Biodegradation of Bacterial Lipopolysaccharides

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Preface

This thesis is the original work of the author.

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SUMMARY

The potential lipopolysaccharide (LPS)-degrading capability of three different systems was investigated. The acellular slime mould Physarum polycephalum was chosen as an example of a phagotrophic microorganism utilizing bacteria as food. Gut juice from the snail Helix pomatia was used because this animal is an example of an invertebrate ingesting materials rich in bacteria. Also the gut juice is a known source of many different enzyme activities. The fate of LPS when incubated with samples of marine mud and sand was investigated because of the abundance of gram-negative bacteria in marine environments, particularly muds.

The main methods used to detect and measure LPS and to obtain evidence of its degradation were: Ketodeoxyoctonate analysis, because of the presence of this substance in almost all LPS preparations; gas-liquid chromatography (GLC) to detect long chain fatty acids; serological methods, including the ability of LPS to sensitize red cells, and haemagglutination inhibition (HAI) tests to demonstrate whether the immunospecific sugars in the oligosaccharide component were still present; and the ability of LPS to trigger complement, as an index of "endotoxic" activity. The anti-complementary activity of LPS was investigated in some detail.

A range of LPS was assayed for anticomplementary (AC) activity against 5 HU_{50} of complement (C) from man, pig and guinea-pig. On average, levels of LPS about 200 times lower were detected with human C than with guinea-pig C and about two times lower than with pig C. There was little variation in C samples from different people in responsiveness to LPS AC activity. Different LPS varied in AC activity over a 10-fold range with each species of C. The rank order of their activities also varied with species of C. With human C, the most active LPS

could be detected down to 2.5 µg. It is suggested that the AC effect of LPS is mediated principally *via* the Alternative Pathway.

Physarum polycephalum appeared to attack only the lipid component of LPS, with a consequent reduction in AC activity and ability to sensitize red cells, while the KDO and HAI values were unaffected. GLC analyses indicated that the lauric, myristic and palmitic acids were split off leaving the β-hydroxymyristic acid still attached to the diglucosamine backbone. Degradation of LPS by gut juice of the snail *Helix pomatia* similarly appeared to affect only the lipid component. When such degraded LPS was analysed on SDS-polyacrylamide gels, one of the characteristic bands of the original LPS was lost or reduced, reflecting loss of fatty acids.

Marine sediments, at different depths and sites, were extracted with phenol-water or trichloroacetic acid (TCA) and yielded more LPS in areas of high organic pollution. KDO was not detected in any of the sediments taken below a depth of 4 cm; AC activity decreased with increasing depth. When killed gram-negative bacteria were incubated with marine sediments, degradation of the LPS was observed. The oligosaccharide component was degraded at a faster rate than the lipid moiety as demonstrated by the loss of KDO and serological specificity. Both amide and ester linked fatty acids of the lipid A were lost.

Most of the previous work on alteration of the LPS molecule has involved chemical treatment. The present studies offer the possibility of more specific procedures. Purification of the *Physarum* and snail gut juice enzyme systems might afford highly selective modes of degradation which would be very useful for increasing knowledge of the structure of

LPS molecules. Also it might be possible to produce altered molecules with different patterns of biological, physiological and immunological properties.

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List of Abbreviations

AC	Anticomplementary
ACU	Anticomplementary unit
AS	Ammonium sulphate
C	Complement
DNA	Deoxyribonucleic acid
EA	Antibody-coated sheep erythrocytes
E-LPS	LPS-sensitized erythrocytes
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycoltetraacetate
ESA	Erythrocyte sensitizing ability
GLC	Gas-liquid chromatography
HAI	Haemagglutination inhibition
HU_{50}, HU_{100}	Haemolytic units based on 50% and 100% Haemolysis endpoints respectively
KDO	Ketodeoxyoctonate
LPS	Lipopolysaccharide
n.t.	not tested
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff
R	Rough (LPS)
RNA	Ribonucleic acid
S	Smooth (LPS)
SDS	Sodium dodecylsulphate
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
VB	Veronal buffer

INTRODUCTION

Brief History of Endotoxin, O-Somatic Antigen and Lipopolysaccharide (LPS)

From cells of gram-negative bacteria can be extracted high molecular weight materials which are more or less water soluble and which always contain fatty acids, divalent cations, phosphorus, carbohydrates and usually other constituents. These substances withstand boiling and after injection into mammals, give rise to striking and varied pathophysiological effects. The characteristic material was called endotoxin because of its association with the bacterial cell rather than the culture fluid. The terms endotoxin, O-somatic antigen and lipopolysaccharide are nowadays used almost interchangeably depending on the focus of interest - whether it is pharmacological, immunological or chemical, respectively.

Panum (1856) is cited by Bulloch (1938) as the first worker to attempt the isolation of the toxin which gave "putrid intoxication." Starting with decomposing tissues, he filtered them clear and then distilled them dry. The activity remained in the residue and could be redissolved in water but not in alcohol; in small doses it produced fever and in high doses, death. The activity was stable to boiling for 11 hours. Billroth (1862) introduced the terms "pyrogen" and "pyrogenic substance" when he stated: "The pyrogenic substances are equally present in dried putrid material and in fresh pus." Billroth also demonstrated for the first time that even distilled water contained a pyrogen, readily detected by the rise in temperature produced on injection into animals. Pfeiffer (1892) was apparently the first to use the term endotoxin (Westphal, 1975). He demonstrated that it was an integral part of the bacterial cell and could be liberated by disintegration of the cells. In the following

year Centanni (1893) extracted endotoxin from typhoid and other organisms, calling his preparations "pirotoxina," to emphasise their high pyrogenic activity. He also stated: "This toxin is ubiquitous to many bacterial genera; it is found in many species, pathogenic as well as non-pathogenic, with always the same properties."

Seibert (1923) showed that fever caused by vaccines and the frequently observed "injection fevers" had a common cause. In systematic investigations she proved that the pyrogenic activity of many substances and injection fluids was due to contamination with traces of bacterial pyrogens. Purification of these pyrogenic and endotoxic substances was greatly advanced by the trichloroacetic acid procedure of Boivin and Mesrobeanu (1933) which was developed primarily to extract O-somatic antigen. Shear and Turner (1943) demonstrated that the tumour-necrotizing component in Serratia marcescens was contained in the endotoxin. This was the first time that the chemical composition was indicated by the name "lipopolysaccharide."

It has only been in the last twenty-five years that the immunochemistry, chemistry and biosynthesis of these complex molecules has been explored in any detail. An historical account of the evolution of the concept of endotoxin or lipopolysaccharide has been provided by Westphal, Westphal and Sommer (1977).

Extraction Of Lipopolysaccharide

As indicated above, the earliest extraction method for endotoxin, in the form of the complete O-somatic antigen, was the trichloroacetic

acid (TCA) method of Boivin and Mesrobeanu (1933). This led to the isolation of the O-antigenic complex containing LPS, protein and phospholipid. Procedures developed by subsequent workers included trypsin digestion followed by TCA extraction (Raistrick and Topley, 1934), anhydrous diethyleneglycol (Morgan, 1937), formamide (Fuller, 1938), urea (Walker, 1940), pyridine-water (Goebel, Binkley and Perlman, 1945), water alone at 80°C (Roberts, 1949) and ethyl ether-water (Ribi, Milner and Perrine, 1959) and others. A comprehensive list of the various procedures has been provided by Nowotny (1963).

The most commonly used method for extracting LPS is the phenol-water method of Westphal, Lüderitz and Bister (1952). This involves treatment of the bacteria with 45% aqueous phenol at 65°-68°C followed by cooling to 10°C. The mixture separates into two phases: an upper 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. There may be some interfacial and sedimented insoluble material. Since LPS exists as large aggregates in water, it can usually be separated from other substances by ultracentrifugation.

Some LPS preparations contain relatively hydrophobic sugar residues or may have lost much of the hydrophilic polysaccharide portion. Such LPS tends to be more soluble in the phenol phase during phenol-water extraction. Extraction with a phenol-petroleum ether-chloroform solvent may be best for the isolation of these LPS (Galanos, Lüderitz and Westphal, 1969). This mild procedure is specific for R (rough) form LPS and glycolipids, and yields preparations in a water-soluble form, minimally contaminated with proteins and nucleic acids. S (smooth) form LPS are excluded from such extracts.

Leive, Shovlin and Mergenhagen (1968) demonstrated that some gram-negative bacteria released about 50% of their LPS when exposed briefly to ethylenediaminetetraacetate (EDTA). These preparations were more immunogenic, and at least as toxic in mice as those obtained by phenol extraction.

Extraction of bacteria with aqueous ether was used by Ribi et al (1961). This method yielded preparations which were distinct from those obtained by the Westphal method, in having exceedingly small amounts of firmly-bound lipid although they showed similar biological activities.

Morrison and Leive (1975) described a milder method for the isolation and purification of LPS involving treatment of aqueous suspensions of bacteria with butanol. This liberated much of the LPS, without releasing much protein or nucleic acid into the aqueous phase.

More recently, Johnson and Perry (1976) used two different cell-disruption procedures before conventional phenol-water extraction. Disruption of cells by grinding with glass beads or by digestion with hen egg white lysozyme before phenol extraction was shown to improve both purification and yield of LPS. Pretreatment of cells with lysozyme and ethylenediaminetetraacetate (EDTA) was the easier of the two methods and also gave the highest yield.

The possibility that LPS may be altered during extraction has been suggested by Nowotny et al (1963) who were particularly critical of procedures involving TCA, phenol and diethylene glycol. They showed that when a range of six extraction methods was applied to different bacteria, there were large variations in the yields of toxic and serologically

Figure 1. Salmonella cell wall with the more detailed structure of the lipopolysaccharide. Region I: the O-specific repeating units; region II: the R-specific core oligosaccharide; region III: lipid A (from Westphal, 1975).

reactive materials. The amounts of nucleic acid released by the different methods varied. Lindberg and Holme (1972) found that for structural studies of LPS involving methylation analysis, the method of choice was phenol-water extraction of purified cell walls made from bacteria killed by γ-radiation. To date, however, procedures which might be best for retention of the native structures have tended to yield LPS preparations contaminated with other cell constituents.

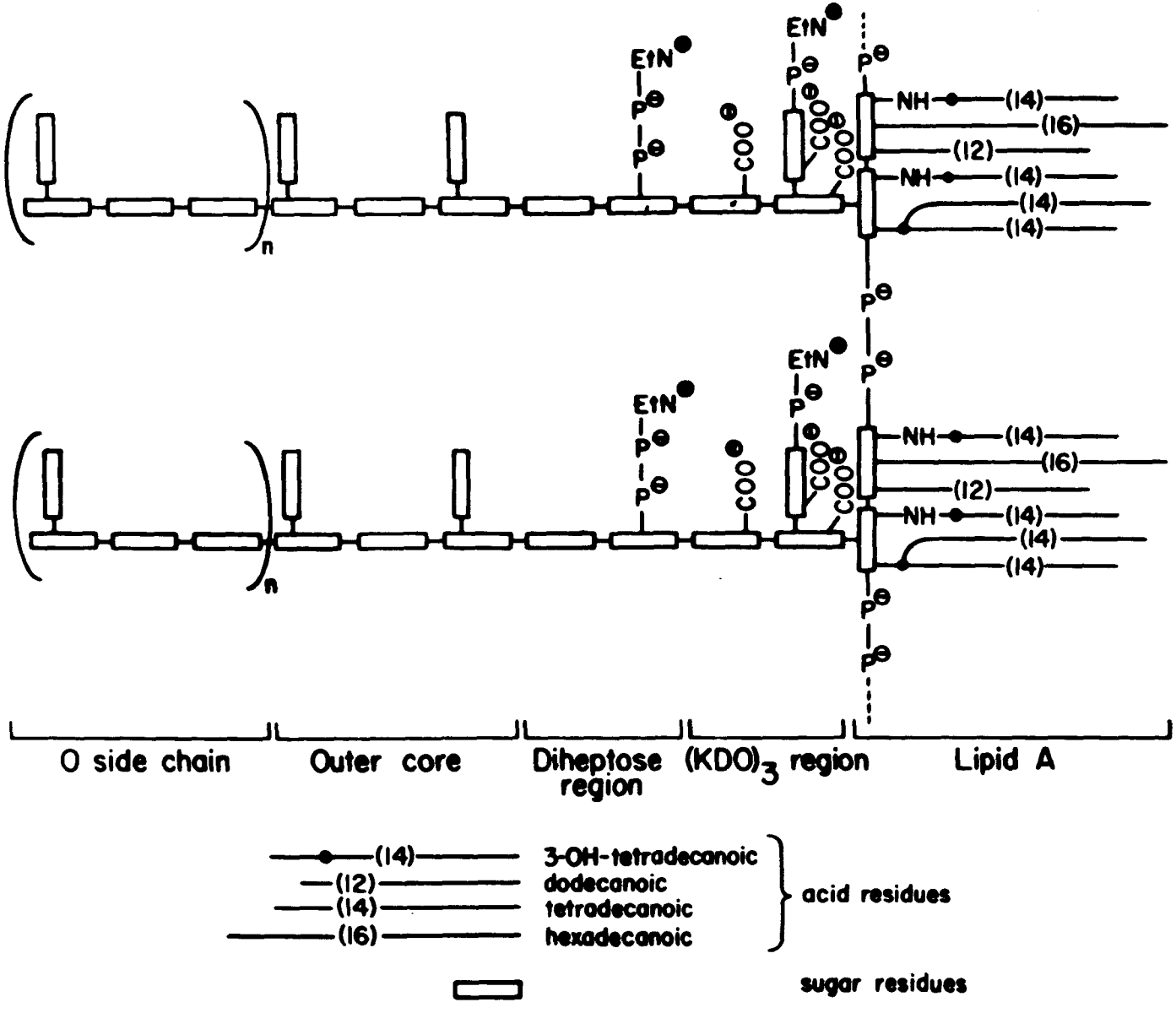
Structure of Lipopolysaccharides

Lipopolysaccharides occur in the outer membrane of the cell envelope of gram-negative bacteria as a high molecular weight component associated with proteins and phospholipids (Fig 1). The purified LPS component of the endotoxin complex itself is made up of two main components with differing physico-chemical characters, ie a heteropolysaccharide and a covalently bound lipid. The polysaccharide component contains two regions: the O-specific chain and the core oligosaccharide. The former, which is represented by a polymer of repeating oligosaccharide units, carries the antigenic determinants responsible for the serological specificity of the LPS and the parent bacteria. The O-specific chain, therefore, is serotype specific. In contrast, the core oligosaccharide is group specific. Thus it is identical in all *Salmonella* species (Lüderitz *et al*, 1970), where it is composed of a main chain substituted at different points by monosaccharides, phosphate, phosphoethanolamine and pyrophosphoethanolamine (Fig 2).

O-antigen

The classical heat-stable polysaccharide antigen of the gram-

Figure 2. A schematic represention of the LPS molecule (from Nikaido, 1973).


EtN
P
COO
NH
(14)
(16)
(12)
n
O side chain
Outer core
Diheptose region
(KDO)3 region
Lipid A
3-OH-tetradecanoic
dodecanoic
tetradecanoic
hexadecanoic
acid residues
sugar residues

negative cell is the O-antigen. The polysaccharide chains which carry the serological specificity usually consist of tetrasaccharide or pentasaccharide repeating units in Salmonella (Hellerquist and Lindberg, 1971) while trisaccharides also occur. The repeating nature of the unit oligosaccharides seems to be a general feature of many Salmonella serotypes, Escherichia coli and Shigella flexneri strains (Lüderitz et al, 1971). This region has diversified greatly during evolution (Nikaido, 1973) and in many strains the repeating unit contains rare sugars such as deoxy- and dideoxyhexoses.

An LPS molecule may carry several different serological specificities (Westphal, 1975). Different parts of the repeating unit serve as determinant groups in the interaction of LPS with O-antibodies. The resolving power of the immunological technique is seen from the fact that S. typhimurium and S. newport LPS show little cross-reaction serologically in spite of the identical monosaccharide composition of their O-repeating units (Lüderitz, Staub and Westphal, 1966).

Morgan and Watkins (1969) demonstrated that serological determinants on polysaccharide blood group antigens had a size range from di- to hexasaccharides.

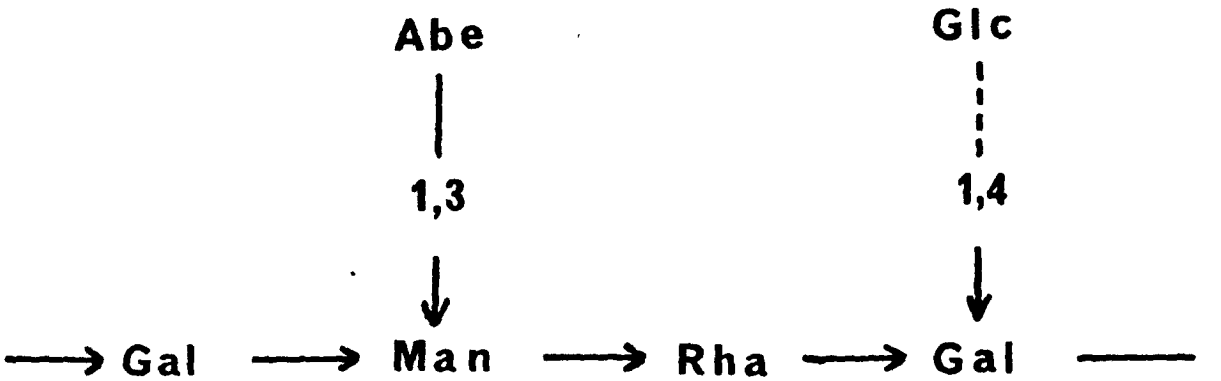
Staub (1964) suggested that enterobacterial O-specific polysaccharides were built up from repeating oligosaccharide units. From partial hydrolysates, oligosaccharides with overlapping sequences were isolated and these allowed the reconstruction of the repeating units of sizes lying between tri- and hexasaccharides. More recently Bjorndal et al (1970) used a combination of gas chromatography and mass spectrometry on

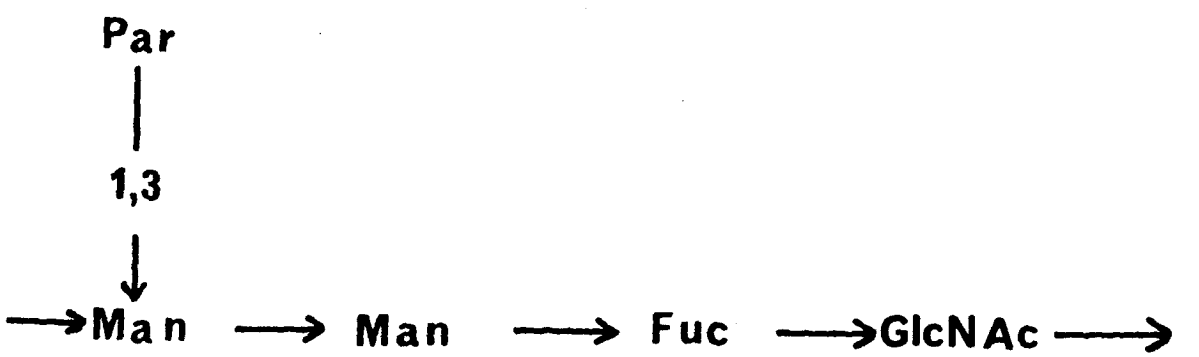
Figure 3. a) Structure of the repeating unit in *Salmonella* group B O-antigen (Westphal, 1975).

Key: Gal (Galactose), Man (Mannose), Abe (Abequose), Rha (Rhamnose) and Glc (Glucose).

b) Structure of the chemical repeating unit of *Yersinia pseudotuberculosis* type 1B LPS (Tomshich *et al*, 1976).

Key: Par (Paratose), Man (Mannose), Fuc (Fucose) and GlcNAc (N-Acetyl-glucosamine.


Abe
Glc
1,3
1,4
Gal
Man
Rha
Gal


Par
1,3
Man
Man
Fuc
GlcNAc

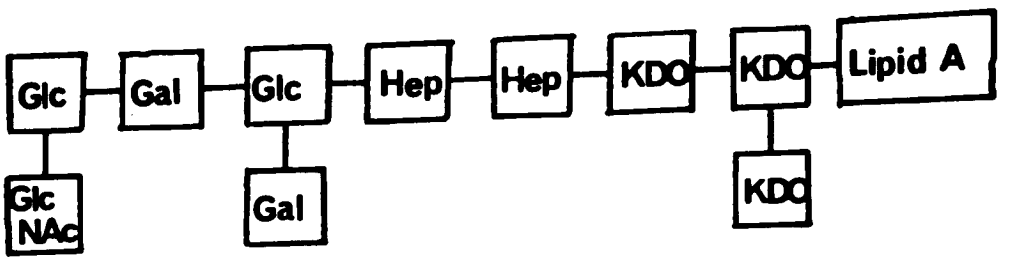
hydrolysates of methylated LPS to analyse the structure of the polysaccharide moiety.

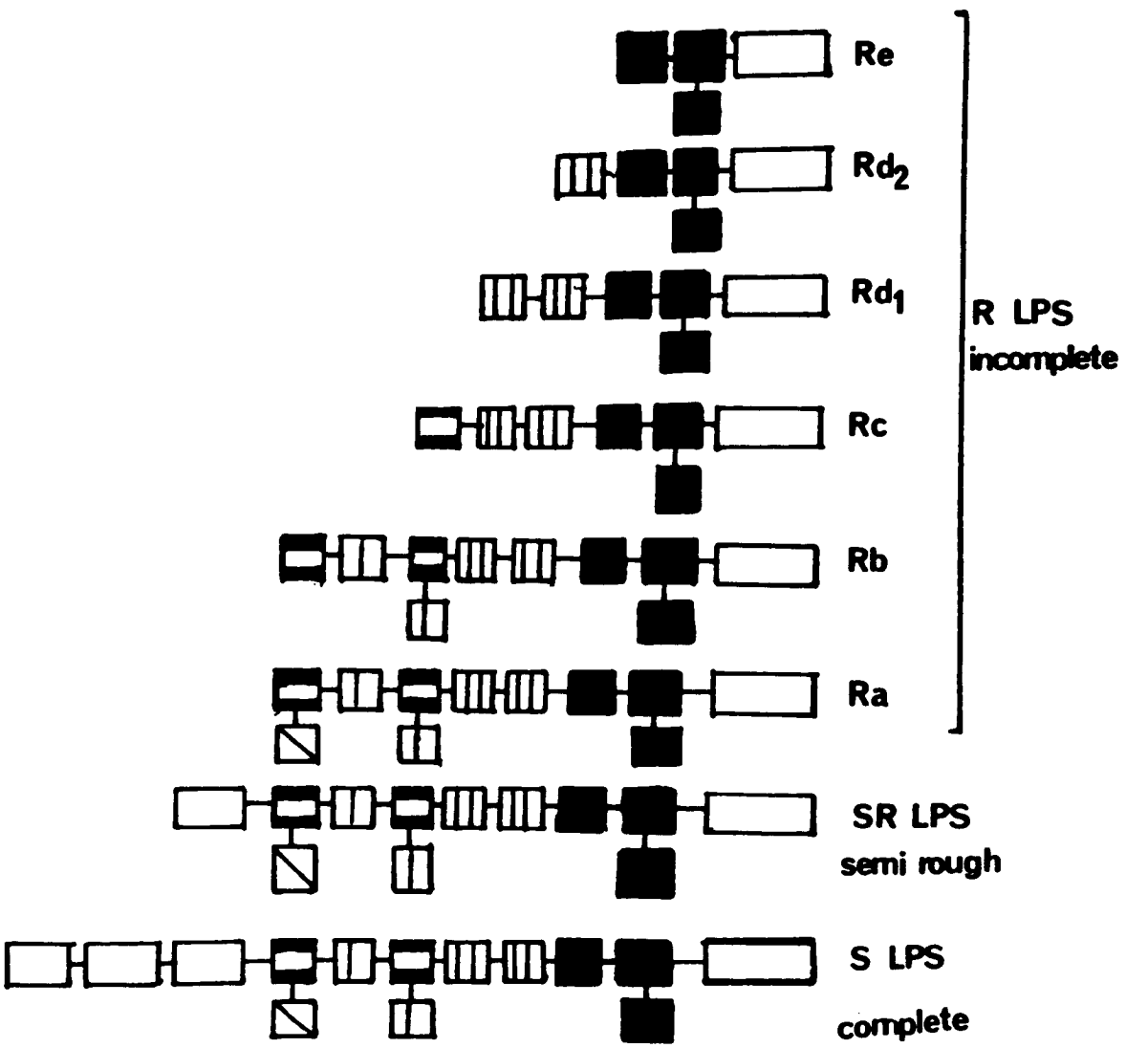
An example of the structure of an LPS repeating unit is shown in Fig 3a (Westphal, 1975). This repeating unit, of Salmonella group B, is a trisaccharide of mannose (Man), rhamnose (Rha) and galactose (Gal) with an α-1-3-linked abequose (Abe) side chain attached to the mannose. Glucose (Glα) may or may not be present on another side chain in α-1-4-linkage. In Salmonella group B polysaccharide, abequose is frequently substituted with O-acetyl in the 2 position. The concept of "immunodominant sugars" was elaborated by Heidelberg and Staub (Lüderitz, Staub and Westphal, 1966), for example, in S. typhi it was the dideoxysugar tyvelose.

During the last 25 years LPS from a wide range of bacteria have been extracted, purified and subjected to structural analysis. While much of this effort has been expended on members of the Enterobacteriaceae, other groups have also been studied in detail. An example of an LPS with a particularly complicated repeating unit is that from Yersinia pseudotuberculosis (Fig 3b) (Tomshich et al, 1976).

LPS from different species, but which have the same monosaccharides in the molecule, are said to belong to the same "chemotype" (Lüderitz et al, 1970). This does not imply serological identity because different inter-sugar linkages give rise to different immunological specificities. For example, S. illinois and S. senftenberg differ only in the linkage of the glucose side group but this gives different immunological properties. Conversely, strains which share little

Figure 4. Structure of the Salmonella Ra to Re LPS mutants (from Westphal, 1975).





core polysaccharide

O polysaccharide

taxonomic affinity for each other may have serologically identical LPS, eg E. coli 058 LPS reacts with antiserum to Shigella dysenteriae type 5 (Dmitriev et al, 1977).

The "Core" oligosaccharide

A major contribution to the study of LPS structure was made by the isolation of mutants defective at various stages of LPS biosynthesis (Lüderitz et al, 1966). The stepwise loss of biosynthesis capability in a series of S. typhimurium mutants from S — Re is shown in Fig 4 . The "innermost" region of LPS, consisting of $(KDO)_3$-lipid A, appeared to be indispensible for the survival of the bacteria, as mutants lacking this structure were not isolated. In contrast to the extreme diversity found in the structure of O-side chains in Salmonella the structure of the core region was relatively constant (Lüderitz et al, 1971) with most of the S-LPS having a core consisting of R-LPS (Simmons, Lüderitz and Westphal, 1965). Other enterobacteria such as E. coli and Shigella spp produced core oligosaccharides with structures slightly different from that of Salmonella spp (Nikaido, 1970). In bacteria unrelated to these three groups, additional variation in the structure of the core occurred. A characteristic component of this portion of LPS, 2-keto-3, deoxyoctonate (KDO or 3-deoxy-D-manno-octulosonic acid) was detected in almost all gram-negative bacteria studied (Ellwood, 1970) and constituted about 0.5% of the cell wall. The wide variation in the amounts of KDO found may reflect differences in the composition of the walls of different bacteria. Among the exceptions which do not have KDO are Flavobacterium spp and Spirillum serpens (Chester and Murray, 1975). Another component, L-glyco-D-manno-heptose, was found in LPS from many gram-negative bacteria although it was absent in a few such as Xanthomonas and Anacystis (Lüderitz et al, 1971).

