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To my parents and my wife, Diane

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THE AFFINITY OF BACTERIAL POLYSACCHARIDES FOR MAMMALIAN CELL SURFACES AND ITS RELATIONSHIP TO ADJUVANT ACTIVITY

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Presented for the Degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow

Department of Microbiology

April, 1974

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"Truth lies within a little and certain compass,
but error is immense ...."

Henry St. John, Viscount Bolingbroke

(1678 - 1751)

ACKNOWLEDGMENTS

To Dr. Duncan Stewart-Tull I express my sincere thanks not only for his conscientious supervision of my research but also for his good humour during some very trying circumstances.

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Finally I would like to thank all the members of the Microbiology Department, both staff and students, who have made the past three years a very pleasant period filled with plenty of good humour.

The Object of the Research

The object of this investigation was to examine the possible relationship between the affinity of bacterial polysaccharides for mammalian cell surfaces and their activity as immunological adjuvants. In 1965, Stewart-Tull, Wilkinson and White, while attempting to find an explanation for mycobacterial adjuvancy, demonstrated a molecular interaction between a mycobacterial glycopeptide and guinea-pig γ_2-immunoglobulin. They suggested that the mycobacterial peptidoglycolipid (Wax D) might combine with newly synthesized γ_2-immunoglobulin and act as a specific derepressor for the ribonucleic acid template in the plasma cell. This hypothesis would require the retention of the mycobacterial component on the cell surface. In a series of initial observations, Stewart-Tull, Dempster and McKean (unpublished observations) showed that the glycopeptide fraction of Wax D was able to bind both to a mammalian cell membrane site and to the γ_2-globulin.

In general the well-defined adjuvant substances are insoluble in water which creates difficulties in measuring their adsorption to cell surfaces. However, lipopolysaccharide (LPS) preparations from Gram negative bacteria were shown by Johnson, Gaines and Landy (1956) to possess adjuvant activity in mice, and Ginsberg, Goebel and Horsfall (1948) showed that lipopolysaccharides were capable of cellular modification. Since LPS preparations can be easily suspended in an aqueous environment the problems associated with the mycobacterial fractions could be avoided.

In this investigation it was proposed to accurately measure (a) the association of bacterial polysaccharides with cell surfaces and

(b) the stimulation of increased antibody levels to a protein antigen in the mouse caused by the bacterial polysaccharides acting as adjuvants. From the results of such an investigation a theoretical approach to the relationship between affinity for cell surfaces and adjuvancy could be attempted.

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Abbreviations Used in the Structural Formulae

Arab	Arabinose
Abe	Abequose
Gal	Galactose
Glu	Glucose
$GalNH_2$	Galactosamine
$GluNH_2$	Glucosamine
Mann	Mannose
NAcMuAc	N-acetyl muramic acid
$NAcGluNH_2$	N-acetyl glucosamine
KDO	2-keto-3-deoxy octonate
Hep	Heptose
Tyv	Tyvelose
Rha	Rhamnose
Ala	Alanine
Glu	Glutamic acid
$GluNH_2$	Glutamine
DAP	Diamino pimelic acid

INTRODUCTION

In order to understand the affinity of bacterial polysaccharides for mammalian cell surfaces and its relationship to their adjuvant activity, it is necessary in the survey of the literature to discuss four distinct topics. A discussion of the chemical composition and structure of the bacterial components together with the structure of mammalian cell surfaces was considered necessary in order to fully understand the complex nature of the binding of bacterial components to cell surfaces. The biological activity of the bacterial components was concerned with the stimulation of an enhanced humoral antibody response and in order to realise the significance of the findings it was necessary to discuss the wider implications of adjuvant activity.

Insert P.18 L4B

"Lymphoid tissue can be divided into two zones, thymus - dependent and thymus independent ....."

Tables 11 - 18

Symbols

N.P. Non-parallel response

* $I.D_{50}$ outside experimental conditions

LPS. Instead, attention will be focused on those features of LPS which have a direct bearing on the subject of this thesis. For general reviews on bacterial LPS the reader is referred to Lüderitz, Staub & Westphal (1966), Simmons (1971), Weinbaum, Kadis & Ajl (1971) and Wright & Kanegasaki (1971).

Lipopolysaccharide occurs widely in Gram-negative bacteria and is present as a complex macromolecular structure in the external layers

of the bacterial cell envelope. In situ the LPS complex is bound non-covalently to the cell-wall mucopeptide (Wheat, 1964), and the maintenance of its tertiary structure is essential for its endotoxic activity (Rudbach, Milner and Ribi, 1964). From the extraction methods used, e.g. Trichloroacetic acid, phenol and ether, it would appear that hydrophobic forces hold the LPS and mucopeptide together. There are exceptions to this, and Wober and Alaupovic (1971) found that the LPS of Serratia marcescens was covalently linked to protein, involving a N-glycosidic link between the reducing-terminal glucosamine of the lipid A and an asparagine residue.

When isolated from the bacillary body, the LPS in aqueous suspension formed a trilaminar or vesicle-like structure (Beer, Braude and Brinton, 1966; Ribi, Anacker, Brown, Haskins, Malmgren, Milner and Rudbach, 1966). This interaction of LPS molecules was by hydrophobic and Mg^{++}-mediated ionic bonds (Rothfield and Horne, 1967; De Pamphilis, 1971). Hence, in dealing with LPS molecules in an aqueous environment, self-assembly to a membrane-like structure occurs. Recently, Katayama, Hattori and Suganuma (1971) showed that this self-assembly process takes place even in lyophilized preparations of LPS. The self-assembly mechanism forms the LPS into a membraneous structure of differing shapes (discs, ribbons, lamellae and vesicles) and Lindberg (1967) and Shands, Graham and Nath (1967) found that the formation of these was dependent on the method of preparation of the LPS.

It appears that the LPS molecule consists of three main regions (Figure 1). An outer region (I), the O-specific polysaccharide, carries the main serological specificity. This is linked to the core

Figure 1: Schematic Diagram of Lipopolysaccharide from Enterobacteriaceae

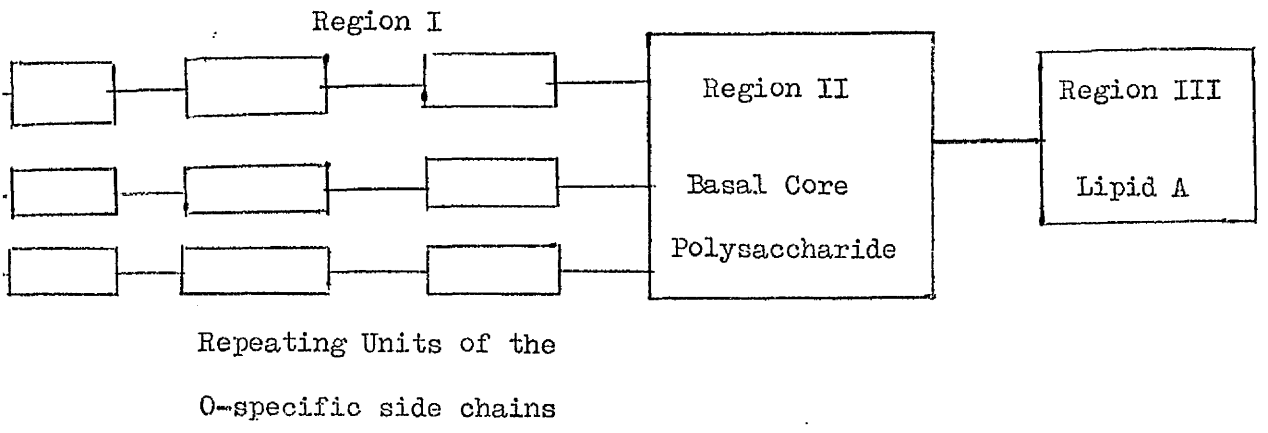
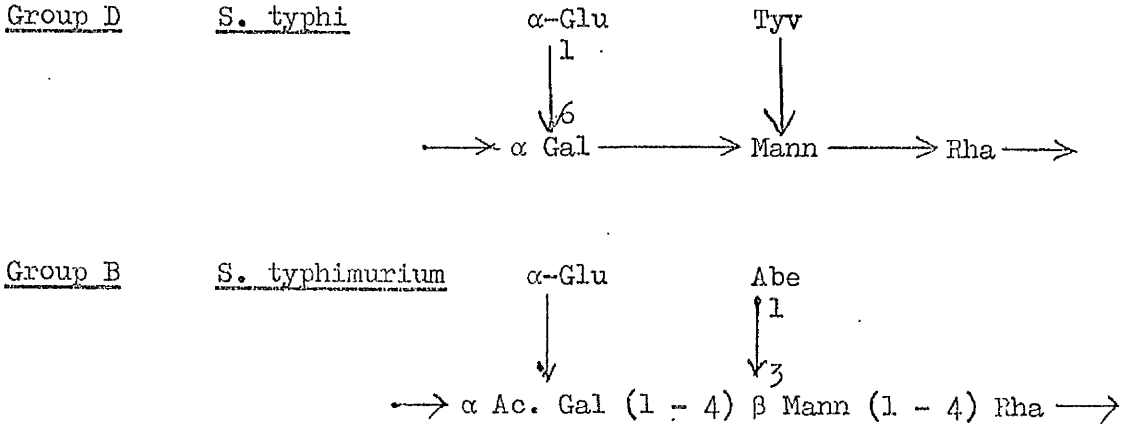


Figure 2: O-Specific Polysaccharide Repeating Units (from Lüderitz, Staub and Westphal, 1966)



polysaccharide (II) which contains the "basal" sugars and which in turn is linked through a ketodeoxyoctonate trisaccharide to the lipid component, lipid A (III). For a particular genus in the Enterobacteriaceae the lipid A and basal core structures are invariable regions, but the O-specific polysaccharide is highly variable, conferring specificity on the organism (Lüderitz, Jann and Wheat, 1968). There is a variable number of O-specific polysaccharide chains per basal core depending on the organism.

Although in an extracted form, LPS appears to consist of three regions, there are further regions or components when the LPS is in situ. Rothfield and Horecker (1964) reported a cephalin type of phospholipid, consisting mostly of palmitic and oleic acid, associated with LPS. This fraction was called lipid B. It was thought to be important in the biosynthesis of the basal core and was associated closely with a protein moiety, which suggested that the protein could be an enzyme complex involved in biosynthesis (Lüderitz, Jann and Wheat, 1968). The protein component not only conferred antigenicity (but not specificity) on the macromolecular complex, but also determined colicin activity (Goebel and Barry, 1958). A wide range of sugars was isolated from LPS, including hexosamines, hexoses, 3,6-dideoxy-hexoses and heptoses (Table 1). All bacteria belonging to a particular genus contain certain sugars in common. Thus various workers (Kauffmann, Lüderitz, Stierlin and Westphal, 1960; Kauffmann, Krüger, Lüderitz and Westphal, 1961) found that in Salmonella spp the O antigens contain the common sugars, heptose, 2-keto-3-deoxy-octonate (KDO), D-glucose, D-galactose and D-glucosamine. Similarly, Simmons (1957; 1958; 1962) found that all Shigella spp poly-

Table 1 . : MONOSACCHARIDE CONSTITUENTS OF ANTIGENS OF SALMONELLA AND RELATED ENTEROBACTERIACEAE

(from Lüderitz, Staub and Westphal, 1966)

Hexosamines	Deoxyhexosamines	Hexoses	6-deoxyhexoses	3,6 dideoxyhexoses
D-Glucosamine	L-Fucosamine	D-Galactose	L-Fucose	abequose
D-Galactosamine	D-Fucosamine	D-Glucose	L-Rhamnose	paratose
	D-Viosamine	D-mannose	6-deoxy-L-talose	tyvelose
				colitose
				ascarylose

Heptoses: L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, D-glycero-D-galactoheptose

Others: KDO, neuraminic acid, ribose, xylose

saccharides contained KDO, aldoheptose, D-galactose, D-glucose, N-acetyl-glucosamine and rhamnose. However, the chemical composition varies depending on the organism and the growth medium (Fukuski, Anacker, Haskins, Landy, Milner and Ribi, 1964). Recently, Fromme and Schlecht (1973) found that in the case of S. typhimurium LPS, the types and amounts of fatty acids and sugars in the complex were independent of the degree of aeration of the culture. However, the number of repeating units of the O-specific polysaccharide tended to vary, so the chains were two to three times longer with low aeration than with high aeration.

O-specific polysaccharides (region I): The O-specific polysaccharide consists of hexose residues arranged in repeating sequences. The nature of the sugar components and their linkages determined the O-specificity of the organism. The O-specific antigenic determinants were not unique to a particular organism, but were shared, so that genera such as Salmonella could be classified into serogroups. Examples of O-specific polysaccharides are given in Figure 2.

