea lipase with a lower peak of activity at pH 5 (Figure 23). This correlated satisfactorily with the known optimum of pH 7.7 with a secondary peak at pH 5.2 (Sigma Chemical Co. Ltd., personal communication) and hence it was assumed that other experimentally determined pH optima were valid. Choosing the most active lipase extract from each bivalve, lipases were listed in terms of potency as follows :- C. cylindracea lipase > Mya fraction 2 > H. pomatia gut juice > Modiolus fraction 2 > Cerastoderma fraction 2 > Mytilus fraction 1.

TABLE 15

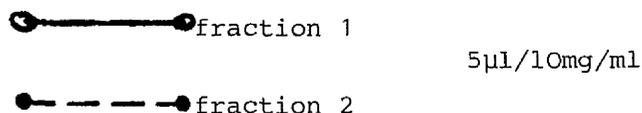
Release of fatty acids from h-LPS by Molluscan extracts

| Molluscan extract | Incubation time (min.) | µg free fatty acids/ml | | |
|----------------------|---------------------------|------------------------|------------------|----------------|
| | | Extract h-LPS | Extract alone | h-LPS alone |
| <u>Cerastoderma</u> | 0 | 48 | 35 | < 2.5 |
| digestive gland | 180 | 58 | 37 | < 2.5 |
| <u>H. pomatia</u> | 0 | 70 | 30 | < 2.5 |
| gut juice | 180 | 100 | 32 | < 2.5 |

extracts incubated in 0.2M citrate/phosphate buffer,
pH 6.0, 22°C or 30°C.

Figure 22 : Variation of lipolysis of tributyrin by marine invertebrate AS extracts with pH

Key :



The diameters of the zones of lipolysis in wells cut in tributyrin (0.2%, w/v, in 0.8% w/v) agar were measured with the help of a low-power microscope. Each data point represents the mean reading of 4 wells. Results are those found in one experiment, which were essentially similar when repeated twice further.

N.B. No lipolysis was detected in either of the Pecten fractions, or in Modiolus fraction 1.

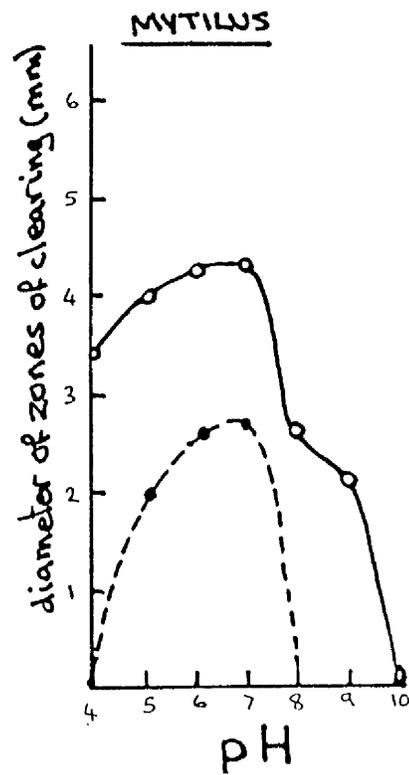
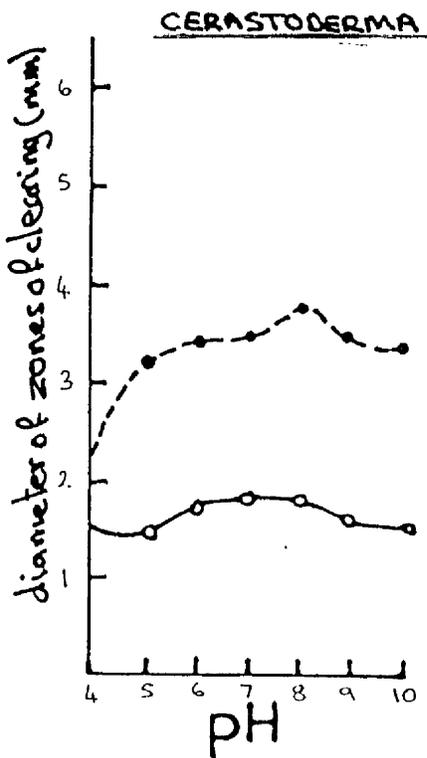
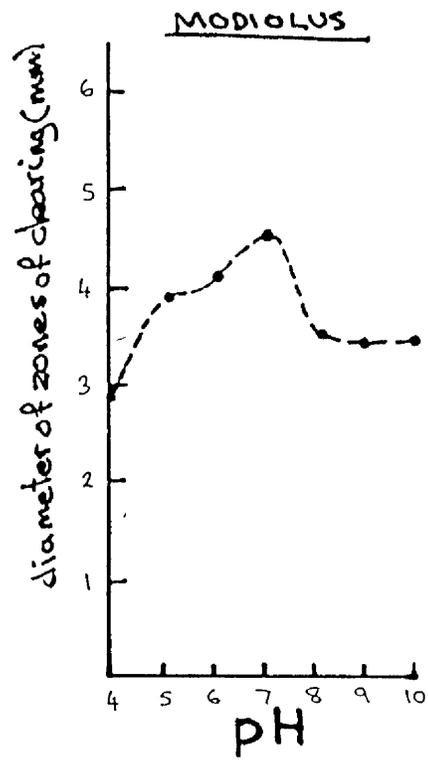
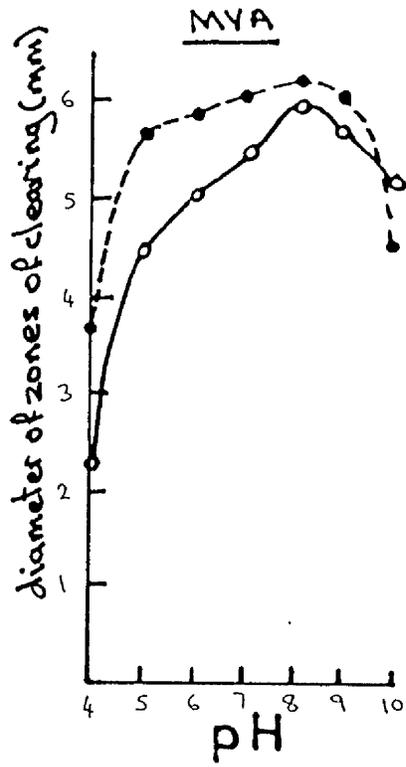
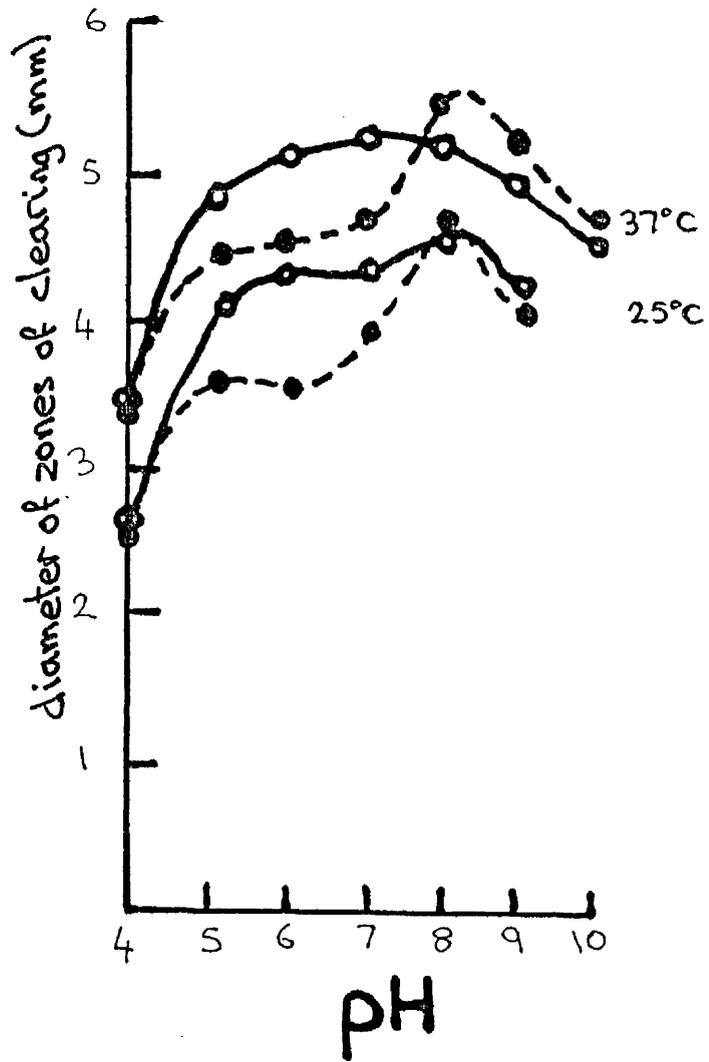


Figure 23 : Variation of lipolysis of tributyrin by H. pomatia gut juice and C. cylandracea lipase with pH and temperature

Key :

○ ——— ○ H. pomatia gut juice (5µl/10mg/ml)
● - - - ● C. cylandracea (5µl/5mg/ml)

For explanation of experimental protocol, see Figure 22 and text.



LPS

Enzyme extracts with the highest lipase activity towards tributyrin, as well as both Pecten fractions, were incubated with LPS at the optimised pH. No release of fatty acids was detected colorimetrically by any of the preparations apart from H. pomatia, which had slight activity. However, when the samples were analysed by TLC, convincing evidence of fatty acid release was obtained. H. pomatia > Cerastoderma > C. cylandracea released fatty acids from LPS of S. minnesota and E. coli O111:B4 (Figure 24). All other extracts were inactive, including Mya fraction 2 which contained a potent lipase for tributyrin.

H. pomatia was the most active of the extracts tested in this system. Of the marine bivalve extracts, Cerastoderma fraction 2 was the only one showing release of free fatty acids from LPS by TLC.