In addition to heptose and KDO, phosphate residues were present in the innermost region closest to the lipid A. Ethanolamine occurred as O-phosphoryethanolamine and O-pyrophosphorylethanolamine in this region. This basal core region, as well as containing the Heptose-KDO "backbone," generally contains a characteristic pentasaccharide. The structure of the Salmonella Ra LPS is shown in Fig 4 (Sutherland, Lüderitz and Westphal, 1965; Nikaido, 1969). It was shown that the biosynthesis of the core differed from that of the O-specific chain with the monosaccharide units being transferred stepwise onto the growing R-core chain (Lüderitz, 1970).

Sometimes there is a third heptose unit on the second heptose. In addition, one of the heptoses may be substituted by diphosphoethanolamine and one of the KDO units by phosphoethanolamine (Fig 2). As each of these R mutant LPS can serve as an acceptor for the next monosaccharide unit in the biosynthesis of the core, Fig 4 also represents the sequence of steps in the biosynthesis of the core.

With Sh. flexneri it was similarly found that mutants of the Ra class synthesize a complete core polysaccharide while those from the Rb class have a core lacking N-acetylglucosamine (Simmons, 1971). Mutations producing defects in the synthetases or transferases of galactose, glucose and heptose result in the classes Rc, Rd and Re respectively (Johnston, Johnston and Simmons, 1968). It may be noted that the LPS depicted in Fig 4 are serologically distinct as might be expected from different terminal structures on the polysaccharide chains (Mayer et al, 1976).

Lipid A

Lipid A, which is a constituent of all LPS is a β1-4 or 1-6

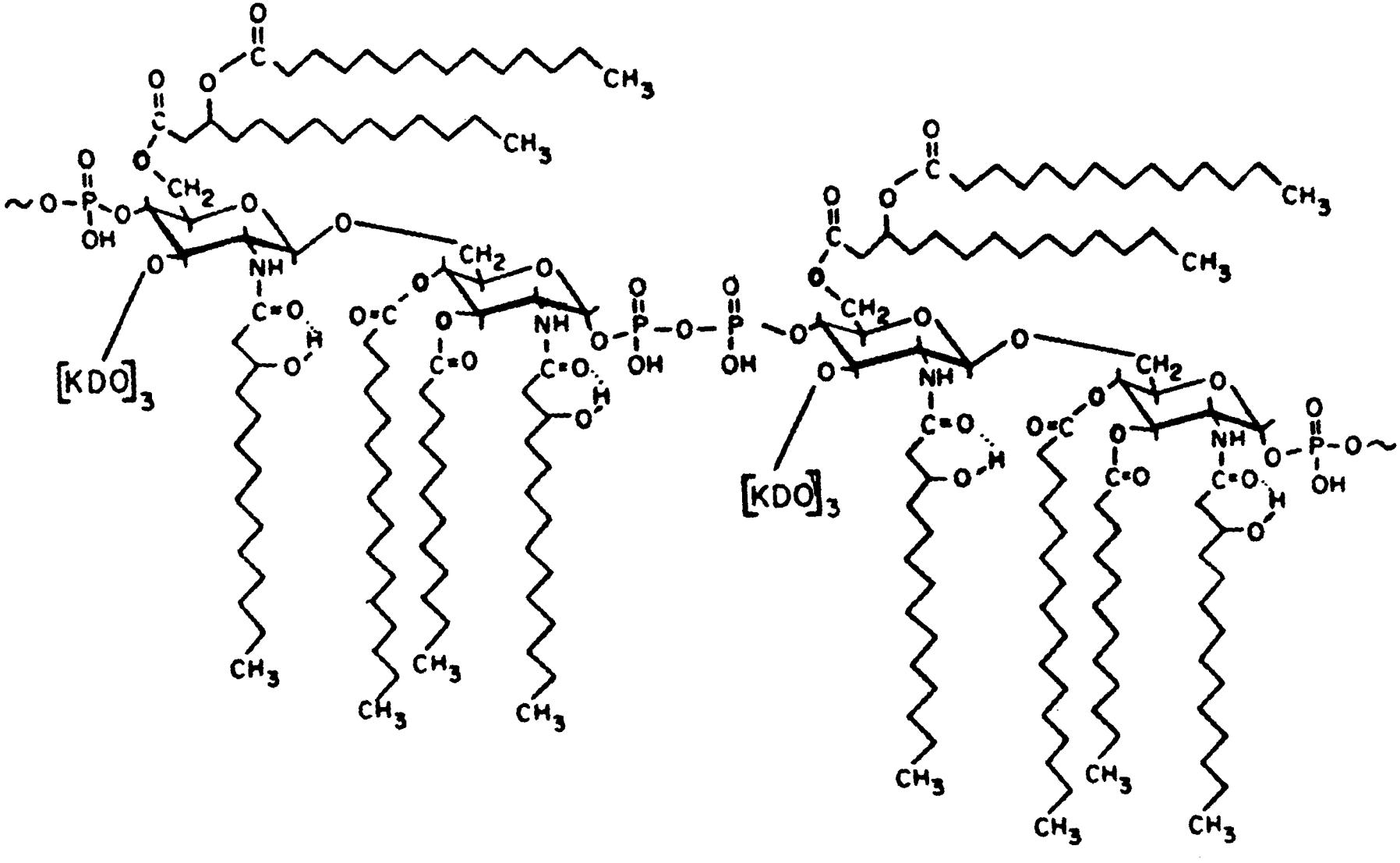

[KDO]3
[KDO]3

Figure 5. Structure of the lipid A component of Salmonella LPS with attached KDO-trisaccharide. (Rietschel et al, 1977).

diglucosamine extensively substituted with fatty acids and phosphate. It is usually isolated by mild acid hydrolysis of LPS, followed by extraction into chloroform. In native LPS, lipid A is covalently bound to the polysaccharide through one or more molecules of KDO, usually three. One of the KDO residues is in turn attached via an acid-labile link to the lipid A.

The lipid A from the glycolipid of S. minnesota Re consisted of β, 1-6-linked D-glucosamine disaccharides, interlinked by phosphate residues in positions 4 and 1, and substituted with long-chain fatty acids in ester and amide linkages (Rietschel et al, 1972). Glucosamine disaccharide units with a β, 1-6 linkage were found in lipid A of many LPS from other species of gram-negative bacteria (Drewry et al, 1973; Hase, Hofstad and Rietschel, 1977) while others with β, 1-4 linked glucosamine residues were identified (Adams and Singh, 1970). The structure of the glucosamine backbones of lipid A from various gram-negative bacteria were published recently by Hase and Rietschel (1976). In Salmonella, the amino groups of the glucosamine disaccharide were substituted with β-hydroxymyristic acid, while the three sugar hydroxyl groups were esterified with lauric (C_{12}), myristic (C_{14}), β-hydroxymyristic (C_{14}-OH) and palmitic (C_{16}) acids. In addition the hydroxyl groups on the ester-bound β-hydroxymyristic acid may be substituted with myristic acid (Rietschel et al, 1972). The exact positions of the ester-linked fatty acids on the diglucosamine backbone have still to be determined. The fourth hydroxyl group is attached to KDO, forming the acid-labile link to the R core and the O-specific chain (Fig 5). Similar results were found by other workers (Romeo, Girand and Rothfield, 1970) when studying the fatty acid composition of a variety of Salmonella LPS (Table 1). Lauric, myristic and palmitic acid were ester-linked, while β-hydroxymyristic acid was

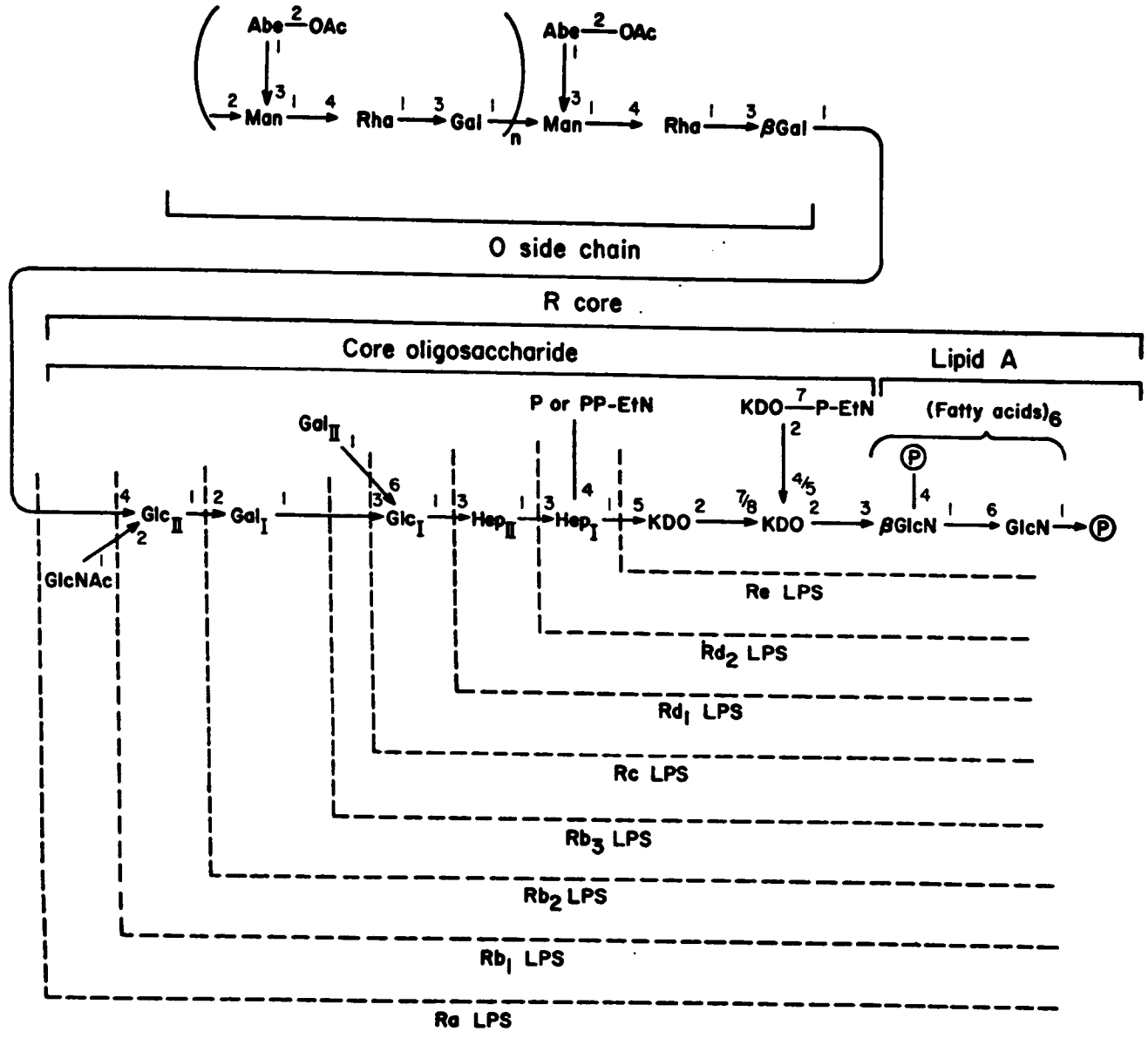
Table 1 Normal constituents of Lipid A (Westphal, 1975)

		Molar Ratio
Glucosamine		2
Phosphate ester		2
fatty acids	β-hydroxymyristic acid 3	6
	Lauric acid C_{16} 1	
	Myristic acid C_{14} 1	
	Lauric acid C_{12} 1	

amide-linked in the lipid A component of LPS of many different species of gram-negative bacteria (Rooney and Goldfine, 1971; Koeltzow and Conrad, 1971). Lipid A from some other organisms, however, present somewhat different structures. That from Rhodopseudomonas viridis had no ester-bound fatty acids (Roppel, Mayer and Weckesser, 1975). Neither β-hydroxymyristic nor β-acyloxymyristic acid are obligatory constituents of lipid A. In myxobacteria (Rosenfelder, Lüderitz and Westphal, 1974) and Xanthomonas (Rietschel, Lüderitz and Volk, 1974) isobranched (3-hydroxy) fatty acids were found and in Veillonella (Hewet, Knox and Bishop, 1971) odd-numbered 3-hydroxy fatty acids were found. Xanthomonas and Pseudomonas lipid A (Wilkinson, Gailbraith and Lightfoot, 1973) contained additional 2-hydroxy fatty acids while Ps. aeruginosa contained 3-hydroxydeconoic, 2-hydroxy and 3-hydroxylauric, and 3-hydroxymyristic acid (Hancock, Humphreys and Meadow, 1970). The ester-bound 3-hydroxy fatty acid was usually 3-O-acylated in enterobacterial LPS from Salmonella (Rietschel et al, 1972) and Shigella (Lugowski and Romanowska, 1974).

The 3-hydroxy fatty acids are the only fatty acids which are amide-bound, in contrast to the wide range of fatty acids which are ester-bound. Usually amide-bound 3-hydroxy acids are straight chained even numbered acids but iso-branched and odd numbered 3-hydroxy acids occur as well. In the few LPS, eg in Pseudomonas and Fusobacterium nucleatum (Hase, Hofstad and Rietschel, 1977), where several homologous 3-hydroxy acids were present, the acid with the longest chain was usually amide-linked though there were exceptions, eg Ps. aeruginosa (Hancock, Humphreys and Meadow, 1970). In myxobacteria where straight chains, fatty acids and isobranched 3-hydroxy acids occur, the one with the longest chain was also amide-linked.

Figure 6. Structure of S. typhimurium LPS (from Nikaido, 1973).


Abe 2 OAc
Man
Rha
Gal
n
Abe 2 OAc
Man
Rha
βGal
O side chain
R core
Core oligosaccharide
Lipid A
P or PP-EtN
KDO 7 P-EtN
(Fatty acids)6
GalII
GlcII
GalI
GlcI
HepII
HepI
KDO
KDO
βGlcN
GlcN
GlcNAc
Re LPS
Rd2 LPS
Rd1 LPS
Rc LPS
Rb3 LPS
Rb2 LPS
Rb1 LPS
Ra LPS

Thus the overall structure of a basic "monomer" unit which is presumably cross-linked with others is shown in Fig 6.

Location, Function and Biosynthesis of Lipopolysaccharides

The gram-negative cell envelope consists of three distinct regions:- a) the outer membrane based on a lipid bilayer structure 7.5 - 8.5 nm thick; b) a layer of variable thickness composed of peptidoglycan; and c) the cytoplasmic membrane. The outer membrane appeared to be similar to the lipid bilayer in the electron microscope (Shands, 1968) but contained proteins, phospholipids and LPS. Muller, Hinckley and Rothfield (1972) showed that LPS molecules readily penetrated the phospholipid monolayer indicating that the outer membrane had a structure typical of biological membranes, i.e. a phospholipid bilayer with proteins penetrating it in a seemingly random fashion. As LPS is an amphipathic molecule it constitutes a part of the bilayer, behaving in a similar manner to phospholipids, with the lipid A component associating with the lipid of the membrane, and the hydrophilic O-antigen chains exposed to the outside (Smit, Kamio and Nikaido, 1975).

The general function of the outer membrane is threefold: structural, diffusion barrier and physical barrier. Peptidoglycan contributes to the rigidity of the gram-positive cell wall but in gram-negatives as this layer is so thin it is unlikely that it is the only rigid layer in the cell wall. Feingold *et al* (1968) showed that lysozyme degraded peptidoglycan in the absence of EDTA in gram-negative organisms with no subsequent production of spherical forms. Passive diffusion is probably the mechanism allowing the entry of many compounds. Leive (1973)

suggested that though the outer membrane was poorly permeable to most compounds, binding proteins might pull certain substances into the periplasmic space. The outer membrane also functions as a barrier against penetration by dyes, bile salts and some antibiotics. Recently, Decad and Nikaido (1976) studied the permeability function of the outer membrane of Salmonella using radioactive non-utilizable oligo- and polysaccharides or polyethylene glycols. They found that only di- and trisaccharides could diffuse into the periplasm, whereas higher-molecular-weight saccharides were non-permeable, suggesting that the cell wall acted as a molecular sieve, with an exclusion limit for sugars between 550 and 650 daltons. Rottem and Leive (1977) demonstrated that LPS, and especially the polysaccharide portion, directly or indirectly restricted the mobility of the lipid hydrocarbon chains observed in the outer membrane. A reduction in either the polysaccharide side-chain length or in LPS concentration increased the fluidity of the outer membrane.

Smit, Kamio and Nikaido (1975) isolated the outer membrane layer from the cell walls of both S. typhimurium LT2 and its rough mutants. They showed that the number of LPS molecules per unit area was constant, regardless of the length of the sugar side-chain in the LPS. In contrast, in rough mutants Re and Rd, possessing LPS with very short sugar-chains, the amount of outer membrane protein per unit surface area decreased to about 60% of the value in the wild type. For the wild type, the amount of phospholipids was slightly less than that needed to cover one side of the membrane as a monolayer. LPS and glycolipid extracted from mutants of S. minnesota (Shands, 1973; Fukushi, Asano and Sasaki, 1977) resembled membrane fragments of various shapes, including ribbons, discs, lamellae

and vesicles, while glycolipid from the R mutants formed onion-like structures. The width of glycolipid leaflets decreased in parallel with the decrease of core polysaccharide component.

The actual biosynthesis of LPS occurs in three different stages. The biosynthesis of lipid A and the O-side chain occur separately, after which they are joined to the core oligosaccharide to complete the molecule (Nikaido, 1973).

In the above process, least is known about the biosynthesis of lipid A. Both Romeo et al (1970) and Rietschel (1971) found the fatty acids, lauric (dodecanoic acid), myristic (tetradecanoic acid), palmitic (hexadecanoic acid) and β-hydroxymyristic (3-hydroxytetradecanoic acid) in the molar ratio of 1:1:1:3. It therefore seems that specific transferases insert each fatty acid into a specific location of the di-glucosamine backbone.

In contrast to the O-side chains which were synthesised independently and then subsequently linked to the R core, the core oligosaccharide was synthesized by the stepwise addition of monosaccharide units to the lipid A complex. The first step was the linkage of KDO to the glucosamine dimer of lipid A (Heath et al, 1966). No information was provided about the addition of the two remaining KDO molecules and heptose residues. Mühlradt (1969) suggested that the phosphorylated heptose molecules were cross-linked via phosphodiester linkages. It was proposed that normally the transfer of glucose preceded phosphorylation of the heptose residues. This was followed by the transfer of phosphorylethanolamine onto the phosphate group. Osborn et al (1964) demonstrated with LPS mutants, that the sequential addition of

galactose, glucose and N-acetylglucosamine occurred after the addition of the first glucose and galactose residues in the core region. The LPS of an R core mutant with the sequence Glc NAc-Glc-Gal-(Gal)-Glc-$(Hep)_2$-$(KDO)_3$-, was synthesized in a cell-free system. Therefore each step in the biosynthetic pathway required the specific transferase, the sugar nucleotide and the acceptor molecule for completion.

The biosynthesis of the O-specific polysaccharide was studied using mutants of S. newington rather than the S. typhimurium of other workers (Wright et al, 1965). Incorporation of sugars into an O-side chain LPS was found when GDP-mannose, dTDP-rhamnose and UDP-galactose were added together to the cell envelope fraction. Dankert et al (1966) showed the presence of a "lipid" carrier or antigen carrier lipid phosphate (P-ACL). In this system, transfer to the R core did not occur until the assembly of the repeating units was complete.

A detailed account of LPS biosynthesis has been provided by Nikaido (1973).

Endotoxic Activities of Lipopolysaccharide

General

Endotoxin exhibits a wide variety of biological activities in mammals (Milner, Rudbach and Ribi, 1970). Parenteral administration is necessary and the properties range from lethality and pyrogenicity to protection against radiation and stimulation of interferon production.

As already indicated, endotoxin is a toxic principle produced

by gram-negative bacteria with endotoxic properties being common to all bacterial LPS irrespective of the source. The relation between LPS structure and endotoxic activity has been studied using R mutants. The various endotoxic reactions which may be elicited both by the complete endotoxin complex and by lipid A complexed with bovine serum albumin (Westphal, 1975) are listed in Table 2. These authors showed that the polysaccharide components of the LPS were not responsible for endotoxicity although they were for the antigenic properties. The polysaccharide component was shown (Galanos et al, 1972) to play an important role in the solubilisation of lipid A and in the conformation of lipid A, and as a result, may exert an indirect influence on endotoxic reactions. Magnusson et al (1977) suggested that the smooth-specific polysaccharide side-chain of S. typhimurium might "blindfold" non-specific host defence mechanisms dependent on hydrophobicity and charge. On the other hand, the R bacteria and R LPS have physico-chemical properties which predispose to interaction with several types of cells, organelles and molecules.

The relation between structure and many endotoxic reactions was shown with a wide range of LPS (Elin et al, 1976). For example, one structural configuration may induce mitogenicity, pyrogenicity and limulus activity while another may be responsible for complement activation.

LPS are negatively charged by virtue of phosphate, pyrophosphate and carboxyl groups. When LPS is extracted from bacteria it is obtained in a salt form containing metal cations and basic amines. Galanos and Lüderitz (1975) demonstrated that when these ionically-bound substances were removed by electrodialysis, acidic polysaccharide was left, which on neutralisation with different bases, yielded preparations with altered physico-chemical and biological properties.

Table 2 Endotoxic reactions produced by both LPS and by lipid A complexed with bovine serum albumin (Westphal, 1975)

Pyrogenicity	Induction of IgG synthesis in newborn mice
Lethal toxicity	Macrophage activation
Toxicity increased by adrenalectomy	Colony-stimulating factor
Toxicity increased by BCG treatment	Complement activation
Schwartzman phenomenon	Hagemann factor activation
Bone marrow necrosis	Plasminogen activator
Embryonic bone resorption	Induction of prostaglandin synthesis
Leukopenia	Induction of interferon production
Leukocytosis	Non-specific resistance to infection
Depression of blood pressure	Early refractory state to endotoxin pyrogenicity
Tumour necrotic activity	
Enhancement of dermal reactivity to epinephrine	Induction of endotoxin tolerance
Adjuvant activity	Limulus lysate gelation
Mitogenic lymphocyte stimulation	

Interactions of endotoxic activity by simple dissociation, eg with detergents, was described by different groups of workers (Ribi *et al*, 1964; Galanos and Lüderitz, 1976). Rudbach *et al* (1966) observed that endotoxin was dissociated and lost its pyrogenicity after treatment with sodium desoxycholate. Dilution or dialysis led to reassociation with the formation of aggregates of a molecular weight of 500,000 daltons, and the recovery of pyrogenicity. It was concluded that a certain micellar organization of subunits, which individually may be inert, leads to an active endotoxin macromolecule. Galanos and Lüderitz (1976) similarly found that only highly-aggregated LPS interacts with complement.

It seems therefore that the toxic form of endotoxin is composed of relatively non-toxic subunits and it is only when incorporated into a macromolecular structure that they provide a "toxic conformation" (Nowotny, 1969). It also appears that long-chain carboxylic acids, ester- or amide-bound to a polysaccharide backbone, play an important role in this "toxic conformation" (Nowotny, 1963).

Further evidence that endotoxic activities are due to the structure and configuration of the lipid A component has been demonstrated by Rietschel and Galanos (1977) who used lipid A antiserum to reduce the endotoxic activity of injected LPS. It was found, however, that the lipid A antiserum had no significant antipyretic effect when it was incubated with lipid A or injected intravenously before lipid A challenge. However fever protection by lipid A antiserum was specific to the lipid A of a particular LPS.

Interaction of lipopolysaccharide with the complement system

It has been known since 1955 (Pillemer et al, 1955) that LPS in the cell wall of gram-negative bacteria, as well as other polysaccharides such as agar and zymosan, inactivate complement in vitro. Serum factors other than classical antibody may be required in this process (Pillemer et al, 1955; Mergenhagen et al, 1969; Gewurz et al, 1970; Dierich et al, 1973) and that the interactions of LPS with complement involved the terminal complement components. Lipid A was found to be the moiety mainly responsible for the AC activity and toxicity while the O-specific chains and core polysaccharide were not necessary (Galanos et al, 1971). More recently, Galanos and Lüderitz (1976) demonstrated that lipid A alone was insufficient for AC activity and that a state of aggregation was required before the endotoxin-complement interaction took place.

Earlier investigations (Pillemer et al, 1955; Gewurz et al, 1968; Galanos et al, 1971) indicated that incubation of LPS with serum led to a decrease in complement activity. Gewurz, Shin and Mergenhagen (1968) demonstrated that the loss of complement activity in serum was due to consumption of C_3 to C_9, but that LPS had a minimal effect, of less than 10% on the C_1, C_2 and C_4 components of complement. This latter observation was also reported in the original work of Pillemer's group (1955). This consumption of C_3 to C_9 in whole serum by LPS was due to activation of the Alternative Pathway which bypasses the C_1, C_2 and C_4 components (Dierich et al, 1973). Apart from the LPS induced activation of the Alternative Pathway, LPS-antibody complexes are assumed to activate the classical pathway, via C_1, C_4 and C_2 (Frank, May and Kane, 1973;

McLean, Townsend and Michael, 1975). Bitter-Suermann et al (1975) showed that the proportion of activation in the two pathways was different when LPS was isolated from a number of strains of the same species of gram-negative bacterium. Füst, Bertók and Juhász-Nagy (1977) demonstrated that irradiation of LPS decreased, in a dose-dependent manner, its ability to activate both pathways. The reduction of Alternative Pathway activation however, was less marked indicating that different parts of the LPS molecule were responsible for activation of the two pathways. Morrison & Kline (1977) suggested that the lipid A region of LPS was mainly responsible for Classical Pathway activation and the polysaccharide region for properdin, or Alternative Pathway activation. Their work agreed with that of Loos et al (1974) which indicated that classical pathway activation by lipid A was independent of antibody to lipid A. However, the polysaccharide portion of the LPS molecule was shown to exert a modifying influence on the potential AC activity of the lipid A. Füst and Ádám (1976) suggested that two different sites on the LPS molecules of 10 different strains of Pseudomonas aeruginosa were responsible for complement activation via the two pathways. Bjornson and Bjornson (1977) used whole cells to demonstrate that other enterobacteriaceae, with the exception of S. minnesota Re (rough), activated the Alternative Pathway in EGTA-treated serum supplemented with $MgCl_2$. However, S. minnesota Re was able to initiate C_3 conversion in unchelated (ie Ca^{++}-containing) human serum, indicating it was capable of activating complement by a different mechanism than the Alternative Pathway. However, Bitter-Suermann et al (1975) found that C_3 was activated via the Alternative Pathway by 10 LPS preparations, including those from rough mutants of Salmonella, as well as lipid A, though the latter were less active than other LPS preparations.

For LPS to interact with complement it had to be in a soluble or dispersed form (Galanos et al, 1971). There were, however, certain lipid A samples which although soluble, did not exhibit AC activity. More recently, Galanos and Lüderitz (1976) showed that LPS interacted with complement only when in a high molecular weight, aggregated state. By converting Na-form smooth LPS into the low molecular-weight triethylamine form, the AC activity was completely lost while reversion to the high molecular weight sodium form restored activity. In contrast, a similar treatment of Re and Rd LPS had no effect on the ability to interact with complement. Both triethylamine and sodium forms were strongly anti-complementary, despite large differences in molecular weight. This was due to the property of R LPS to reaggregate into a large molecular weight form through absorption of Mg^{++} and Ca^{++} ions present in guinea-pig serum used as the complement source. LPS derived from the Ra and Rb classes showed only negligible anticomplementary activity even when converted into a high molecular weight form. This low activity was not due to an inactive lipid A.

The molecular basis of the interaction of LPS with C is not understood completely. Possibly unknown structural and chemical differences in the intact LPS may be involved as well as a state of high aggregation, a property which is shared by other preparations known to interact with complement such as pneumococcal cell walls (Winkelstein and Tomasz, 1977), water-insoluble glucans of Streptococcus (Inai et al, 1976) and zymosan (Rother, Hadding and Till, 1974).