Basal Core (region II): Simmons (1966) showed that Salmonella and Shigella basal core polysaccharides contained the same constituents although their structures were not identical. Similarly, the majority of E. coli LPS contained the basal sugars (Lüderitz, Jann and Wheat, 1968). The sugar sequences were similar in E. coli and Salmonella with respect to galactose, glucose and N-acetyl glucosamine (Edstrom and Heath, 1964; Edstrom and Heath, 1965; Heath, Mayer, Edstrom and Beaudreau, 1966), although there were distinct differences. Osborn, Rosen, Rothfield, Zeleznick and Horecker (1964) and Sutherland, Lüderitz and Westphal (1965) proposed that the Salmonella basal core consisted of N-acetyl α-D-

glucosamine-α-D Glucosyl-α-D-galactosyl (1 - 3) D glucose, with a galactose unit α(1 - 6) linked to the reducing glucose unit. Johnston, Johnston and Simmons (1967) have proposed that the Shigella analogue had the structure, N-acetyl-α-D-glucosaminyl (1 - 4) α-D-galactosyl (1 - 3) D glucose, with α-glucose substituted on the C_3 and C_4 position of galactose and glucose respectively (Figure 3).

Lipid A (region III): Lipid A has an N-β-hydromyristoyl-glucosamine-phosphate backbone in which all the available hydroxyl groups in the hydroxy acid and glucosamine are esterified with long chain fatty acids (Nowotny, 1961; Burton and Carter, 1964) (Figure 4). It has been generally assumed that lipid A is the active centre of endotoxic activity, although Ribi and his coworkers have cast doubts on this assumption (Ribi, Anacker, Fukushi, Haskins, Landy and Milner, 1964). Recently, Rank, Di Pauli and Flügge-Rank (1972) found that LPS from Shigella, Salmonella and E. coli and their lipid A fractions, were able to evoke immune responses which exhibited a high degree of cross-reactivity. This cross-reactivity component appears to lie in the lipid A, and they suggested that lipid A induced antibodies responsible for the observed endotoxic effects.

The fatty acids found in lipid A are unique and are generally not encountered anywhere else in the cell. The fatty acids, lauric, myristic, palmitic and 3-hydroxymyristic acid are either ester-linked or amide-linked, and Neter, Westphal, Lüderitz, Gorzynski and Eichenberger (1956) showed that removal of the esterified fatty acids reduced the biological activity of the LPS.

Figure 3: The basal core structure of Shigella flexneri (Simmons, 1971)

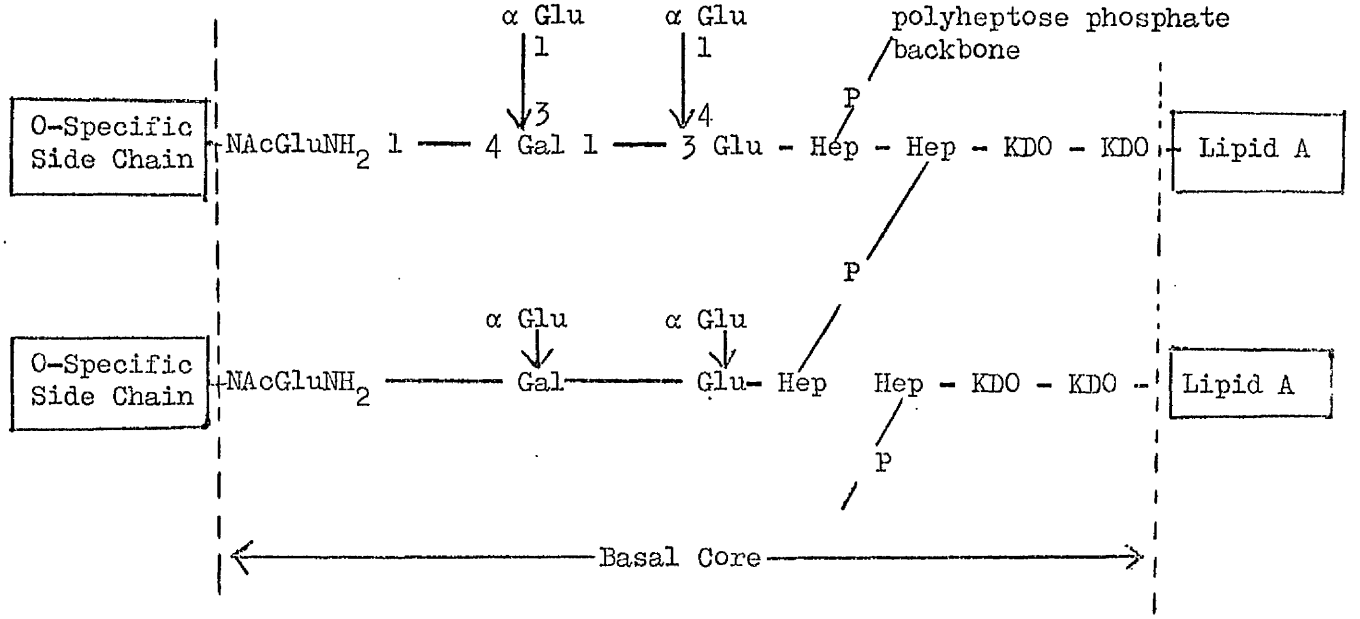
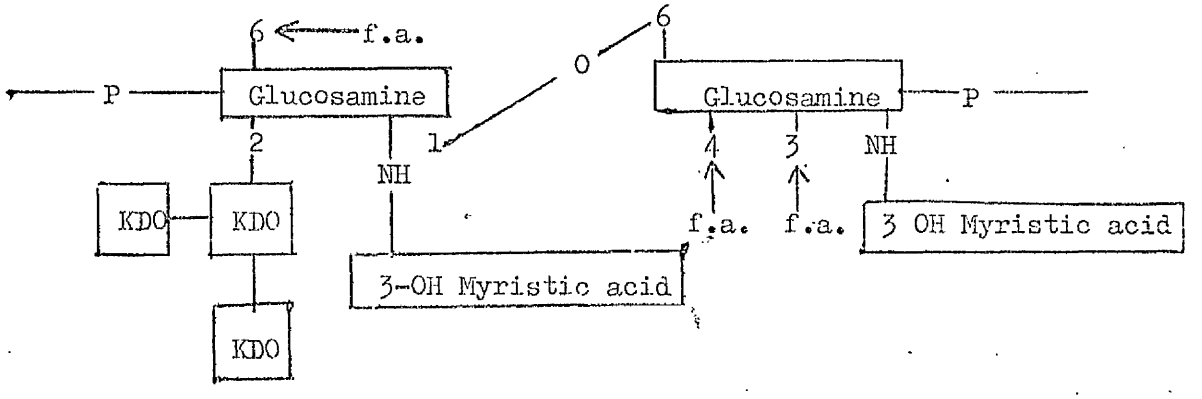


Figure 4: Lipid A Structure of S. minnesota R595 (from Lüderitz, Galanos, Lehmann, Nurminen, Rietschel, Rosenfelder, Simon and Westphal, 1973)



GLYCOPEPTIDE AND ALLIED SUBSTANCES FROM MYCOBACTERIA

During mycobacterial growth, numerous polysaccharides are released into the culture medium (Siebert, Pederson and Tiselius, 1938; Siebert, 1949; Siebert, Soto-Figueroa and DuFour, 1955; Stacey, 1955). In addition, there is also a wide variety of polysaccharides that remain in association with the bacillary body. Haworth, Kent and Stacey (1948a) and Bayly, Jones and Stacey (1955) showed that the most frequent occurring sugars in these latter polysaccharides were arabinose, mannose, galactose and glucosamine. They further reported that rhamnose, ribose and hexosamines (besides glucosamine) could also occur. In some cases the polysaccharide was isolated in conjunction with lipids. For example, Lee (1966) isolated a lipopolysaccharide from the cell-wall of Mycobacterium phlei and its structure, subsequently determined by Ferguson and Ballou (1970), Grellert and Ballou (1972) and Gray and Ballou (1972), bore no relationship to LPS isolated from Gram-negative bacteria.

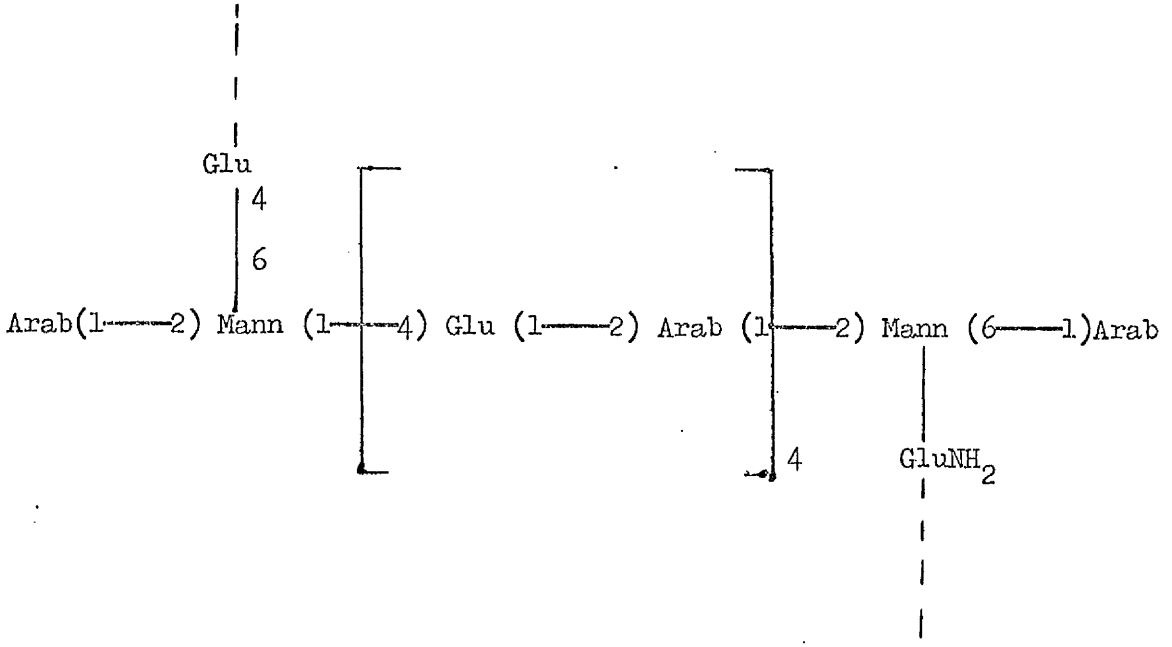
Besides an extensive range of "simple" polysaccharides and the above LPS's, Mycobacteria also contain an array of complex lipid-bound polysaccharides known as Waxes A, B, C and D. White, Bernstock, Johns and Lederer (1958) found that the adjuvant activity of Mycobacteria was associated with the Wax D fraction. The latter extracted by a modified Anderson's method (Asselineau and Lederer, 1960), was shown by Asselineau, Buc, Jollès and Lederer (1958) and Lederer (1961) to be a peptidoglycolipid. The lipid component of this structure is mycolic acid. Asselineau, Buc, Jollès and Lederer (1958) found that the polysaccharide moiety of Wax D contained D-arabinose, D-mannose, D-galactose, D-glucosamine and D-galactosamine. This polysaccharide was thought to resemble

the lipid-bound polysaccharide isolated by Haworth, Kent and Stacey (1948b) (Figure 5). Also, in 1958, White, Bernstock, Johns and Lederer showed by fractionation studies on Wax D that adjuvant active fractions always contained a characteristic polypeptide of meso α,α' diaminopimelic acid (DAP), D-glutamic acid, D-alanine and L-alanine. The Wax D extracted from human Mycobacteria occurs in two forms; the major component (> 90%) can be represented as Myc-Polysacc-Polypep and the minor component as Myc-Polysacc . The abbreviations refer to mycolic acid, polysaccharide and polypeptide respectively (Jollès, Samour and Lederer, 1962; White, Jollès, Samour and Lederer, 1964).

Wax D is considered to be an integral part of the cell wall mucopeptide complex and several structures have been proposed (Kanetsuna and San Blas, 1970; Lederer, 1971; Markovits, Vilkas and Lederer, 1971) (Figure 6).

By acid hydrolysis of Wax D a hydrosoluble glycopeptide was formed which lacked mycolic acid and adjuvant activity, despite containing the characteristic peptide of alanine, glutamic acid, DAP and glycine (White, Jollès, Samour and Lederer, 1964). However under the appropriate conditions the hydrosoluble glycopeptide has since been shown to possess adjuvant activity (Stewart-Tull et al., 1974).
A tentative structure for this glycopeptide (Figure 7) was proposed by Stewart-Tull and Wilkinson (1973). Recent studies indicated that there is a wide range of hydrosoluble adjuvant-active complexes extractable from mycobacteria (page 37). These are generally glycopeptides and are structurally related to the hydrosoluble moiety of Wax D.