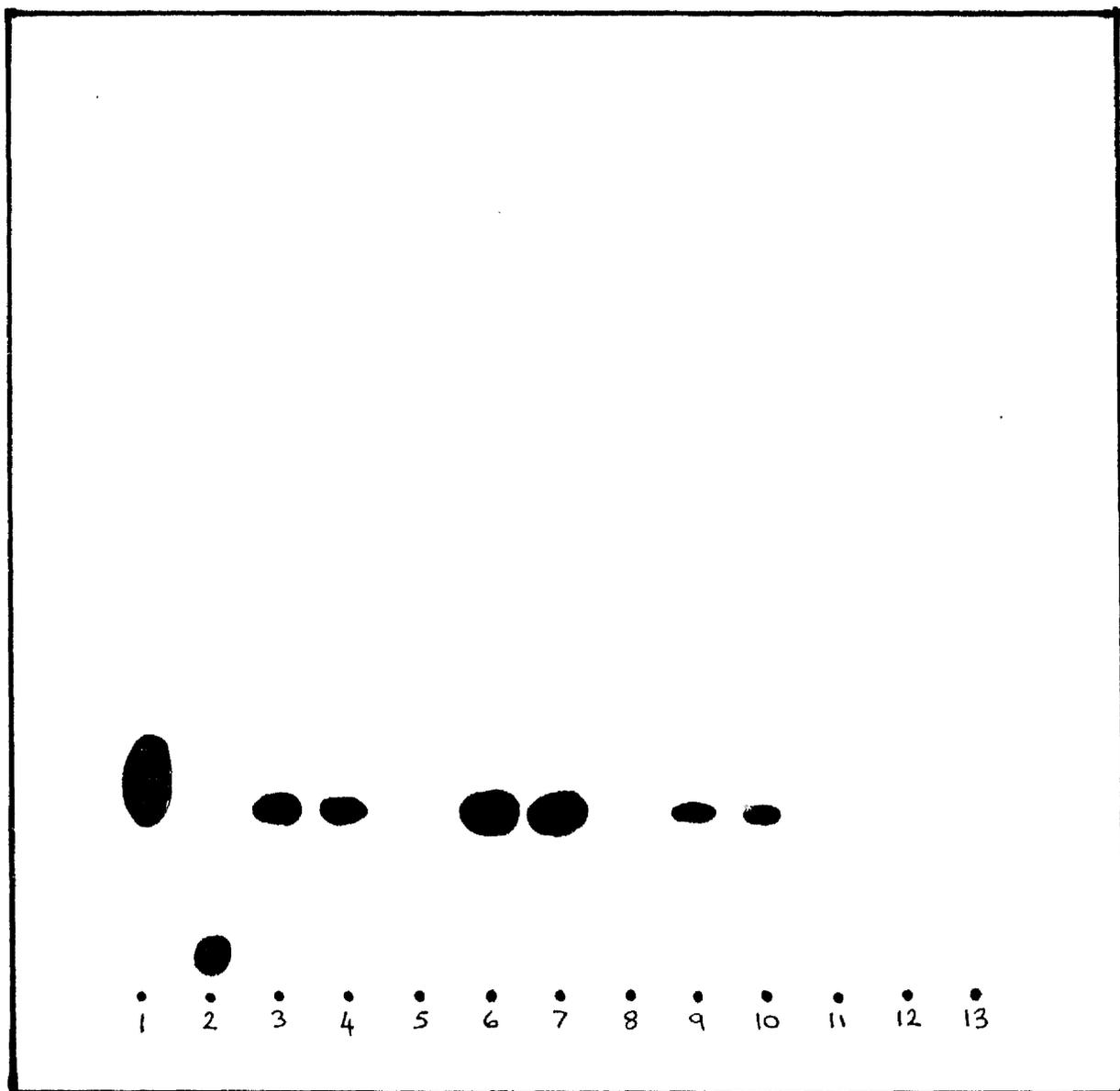
Those extracts that were active against LPS produced spots of similar intensity by TLC when incubated with either of the LPSs tested (Figure 24). The detection method is quantitative, with the intensity of the spot being proportional to the fatty acids present (Fewster et al., 1969). Modiolus fraction 2 contained large amounts of material with identical migratory pattern to triglycerides which may have acted as a competitive substrate, thereby protecting LPS.

The solvent system in the TLC allowed differentiation between hydroxylated and non-hydroxylated fatty acids. Thus it was possible to conclude that no 3-hydroxytetradecanoic acid was released by any of the enzyme preparations. The non-hydroxylated fatty acids released could not be further characterised by this method.

Figure 24 : TLC of fatty acids released from LPSs by lipases

Key :

1. tetradecanoic acid
2. 3-hydroxytetradecanoic acid
3. Cerastoderma fraction 2 + S. minnesota LPS
4. Cerastoderma fraction 2 + E. coli O111:B4 LPS
5. Cerastoderma fraction 2 alone
6. H. pomatia gut juice + S. minnesota LPS
7. H. pomatia gut juice + E. coli O111:B4 LPS
8. H. pomatia gut juice
9. C. cylandracea lipase + S. minnesota LPS
10. C. cylandracea lipase + E. coli O111:B4 LPS
11. C. cylandracea lipase alone
12. S. minnesota LPS alone
13. E. coli O111:B4 LPS



Section 2 - Degradation of LPSs by Marine Bacteria

LPS-Degrading Bacteria Isolated by Saddler and Wardlaw (1980).

Cell-free LPS-degrading enzymes were investigated in the previously isolated strains of Saddler and Wardlaw (1980). Strain A (a Bacillus sp.) was chosen for the study of its ability to degrade lipid A by lipolysis. Strain B (a Coryneform) was selected as it had been reported to be saccharolytic towards LPS.

Although the strains could be sub-cultured in the LPS/seawater medium of Saddler and Wardlaw (1980), growth was sparse and unsuitable for the bulk preparation of bacteria for enzyme extraction. The low nutrient content of the medium was also manifested by Strain A undergoing sporulation in it.

To obtain more abundant growth of the bacteria, batches of each isolate were grown in ASWYP and the supernates fractionally precipitated by AS at 20, 40, 60 and 80% saturation, dialysed and freeze dried. The fractions from Strain A were tested for the ability to release fatty acids from LPS by incubating with LPS for up to 24h and assaying for fatty acid release colorimetrically, but all experiments were negative. Extracts from Strain B were tested for attack on the polysaccharide moiety of LPS by analysis of incubation mixtures by SDS-PAGE (Saddler et al., 1979b; Czako et al., 1983) however no degradation was detected.

Unexpectedly, it was also found that Strain A, although reported by Saddler and Wardlaw (1980) to be lipolytic towards lipid A, was not lipolytic when cultured on plates with an overlay of tributyrin agar. It was therefore decided to attempt to isolate fresh LPS-degrading bacteria. Sand and mud samples were collected near the Dunstaffnage Marine Laboratory, Oban. Initially the sub-culturing procedure of Saddler and Wardlaw (1980) was followed and, after sub-culturing every 7 days for 6 weeks at 25°C in 4mg/ml LPS in seawater and subsequent inoculation onto ASWYP agar, three distinct

colony types (differentiated by colour :- cream; yellow; orange) could be identified. Cell-free AS fractions of culture supernates were prepared as before but no degradative activities were detected when assayed as previously for lipolytic or saccharolytic activity towards LPS.

The LPS-containing culture fluid, after removal of isolates, was analysed for residual alkali-releasable fatty acids and by SDS-PAGE for gross alteration or lowering in quantity of LPS, but no difference was detected from uninoculated LPS-containing medium. Thus it seemed that the bacteria which were isolated could degrade LPS to release nutrients sufficient to allow enough multiplication so that a loopful from the culture contained enough organisms to establish growth in fresh medium. Yet the same bacteria grown in a rich medium did not release detectable LPS-degrading enzymes into the culture supernate.

Lipolytic Isolates

Since, as suggested in the Introduction, enzymes capable of releasing fatty acids from LPS would be of considerable interest, a primary isolation was made of bacteria which were lipolytic towards tributyrin. From such a collection, there could then be a second stage of selection of strains that were lipolytic against LPS.

Sediment samples from Dunstaffnage were inoculated onto ASWYP agar with an overlay containing 1% (w/v) tributyrin, and lipolytic colonies were selected after incubation at 25°C. A total of ten lipolytic organisms (all Gram-negative) was selected, with no more than one isolate being taken from each plate, to minimise duplication of isolates. (The isolates were labelled P1-P10).

Lipid A, because of its hydrophobicity, is highly insoluble in water. Purified lipid A was thus prepared from LPS of S. minnesota by mild acid

hydrolysis after which it was incorporated (1%, w/v) into an overlay agar layer on ASWYP agar and the ten lipolytic isolates tested for the ability to create a zone of clearing around the lipid A which indicated release of fatty acids from lipid A (cf. tributyrin agar). Only one isolate (P8) had this ability and the effect could also be observed against whole LPS incorporated into culture plates. However, this clearing was less obvious because LPS is more water-soluble than lipid A.

When P8 was grown in LPS-containing liquid medium with traces of yeast extract and peptone to stimulate growth, the amount of alkali-releasable fatty acids remaining in the culture fluid after removal of bacteria was considerably lower than in uninoculated control medium (Table 16). Unfortunately, AS fractions of culture supernates, although strongly lipolytic towards tributyrin, as tested by zones of clearing in tributyrin agar diffusion plates, had no detectable lipolytic effect on LPS or lipid A even when they were incorporated into agar diffusion plates as for tributyrin (data not shown).

Bacteriolytic Isolates

It was observed that occasionally in some culture plates of marine sediment, a spreading Gram-negative bacterium was apparently being lysed by neighbouring colonies. Therefore the lysable bacterium was isolated, batch-cultured and heat-killed so as to avoid lysis. After incorporation into solid medium several bacterial isolates produced a broad zone of bacteriolysis after inoculation and subsequent incubation at 25°C (Whiteside and Corpe, 1968). However, all were negative when tested for their ability to degrade LPS extracted from the organism by the phenol/water method or from its lipid A prepared by acid hydrolysis when either were incorporated into solidified media. The previously isolated P8, used as control, did produce a zone of clearing on lipid A containing plates.

TABLE 16

Alkali-releasable fatty acid in LPS-containing culture media, with and without growth of the LPS-degrading bacterial isolate, P8.

| Test Sample | ASWYP (1/10) | LPS | Bacterial isolate P8 | µg free fatty acid |
|-------------|-----------------|-----|-------------------------|-----------------------|
| 1 | + | - | - | 0 |
| 2 | + | - | + | 0 |
| 3 | + | + | - | 1000 |
| 4 | + | + | + | 600 |

Medium (ASWYP) was diluted 1/10 with ASW and extracted three times with diethyl ether to remove free fatty acids. After growth of P8, extraction was repeated and the medium was treated with NaOH to release fatty acids which were then estimated colorimetrically. LPS (from S. minnesota) was incorporated in the medium as 4 mg/ml.

Experiment was carried out twice, in duplicate and the figures for fatty acid have been rounded to the nearest 10 µg.

Isolation on LPS-Containing Solid Medium

Due to LPS being of limited solubility in salt containing solutions, an attempt was made to isolate bacteria from marine sands and muds on solid medium containing LPS as an indicator - clear zones around growth were assumed to represent LPS degradation to more soluble products. Alternatively, release of lipid A from LPS might give a zone of opacity.