Galanos et al (1971) showed that LPS molecules varied substantially

in AC activity. As little as 4 μg of some LPS preparations inhibited C-mediated haemolysis of sensitized red cells. Other preparations, however, showed lower activity and a few were virtually inactive.

Biodegradation of Lipopolysaccharides

Although much is known about the structure and biosynthesis of LPS, there is comparatively little information about its degradation in nature. Considering how helpful it would be to have specific LPS-degrading enzymes for structural analysis and isolation or destruction of biologically active groups, it is surprising how little effort has been expended in this area. The few LPS-degrading systems reported in the literature were derived from phages, the slime mould Dictyostelium discoideum and mammalian plasma. The related question of whether or not LPS accumulates in the environment has received scanty attention.

Bacteriophage

So far, all the enzymes active against the polysaccharide portion of LPS appear to be associated with phage infection of the host bacteria. Such enzymes, however, are active in removing only the O-antigenic side-chains or attached acyl groups (Sutherland, 1977). There have been no reports of enzymes active on the core polysaccharide.

Bacteriophages from 5 strains of Enterobacteriaceae degraded smooth LPS in situ in the host cell-wall. The Salmonella phages G^{15} (Kanegasaki and Wright, 1973; Takeda and Uetake, 1973), P_{22} (Iwashita and Kanegasaki, 1973) and G^{84} (Iwashita and Kanegasaki, 1975; Taketa, Uetake and Toyama, 1975) as well as the E. coli phage Ω8 (Reske, Wallenfels

and Jann, 1973) cleaved glycosidic linkages with resultant depolymerisation of the O-specific side-chains. Salmonella phage C_{341} appeared to split off O-acetyl groups. All five bacteriophages required the O-specific side-chains of the respective host LPS for attachment (Lindberg, 1973), while in ε^{15}, ε^{34}, P_{22} and C_{341}, less enzymic activities were associated with the tail spikes of the phages.

Kanegasaki and Wright (1973) showed that the LPS of S. anatum containing the sequence - gal - man - rha - was the substrate for a phage hydrolyase. The ε^{15} phage destroyed O-specificity of S. anatum. With ^{14}C-labelled bacteria there was a rapid release of radioactive material, in amounts proportional to the concentration of the bacteriophage. When lysates were ultracentrifuged, the phage-depleted supernates were found to contain the enzyme. The terminal reducing sugar rhamnose was released from the oligosaccharide so the enzyme was therefore an endo-rhamnosidase acting on the α-rhamnose 1-3-galactose-linkage in the oligosaccharide. The high specificity of the enzyme was shown by the fact that the polysaccharide from the lysogenic AIε^{15} strain, which has this linkage, was not hydrolysed due to the presence of β-galactosidic linkages. The enzyme, which is an endorhamnosidase, hydrolyses random α-rhamnosyl 1-3 galactose linkages to produce disaccharide mannosylrhamnose, pentasaccharide, octasaccharide and higher oligosaccharides of rhamnosylgalacosylmannose.

E. coli 08 LPS whose O-specific side-chain consisted of trisaccharide repeating units of - 3-man 1-2 man 1-2 man 1 - lost its characteristic opalescence after phage treatment (Reske et al, 1973). Following ultracentrifugation, the supernatant fluid contained 55% of the polysaccharide while the pellet contained bacteriophage, unreacted LPS and

an insoluble sediment produced by phage action. Reaction products ranged from trisaccharides to dodecasaccharides but were mainly nonasaccharides and hexasaccharides in the ratio of about 2:1. Similar results were obtained when lipid-free polysaccharide was used as the substrate, indicating that the enzyme was not affected by absence of lipid A.

Klebsiella aerogenes O5 LPS has a structure similar to that of E. coli O8, being composed of repeating units of five mannose residues, two being 1-3 linked and three 1-2 linked. Lindberg (1973) showed that the polysaccharide chain was similar in size to the E. coli O8 polysaccharide, with 13 repeating units, but was terminated by a 3-O-methyl-D-mannose residue. Degradation of the oligosaccharide was demonstrated, but the products were not analysed.

More recently six smooth-specific bacteriophages were isolated from sewage by making use of pairs of smooth and rough forms of enterobacterial species (Reiger-Hug, Choy, Schmidt and Stirm, 1977). The six bacteriophages isolated and another smooth specific phage were tested after purification and four of them were shown to liberate reducing groups from pure LPS. In each case glycosidic linkages within the O-specific sidechain were split. This glycanase activity liberated di- to nanosaccharides but there was no esterase activity as evidenced by absence of acetate liberation. Two virus-associated glycanases active on LPS may be quite common in smooth-specific Enterobacteraceae phages, although esterases hydrolysing O-acetyl groups are uncommon.

Dictyostelium discoideum

Degradation of LPS by amoebae of the phagotrophic slime mould Dictyostelium discoideum was reported by Malchow et al (1967). Growth of this organism in submerged culture on Salmonella or E. coli B cells as the sole nutrient, released into the medium partially-degraded LPS which could be isolated by the phenol-water procedure as a high molecular weight, water-soluble product. This material contained all the sugar constituents and exhibited the serological specificity of the native LPS. However, it differed from the native molecule by lacking β-hydroxymyristic acid and the other long-chain fatty acids of the lipid A.

Further analytical work by Malchow et al (1969) showed that the degraded LPS contained all the sugar constituents of the parent molecule, including the glucosamine backbone of lipid A, as well as O-acetyl groups on the galactose units of the O-specific chains, and the N-acetyl groups of the glucosamine in the core. However, the degraded polysaccharide was completely devoid of the ester and amide-linked long-chain fatty acids of lipid A. The degraded polysaccharide, although retaining its serological O specificity, failed to sensitize erythrocytes for passive haemagglutination. It was much less toxic in mice than the original LPS, $LD_{50} \equiv 100$ µg/mouse compared to $LD_{50} \equiv 2$ µg for source LPS (Tanaka, unpublished observations cited from Malchow et al, 1969). The Dictyostelium-degraded polysaccharide derived from E. coli B gave similar results. Later work by Nigam et al (1970) gave similar results but little β-hydroxymyristic acid was released. Thus the mode of action of D. discoideum is to cleave the long-chain fatty acids from the lipid A component of bacterial LPS by means of esterases and amidases.

Mammalian systems

In a manner possibly similar to the action of D. discoideum, enzymic deacylation is thought to be the mode of detoxification of LPS by mammalian serum in vivo and in vitro (Rudbach et al, 1966). Early investigators (Goodale et al, 1956) demonstrated that human and horse sera inactivated the pyrogenic property of LPS during prolonged incubation (for about 12 h) at 37°C. Incubation of LPS in serum or plasma also altered its antigenic characteristic (Stauch and Johnson, 1959). The detoxifying effect of serum from a number of species also reduced the lethal action of endotoxin as well as the Schwartzman reaction and tumour necrotizing properties (Landy et al, 1957; Ho and Kass, 1957). Early work by Skarnes et al (1958) indicated that the detoxifying property in serum or plasma was destroyed by heating at 56°C for 60 min and the term "endotoxin detoxifying component" (EDC) was proposed. The persistence of intravenously injected LPS in the circulation of experimental animals, which circulates in the blood for at least 6 hours after intravenous injection of sublethal doses (Chedid et al, 1963), implied that the rate of detoxification in vivo proceeds slowly and that this process takes place mostly in the vascular compartment. Skarnes (1966) attempted to isolate and characterise the EDC activity and found that an α-globulin fraction separated on DEAE-cellulose was able to disaggregate or partially degrade LPS without detoxifying it. This globulin which was anion-dependent proved to be an endotoxin-binding protein associated with an α_1-lipoprotein. It seemed that a two-stage reaction was required for ultimate detoxification of LPS. First there was the anion-dependent "binding and degradation" of endotoxin by a heat-stable α_1-lipoprotein and this was followed by enzymic

detoxification by a heat-labile α_1-globulin. Immunodiffusion and immunoelectrophoretic methods demonstrated that the α_1-globulins which bind endotoxins in the presence of anions were organophosphate-resistant esterases of the carboxylic acid type. Greisman, Young and De Buy (1969) demonstrated that the pyrogen-tolerant state which followed repeated injection of LPS was mediated by two distinct mechanisms. There was an early transient cellular refractory state and a later production of antibody which assists the reticuloendothelial system with clearance and destruction of the molecule. O-specific antibodies appeared to play the major role in the late phase of pyrogen tolerance following a single intravenous injection of endotoxin. More recently, Johnson et al (1977) isolated a protein, termed the LPS inactivator (LPS-I), from normal human serum. LPS treated by LPS-I lost its toxicity for mice and reactivity in the Limulus assay and appeared to be irreversibly disaggregated. This process was temperature and time dependent and was not blocked by inhibitors of serine esterases. The LPS inactivator migrated as an α-globulin in whole serum but its relation to α-globulin described by Skarnes was not clarified.

Rutenberg et al (1967) showed that the reticuloendothelial cells of the spleen take up and rapidly detoxify LPS but only when the spleen was denervated. Spleen homogenates yielded an esterase (Skarnes et al, 1968) with detoxifying activity. This esterase was found to be a carboxylic, organophosphate-resistant enzyme though differing physiochemically from the serum esterases which also detoxify LPS.

An esterase-rich fraction from rabbit polymorphonuclear leukocytes was found to abolish the lethal action of endotoxins (Skarnes,

1970). Gimber and Rafter (1969) incubated radioactive LPS with intact leukocytes and discovered that labelled fatty acids were liberated. This indicated that a leukocyte lipase acts on endotoxin. Filkins (1971) demonstrated that sonicates of macrophages from liver, lung and peritoneal exudates displayed marked endotoxin detoxifying ability. Gans and Wendell (1976) suggested that an enhanced blood clearance rate during endotoxin tolerance was due to an increase in the number of specific membrane binding sites on the macrophage, rather than to effects mediated by humoral factors. Thus LPS in the mammalian body was detoxified by carboxylic esterases in the plasma, in granulocytes and in spleen. Although the inactivation of LPS in most of the above systems was time and dose-dependent, no direct evidence for enzymatic alteration of LPS was obtained.

Autolysis

Loss of LPS during the developmental cycle of a gram-negative organism was demonstrated by Sutherland (1976) working with Myxococcus xanthus. In the developmental cycle of this and related species, a simple fruiting body was formed in which spherical myxospores were surrounded by extracellular polysaccharide slime. When the entire bacillus underwent conversion to the myxospore form, there was no apparent loss of cell material which was observed during the morphogenesis of gram-positive bacilli to endospores.

Thus while LPS made up 0.7 to 1.1% of the dry weight in the bacillary forms of two strains of M. xanthus and was extractable by the hot phenol procedure, attempts to obtain LPS from the myxospores of either

strain were unsuccessful. Neither was carbohydrate-containing material detectable in the phenol-phase after extraction of myxospores as would be expected if the polymer had become more hydrophobic through loss of polysaccharide. Chromatographic analysis of myxospore hydrolysates showed that in one strain, the mannose of the LPS had almost disappeared and the ratio of glucose and galactosamine to glucosamine increased greatly. In the other strain of M. xanthus, the LPS contained two sugars, mannose and galactose, both of which were lost almost entirely from the myxospores with concomitant increase in glucose and galactosamine. Both strains contained 2-keto-3-deoxyoctonate in the LPS of the vegetative cells but none could be detected in hydrolysates of myxospores. The fate of the lipid A during morphogenesis of M. xanthus was, however, not elucidated. Thus there appeared to be a selective loss of the polysaccharide component, but whether this was due to excretion or to degradation and assimilation was not established.

Isolation of Lipopolysaccharide-degrading Microorganisms from Soil

So far the only workers to isolate an LPS-degrading bacterium are Voets and Beyaert (1970). They inoculated soil particles into a minimal medium containing yeast extract, LPS and agar and made serial transfers to enrich and purify the cultures obtained. An organism identified as Bacillus firmus was isolated. It grew on LPS agar without the yeast extract. Further investigations (Voets, Vandamme and De Maerteleire, 1972) with LPS from three different bacteria resulted in the isolation of only a few organisms of B. macerans on only one of the LPS. Growth was very sparse unless glucose was added. From thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) results it was

shown that the LPS was hydrolysed first to lipid A and a polysaccharide fraction; the latter was not further hydrolysed. However, free fatty-acids and phosphate were liberated from the lipid A thereby revealing the free amino groups of the disaccharide units. No free glucosamine could be detected however. The bacillus was thus displaying lipolytic activity with growth on the fatty acids liberated from lipid A. The authors did not however investigate the possible biological changes which may have occurred in the LPS.

Environmental Distribution of Lipopolysaccharide

Linked in a reciprocal fashion with the biodegradation of LPS is the question of distribution and persistence of LPS in nature. There have been few extensive studies on this point, although with the advent of the Limulus test, surveys of the endotoxin concentration in waste waters, rivers, lakes and the sea are appearing with increasing frequency (Enzinger and Cooper, 1975; Gerba and McLeod, 1976; Sullivan, Valois and Watson, 1976; Jorgensen, Lee and Pahren, 1976). Gram-negative bacteria have long been recognised as abundant in soils, natural waters and in fresh water and marine sediments. It might be expected therefore that LPS should be detectable in these locations and indeed this is the case. Sullivan et al (1976) have used the Limulus assay, in conjunction with ATP measurements to estimate both total biomass in the sea (Cheer, Gentile and Hegre, 1974) and bacterial biomass. The majority of marine bacteria are gram-negative and these studies showed that over 50% of the total biomass in the marine environment consists of bacteria. This startling claim still has to be confirmed. The persistence of LPS in the environment was also indicated

when the Limulus assay was used to detect endotoxins in potable waters and from reclaimed waste water effluents, with values from 0.78 ng/ml to 1,250 ng/ml being obtained (Jorgensen, Lee and Pahren, 1976). However it has been shown that the Limulus test can respond to both intact and partially degraded LPS in some cases (Wachtel and Tsuji, 1977).

OBJECT OF RESEARCH

In contrast to the wealth of information on the structure, biosynthesis and immunological and physiological properties of bacterial lipopolysaccharides (LPS), there are few studies on the fate of LPS in nature.

This work has been directed at examining the potential LPS-degrading capability of three systems each of which exemplifies broad categories of agents or situations in which LPS might be decomposed.

The acellular slime mould Physarum polycephalum was chosen because it belongs to the large class of phagotrophic microorganisms which utilize bacteria as food.

Gut juice from Helix pomatia was used to investigate LPS degradation because this snail is an example of an invertebrate ingesting materials rich in bacteria.

Finally, the fate of LPS when incubated with samples of marine mud and sand was explored because of the ubiquity of gram-negative bacteria in marine environments, particularly in muds.

A considerable part of the work was concerned with comparing the suitability of the Westphal and Boivin methods for extracting LPS from complex mixtures and with the utilization of anticomplementary activity, ketodeoxyoctonate analysis and gas-liquid chromatography for characterizing LPS.

MATERIALS AND METHODS

Lipopolysaccharides and Organisms

LPS from Escherichia coli $O_{111}B_4$, E. coli $O_{55}B_5$, Shigella flexneri and Salmonella minnesota, extracted by the Westphal procedure, were purchased from Difco Laboratories, Michigan, U.S.A.

E. coli $O_{111}B_4$ NCTC 800 7D433 was obtained from the National Collection of Type Cultures, Colindale, London. E. coli Lilly and E. coli O_{125} were obtained from Professor A.C. Wardlaw. One S. minnesota smooth strain and seven rough strains were obtained from Dr. G. Schmidt of the Max Planck Institut für Immunbiologie, Freiburg, Germany. Cultures were maintained on nutrient agar slopes.

For preparation of LPS, the organisms were grown in Oxoid Nutrient Broth No. 2 (code CM67) for 18 h at 37°C to give starter cultures. These in turn were used to inoculate fifteen litre fermenters containing nutrient broth and actively aerated at 37°C for 24 h. The cells were killed by adding formaldehyde or phenol to final concentrations of 0.5% and 0.1% respectively and were harvested in a Sharples continuous centrifuge (Sharples Centrifuges Ltd., England) at 20,000 rpm, with a flow rate of 250 ml/min. After three washes in physiological saline, the organisms were lyophilised.

Physarum polycephalum was obtained from Dr. J.G. Coote of this department. Cultures were grown at 26°C as a shaken suspension of microplasmodia in Carlile's (1971) medium which is referred to here as "Complete medium." When bacteria or LPS were included in this medium, peptone and yeast extract were omitted to give Basal medium.

Extraction of Lipopolysaccharide from Bacteria

Westphal extraction

The procedure of Keleti and Lederer (1974) was used, modified from Westphal *et al* (1952). Dried bacteria (20 g) were suspended in 350 ml water in a 68°C water bath; 350 ml of 90% (w/w) phenol, preheated to 68°C, was added with vigorous shaking for 30 min; the mixture was cooled to about 10°C, centrifuged at 7,000 rpm in an MSE high speed 25 centrifuge for 45 min and the upper aqueous layer collected. To the lower phenol layer was added another 350 ml of 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 5,000 rpm for 15 min to remove particulate impurities, the supernatant fluid was centrifuged at 40,000 rpm in an MSE superspeed 65 for 4 h. The sedimented pellet was resuspended in water and the ultracentrifugation procedure repeated twice. The resuspended pellet was examined by UV spectrophotometry to ensure that contaminating nucleic acid was minimal, then lyophilised.

Note: The term "Westphal Extraction" is used throughout this thesis for the overall procedure of phenol-water extraction, dialysis, ultracentrifugation, UV spectrophotometry and lyophilisation.

Boivin extraction

The procedure followed was modified from Boivin *et al* (1933). Dry bacteria were suspended in distilled water at 0-4°C to a concentration of 2% w/v. An equal volume of cold 1.0N trichloroacetic acid (TCA) was added and the mixture stirred for 8 h at 4°C. Cell debris was removed by centrifugation at 10,000 rpm for 30 min and the supernate dialysed for 48 h against running tap water followed by 24 h against distilled water. The

dialysate was concentrated by rotary evaporation to about 1% (w/v) assuming that the TCA extract contained about 3% of the starting material. LPS was precipitated with 2 volumes of 95% ethanol at 0°C by pouring the cooled solution into 95% ethanol cooled to 0°C. This was left to stand at 0°C overnight then centrifuged at 7,000 rpm for 30 min. The pellet was redissolved in water and lyophilised.

Phenol:chloroform:petroleum ether extraction of glycolipids

This procedure was essentially that developed by Galanos et al (1969). Bacteria were collected by centrifugation at 7,000 rpm for 45 min, washed with water and successively with 95% ethanol, acetone and ether with centrifugation at 7,000 rpm for 10 min at each stage. The final pellet was lyophilised. To 25 g of washed dried bacteria was added, 100 ml of the extraction reagent (90% phenol:chloroform:petroleum ether; 20:50:80 by volume). The mixture was homogenized for 2 min at 4°C and stirred for a further 10 min. After centrifugation at 5,000 rpm for 15 min at 4°C the supernate containing the glycolipids was filtered through Whatman No. 1 paper into a round-bottomed flask. The extraction procedure was carried out twice on the pellet and the supernatants were bulked. The petroleum ether and chloroform were removed in a rotary vacuum evaporator at 35°C and the residue, containing phenol, was transferred to a glass centrifuge tube and water was added dropwise until the glycolipid was precipitated. It was collected by centrifugation at 3,000 rpm for 10 min at 9°C, the supernatant was discarded and the inside of the tube was dried with a filter paper. The precipitate was washed three times with 2.5 ml of 80% (w/w) phenol followed by three centrifugation washes with ether (9,000 rpm for 10 min at 4°C). The final precipitate was lyophilised.

Extraction of Lipopolysaccharide from Degradative Systems

Westphal extraction of marine sediments

Distilled water (20 ml) was added to 100 g (equivalent dry weight) of moist sediment and the mixture was placed in a 68°C shaking water-bath. Twenty millilitres of 90% phenol (preheated to 68°C) was added and the mixture was shaken vigorously for 30 min. After cooling at 10°C, it was centrifuged at 7,000 rpm for 45 min and the upper water-layer collected. To the phenol-layer and insoluble residue was added another 20 ml of distilled water. The mixture was shaken and heated to 68°C and the procedure repeated. The aqueous layers were combined, dialysed against running tap water for 3 days and distilled water for 1 day. Particulate impurities were removed by centrifugation for 15 min at 5,000 g and the supernatant containing crude LPS centrifuged at 40,000 rpm for 4 h. The UV absorbance was checked to ensure that contaminating RNA was less than 2% of the material which was then lyophilised.

Boivin extraction of sediments

To 100 g of sediment was added 100 ml of 0.5N TCA with shaking and cooling to 4°C. The mixture was shaken for 8 h at 4°C and thereafter the extraction procedure was as described above for bacterial cells.

Westphal extraction of *Physarum* cultures and snail gut juice mixtures

Phenol was added to cultures of *Physarum* and to snail gut-juice mixtures (containing LPS) until a 45% (w/w) solution was obtained. The mixtures were shaken at 68°C for 30 min and the extraction completed as described above with other Westphal extractions. Samples were made up to

a constant volume in veronal buffer (V.B.). In later experiments with Physarum, the final dialysed solution was treated with an equal volume of 50% ethanol at 4°C to precipitate slime while ensuring that LPS itself was not precipitated. The supernate from this treatment was dialysed again and then lyophilised.

Preparation of Cell Extract and Supernate from Physarum Cultures

Physarum was grown for 4 days on 20 ml basal medium containing 200 mg of killed bacterial cells. The microplasmodia were collected by centrifugation at 15,000 rpm for 30 min and the supernate retained. Bulked pellets were suspended in 0.2M KH_2PO_4 buffer pH 6 with 1 ml of buffer for every 10 ml of original culture. The microplasmodia were disintegrated by two passages through an LKB X-Press at -25°C and the supernate collected after centrifugation at 15,000 rpm for 30 min.

Ammonium sulphate precipitates from cell extracts and supernates

Supernates and cell-free extracts obtained above were treated with ammonium sulphate (AS) to 25% saturation. The mixtures were stirred for 3 h at 4°C, centrifuged at 3,000 rpm for 10 min and the pellets collected. The 25% supernate was adjusted to 50% saturation with more ammonium sulphate and after removal of precipitate, the levels were increased to 75% and 100% saturation. The pellets were redissolved in fixed amounts of 0.2M phosphate buffer pH 6 and used directly for LPS-degradation. In initial experiments the precipitates were dialysed first for 3 days against 0.2M phosphate buffer pH 6, but subsequent experiments showed that precipitates could be used directly after dissolving in a small amount of buffer.

Chemical Analyses

Ketodeoxyoctonate determination

KDO was determined by the Keleti and Lederer (1975) modification of the thiobarbituric acid method of Waravdekar and Saslaw (1959). KDO-1,4-lactone from BDH Ltd., England, was used as standard. The absorption at 548 nm was read and calibrated against a standard curve to obtain the value for KDO. The lactone (m.w. 220) gave an absorption spectrum and E_{max} which appeared to be indistinguishable from that of the acid (Ellwood, 1970). The values given by LPS were expressed in terms of KDO itself (m.w. 238).

Paper chromatography

Samples of LPS were hydrolysed in 2 ml 1N HCl in sealed tubes for 24 h at 100°C. Hydrochloric acid was removed by repeatedly evaporating to dryness over phosphorous pentoxide and sodium hydroxide pellets *in vacuo*, remoistening and re-evacuating. Residues were finally taken up in a small amount of water to give a concentration of approximately 100 mg/ml in terms of original LPS. Samples containing 15 µl (1000 to 1500 µg) hydrolysed LPS or 5 to 20 µg standard sugar were spotted on to sheets of Whatman No. 1 paper (23 x 56 cm). Descending chromatography with n-butanol-pyridine-water (5:5:1:3) was done, the papers first being allowed to equilibrate in the chromatography cabinets for 18 h. After 18 h chromatography, the papers were dried in a fume cabinet and developed using the alkaline silver nitrate method (Trevelyan, Proctor and Harrison, 1950).

Thin layer chromatography (TLC)

TLC analysis of fatty acids was done with mild acid hydrolysates of lipid A. To 10 mg of LPS or glycolipids in 6 ml water was added 6 ml of 0.2N acetic acid, mixed vigorously, then heated in a sealed tube in a boiling water bath for 4 h. The pellet was collected after centrifugation at 15,000 rpm for 25 min at 4°C and washed three times with water and once with acetone, and finally dried over $CaCl_2$. The fatty acids were liberated with 4N NaOH at 100°C for 4 h, acidified, extracted into chloroform and adjusted to a constant volume. Samples were run on Polygram Sil N-HR (Machery-Nagel & Co.) with n-butanol-n-propanol-water (2:10:5) and petroleum ether-diethyl ether-acetic acid (35:15:1). The plates were developed by exposure to iodine vapour.

Gas-liquid chromatography (GLC)

Fatty-acids in lipid A acid hydrolysates, were methylated with diazomethane in ether and made up to a constant volume with methanol (Kates, 1972). Diazomethane was prepared by adding 1 ml of ethylene or propylene glycol and 10 ml ether to 0.5 g of Nitrosan. The reaction was started by adding 1 ml 40% NaOH and allowed to proceed in a fume cupboard at ambient temperature and the distillate collected in chilled ether (giving a characteristic yellow colour). This was added to the dried fatty acids which were methylated. After evaporation of the ether, methanol was added to a constant volume. Different amounts of the solutions were injected into a column (1 m x $\frac{1}{8}$ in) containing 5% FFAP on Chromosorb G (80 to 100 mesh) at 200°C with nitrogen (20 m/min) as carrier gas. The column was contained in a Perkin-Elmer fractometer 20 with a flame ionization detector (250°C).

Slab gel electrophoresis

Slab gel electrophoresis of intact LPS, and LPS after exposure to degradative systems was done in polyacrylamide gels with a discontinuous SDS buffer system based on the method of Laemmli (1970) as modified by Ames et al (1974). Separating and stacking gels contained 0.1% (w/v) SDS. To disperse the LPS, a 0.5 ml sample was added to 0.5 ml of 0.125M-tris-HCl buffer pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue, and the mixture heated to 100°C for 5 min. The volume of sample mixture applied to the gels was 50 µl (50 µg LPS) and bands were rendered visible by the periodic acid-Schiff (PAS) staining procedure of Zaccharius et al (1969), as modified by Huang and Evans (1973).

Serological Assays

Sensitization of sheep erythrocytes with lipopolysaccharide

Citrated sheep's blood was washed three times in saline by centrifugation, and a 2% (v/v) suspension made in veronal buffer (VB) (Kabat and Mayer, 1961). This suspension was mixed with an equal volume of 0.1 mg/ml of LPS previously activated by incubation for 18 h at 37°C in 0.01N NaOH in saline (Neter, 1956) and adjusted to pH 7 with dilute HCl. The cells and LPS were incubated for 1 h at 37°C, centrifuged and washed 3 times in VB. The sensitized erythrocytes were resuspended in VB to make a 2% suspension for haemagglutination tests. The sensitizing power of LPS extracted from degradation mixtures was tested in the same manner.

Haemagglutination tests

These were done in microtitre trays with dropping pipettes which delivered 0.025 ml per drop. Serial dilutions of antiserum were made, 0.05 ml in each well and 0.05 ml of sensitized erythrocytes added. Mixtures were shaken and results were read after 12-18 h at room temperature. Controls with normal serum and with a known positive antiserum were included. The highest dilution showing complete agglutination of LPS-sensitized erythrocytes was taken as the end-point.

Haemagglutination-inhibition tests

Serial dilutions of antigen, ie LPS or potentially degraded LPS, were made (0.05 ml volumes) in microtitre trays and 0.05 ml of a suitable dilution of antiserum containing 5 haemagglutinating units (5 HU) (5 times the titre as previously determined) added to each well. Mixtures were allowed to stand at room temperature for 1 h and 0.05 ml LPS-sensitized erythrocytes (E.LPS) added. Controls of antiserum plus E.LPS and saline plus E.LPS were included. Results were read after 18 h at room temperature. The well in which there was 50% inhibition of haemagglutination in the presence of 5 HU of antibody was taken as the end-point.