Figure 5: Structure of the Lipid-bound Polysaccharide isolated by Haworth, Kent and Stacey (1948b)



Fatty acids could be attached to the terminal arabinose residues

Figure 6: Hypothetical Structure of the Wax D Monomer

Proposed by Markovits, Vilkas and Lederer (1971)

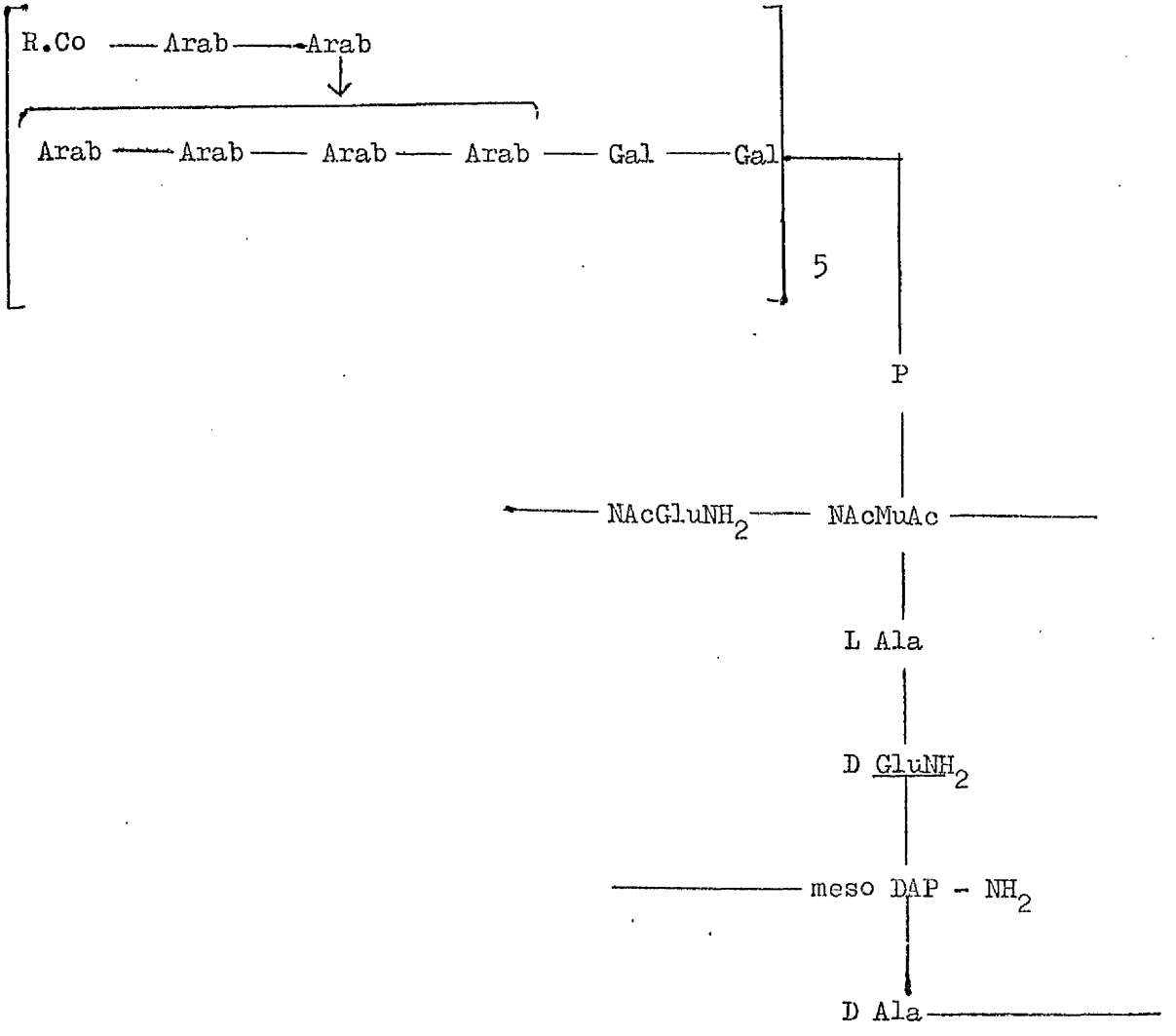
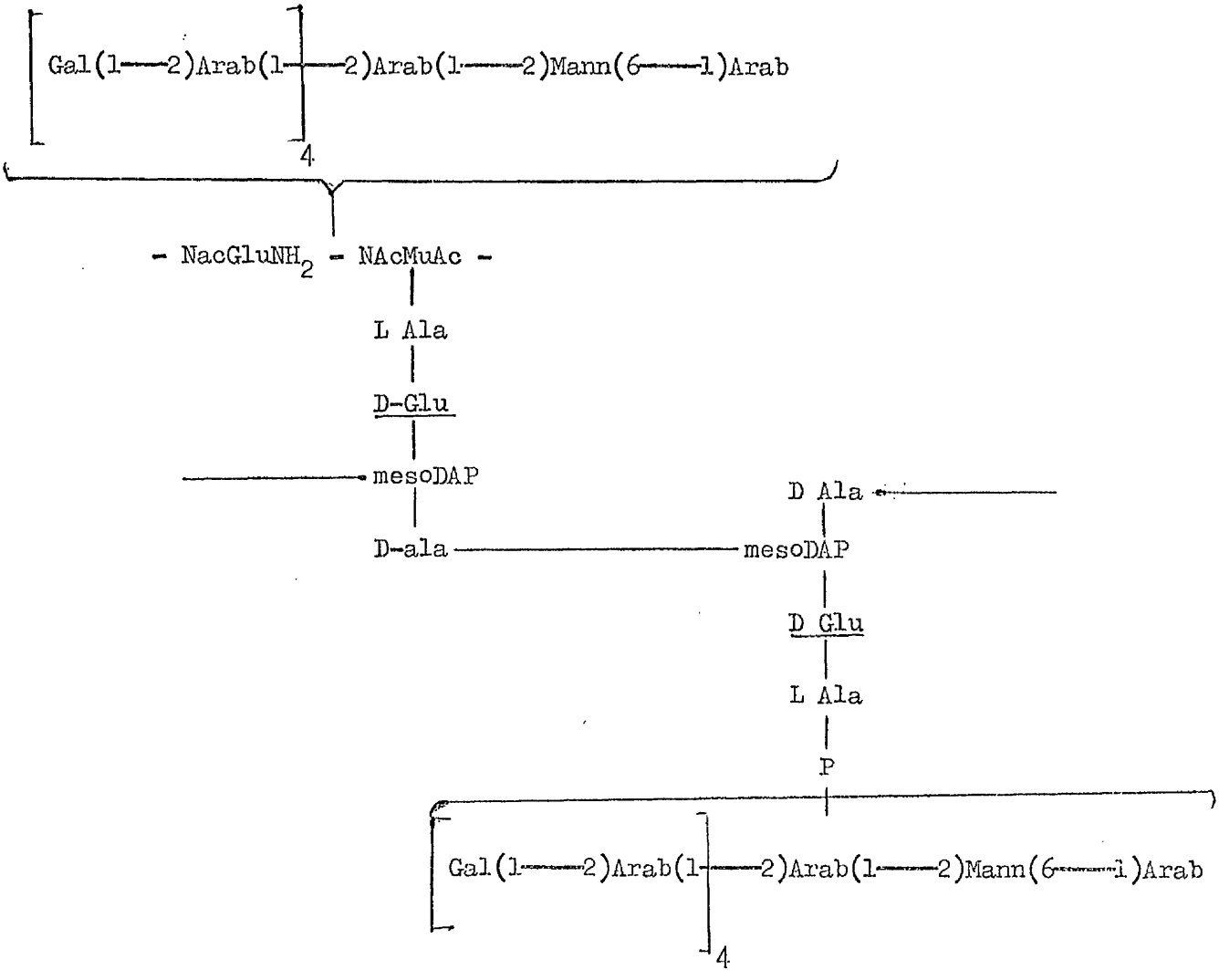


Figure 7: Tentative structure of the glycopeptide monomer (from Stewart-Tull and Wilkinson, 1973)



Proposed by Stewart-Tull and White (1964) and Haworth, Kent and Stacey (1948)

II MAMMALIAN CELL MEMBRANES

General Account

The mammalian cell membrane can be conveniently considered in two parts, the plasma membrane and an outer layer containing periodic acid-Schiff (PAS) positive material which was presumed to be carbohydrate. There is now evidence that this outer layer is present on the surface of many mammalian cells (Brandt, 1962; Fawcett, 1965; Ito, 1965; Lovell, Clark and Curran, 1966). This outer layer differed in composition, form and resistance to lytic agents according to the cell type, and hence is given different names. For example, in epithelial cells it is termed the "surface coat" (Ito, 1965; Revel and Ito, 1967), or "glycocalyx" (Bennett, 1963; 1969), and "cell coat" describes a layer believed to cover ascites tumour cells (Gasic and Gasic, 1963).

The plasma membrane of all mammalian cells consists of proteins and lipids, and each membrane is characterized by the amount and kind of each protein (including enzymes) and lipid (phospholipids, glycolipids, cholesterol) that it contains. Small amounts of carbohydrate may also be present.

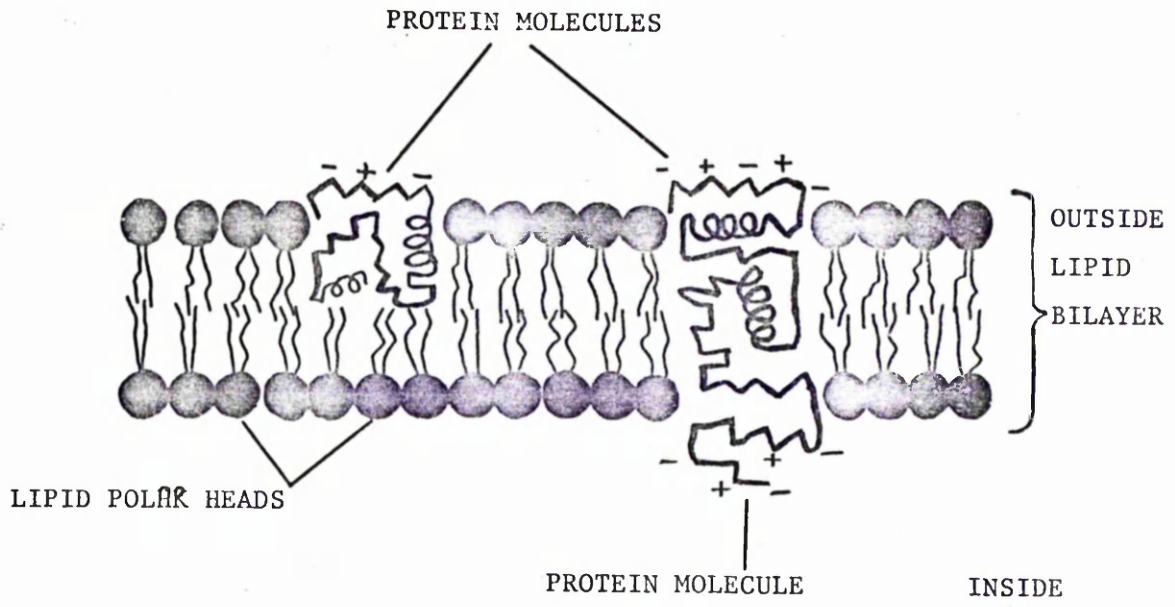
Various studies have shown that the plasma membrane is a lipid bilayer (Danielli and Davson, 1935; Davson and Danielli, 1952; Gorter and Grendel, 1925). Robertson (1959, 1964) found that the plasma membrane of myelin was a triple-layered structure 75Å thick and clearly visible in the electron microscope. The membrane consisted of two outer electron-dense areas 20Å thick, separated by an electron transparent area 35Å wide. This structure was termed the "unit membrane". However

it was found that the width of the membrane and the dimensions of the individual layers varied for plasma membranes of different cells (Sjostrand, 1963a, 1963b; Yamamota, 1963; Stoeckenius, 1964). Initially, it was thought that there was a protein monolayer on the polar heads of the lipids (Danielli and Davson, 1935) but there is little evidence to suggest that a protein monolayer in the extended β-configuration exists (Maddy and Malcolm, 1965, 1966). However, Lenard and Singer (1966) found that such protein was present in small amounts on erythrocyte membranes. A globular structure for the majority of protein was proposed by Sjöstrand (1963a, 1963b), Nilsson (1964, 1965) and Robertson (1965, 1966a, 1966b).