(a) Extraction and Purification of *Methylophilus methylotrophus* LPS

Because of the large quantities of LPS needed for these studies, it was necessary to find a cheap and convenient source, and for this purpose the ICI product 'Pruteen' made from *Meth. methylotrophus* was chosen. Although 'Pruteen' is not a rich source of LPS, large quantities were available and therefore relatively large amounts of LPS could be obtained (Table 17). The phenol/water method gave the highest yield of LPS, and the preparations were essentially free of nucleic acid contamination (< 2.5%, w/v) (Figure 25). In contrast, EDTA crude extracts are reported to be commonly contaminated by membrane protein under the extraction conditions followed here, but nucleic acid contamination was expected to be minimal. However, Figure 26 shows that after initial release of LPS, the nucleic acid content was considerable (25%, w/w). Its apparent contamination increased after Amicon ultrafiltration due to the removal of low m.w. material but was decreased by ultracentrifugation to around 15% (w/w). Thus although Amicon ultrafiltration was ineffective in removing nucleic acid contamination it provided a useful method of concentrating the LPS-containing extracts for ultracentrifugation with the most effective purification step. The Galanos extraction was carried out because of the low yield obtained by the phenol/water method. It is known that some LPSs, usually of the Rough phenotype are extracted more efficiently by the Galanos method. However, it was found that this method extracted LPS less efficiently (Table 17). It was finally concluded that

Figure 25 : Purification of LPS from Methylophilus methylotrophus
extracted by the phenol/water method

Each 'LPS' sample contained 1mg/ml lyophilised material dissolved in distilled water.

- . — . — . aqueous layer of phenol/water extraction
after dialysis and centrifugation to remove
particulate material.
- ultracentrifuged material treated with
nuclease and pronase (final product)
- salmon testes DNA (25µg/ml)

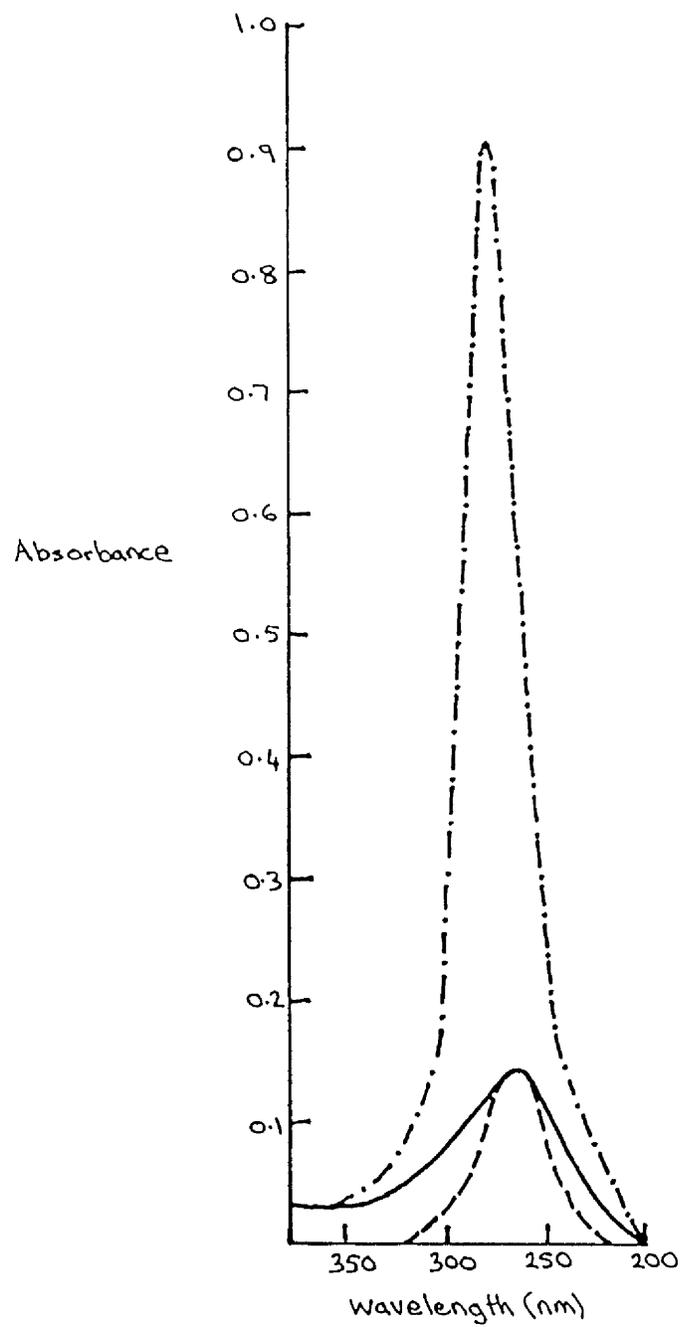


Figure 26 Purification of LPS from Methylophilus methylotrophus
extracted by the EDTA method

Each sample contained 250µg/ml lyophilised material dissolved in distilled water.

- cell-free supernate after EDTA treatment and dialysis
- Amicon filter retentate
- pellet obtained after ultracentrifugation (final product)
- salmon testes DNA (100µg/ml)

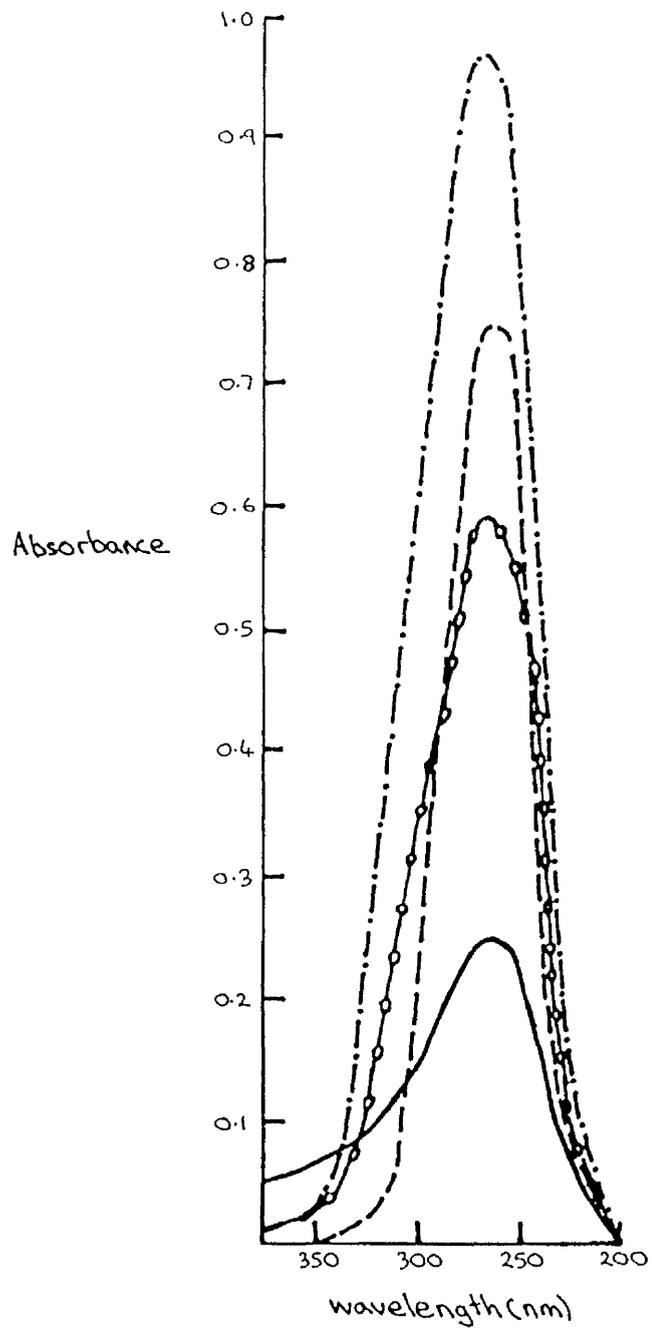


TABLE 17

Comparison of yield and nucleic acid content of LPS extracted from 'Pruteen'
by different methods.

| Extraction method | mg LPS obtained/100g 'Pruteen' | % nucleic acid (w/w) contamination in lyophilised material |
|----------------------|-----------------------------------|--|
| Westphal* | 500 | 2.5 |
| Galanos + | 121 | N.D. |
| EDTA o | 90 | 16 |

N.D. = Not Done

* mean of approx. 30 extractions (occasionally only 50% of this yield obtained)

+ mean of 2 extractions.

o mean of 3 extractions.

the phenol/water method was the best method of extraction of LPS from 'Pruteen'.

(b) Characterisation of Isolates

Marine sediments collected from three different geographical locations were sampled for LPS-degrading bacteria by incubating for 4 days at 25°C as described (Table 18). A total of nine isolates were obtained (M1-M6, HB1-HB3) although, surprisingly, none could be isolated from the Dunstaffnage mud (DMS), from which several lipolytic, bacteriolytic and one LPS-degrading bacterial strain (P8) had previously been isolated. All isolates produced zones of clearing on a medium containing Meth. methylotrophus LPS (Figure 27). None of the isolates grew on agar-solidified ASW alone, while all of them grew on silica gel medium containing LPS. In this medium however, LPS was completely soluble since gelling can not be done at salt strengths equivalent to ASW and therefore distilled water had to be used. Hence, no zones of clearing were observed although the growth of colonies indicated that the LPS was being used as a nutrient source. All isolates were Gram-negative rods and grew well on ASWYF agar, on which they were maintained.

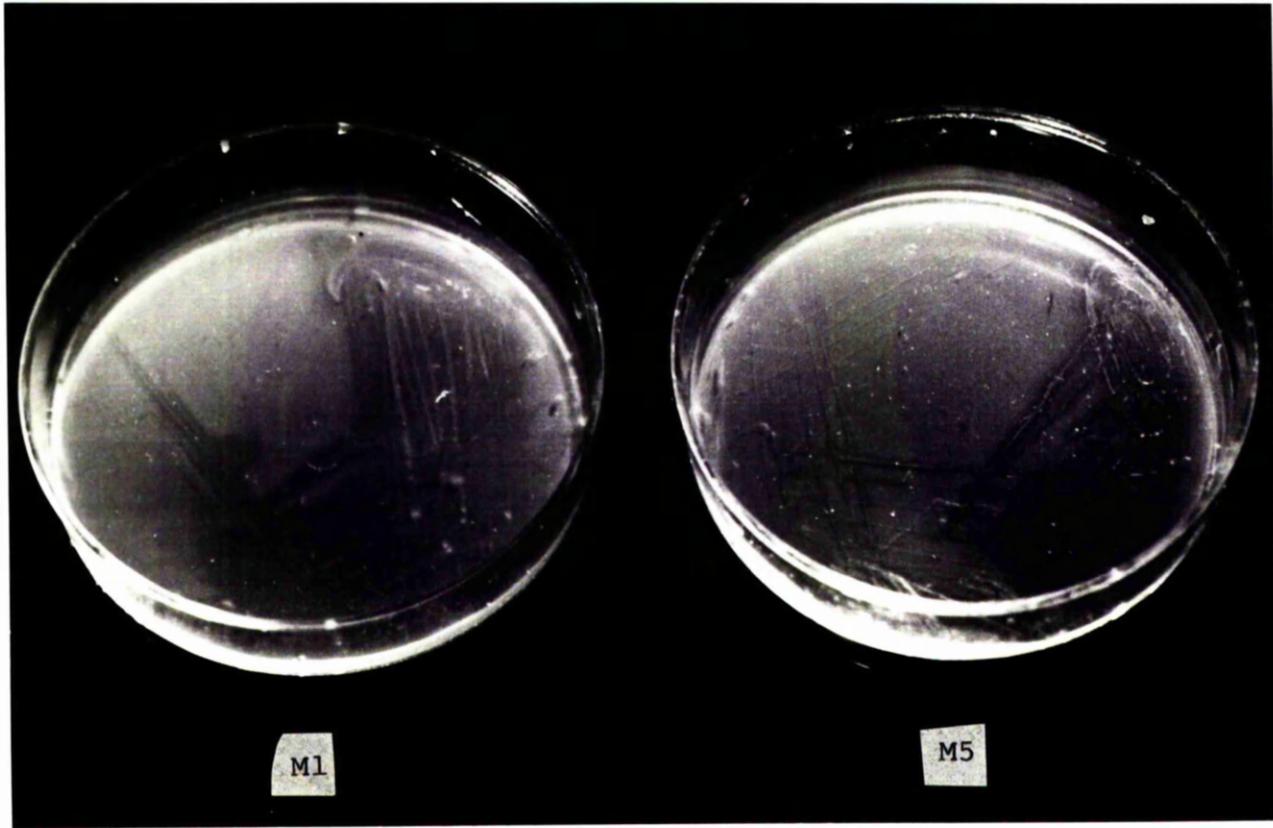
To attempt to characterise the degradative activity that was responsible for the zones of clearing on the LPS agar, their ability to degrade other substances were tested :- lipid breakdown was tested since a possible explanation may have been lipolysis towards lipid A; production of nuclease and protease were tested for; since it was possible that the clear zones represented breakdown of contaminants of the LPS preparations (although these were known to be low); starch degradative ability was tested for in order to detect saccharolytic activity which might have some effect on LPS.