Assays Involving Complement

Complement

Freeze-dried guinea-pig C was obtained from Wellcome Research Laboratories, Beckenham. Fresh pig serum, from an adult boar, was provided by the Veterinary School, Glasgow University. For the initial

Table 3 Protocol of a haemolysin titration with typical results

Tube	1	2	3	4	5	6	7	8	9
Veronal buffer (ml)	0	2.0	1.5	1.75	1.88	1.94	-	2.0	2.5
1/3000 Haemolysin (ml)	2.0	1.0	0.5	0.25	0.125	0.06	2.0	-	-
1/30 Guinea pig complement (ml)	0.5	0.5	0.5	0.5	0.5	0.5	-	0.5	-
2% Erythrocytes (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Result:									
% Haemolysis	100%	100%	75%	20%	0	0	0	0	0

taking 100% haemolysis as the end point, the haemolysin titre is 3000 HU_{100}/ml.

Table 4 Protocol of a complement titration with typical results

Tube	1	2	3	4	5	6	7	8
Veronal buffer (ml)	-	1.0	1.5	1.75	-	1.0	1.5	2.0
1/120 Guinea pig complement (ml)	2.0	1.0	0.5	0.25	-	-	-	-
1/1920 Guinea pig complement (ml)	-	-	-	-	2.0	1.0	0.5	-
1% EA (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Result:								
% Haemolysis	100	100	90	50	0	0	0	0

For both human and pig complements the corresponding dilutions were 1/12 and 1/192.

Taking 50% haemolysis as the end point, the complement titre in this example is 480 HU_{50}/ml.

work, samples of fresh human serum were obtained from laboratory personnel. However, most of the work was done with two batches of 200 ml of freshly collected human serum supplied by Dr. J. Wallace of the West of Scotland Blood transfusion service, Law Hospital, Lanarkshire. The pig and human sera were stored in small aliquots at $-196^{\circ}C$.

Titration of complement

Serial two-fold decreasing volumes of serum, or diluted serum, were prepared in 12 x 100 mm tubes held in an ice bath and the volumes equalised to 2.0 ml with VB. Sheep erythrocytes sensitized with 2 HU_{100} of rabbit haemolytic serum (Wellcome Research Laboratories) were added as 1 ml of a 1% (v/v) suspension to each tube and the mixtures were incubated for 30 min at $37^{\circ}C$ (see Table 3 for method of standardization). The unhaemolysed cells were spun down at 3,000 rpm in an MSE bench centrifuge for 5 min and the 50% haemolysis end-point established by visual comparison with a tube containing 0.5 ml of 1% sensitized cells and 2.5 ml distilled water. In this system, human, pig and guinea-pig C contained respectively about 50, 50 and 200 HU_{50} per ml of undiluted serum. Table 4 shows the protocol of a complement titration with typical results.

Assay of anticomplementary activity

To economise on the consumption of LPS and C, the anticomplementary (AC) tests were done on test mixtures of final volume one-half of that used for the C titration. Each LPS sample, dissolved in VB was delivered in a series of 2-fold decreasing volumes to 12 x 100 mm tubes and the volumes equalised to 0.9 ml with VB (Table 5). To each tube was added

Table 5 Protocol of an anticomplementary titration with typical results

Tube	1	2	3	4	5	6	7	8	9
Veronal buffer (ml)	-	0.45	-	0.45	-	0.45	0.9	0.95	0.975
E. coli $O_{111}B_4$ LPS 200 µg/ml	0.9	0.45	-	-	-	-	-	-	-
" " " 50 µg/ml	-	-	0.9	0.45	-	-	-	-	-
" " " 12.5 µg/ml	-	-	-	-	0.9	0.45	-	-	-
5 HU_{50} human C (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.05	0.025
1% EA (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Result:									
% Haemolysis	0	0	0	50	90	100	100	40	0

Taking the 50% haemolysis end point, *E. coli* $O_{111}B_4$ LPS has 44 ACU/mg.

0.1 ml C dilution containing 5 HU_{50}. In calculating this dose of C, account was taken of the smaller volume of the AC test as compared with the C titration. An example of the calculation is as follows:

End point in C titration : 0.5 ml of 1/25 C

$\therefore$ 1 HU_{50} is contained in 0.5 x 1/25 ml of undiluted C

$\therefore$ 5 HU_{50} is contained in 5.0 x 0.5 x 1/25 ml of undiluted C

= 0.1 ml of undiluted C.

For the AC test, because of the smaller volume of test mixture, one-half this amount, ie 0.05 ml, is needed but should be delivered in a 0.1 ml volume. Thus the 5 HU_{50} dose of C in each AC tube would be 0.1 ml of a $\frac{1}{2}$ dilution.

To check the potency of C, tubes containing 0.1, 0.05 and 0.025 ml diluted C, without LPS, were included in each series of tests. The tests were incubated at 4°C for 18 h followed by 1 h at 37°C, when 0.5 ml of 1% sensitized erythrocytes was added before a further incubation at 37°C for 30 min to detect residual C.

To facilitate the spectrophotometric estimation of haemolysis in these relatively small-volume test mixtures, a slight modification of the procedure of Macmorine, Wardlaw and Weber (1965) was used: the tubes were centrifuged for 5 min at 3,000 rpm and the supernatant fluids were poured off the pelleted red cells and discarded. The residual, unhaemolysed erythrocytes were then haemolysed in 3 ml distilled water and the optical density read in an EEL colorimeter with 430 nm filter. A graph of optical density (OD) against log dose of LPS was plotted and the

end-point taken at 50% haemolysis, ie that OD given by 0.025 ml of 1% sensitized cells diluted to 3.0 ml in distilled water. The AC value of the LPS was determined from the graph by reading off the interpolated number of µg corresponding to 50% haemolysis. One Anticomplementary Unit (ACU) was defined as that weight of LPS which, when incubated with 5 HU_{50} of C under the above conditions, gave 50% haemolysis. To facilitate comparison of the different LPS and the different species of C, the AC activity was expressed as the number of ACU per mg of LPS (ACU/mg).

Preparation of anti-lipopolysaccharide sera

The LPS suspension was made up at a concentration of 1.5 mg/ml in distilled water and 1.0 ml emulsified with 1.0 ml Bayol F (Esso) containing 1.0 mg heat-killed tubercle bacilli. Arlacel A (0.5 ml) was added and the mixture was ultrasonicated. Two 1.0 ml amounts of this mixture were injected intramuscularly into the hind-limbs of New Zealand White rabbits. After one month a similar pair of injections, minus the tubercle bacilli, was given intraperitoneally. Blood was collected 10 days after the second injection and the serum separated and stored at -20°C.

Experimental Systems for Studying Lipopolysaccharide Degradation

Degradation of lipopolysaccharide by Physarum

Preliminary work showed that if the nutrient sources in Carlile medium (1971) ie glucose, yeast extract and peptone, were left out and killed bacteria added instead, the slime mould responded by migrating up the side of the glass flask. This could be prevented by use of "Basal medium" (BM)

consisting of Carlile medium from which the peptone and yeast extract were omitted but which still contained the glucose. To 20 ml of BM was added measured amounts of either killed bacterial cells or LPS. Physarum was inoculated from an actively growing culture in Carlile medium and the incubation mixture was shaken at 100 rev/min at 26°C in the dark. After a stated time the reaction was terminated by phenol-water extraction.

With cell-free extracts of Physarum or culture supernatant fluids, fixed volumes were usually incubated at 30°C with a known initial amount of LPS, the reactions being terminated either by phenol-water extraction or by heating in a boiling water bath for 5 min. These procedures were similarly used with ammonium sulphate precipitates of the Physarum extract and the culture supernatant fluids. The precipitates were made up to a fixed volume with 0.2M phosphate buffer pH 6 and 1 ml amounts of this material were incubated with LPS. The reactions were terminated by placing the reaction mixtures in a boiling water bath for 5 min.

Degradation of lipopolysaccharide by gut juice from the snail Helix pomatia

To 4 mg of LPS was added variable quantities of gut juice from the snail Helix pomatia (Sigma Chemical Co., St. Louis, U.S.A.) made up to a final volume of 4 ml with 0.2M phosphate buffer pH 5. After incubation at 30°C for 3 h, the reaction mixtures were extracted by the Westphal method and adjusted to a final volume of 1 ml.

Degradation of lipopolysaccharide by marine sediments

Using a simple coring device (a metal tube of diameter 11 cm) samples of marine mud were collected from the intertidal zone of the Clyde

Estuary near to Dumbarton (Grid reference: NS 424 740). Samples of mud and sand were obtained from Kames Bay (NS 171 550) and White Bay (NS 176 591) on Great Cumbrae Island in the Clyde Estuary. Sediment samples were used either when fresh or after a period of "ageing" for several months at room temperature in the laboratory. The "ageing" was done simply by leaving the sediment in an open vessel and periodically adding water to replace that lost by evaporation.

The cores in some cases were sectioned into different layers of depth. Samples were sieved through a 2 mm mesh sieve to remove large particles of mineral material, plant debris, shells, worms, etc. Aliquots of each sample of sediment under test for LPS-degrading activity were extracted with phenol-water or TCA, or both, in order to obtain an estimate of the amount of endogenous LPS present. The degradation tests were done by placing a weighed sample of sediment in a 300 ml screw-cap centrifuge bottle, adding varying amounts of killed bacteria and incubating at 22°C. Samples were incubated either aerobically or anaerobically. The former was done by loosening the caps and shaking the vessels on an orbital shaker at 100 rev/min while the latter was performed by static incubation with the caps screwed tight.

Isolation of lipopolysaccharide-degrading bacteria from sediment

This was done by serial transfer in seawater-LPS medium. A sample of sediment previously shown to degrade LPS, was added to 5 ml of sterile sea water containing *E. coli* $O_{111}B_4$ LPS at a concentration of 4 mg/ml. After incubation at 20°C for 7 days, 0.05 ml was taken from this sample and subcultured to a further 3 ml of sterile sea water containing the same LPS at a concentration of 4 mg/ml. Similar transfers were made serially 7 times

and samples from the eighth culture were plated out on silica gel plates which contained 4 mg/ml *E. coli* $O_{111}B_4$ LPS as sole carbon and energy source. The silica plates were prepared according to Thatcher and Weaver (1974) immediately prior to use as synergesis caused shrinking of the gel after about five days. Isolates were subcultured twice on silica-LPS and were finally stored on nutrient agar (Oxoid Ltd., England) slopes. Only gram-positive and gram-variable organisms were selected for degradative experiments since it was desirable to avoid organisms which would themselves contribute LPS to LPS-degradation tests.

Incubation of bacterial isolates with lipopolysaccharides

The potential LPS-degrading bacterial isolates were taken from cultures growing on silica gel-LPS plates. They were inoculated into 1 ml sterile sea water containing 0.1% ammonium phosphate and 4 mg of LPS. The incubation mixtures were grown at 22°C for 10 days after which the samples were assayed for LPS either directly or after Westphal extraction. Bacterial isolates were stored in freeze-dried ampoules or on slopes of Zobell marine agar (Difco Ltd.).

RESULTS

Anticomplementary Assay for Lipopolysaccharide

Although all lipopolysaccharides (LPS) possess certain general features in their chemical composition and most of them produce the diverse physiopathological changes designated "endotoxin reactions," there may be substantial quantitative differences between different preparations. In short, there is probably no simple but fully satisfactory assay method for detecting and measuring unknown LPS in complex mixtures. At the outset of this investigation, various methods were considered for assaying LPS in order to study the degradation of its endotoxic activity by slime mould cultures, marine sediments and invertebrate gut juice. The three most attractive possibilities were the Limulus Lysate Test, the Chick Embryo Lethality Test and the Anticomplementary (AC) Test. The prime considerations were rapidity, cheapness, convenience, reproducibility and specificity; high sensitivity was not thought essential since relatively large amounts of LPS would be used in each degradative system. After weighing the various factors, the AC test was selected for evaluation and application to the main project. The following pages deal with the development of the AC test and its performance with a variety of LPS. One of the main points to emerge was that human serum was a much better source of complement (C) than the serum of guinea-pig or pig for detecting low levels of LPS.

Anticomplementary dose-response curve

AC activity was assayed by incubating dilutions of LPS in VB with a constant dose of C and then detecting residual C by adding antibody-coated sheep erythrocytes (EA). Fig 7 shows a typical log dose response

Figure 7. Typical log dose response curve for determining anticomplementary activity of LPS towards human complement.

Table 6 Parallel titrations of anticomplementary activities of different species of LPS towards different species of complement in a single test

LPS	Anticomplementary activity (ACU/mg) towards complement from:		
	Man	Pig	Guinea-Pig
Escherichia coli $O_{111}B_4$	40	20	0.2
E. coli O_{125}	200	20	0.8
E. coli O_{55}	200	40	0.66
Shigella flexneri	200	143	0.66
Bordetella pertussis	200	80	4.0
E. coli Lilly	333	200	0.5
Salmonella typhi	400	83	0.5

curve for determining the AC activity towards human C. The 50% haemolysis end-point for *E. coli* $O_{111}B_4$ LPS corresponded to an interpolated $\log_{10}$ dose of 1.7 (ie 50 µg of LPS), giving an AC activity of 20 ACU/mg.

Effect of different lipopolysaccharide and complement species

A collection of 7 different LPS was titrated repeatedly for AC activity against constant test doses (5 HU_{50}) of C from man, pig and guinea-pig (Table 6). The values recorded in the table were geometric means of at least 2, and usually 3, estimations. The LPS preparations are arranged in order of increasing AC activity towards human C and span a 10-fold range of activity. *E. coli* $O_{111}B_4$ LPS was the least anti-complementary and *S. typhi* the most. With guinea-pig C, the AC potencies were between 50 and 800 times lower (geometric mean 200); *E. coli* $O_{111}B_4$ was the least active again. However, *B. pertussis* LPS was the most actively AC with guinea-pig C. Pig C responded similarly to human C, although the comparative range of ACU mg/ml values were about one-half with *E. coli* "Lilly" the most active LPS. It will be noted that although the rank-order of activity of the 7 LPS was different with the C from the three species, the difference between the most and least active within each species of C was in the order of 10-20 fold.

When different batches of the same LPS were tested for AC activity against sera from human, pig and guinea-pig, over an 18 month period, variations in activity were observed (Table 7). With most preparations this was not great. One particular batch of *Sh. flexneri* LPS however was much less active than other batches obtained from the same supplier. This was similarly found for one batch of *E. coli* O_{55} LPS as shown by the

Table 7 Anticomplementary activities of different batches of LPS from various species of bacteria towards complement from the pig, guinea-pig and human being. These data were accumulated over an 18 month period.

LPS	Anticomplementary activity (ACU_{50}/mg) and (geometric mean) toward complement from:		
	Man	Pig	Guinea-pig
Escherichia coli O_{55}	50, 40, 31 (41)	33, 33 (33)	0.7, 0.7, 0.6 (0.7)
Salmonella minnesota smooth	50, 40, 36 (42)	12, 22 (17)	4, 4 (4)
Shigella flexneri	200, 20, 10 (75)	143, 40, 20 (67)	0.7, 0.6, 0.5
E. coli O_{125}	33*, 31*, 100, 143 (77)	33, 33 (33)	25, 20 (22)
E. coli $O_{111}B_4$	62*, 67*, 125, 100 (88)	67, 33, 20 (40)	1, 0.2, 0.2 (0.5)
Bordetella pertussis	200, 125 (162)	80, 40 (60)	7, 5 (6)
E. coli Lilly	100*, 100*, 333, 200 (183)	200, 120 (160)	6, 5, 4 (5)
S. minnesota rough (R595)	200, 180, 180 (187)	72, 60 (66)	12, 10 (11)
S. typhi	333, 333 (333)	10, 22 (16)	2, 1 (1.5)

*Samples were extracted by the Boivin method.

Table 8 Parallel titrations of anticomplementary activities of different species of LPS towards human sera from different individuals (A - D). Columns 1 and 2 refer to different bleedings from each individual 2 months apart.

LPS	Anticomplementary Activity (ACU/mg) towards serum							
	A		B		C		D	
	1	2	1	2	1	2	1	2
E. coli $O_{111}B_4$	33	33	10	20	33	21	33	33
E. coli O_{55}	10	42	10	20	33	21	33	33
B. pertussis	100	250	100	80	100	40	100	100
E. coli Lilly	200	67	220	67	220	33	220	222
Sh. flexneri	250	250	170	333	200	250	170	166

Table 9 Anticomplementary activities of LPS from different species of bacteria towards complement from different human sources stored for various periods

All the titrations were done in parallel on the same day

LPS	Anticomplementary activity (ACU/mg) towards complement from:		
	A	E	F
Escherichia coli $O_{111}B_4$	56	24	24
E. coli O_{55}	20	52	20
Shigella flexneri	50	48	200
Bordetella pertussis	100	58	52
E. coli Lilly	150	10	10
Salmonella minnesota smooth	80	180	50
S. minnesota rough	128	96	128

A: Author's own complement; tested fresh.

E: Stock complement, stored at -196°C for 1 year.

F: Stock complement, stored at -196°C for 1 week.

different values in Table 6 and Table 8 . LPS extracted by the Boivin method was shown to be about one-half as AC as that extracted by the Westphal method. This comparison however was restricted to E. coli LPS.

Samples of serum obtained from 4 of the laboratory staff on 2 occasions (2 months apart) were used for AC tests with various LPS (Table 8). These results showed that there were no substantial changes in the sera obtained on the two occasions.

Further work using the AC assay required large amounts of human serum which was obtained by courtesy of Dr. Wallace, West of Scotland Blood Transfusion service, Law Hospital, Lanarkshire. All sera were stored in small aliquots in liquid nitrogen until required. Table 9 indicates that the AC responsiveness was not affected during storage over 1 year. The variation in AC activity of Sh. flexneri and S. minnesota smooth LPS between sera E and F may be due to different immunological histories of the individuals involved.

Degradation of Lipopolysaccharide by Physarum polycephalum

Lipopolysaccharide in heat-killed bacteria

Experiments to investigate the possible degradative action of Physarum microplasmodia on the LPS in heat-killed bacteria were made by growing the slime mould for 7-10 days in flasks where two essential components of the medium (peptone and yeast extract) were replaced by 400 mg dry weight of either E. coli $O_{111}B_4$ or S. minnesota. Previous attempts to leave out glucose as well as tryptone and yeast extract from

Table 10 Degradation of LPS in killed cells of *E. coli* and *S. minnesota* during incubation with *Physarum* for 7 days (Expt 1) and 10 days (Expt 2)

Westphal extract from culture flask containing		Residual quantity per culture flask after incubation and extraction*					
		Anticomplementary activity (ACU)		KDO (μg)		Haemagglutination-inhibition† (reciprocal HAI titre)	
Physarum	Bacteria (400 mg)	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	*E. coli* $O_{111}B_4$	265	325	347	385	2,560	2,560
+	*E. coli* $O_{111}B_4$	30	70	354	382	640	1,280
0	*S. minnesota*	360	420	176	201	40	40
+	*S. minnesota*	20	100	185	174	10	10
+	none (complete medium)	20	20	23	17	0	0

* each culture was extracted by the Westphal method.

† in system containing 5 HA units of homologous antiserum and E_{LPS}.

the components of Carlile's medium (complete medium) resulted in the migration of the Physarum up the side of the flask walls, out of the medium. Thus a basal medium without tryptone and yeast extract but containing glucose was used. As a control, Physarum was also grown on the complete medium alone where the growth was usually more abundant than on the bacteria. At the end of the exposure period, the entire contents of each culture flask, including Physarum adhering to the walls, was extracted by the Westphal procedure.

Three features of LPS were measured in order to detect possible degradation, namely AC activity, KDO content and ability to inhibit the agglutination of E.LPS by specific antiserum. From the AC results (Table 10) it will be seen that Physarum grown on complete medium (no added bacteria) yielded an extract with only slight AC activity. This activity may be due to the polysaccharide slime, a sulphated polygalactan which the organism produces in abundance. The bacteria alone in the doses used yielded highly AC extracts, but extracts of bacteria on which Physarum had grown for 7-10 days showed residual AC activity only slightly higher than that given by Physarum grown in the absence of bacteria. It was apparent therefore that the slime mould was able to degrade that feature of the bacterial LPS responsible for AC activity. From the work of Galanos (1975) this is taken to be mainly lipid A.

KDO analyses of the same extracts showed that this component of the LPS, either from E. coli or S. minnesota, was not significantly affected by 7-10 days exposure to Physarum. A small amount of KDO or KDO-like material was extracted from the Physarum alone grown on complete medium. This was thought to have arisen from the polysaccharide slime produced by

Table 11 Degradation of LPS in killed cells of *E. coli* $O_{111}B_4$ (A) and *S. minnesota* R60 (B) (500 mg) during incubation for different periods with *Physarum*

Westphal extract from culture flask of *Physarum* + bacteria on day	Residual quantity per culture flask after incubation and extraction*							
	Anticomplementary activity (ACU)		KDO (µg)		Reciprocal Haemagglutination (HAI) titre		Erythrocyte sensitizing ability (ESA) of extract†	
	A	B	A	B	A	B	A	B
0	500	390	264	83	256	-	64	-
1	530	190	240	76	128	-	64	-
2	140	25	278	81	64	-	16	-
3	50	20	220	87	128	-	4	-
6	40	20	250	93	64	-	4	-
9	40	20	260	89	128	-	2	-
Controls:								
Bacteria alone on day 0	520	530	276	98	256	-	120	-
Physarum in complete medium on day 9	20			< 5	0		0	

*by Westphal method

†HA titre of rbc sensitized with extract at (0.05 ml from stock of 5 ml at 1/1).

the organism. In subsequent experiments, any residual slime remaining after phenol-water extraction was precipitated out by adding ethanol whereupon the KDO-like material was eliminated.

There appeared to be no extensive degradation of the immunodominant sugars in either E. coli or S. minnesota LPS, since the HAI titres only dropped by a factor of between 2 and 4 during exposure to Physarum.

Information on the rate of degradation of the LPS in situ in E. coli whole cells was obtained by setting up a series of replicate flasks of heat-killed bacteria and Physarum as before, but terminating the reaction by phenol-water extraction of individual flasks on different days. The results showed that maximum loss of ACU occurred between day 3 and day 6, but that even by day 9 there had been no significant loss of KDO or HAI activity (Table 11). Under these conditions Physarum reached the stationary phase of growth by day 4 (MacLeod and Coote, personal communication).

Another index of attack on the lipid portion of the LPS was provided by tests of the ability of the extracts to sensitize red blood cells to agglutination by antiserum (ESA activity). This property was largely destroyed by exposure to Physarum (Table 11).

Action on extracted lipopolysaccharide

Experiments similar to the above were done in which Physarum was incubated with purified LPS rather than with killed bacteria. The results in Table 12 show that after 5 days incubation the ACU in Westphal extracts of the culture contents had greatly declined, while the KDO levels

Table 12 Degradation of purified LPS by *Physarum* during 5 days incubation

Westphal extract from culture flask containing		Residual quantity per culture flask after incubation and extraction*	
Physarum	LPS (5 mg)	ACU	KDO (µg)
0	*E. coli* $O_{111}B_4$	570	532
+	*E. coli* $O_{111}B_4$	25	536
0	*S. minnesota*	750	351
+	*S. minnesota*	80	275
+	none (complete medium)	15	21.5

*by the Westphal method.

Table 13 Degradation of a variety of LPS by Physarum

Composition of reaction mixture			Residual quantity per culture flask after incubation and extraction*			
Basal medium	LPS (20 mg)	Physarum	ACU	KDO (µg)	reciprocal HAI titre	ESA titre
+	-	+	200	25	0	0
+	S.minnesota smooth (SmS)	+	800	810	1024	64
-	S.minnesota smooth (SmS)	-	2200	750	2048	1024
+	E. coli $O_{111}B_4$	+	800	1820	128	32
-	E. coli $O_{111}B_4$	-	1800	2580	256	512
+	E. coli $O_{55}B_5$	+	700	1340	256	32
-	E. coli $O_{55}B_5$	-	2000	1320	256	512

*Cultures were incubated for 5 days then extracted by the Westphal method.

remained essentially unchanged. The AC activity of *E. coli* $O_{111}B_4$ was reduced by over 90% while that of *S. minnesota* was about 80%. There was no significant loss of KDO, indicating its escape from attack. The value for KDO obtained from Westphal extract of *Physarum* grown on complete medium without added LPS was thought to be due to the extracellular slime, a sulphate polyglucan, which is produced in large amounts. When the above degradation experiment was repeated (Table 13) and the *Physarum* control was grown on basal medium rather than on complete medium, there was little slime produced, as reflected by less colour in the KDO test. Each of the three LPS showed a loss of 60 to 80% of their AC activity after incubation with *Physarum*. Again the KDO values were little affected. The HAI titre was similarly unaffected indicating that the immunodominant sugars in the oligosaccharide part of the LPS molecule were not attacked. The reduction in ESA titre, which depends on the ability of LPS to coat red cells, indicated an alteration of the lipid component of the LPS molecule. The AC activity and ESA titre were decreased to about the same extent for each of the LPS tested.

Location of the degradative enzymes

To find out whether degradation of LPS in *Physarum* cultures was mediated by intra- or extra-cellular enzymes, LPS was incubated for 48 h with the culture supernatants or a cell extract prepared from *Physarum* previously grown on *E. coli*. The LPS-degrading activity was labile to heat at 100°C for 5 min, indicating its enzymic nature. The results in Table 14 show that both culture supernates and cell-extract were able to reduce the ACU in the LPS, although a clear demonstration of this effect

Table 14 Degradation of LPS by culture supernate and cell extracts of Physarum after 24 h (Expt 1) and 48 h (Expt 2) incubation at 30°C

Each reaction mixture contained LPS (4 mg) made up to a total volume of 4 ml with either the cell extract which had been diluted in 0.2M KH_2PO_4 buffer pH 6, or with the culture supernatant.

Reaction mixture		Residual quantity per mixture after incubation and extraction*			
Culture fraction	*E. coli* LPS added	ACU	KDO (µg)	reciprocal HAI titre	ESA titre
Expt. 1					
-	$O_{111}B_4$	500	290	512	128
supernatant	$O_{111}B_4$	170	269	512	32
cell extract	$O_{111}B_4$	130	220	256	8
Expt. 2					
-	$O_{55}B_5$	300	142	1024	-
supernatant	$O_{55}B_5$	30	122	512	-
cell extract	$O_{55}B_5$	170	144	256	-
supernatant	-	20	65	512	32
cell extract	-	170	60	32	2

*by the Westphal method.

Figure 8. GLC trace of methyl esters of fatty acids remaining after incubation of S. minnesota LPS with Physarum extracts

(A) S. minnesota LPS alone

(B) S. minnesota LPS after incubation with cell-free extract (CFE) of Physarum

(C) S. minnesota LPS after incubation with culture supernate of Physarum

Peaks correspond to (1) lauric; (2) myristic; (3) palmitic; (4) β-hydroxymyristic.