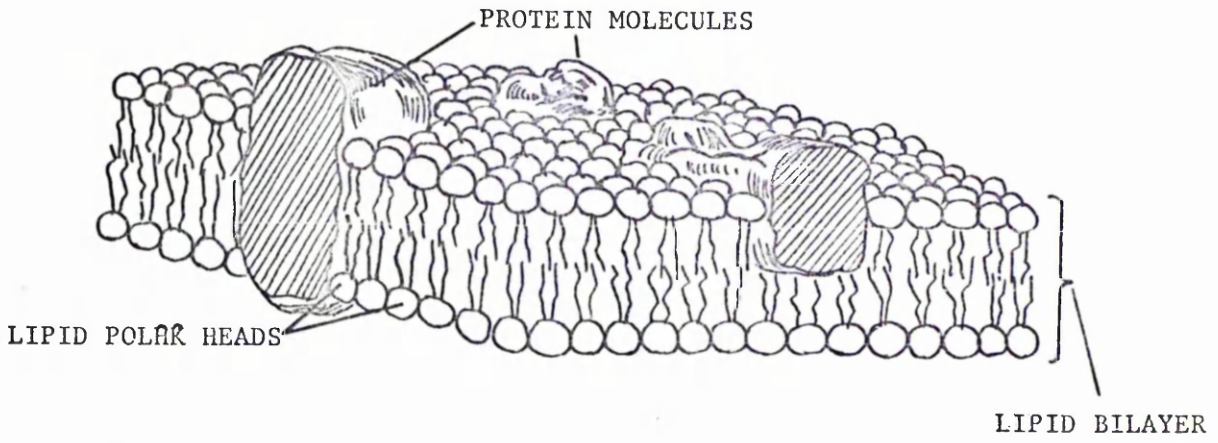
In the classical model, Danielli and Davson (1935) proposed that the protein-lipid interactions were ionic. However, there is evidence for hydrophobic protein-lipid interactions between the aliphatic amino acids of the hydrophobic portion of the proteins and the non-polar fatty acids of the phospholipids. It was visualized that the hydrophobic part of the proteins was on the inside of the membrane. The polar groups of the phospholipids, the ionogenic amino acids and the sialic acid residues were in contact with the aqueous phase (Wallach and Zahler, 1966; Lenard and Singer, 1966). Hence it was proposed by Lenard and Singer (1966) that the membrane consisted of inner and outer clusters of phospholipids interspaced by hydrophilic protein, and an intermediate layer of hydrophobic proteins and lipids. This membrane model is represented in Figure 8.

The outer coat, carbohydrate in composition, had a filamentous appearance in electron micrographs (Ito, 1965; Revel and Ito, 1967;

Figure 8: Proposed Models for Mammalian Cell Membranes



OUTSIDE



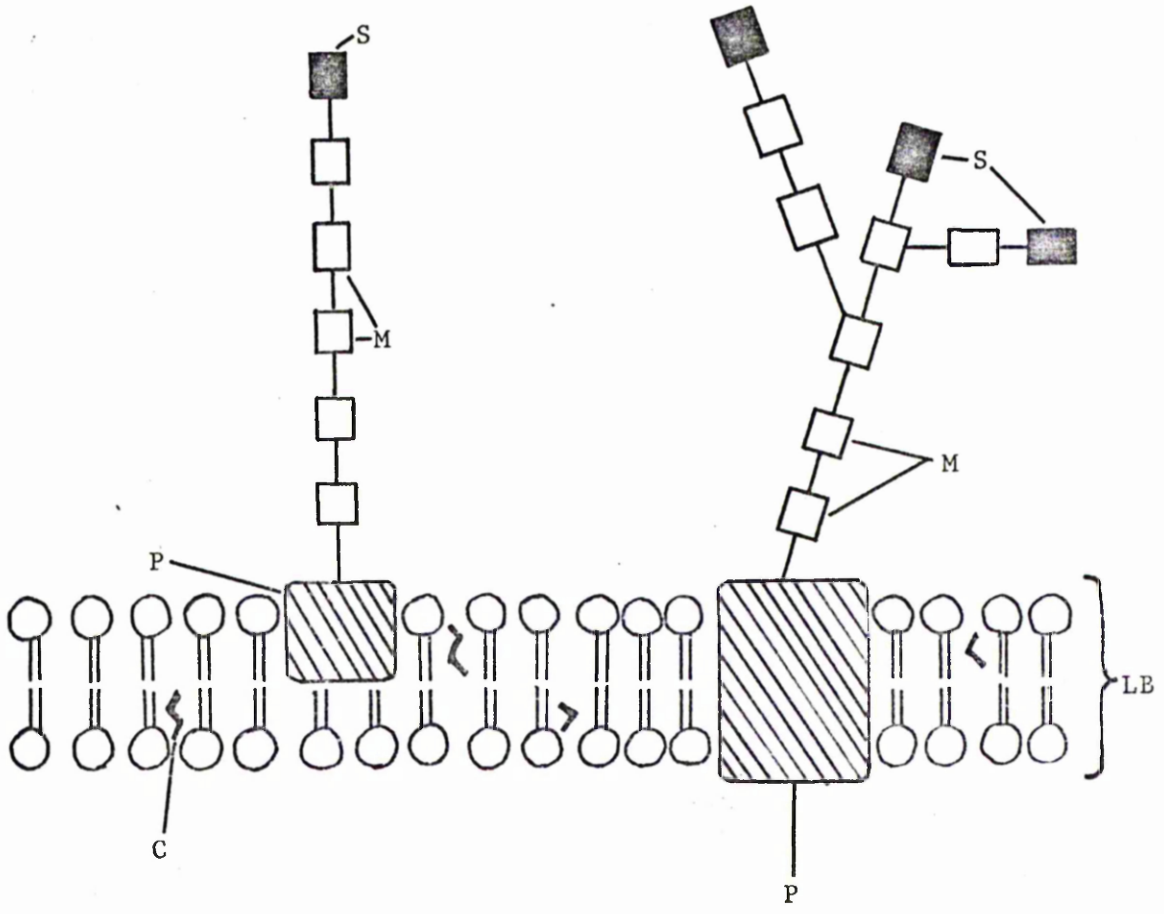
INSIDE

Models presented by Singer and Nicholson (1972)

Thiery, 1967). Granules 200Å apart were also visible on the outside of the membrane. This outer coat consisted of sialic acid rich glycoproteins, which are defined as conjugated proteins containing as prosthetic groups one or more heterosaccharide repeating units. These are bound covalently to a polypeptide chain (Gottschalk, 1966; Simkin, 1970; Spiro, 1970). Also present were mucopolysaccharides, linear molecules composed of small repeating units, in which hexosamines and hexuronic acid were arranged in an alternating manner (Figure 9). The link with the protein varied, ionic for hyaluronic acid and chiefly covalent (O-glycosidic) for chondroitin sulphates (Brimacombe and Weber, 1964). The mucopolysaccharides differed from the glycoproteins in that the latter tend to have a branched configuration due to the attachment of one or more carbohydrate side chains to the protein backbone.

In the glycoproteins the side chains consist of two to fifteen monosaccharide units containing two to six different types of sugar residues depending on the cell type. The hexosamines, glucosamine and galactosamine, were present in the N-acetyl form, and were usually attached to the protein by O-glycosydic linkages involving seryl and threonyl residues or by glycosidic ester bonds involving glutamic and aspartic acid residues. The sialic acids are in the N-acetyl, N-glycolyl and sometimes O substituted forms. The sialic acid residues were present as N-acetyl neuraminic acid (NANA), which was usually found at the end of the side chains together with L-fucose. The other monosaccharide units were the neutral sugars, galactose, mannose and fucose, which appeared throughout the chains where mannose was considered as the branching points (Rambourg, 1971).

Figure 9: Diagrammatic Representation of Cell Membrane and Surface of a Mammalian Cell



The monosaccharide structure on the left of the model represents a ganglioside molecule; the structure on the right a glycoprotein.

Symbols

S : Sialic acid residues

M : monosaccharide residues e.g. glucose, mannose

P : protein molecule

LB : lipid bilayer

C : cholesterol

Data from Bara et al., (1973)

The sialic acid content of plasma membranes was found to be higher than other cell membranes, and it was considered that this had functional significance. Sialic acid residues have been associated with receptor sites for viruses (Cohen, 1963) and serotonin (Woolley and Gommi, 1964), potassium transport (Glick and Githens, 1965) and protein release from cells (Glick, Goldberg and Pardee, 1966). They were also found to contribute to the net negative surface charge of the cells (Cook, Heard and Seaman, 1961; Ambrose, 1965). Hence the sialic acid of the glycoproteins was related generally to cell permeability. These cell coat glycoproteins also formed part of the antigenic mosaic of the cell concerned.

The Erythrocyte Cell Surface

The structure of the erythrocyte plasma membrane was shown to be compatible with the bileaflet models so far proposed (Finean, Coleman, Green and Limbrick, 1966). The lipid content varied in erythrocytes depending on the diet and pathological conditions. Sardet, Hansma and Ostwald (1972) showed that guinea-pigs fed cholesterol had an increased cholesterol content in their erythrocyte membranes which led to an altered morphology. Other workers found lipid changes in erythrocyte membranes, in rabbits fed cholesterol (Westerman, Wiggans and Mao, 1972) and rats fed orotic acid (McBride and Jacob, 1970). Similarly, in human beings with a variety of pathological conditions, changes in erythrocyte morphology occurred (Cooper, 1970; Cooper and Jandl, 1968; Nordenand Gjone, 1968).

A wide variety of lipids are found in erythrocyte membranes,

and the proportions vary according to the erythrocyte species (Hanahan, 1969). The relation that these lipids have to proteins also varies depending on the erythrocyte species. The relation of lipid to protein was variable between erythrocytes and other cell types (Korn, 1966).

If human erythrocytes were treated with trypsin, sialoglycopeptides were released (M.W. > 31,000) containing 78% carbohydrate with the MN specificity (Ohkuma and Ikemoto, 1966; Winzler, Harris, Pekas, Johnson and Weber, 1966; Thomas and Winzler, 1969). Some of the polysaccharides were linked to the peptide via an O-glycosidic link between N-acetyl galactosamine and the hydroxyl groups of serine and threonine (Winzler, Harris, Pekas, Johnson and Weber, 1966; Kathan and Adamy, 1967). Winzler (1970) showed that the amino acids of the glycopeptide were more hydrophilic than the total glycoprotein. The latter was attached to the membrane via a peptide segment with a high content of apolar amino acids. The antigenicity of these glycopeptides was attributed to their polysaccharide moiety (Cook and Eylar, 1964, 1965) although free amino groups, most probably lysine ε-NH_2 groups, were indispensable for the blood group activity of MN glycopeptides (Lisowska and Morawiecki, 1967).

Not all the antigenic determinants are exposed on the erythrocyte surface, since they are found to be distributed in the deeper layers of the membrane and on different membrane proteins. Tucker and Ellory (1970) found that erythrocytes retained blood group L and M activity after treatment with chymotrypsin, papain and neuraminidase, indicating that these substances were not superficial protein, peptide or sialic acid linked molecules. As well as the sialoglycoproteins there were also

proteins exposed to the environment capable of binding mono- and di-saccharides, amino acids and inorganic ions, and transporting them across the membrane (Heppel, 1969; Kalckar, 1971; Lin, 1970). The binding sites varied for the different classes of substances and Zimmer, Lacko and Gunther (1972) noted that there was variation for different monosaccharides and amino acids, hence glucose bound to a different protein moiety in the membrane than sorbose. Some of the surface polypeptides were shared amongst various cell types. There were seven polypeptides common to rat liver and kidney and three of these were also found in the erythrocyte membrane (Neville and Glossman, 1971).

THE SURFACES OF CELLS INVOLVED IN THE PRODUCTION OF AN IMMUNE RESPONSE

The immune response involves the plasma membrane, first as a carrier of specific groups of surface topography which distinguishes between (i) "self" and "non-self", (ii) one tissue from another tissue, (iii) one stage of differentiation from another and (iv) one particular area on the cell surface from another area (Wallach, 1973). Secondly, the plasma membrane of immuno-competent cells must also be able to elicit a response to the appropriate stimuli so that cell activation occurs. Thirdly, the plasma membrane must act as the target for killer cells in cell-mediated cytotoxicity.

The clonal selection theory of Burnet (1959) postulated that immunocompetent cells were predestined to respond to a limited set of antigens. This response was determined by specificity of the antigen receptors on the plasma membrane (Burnet, 1970; Jerne, 1971; Siskind and Benacerraf, 1969). It was implied that these antigen-receptors

were antibody-like structures. Considerable evidence has been obtained showing that predestined lymphocytes exist and have immunoglobulin-like structures on their plasma membranes.

It was reported that lymphocytes from non-immune animals were able to bind radioactively-labelled antigen at low levels (Naor and Sultzinau, 1967; Byrt and Ada, 1969; Davie and Paul, 1971; Davie, Rosenthal and Paul, 1971). If lymphocytes from non-immune animals were treated with I^{121}-antigen, these cells lost the capability of transferring immune responsiveness to isogeneic irradiated recipients, presumably due to specific irradiative killing of the antigen-binding cells (Davie, Rosenthal and Paul, 1971). Similar observations were made after passing the lymphocytes through columns containing glass beads bearing specific antigen (Wigzell and Anderson, 1971). These receptor-bearing cells were highly specific for haptens (Davie and Paul, 1971) and immunoglobulin receptors were mostly of the $gamma_2$-heavy chain type (Taylor, Duffus, Raff and De Petris, 1971). However it was reported that at one stage, at least, in the immune response more than one type of receptor was present. During the early phase of a primary response in rabbits to sheep red cells, Bona, Trebiciavsky, Anteunis, Heuclin and Robineaux (1972) showed two classes of immunoglobulin were present on the surface of the same cell. Sell (1967) found that 25% of transformed rabbit lymphocytes carried immunoglobulins belonging to at least two classes. Bona et al., (1972) also reported that between one and five days of the immune response, significant numbers of cells carrying two classes of immunoglobulin were present and that in non-immune animals, the antigen reactive cell carried one immunoglobulin class on the plasma membrane.