Table 19 shows that all of the isolates were lipolytic on tributyrin and tripalmitin agar and had the ability to grow on tributyrin agar with no

Isolation of LPS-degrading bacteria from marine sediments

| location of sampling site | description of sampling site | Isolation technique | abundance of LPS-degrading bacteria | Isolates |
|--|--|---|-------------------------------------|-----------------|
| University Marine Biological Station (UMBS), Millport, Isle of Cumbrae | adjacent to sewer outflow of UMBS (MMS1) decomposing shellfish remains in ditch, East of Specimen Supply Dept., UMBS (MMS2) Enteromorpha rock pool, to West of Specimen Supply Dept. UMBS (MMS3) Enteromorpha rock pool Farlan Point (MMS4) | Inoculation of loopful of sediment on to ASWYP overlaid with ASWLPS agar, followed by subculture on to ASWLPS agar for selection of individual colonies | + + + + | M3 - |
| Helensburgh | adjacent to sewer outflow West of town (HBMS) | Primary enrichment by growth in ASWLPS liquid medium, followed by inoculation on to ASWLPS agar and selection for individual colonies | + + | HB1, HB2 HB3 |
| Dunstaffnage, Marine Laboratory, Oban | adjacent to outflow, Marine Laboratory (DMS) | | 0 | None |
| + + | 20 per plate | | | |
| + | 1 -20 per plate | | | |
| 0 | none | | | |

Figure 27



LPS-degrading bacterial isolates (M1,M5) producing zones of clearing,
when grown for 4 days at 25°C on ASW agar containing 4mg/ml LPS from
Meth. methylotrophus as overlay.

TABLE 19

Ability of LPS-degrading bacterial isolates to degrade substances other than LPS

| ISOLATE | * TRIBUTYRIN | | DNA | | STARCH | | CASEIN | | TRIPALMITIN | |
|---------|--------------|-----|-------|-----|--------|-----|--------|-----|-------------|-------|
| | ⊕ ASWYP | ASW | ASWYP | ASW | ASWYP | ASW | ASWYP | ASW | ASWYP | ASWYP |
| M1 | + | + | 0 | 0 | + | + | 0 | + | + | |
| M2 | + | + | + | + | + | + | + | + | + | |
| M3 | + | + | + | + | + | + | + | + | + | |
| M4 | + | + | + | + | + | + | + | + | + | |
| M5 | + | + | + | + | + | + | + | + | + | |
| M6 | + | + | + | + | + | + | + | + | + | |
| HB1 | + | + | + | 0 | + | + | + | + | + | |
| HB2 | + | + | + | + | + | + | + | + | + | |
| HB3 | + | + | + | + | + | + | + | + | + | |

Key : * upper layer (substrate (1% w/v) in ASW agar)

⊕ lower layer (solidified with 1.5% agar)

0 = no hydrolysis

± = partial hydrolysis

⊕ = definite hydrolysis

other organic nutrients. Tripalmitin was less readily degraded. Most isolates produced DNase and could utilize DNA as a sole organic source, although M1 did not produce DNase at all, and HBl required other nutrients for DNA hydrolysis. All isolates were proteolytic towards casein, apart from M1. Starch was readily hydrolysed by all isolates apart from M1 and M5 which brought about only partial hydrolysis. All isolates could utilize starch as their sole carbon source.

Figure 25 shows that nucleic acid contamination of LPS preparations was around 2.5% (w/w). None of the isolates gave visible growth on medium containing an amount of DNA equivalent to even 10% (w/w) contamination and when this level of DNA was provided as an overlay on ASWYP agar (which did provide growth) there was not enough acid precipitable DNA to detect degradation by this method.

Thus DNA degradation was ruled out as a possible explanation of visible bacterial colonies surrounded by zones of clearing which the isolates produced on LPS medium.

The isolates which produced the most growth and the largest zones of clearing on LPS plates were selected for studies on cell-free enzyme activity. It was assumed that such activity would not be cell-associated, since the zones of clearing often extended several mm from the bacterial growth. Strains M1, M4 and M5 were therefore grown (as described in Materials and Methods) and the proteins in the supernate were precipitated by AS as before, yielding < 10mg of protein/500ml of culture.

Although lipase activity towards tributyrin was readily demonstrable by agar diffusion in tributyrin plates, lipase activity towards LPS was not detected either colorimetrically or by TLC. However, one extract (70% saturation AS fraction of M1) appeared to degrade the polysaccharide moiety, since there was an increase in the reducing sugar equivalents of enzyme/LPS mixtures

TABLE 20

Formation of reducing sugar equivalents from Meth methylotrophus
LPS by culture supernate extract of the LPS-degrading bacterial
isolate, M1.

| INCUBATION | (h) | µg reducing sugar equivalents/ml | | |
|------------|-----|----------------------------------|----------------------------|-----------|
| | | * Extract alone | Extract + LPS [⊕] | LPS alone |
| 0 | | 170 | 210 | 30 |
| 24 | | 175 | 305 | 30 |

⊕ Meth. methylotrophus LPS, 4 mg/ml.

* 70% AS extract (5 mg/ml) of culture supernate.

Incubations in ASW (pH 7.0) for 24h at 25°C.

after overnight incubation at 25°C (Table 20).

Isolates (M1, HBl, M5) were grown in liquid culture containing LPS as the sole carbon source and aliquots were removed and assayed for free fatty acids, total sugar and reducing sugar equivalents. Of these criteria of LPS degradation, only total sugar, which decreased to varying extents with bacterial growth, showed evidence for degradation.

Therefore, although several Gram-negative bacteria were isolated by a selection method which demonstrated LPS degradation, no other evidence of LPS degradation was obtained, apart from the indication of degradation of the polysaccharide moiety which would by itself be expected to give a zone of opacification by release of lipid A. Hence the clearing effect observed on LPS incorporated into agar is not immediately explicable.

Anaerobic LPS Degradation

Because anaerobic bacteria may play an important role in LPS degradation in the sub-surface of marine sediments (i.e. between 1-4cm depth, where conditions are anaerobic) and because all previous isolation attempts had yielded only Gram-negative bacteria, such sediments were hoped to yield Gram-positive isolates (e.g. Clostridium sp.). These would provide a potential source of enzymes which would not be contaminated by LPS and thus would not be a problem in subsequent analytical work.

Therefore attempts were made to detect and isolate marine anaerobic LPS-degrading bacteria. Meth. methylotrophus LPS incorporated into solid media was used as an indicator of degradation, with plates being incubated at 25°C in an anaerobic jar. Despite several attempts with all of the sediments in Table 18 no LPS-degrading bacteria were obtained and the total numbers of colonies was low.

LPS degradation was also studied in anaerobic liquid cultures developed from marine sediments. LPS was incorporated into discs of gel (agar or silica

gel) which were dropped into the liquid medium and inoculated with sediments from the four sites sampled from the Isle of Cumbrae (Table 18).

In preliminary experiments with LPS incorporated into agar discs, when discs were removed from cultures inoculated with sediments MMS1 and MMS3 they had become transparent and indistinguishable from non LPS-containing discs. Discs from MMS2 and MMS4 cultures retained their opacity (due to LPS), as did LPS-containing discs immersed and incubated in uninoculated medium. However, discs removed from all inoculated vessels were slightly smaller, and their edges were less sharp than discs removed from uninoculated vessels. This was assumed to represent agar digestion by the bacterial cultures. Nevertheless, this did not obscure the main result that LPS apparently was being degraded under anaerobic conditions.

To avoid this disintegration of the matrix, LPS was therefore incorporated into silica gel discs^{to} provide a more inert matrix to hold the LPS. However, this presented the problem already cited, of LPS not giving opacity to such discs even at 5mg/ml. Various dyes were tested for their ability to stain LPS-containing discs :- Sudan Black for lipid, aqueous methylene blue for carbohydrate and the carbocyanine dye of Janda and Work (1971) for LPS. Only Sudan Black effectively stained LPS within gel discs. However, although LPS-containing discs could be distinguished from non-containing discs, the difference was not clear enough to give a usable test. Further experiments showed that the discs containing 500µg/ml of LPS could satisfactorily be stained with silver by the method of Tsai and Frasch (1981), and this was used.

After inoculation and incubation at 25°C for 7 days (sediments MMS1 and MMS3 only), discs were removed and stained for LPS. The amount of LPS diminished (as determined by staining ability) to levels similar to silica gel itself. Control, LPS-containing gels in uninoculated media retained normal stained appearance (Table 21).

TABLE 21

Biodegradation of LPS, incorporated into silica gel discs, by anaerobic
broth cultures of marine bacteria.

| Content and treatment of disc | Intensity of staining after incubation | | |
|--|---|--------|---------|
| | 0 | 7 days | 14 days |
| Plain silica gel uninoculated | 0 | 0 | 0 |
| Silica gel + LPS uninoculated | ++ | ++ | ++ |
| Plain silica gel inoculated with MMS1 | 0 | 0 | 0 |
| silica gel + LPS inoculated with MMS1 | ++ | ± | ± |
| Plain silica gel inoculated with MMS3 | 0 | 0 | 0 |
| silica gel + LPS inoculated with MMS3 | ++ | ± | ± |

KEY : 0 colourless
+ very faint brown
++ dark brown

In order to determine whether 7 days incubation was indeed required to bring about this extent of degradation, discs were removed and stained each day after incubation over a period from 1 to 7 days. Although intermediate levels of staining intensity were found, results were erratic and difficult to reproduce. Similarly, attempts to construct an index of the LPS content of discs, by incorporating graded amounts of LPS in discs followed by staining were only moderately reproducible. This was perhaps due to the fact that each group of replicate discs had to be stained in isolation from other discs, and thus it was difficult to apply an identical treatment to each group of discs. However, it was clear that no LPS remained in the discs after 3 days incubation in media inoculated with either sediment MMS1 or MMS3 but remained in discs in uninoculated media for at least 14 days.