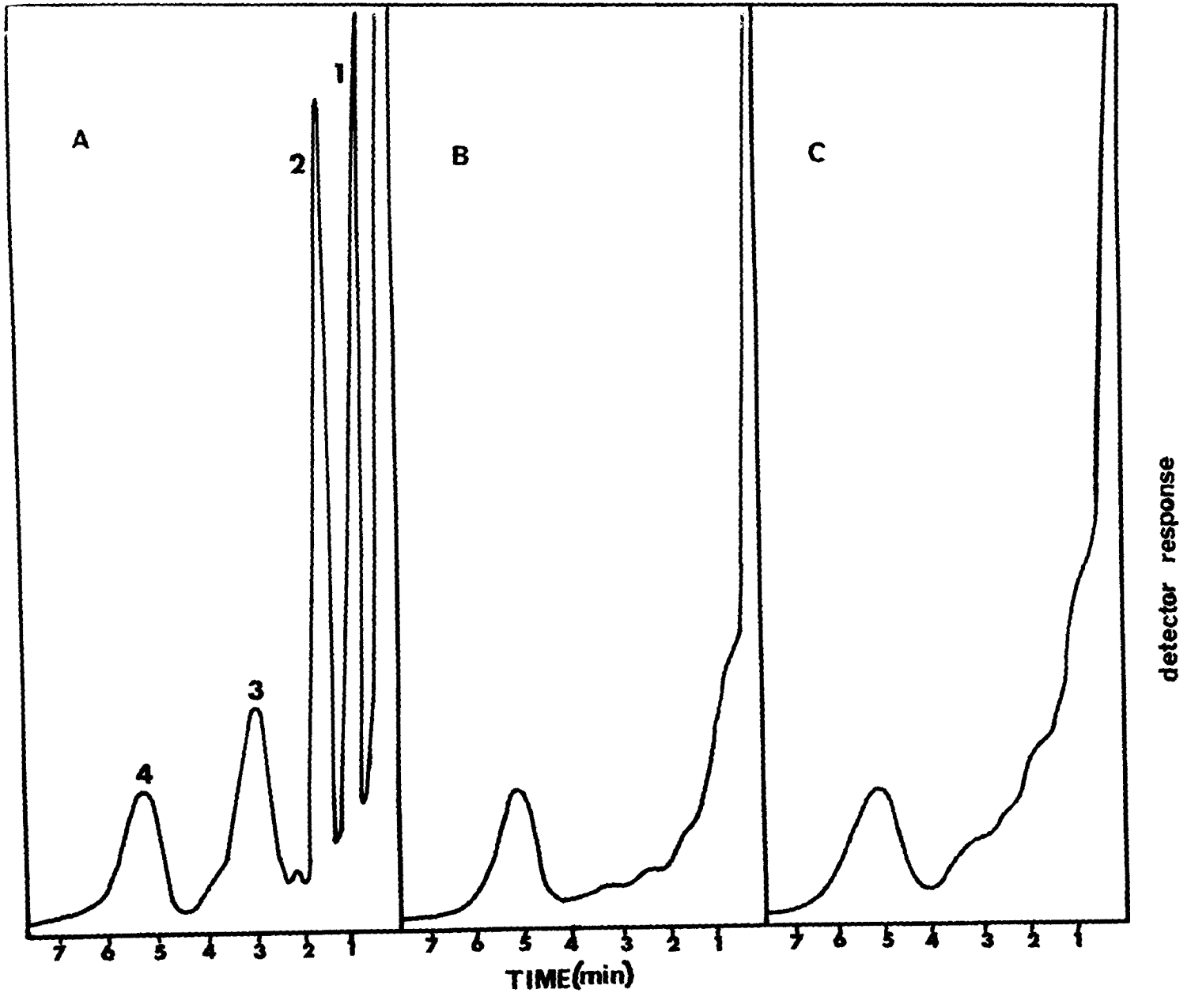
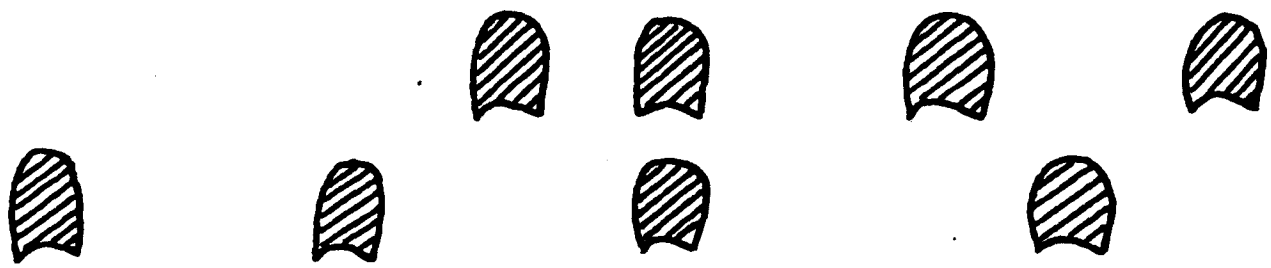

A
B
C
1
2
3
4
7 6 5 4 3 2 1
7 6 5 4 3 2 1
7 6 5 4 3 2 1
TIME(min)
detector response

Figure 9. TLC of *Physarum*-degraded LPS after hydrolysis and extraction into chloroform.

(C.F.E. = *Physarum* cell-free extract).



β-OH Myristic acid	Super-nate	Super-nate + LPS	Lauric acid	S.minne-sota LPS alone	C.F.E. alone	Palmitic acid	C.F.E. + LPS	Myristic acid

with the cell extract was hampered by the high background of AC activity. Neither KDO nor HAI activity was noticeably affected.

Loss of fatty acids from the lipopolysaccharide

To investigate the possible removal of fatty acids from LPS during incubation with Physarum, culture supernatants and Physarum extracts were incubated with LPS as detailed in Table 14. The reaction mixtures were subjected to Westphal extraction, dialysis and freeze-drying. Lipid A was released by mild acid hydrolysis and fatty-acids split off with alkali. The methyl esters were analysed by gas-liquid chromatography, with the results shown in Fig 8. Control preparations of lipid A gave clearly separated peaks corresponding to lauric, myristic, palmitic and β-hydroxymyristic acids. In contrast, lipid A from LPS with Physarum supernates or cell extract showed little or none of the first three although the β-hydroxymyristic was still present. TLC analyses confirmed GLC results in that lauric, myristic and palmitic acids were absent from hydrolysates of Physarum-treated LPS (Fig 9). It therefore appeared that the principal effect of Physarum on LPS was to remove the non-hydroxylated long-chain fatty acids.

Effect of temperature

Physarum was cultivated at 26°C in shake flasks. The cell extract and culture supernate were prepared as in the previous experiments but the substrate bacteria used was S. minnesota R595. After incubation of the extracts with S. minnesota smooth (SmS) LPS for 24 h at various temperatures, the reactions were terminated by phenol-water extraction (Table 15). For both the cell extract and the supernatant fluid, high

Table 15 The effect of temperature on degradation of LPS by *Physarum* extract

Composition of reaction mixture		temperature of incubation C°	Residual quantity per mixture after incubation and extraction*			
Physarum† extract	*S.minnesota* SmS LPS		ACU	KDO	HAI µg	ESA
-	+	-	300	37	128	128
S	-	30	40	25	8	2
S	+	20	100	61	64	8
S	+	30	60	54	64	4
S	+	37	150	56	128	16
CFE	-	30	100	19	4	0
CFE	+	20	60	27	128	8
CFE	+	30	100	24	128	8
CFE	+	37	240	69	128	16

*Reaction mixtures were incubated for 24 h then terminated by Westphal extraction.

†Where S is culture supernate and CFE is *Physarum* cell extract from cultures grown on bacteria.

Table 16 Degradation of two *E. coli* and two *S. minnesota* LPS by extracellular components from *Physarum*

Composition of reaction mixture		Residual quantity per mixture after incubation and extraction*		
Physarum supernate	LPS	ACU	KDO µg	HAI
+	-	110	12	32
+	*E. coli* $O_{111}B_4$	260	255	1024
-	*E. coli* $O_{111}B_4$	560	287	4096
+	*E. coli* $O_{55}B_5$	220	121	1024
-	*E. coli* $O_{55}B_5$	660	145	2048
+	*S.minnesota* smooth	220	56	512
-	*S.minnesota* smooth	670	78	1024
+	*S.minnesota* R595	230	319	512
-	*S.minnesota* R595	830	355	1024

*3 ml of the culture supernate was incubated for 3 h at 30°C with 5 mg of each LPS. The reactions were terminated by Westphal extraction.

background values for each of the parameters studied, ie ACU, KDO, HAI and ESA, occurred. This was probably due to undegraded LPS from the substrate bacteria on which the Physarum had been grown. The KDO values for the LPS incubated at each of the temperatures did not decrease. The KDO content of CFE and supernate is probably due to LPS from the rough S. minnesota on which the Physarum had previously been grown. There was similarly, at each temperature, very little decrease in the HAI titre, with either the cell extract or the supernate reaction mixtures. Incubation of LPS with the supernate at 20°C produced a 66% loss of ACU, while at 30°C there was an 80% loss. Incubation of the cell extract with LPS at 37°C similarly showed the smallest drop in ACU with only a 20% reduction occurring. Incubation at 20°C was best for degradation by the cell extract. The changes in ESA titres paralleled the AC changes, with incubation at 37°C being the least effective. Thus the optimum temperature for degradation seems to be between 20° and 30°C.

When the culture supernate from Physarum grown on S. minnesota R595 cells was incubated with various LPS at 30°C (Table 16) there was a similar reduction in the ACU of both E. coli and S. minnesota LPS. Again the KDO and HAI values for each of the LPS were not greatly affected although there was consistent reduction in each of the values after incubation with the supernate .

Rapidity of degradation

Physarum cell extract and supernate were prepared as previously, after growth on E. coli $O_{111}B_4$ bacteria. The incubation of the LPS with the cell extract and supernate was terminated at timed intervals by phenol-

Table 17 Rapidity of LPS-degradation by different *Physarum* extracts

Reaction mixture		Residual activity after incubation for various times (h)															
		ACU				KDO µg				ESA				HAI			
Physarum extract*	*E. coli* $O_{111}B_4$ LPS	0	2	8	24	0	2	8	24	0	2	8	24	0	2	8	24
-	+	420	-	-	-	249	-	-	-	64	-	-	-	512	-	-	-
CFE	-	380	-	-	-	37	-	-	-	8	-	-	-	128	-	-	-
S	-	300	-	-	-	33	-	-	-	16	-	-	-	256	-	-	-
CFE	+	760	380	330	280	257	251	217	208	64	32	8	4	1024	512	256	256
S	+	680	320	280	280	284	262	214	232	64	32	16	8	1024	1024	512	512
CFE†	+	720	-	-	800	237	-	-	225	32	-	-	32	512	-	-	512
S†	+	720	-	-	680	233	-	-	222	32	-	-	16	1024	-	-	1024
CFE‡	+	720	-	-	600	202	-	-	193	64	-	-	32	512	-	-	512
S‡	+	880	-	-	600	235	-	-	187	64	-	-	32	512	-	-	512

*Cultures had been grown on basal media + 200 mg *E. coli* $O_{111}B_4$ dried cells for 2 days before separation by centrifugation.

†Heated to 100°C in a water bath for 15 min.

‡From *Physarum* grown on complete medium.

water extraction (Table 17). Controls of cell extract and supernate inactivated by heating and cell extract and supernate from Physarum grown in the absence of bacteria on complete medium, showed little ability to reduce the ACU, KDO, ESA and HAI values for the LPS.

The LPS incubated with cell extract and supernate from Physarum grown on E. coli showed a loss of AC activity after only 2 h. During this time the AC activity fell to the background values obtained from cell extract and supernate alone. Incubation of LPS with both cell extract and supernate for up to 24 h resulted in a more gradual reduction in ACU. The ability of LPS to sensitize erythrocytes, indicated by the ESA titre, also showed a reduction with time, and paralleled the loss of AC activity. There was no real change in KDO content after 2 h but after 24 h both samples showed a loss of about 20% of their KDO. Similarly there was only a slight reduction in HAI titre, 2 to 4-fold, after 24 h incubation. Again the high ACU values for cell extract and supernate alone were thought to be due to LPS from the bacteria used for Physarum growth.

Ammonium sulphate precipitation of Physarum extracts

Physarum was grown for 3 days in 6 flasks containing basal medium and killed E. coli. Cell extracts and culture supernateswere subjected to fractional precipitation with increasing amounts of ammonium sulphate. In preliminary work the precipitates were dialysed against buffer prior to use, but latterly they were made up to a constant volume with buffer and used directly. Table 18 indicates that for both the cell extract and the culture supernatant fluid, proteins precipitating at 25% and 50% ammonium sulphate concentration seemed to be the most active in

Table 18 Concentration, by ammonium sulphate precipitation, of the LPS-degrading enzymes of *Physarum*

Physarum component	% ammonium sulphate precipitation	fraction alone		Residual activity after incubation of fraction with LPS (8 mg)*			
				0 h		24 h	
		ACU	KDO	ACU	KDO	ACU	KDO
cell extract	25	0	0	480	100	0	108
"	50	0	1	490	60	0	40
"	75	0	0	490	40	240	20
"	100	0	0	510	60	280	56
supernate	25	160	52	720	188	160	68
"	50	320	76	800	198	320	124
"	75	320	0	720	120	400	100
"	100	208	0	720	156	368	132
LPS alone O_t		640	126				

*Ammonium sulphate fractions were dialysed against phosphate buffer pH 5 and 4 ml of each fraction incubated with 8 mg of *S. minnesota* SmS LPS. Reactions were terminated by heating at 100°C for 15 min.

Table 19 Degradation of LPS by ammonium sulphate precipitates of *Physarum* culture supernates

Reaction mixtures were incubated at pH 5 at 30°C for 3 h then terminated by heating (100°C, 15 min)

LPS (2 mg)	Residual quantity per mixture after incubation* for 3 h at 30°C and pH 6											
	ACU				KDO (µg)				HAI			
	†1	2	3	4	1	2	3	4	1	2	3	4
E. coli $O_{111}B_4$	35	50	93	100	120	131	134	131	256	512	256	512
E. coli $O_{55}B_5$	32	49	92	104	50	75	72	72	64	128	128	256
S. minnesota SmS	20	82	196	220	27	40	45	44	128	128	128	256
Sh. flexneri	26	72	145	160	37	57	58	58	2048	4096	2048	4096
-	<5	<5	<5		5	0	3		0	0	0	

† 1. LPS + 90% AS precipitate of the supernate from *Physarum* grown on bacteria.

2. LPS + 90% AS precipitate of the supernate from *Physarum* grown on complete medium.

3. LPS + 1 (which had been heated at 100°C for 15 min).

4. LPS alone.

reducing the AC activity of the LPS after 24 h incubation. Both these protein preparations reduced the AC activity after 24 h incubation to the background level of ACU. The remaining proteins precipitated with 75% and 100% ammonium sulphate saturation were less active. Although these latter precipitates from the cell extract showed very little activity, the 75% and 100% precipitates from the supernate were still quite active, with the LPS losing about 66% of its activity. The background ACU values for the supernate precipitations may be due to undegraded LPS from the substrate bacteria on which the Physarum had been grown. None of the cell extract preparations had any effect on the KDO content. However, there was some reduction in KDO by the supernate subfractions, especially with the 25% AS precipitate which reduced KDO by about one-half; the others reduced the KDO by about a quarter.

Most of the enzyme activity from a Physarum culture supernate grown on S. minnesota smooth bacteria, was precipitated by 90% ammonium sulphate. In this way, it was hoped to concentrate all the enzymes active against LPS. Table 19 indicates that the LPS most affected by the enzyme preparation was S. minnesota smooth LPS, where the AC activity was reduced by over 90%. The KDO values showed a slight drop while the HAI titre was unaffected. Both E. coli $O_{111}B_4$ and E. coli $O_{55}B_5$ LPS lost about 66% of their AC activity, while the LPS of Sh. flexneri lost about 80%. The values for KDO also dropped slightly for the three LPS, while the HAI titres were unaffected. The enzyme preparations inactivated by heating showed no ability to reduce the ACU, KDO or HAI of any of the LPS. The preparation from the supernate of Physarum grown on complete medium degraded the LPS. It was shown to be about one-half as active in reducing

Table 20 Effect of temperature on degradation of S. minnesota SmS LPS by ammonium sulphate precipitates from Physarum culture supernates

Reactions were terminated after 3 h by heating (100°C, 15 min).

Temperature °C	Residual quantity per mixture after incubation for 3 h at pH 5											
	ACU				KDO (µg)				HAI			
	†1	2	3	4	1	2	3	4	1	2	3	4
4	196	189	196		36	40	38		128	128	256	
22	40				38				128			
30	62	176	180	200	37	39	38	42	128	128	256	256
37	106				36				128			
56	200	196	206		40	37	38		256	128	128	

† 1. LPS + 90% AS precipitate from supernate of Physarum grown on bacteria.

2. LPS + 90% AS precipitate from supernate of Physarum grown on complete medium.

3. LPS + 1 (which had been heated at 100°C for 15 min).

4. LPS alone.

Table 21 Effect of pH on degradation of S. minnesota SmS LPS (2 mg) by ammonium sulphate precipitates from Physarum culture supernates

Reactions were terminated after 3 h by heating (100°C, 15 min)

pH	Residual quantity per mixture after incubation for 3 h at 30°C											
	ACU				KDO (µg)				HAI			
	+1	2	3	4	1	2	3	4	1	2	3	4
3	188				36				128			
5	38	166	194	200	37	37	38	42	128	128	128	256
7	46				40				128			
9	162				36				128			
11	190	186	196		39	38	39		128	128	128	

+ 1. LPS + 90% AS precipitate from supernate of Physarum grown on bacteria.

2. LPS + 90% AS precipitate from supernate of Physarum grown on complete medium.

3. LPS + 1 (which had been heated at 100°C for 15 min).

4. LPS alone.

the AC activity of the LPS when compared to the preparations from Physarum grown on bacteria. However, it did not reduce the KDO value nor was the HAI titre affected.

To find the optimum temperature and pH for LPS degradation, a crude enzyme preparation was again obtained by 90% ammonium sulphate precipitation. S. minnesota smooth LPS was incubated for 2 h at various temperatures as shown in Table 20. The reactions were terminated by heating and the samples assayed. There was no decrease in ACU, KDO or HAI for samples incubated at 4°C or 56°C. The optimum temperature for degradation was between 22°C and 30°C where 80% and 70% of the AC activity was lost respectively. The boiled enzyme preparation showed no activity, while the enzyme preparation from Physarum grown on Complete Medium was able to reduce the AC activity by only 15% after incubation at 30°C. It would therefore appear that the LPS-degrading enzyme(s) of Physarum may be inducible by growth on bacteria but further work would be required to establish this.

When degradation of LPS was studied over a range of pH's (Table 21), maximum activity was observed between pH 5 and 7.0. The values for KDO and HAI were unaffected at any of the pH's tested. The heat-inactivation controls had no effect, while the enzyme preparation from complete medium destroyed about 17% of the LPS AC activity at pH 5.

Degradation of Lipopolysaccharide by Gut Juice from the Snail Helix pomatia

Gut juice of the snail, Helix pomatia, has long been known as a source of diverse enzyme activities especially towards sugar substrates (Holden and Tracey, 1950). The snail was selected as an example of an invertebrate which, due to its mode of life, was constantly ingesting materials rich in bacteria. It therefore seemed a likely source of LPS-degrading enzymes.

Varying quantities of snail gut juice (SGJ) were incubated with LPS from E. coli, Sh. flexneri and S. minnesota and the mixtures were subjected to Westphal extraction, dialysis and freeze drying. Table 22 shows that the KDO was not significantly altered but AC activity was greatly reduced. Additional evidence for the degradation of LPS by SGJ was obtained by electrophoretic examination after dispersion in SDS. Undegraded LPS showed a characteristic pattern of Schiff-positive bands (Fig 10a). With degraded LPS a different pattern was observed (Fig 10b). The major fast moving band had entirely disappeared and two new, slower moving bands were visible, one of which was in the stacking gel. When lower concentrations of SGJ were used, this fast moving band was markedly reduced but did not disappear. Other LPS gave similar results. A small amount of Schiff-positive material in the snail gut juice alone did not contribute significantly to the overall band pattern.

The polysaccharide component of LPS was apparently undegraded as there was no reduction in HAI. Paper chromatography similarly showed an absence of any liberated sugar units which might have appeared if the oligosaccharide had been partially or totally degraded.

Table 22 Effect of snail gut juice on the anticomplementary activity and KDO content of LPS from various bacteria

Each reaction mixture contained 4 mg LPS with variable quantities of SGJ made up to a final volume of 4 ml with 0.2M phosphate buffer pH 5.

Reaction mixture		Residual quantity per mixture after incubation and extraction*	
SGJ (ml)	LPS	ACU	KDO (µg)
-	E. coli $O_{55}B_5$	560	160
0.1	"	150	177
0.025	"	310	200
0.01	"	700	157
-	E. coli $O_{111}B_4$	650	393
0.025	"	290	380
-	Sh. flexneri	550	107
0.1	"	85	106
0.025	"	350	100
-	S. minnesota	700	126
0.025	"	550	98
0.01	"	475	93
0.1	-	10	12

*After incubation at 30°C for 3 hr the reaction mixtures were extracted by the Westphal method.

Figure 10. Line drawing of the band patterns observed after SDS-polyacrylamide gel electrophoresis of E. coli $O_{55}B_5$ LPS a) alone and b) after incubation with snail gut juice, as described in Table 22. Bands were visualised by the PAS staining procedure.

Occurrence and Degradation of Lipopolysaccharide in Marine Sediments

Evaluation of the Boivin and Westphal methods for extracting LPS from marine sediments

Although there are several methods for extracting LPS from pure cultures of gram-negative bacteria, it was not known which of these would be best for obtaining maximum yields from the endogenous bacteria of marine sediments. Therefore before embarking on experiments to monitor the possible degradation of LPS in sediments, it was first necessary to develop and evaluate methods of extraction. Attention was focused on the Boivin and Westphal methods which were applied in parallel to sediment samples from different sites and depths and stored for various periods in the laboratory. Extractions were done on mixtures of sediment with added amounts of particular bacteria to determine whether components of sediment might interfere with LPS-extraction from a known source. The criteria used as indicators of LPS were as before, namely AC activity, KDO, GLC analysis for fatty acids, HAI and ESA.

Dumbarton foreshore on the estuary of the River Clyde (grid reference: NS. 424 740) was the initial site for the collection of marine sediments. For comparative extraction experiments (without degradation), the top 5 cm of sediment near the low water mark was collected and used, both fresh, and after "ageing" for several months in the laboratory. Fresh marine sediment was used in experiment 1 (Table 23) and both Boivin and Westphal methods were applied to three tests systems, viz:

Sample A : sediment plus a known amount of heat-killed bacteria

Sample B : sediment alone

Sample C : bacteria alone.

The object of these experiments was 1) to compare the efficiency of Boivin and Westphal methods; 2) to determine whether sediment interfered with the recovery of a known added amount of LPS in the form of a particular bacterial species; 3) to measure the level of endogenous LPS in the sediment.

In experiment 1 where the sediment was used fresh and where 0.5 g of E. coli $O_{111}B_4$ was added to 100 g sediment the results were as set out in Table 23 and permit the following conclusions: with bacteria alone, the Westphal method yielded about 10% more LPS than the Boivin, as indicated by recovery of KDO and the measurement of ACU; with sediment alone, both the Boivin and Westphal methods yielded KDO in small but easily detectable amounts (67 and 75 µg respectively, the limit of sensitivity of the test being about 5 µg). Here again, the Westphal method yielded about 10% more material than the Boivin technique. However, there was a major difference in the amount of AC substance extracted from sediment, the Westphal method giving nearly 20 times the yield of Boivin. In the mixture of sediment plus bacteria both the Boivin and Westphal methods appeared to extract ACU and KDO at levels expected from those obtained from the separate components.

In experiment 2 a similar sample of sediment was aged for 3 months at room temperature before testing. Here again, the bacteria were E. coli $O_{111}B_4$ but the amount used was increased from 0.5 to 0.8 g.

Table 23 Protocols and results of experiments to compare the efficiencies of the Boivin (B) and Westphal (W) methods for extracting LPS from mixtures of bacteria and marine sediments

Expt.	Bacterial strain	Sample	Marine* sediment dry weight (g)	Bacterial dry weight (g)	Yield from each test mixture			
					ACU		KDO (µg)	
					B	W	B	W
1	E. coli $O_{111}B_4$	A	100	0.5	845	6250	279	277
		B	100	0	280	4875	67	75
		C	0	0.5	510	580	175	222
2	E. coli $O_{111}B_4$	A	100	0.8	820	1250	341	390
		B	100	0	156	600	27	30
		C	0	0.8	610	660	321	362
3	S.minnesota R595	A	100	1.0	860	1800	83	87
		B	100	0	180	1100	5	5
		C	0	1.0	750	850	75	97

*A separate batch of marine sediment was used in each experiment; in Expt 2 and 3 the sediment had been "aged" in the laboratory for 3 months and 4 months respectively, while Expt 1 was done with a fresh sample of sediment.

As before, the Westphal method yielded about 10% more ACU and KDO from bacteria alone and about 10% more KDO from sediment alone. However, there was still a large difference in the ACU of the Westphal extract in sediment, although the process of ageing had reduced it from 20-fold over Boivin to 4-fold. When mixtures of sediment plus E. coli were extracted there was again good recovery of the KDO and ACU values exhibited by the separate components.

In the third experiment a 4-month "aged" sediment was used and 1 g S. minnesota R595 was added. As in the two preceding experiments, Westphal extracts of bacteria alone gave appreciably more ACU and KDO than the Boivin method; sediment alone had barely detectable amounts of KDO, but the ACU were about 6 times higher in the Westphal extracts than in the Boivin. Additivity was satisfactory.

Distribution of lipopolysaccharide in samples from different sites and depths

After the above preliminary experiments, a further set of sediment samples was collected at the Dumbarton site but in slightly different locations in relation to a sewage outfall (Fig 11). A simple coring technique was used and each sample divided into a top 2 cm-thick layer, a middle 2-6 cm deep layer and a bottom 6-10 cm zone. Each sample was extracted while fresh as a 500 g dry weight (about 2 kg moist weight) specimen by both the Boivin and Westphal methods. In Table 24 the yields of ACU and KDO are expressed per kg dry weight. The following points may be noted:

1) KDO levels fell off dramatically with depth of burial but with the

Figure 11. Site of sediment samples collected at Dumbarton foreshore (grid ref: NS 424 740) at slightly different locations in relation to a sewage outfall.

X - High water mark, 100 m from sewage outfall

Y - Low water mark, 100 m from sewage outfall

Z - Low water mark, beside sewage outfall.