The distribution of the immunoglobulin receptors on the surface tended to confirm the idea that the plasma membrane of mammalian cells was fluid in nature and not static. The receptors were apparently scattered randomly over the membrane of resting lymphocytes, but if these cells were treated with anti-immunoglobulin antisera polar distribution was induced, after which the immunoglobulin molecules were "interiorized" (Taylor, Duffus, Raff and De Petris, 1971). This process was temperature dependent and required energy. At $0^{\circ}C$ a ring-like formation was produced, and at $37^{\circ}C$ a "cap" was formed. "Ring-cells" from $0^{\circ}C$ will change into "cap cells" if they are placed at $37^{\circ}C$.

Besides the specialized functions, the plasma membranes of immuno-competent cells still function as classical membranes previously described. The bursal and thymic lymphocytes carried polypeptides on their surface which were unique to each cell type (Jankovic, Isakovic, Petrovic and Vujic, 1970; Toben and St. Pierre, 1972). The mature thymic lymphocytes carried the θ antigen (Raff, Sternberg and Taylor, 1970), while the immunoglobulin was the marker for mature bursal lymphocytes (Raff, Sternberg and Taylor, 1970). Ragland, Pace and Doak (1973) isolated 32 separate proteins from bursal lymphocyte membranes, 31 from thymic lymphocyte membranes and 30 from circulating lymphocyte membranes. The immature lymphocytes (bursal and thymic) contained two proteins not expressed in mature cells. The bursal lymphocyte membranes had four unique proteins, two of which were expressed in circulating lymphocyte membranes. Similarly, the thymic lymphocyte membranes had three unique proteins, one of which was expressed in circulating lymphocyte membranes. The circulating lymphocyte membranes possessed one unique protein.

The integrity of cell surface glycoproteins was essential for the maintenance of normal lymphocytes. If the surface of these cells was treated with glycosidases, glycoproteins were removed and the lymphocytes were eliminated from circulation by reticuloendothelial cells (Gesner and Ginsberg, 1964). It was suggested that the sugars of the glycoprotein on the lymphocyte cell surface allowed them to traverse the barriers and follow their unique route throughout the body. The sugars, acted as sites recognized by complementary structures on the surface of reticuloendothelial cells in the postcapillary venules of the lymphoid tissue which prevented their destruction.

From the work on mammalian cell membranes it can be concluded that the membrane is a "mobile, fluid" structure and not a static, rigid construction as was originally supposed. Investigative work on membranes should take into account the fact that the surface of the mammalian cell is constantly changing so that the different receptor sites vary both in number and position on the cell surface.

III CELLULAR IMMUNOLOGY AND ADJUVANTS

When a foreign antigenic substance is introduced into the body either a specific immune response is elicited, or depending on the signal received by the lymphoid tissue, a state of tolerance is induced. The specific immune response results in the production of antibodies (humoral response) and/or the development of cell-mediated immunity (CMI) (cellular response).

The humoral response results in the increased production of certain classes of specific immunoglobulins reacting with the antigen forming immune complexes, which are eliminated from the body. After initial contact with antigen, there is an initiation lag period before the production of low levels of antibody which rapidly decline. This is termed a primary response which stimulates a specific form of memory, so that on repeated exposure to the antigen, a secondary response is elicited. This secondary response has no lag period, and higher levels of antibody are produced which persist for a longer period of time.

Cellular immunity is mediated by specifically stimulated small lymphocytes and is dependent on the presence of the thymus at birth. This type of immunity is responsible for reactions such as allograft rejection and delayed-type hypersensitivity (DTH).

When an antigen is introduced into the body, it is rapidly disseminated through the blood and lymphatic systems, and is localized in the lymphoid tissues. The most important tissues here are the lymph nodes and the spleen. In the former, the antigen is either taken up by the medullary macrophages, where it is sequestered in lysosomal inclusions and rapidly degraded, or it is retained extracellularly on or between the opposing cell membranes of the dendritic cells of the lymphoid follicles (White, 1963). In the spleen there is also extracellular association of the antigen but the role of the macrophage is less prominent (Nossal and Ada, 1971).

Upon stimulation the lymphoid tissues become hyperplastic, and the lymphocytes tend to cluster around the macrophages and dendritic cells, with plasma cells becoming conspicuous. It is believed that the plasma cells are responsible for the production of antibodies.

MECHANISMS FOR THE PRODUCTION OF IMMUNE RESPONSES

Involvement of Specific Cells

It is recognised that lymphocytes play an important role in both humoral and cell-mediated immunity. Lymphocytes are divided into three classes, small, medium and large and it is the former which are dominant in immune responses. There are two distinct populations of small lymphocytes. The minor population arise as stem cells in the bone marrow, divide and give rise to lymphocytes. Upon appropriate antigenic stimulation these differentiate into antibody-producing cells. This sub-population is found in the medulla of the lymph nodes and spleen, and is produced independently of the thymus (Humphrey and White, 1967). These cells came from the Bursa of Fabricius or its mammalian equivalent and were termed B cells or Thymus independent cells (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969). The major population arise as stem cells in the bone marrow, travel to the thymus where they differentiate into small lymphocytes (thymocytes) and then migrate to the peripheral lymphoid centres. These cells are stimulated to replicate on contact with an antigen. Since their development is under the control of the thymus they are termed T cells or Thymus dependent cells (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969; Miller and Osoba, 1967; Gowans and McGregor, 1965). The properties of T and B cells are summarized in Table 2, and T cells and B cells migrate to the appropriate zone (Parrott and De Sousa, 1971).

It was found that in birds (Cooper, Peterson, South and Good, 1966) and rodents (Miller and Osoba, 1967), that if the thymus was

TABLE 2: PROPERTIES OF T AND B LYMPHOCYTES* (taken partially from Katz and Benacerraf, 1973)

Property	B Lymphocytes	T Lymphocytes	Key to References
1. Morphology	Small cells 6 - 8 μ diameter	Large cells 8 - 10 μ diameter, more dense	(1) Sprent and Basten (1973)
2. Generation Time	Short-lived, average life span 5-7 weeks in mouse (1)	Long-lived, average life span 4 - 6 months in mouse (1)	(2) Martin and Miller (1968)
3. Effect of Anti-lymphocyte antiserum (ALS)	Only slight effect.	Preferentially depleted (a)	(3) Andersson, Möller and Sjöberg (1972)
4. Response to mitogens	Stimulated to proliferate by PWM. No response with ConA, PHA and lentil, although those compounds are bound by the cells.	Stimulated by PWM, ConA, PHA, Lentil (3)	(4) Claman and Chaperon (1969)
5. Functional sensitivity to Xirradiation and corticosteroids	Xirradiation sensitive (4). Cortisone sensitive only when located in peripheral lymphoid tissue, resistant in bone marrow (5)	Xirradiation resistant w.r.t. helper activity (6) cytotoxic activity (7), DTH (8). Cortisone sensitivity - distinguishes two populations. A sensitive population (90-95%) found in thymic cortex, and a resistant population (5-10%) found in medulla (9)	(5) Levine and Claman (1970) (6) Katz, Paul, Goidl and Benacerraf (1971) (7) Moller and Moller (1965) (8) Asherson and Loewi (1967) (9) Ishidate and Metcalf (1963)
6. Surface antigenic markers	Mouse B lymphocyte antigen (MBLA) (10)	θ-antigen (11)	(10) Raff, Nase and Mitchison (1971)
7. Surface immunoglobulin (I_G) determinants.	High density of immunoglobulin (10^5 molecules/cell) (12)	Low density (10^3 mols/cell) (12)	(11) Raff and Wortis (1970)
8. Surface receptors	Specific antigen receptors (13) C_3 receptor (14) Receptors for immune complexes (15)	Specific antigen receptors (16) No C_3 receptors (14) No receptors for immune complexes (15)	(12) Unanue, Grey, Rabellino, Campbell and Schmidtke (1971) (13) Naor and Sulitzeanu (1967) (14) Nussenzweig, Bianco, Dukor and Eden (1972) (15) Basten, Sprent, Mandel, Miller and Pye (1972)
9. Peripheral Localization (as % of lymphocytes) (a) Blood (b) Thoracic duct (c) Lymph Node (d) Spleen	 10% or less (11) 15-20% (11) 25% (11) 60-65% (11)	 90% or more (11) 80-85% (11) 75% (11) 35-40% (11)	(16) Roelants and Askonas (1971)
10. Response to Antigen	Differentiate into antibody producing cells; usually require T-cell influence. Not specifically involved in CMI reactions. Establish immunological memory and be rendered specifically tolerant.	Recognize and bind antigen; undergo mitotic proliferation; exact regulatory influence on B-cells. Do not synthesize antibody. Specifically involved in CMI reaction. Establish specific memory and can be rendered tolerant.	(17) Paul (1970)
11. Specificity	Hapten specific (17)	Carrier specific (17)	

* Figures in brackets refer to references

removed neonatally cell mediated immunity was impaired, while the effect on humoral immunity was not so marked. In birds, if the Bursa of Fabricius was removed, humoral activity was diminished, whereas cell-mediated reactions were not impaired (Cooper, Peterson, South and Good, 1966). As yet the mammalian equivalent of the Bursa has not been identified, but there is evidence to suggest that in rodents, the lymphocytes are produced in haematopoietic tissue. This tissue is the liver in the embryo and the bone marrow in the adult. Everett and Caffrey (1966) suggested that it was possible that these were the sites where stem cells differentiated into B cells. Also, most thymocytes leaving the thymus are immunoincompetent, suggesting that they are influenced prior to T cell function. This influence could be under the control of thymic humoral factors or hormones, e.g. thymosin (Stutman, Yunis and Good, 1969). It appears that there are T cell subpopulations within the thymus and the peripheral lymphoid tissues (Raff, 1975).

T cells play an important role in cell mediated immune reactions (Davies, 1969; Claman and Chaperon, 1969) while the B cells are important in humoral immunity, being transformed specifically into antibody-producing cells on contact with an antigen (Miller and Mitchell, 1969; Davies, 1969). Besides the T and B lymphocytes, macrophages are important in antigen trapping and processing. Macrophages are defined as "cells of the reticuloendothelial system (RES), characterized by their capacity to phagocytose and retain foreign particulate and colloidal particles." (Herbert and Wilkinson, 1971). They adhere readily to glass surfaces and are important in non-specific immunity.

An optimum immune response to an antigen requires the interaction of at least three cell types, the T and B lymphocytes and the macrophages. The latter interact with a multiplicity of antigens, catabolizing and destroying some of them, and functioning as a helper cell in the inductive process by presentation of some of the antigen to the immunocompetent cells (Unanue, 1972). The helper activity of macrophages is related to (a) their capacity to remove extracellular antigen which might interact with and eliminate isolated T and B cells, (b) their ability to retain antigen in lymphoid tissues, and (c) the promotion of antigenic interaction with the small lymphocytes (Unanue, 1972). There is a relationship between the degree of uptake of an antigen by the macrophages and the immunogenicity of that antigen. Hence, erythrocytes and bacteria which are readily taken up by macrophages evoke a strong immune response, while soluble proteins and polysaccharides show the opposite reaction (Dresser, 1962; Spiegelberg and Weigle, 1967; Golub and Weigle, 1969). The type of macrophage is important to a well balanced immune response. Hence if injections are given intraperitoneally the response is lower than if the antigen had been administered intravenously. This is thought to be due to the large concentration of peritoneal macrophages (Perkins and Makinodan, 1964; Perkins, 1970) which completely catabolize the antigen. The same is true of lymphoid macrophages but they are anatomically in a better location to interact with the lymphocytes.

When antigen is administered to an animal, 80 - 90% of the injected material is taken up by the liver and spleen cells, with the majority being fixed by cells of the RES in the liver (Benacerraf,

Biozzi, Halpern, Stiffel and Mouton, 1957; Benacerraf, 1964). The remainder of the antigenic material may be taken up by free macrophages. There are two recognized stages in antigen uptake: first the attachment of the antigen to the cell surface, and second the endocytosis of the fixed material. Once inside the cells, the pinocytic and phagocytic vesicles containing the antigen fuse with lysosomes and the antigen is enzymatically degraded. The initial antigen uptake by the macrophage depends on the ability of the antigen to react directly with the macrophage membrane, or indirectly as an immune complex formed with macrophage-cytophilic antibody produced by lymphocytes (Philips-Quagliata, Levine and Uhr, 1969; Philips-Quagliata, Levine, Quagliata and Uhr, 1971). Some bacteria do not bind directly to macrophages, e.g. pneumococci, some salmonella (Jenkin and Benacerraf, 1960) and staphylococci (Mackaness, 1960). The macrophage cytophilic antibody belongs to different classes depending on the animal species, e.g. in guinea-pigs the $gamma_2$-immunoglobulin is macrophage cytophilic (Berken and Benacerraf, 1966). It appears that molecules of the antigen persist for some time on the macrophage surface (Unanue and Cerottini, 1970).