To test whether the loss in LPS-content of the discs was due to an alteration of the pH of the medium caused by bacterial growth, which may have caused relatively unaltered LPS to leave the gel matrix, the pH of the culture fluid was monitored over the period of 1 to 7 days after inoculation. The pH dropped gradually from pH 7.3 to pH 6.0. Discs incubated in uninoculated medium adjusted to within this pH range stained normally.

DISCUSSION

GENERAL DISCUSSION

The overall conclusion from these studies is that although bacterial lipopolysaccharides are biodegradable, and perhaps readily biodegradable under the right conditions, to demonstrate the phenomenon by simple and convincing methods is difficult. It is certainly much more difficult than to show biodegradation of lipids such as tributyrin or polysaccharides such as starch. Thus a variety of biochemical and cultural procedures which convincingly demonstrated the activity of lipases, glycosidases, proteases and DNases were unyielding of reproducibly positive results when applied to LPS.

The first part of the study focussed attention on trying to demonstrate biodegradation of LPS in extracts of digestive glands from five species of marine molluscs. These animals were chosen because their mode of nutrition suggested that bacteria might contribute to their diet and thus they might contain enzymes capable of degrading LPS. Because particular attention was paid to degradation of the lipid A moiety of LPS, which is the most conservative sub-structure in the molecule, well-characterised LPSs from Salmonella spp. and E.coli were used in preference to the less well-studied and less freely available LPS from marine bacteria.

The second part of the study investigated the biodegradation of LPS by marine microorganisms. Although various shoreline environments were sampled, emphasis was given to the study of sediments contaminated with sewage pollution which should provide a large reservoir of potentially biodegradable LPS and could sustain a large population of LPS-degrading bacteria. Unfortunately, although it was apparent that such bacteria were indeed readily isolated, conclusive evidence of their affect on LPS could not be convincingly demonstrated.

Degradation of LPS by Marine Molluscs

All of the marine mollusc digestive gland extracts tested and the gut juice of the terrestrial mollusc H. pomatia had the ability to degrade the polysaccharide moieties of LPSs from three different bacteria. The amount of reducing sugar released from each LPS type was proportional to the content of polysaccharide. Thus extracts released most reducing sugar from the most polysaccharide-rich LPS (S. minnesota) and least from the LPS with lowest polysaccharide content (E. coli O111:B4). H. pomatia gut juice is rich in glycolytic enzymes (Holden and Tracey, 1950) but a previous study stated that it did not degrade the polysaccharide moieties of various LPSs, although there was inter-batch variation in this commercially supplied enzyme source (Saddler et al., 1979b).

Although initially reaction mixtures were incubated for 24h it was found that for Mya fraction 2 at least, this could be shortened to 3h and thus in any further experiments to determine other characteristics of the hydrolysis, such as optimum temperature and ionic strength, the incubation time could be shortened appropriately. The rate and extent of this reaction (500µg glucose equivalents /4mg LPS, released after 3h) were very similar to that obtained by Reiger-Hug et al. (1977) from the depolymerisation of LPS from several Smooth enterobacterial strains by bacteriophages. They found that depolymerisation reached a peak after 3h and the amount of released glucose equivalents ranged from 200-500µg/14mg of LPS after incubation with $2-4 \times 10^{10}$ pfu of purified phage particles. However, the extent of liberation of reducing sugars from LPS by Mya and all other extracts tested was considerably greater than that determined for the degradation of Salmonella anatum LPS by phage ϕ^{15} (Takeda and ~~W~~etake, 1973); purified phage particles (5×10^{10} pfu/ml) released only ~70µg reducing sugar equivalents from 6.5mg of LPS after 12h, after which there was no further degradation. However, phage particles depolymerise LPS to di and tri repeating units (Chaby and Girard, 1980), whereas the extracts

prepared here degraded LPS to the monosaccharide level which should thus contribute more to the released reducing material.

Bacteriophages are the most widely used source of enzymes which are glycolytic towards LPS; such enzymes have potentially useful applications. They have been used to produce non-toxic LPS haptens which were conjugated to proteins thus providing vaccines against Gram-negative bacteria. Protection against Salmonella typhimurium infection in cattle has been achieved by this method (Svenson and Lindberg, 1980; 1983). However all conjugates thus far examined have been strain-specific and thus have no general applications. Due to the relative uniformity of structure, the core region would provide a better hapten, however no phage associated enzymes have been shown to be active against this moiety (Lambden and Heckels, 1982). Chemically released core structures from N. gonorrhoeae were immunogenic when conjugated to protein but their protective ability was not tested (Lambden and Heckels, 1982). The sequence of monosaccharides in the O-chain of Shigella flexneri LPS was determined by the study of oligosaccharides derived by phage-mediated enzymatic hydrolysis of LPS (Carlin et al., 1984). Thus the glycolytic activities of the marine molluscan extracts, may be more useful than phage-associated enzymes since they have demonstrably higher activity.

The comparative activities of each extract varied depending on whether activity was expressed per mg of lyophilised material or per mg of protein. When compared as per mg of protein, their specific activities were generally higher than the commercial source of glycosidases, H. pomatia gut juice. Thus the glycosidases from marine bivalves may have commercial potential, since they are also stable to lyophilisation. This has previously been suggested by Santoro and Dain (1981).

It is difficult to assess which of the bivalves may have the most potential for the purification of LPS-degrading glycosidases. The extracts contained

quantities of polysaccharide, although it is not known whether this is covalently linked to protein having enzymatic activity. Hence some or all of the extracts may have contained glycoprotein enzymes and in others, the sugar component may have been non-covalently linked contamination. Thus the latter type of extract would have potential to increase in activity by purification, whereas a glycoprotein-rich extract would not have this potential. However Mya fraction 2, which was isolated as a protein rich fraction, in considerable quantities and was of high activity, was the most promising of the molluscs tested, for future work.

Degradation of S. minnesota LPS released glucose, galactose and/or N-acetylgalactosamine and N-acetylglucosamine with glucose being the major monosaccharide product. In this LPS glucose is present only in the core-oligosaccharide, where it is not present as a side-chain residue (Figure 3). Thus release of the glucose residue distal to lipid A should require cleavage of the linkage unit of the O-chain to the core region and subsequent release of N-acetylglucosamine in equimolar quantities to glucose. Release of the glucose residue proximal to lipid A should release similar amounts of galactose. N-acetylglucosamine was not as abundant as glucose, but standard N-acetylglucosamine stained poorly by the method used and hence it may have been present in higher quantities than was apparent. Due to the heterogeneous nature of the hydrolysis mixtures, and the low concentration of monosaccharides, specific colorimetric assays were not carried out because of the expected cross-reactions.

Most Smooth LPS preparations are heterogeneous and contain biosynthetic precursors of complete LPS (Tsai and Frasch, 1981). Thus Rb and Rc chemotypes (Figure 3) which would be expected in an LPS extract, even from a Smooth strain, would allow the release of glucose without concomitant release of other sugars. The finding of galactose and/or N-acetylgalactosamine and N-acetylglucosamine indicated degradation of the O-chain (Figure 2). Unexpectedly, two sugars were also released which could not be identified as belonging to this LPS.

It is possible that they are present only in small amounts and were more labile to the usual degradative procedures used in analysis. For example, colitose and abequose are particularly sensitive to such treatments (Edstrom and Heath, 1965; Svenson and Lindberg, 1983). The purity of the LPSs used in the present study was greater than 98%, but the identity of any impurities was not determined (personal communication, Sigma Chemical Company Ltd.). Thus it is unlikely that contaminating polysaccharide of nucleic acid was the source of the unidentified spots. Alternatively, the spots might have represented breakdown products of known sugars which had been released from LPS.

Molluscan enzyme hydrolysis of E. coli O111:B4 LPS released glucose and N-acetylglucosamine as the main monosaccharide products. Although no standard colitose was available to help to detect its release from LPS, it was concluded that no sugars with similar expected migratory pattern were present. Thus it is unlikely that most of the monosaccharide products were derived from the core-oligosaccharide which is basically the R3 type (Figure 4) but may contain residues attached to the heptose residues. It can be noted that this core contains chemical linkages which are identical or similar to some found in S. minnesota LPS core : $\text{Glc} \xrightarrow{\alpha 1,3} \text{Hep}, \text{Glc} \xrightarrow{\alpha 1,2} \text{Glc/Gal}$ and $\text{GlcNac} \xrightarrow{\alpha 1,3/2}$ linked. Thus the release of glucose and N-acetylglucosamine from these LPSs may be the result of similar enzymes. A sugar migrating as rhamnose was detected as a hydrolysis product but has not been reported in this particular strain of E. coli although it has been found in the core region of other E. coli strains (Figure 4, K-13).

Glucose was the major monosaccharide product released from Meth. methylo-
trophus LPS by all extracts. Although the structure of this LPS has not been well characterised, it contains glucose, galactose, mannose, 2-deoxyglucose, fucose, rhamnose and N-acetylglucosamine as the main saccharide components (Collins, 1984).

The pH optima of the extracts fell into the expected range, with most fractions having a peak at pH 4.5. All three LPSs had shown similar extents of degradation at particular pH values by each extract. The scallop Placopecten magellanicus (not used here) is reported to have different pH optima for individual digestive gland enzymes (Wojtowicz, 1972) :- α -glucosidase (pH 4.0), β -glucosidase (pH 4.5), β -galactosidase (pH 4.0), laminarinase (pH 5.5) and chitinase (pH 4.5). This may suggest that a limited number of enzymes was involved in LPS degradation. Alternatively, it is possible that optimum LPS degradation was detected at a particular pH which caused a conformational change in the LPS molecules or an alteration in their aggregation, thus rendering certain glycosidic bands more susceptible to enzymatic hydrolysis.

It was remarkable that only Modiolus fraction 1 had demonstrable endoglycolytic activity towards LPS. The tetrasaccharide products from E. coli O111:B4 and S. minnesota LPS could not be further characterised and it is not known from which portion of the respective LPS that they were derived, or whether each tetrasaccharide actually consisted of a series of different structures with similar m.wt. Due to the similarity of the products released from both LPSs, it is probable that the core-oligosaccharide was the site of attack since there is some structural homology (Figure 3, Ra; Figure 4, R3).