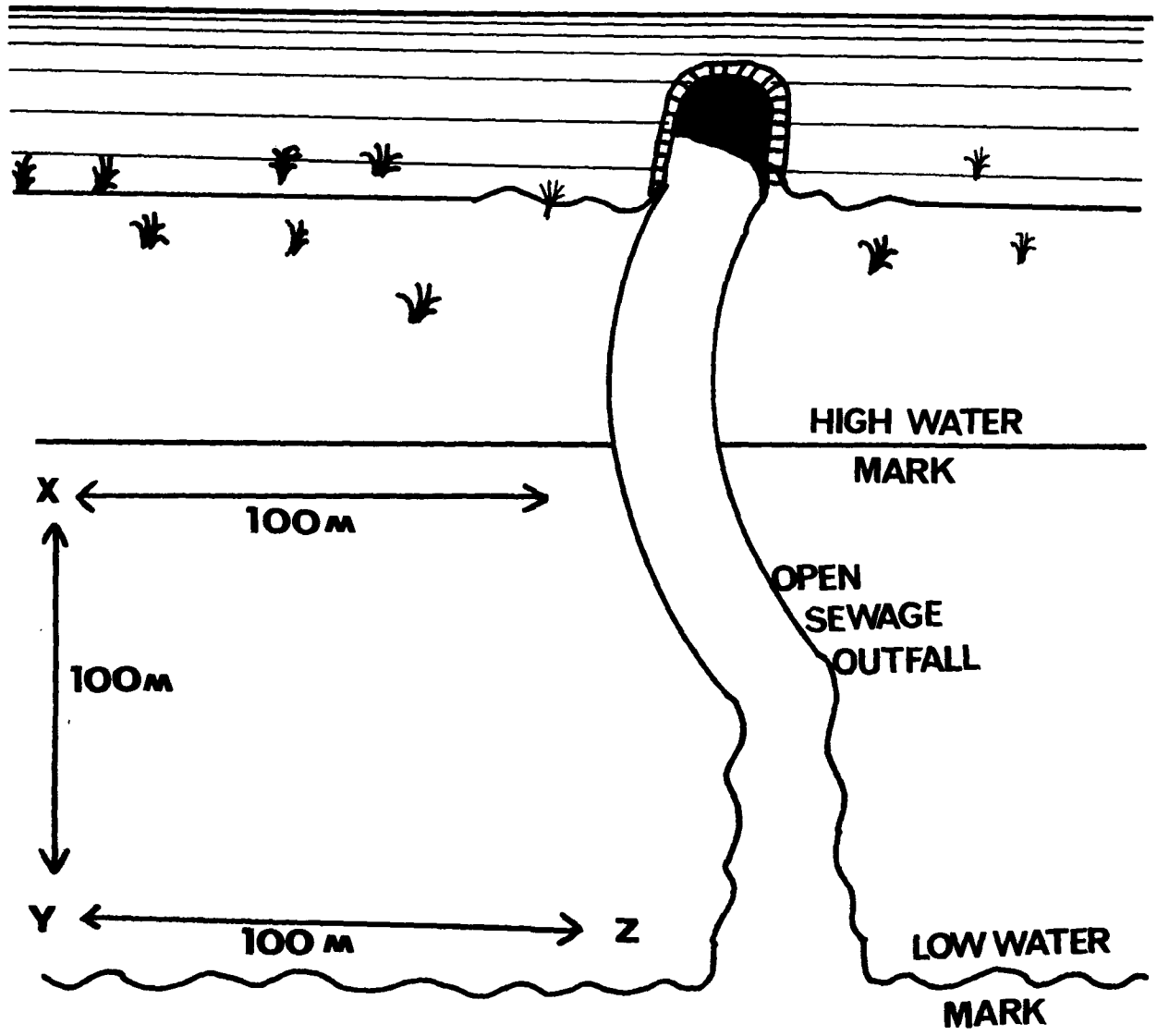


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Table 24 Extraction of LPS from Dumbarton foreshore sediments obtained from different sites and at different depths and subjected to B and W procedures in parallel

Source of Sediment	Core layer (cm from top)	Yield per kg			
		ACU		KDO (µg)	
		B	W	B	W
High water mark, 100 m from sewage outlet **X**	0 - 2	10,000	61,000	206	230
	2 - 6	8,600	30,000	10	21
	6 - 10	5,400	14,500	<1	<1
Low water mark, 100 m from sewage outlet **Y**	0 - 2	8,700	57,000	201	237
	2 - 6	5,100	17,000	10	30
	6 - 10	2,800	8,000	<1	<1
Low water mark, beside sewage outlet **Z**	0 - 2	12,000	60,000	253	270
	2 - 6	9,700	37,500	31	35
	6 - 10	5,500	15,000	<1	<1

Westphal method consistently yielding more KDO than the Boivin.

2) There was no great differences in ACU in the top 2 cm close to the outfall and 100 m distant.

3) The Westphal method yielded much higher amounts of ACU than the Boivin method but the divergence diminished with depth of burial, being about 6-fold in the top layer and 3-fold in the lowest layer.

4) The decline in AC activity with depth was much less steep than the decline in KDO. Thus while ACU diminished by a factor of 3-fold to 7-fold between top and bottom layers, KDO diminished by over a 100-fold.

In the next series of experiments a comparison was made of the yield of LPS from relatively clean sandy beaches and the grossly polluted estuarine mud of the Dumbarton foreshore. The cleanest of the beaches was White Bay at the north end of Great Cumbrae Island (grid reference: NS 176 591) while Kames Bay, Millport (grid reference: NS 171 550) on the same island should probably be regarded as moderately polluted. Again core samples were obtained and sectioned into surface, middle and bottom layers, down to 6 cm. The ACU and KDO results obtained with parallel Boivin and Westphal extracts are presented in Table 25. These data provide further confirmation of the previous results and in addition show that even relatively clean sandy beaches contain detectable amounts of KDO and ACU in the top 1cm. The amounts of KDO and ACU however were respectively about 20% and 7% of these observed in the Dumbarton samples. Again KDO diminished with depth much more rapidly than did ACU.

Sequential Westphal and Boivin treatments

It was apparent from the above that while the Westphal technique gave the greater yield of LPS (as measured by KDO) it also seemed to extract

Table 25 Comparison of LPS yields from grossly-polluted sediments (Dumbarton) and from relatively clean sandy beaches (White Bay and Kames Bay) per 500 g of sediment sample

Source	core layer (cm from top)	ACU		KDO	
		B	W	B	W
Dumbarton					
grid ref: NS 424 740	0 - 1	1,360	42,000	120	153
	1 - 4	1,160	16,000	4	12
	4 - 6	600	13,880	< 2	6
White Bay					
grid ref: NS 176 591	0 - 1	96	2,800	12	22
	1 - 4	88	880	< 2	< 2
	4 - 6	48	500	< 2	< 2
Kames Bay					
grid ref: NS 171 550	0 - 1	1,400	6,400	19	34
	1 - 4	408	2,800	< 2	< 2
	4 - 6	252	1,400	< 2	< 2

large amounts of AC activity which could not be attributed to LPS. In fact, it was obvious to the naked eye that Westphal extracts of sediments contained much extraneous material because they were yellow and viscous whereas the Boivin extracts similarly concentrated were non-viscous, opalescent fluids resembling purified LPS. It was decided therefore to explore a two-step extraction procedure, i.e. to apply the phenol-water technique first and then to treat the dialysed water layers with TCA to precipitate non-LPS material. The converse procedure of TCA extraction followed by phenol-water was also explored. It should be noted that unlike previous experiments there was no ultracentrifugation step applied and this should be kept in mind when considering the results. The various procedures were done on bacteria alone, sediment alone and mixtures of bacteria plus sediment. Smaller amounts of sediment were used while the amount of added bacteria was increased (Table 26). Westphal and Boivin extraction of E. coli cells alone gave similar results for ACU, KDO and HAI but with the Westphal values about 10% higher.

Boivin extraction of sediment alone gave a value of 40 ACU per 50 g. When this material was phenol-water extracted and re-assayed, similar values for ACU and KDO were found. The phenol-water extraction of the sediment alone and sediment plus bacterial cells already extracted by the Boivin method, procedure 4, showed similar results, with the phenol-water extraction of the Boivin extracts giving corresponding values for ACU, KDO and HAI to those that were found in the original Boivin extracts.

Westphal extraction of marine sediment yielded samples, with 6 times as much AC activity and 5 times as much KDO-like material as sediment extracted by the Boivin method. When the Westphal extracted

Table 26 Sequential application of Westphal and Boivin procedures to marine sediment and added *E. coli*

Reaction mixture	ACU/sample				KDO (μg)				HAI			
	*W	W/B	B	B/W	W	W/B	B	B/W	W	W/B	B	B/W
1. 50 g Dumbarton sediment alone	2480	1980	40	40	600	168	115	120	4	0	0	0
2. 50 g Dumbarton sediment + 2.0 g *E. coli* $O_{111}B_4$ cells	3400	2860	710	640	3720	3160	2730	2300	256	128	128	128
3. 2.0 g *E. coli* $O_{111}B_4$ cells	800	760	680	620	3070	2740	2640	2620	256	256	256	128

* Procedure W : Westphal extraction

" W/B : TCA precipitation of Westphal extract

" B : Boivin extraction

" B/W : Westphal extraction of Boivin extract.

Table 27 Sequential application of Westphal and Boivin procedures to marine sediment and added S. minnesota

Reaction mixture	ACU/sample				KDO (µg)				HAI			
	* W	W/B	B	B/W	W	W/B	B	B/W	W	W/B	B	B/W
1. 50 g Dumbarton sediment alone	3610	3300	180	120	160	80	65	50	0	0	0	0
2. 50 g Dumbarton sediment + 2.0 g S. minnesota smooth cells	4760	4350	1440	1350	1695	1450	1565	1610	4096	2098	2048	1024
3. 2.0 g S. minnesota smooth cells	1400	1360	1250	1200	1525	1320	1455	1410	4096	2048	4096	1024

*Procedure W : Westphal extraction

" W/B : TCA precipitation of Westphal extract

" B : Boivin extraction

" B/W : Westphal extraction of Boivin extract.

samples were subjected to TCA precipitation, procedure W/B (Table 26), the sediment samples lost about 20% of their ACU activity and about 75% of their KDO-like material. In contrast the E. coli cells alone showed little change in ACU or KDO when the Westphal extract was subjected to TCA precipitation. Comparison of the HAI titres after each of the extraction procedures indicated that they were almost equally as efficient at extracting the LPS from added bacteria, whether in the presence of sediment or alone.

When the above 4 procedures were repeated with a new sample of sediment and a different strain of added bacteria, Table 27, the results were essentially the same. Both extraction methods of sediment alone had higher values for AC activity than did the samples in Table 26, but the KDO values in this case were lower. Comparison of the HAI titres showed that the 4 extraction procedures had similar efficiencies in extracting LPS.

GLC analyses of esturine mud extracts

Previous results indicated that Westphal extraction was approximately 10% more efficient at extracting LPS from bacteria than the Boivin method. Similarly, values obtained for KDO or KDO-like material in extracts of sediment alone or sediment plus bacteria indicated that the Westphal extraction was about 10-20% more efficient at extracting LPS. However the large differences in the AC activity of sediments extracted by the different methods was far larger than could be explained by this difference in efficiency. Therefore, to find if the high AC activity of Westphal extracted sediment was due to LPS alone, extracts were hydrolysed and the liberated fatty acids were then methylated for GLC analysis. It

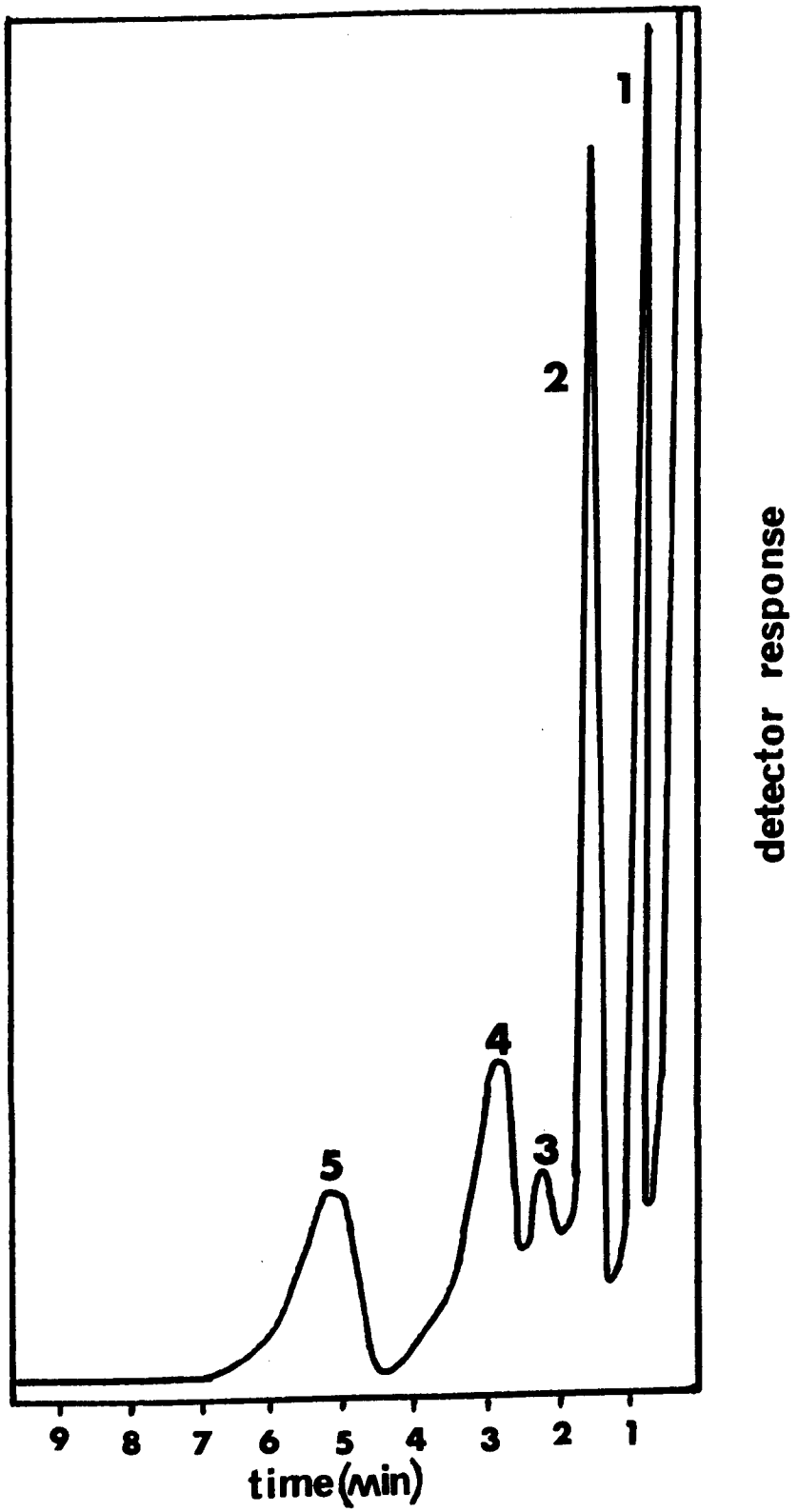
Table 28 Retention times and quantitation of fatty acids from LPS of *E. coli* $O_{111}B_4$ cells after mixing with Dumbarton sediment followed by extraction, dialysis and hydrolysis

peak number	fatty acid methyl esters	t_r	Total amount of fatty acid released after hydrolysis (μg)† Boivin Extraction			Westphal extraction		
			cells alone	sediment + cells*	sediment alone	cells alone	sediment + cells	sediment alone
1	Lauric	0.54	205	207	3	229	235	7
2	Myristic	1	196	209	5	211	217	8
3	Palmitic	2.16	220	227	8	199	206	6
4	β-Hydroxy-myristic	3.91	624	638	17	672	665	15

*From 50 g Dumbarton sediment (equivalent dry wt), and 2.0 g of *E. coli* $O_{111}B_4$ cells (dry wt).

†Weights of fatty acids were calculated from responses of standards at known concentrations.

Figure 12. GLC trace of methyl esters of the fatty acids released after hydrolysis of *E. coli* $O_{111}B_4$ LPS. The peaks correspond to: 1, lauric; 2, myristic; 3, Δ^2-tetradecanoic; 4, palmitic; 5, β-hydroxy-myristic acid.


1
2
3
4
5
9 8 7 6 5 4 3 2 1
time(min)
detector response

was hoped that differences in the amounts of fatty acids, characteristic of the lipid A of LPS, would indicate whether this high AC activity was due to more lipid A, and thus more LPS, being present in the Westphal extracted sediment. Figure 12 indicates the trace obtained for the methyl esters of the fatty acids from the lipid A component of E. coli $O_{111}B_4$ LPS. When each of the different extracts had been methylated, quantitative examination using known amounts of standard fatty acids indicated that both extraction procedures yielded similar amounts of fatty acids (Table 28). When the values for cells alone were added to those from sediment alone, they corresponded to the values for sediment plus cells in both extraction procedures.

The values obtained for lauric, myristic, palmitic and β-hydroxymyristic acid from sediment alone indicated that about 1/3000 of the dry weight of the Dumbarton sediment could be ascribed to gram negative bacteria (assuming that β-hydroxymyristic acid is 0.1% of the dry weight of an average Gram-negative bacterial cell). Thus comparison between Table 26 and Table 28 indicates that a large amount of the AC activity shown by Westphal extraction of sediment is not due to bacterial LPS alone. Lauric, myristic, palmitic and β-hydroxymyristic acids were the only fatty acids which were assayed for quantitatively in these extracts although very small amounts of other fatty acids such as C_{10} and C_{18}, which are known to occur in the lipid A of some LPS, may also have been present.

Anticomplementary activity and KDO analysis of various polymers and lipopolysaccharides

As the above results seemed to indicate that some highly AC

material other than LPS was being extracted from marine sediments by the Westphal procedure, a range of known compounds which might contribute to this contamination were assayed for their AC activity and for their ability to react in the thiobarbituric acid test for KDO. Polymers which were thought likely to occur in marine sediments, substances known to be highly anticomplementary and a range of LPS were assayed. Table 29 indicates that some polymers which might occur in marine sediments such as agar, RNA and alginate are all anticomplementary. However, when these substances were Westphal extracted, the only one to yield much AC activity was RNA. This "extract" of RNA was, however, less than 5% as anticomplementary as the starting material, indicating that more than 95% of the RNA was eliminated during Westphal extraction. Thus the ultracentrifugation of the aqueous layer in the Westphal procedure would seem to have eliminated the main part of the RNA which might contaminate LPS preparations.

When these polymers were examined in the thiobarbituric acid test, various adsorption spectra were obtained (Fig 13). KDO absorbs maximally at 548 nm giving a characteristic absorption pattern. DNA also reacts strongly with the thiobarbituric reagent to give an absorption maximum at 532 nm. Compounds were reacted with the thiobarbituric test and the absorption values obtained at 548 nm were quantitated to give the % of KDO that could be, or which was present, equivalent to this absorption value (i.e. 11.5% of the S. minnesota R595 LPS molecule is KDO). The highly AC polymers, agar, RNA and alginate showed little absorbance at 548 nm after reacting with the thiobarbituric reagents. DNA, which does not exhibit anticomplementary activity, reacted with the thiobarbituric reagents to give an absorption spectrum similar to that of KDO. DNA was

Table 29 Anticomplementary activity and KDO equivalent of various polymers and lipopolysaccharides

Sample	ACU/mg	% equivalent KDO
E. coli $O_{111}B_4$ LPS	80	6
E. coli $O_{55}B_5$ LPS	80	5.5
S. minnesota SmS LPS	90	3.1
S. minnesota R595 LPS	200	21.5
Agar	84	0.1
Agarose	2.4	0.01
DNA	< .02	2
RNA	80	0.03
Heparin	120	0.01
Alginate	104	0

Figure 13. Absorption spectra of various polymers, LPS and sediment extracts in the thiobarbituric acid test.

a)	1.	KDO	1 mg/ml
	2.	*E. coli* $O_{111}B_4$ LPS (contains colitose)	5 mg/ml
	3.	*S. minnesota* R595 LPS	5 mg/ml
	4.	*S. minnesota* SmS LPS	5 mg/ml
b)	5.	Agar	40 mg/ml
	6.	RNA	40 mg/ml
	7.	Agarose	40 mg/ml
	8.	Heparin	40 mg/ml

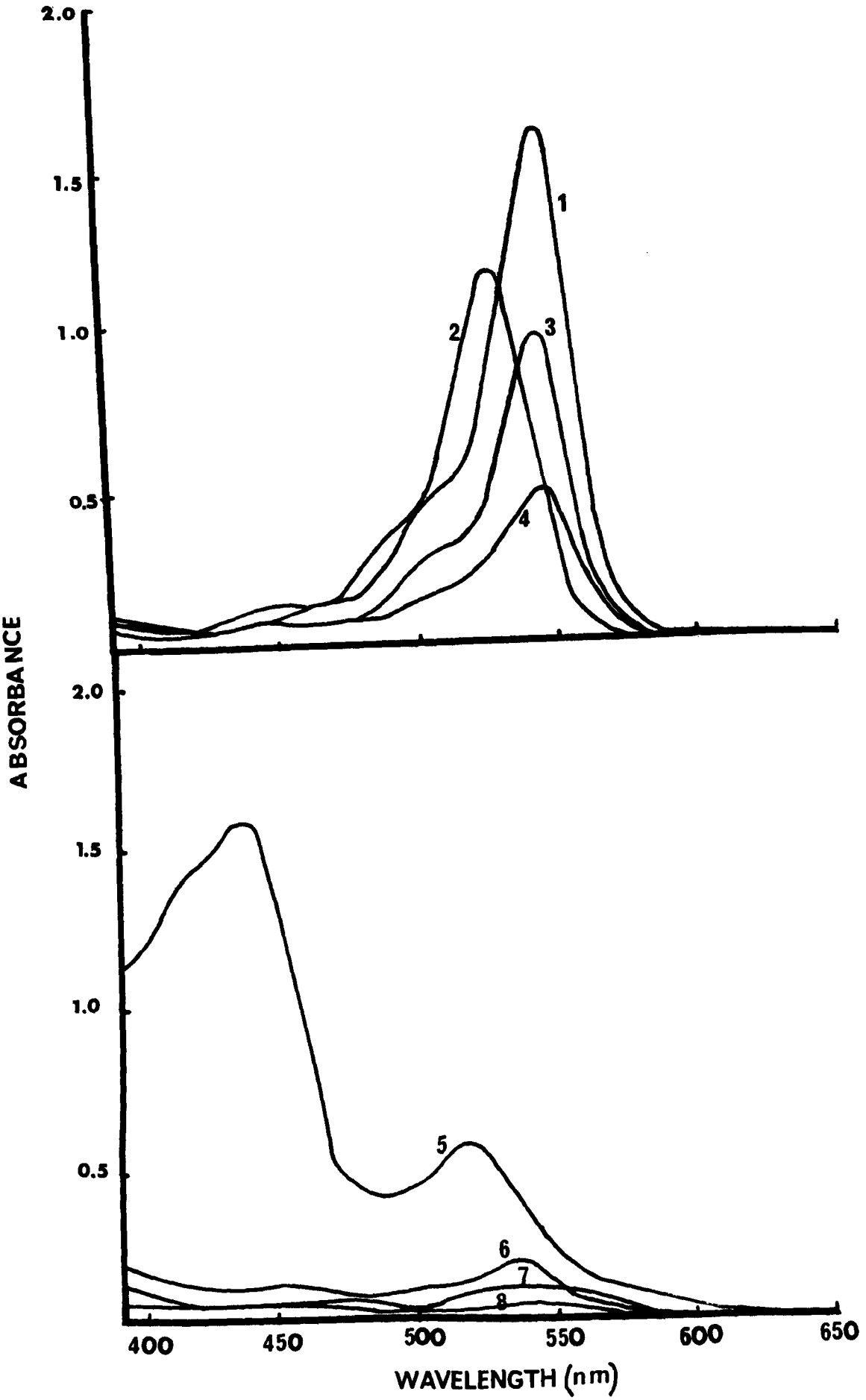
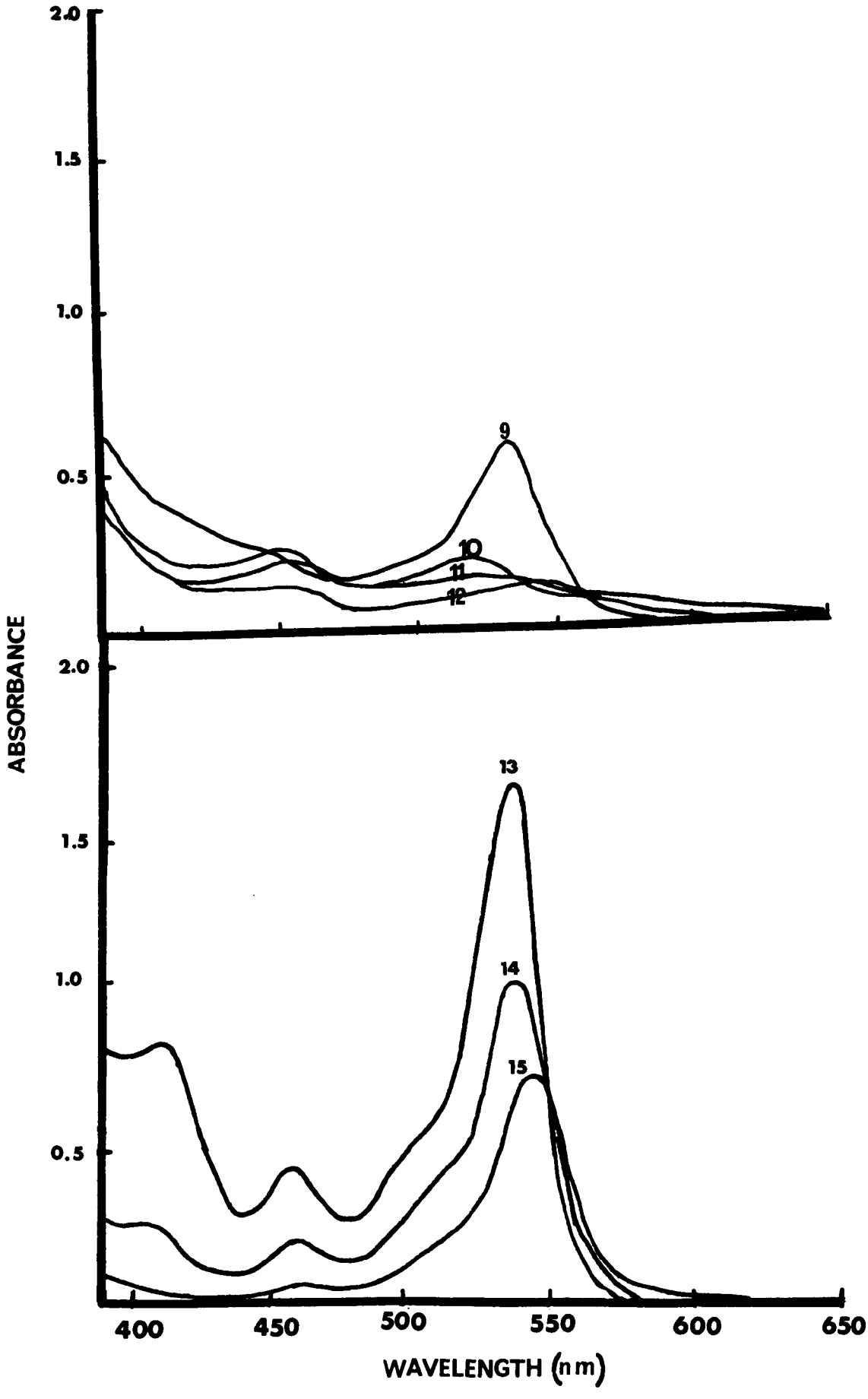

2.0
1.5
1.0
0.5
1
2
3
4
2.0
1.5
1.0
0.5
5
6
7
8
ABSORBANCE
400
450
500
550
600
650
WAVELENGTH (nm)

Figure 13. Absorption spectra of various polymers, LPS and sediment extracts in the thiobarbituric acid test.

c)	9.	Concentrated slime from *Physarum*	40 mg/ml
	10.	Galactose	100 mg/ml
	11.	Glucose	100 mg/ml
	12.	Alginate	40 mg/ml
d)	13.	Salmon sperm DNA	10 mg/ml
	14.	Westphal extract of Dumbarton sediment (50 g)	
	15.	Boivin extract of Dumbarton sediment (50 g)	


2.0
1.5
1.0
0.5
9
10
11
12
2.0
1.5
1.0
0.5
13
14
15
ABSORBANCE
400
450
500
550
600
650
WAVELENGTH (nm)

however at least ten times less active in reacting with the thiobarbituric acid reagents and the absorption spectrum, though similar, had an absorption peak at 532 nm while two additional peaks, which were not given by KDO, were obtained at 415 nm and 460 nm.

Figure 13d shows the absorption spectra of Westphal and Boivin extracted sediments in the thiobarbituric acid assay. Boivin extracted sediment gave an absorption pattern similar to that of KDO alone while the Westphal extract seemed to contain material other than just KDO, which reacted to give a small secondary peak at 460 nm.

Slime from Physarum polycephalum was concentrated by ethanol precipitation and freeze-dried. Figure 13c shows its absorption spectrum after reacting with the thiobarbituric acid reagents, giving an absorption maximum at 535 nm. A range of monosaccharides tested gave no AC activity at concentrations/100mg/ml and negligible absorbance when reacted with the thiobarbituric reagents at 100 mg/ml.