The antigen tends to localize in lymphoid tissue, and this localization was greater if associated with macrophages (Nossal and Ada, 1971). The antigen localizes either in the medullary macrophages or in the lymphoid follicles. The latter has a web of interdigitating cell processes from lymphocytes, at various stages of differentiation, and non-lymphocytes (dendritic macrophages, reticulum and dendritic cells) (White, 1963). The follicles also contained the B cells which have C^1_3 and immune complex receptors on their surfaces (Bianco, Patrick and

Nussenzweig, 1970). The antigen is maintained between closely packed cells on the membranes of the specialized dendritic type cells (Mitchell and Abbott, 1965; Nossal, Abbot and Mitchell, 1968; Nossal, Abbot, Mitchell and Lummus, 1968) where it persists for a prolonged period. Frequently lymphocytes and plasma cells have been observed clustering around the macrophages in perifollicular areas (Thiery, 1962; Miller and Avrameas, 1971) without apparent cytoplasmic fusion (McIntyre, Niblack, Prater and Lavia, 1971). However, it was reported that macrophage-associated antigen interacted with both T and B cells (Unanue and Feldman, 1971) but there was a preferential attachment to B cells due to interaction between the Ig receptor on B lymphocytes with the Fc cytophilic receptor on macrophages (Schmidtke and Unanue, 1971). The macrophage could transfer antigenic information to the immunocompetent cells (Yokomuro, Mabuchi and Kimura, 1972) and it was reported that LPS from Salmonella was transferred from the macrophages to the lymphocyte in order to elicit a response (Bona, Robineaux, Anteunis, Heuclin and Astesano, 1973).

Effect of Antigenic Stimulation on Immunocompetent Cells

During the induction of Graft-versus-host (GVH) reactions (Pederson and Morris, 1970) and during the sensitization of normal guinea-pigs to chemical allergens (Oort and Turk, 1965; Turk, 1967) a morphological transformation of small lymphocytes to large pyroninophilic or blast cells was observed. This process of antigenic stimulation transformed the lymphocytes both morphologically and functionally (Naspitz and Richter, 1968). In the resting lymph node, the proportion

of cells was 80% small lymphocytes and 20% large lymphocytes, but after antigenic stimulation the plasma cell count increased to between 3 and 20% of the total (Davis, Dulbecco, Eisen, Ginsberg and Wood, 1968).

The changes observed during lymphocyte transformation are complex and were summarized by Bloom (1971). Transformation of lymphocytes occurred not only by antigenic stimulation but also by the action of other agents (Naspitz and Richter, 1968) e.g. Phytohaemagglutinin (PHA), Conconavalin A (ConA), Streptolysins, antilymphocyte sera (ALS), anti-immunoglobulin sera and pokeweed mitogen (PWM). PHA and ConA bind to specific polysaccharide receptors on the cell membrane. Novogrodsky and Katchlaski (1971) showed that chemical modification of the lymphocyte membrane by sodium periodate also triggered cellular transformation.

Both in vitro and in vivo it was observed that antigenic stimulation caused an asynchronous development of transformation. This could be explained by the inability of the antigen to interact directly with the lymphocyte membrane, and that some form of antigen processing was required. Cline and Swett (1968) together with Hersh and Harris (1968) showed that the removal of macrophages and monocytes from lymphocyte cultures abolished the ability of PPD to cause transformation. The contact of antigen with the lymphocytes in vivo, initially stimulated small numbers of cells. However, once stimulated they are divided by clonal proliferation with each daughter cell dividing with constant generation and doubling times of about eight and thirteen hours (Marshall, Valentine and Lawrence, 1969).

Mention should also be made of the effect of injected antigen on lymphocyte migration patterns. Ford (1972) showed that rat spleens

stimulated by tetanus toxoid initiated a secondary response which had two effects on circulating lymphocytes as they migrated through the splenic pulp. The antigen-sensitive cells (ASC) were selected from the population of immune cells, and were retained in the spleen, and although transient, this was at the expense of the lymph nodes.

Cellular Interactions in the Immune Response

Initial models of immune responses based on histological evidence of the localization of injected antigens led to the conclusion that at least two types of cells were required for a balanced immune response. This developed into the concept of cooperation between macrophages (which digested and processed the antigen) and a cell which responded to this modified antigen by producing antibodies (Fishman, 1961; Fishman and Adler, 1963, 1967; Gallily and Feldman, 1967). This cellular interaction was demonstrated for a wide range of antigens.

Miller (1961, 1962) using neonatally thymectomized mice was able to show that interactions of T and B lymphocytes were required to elicit an immune response, when a thymus graft, partially restored the response. It would seem that both T and B cells were necessary, but Osoba and Miller (1963) were able to demonstrate the partial restoration of immune responses in neonatally thymectomized mice when the thymus graft was placed in a Millipore chamber which indicated a possible role for a soluble factor(s) produced by the thymus. Also, Doria, Agarossi and Di Pietro (1971) found that spleen cell cultures from neonatally thymectomized mice required the addition of thymocytes in order to produce a measurable immune response. This cellular cooperation was prevented

if the spleen cells were treated with antiserum to mouse serum proteins. If the thymus cells were treated, there was no inhibition of cooperation which seemed to indicate that the T-cells lacked receptors present on B-cell membranes.

The antigen-sensitive cells (ASC) are committed to the expression of a unique antibody and respond to the antigen either by differentiation and proliferation to antibody-forming cells (AFC) or they are inactivated, in that they are no longer inducible (i.e. immunologically tolerant or paralysed). The interaction of the antigen (or its processed determinants) with the membrane of the ASC is common to both pathways. Hence, T-cell influence over B-cells was necessary to complete the signal to B-cells so that induction of antibody synthesis took place at antigen concentrations that would normally induce tolerance. Grumet (1970) further showed that T-cells enhanced the B-cell response of all antibody classes especially IgG and that without such influence the switch of antibody production from IgM to IgG failed to occur. Hence, this means that it is possible that T-cells also control antibody levels by means of suppressive effects on the AFC. This suppressive role could also be played by the actual antibodies formed either by competing with the lymphocyte receptors for the antigenic determinants, or by forming antibody-antigen complexes which are tolerogenic to AFC. It is also possible that antibodies could form complexes with antigens which are highly immunogenic and this could in part explain some aspects of the secondary immune response.

Induction or tolerance could be dependent on the signals received by the lymphocyte immunoglobulin (Ig) receptors and consequently

how these receptors behave. Evidence from Sela and Mozes (1966) showed that the electrostatic charge of surface Ig receptors appeared to attract antigens of different types. Also the valency of the actual receptor was vital, so that multivalent receptors of the IgM type were more efficient at "concentrating" antigen than divalent IgG receptors. Also the movement and endocytosis of these receptors (see page 15), was not sufficient to trigger primed lymphocytes in the absence of antigenic stimuli. However, after treatment with Ig antiserum the efficiency of the antibody response of these lymphocytes was significantly increased on antigenic stimulation (Katz and Unanue, 1972).

The actual mode of action of T-cells on B-cells is still uncertain. Various suggestions were made that the T-cells transported and presented the antigen to the B-cells (Mitchison, 1969). The latter idea was termed "antigen focussing", and indicated a passive role for the T-cells. However, Miller, Sprent, Basten, Warner, Breitner, Rowland, Hamilton, Silver and Martin (1971) and Katz, Paul and Benacerraf (1970) have evidence to the contrary. This activity of T-cells is possibly a minor function, as it would appear to be a wasteful duplication of macrophage function.

It was further suggested that genetic transfer occurred during cooperation. However, there is little evidence for this, with numerous antagonistic reports (Potter and Lieberman, 1967; Mitchison, 1971). It should be noted however that Rosenstreich, Shevach, Green and Rosenthal (1972) observed that in "resting" animals approximately 40% of peritoneal lymphocytes formed uropods whereas thymocytes and lymph node cells formed a few. After antigenic stimulation the incidence of uropod formation

increased, so that there was an association between antigen reactivity and uropod formation. It also appeared that the majority of uropod-bearing cells were thymus derived. It was assumed that uropod-formation was also linked to direct cytotoxicity of small lymphocytes. Mention should be made of work by Yokomuro and Nozima (1972) who demonstrated cytoplasmic bridges between macrophage and macrophage and "filamentous" bridges between macrophages and lymph node cells.

The other influence of T-cells currently gaining support, is through the action of soluble mediator(s), which react rapidly, with a short half-life. There are three main sources of evidence to support this concept. The first comes from non-specific stimulation of primed lymphocytes by mitogens in absence of known further antigenic stimuli (Tao, 1964; Mäkelä and Pasanen, 1969; Katz and Unanue, 1972). Secondly, evidence for mediators was obtained using in vitro culture techniques. Supernatant fluid from T-cell cultures (Gorczynski, Miller and Philips, 1972) and peritoneal lymphocyte cultures (Kennedy, Treadwell and Lennox, 1970) were able to partially restore the immune response in neonatally thymoctomized mice. This restoration was antigen specific but not ~~but not~~ species specific. Thirdly, graft-versus-host (GVH) reactions were found to affect several parameters of the immune response (Katz, Davie, Paul and Benacerraf, 1971; Katz, Goidl, Paul and Benacerraf, 1971; Katz, Paul and Benacerraf, 1971) and this was termed the "allogeneic" effect. Katz and his colleagues found that GVH reactions increased antibody synthesis without further antigenic stimulation, and that the intensity of this response was related to the intensity of the GVH reaction. Also, if the correct time interval was observed upon secondary antigenic stimulation an enhanced secondary response was observed. They

also found that hapten specific memory, a property of B-cells, was enhanced and that they were able to elicit an antibody response to antigens for which it was presumed that no T-cells existed if the antigen was administered during the GVH reaction. This work was performed in guinea-pigs, but Hirst and Dutton (1970) obtained similar results with mice. During a GVH reaction, there is specific immunological attack by the grafted cells on host cells which may cause proliferation of host T-cells so that sufficient numbers are available at the time of secondary challenge, or the GVH reaction may exert some influence on AFC-precursors of host origin and alter their response. It is thought that the second explanation is the most feasible and that soluble mediator(s) are produced during the course of a GVH reaction.

It should be mentioned that with respect to T/B cell interactions in immune responses, there is a class of antigens which are able to stimulate immune responses in the absence of T-cell influence. These antigens are thymus-independent antigens, and possess this property due to their unique tertiary structure. These antigens are polymerized flagellin (Armstrong, Diener and Shellam, 1969), Pneumococcal polysaccharide (Humphrey, Parrott and East, 1964), E. coli endotoxin and polyvinyl pyrrolidone (Andersson and Blomgren, 1971) and various viruses (Basch, 1966). They stimulate predominantly IgM antibody which is thought to represent the limited response that B-cells can achieve in the absence of T-cells. In this type of system, T-cells could depress the response (Armstrong, Diener and Shellam, 1969).

Cell-Mediated Immunity (CMI)

During the development of an immune response, the B-cells on

appropriate antigenic stimulation differentiate and proliferate to form clones of AFC. The T-cells involved in such a reaction also undergo blast transformation to form colonies of specifically sensitized small lymphocytes, which, when stimulated further, mediate cellular immunity either directly, or through the production of a series of soluble factors (Lymphokines). These sensitized lymphocytes represent the body's line of defence against viral infections and some bacterial attacks e.g. M. tuberculosis. However, under the appropriate conditions a cellular state of over-reactivity to injected antigens can be achieved, i.e. delayed hypersensitivity. This hypersensitive state could be achieved using adjuvants and Coon and Hunter (1973) using bovine serum albumin in guinea-pigs related the production of delayed hypersensitivity to the ability of the adjuvant to localize the antigen in thymus-dependent areas.

Another manifestation of CMI is the Graft-versus-Host (GVH) reactions where sensitized small lymphocytes in the graft react to cells of host origin. Cantor and Asofsky (1972) showed that there was T/T cell interaction. One of the T-cells involved was of a non-circulating type and determined the specificity of the response. This was termed the 'effector cell', and the other T-cell is a recirculating type which amplifies this response and is generally known as the 'amplifier cell'.

It is assumed that during CMI responses T-cells have the assistance of macrophages, presumably through the action of secreted lymphokines. Macrophages were the dominant cells in all these reactions (Lubaroff and Waksman, 1968) although T-cells also responded by directly killing target cells (Raff, 1969; Bloom, 1971). Dennert and Lennox (1972) further suggested a major role of the T-cell in CMI reactions was

to trigger the B-cell to produce target cell specific antibody. This antibody attached to the target cells which were subsequently attacked by macrophages.

Soluble Factors Produced as Immune Response Mediators

Apart from antibodies, the main battery of soluble factors are those produced by sensitized lymphocytes upon secondary antigenic stimulation. These factors were collectively called "lymphokines" by Dumonde, Wolstencroft, Panayi, Matthew, Morely and Howson (1969). There were at least eleven different activities assigned to the lymphokines, but whether these represent eleven different activities is not known. These are summarized in Appendix IX.