Previous investigators have reported that the polysaccharide moieties of LPSs are highly resistant to degradation by commercially available glycosidases. Thus Lehrer and Nowotony (1972) found that the LPS of Serratia marcescens O8 was resistant to lysozyme, chitinase and α and β -amylase. Also cellulase, which should have hydrolysed the terminal β 1-4 linked glucose unit of the O-chain was inactive (Figure 2). Duncan and Morrison (1984) found that the polysaccharide portion of E. coli O111:B4 LPS was undegraded after its uptake by murine macrophages (although lipid A was degraded). Physarum polycephalum

is known to be rich in glycosidases including α and β -glucosidases and α and β -galactosidases and N-acetylglucosaminidase (Kilpatrick and Stirling, 1977) but did not degrade the polysaccharide moieties of LPSs (Saddler *et al.*, 1979a). It is possible that the resistance of O-chains to degradation is due to their side-chain residues. Edstrom and Heath (1967) found that the α 1-4 linked glucose residue of E. coli O111:B4 LPS O-chain (Figure 2) was resistant to α -glucosidase due to adjacent colitose and N-acetylglucosamine residues, although β -N-acetylglucosaminidase had previously been shown to efficiently release N-acetylglucosamine efficiently from tetrasaccharides lacking the α 1-6 linked colitose residues (Edstrom and Heath, 1965). Moreau *et al.* (1984) reported that the core-oligosaccharide of Bordetella pertussis LPS having a terminal β -D-glucose residue was particularly resistant to β -glucosidase. Despite repeated attempts by these investigators, only about 10% of the glucose present was released. Two core-oligosaccharides of Shigella sonnei with terminal α -galactose residues were highly resistant to α -galactosidase. Although dephosphorylation rendered one of the oligosaccharides susceptible to the enzyme, the other remained resistant, possibly due to it having an adjacent trisaccharide unit side-chain (Gamian and Romanowska, 1982).

Thus polysaccharide moieties may resist degradation because of steric hinderance by side-chains or, in the case of core-oligosaccharides, be rendered resistant by phosphate groups. The presence of acidic sugars inhibits the action of glycosidases (Levy *et al.*, 1964). Teichoic acids are phosphorylated polymers found in Gram-positive bacterial cell walls and there have been few reports of their biodegradation in nature (Duckworth, 1977). As will be discussed, there may have been some correlation between the ability of extracts to dephosphorylate and to hydrolyse sugars from LPS.

Although apparently only S. minnesota LPS degradation released monosaccharides from the O-chain, it is not known if the O-chain of Meth. methylo-
trophus LPS was degraded since there is less information available on its

structure. Although no positive evidence for E. coli O111:B4 LPS O-chain degradation to the monosaccharide level was detected, analysis of enzyme hydrolysates of LPS by TLC for both monosaccharides and oligosaccharides revealed streaks of unresolved saccharides, probably representing the products of random endoglycolytic activity.

Thus the extracts described here compare favourably, in potency against the polysaccharide moieties of LPSs, with any other cell-free activities previously described. It is possible that even more extensive breakdown of the polysaccharide moieties could have been detected if the extracts had not been partially fractionated by the use of AS. Thus an enzyme present in one fraction may have been active against a substrate that was a product of enzymatic degradation of LPS by an enzyme that was present in another fraction.

Since most fractions did not release oligosaccharides from LPSs, it must be concluded that degradation was by the action of exoglycosidase. The exception to this was a fraction from Modiolus. The endoglycolytic activity in this extract may have potential applications in the preparation of artificial vaccines against Gram-negative bacteria. However the activity was weak and a better source of enzymes may be bacteriophage. By isolating bacteriophage which are specific for certain fish pathogens, LPS O-chain or core-oligosaccharide would be typically degraded by phage-associated endoglycolytic activity and the derived oligosaccharide could be isolated; conjugated to protein, and used as vaccines against bacterial infection of farmed fish.

In experiments to detect the release of KDO from LPS, no clear evidence of such release was found. KDO determination by the TBA assay is sensitive to various interfering substances (Weissbach and Hurwitz, 1959; Wahlström et al., 1984). In particular, deoxysugars cross-react, and give an absorption peak at 532nm (Waravdekar and Saslaw, 1959), and a KDO derivative (2-Keto-3-deoxy-

ctitol) is known to have an absorption peak at 532nm (Moreau et al., 1984) and thus may represent a post-hydrolytic product of KDO. The sensitivity of the chromophore to boiling has been reported by Brade et al. (1983a) who suggested that for each LPS, the KDO value reached a maximum under particular chemical hydrolysis conditions.

Although no readily detectable KDO was released, it is possible that KDO-containing linkages were hydrolysed. It is known that KDO residues that are substituted at position C4 or C5 react weakly in the assay. Thus liberated KDO side-chain disaccharides (Figure 3) would react weakly, as would molecules bearing KDO trisaccharide that had been released by cleavage at the point of linkage to lipid A (Banaub et al., 1983).

All marine invertebrate extracts tested, apart from Modiolus had very strong phosphatase activity towards LPS, in pH 4.0 buffer. Phosphorus normally constitutes between 1.5-2.5% of LPS (Le Dur et al., 1978; Kuwae and Kurata, 1983). Mya extract released almost 100% of the phosphorus in S. minnesota LPS. E. coli O111:B4 LPS contains relatively low levels of phosphorus (Morrison and Leive, 1975) and less phosphorus was released from this LPS by all extracts than from S. minnesota LPS (Table 8).

Although all extracts released phosphate from LPS, they were not tested for similar activity on purified lipid A. It has been suggested that mono-phosphoryl lipid A preparations are non-toxic (Amano et al., 1982). Thus a purified phosphatase preparation that specifically removed the glycosidically linked phosphate at position C1 of the diglucosamine backbone may have potential applications in the detoxification of various preparations that are contaminated with endotoxin. Phosphatases have been used on purified LPS sub-structures. Hase and Rietchel (1977) used alkaline phosphatase to remove the ester-linked phosphate at position C4 of biphosphoryl glucosaminyl glucosaminitol disaccharide. Rosner et al. (1979b) reported that only negligible amounts (~6%) of inorganic

phosphate was released from whole LPS by alkaline phosphatase under conditions which resulted in dephosphorylation of LPS derivatives which lacked esterified fatty acids. Phosphate at C1 became sensitive to phosphatase treatment after enzymatic removal of the adjacent amide linked 3-hydroxytetradecanoic acid residues. The resistance of the C1-phosphate to hydrolysis by phosphatase has also been observed for Salmonella lipid A precursor (Rick et al., 1977) and may be due to steric hinderance. Verret et al. (1982b) used alkaline phosphatase to remove C4¹-phosphate from a lipid A derivative lacking esterified fatty acids.

It is worth noting that the relative phosphatase activities of each extract paralleled their relative glycosidase activities towards LPS. Gamian and Romanowska (1982) found that Shigella sonnei LPS core-oligosaccharide was rendered sensitive to α -galactosidase only after desphorylation. Thus it is possible that the ability to dephosphorylate the core region of LPS may be a pre-requisite for the further biodegradation of the molecule. This is best exemplified by comparing the glycosidase and phosphatase activities of Mya and Modiolus fractions¹(Figure 11, Table 8). Degradation of S. minnesota LPS appeared to release monosaccharides from both the O-chain and core region, whereas degradation of E. coli O111:B4 LPS released monosaccharides only from the latter. Modiolus fraction 1 (which was low in phosphatase) degraded S. minnesota LPS 6 times more effectively than it degraded E. coli O111:B4 LPS. Mya fraction 1 (high in phosphatase) degraded S. minnesota LPS only about twice as effectively as it degraded E. coli O111:B4 LPS. This suggests that the degradation of those LPSs which have a high proportion of core-oligosaccharides, is enhanced by phosphatase treatment. Such sequential enzymatic degradation of LPS may be indicative of the specialisation of the marine bivalve digestive system to the breakdown of this particular bacterial polymer. Mytilus edulis digestive gland contains enzymes that readily degrade DNA, which is also rich in phosphate (Birkbeck and McHenry, 1982).

It was expected that free ethanolamine would be released from LPS by at least some extracts since the molecule is normally linked to LPS in the form of biphosphorylated ethanolamine. Hence, if the majority of phosphate residues were released in the free form, some ethanolamine should have been detected. However, the detection method used was relatively insensitive and even purified standard ethanolamine was not readily detected unless applied in relatively large quantities (10 μ g). By providing more LPS substrate, (10mg/ml) it is possible that free ethanolamine might have been detected.

At least some proportion of the nutrition of marine filter-feeding bivalves is derived from Gram-negative bacteria. Thus, they may produce enzymes that release fatty acids from the lipid A moiety of LPS as this would both substantially degrade free LPS, and may also release LPS from the outer membrane of whole bacteria. In order to detect such enzymes, several marine molluscs were tested for the ability to release fatty acids from LPS.

Extensive studies with crude digestive gland homogenates from 5 different species of marine invertebrates revealed that fatty acids were not readily released from LPS but that Cerastoderma extracts provided most promise for further work. The incubation conditions were those that had been used by Saddler and Wardlaw (1979b) for LPS degradation by the mollusc H. pomatia. However, even this commercial snail gut juice released only small amounts of fatty acids from LPS. The possibility of batch to batch variation cannot be excluded as an explanation as to why these preparations did not have pronounced activity against LPS in the present work.

The ability of Mytilus digestive gland extracts to lyse whole bacterial cells was reported by Birkbeck and McHenry (1982). Their method when applied here yielded extracts from Cerastoderma digestive gland and crystalline style that were lipolytic towards whole heat-killed cells of E. coli, with the crystalline style extracts having weak activity. Reid (1968) also found

crystalline style lipases to be weak, but George (1952) reported this to be the strongest source of lipase in bivalves. In preparing the cells of E. coli, care was taken to avoid bacteriolysis prior to lipase estimation, hence it is possible that at least some of the fatty acids released were derived from LPS, since the accepted model of Gram-negative outer membrane architecture (Smit et al., 1975, Figure 6) suggests that the outermost leaflet of the membrane contains little or no phospholipids, thus the LPS-fatty acids would be the most accessible to lipases. When tested against free LPS of S. minnesota, these extracts were however only weakly lipolytic.

It is normal practice to use detergents as substrate-dispersants in the assay of lipases (Jürgens et al., 1981). In an attempt to increase the access of lipolytic enzymes to lipid A fatty acids various detergents were examined in these studies but were found to be ineffective in increasing lipolysis of LPS by the extracts. Hall and Mumford (1983) found that the deacylating activity of extracts from human neutrophils towards LPS from S. typhimurium was increased 3-5 fold by the addition of Triton X-100 (0.1%, v/v) to the incubation mixture. However, they used an R-mutant in which the LPS would have been very hydrophobic compared with the Smooth LPS used here. Evidence for fatty release from LPS without the use of detergents has been obtained by several other workers (Voets et al., 1973; Nigam et al., 1970; Verret et al., 1982 and Saddler et al., 1979b)

Mild alkaline treatment is known to release some ester-linked fatty acids from LPS, leaving a structure which might be more accessible to lipases (Warren et al., 1977). However, it is not known if specific fatty acids are released by this method, which may actually diminish the apparent activity of an enzyme extract towards LPS if it had specificity towards the fatty acids that were lost. Alternatively, the treatment may randomly

release fatty acids, producing an even more heterogeneous mixture of lipid A structures than is normally encountered, but with the total fatty acid content lowered. Extracts from Cerastoderma and H. pomatia had low activity against this substrate. However, it was noted that the molluscan extracts and the LPS prior to incubation had fatty acid contents greater than would be expected upon addition of both materials, suggesting that perhaps some fatty acid release had taken place before the 'non-incubated' control samples could be terminated (by the addition of chloroform).