Degradation of lipopolysaccharide in marine sediments

It appeared from the above that although LPS could be extracted from sediment, nucleic acids and/or other compounds might be present and might contribute, particularly after Westphal extraction, to the ACU and KDO assays. However, by using controls of bacterial cells alone, sediment alone and autoclaved sediment plus bacteria, it was hoped to minimize this problem of interference.

In preliminary experiments to investigate LPS degradation by marine sediment, it was decided to use anaerobic conditions since these are

Table 30 Degradation of LPS during anaerobic incubation of heat-killed *E. coli* $O_{111}B_4$ with marine sediment

All samples were extracted by the Boivin method.

Experiment	Reaction mixture		ACU		KDO (µg)	
	Sediment ‡ weight (g)	Bacteria dry wt (g)	extracted immediately	incubated*	extracted immediately	incubated*
1	500	5	8,500	840	1,448	180
	500	0	900	1,000	28	24.8
	500†	5	n.t.	8,110	n.t.	1,347
	0	5	7,000	n.t.	1,370	n.t.
2	500	5	8,000	830	1,337	120
	500	0	400	450	23	24
	500†	5	n.t.	7,800	n.t.	1,260
	0	5	7,000	n.t.	1,389	n.t.

* In Expt 1 the incubation was for 21 days and in Expt 2 for 13 days.

† Autoclaved sediment + toluene.

‡ A separate batch of marine sediment was used in each experiment; in Expt 1 and 2 the sediment was "aged" in the laboratory for 3 months and 4 months respectively.

Table 31 Degradation of LPS during anaerobic incubation of *E. coli* $O_{111}B_4$ and *E. coli* Lilly with fresh marine sediment. All samples were extracted by the Boivin method.

Experiment and bacteria	Reaction mixture		ACU remaining at day:				KDO (μg) remaining at day:			
	Sediment weight (g)	Bacteria dry wt (g)	0	4	10	21	0	4	10	21
1	50	0.8	1000	560	345	196	353	88	30	0
E. coli	50	0	90	n.t.	n.t.	75	30	n.t.	n.t.	0
$O_{111}B_4$	50*	0.8	n.t.	n.t.	n.t.	980	n.t.	n.t.	n.t.	358
	0	0.8	690	n.t.	n.t.	n.t.	313	n.t.	n.t.	n.t.
2	50	0.5	1150	800	410	188	355	77	10	0
E. coli	50	0	50	n.t.	n.t.	78	20	n.t.	n.t.	0
Lilly	50*	0.5	n.t.	n.t.	n.t.	1050	n.t.	n.t.	n.t.	357
	0	0.5	900	n.t.	n.t.	n.t.	309	n.t.	n.t.	n.t.

*Autoclaved sediment + toluene.

the conditions likely to prevail *in situ*. Samples were incubated in closed bottles at 22°C for variable periods of time, after which degradation was stopped by Boivin extraction. In experiment 1, Table 30 the sediment which was aged for 3 months produced a reduction in the AC activity of about 90% and in the quantity of KDO 87%. The test mixture containing autoclaved sediment and bacterial cells showed no decrease in either AC activity or in the amount of KDO. Sediment alone showed a slight increase in AC activity and a slight drop in KDO content. When this procedure was repeated with sediment which was aged for four months (experiment 2, Table 30) similar results were obtained. Both AC activity and KDO values for the LPS were greatly reduced after incubation with the sediment.

In the next experiment it was decided to repeat this procedure but with smaller amounts of both sediment and added bacteria to facilitate extractions. Also the test mixtures were replicated so as to permit extractions at different times (Table 31 , experiment 1). Half of the added bacterial AC activity was lost after 4 days incubation with sediment and by 21 days, 80% of the AC activity had gone. KDO was eliminated more quickly, having largely disappeared after 4 days incubation while at 21 days, virtually no KDO could be detected.

Degradation of a rough strain of *E. coli* was studied to see if a different pattern might emerge. The results in experiment 2 (Table 31) were similar, however, with 30% of the AC activity lost after 4 days and 60% after 21 days. KDO again disappeared more quickly, to the point where nothing was detectable after 21 days. There was little change in the AC

Table 32 Degradation of LPS during anaerobic incubation of S. minnesota SmS with fresh marine sediment

Samples were Westphal-extracted.

Reaction mixture		ACU remaining at day:				KDO (µg) remaining at day:			
Sediment weight (g)	Bacteria dry wt (g)	0	3	6	9	0	3	6	9
50	1.0	5,000	3,750	3,150	2,900	367	220	120	48
50	0	2,800	n.t.	n.t.	1,850	40	n.t.	n.t.	40
50*	1.0	n.t.	n.t.	n.t.	5,500	n.t.	n.t.	n.t.	321
0	1.0	1,000	n.t.	n.t.	n.t.	360	n.t.	n.t.	n.t.

*Autoclaved sediment + toluene.

or the KDO-like activities of the sediment alone controls in both experiments suggesting that the contributing substance(s) were much more stable to degradation than was LPS.

These degradation experiments were repeated using fresh marine sediment (Table 32). As previous experiments had indicated that Westphal extraction was slightly more efficient than Boivin, the reaction was terminated by applying this procedure. Results similar to those of the previous experiments were obtained, with 50% of the AC activity and 80% of the KDO lost by day 9. Again Westphal extraction of sediment alone yielded extracts with much higher AC activity than had been obtained by Boivin extraction of similar sediment samples. This was likewise found for the KDO values of the different extracts. That LPS had been degraded during the 9 days incubation was indicated by the reduction of AC activity and KDO of the LPS plus sediment mixture down to levels similar to those given by sediment alone. The autoclaved sediment plus killed bacterial cells showed no decrease in either AC activity or KDO content after incubation.

Comparison of anaerobic and aerobic incubation of lipopolysaccharide with marine sediments

Killed bacteria were incubated with marine sediment aerobically and anaerobically in parallel to determine which conditions would give most rapid degradation of LPS. The reactions were terminated at different times by either Westphal or Boivin extraction. Westphal-extracted 7 day samples (Table 33) showed no decrease in either AC activity or in the ability of the incubated LPS to sensitize red cells (ESA titre). Aerobic incubation resulted in 25% of the KDO being lost after 7 days while

Table 33 Application of Westphal extraction to investigate LPS degradation under aerobic and anaerobic conditions

Time	Reaction mixture*	aerobic (A) anaerobic (AN)	ACU	KDO (µg)	HAI	ESA
0	sediment + cells	-	10,200	400	2,048	2,048
0	sediment alone	-	7,750	32	32	32
0	cells alone	-	570	358	2,048	2,048
7 d	sediment + cells	A	11,000	276	512	2,048
7 d	"	AN	10,600	377	512	2,048
18d	"	A	10,230	192	128	1,024
18d	"	AN	8,100	268	256	512
25d	"	A	8,700	82	64	512
25d	"	AN	7,400	136	256	256
25d	sediment alone	A	6,400	26	8	16
25d	"	AN	6,300	27	8	16
25d	autoclaved sediment + cells	-	13,800	367	512	2,048

*100 g fresh Dumbarton sediment and/or 1.0 g of E. coli $O_{111}B_4$ heat-killed cells

Table 34 Application of Boivin extraction to investigate LPS degradation under aerobic and anaerobic conditions

Time	reaction mixture*	aerobic (A) anaerobic (AN)	ACU	KDO (µg)	HAI	ESA
0	sediment + cells	-	1,520	322	2,048	512
0	sediment alone	-	660	27	0	2
0	cells alone	-	500	337	2,048	512
7 d	sediment + cells	A	1,440	255	256	256
7 d	"	AN	1,400	289	512	256
18d	"	A	600	127	16	128
18d	"	AN	540	153	64	64
25d	"	A	520	78	0	32
25d	"	AN	500	89	4	16
25d	sediment alone	A	280	21	0	4
25d	"	AN	360	12	4	4
25d	autoclaved sediment + cells	-	1,200	310	256	512

*100 g fresh Dumbarton sediment and/or 1.0 g of *E. coli* $O_{111}B_4$ heat-killed cells.

anaerobic incubation had no effect. Both incubation mixtures, however, showed a decrease in HAI titre. After 25 days, both aerobic and anaerobic incubation showed a loss of AC activity with anaerobic incubation giving the greatest drop. There was a similar decrease in ESA. Values for KDO and HAI titre indicated that aerobic incubation permitted greater breakdown of the oligosaccharide component of the LPS.

When the incubation mixtures were extracted by the Boivin method (Table 34), similar results were found. The Boivin extracts, however, did not have such high background AC activities from the sediment itself. Anaerobic incubation resulted in a greater loss of AC activity and ESA titre while aerobic incubation resulted in a greater loss of KDO and decrease in the HAI titre. Both extraction procedures demonstrated a loss of AC activity and detectable KDO in the sediment-alone samples after both aerobic and anaerobic incubation, possibly indicating degradation of the endogenous LPS.

In an attempt to diminish the effect of the AC-interfering material in Westphal extracts of sediment, a higher proportion of bacteria relative to sediment was used in a new set of test mixtures. Table 35 indicates that Westphal extraction of sediment alone yielded highly AC material. The lipid component of the added LPS seemed to be more readily degraded under anaerobic conditions with a greater reduction in ACU. In contrast, aerobic incubation had a greater effect on the oligosaccharide component, as indicated by the reduction in KDO and HAI titre. About 50% of the KDO was lost during incubation, while the HAI titre was also greatly reduced. Very high levels of KDO-like material were detected in the

Table 35 Attempt to reduce interference by non-LPS anticomplementary material when the Westphal technique was applied to monitoring LPS-degradation in marine sediments

Reaction mixture*	ACU			KDO (μg)			HAI		
	0_t	21 d		0_t	21 d		0_t	21 d	
		A†	AN		A	AN		A	AN
Sediment alone	2480	1860	2040	600	650	675	4	0	0
Sediment + bacteria	3400	2700	2610	3720	1710	2100	256	8	32
Autoclaved sediment + bacteria	-	-	3640	-	-	3560	-	-	512
Bacteria alone	800	-	780	3070	-	2970	256	-	512

* 50 g Dumbarton sediment and/or 2 g heat-killed *E. coli* $O_{111}B_4$.

† Where A is aerobic and AN is anaerobic growth for 21 days at 22°C.

Table 36 Attempt to reduce interference by non-LPS anticomplementary material when the Boivin technique was applied to monitoring LPS-degradation in marine sediments

Reaction mixture*	ACU			KDO (µg)			HAI		
	0_t	21 d		0_t	21 d		0_t	21 d	
		A†	AN		A	AN		A	AN
Sediment alone	40	75	50	115	112	150	0	0	0
Sediment + bacteria	790	280	250	2730	825	1050	128	8	32
Autoclaved sediment + bacteria	-	-	700	-	-	2860	-	-	256
Bacteria alone	680	-	675	2640	-	2600	256	-	256

*50 g Dumbarton sediment and/or 2 g heat-killed E. coli $O_{111}B_4$

†Where A is aerobic and AN is anaerobic growth for 21 days at 22°C.

sediment alone samples both at zero time and after 21 days incubation. However, when the absorption spectrum of the coloured reaction products was assayed after the thiobarbituric test, there was evidence of high levels of endogenous DNA contributing to the value for KDO. This high level of DNA was also indicated by the high absorbance obtained at 260 nm when the extracts alone were examined. Incubation for 21 days reduced this value by about 20%. As before, the high background AC activity masked the reduction in AC activity of the added LPS. However, the AC activity of sediment plus cells after 21 days incubation was almost reduced to the levels obtained from extracts of sediment alone. In contrast, Boivin extraction of sediment produced little AC material (Table 36). After incubation of bacteria with sediment for 21 days about 66% of the AC activity and 66% of the KDO was lost. Aerobic incubation was more active in reducing the KDO content and HAI titre, but less active in reducing AC activity when compared to anaerobic incubation.

To see if there was a reduction in the fatty acid content of the lipid A from the LPS, corresponding to the loss of AC activity, Boivin and Westphal extracts of the previous degradation experiments were hydrolysed and the fatty acids of the lipid A were methylated and assayed by GLC. Table 37 shows that about one-half of the total fatty acids normally found were lost. The high AC activity of Westphal extracted sediment was again shown not to be due to LPS alone. Both Boivin and Westphal extracted samples of sediment contained only small amounts of the characteristic fatty acids of lipid A. Only the occurrence of lauric, myristic, palmitic and β-hydroxymyristic acids were studied quantitatively, although other fatty acids are known to be present in some LPS (Rietschel

Table 37 Demonstration of LPS degradation in marine sediment* by GLC analysis of characteristic fatty acids of lipid A

Peak number	fatty acid methyl esters	t_r	Total fatty acid (µg)									
			Westphal extraction					Boivin extraction				
			cells alone	sediment + cells		sediment alone		cells alone	sediment + cells		sediment alone	
			0_t	0_t	21 d	0_t	21 d	0_t	0_t	21 d	0_t	21 d
1	Lauric	0.54	252	255	131	9	7	243	250	117	6	12
2	Myristic	1	231	247	109	12	8	201	228	97	6	6
3	Palmitic	2.16	241	253	123	11	15	222	237	101	2	7
4	β-Hydroxymyristic	3.91	591	613	213	20	17	573	630	197	13	18

*Taken from samples shown in Table 35 and 36 after anaerobic incubation.

et al, 1977). Approximately 50% of the lauric, myristic and palmitic acids of the LPS were lost during incubation with the sediment, while about 66% of the β-hydroxymyristic acid was lost. The ratio of lauric, myristic and palmitic acid to β-hydroxymyristic acid which was 1:1:1:2.4 in the undegraded LPS changed to approximately 1:1:1:1.9 after degradation. The similar values for fatty acid content of the Boivin and Westphal extracts of each of the samples demonstrated that reduction in AC activity could be attributed to degradation of the lipid A component of the LPS and at the same time indicated that the high AC value obtained by Westphal extraction of sediment was not due to LPS alone.

Bacterial isolates from marine sediments

As enzymes capable of degrading LPS could originate from a variety of sources in marine sediments, attempts were made to isolate bacteria with this capability. Three bacterial isolates, none of which was gram-negative, were isolated and identified as, a gram-positive Bacillus, isolate A, and two gram-variable Coryneform organisms, isolates B and C. Several gram-negative organisms were observed growing on the silica gel containing LPS but no attempt was made to isolate or classify them. The bacterial isolates were incubated for different periods of time with LPS to find how quickly they utilised the substrate LPS and brought about the consequent degradation (Table 38). Isolate B seemed to attack the oligosaccharide component of the LPS, resulting in complete loss of serological specificity and a loss of about 60% of the KDO. Isolate B however, did not seem to attack the lipid component of the LPS as there was no great drop in AC activity. Isolates A and C possessed both

Table 38 Degradation of LPS by bacterial isolates obtained from Dumbarton sediment

isolate	length of* incubation wks	ACU	KDO (μg)	HAI
A	0	440	383	2048
"	1	260	299	16
"	2	220	215	4
"	3	160	170	0
B	0	460	390	2048
"	1	400	208	256
"	2	400	177	64
"	3	400	131	2
C	0	480	375	2048
"	1	280	355	512
"	2	240	276	128
"	3	200	239	82

*Reactions were terminated by Westphal extraction after incubation with 2 ml of sterile sea water containing 5 mg/ml of E. coli $O_{111}B_4$ LPS + 0.1% ammonium phosphate at 22°C.

Table 39 Degradation of LPS by two isolates from marine sediment (strains C and B)

Substrate *	ACU						KDO (µg)						HAI					
	C			B			C			B			C			B		
incubation/ wk	0	1	3	0	1	3	0	1	3	0	1	3	0	1	3	0	1	3
S.minnesota SmS	250	190	120	250	210	180	50	25	13	50	26	19	64	32	16	64	16	8
S.minnesota SmS + glucose	270	172	112	270	196	206	50	31	17	50	30	15	64	32	16	64	16	8
Glucose	0	6	12	0	3	6	0	0	0	0	0	0	0	0	0	0	0	0
Sh.flexneri	250	197	141	250	220	202	50	33	25	50	22	10	64	32	8	64	32	2
Sh.flexneri + glucose	267	196	135	267	218	171	50	44	28	50	28	12	64	32	32	64	16	4

*Incubation mixture consisted of 4 mg/ml LPS in 1 ml sterile sea water + 0.1% ammonium phosphate at 30°C.

Reactions were terminated by heating 100°C, 15 min.

saccharolytic and lipolytic properties with the substrate LPS losing about 50%-60% of its AC activity. Isolate A seemed to be more active in its ability to degrade the oligosaccharide component than isolate C. The degraded LPS lost its serological specificity and about 60% of its KDO after three weeks incubation. LPS degraded by isolate C lost about 30% of its KDO while the HAI titre was greatly reduced.

Isolates B and C, which had previously shown different modes of degradation of LPS, were grown in medium containing glucose and LPS. It was hoped that the presence of a more utilisable substrate would allow greater growth of the isolates and result in greater degradation of the LPS (Table 39). However the addition of glucose did not appear to increase the efficiency of degradation.

To show that degradation of lipid A was actually occurring, both the phenol and aqueous phases were assayed for AC activity. This was to ensure that loss of AC activity was not due to the lipid A separating into the phenol phase during Westphal extraction, as a result of losing the hydrophilic oligosaccharide component by degradation. Table 40 indicates that some AC material could be detected in the organic phase. It would seem that degradation of the sugar component of the LPS occurred at a faster rate, resulting in some of the lipid A separating into the phenol layer. Degradation of the lipid A did occur, however, as indicated by the AC activities of the phenol and water phases added together compared with the undegraded LPS. Isolate C which was isolated on E. coli LPS was capable of degrading the sugar components of Shigella and Salmonella LPS as well. The different bacterial isolates seem to preferentially degrade different components of the LPS molecule, using the degradation products

Table 40 To investigate whether the lipid component of LPS is degraded by the bacterial isolate C after 3 weeks incubation at 22°C. Both the phenol layer and the water layer of Westphal extracts were dialysed and analysed.

Substrate (LPS 2 mg)	ACU in		KDO (µg)		HAI	
	phenol layer	water layer	phenol layer	water layer	phenol layer	water layer
S.minnesota SmS LPS	10	60	< 5	23	0	16
S.minnesota SmS + glucose	22	70	< 5	21	0	16
glucose alone	< 5	< 5	< 5	< 5	0	0
Sh. flexneri	12	36	< 5	10	0	6
Sh. flexneri + glucose	10	30	< 5	25	0	4
S. minnesota SmS control	< 5	100	< 5	25	0	512
Sh. flexneri control	< 5	78	< 5	32	1	256

as their carbon source. In each of the cases studied the oligosaccharide component of the LPS was always degraded at a faster rate than the lipid component.

DISCUSSION

Detection of Lipopolysaccharides

Bacterial lipopolysaccharides exhibit an array of chemical and biological properties which, taken in aggregate, are unique, although each separately may be shared by other substances. For example, although KDO is specific to LPS, the thiobarbituric acid test is not completely specific for KDO. Likewise, AC activity, although exhibited by most LPS, is also a feature of many other macromolecules including RNA and some polysaccharides. Perhaps the most reliable chemical marker for LPS is β-hydroxymyristic acid which occurs in all of the enterobacterial LPS so far studied. However, even this is replaced by other β-hydroxy fatty acids in some Veillonella, Xanthomonas and Myxobacteria species while Brucella lipid A does not contain any β-hydroxy fatty acids at all (Rietschel et al, 1977). Because of these considerations, most of the experiments in this thesis involved an initial extraction of LPS (with phenol-water or TCA) followed by a series of analytical procedures. It was hoped by this combination of methods to provide valid measurements of LPS in degradation systems, some of which were exceedingly complex.

The phenol-water extraction method was the primary procedure adopted here, because of its previous widespread use by other workers and because it was reported to give greater yields of LPS than other methods. It also provided a convenient way of terminating degradation experiments. The procedure is not without shortcomings, however: nucleic acids and polysaccharides may be co-extracted with LPS; some LPS do not localise in the water layer (Lüderitz et al, 1970) and some LPS may not be sedimented by ultracentrifugation of the dialysed water layer. Finally, there is a

dearth of direct studies on the absolute efficiency of phenol-water extraction, in that few if any, authors have reported analyses of the LPS content of whole cells and of residues left after phenol-water extraction. Because of these uncertainties, each degradation experiment in this investigation was provided with various controls for internal reference.

Several considerations dictated the choice of the analytical procedures used for measuring LPS. Fatty-acids and KDO were selected as specific indices of the lipid and polysaccharide components of the molecule respectively; HAI tests allowed detection and semi-quantitation of the immunodominant sugars of the polysaccharide moiety, while ESA depended on the lipid A component being present and able to attach to erythrocytes (Neter, 1956). As discussed below, AC activity was probably a function mainly of lipid A.

Thethiobarbituric acid test for KDO is highly specific for deoxysugars, and positive reactions are therefore obtained not only with KDO but also with DNA. The non-sugar component 2-aminopyrimidine is also reactive (Waravdekar and Saslaw, 1959). The absorption maxima of the red complexes vary slightly, however, for KDO it was at 548 nm while for DNA it was at 532 nm. No colour was generated by common pentoses, hexoses or heptose. Agar in great excess gave some colour at 548 nm but this was the shoulder of an absorption curve with a minor peak at 530 nm and a major peak at 435 nm. The polysaccharide slime from Physarum polycephalum gave absorption at about 530 nm. Despite these potential complications there was rarely any difficulty in interpreting the results of the KDO assays.

Although KDO may be taken quantitatively as one of the most specific indexes of LPS, there are a few LPS, eg from Flavobacterium acidificum and Shigella shigae, which completely lack this compound (Ellwood, 1970). Because of the wide variation in KDO content of LPS (from 1-20%) it is not feasible to estimate the concentration of an unknown LPS simply from a KDO analysis.

Considerable effort was expended on developing a test for AC activity - this property being taken as an index of "endotoxin" potency. Previous workers used a method different to that adopted here, the essential difference being their use of undiluted or only slightly diluted serum (Pillemer et al, 1956; Gewurz et al, 1968; Galanos et al, 1971). The incubated mixtures with different amounts of LPS were then serially diluted in order to titrate residual haemolytic C or individual C components. Such methods were not adopted here for several reasons: they were wasteful of serum; they were not as sensitive as tests employing a smaller haemolytic dose of complement; and perhaps most important, they were not well adapted to the comparative assay of AC activity of different LPS. An alternative method based on the complement-fixation test was therefore developed in which the serum was used at a dilution which provided 5 HU_{50} of C in each test mixture initially, and the AC end-point was taken as the dose of LPS which left 1 HU_{50} of residual C. Using this system, human C was found to be about 200 times more sensitive than guinea-pig C for detecting LPS. Pig C was close to human C but still only one-half as active. Although all three species of C were used by previous investigators, parallel tests at constant haemolytic unitage, as done here, had not been made. For example,

Pillemer's work was done entirely with human C; Galanos et al (1971) used pig and guinea-pig C but did not comment on differences in sensitivity. Gewurz et al (1968) investigated four species of C (guinea-pig, mouse, rabbit and human) and in apparent contradiction to the work reported here, found guinea-pig to be the best for detecting minimal levels of LPS. A possible solution to this paradox may be that activation of the Alternative Pathway requires a higher concentration of certain complement components than is needed for the Classical Pathway (Fine, 1974). Thus the lower sensitivity of guinea-pig C, compared with human C, in tests containing 5 HU_{50} may simply be due to the lower concentration of guinea-pig serum, which might be unfavourable for the Alternative Pathway.

In the present investigation the AC activities of a variety of purified LPS were found to vary over a 10-fold range when titrated against human C. The basis of this variation is obscure and is probably multi-factorial, depending for example, on the content of lipid A (Morrison and Kline, 1977), the state of dispersion of the LPS (Galanos et al, 1971), the salt form (Galanos and Lüderitz, 1976) and perhaps on the amount of specific anti-LPS antibody in the serum used as a C source. A further complicating factor was the observation on a few occasions that different batches of the same LPS purchased from Difco Laboratories varied by so much as a factor of 5 in AC potency. On the other hand, remarkably uniform titres of AC activity were obtained with C from different individuals who donated blood on different occasions. This observation suggests that the Alternative Pathway is the primary mechanism by which LPS exerts its AC activity in this test system; for if specific antibodies

were involved as a major factor, one would expect greater differences between individuals bled on different occasions. It has been demonstrated (Loos et al, 1974; Morrison and Kline, 1977) that LPS may also activate C directly via the Classical Pathway but by an antibody-independent mechanism. From the work of Galanos et al (1971), it would seem that the lipid A is the moiety mainly responsible for AC activity.

As compared with other methods which might have been used to measure the "endotoxin" activity of LPS, the AC test has the merits of technical convenience, inexpensive reagents and reproducibility. However, it has the drawbacks of being somewhat less sensitive than certain animal lethality tests and much less sensitive than the Limulus test or the Pyrogen test in rabbits (Noordwijk and de Jong, 1976). It is probably also less specific than the two latter methods. The experiments reported here were designed to minimise these limitations. The sensitivity problem was avoided by using in each degradation system a sufficient dose of added LPS to be readily detectable by its AC activity; the specificity problem was tackled by making a phenol-water extract of the degradation reaction mixture prior to AC testing. This worked well except for marine sediments where the TCA procedure offered distinct advantages.

The erythrocyte-sensitizing activity (ESA) of LPS is mediated by the lipid A which provides the attachment site to the erythrocyte membrane; the immunodominant sugars of the polysaccharide extend into the aqueous phase and react with specific antibody to produce agglutination. The lipid A of freshly extracted LPS may only have slight attaching activities and "stickiness" can usually be greatly enhanced by the standard

procedure of incubating the LPS with dilute alkali at 37°C or 56°C (Neter, 1956). This treatment was shown to cause partial loss of ester-linked fatty acids, but did not affect the amide-linked β-hydroxy fatty acids. The exact nature of the chemical changes produced by NaOH activation are not clear and it may be that the partial loss of ester-linked fatty acids is not the most important feature (Niwa et al, 1968). For example, cleavage of phosphodiester linkages between lipid A and heptose may occur and may be of greater consequence. Hammerling and Westphal (1967) showed that pure polysaccharides do not fix to erythrocytes but can be rendered active by introducing small amounts of long-chain fatty-acids as esters.

Degradation of Lipopolysaccharide by Physarum polycephalum

The results presented here show that LPS from a variety of bacteria are susceptible to partial degradation by Physarum. The slime mould was able to exert its effects both on purified LPS and on LPS localized in heat-killed bacteria. In all cases a marked loss of AC activity and ESA occurred. The loss of ESA was more extensive, proportionately, than the loss of AC activity. In contrast, there was little or no change in KDO concentration and the HAI titre never changed by more than 2 or 4-fold. TLC and GLC analyses showed that lauric, myristic and palmitic acids were lost while β-hydroxymyristic acid was retained. However, the analyses were insufficient to determine whether no β-hydroxymyristic acid at all was removed. Rietschel and coworkers (1972) showed that some β-hydroxymyristic acid was ester-linked in the lipid A of Salmonella. Thus Physarum-degraded LPS may still contain some

ester-bound β-hydroxymyristic acid as well as that which is in amide linkage as GLC analysis indicated that there was no apparent loss of this fatty acid. The GLC trace of undegraded LPS also indicated that a small amount of unsaturated myristic acid was present. This fatty-acid was shown by other workers (Rietschel et al, 1972) to derive from β-hydroxymyristic acid as an artefact during hydrolysis. The comparatively small amount of this material relative to that reported by Rietschel et al (1972) was probably a result of the milder hydrolysis used here to liberate fatty-acids. No diffusible sugar fragments were detected by paper chromatography of highly concentrated dialysates of reaction mixtures in which extensive changes in ESA had occurred. LPS which had been partially degraded by Physarum (eg with extensive loss of ESA) was still of high molecular weight as shown by its sedimentation at 40,000 rpm and by its retention within Visking tubing.