ADJUVANTS AND THE IMMUNE RESPONSE

General Account

The extent of an antibody response elicited by an antigen is dependent on several factors. The molecular weight of the antigen is important, together with the dose level and the number of injections. Antigens that are easily digested are not so immunogenic as substances that remain in the body for a long time (Wilson and Miles, 1957). Another important factor is the presence or absence of an adjuvant in the injection mixture. In 1967, White described an adjuvant as a substance able to (a) convert apparently non-antigenic material into an effective antigen, (b) increase the levels of circulating antibody, (c) lead to the production of delayed hypersensitivity or its increase,

and (d) lead to the production of certain disease states. These four criteria may or may not apply to biological adjuvants, but chemical adjuvants do not show all of these effects. The adjuvant response elicited by a particular substance can be affected by the amount and type of antigen (Weigle, Dixon and Deichmiller, 1960; Shaw, Alvord, Fahlberg and Kies, 1962), the route of administration (Lipton and Freund, 1953), the number of injections (Miles and Pirie, 1939; Farthing and Holt, 1962), the animal species (Rice, 1947) and the animal's age (Miles and Pirie, 1939).

The general mode of action of adjuvants has been reviewed by numerous workers (Munoz, 1964; Neter, 1971). These included the effect on the antigenic stimulus, either by its slow release from a depot, or its protection from destruction and rapid excretion, thus improving its distribution through the body. The antigenic processing can be affected in the presence of adjuvants by mobilization of operative cells or by facilitation of the interaction of antigens with cell membranes. Further, antibody-producing cells could be stimulated to proliferate with an alteration in their protein synthesis. This represents a general effect on control mechanisms in the body. Finally, the adjuvant could affect cells not involved in the immune process, so that breakdown products are released which affect immunocompetent cells. These are general mechanisms, and probably do not apply to all adjuvants.

In 1935 Ramon and Falchetti suggested that the adjuvant activity of oil emulsions and tapioca was due, in part, to their ability to cause an inflammatory response. The slow release of antigen from the injection site was attributed to the action of alum (Holford, Ludden and

Stevens, 1943; White, Coons and Connolly, 1955a) and water in oil emulsions (White, Coons and Connolly, 1955b) as a means of increasing the immunogenicity of the antigen. Similarly, Freund (1956) decided that the adjuvant made the antigen more effective by protecting it from destruction and improving its distribution throughout the body so providing a more prolonged antigenic stimulus to the immunocompetent cells.

There have been numerous reports implying that adjuvants exerted a beneficial effect on the antibody-forming cells (AFC). White, Coons and Connolly (1955a, 1955b) showed that Freund's adjuvant and alum stimulated the AFC. There was a redistribution of precursor and helper cells with an influx of macrophages into the injection site. Kessel and Braun (1965) showed that adjuvants also affected macrophages by causing changes in the activity and level of intracellular enzymes. Spitznagel and Allison (1970) using mice, found that if the antigen was injected together with macrophages, the resulting immune response was greater than the response elicited by antigen alone. Similarly, Munder and his colleagues (Munder, Ferber, Modolell and Fischer, 1969; Munder, Modolell, Ferber and Fischer, 1970) found that all substances tested, except endotoxin, caused changes in macrophages metabolism with the resulting formation of lysophosphatides. From this work Munder and Modolell (1973) postulated and proved that if the endogenous formation of lysophosphatides was the lowest common denominator in the mediation of an enhanced response, then lysolecithin should act as an adjuvant.

In 1972 Maillard and Bloom found a soluble factor, produced

by adjuvant-primed T-cells, which enhanced a B-cell response. It is possible that adjuvants release substances from cells which affect the immune response, and this was suggested by Jaroslow and Taliaferro (1956). There have been numerous reports that nucleic acid breakdown products (oligodeoxyribonucleotides) released from cells due to the "toxic" activity of adjuvants enhanced the antibody response. Freedman and Braun (1965) suggested that oligodeoxyribonucleotides stimulated phagocytic activity of the RES, and Johnson and Johnson (1968) indicated that they might affect antigen processing by macrophage. Braun and Nakano (1965) found that these nucleic acid fragments increased the number of AFC in mice spleens, but to be effective as an adjuvant by themselves, they had to be injected concurrently with the antigen. Campbell and Kind (1971) found that polynucleotides enhanced the number of plaque-forming cells (PFC) and appeared to act by increasing mitoses in bone marrow cells and the number of bone marrow derived cells that interacted with T-cells. Hence, it seemed that polynucleotides have B-cells as targets, a hypothesis verified by Jaroslow and Ortiz-Ortiz (1972).

It should be mentioned that stimulated and sensitized T-cells produced a range of soluble factors, one of which was interferon. Braun and Levy (1972) were able to get increased numbers of spleen AFC using interferon preparations and postulated that this action could involve cyclic-AMP.

It is probable that adjuvants act through a variety of mechanisms, and different adjuvants have different modes of action. From Table 3 , which contains a list of substances possessing adjuvant activity, it is apparent that the compounds are so diverse that the chance

TABLE 3: A SUMMARY OF SUBSTANCES POSSESSING ADJUVANT ACTIVITY

I Chemical adjuvants

Alum (Glenny, Pope, Waddington and Wallace, 1926)
Aluminium hydroxide (Hektoen and Welker, 1933)
Lanolin in oil, cholesterol, Mg and Ca salts (Ramon, Lemetayer and Richou, 1935)
Tapioca (Ramon, 1937; Schmidt and Steenberg, 1936)
Phosphorylated hesperidin (Moss, Beiler and Martin, 1956)
Calcium alginate (Amies, 1959)
Polyvinylphrrolidone (PVP) (Amies, 1962)
Vitamin A (Dresser, 1961)
Silica (Vigliani and Pernis, 1959; Antweiler, 1959)
Carbon, tetrachloride-induced cell destruction (Heuer, Pernis and Braun, 1962)
Pentosan Sulphate (Diamanstein, Stork and Malchús, 1973)

II Miscellaneous Methods and Agents

Serum containing heterophile antibody
Diffusion chambers in peritoneal cavity (Adler and Fishman, 1962)
Antigen-antibody complexes made in the region of antigen excess (Terres and Wolins, 1961; Terres and Stoner, 1962)
Removal of adrenal gland (Murphy and Sturm, 1947; Char and Kelley, 1962)
Damage or removal of liver (Havens, 1959)
Interferon preparations (Braun and Levy, 1972)
Mycobacterial RNA (Gumbiner, Paterson, Youmans and Youmans, 1973)
Synthetic polynucleotides (Campbell and Kind, 1971; Gumbiner, Paterson, Youmans and Youmans, 1973)
Streptococcal pyrogenic exotoxin (Hanna and Watson, 1973)
Abscess formation (Ramon, 1926)
Staphylococcal toxin (Burky, 1934; Schultz and Swift, 1934)
Cl. botulinum A toxoid adjuvant for Cl. botulinum B toxoid (Rice, 1947)
Lipids from Listeria monocytogenes (Jakonivk, Borowski, Szpak and Jarzyna, 1973)

III Oil in Water Emulsions (Freund's Adjuvant)

Oil in water emulsions initially contained Mycobacterium tuberculosis (Freund, Casals and Hosmer, 1937; Freund and McDermott, 1942; Freund and Bonanto, 1946)
Lipid extracts replace whole cells (Freund, Thompson, Hough, Sommer and Pisani, 1948; White, Coons and Connolly, 1955a; White, 1959)
Freund's adjuvant shown to be active with a wide variety of organisms:
M. butyricum M. tuberculosis (human, bovine and avian strains), M. phlei.
M. smegmatis (Freund, 1947; White, 1959)
M. leprae (Stewart-Tull and Davies, 1972)
C. parvum
Nocardia asteroides (Freund and Lipton, 1948)
Ps. pseudomallei (Steigman and Lipton, 1960)
S. typhosa (Kabat, 1957)
S. typhimurium (Katsh, 1959)
Coccidioides immitis (Kabat, 1957)
Streptococcus sp. (Campbell, 1962)
C. rubrum E. coli Ps. aeruginosa (Shaw, Alvord, Fahlberg and Kies, 1962)

IV Lipopolysaccharide Adjuvants

S. enteritidis (Tarmina, Milner, Ribi and Rudbach, 1968)
S. abortus equi (Luecke and Sibal, 1962)
N. meningitidis (Condie, Zak and Good, 1955)
S. typhi, E. coli, Ps. aeruginosa, Pr. vulgaris, Br. Melitensis (Johnson, Gaines and Landy, 1956)
Bd. pertussis, E. coli (Farthing and Holt, 1962)
Serratia marcescens (Margherita and Friedman, 1965)

of a common mode of action is slight, unless that mechanism lies at a membrane or intracellular level.

Mycobacterial Fractions and the Immune Response

Lewis and Loomis (1925) together with Dienes (1927, 1928) observed that higher antibody titres were obtained in tuberculous guinea-pigs, as opposed to normal animals. Several years later Freund and his colleagues conducted a series of experiments in which they raised high titre antisera by administering the antigen in a water-in-oil emulsion containing killed Mycobacterial whole cells (Freund, Casals and Hosmer, 1937; Freund and McDermott, 1942; Freund and Bonanto, 1946; Freund, Thompson, Hough, Sommer and Pisani, 1948); delayed-type hypersensitivity was also stimulated by this method. Freund (1956) found that to be effective a water-in-oil emulsion was required so that the antigen was in the aqueous phase, surrounded by an oil droplet; oil-in-water emulsion was ineffective. If the water-in-oil emulsion was used without added Mycobacterial cells it was termed Freund Incomplete Adjuvant (FIA). The addition of Mycobacteria to the emulsion produced a more effective adjuvant and was termed Freund Complete Adjuvant (FCA). For Freund adjuvant to be effective it must be administered concurrently with the antigen. Recently, Finger, Emmerling, Hof and Plager (1973) found that injection of the emulsion prior to the antigenic stimulation caused a significant suppression of the primary response both at the humoral and cellular level. This suppression, it was suggested, was due to a deficiency of the antigen at the site of the immunocompetent cells, caused by competition between the oil droplets and antigen for available

macrophages. Similarly it was found that the antibody levels produced in response to an antigen administered in Freund Complete Adjuvant was depressed if the antigen had previously been given without Freund Adjuvant. This phenomenon was termed immune deviation, and was thought to be due to suppressive effects caused by antibody.

The sensitization produced depended on the antigen and adjuvant being administered at the same site (Freund, 1956), and consisted of an alteration in the hypersensitive response from the immediate to the delayed-type (Couland, 1935; Saenz, 1937; Freund, 1956). Bloom (1970) stated that the production of cell-mediated immunity towards the antigen was critical for an adjuvant effect. In the guinea-pig, the administration of an antigen in Freund Complete Adjuvant caused an increase in the γ_2-immunoglobulin antibody titre (Boyden, 1964), and this increase correlated with a state of delayed hypersensitivity in the animal.

It was considered that Freund complete Adjuvant was the most powerful known, in the stimulation of antibody responses in animals generally considered to be poor antibody producers (Anacker and Munoz, 1961). Also Benedict and Tips (1954) found that both FCA and FIA were effective in producing anaphylactic sensitization in animals considered resistant to anaphylaxis.

White, Coons and Connolly (1955) and White and Marshall (1958) found that during an adjuvant response, lymphocytes, macrophages, plasma cells and epitheloid cells proliferated at the injection site and in the regional and distal lymph nodes. It should be mentioned that paraffin oil attracts to the antigen such cells as monocytes, lymphocytes and

plasma cells so that local proliferation tended to occur. Henle and Henle (1945) found that this latter phenomenon was dependent on the viscosity of the oil; high viscosity oils tended to favour local cellular reactions. Steiner, Langer and Schatz (1960) reported that these local cellular reactions could be produced by Mycobacterial fractions, but with the addition of oil these were enhanced. Janicki, Aron, Schechter and McFarland (1972) tested a variety of tuberculoproteins and polysaccharides from M. tuberculosis for their ability to induce lymphocyte blastogenesis. They found that the tuberculoproteins induced a high degree of blastogenic activity, but the polysaccharides were completely inactive.

As early as 1948, Freund found that lipid extracts of Mycobacterial cells could replace the whole cells in FCA (Freund, Thompson, Hough, Sommer and Pisiani, 1948). Since then other workers have verified this finding (Freund and Stone, 1959; White, 1959; White, Coons and Connolly, 1955). In 1949, Raffel, Arnaud, Dukes and Huang found that the Wax D fraction of Mycobacterial cells, either alone or in water-in-oil emulsion was capable of inducing delayed hypersensitivity to ovalbumin. Contact sensitivity to picryl chloride could also be induced by this method (Raffel and Forney, 1948). Although Freund and Stone (1959) found that Wax D was less active in a purified form than when it was associated with the cell, White, Coons and Connolly (1955) together with Freund (1956) demonstrated that Wax D caused a cellular reaction that was indistinguishable from that caused by whole cells. Also Tanaka, Ishibashi, Sugiyama and Takamoto (1971) stated that FCA was a disadvantage for weak antigens or small amounts of antigen, due to antigenic competition from the Mycobacteria and have prepared an

acetylated form of the active Mycobacterial component, which possessed adjuvancy but was non-antigenic and produced no inflammation. The competing antigenicity was thought to be present in the polysaccharide moiety. However, White in 1967 reported that biologically active material became completely inactive once acetylated. The activity was restored by careful de-acetylation of the inactive material.