Prior to testing AS fractionated preparations from marine molluscs, their activity towards tributyrin was characterised. Preliminary experiments showed that activity increased with decreasing salt concentration (from 3.5% NaCl). Thus further experiments were carried out in 0.05M buffer. Although the glycosidase activities of the molluscan extracts had acidic pH optima, the lipase activities were most potent in the pH range 7-8. This is in agreement with Patton and Quinn (1973) who reported an optimum at pH 8.0 for crystalline style lipase from the surf clam Spisula solidissima.

In regard to species differing in lipase activities, Reid (1968) reported that Mya had strong lipase activity whereas Pecten was very weak, Modiolus and Cerastoderma were intermediate. In the present study Mya had highest specific activity of those tested whilst Modiolus, Cerastoderma (and Mytilus) had intermediate activities but Pecten had none (although fraction 3 was not tested). Thus there is good agreement with this previous report and the present work.

When the most active fraction from each bivalve, along with commercially supplied lipase from Candida cylindracea and H. pomatia gut juice were tested for the released fatty acids from LPS, only Cerastoderma, and the two commercial products, released fatty acids from the LPS of E. coli O111:B4 or S. minnesota.

There were similar amounts of fatty acids released from both LPSs by each active preparation, E. coli O111:B4 LPS might have been expected to give greater fatty acid release since it has a higher lipid content but this was not found. The active preparations released exclusively non-hydroxylated fatty acids from LPS. Thus their mode of action was to release the ester-linked acyl residues of amide and ester-linked acyloxyacyl residues. These residues are more abundant in S. minnesota lipid A than in E. coli LPS (Figure 5). This may compensate for S. minnesota LPS having less overall lipid content.

Acyloxyacyl residues have been reported in the lipid As of several Gram-negative bacterial LPSs (Wollenweber et al., 1984). The granule fraction of human neutrophils deacylated S. typhimurium lipid A by removing the nonhydroxylated fatty acids, leaving almost all of the 3-hydroxytetradecanoyl residues linked to glucosamine (Hall and Mumford, 1983) thus having similar activity to Cerastoderma digestive gland and H. pomatia gut juice reported here, and to P. polycephalum extracts (Saddler et al., 1979b) towards LPS.

C. cylindracea lipase, which was the most potent against tributyrin, was weakly effective against LPS. Conversely, Cerastoderma was of moderate potency towards tributyrin among the bivalve extracts tested, but was active against LPS, while Mya extract which was the strongest, had no effect on LPS. Lehrer and Nowotny (1972) used 6 commercially available lipases at their optimum conditions against LPS from Serratia marcescens O8 and found no evidence for fatty acid release by gas-liquid chromatography. Thus there is a high degree of specificity in lipases that are active against LPS. Most of the reports of biodegradation of LPS with release of fatty acids by eukaryotic enzyme extracts have come from sources where it would be expected that the experimentally found release of fatty acids should have a physio-

logical role e.g. the phagotrophic slime moulds D. discoideum and P. polycephalum (Nigam et al., 1970; Saddler et al., 1979a), where they may provide nutrition (although it has been suggested by Verret (1984) that in vivo, the function may be detoxification) or in the liver and spleen of mammals (Freudenberg et al., 1984, where a role in detoxification would be more likely). Thus it appears that commercial lipases (e.g. the wheat-germ lipase) do not degrade lipid A and it can be speculated that Cerastoderma may have a physiological use for fatty acids from bacterial LPSs.

Potentially, assuming the suggested mode of attack on lipid A is correct, Cerastoderma extracts might be expected to degrade lipid A to the level of the chemically synthesised structure in Figure 7 (406), which would exhibit most of the spectrum of endotoxic properties except for pyrogenicity or Schwartzman reactivity (Galanos et al., 1984). In order to bring about complete detoxification of lipid A by enzymatic means, 3-hydroxytetradecanoyl residues would have to be removed. Although this activity was not detected in Cerastoderma extracts, it is possible that further investigation may yield alternative enzymatic activities towards lipid A. Extracts from D. discoideum, although having mainly amidase activity towards lipid A of E. coli, contained esterase activity towards this substrate (Verret et al., 1982a). Conversely, P. polycephalum extracts released non-hydroxylated fatty acids from lipid A, although the possibility that ester-linked 3-hydroxytetradecanoyl residues were also released could not be ruled out (Saddler et al., 1979a). Hall and Munford (1983), although finding that the major activity of the granule fraction of human neutrophils towards LPS was the removal of nonhydroxylated fatty acids, found that removal of 3-hydroxytetradecanoic acid also occurred, although at a much slower rate.

Despite the numerous reports of chemical detoxification of endotoxins, these methods are too severe to be applied to the removal of endotoxins in materials where its presence is unwanted, such as biological fluids for intravenous injection. Although various methods of physically separating endotoxins from biological fluids are available and should be adequate for the removal of endotoxins from most parenteral solutions, endotoxins are 'sticky' and bind to some protein preparations for intravenous injection, such as human growth hormone, produced in E. coli (Axelsson, 1985).

Thus a highly purified enzyme preparation could be immobilised by an appropriate technique (Sundaram et al., 1972) and would have useful applications where endotoxin elimination is particularly critical. Likely sources of such enzymes may be Cerastoderma digestive gland and H. pomatia gut juice (described in this investigation) or D. discoideum, which has amidase, fatty acyl esterase and phosphatase activities against LPS, and from which two amidases have already been purified (Verret et al., 1982a).

Enzymatic release of hydroxy fatty acids from lipid A has also been suggested as a potential improvement to the method of Maitra (1978) for the detection of LPS in serum; Verret (1984) suggested that due to the specificity of such enzymes, artefacts encountered by chemical release would be avoided.

Degradation of LPSs by Marine Bacteria

Saddler and Wardlaw (1980) isolated three strains of marine bacteria which degraded LPS. They used anticomplementary activity, haemagglutination inhibition and KDO as indicators for lipid A, O-chain and core-oligosaccharide degradation respectively. Although two of these particular isolates were provided to me in pure culture, they were found to show

poor growth in the medium described by the authors for their isolation. The Gram-positive Bacillus sp. (strain A) underwent sporulation in the recommended medium, suggesting that the medium was of low nutritive value to the organism (although this may have been an indication that the organism had utilised all the available LPS derived nutrients). However, both strains were able to multiply to the extent that they could be repeatedly sub-cultured in seawater containing LPS as the sole carbon source.

AS extracts of culture supernates of the organisms failed to degrade LPS, however it is possible that the activity was cell-associated. Cortez (1976) and Apicella et al. (1978) used whole-cell extracts to detect LPS degradation in vitro, although the latter authors suggested an extracellular role for the degradative enzymes.

When the bacterial isolation procedure of Saddler and Wardlaw (1980) was followed, three separable microorganisms (by colony morphology) were obtained. However after culture of these organisms in ASW-LPS medium, no alteration could be detected in the LPS that remained in the supernate after the removal of cells by centrifugation.

Shoham et al. (1983) studied the biodegradation of the bacterial polymer emulsan, which has a hydrophilic heteropolysaccharide moiety linked to hydrophobic fatty acids which are amide and ester-linked, and is thus very similar to LPS. They found that a mixed population of bacteria capable of degrading it was readily obtained by enrichment. However, extensive screening was required to isolate an emulsan-degrading strain in pure culture. This strain, when it was isolated, was found to be a minor constituent (< 1%) in the mixed population from which it came. It grew very slowly on nutrient agar and formed small colonies only after 4 to 5 days. Thus it is perhaps likely that although, in the work

reported here, three different colony types were found, there may have been at least one other organism present which was responsible for degrading LPS. Alternatively, LPS degradation may have required the cooperative growth of a mixed community of degradative organisms such as has been demonstrated with various recalcitrant molecules such as ioxynil (Itsu and Camper, 1976) and monosubstituted phenols (Shimp and Pfaender, 1985). Although preliminary experiments using a mixed community of the three isolated colony types did not support this explanation, it is possible that the original mixed population of LPS degrading organisms contained several other bacterial types which had not grown sufficiently to form visible colonies.

Lehrer (1971) isolated by enrichment from soil, several microorganisms which could utilise LPS as their sole organic nutrient. These bacteria and fungi were able to grow in LPS mineral salts medium, but culture filtrates did not reduce endotoxin lethality. The effect on other pharmacological activities was not tested.

Attempts were made in the present investigation to isolate, specifically, bacteria from marine sediments which degraded LPS by attacking the fatty acids of the lipid A moiety. Preliminary plating out on tributyrin agar, without enrichment, provided ten isolates which were lipolytic towards this substrate. However, only one of these isolates (P8) had convincing activity towards LPS as manifested by the production of clear zones around the purified lipid A of S. minnesota incorporated into agar and inoculated with the isolates.

When this Gram-negative bacterium was incubated in ASW-LPS liquid medium with small amounts of other nutrients, the amount of alkali-releasable fatty acids in the LPS diminished after growth of the organism. Thus P8 had similar activity to B. marcerans isolated by Voets (1973) and strains A and C

isolated by Saddler and Wardlaw (1980) in that the main mode of action was to release fatty acids from LPS.

No lipase activity towards lipid A or LPS was detected in AS extracts of culture supernates from this organism, but the extracts did have lipolytic activity towards tributyrin. This may have been an expression of the same enzyme activity which at a higher concentration would be active against LPS or lipid A.

Several bacteria were isolated from marine sediments which were capable of lysing heat-killed cells of an unidentified Gram-negative marine bacterium. The emulsan-degrading isolate of Shoham et al. (1983) degraded cell-bound emulsan, producing a zone of lysis similar to that observed with the isolates described here. However, when LPS or lipid A was prepared from the degraded bacterium and incorporated into solid medium, no zones of clearing were detected when any of the isolates were tested. Whiteside and Corpe (1969) isolated a Pseudomonas sp. which had bacteriolytic activity against whole cells or cell envelopes of the Gram-negative organism Chromobacterium violaceum. Several enzymes were extracted from culture supernates of the Pseudomonas spp. which were capable of releasing fatty acids, carbohydrate and protein from cell-envelopes. It was suggested that phospholipids were the main source of the fatty acids and that since no heptose was present in the released carbohydrate, that the core-oligosaccharide of LPS was not degraded. The O-chain was not considered by the authors as a potential substrate for the degradative enzymes.