It appears from the above that the main effect of Physarum on LPS is esterolytic, with cleavage of the ester-linked long-chain fatty-acids from the lipid A. This would appear to be the first report of esterase activity in Physarum: esters containing each of the three ester-linked fatty-acids appeared to be similarly susceptible. The fact that LPS was susceptible to attack when presented to the slime mould as heat-killed bacteria suggests that Physarum may also have phospholipases capable of degrading the phospholipids of the outer membrane of gram-negative bacteria where the lipid A is buried. However, this remains to be investigated.

Loss of AC activity after exposure of LPS to Physarum

is consonant with loss of the fatty-acids of lipid A. Gewurz et al (1968) showed that certain chemical treatments (trans-esterification, deacylation, treatment with alkali and treatment with pyridinium formate) which cleaved off the ester-linked long-chain fatty acids caused a marked loss of AC activity, particularly loss of the ability to inactivate C3. This latter property is characteristically a manifestation of Alternative Pathway activation, which in turn is effected mainly, but not exclusively by lipid A. Thus extensive loss of ester-linked fatty acids from lipid A would be expected to produce an extensive, but not complete, loss of AC activity which in fact was observed. The retention of a small amount of AC activity would be in accord with the view that the undegraded polysaccharide component may also contribute to this activity (Morrison and Kline, 1977).

The loss of ESA, but with little change in HAI, in Physarum-treated LPS again points to changes in the lipid A component. During prolonged incubation, ESA was almost totally eliminated, as were the ester-linked fatty-acids, although the β-hydroxymyristic acid, presumably in amide linkage, remained. This suggests that the residual β-hydroxymyristic acid was insufficient for erythrocyte attachment. Alternatively the Physarum may have produced other structural alterations in the lipid A, for example, cleavage of the glycosidic linkage between the glucosamine residues. The organism has been reported to produce N-acetyl glucosaminidase (Kilpatrick and Stirling, 1977).

In contrast to the obvious attack on the lipid A, there was little evidence of degradation in the polysaccharide component, although

subtle changes might have escaped detection by the methods used. The relatively small drop in HAI activity - usually 2-fold and never greater than 4-fold - which was regularly observed after Physarum treatment might indeed be due to partial fragmentation of the polysaccharide, with consequent loss during dialysis. However no sugars were detected in paper chromatograms of concentrated diffusates, and it therefore seems more likely that the loss of fatty-acids from the lipid A might have affected the dispersion of the LPS so as to alter slightly its antibody-binding activity in HAI tests. However, because the change in HAI was so close to the limit of sensitivity, the question was not further explored.

There was no significant loss of KDO during Physarum treatment of LPS, even after the longest incubation periods. This would mean that if the polysaccharide chain had been attacked, the KDO must have remained attached to a large molecule since all KDO assays were carried out on fractions which had been obtained by Westphal extraction and exhaustive dialysis. A problem in these KDO tests was that extracts from Physarum grown on complete medium without bacteria, gave absorption at 548 nm after reacting with thiobarbituric acid. This colour reaction was due to an extracellular slime produced by the organism. This slime which is precipitated by ethanol (at lower concentrations than can precipitate LPS)' is composed of a sulphated polygalactan. However, Waravdekar and Saslaw (1959) showed that galactose itself did not react with the thiobarbituric reagent indicating that the colour reaction observed may be due to some other component in the slime.

The above tentative conclusion that the polysaccharide component of LPS is not appreciably affected by Physarum is surprising. Enzymes

which degrade carbohydrates are known to be produced by Physarum, particularly acetyl-glucosaminidases and glycosidases (Kilpatrick and Stirling, 1977). But it must be emphasised that the results presented here do not exclude the possibility of subtle changes in the polysaccharide moiety, eg the cleavage of monosaccharides from the branch chains of tetra- or pentasaccharide units, or cleavage of the units themselves. However the major change is clearly in the lipid A.

The action of Physarum on LPS was investigated both with live cultures of the slime mould and also with supernates and extracts. With live cultures, maximum degradation occurred during the exponential phase of growth when maximum production of extracellular glycosidases is known to occur (Kilpatrick and Stirling, 1977). Under the conditions used here, the cultures reached stationary phase by day four as had been previously found by other workers (MacLeod and Coote, personal communication). This coincided with the time when degradation of LPS was maximal.

Microscopical examination of Physarum cultures growing on formaldehyde-killed E. coli showed a one-day lag phase during which the majority of the bacteria remained intact. Thereafter, the number of intact bacteria progressively diminished and were increasingly obscured by what appeared to be cell debris. However, it was difficult to determine whether the bacteriolytic effect was extracellular or dependent on phagocytosis. LPS-degrading activity was found in both culture supernates and in cell-free extracts of the plasmodium after three days growth.

The enzymic nature of these activities was suggested by the temperature optimum (for destruction of AC activity) between 30 and 37°C

and the close dependence on pH, values between 5.0 and 6.0 being optimal. Such a pH optimum was close to those reported by Kilpatrick and Stirling (1977) for the Physarum glycosidases.

Fractional precipitation with ammonium sulphate yielded concentrated enzyme preparations largely free from the polysaccharide slime whose removal was desirable because of the colour generated with thiobarbituric acid. These enzyme preparations produced extensive degradation of purified LPS during 24 h at 25°C. In some circumstances the AC activity was completely abolished without greatly affecting the KDO. This essentially complete abolition of AC activity suggests degradation of both the lipid and polysaccharide components, but it must be emphasised that this was only observed with concentrated cell-free extracts of Physarum and direct chemical evidence of polysaccharide degradation was not obtained. It may be noted that one or two of the enzymes detected by Kilpatrick and Stirling (1977)β- N-acetylglucosaminidase and galactosidase - would be expected to cleave off the N-acetylglucosamine and galactose which occur as side groups of the core in the S. minnesota LPS used. It seems likely that with an LPS chosen so that its sugars and sugar linkages corresponded to the glycosidases reported by Kilpatrick and Stirling, extensive degradation of the polysaccharide might be obtained.

There are many points of similarity between these studies with the acellular slime mould Physarum and the previous work of Malchow et al (1969) and Nigam et al (1970) with the cellular slime mould Dictyostelium discoideum. In the initial work with Dictyostelium, the principal effect

on LPS was removal of both amide and ester-linked long-chain fatty-acids. However, in later work it was shown that the amide-linked β-hydroxy-myristic acid was sometimes retained. As with Physarum, there was no apparent effect on KDO, nor was the LPS attacked so as to transfer the KDO to a diffusible fragment. The polysaccharide chains of the two LPS studied (S. london and E. coli B/r) appeared to be unaffected as monitored by HAI. The glycosidic linkage between the glucosamine residues in the lipid A likewise remained intact. The Dictyostelium-degraded poly-saccharides, which still possessed full serological O-specificity were shown to be 50 times less toxic for mice (Tanaka, unpublished observations cited from Malchow et al, 1969) a result which may be compared with the similar loss in AC activity observed here with Physarum. It is clear from the above that there is considerable scope for further investigation in this area. Not only is there the need for further definition of the chemical changes produced by the two slime moulds, but the number, nature and purification of the enzymes involved would also be desirable. It may also transpire that different LPS may vary markedly in degradation sensitivity of both the polysaccharide and lipid moieties. For example, it would be interesting to determine whether lipid A containing C_{10} or C_{18} fatty-acids would be as readily degraded as the C_{12}, C_{14} and C_{16}-containing molecules studied here.

Action of Snail Gut Juice on Lipopolysaccharide

Gut-juice from the snail, Helix pomatia, was selected for investigation as a possible source of LPS-degrading enzymes because the animal itself is constantly ingesting materials rich in bacteria. Moreover

this gut juice has long been recognised as a potent source of hydrolytic enzymes. Indeed thirty or more enzyme activities were reported by Holden and Tracey (1950), about 20 being carbohydrases. Snail gut juice, because of its degradative properties, is used routinely in the preparation of protoplasts from yeast cells (Eddy and Williamson, 1957), again indicating its action against carbohydrates. It was hoped in the present study that snail gut juice might contain enzymes active against LPS, and against the polysaccharide component in particular.

Unfortunately, however, it was not possible to obtain a full answer to this question because supplies of the commercial SGJ were erratic. Thus although certain definite changes in LPS after SGJ action were observed, it was not possible to characterize these as fully as was wished. There appeared to be no significant effect on the immunodominant sugars of four different LPS; nor was there extensive cleavage of the oligosaccharide chains since the serological specificity remained in a high molecular weight form. Likewise the KDO was unaffected by snail gut juice action on LPS. Anticomplementary activity was extensively reduced and this may be attributed to loss of fatty-acids from the lipid A. Alternatively, there could have been cleavage between KDO and lipid A which would cause the latter to move into the phenol layer in the phenol-water extraction. Direct chemical analyses would be required to distinguish these possibilities. SDS-polyacrylamide gel studies likewise showed that LPS was affected by SGJ but again did not define the exact mode of attack. In SDS-PAGE, rough LPS moves rapidly and separates as a single Schiff-positive band, while smooth LPS separates into two components, one of which has the same rapid mobility as rough LPS and the

other is slower. According to Jann, Reske and Jann (1975) the mobility of LPS in these gels is determined both by molecular weight and by the presence of the fatty-acids in the lipid A. After SGJ treatment of smooth LPS, the fast band was greatly reduced while the slow band was scarcely affected. In addition, a new, non-migrating band appeared and this would seem to come from the rough component. It could have arisen either by removal of fatty-acids from the lipid A or by cleavage at the acid-labile KDO linkage. Both of these modes of attack would lead to loss of AC activity and failure to migrate in SDS-PAGE.

Although these studies with SGJ were rather preliminary it was still surprizing that no degradation of the oligosaccharide component was detected by chemical or serological methods in view of the diversity of saccharolytic enzymes known to be present in the gut juice. Clearly further analytical work would be required to determine the mode of action of SGJ on LPS.

Occurrence of Lipopolysaccharide in Marine Sediments

Although Physarum and snail gut juice were useful laboratory systems for studying LPS degradation, they could scarcely be considered as major determinants of the fate of LPS in nature. Since gram-negative bacteria are abundant in marine environments, especially sediments, a preliminary survey was made of the occurrence and vertical distribution of LPS in samples of coastal muds and sands. Persistence of LPS, eg with depth of burial, would indicate relative non-biodegradability. Conversely, the rapid loss of LPS with depth would suggest that sediments contain LPS-degrading systems.

In the initial experiments to extract LPS from estuarine mud, both Westphal and Boivin procedures were used in the expectation that one of them would prove to be clearly superior. However a definite decision proved difficult to reach and, as a result, both methods were applied in each of the several experiments with marine sediments. In short, the Westphal procedure was more efficient but less specific than the Boivin method. For example, the yield of AC activity was sometimes 10-fold greater with Westphal than with Boivin, and the yield of thiobarbituric acid reactive substances was up to 5-fold greater. On the other hand, the yield of the characteristic fatty acids of lipid A was, at most, only about 20% higher in Westphal extracts than in Boivin, suggesting that the former contained much extraneous material. Indeed this was apparent by simple inspection, for whereas the concentrated Boivin extracts had the characteristic milky opalescence of purified LPS, the Westphal extracts were often cloudy, yellowish-brown and viscous. When samples of sediment were subjected to "ageing" at room temperature for several months, most of the interfering substances which found their way into Westphal extracts disappeared. Such sediments, however, still retained the ability to degrade added LPS, probably due to viable spore-forming bacilli.

Although no attempt was made to analyse directly the non-LPS anticomplementary and KDO-interfering material in Westphal extracts of sediments, a variety of tests were made with known substances which might have been present in the samples. These substances, for example, purified DNA, RNA, alginate and agar, were all shown to have significant AC activity and to contribute at least some colour in the KDO test. However, when they were subjected to Westphal extraction, only RNA yielded an extract

with significant AC activity and only DNA an extract with significant KDO-like activity. It was concluded therefore that the non-LPS AC activity and KDO in Westphal extracts of sediments was not due to alginate or agar but that RNA and DNA respectively probably did contribute to some extent. It also seems likely that the yellow humic material, which is only slightly degraded in the marine environment, might contribute to AC activity. This was suggested by the persistent yellow colour and relatively high AC activity in Westphal extracts of deep layers of sediments - activity which KDO analysis showed could not be attributed to LPS.

In order to take advantage of the higher efficiency of phenol-water extraction together with the better specificity of TCA extraction, the two procedures were applied in series, i.e. sediment was extracted first with phenol/water and the product subjected to precipitation of impurities by TCA. This two-step operation was found to get rid of the nucleic acids and most of the yellowish humic material, without loss of LPS. However, these "two-step extracts" still seemed to contain some non-LPS material, since the AC activitiy remained higher than could be explained from the KDO content. Until the nature of this interfering material has been determined, it would appear best to use the Boivin method to extract LPS from marine sediments.

Concurrently with these efforts to devise a satisfactory LPS-extraction method for marine sediments, surveys were made of the LPS content of sediments from different sites and from different depths at each site. Even allowing for the above uncertainties, some firm

conclusions are warranted: LPS is readily detectable in 50 g samples of surface layer estuarine sediment; sewage-contaminated beaches contain more LPS than a clean beach; the concentration of LPS drops off steeply with depth of burial, indicating fairly rapid biodegradation under natural conditions; the KDO disappears with depth much more rapidly than the AC activity. The data allow rough estimates to be made of bacterial biomass in the marine sediments. In the top 1 cm layer of Dumbarton foreshore at low tide and close to a sewage outfall, the AC activity in Boivin extracts correspond to about 4 g dry weight of gram-negative bacteria per kilogram dry weight of marine sediment. This is based on the assumptions that the LPS has an average AC activity of 90 ACU/mg, that LPS constitutes on average 1% of the dry weight of a bacterial cell and that the moist weight of bacteria is 5 times the dry weight. Alternatively if we take β-hydroxymyristic acid as the index and assume that an average LPS contains 10% of this substance, the gram-negative bacterial biomass per kilogram of the top 1 cm of the above sediment would be about 300 mg. These values are probably maximal in view of the heavily polluted nature of the site. On the relatively unpolluted sand beach at Kames Bay, Millport, the values were about one-fifth of those found at Dumbarton, while on the clean sands at White Bay on Cumbrae Island the values were about one-tenth.

As already indicated, LPS disappeared rapidly with depth of burial: the top 1 cm of sediment usually having about 3 times as much AC activity per gram as the layer from 1-4 cm and about 6 times as much as the 6-10 cm layer. With KDO however, the top 1 cm sediment usually had about 10-20 times as much as the layer from 1-4 cm, while virtually none could be detected in the 6-10 cm layer.

From the Dumbarton values it can be calculated that the amount of bacteria in the sediment is about 4 g dry weight per m^2 of sediment surface and taken down to a depth of 10 cm. This figure is 160 times higher than given by Tait (1977) who quoted 0.025 g/m^2 for the average bacterial biomass "in shallow water deposits around the British Isles." The benthic fauna found in such deposits was credited with an average biomass of 10 g/m^2 which led Tait to conclude that "the biomass of benthic bacteria is obviously very small compared with the larger organisms." However the results presented in this thesis, while in no way definitive or comprehensive, suggest that the biomass of bacteria in marine sediments may be one or two orders of magnitude higher than Tait suggested and thus closer to the estimate of Sullivan et al (1976) that "over 50% of the total biomass in the marine environment is composed of bacteria."

Degradation of Lipopolysaccharide added to Marine Sediments

It was clear from the foregoing that the endogenous LPS of marine sediments were subject to degradation in situ and experiments were therefore made to imitate the process in the laboratory. Heat-killed bacteria were incubated with sediment samples for specified times, after which the reaction was terminated by Westphal or Boivin extraction. In the initial experiments using sediments which had been "aged" in the laboratory, both rough and smooth strains of gram-negative bacteria, after incubation for 21 days showed an 80-90% reduction in AC activity of the extracts. KDO disappeared more rapidly than the AC activity, suggesting that the breakdown of the oligosaccharide component of LPS, or at least degradation of KDO, occurs faster than degradation of lipid A. Similar

results were obtained when bacteria were added to fresh sediment samples although certain aspects of the data were harder to interpret because of the extraneous material in Westphal extracts. In many respects these results parallel the observations of Martin (1971) on soils. He showed that most plant or microbial polysaccharides, whether present in complex organic residues or added individually to soils were largely decomposed within a few weeks to a few months. It was shown however that a few microbial polymers were more resistant and entered into combination to form a complex mixture of various microbial and plant polysaccharides. These polysaccharides isolated from soil normally contained ten or more major sugars with others being present in smaller concentrations, although KDO was not detected among them (Swincer, Oades and Greenland, 1969). In the work reported here however, the loss of KDO and the reduction in HAI titre indicates that the oligosaccharide from the LPS is broken down with the composite units possibly being utilised as an energy source.

In the LPS-degradation studies with marine sediments, both aerobic and anaerobic conditions were tested and both appeared almost equally effective. Only degradation of the oligosaccharide component as measured by the loss of HAI titre and KDO was marginally more efficient aerobically. When fresh sediments were used to carry out degradation of added LPS, considerable difficulties were encountered from the endogenous interfering substances in the sediments. Eventually this was minimised by using a relatively small quantity of sediment (in terms of AC activity and KDO yield) and a large amount of heat-killed bacteria as the source of LPS. For example, using Boivin extraction it was customary to recover

700 ACU from the added bacteria at zero time and 50 ACU from the sediment alone. After incubation for 21 days the total ACU from the reaction mixture had commonly dropped to about 250. This was interpreted as indicating significant degradation of the lipid A of added LPS since the endogenous AC activity of sediment incubated alone was stable. The oligosaccharide component of the added LPS was more extensively degraded as shown by the 80% fall in HAI titre and 70% drop in recoverable KDO. GLC analyses showed loss of about 50% of both the ester-linked fatty acids, lauric, myristic and palmitic and of the amide-linked β-hydroxy-myristic. This percentage loss corresponded closely to the loss of AC activity and, taken with the other data, indicated that the polysaccharide component of LPS was more rapidly degraded than the lipid. It may be mentioned also that the gas-liquid chromatograms of sediment samples had peaks corresponding to fatty-acids additional to the four mentioned above and which have been reported to be present in the lipid A of other LPS preparations.

Isolation of Lipopolysaccharide-Degrading Bacteria

A serial enrichment technique in which samples of marine mud were inoculated into sterile sea-water containing 4 mg/ml of LPS as the sole source of carbon was used to isolate LPS-degrading bacteria. After 7 transfers, with 1 week at room temperature between each transfer, inocula were put on to silica gel plates containing LPS as the sole carbon and energy source. After incubation single colonies were picked off and transferred to LPS-seawater-ammonium phosphate for propagation. Only gram-positive organisms were selected so as to avoid any LPS contribution

in degradation experiments where the strain was incubated with added LPS. Three isolates were selected for further study; one Bacillus spp (isolate A) and two Coryneforms (B and C). All grew in LPS-seawater with $(NH_4)_2PO_4$ under aerobic conditions at 20°C. Isolates A and C both showed lipolytic and saccharolytic enzyme activities. Incubation of LPS with these isolates for 3 weeks resulted in the loss of 66% of the AC activity while the serological specificity, i.e. HAI titre, was virtually abolished. The faster rate of degradation of the oligosaccharide component of LPS was shown when LPS degraded by C was phenol-extracted and AC activity detected in the phenol layer. This was probably due to the loss of the hydrophobic sugar component causing the lipid moiety to separate into the phenol layer. Isolate B seemed, however, only to attack the sugar component of the LPS leading to a virtual complete loss of its serological properties, i.e. HAI titre. The loss of KDO would seem to indicate that there had been some breakdown of the individual sugar molecules or oligosaccharides which subsequently may have disappeared during dialysis or ultracentrifugation. Other workers have shown that saccharolytic enzymes are produced by bacteria isolated from soil (Ito, Muramatsu and Kabata, 1975): for example, the culture fluid of Clostridium perfringens could be used as a source of various exoglycosidases and endoglycosidases such as endo-β-N-acetylglucosaminidases. Thus a wide range of saccharolytic enzymes from various bacteria isolated from soil and sediment are known to be produced, acting on a range of polysaccharides, from LPS to mannan (Nakajima, Maitra and Ballou, 1976) and producing a rapid degradation of these polymers.

There appears to be only one other laboratory where bacteria

capable of degrading LPS have been isolated (Voets et al, 1972). These workers isolated a Bacillus macerans strain and also a strongly lipolytic Micrococcus spp both from soil. Both strains acted similarly, splitting LPS into lipid A and polysaccharide. In addition, free fatty-acids were liberated from the lipid A but the polysaccharide component was not further hydrolysed.

A Pseudomonas species which may be of interest in the present context was isolated from soil by Whiteside and Corpe (1969) and was shown to attack gram-negative cells through the action of potent lipases. There was a loss of 37% of total lipid from the outer membrane of Chromobacterium violaceum used as target organism. KDO and heptose content of the cells remained unchanged and it is not clear therefore whether LPS was in fact attacked. The cell envelopes after treatment retained their characteristic shapes.

It seems therefore that both saccharolytic and lipolytic enzymes are produced by a variety of organisms in soil and sediments. However each isolate seems to produce a specific enzyme such that for complete degradation of a complex molecule such as LPS, the action of a range of enzymes from a variety of organisms may be required.

Conclusions and Perspectives

LPS is subject to biodegradation in nature by a wide variety of organisms. It has been shown both in this work and that of others that various bacteria degrade LPS, possibly utilising the degradation products. So far, mainly gram-positive organisms have been isolated. Due to the ubiquity of gram-negative bacteria in the marine environment

it would be expected that possibly many more organisms could degrade LPS utilising the lipid or the oligosaccharide component as a carbon and energy source.

Physarum was shown to degrade the lipid component of LPS in a mechanism analogous to that reported by Malchow et al (1969) for Dictyostelium. As both these species feed on bacteria it would possibly be expected that LPS degradation would be exhibited by these organisms and probably by many others that feed on bacteria.

From the work with snail gut juice, it would be of interest to investigate other invertebrates, such as marine filter and detritus feeders, which ingest gram-negative bacteria to see if LPS-degrading enzymes are produced. It has already been established that various marine invertebrates produce lysozyme (Hylleberg Kristensen, 1972; Hardy, Fletcher and Gerrie, 1976).

There have been no reports of characterization of enzymes active in LPS degradation. Further work on the specificity and activity of such LPS degrading enzymes would be invaluable in structural studies. Selective degradation of different features of the LPS could possibly remove the endotoxic activity which might have application in the production of enzymes and vaccines from gram-negative bacteria. Finally, further surveys on the occurrence of LPS in nature might have a bearing on the genesis of fossil fuels, particularly petroleum (Davis, 1967).

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APPENDICES

I Assay for 2-keto-3-deoxyoctonate (KDO) (Keleti and Lederer, 1974)

To 0.1 ml of sample was added 0.1 ml of 0.5N H_2SO_4 and the mixture shaken and placed in a boiling water bath for 8 min (this step was omitted when using pure KDO but was required with LPS). The tubes were cooled to room temperature and 0.1 ml of H_5IO_6, reagent A, was added. This was shaken and left to stand at room temperature for 10 min. After this time, 0.4 ml of arsenite reagent B was added and the reaction mixture was then shaken well. Thiobarbituric acid, reagent C (1.6 ml), was added and the mixture was again shaken. The mixture was placed in a boiling water bath for 10 min then cooled to room temperature under running tap water. Butanol, reagent D, was added (1.0 ml) and shaken. The mixture was centrifuged at 2,000 rpm for 10 min at 4°C after which 1 ml of the upper butanol layer was taken and the absorption at 548 mμ measured.

Reagents

A. 2.28 g H_5IO_6 (O-paraperiodic acid) dissolved up to 100 ml with water (0.1N). (Store in dark bottle).

B. 4 g of $NaAsO_2$ dissolved up to 100 ml with 0.5N HCl (4.0%).

C. 600 mg of 2-thiobarbituric acid dissolved up to 100 ml in boiling water (0.6%) and cooled to room temperature. Prepare prior to use.

D. 5 ml concentrated HCl added to 95 ml n-butanol.

II Buffers

Veronal Buffer (Kabat and Mayer, 1961)

To 85 g of NaCl was added 3.75 g of sodium 5,5-diethyl barbiturate which was then dissolved in 1400 ml of distilled water. To this was added barbituric acid (5.75 g) dissolved in 500 ml of hot water and 5 ml of stock solution C and the mixture made up to a total of 2 l. This gives 5x concentrated VB which is stored at -20°C.

Stock solution C:

1.00M $MgCl$	3.33 g $CaCl_2$)	
	)	in 100 ml
3.00M $CaCl_2$	20.32 g $MgCl_2.6H_2O$)	

Phosphate Buffer (Cruickshank et al, 1973)

Stock solutions

A: 0.2M solution of monobasic sodium phosphate (31.2 g NaH_2PO_4, $2H_2O$ in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (28.39 g of Na_2HPO_4 or 71.7 g of $Na_2HPO_4.12H_2O$ in 100 ml)

X ml of A + Y ml of B dilute to 200 ml

pH	X	Y
6	87.7	12.3

Citrate-Phosphate Buffer (Cruickshank et al, 1973)

Stock solutions

A: 0.1M solution of citric acid (19.21 g in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (28.39 g of Na_2HPO_4 or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml)

X ml of A + Y ml of B, diluted to a total of 100 ml

pH	X	Y
5	24.3	25.7
6	17.9	32.1

Universal Buffer (Dawson et al, 1969)

1 l of mixture for titration contains citric acid (6.008 g), KH_2PO_4 (3.893 g), H_3BO_3 (1.769 g), diethylbarbituric acid (5.266 g). 100 ml of this mixture is titrated with X ml of 0.2N NaOH to give the required pH.

pH	X
3	6.4
5	27.1
6	38.9
7	50.6
9	72.7
11	86.0

III Endogenous lipopolysaccharide in Marine Sediments

all quantities per g dry weight

Particulars of Sediment	cm from top	ACU_{50} B	ACU_{50} W	KDO (mg) B	KDO (mg) W	fatty acids (mg) L. M. P. BHM
Fresh White Bay						
(N.S.176 591)	0-1	0.2	6	24	44	
	1-4	0.2	2	2	2	
(1/2/77)	4-6	0.1	1	2	2	
Fresh Kames Bay						
(N.S.171 550)	0-1	3	13	38	68	
	1-4	1	6	2	2	
(1/2/77)	4-6	0.5	3	2	2	
Fresh Dumbarton foreshore (N.S424 740)	0-1	3	84	240	306	
(1/2/77)	1-4	2	32	8	24	
	4-6	1	28	2	12	
(4/10/76)	1-5	3	49	670	750	
(27/9/77)	1-5	4	70	130	330	
(24/5/76)	1-5	2		150		
(18/8/77)	1-5	1	56	156	800	
(26/1/77)	1-5	7	78	270	320	B 240 120 140 360 W 140 160 300 340
(18/8/77)	1-5	1	50	230	1200	B 60 100 160 340 W 140 160 120 330
(22/9/76)	0-2	10	61	206	230	
High water mark 100 m from sewage outlet	2-6	9	30	10	21	
	6-10	5	15	1	1	
Low water mark 100 m from sewage outlet	0-2	9	57	201	237	
	2-6	5	17	10	30	
	6-10	3	8	1	1	

Patriculars of Sediment	cm from top	ACU_{50} B	ACU_{50} W	KDO (mg) B	KDO (mg) W	fatty acids (mg) L. M. P. BHM
Fresh Dumbarton foreshore (N.S.424 740)						
Low water mark beside sewage outlet	0-2	12	60	253	270	
	2-6	10	37	31	35	
	6-10	6	15	1	1	
Aged Dumbarton sediment (N.S.424 740)						
3 months (13/8/76)	1-5	2	11	270	300	
4 months (20/8/76)	1-5	2	6	50	50	
3 months (26/3/76)	1-5	2		56		
4 months (26/3/76)	1-5	1		48		