As an alternative to the serial subculture method for isolating LPS-degrading bacteria, the production of clear zones around bacterial colonies on LPS-agar plates was considered. However this would require large quantities of LPS, since to produce opacity by adding LPS to agar, approximately $3\text{-}5\text{mg/ml}^{-1}$ is needed. Thus a cheap and convenient source of LPS was required.

Possible source materials for LPS are heavily contaminated marine muds,

raw sewage, cattle faeces and Gram-negative bacterial cell pastes. However, it was considered that 'Pruteen' (Meth. methylotrophus cells) which was provided free by I.C.I., was easy to handle, was of consistent quality and was available in sufficient quantity should be the most suitable.

Extraction of 'Pruteen' by the phenol/water method was disappointingly inefficient. Since it is known that the method of Galanos et al. (1969) may be more favourable for some LPSs, this method was also applied, but even lower yields of LPS were obtained (Table 17).

Thus the Westphal method was preferred, as the poor percentage yield could be counteracted by using large amounts of starting material. An alternative method using EDTA was also used (Leive and Morrison, 1972). It was quicker than the phenol/water method but provided less pure LPS preparations (Table 17). These were used only in large-bulk liquid cultures.

EDTA-extracted LPS preparations were heavily contaminated with nucleic acid, which was unexpected under the conditions employed. This result may have been a peculiarity of 'Pruteen'. When attempts were made to concentrate and simultaneously purify the aqueous extract by Amicon ultrafiltration through a 100,000 D filter, there was an apparent increase in the nucleic acid content of the material. This was due to the aqueous extract being 'enriched', by ultrafiltration, for higher molecular weight material. Most of the remaining nucleic acid contamination could be removed by ultracentrifugation. Similarly with LPS extracted by the Westphal method, nucleic acids (which were relatively low in the crude extract) could be removed to acceptable levels (< 2.5%) by ultracentrifugation.

When 'Pruteen' LPS was extracted, purified and incorporated into solid medium at 4mg/ml^{-1} , the agar was made opaque by the LPS. On this medium, several marine bacterial isolates produced zones of transparency around the areas of growth. This was presumed to represent LPS degradation. All these

strains were Gram-negative.

Marine sediments from the Isle of Cumbrae and Helensburgh were abundant in such LPS-degrading bacteria. However, none was isolated from the Dunstaffnage sample.

Four different sites were sampled from the Isle of Cumbrae (Table 19). Samples MMS1 and MMS2 were from environments that might be expected to be high in saprophytic microbial activity, and thus may have been higher than samples MMS3 and MMS4 in readily degradable carbon substrates such as amino acids, lipids and monosaccharides. Other workers (Horvath, 1972; Shimp and Pfaender, 1985) suggested that a compound that is normally stable and incapable of supporting bacterial growth can be partially degraded, but not for carbon or energy, when a readily degradable secondary source of carbon is also available. Although the experiment was not done strictly quantitatively, it thus may be significant that MMS1 and MMS2 yielded more LPS-degrading bacteria than MMS3 and MMS4. It could also be speculated that many of those isolated, which initially may have apparently been able to grow on medium containing only LPS as the sole nutrient, may lose the ability on repeated subculture on medium lacking more readily degradable substrates. Thus isolates capable of using LPS as their sole organic nutrient may not have been as common as may have been apparent.

Marine sediments HBMS and DMS were from similar environments, but they varied in the abundance of LPS-degrading bacteria, the latter having none that could be isolated despite the fact that identical isolation procedure was followed for both samples. Thus the potential ability of a sediment to degrade LPS cannot be predicted by examination of simple parameters of the environment. A wide variety of factors should influence the biodegradation of LPS in the marine environment. These include temperature, salinity, pH and the availability of inorganic and organic nutrients, oxygen and on the types

of organic materials that the microbial community has encountered in the past (Shimp and Pfaender, 1985). Fine-grained sediments are known to be higher in microbial activity (Zobell, 1938) reflecting biodegradation of detrital carbon associated with these particles (Atlas et al., 1983).

Although my isolates had several other degradative properties, including the breakdown of common contaminants of LPS (nucleic acid, protein), these could not be responsible for the clear zones produced on LPS-agar :- neither material was present in sufficient concentration in LPS preparations to either support growth or produce an opaque background, and not all of the LPS-degrading bacteria degraded DNA or protein.

All isolates grew on tributyrin or tripalmitin as their sole organic nutrient, producing zones of lipolysis, although this does not mean that lipolysis was their mode of action on LPS, it does suggest that they could grow on the products of such activity. Marine bacteria are known to show definite specificity in their lipolytic activities. In an extensive study by Tom and Crisan (1975) on 170 lipolytic isolates, 14 were only active on fish oil, 11 could hydrolyse fish oil and Tween 80 and/or tributyrin and 145 isolates could only hydrolyse one or both of the non-marine lipids. In a study of a fresh-water stream, lipolytic bacterial counts were 100-fold higher downstream from a sewage outfall than was recorded upstream (Blaise and Armstrong, 1977). Nitkowski et al. (1977) found lipolytic bacterial counts to be 4 times higher in marine sediments polluted by sewage than in unpolluted sediment, 76% of the isolates were Gram-negative.

All of my isolates were also capable of degrading starch; however M1 and M5 had relatively weak amylase activity but had full activity against LPS. Purified amylases have no effect on LPS (Lehrer and Nowotony, 1972). Thus the isolates need not necessarily have had a saccharolytic mode of attack on LPS.

When AS extracts were prepared from culture supernates of some of my isolates, lipase against tributyrin could be detected in vitro but no lipolytic activity towards LPS was found. AS extracts did however degrade the polysaccharide moiety of LPS, although the activity was weak. This activity would not be expected to produce a clear zone in LPS in agar, since lipid A, which might be expected as a product, is even more insoluble than LPS and should have produced a zone of opacification.

Voets et al. (1973) showed that when B. macerans degraded LPS in liquid medium containing LPS as the sole organic nutrient, releasing fatty acids and phosphate, these were detected in the supernate. In analagous experiments, reported here, the total sugar content of supernates of cultures of the isolates in ASW-LPS fell by about one half. However, this was not preceded by initial increase in the reducing sugar content of the supernate which would have indicated polysaccharide breakdown and subsequent utilisation. It is possible that the bacteria bind LPS in a high molecular weight form to their cell wall and degradation may be coupled to uptake of the breakdown products.

The most likely explanation for the zones of clearing around growth of the isolates on ASW-LPS agar (Figure 27) is degradation of the polysaccharide moieties. However, since lipolysis would be more likely to cause a clearing of the medium, the effect observed is not fully explicable as LPS degradation.

It is possible that the clear zones that were observed around the growth of bacterial isolates on LPS-containing solid medium was due the activity of a surfactant produced by the isolates. Thus the LPS may have been solubilised, and as evidenced by the growth of the bacteria, may have been subsequently utilized by some unknown mechanism. An intracellular location of degradative enzymes from the isolates was not suspected, since the zones of LPS hydrolysis were several mm from the bacterial growth. Since the apparent LPS degradation was more readily detected on solid medium, it is possible that LPS degradation

required the presence of a solid surface, as suggested by Cortez (1977b).

The use of more sensitive methods for the detection of hydrolysis products from LPS, particularly the use of radioactively labelled LPS, might provide information on the mode of attack of the bacterial enzymes. Although degradation of the polysaccharide moieties of LPS have been reported in vitro by extracts from bacteria, there have been no reports of bacterial enzymes which degrade lipid A with the release of fatty acids in vitro.

Unexpectedly, no anaerobic LPS-degrading bacteria were isolated from any of the marine sediments tested when isolation was attempted on agar plates containing LPS, incubated in anaerobic jars. Since the total numbers of bacteria isolated were lower than those obtained by aerobic culture, it was possible that some sensitive anaerobes failed to survive the isolation procedures. In ocean sediments in the top 3-5cm from water depth of 800m, anaerobic bacteria have however been reported to be 20% less abundant than aerobic bacteria (Zobell, 1938).

To test whether there would be better recovery of bacteria in liquid media, LPS degradation was studied in cultures developed in Robertson's Cooked Meat medium prepared with ASW. To achieve this, LPS was incorporated, initially, into sterile agar discs which were added aseptically to the medium. Diminution of opacity was used as an index of LPS degradation.

After inoculation with marine sediments and subsequent incubation, turbid growth developed. However, although LPS degradation was observed in discs exposed to cultures from two of the four sediments, there was also evidence of digestion of the agar matrix. To overcome this, LPS was incorporated into silica gel discs. Inoculation and incubation of medium containing these discs brought about degradation of LPS as indicated by the silica gel discs failing to stain by the silver technique of Tsai and Frasch (1982).

Although the most probable explanation is that the LPS was broken down to low molecular weight components that were free to leave the gel matrix, it is also possible that relatively unaltered LPS was leached from the gels. However, the role of pH, which is perhaps the most likely source of such an effect was ruled out.

Assuming that LPS was indeed degraded, it would be expected that the polysaccharide moieties should contribute most to the stained appearance of the discs, and thus their degradation may account for the failure of the discs to be stained.

Although not attempted, it would have been expected that sub-cultures from the liquid cultures would have yielded anaerobic LPS-degrading bacteria, although co-metabolic processes may have been responsible and thus the organisms may not degrade LPS when it is supplied as the sole organic nutrient source.

Whether the degradation was due to lipolytic or saccharolytic attack was not determined. Seki (1967) suggested that in anaerobic marine sediments, carbohydrates may act as a hydrogen acceptors for lipolytic bacteria. Saddler and Wardlaw (1980) reported similar extents of degradation of LPS under aerobic and anaerobic conditions, although aerobically, the polysaccharide moieties were degraded more rapidly.

Fogel et al. (1982) incubated the insecticide methoxychlor anaerobically with garden soil and subsequently transferred the incubation mixture to an aerobic atmosphere. Biodegradation occurred at a rate which was as much as 70-fold greater than when it was exposed to solely aerobic incubation. Thus it is possible that in marine sediments, which are subject to water-logging and consequently oxygen depletion, that anaerobiosis may play a role in 'preparing' LPS for degradation under subsequent aerobic conditions.

Although the method used to detect anaerobic degradation of LPS was rather indirect, there was enough indication that some sediments had an anaerobic microbial community which was capable of degrading LPS to warrant further research into characterisation of the types and numbers of micro-organisms involved.

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APPENDIXArtificial Seawater (ASW)

| | |
|---|---------|
| NH_4NO_3 | 0.002g |
| H_3BO_3 | 0.027g |
| CaCl_2 | 1.140g |
| FePO_4 | 0.001g |
| $\text{MgCl}_2 \cdot (6\text{H}_2\text{O})$ | 10.990g |
| KBr | 0.100g |
| KCl | 0.690g |
| NaHCO_3 | 0.200g |
| NaCl | 24.320g |
| NaF | 0.003g |
| Na_2SiO_3 | 0.002g |
| Na_2SO_4 | 4.060g |
| $\text{SrCl}_2 \cdot (6\text{H}_2\text{O})$ | 0.438g |

Distilled water 1,000ml