In 1964, White, Jolles, Samour and Lederer showed that the active fraction contained a peptide composed of D- and L-alanine, D-glutamic acid and meso-DAP. The active fraction was a peptidoglycolipid containing mycolic acid and corresponding closely in amino acid, amino-sugar and hexose content to the mucopeptide of bacterial cell walls. In 1959, Lederer had found that delayed hypersensitivity could be induced to by a variety of mycolic acid esters of carbohydrates, or even a mycolate of a hexose, although these were not effective in the production of isoallergic diseases or antibody formation. The major part of the adjuvant work has been done with Wax D, but some workers are now measuring adjuvant activity with other fractions from Mycobacteria, although the structural relationship of these fractions to Wax D has not been determined. Pound and O'Rourke (1971) have produced enhanced antibody response to sheep red cells using a lipid in a saline emulsion. This bacillary lipid corresponded to the "hard wax" fraction of Anderson (1927). It should be noted that White, Coons and Connolly (1955b) found that Wax D in a saline emulsion was inactive.

Migliore-Samour and Jolles (1973) obtained a wide range of low molecular weight hydrosoluble fractions which possess adjuvant activity when incorporated into FIA. Hiu (1972) isolated a lipid free water-

soluble fraction that stimulated antibody production and induced delayed hypersensitivity in saline solution.

Although the mode of action of FCA has been extensively reported no one single mechanism can be cited as the true role of FCA in an adjuvant response. The oil contributes to the activity by acting as an antigen depot so that the antigen is slowly released, thus providing a prolonged stimulus to the immunocompetent cells (Halbert, Mudd and Smolens, 1946; White, Coons and Connolly, 1955b; Freund, 1956; Holub, 1957). The cellular response at the injection site (granuloma formation) was increased by the addition of Mycobacteria (Fischel, Kabat, Stoerk and Bezer, 1952). It should be noted that granulomas were found at other sites, distant from the injection site (Fischel, Kabat, Stoerk and Bezer, 1952; Freund, 1956). In 1963, Bendixen concluded that the oil served only as a solvent for the tubercle wax fraction, although various workers have since shown that antigen distribution throughout the body was affected by the oil so that there was a decreased rate of drainage of the antigen from the injection site (McDevitt, Askonas, Humphrey, Schechter and Sela, 1966).

The FCA also affected the antibody-forming cell precursors with an increase in the numbers, which possessed enhanced antibody-producing capacity (Koga, Ishibashi, Sugiyama and Tanaka, 1969). This stimulatory effect was observed in the regional and distal lymph nodes and in the spleen (Steiner, Langer and Schatz, 1960). Fischel, Kabat, Stoerk and Bezer (1952) correlated increase in antibody production not only with hyperplasia in the lymphoid tissue but also with the granuloma formation. The granuloma formation could result in "local" antibody

production, and several workers showed that antibody-antigen reactions, at the time of the antigenic stimulus to the immunocompetent cells of lymph nodes and spleen increase antibody formation (Good, Condie, Thompson and Jensen, 1957; Terres and Wolins, 1961; Terres and Stoner, 1962).

Cellular damage resulting from the use of FCA was proposed as an important step in the production of an adjuvant response. Jones and Roitt (1961) thought that the cellular damage made antigens more accessible to the AFC. Weissman (1964) proposed that lysosomal enzymes released during this damage "dissolved or lysed cells." During this lytic process, oligodeoxyribonucleotides could be released.

An effect on the reticuloendothelial system (RES) was observed. Besides the migration of macrophages into the injection site, Unanue, Askonas and Allison (1969) found the antibody response was better depending on the association of the adjuvant with the macrophages containing the antigen. However, Askonas and Humphrey (1958) had previously shown that the presence of the adjuvant did not affect the catabolism or retention of the antigen by the macrophage. Vacher, Deraedt and Benzoni (1973) observed that changes in the activity of the RES affected the development of adjuvant-induced polyarthritis.

A further effect established by Coates and Lennon (1973) was that in the presence of FCA during the production of an immune response to myelin, a greater proportion of the splenic lymphocytes were found to be binding antigen. This binding capacity was related to the γ_2-antibody in the guinea-pig. Boyden (1964) found that the formation of

cytophilic antibodies required the mandatory use of FCA. In 1966, Berken and Benacerraf demonstrated that the cytophilic antibody was the γ_2-globulin.

Hence the mechanisms involved in the production of both humoral and delayed hypersensitivity responses are numerous. It would seem that these responses depend on a combination of effects concerned with antigen processing and stimulation of the appropriate immunocompetent cells.

Lipopolysaccharides (Endotoxins) and the Immune Response

The effects of endotoxins on the immune response are as complex as those of mycobacterial fractions. It was observed by Condie, Zak and Good (1955) that large amounts of endotoxin acted as an immunosuppressant; this property was in direct contrast to their ability to act as immunological adjuvants. Although the two properties may be closely related only their ability to stimulate an increased immune response will be discussed.

As far back as 1926 Ramon and Zoeller studied the adjuvant activity of endotoxins. Later, MacLean, Edin and Holt (1940) found that up to five times more tetanus antitoxin could be produced in human beings, if the injection of tetanus toxoid was given together with heat-killed Salmonella typhi and paratyphi. Johnson, Gaines and Landy (1956) identified the lipopolysaccharide (LPS) as the active fraction in stimulated antibody production by S. typhi, E. coli, Ps. aeruginosa and Pr. vulgaris. Besides increasing antibody levels upon injection with

antigen, LPS was found to increase antibody levels in the absence of a known antigenic stimulus (Michael, 1966). LPS also induced increased anaphylactic sensitivity in animals that were originally thought to be relatively insensitive (Malkiel and Hargis, 1959; Munoz, 1963). Similarly, LPS was able to break immunologic unresponsiveness (tolerance) to various antigens, such as pneumococcal polysaccharide, when injected into mice (Brooks, 1965). Neeper and Seastone (1963) suggested this breaking of tolerance was by a non-specific mechanism, as was the increase in antibody levels in the absence of known antigenic stimuli.

Ward, Johnson and Abell (1959) found that endotoxin potentiated all phases of the immune response from the shortening of the lag period to increasing a prolonged response. Various workers (Turowska and Turowska, 1965; Sibal, Fink, Robertson and Cowles, 1968) used LPS to produce high titred antisera. Pierce (1967) found that LPS enhanced the primary response with production of 19S antibodies. However in splenectomized animals LPS acted as an adjuvant in the primary and secondary responses, with the production of 7S antibodies. Pierce implied from this that adjuvant action was due to an interference in the feedback inhibition pathway of antibody production. Taliaferro and Talmage (1955) thought that endotoxin affected a critical early step in antibody synthesis before the actual incorporation of amino acids in the antibody, and Kind and Johnson (1959) supported this vague postulate. Although evidence points to the involvement of endotoxin in the primary response (Johnson, Gaines and Landy, 1956) reports notably by Rethy (1967) indicated that endotoxin acted as an adjuvant in both the primary and secondary immune responses.

Westphal (1957) and Kabat (1961) postulated that the lipid content of endotoxin was important for its activity, but Ribi, Haskins, Landy and Milner (1961) concluded that the endotoxic active moiety contained little or no lipid and polypeptide. Lipid A, although less toxic, enhances immunogenicity although the effect is less than that produced by the whole LPS molecule (Farthing, 1961). The adjuvant activity of lipid A from B. pertussis and E. coli LPS was further confirmed by Farthing and Holt (1962). In 1973, Peavy, Shands, Adler and Smith decided that the mitogenic effect of endotoxin on lympho-reticular cells was due to the lipid A fraction. This mitogenic activity was independent of the thymus, and it was postulated as the reason for the thymus independent nature of endotoxin as an antigen. Watson, Trenkner and Cohn (1973) decided that induction of antibody synthesis resided in the ability of the lipid A to bind to the lipid bilayer of cell membranes.

Despite the lipid A findings, Condie and Good (1956) related adjuvant action to the toxic properties of the endotoxin, since in tolerant rabbits no adjuvant activity was observed; this was verified by Johnson, Gaines and Landy (1956). However, in mice which are rather resistant to endotoxic activity, Merritt and Johnson (1962) demonstrated an adjuvant effect; this was also observed in chickens (Luecke and Sibal, 1962). Several years earlier Rauss, Ketyi and Rethy (1958) showed that if the toxicity of endotoxin was reduced by aluminium hydroxide treatment, there was an increase in adjuvancy. However Johnson and Nowotny (1964) found this increased adjuvancy was dependent on the method used to prepare the endotoxoid. Further to this, Tarmina, Milner, Ribi and

Rudbach (1968) suggested that molecular integrity of endotoxins was important for biological activity, since there was a decrease in adjuvancy if the endotoxin was treated with deoxycholate. Finally Kawakami, Hara and Osawa (1971) suggested that a basal core polysaccharide of LPS was important for some activities.

Endotoxins do not appear to act as adjuvants either by local proliferation of cells at the injection site, or by a depot effect, since the injection of endotoxins does not have to be concurrent with that of the antigen (Luecke and Sibal, 1962; Freedman, Fox and Schwartz, 1967). However the distribution and retention of the antigen could be influenced by the haemodynamic changes caused by the endotoxin (Condie, Zak and Good, 1955). Cohen, Crosby and Talmage (1964) suggested that the non-specific ability of endotoxins to break tolerance, exerted an influence in adjuvancy by making available more responsive cells to the antigen. As antibodies are produced the antigen was eliminated more efficiently from the body, and Solliday, Rowley and Fitch (1967) thought that the endotoxin overcame this immunosuppressive effect of antibodies.

As with FCA, hyperplasia in the lymphoid tissue was observed (Farthing and Holt, 1962) and Freedman, Nakano and Braun (1966) believed that this hyperplasia was due to an increase in the number of AFC, an important factor in adjuvancy. A similar effect was observed with thymic lymphocytes when Rowlands, Claman and Kind (1965) and Kind, Campbell and Rowlands (1967) observed an apparent loss of thymic lymphocytes, coupled with an increase in the number of pyroninophilic cells. From recent work (Jones and Kind, 1972; Schmitdke and Dixon,

1972; Watson, Epstein, Nakoinz and Ralph, 1973) LPS appeared to stimulate DNA synthesis and cell division of B-cells (even in the absence of T-cells) and it was suggested that this was related to adjuvant action. It was suggested that the mode of action of endotoxin was independent of the thymus, provided that the latter was not removed neonatally (Campbell, Kind and Rowlands, 1965; Campbell, Rowlands, Harrington and Kind, 1966). Even in T-cell culture, endotoxin was unable to stimulate DNA synthesis (Andersson, Möller and Sjöberg, 1972). Andersson, Sjöberg and Möller (1972) noticed that endotoxin stimulated all B-cells in the culture. In 1973 Watson, Trenkner and Cohn considered that B-cells required two signals for induction, the first being provided by the antigenic determinant and the second by the cooperating cell system. In the absence of T-cells, LPS was able to supply the second signal by directly interacting with the cell membrane of the B-cells. In an adjuvant response, LPS could still initiate this second signal so that induction is imposed on all possible cells including those that might remain unresponsive if the antigen was administered alone.

Other approaches to the adjuvant mechanisms have been proposed. Nakano, Uhiyama and Saito (1973) found that LPS affected both the thymus-derived antigen-reactive cells (ARC) and the bone marrow derived antigen-sensitive cells (ASC) by actually facilitating cooperation between these cells. In 1963, Talmage and Pearlman postulated that complement might act as an inhibitor of cell division, which led Pearlman, Sauers and Talmage (1963) to put forward the theory that the fixation inactivation of complement by LPS could be important in adjuvancy. It

was shown that oligonucleotides acted as adjuvants in the immune response and this led Jaroslow (1960), Merritt and Johnson (1965) and Plescia and Braun (1968) to advance the theory that endotoxin adjuvants caused cellular damage similar to FCA with the subsequent release of nucleic acid breakdown products.

The action of endotoxins followed a series of complex reactions, the most apparent concerned with the reticuloendothelial system (RES). Biozzi, Benacerraf and Halpern (1955) and Rubenstein, Fine and Coons (1962) suggested that this might be responsible for some adjuvant activity. Jenkin and Palmer (1960) found that endotoxin increased the phagocytic activity of macrophages, and Feldman (1972) showed that LPS was bound to cells of the accessory cell system, especially macrophages. It was possible that the LPS induced these cells to release humoral factors which interacted with the B-cells and completed the second signal for induction. Such factors were produced by T-cells (Dutton, Falkoff, Hirst, Hoffmann, Kappler, Kettman, Lesley and Venn, 1971; Gorczynski, Miller and Philips, 1973).

Permeability changes produced by endotoxins (Rose, 1959; Munoz, 1961) were thought to be involved in adjuvancy together with changes in the adrenal glands, liver damage resulting in the release of enzymes (Havens, 1959; Weissman and Thomas, 1962), increase in blood lysozyme levels (Hook, Carey and Muschel, 1960) and an increase in the permeability of blood-brain barriers (Eckman, King and Brunson, 1958). The latter was suggested as the cause of the isoallergic disease of experimental allergic encephalomyelitis, coupled with the fact that tissue antigens became modified after linkage to LPS (Davies, Gery,

Rosenmann and Laufer, 1963).

Some of the terms used in this section are defined and explained in Appendix X.

IV THE AFFINITY OF BACTERIAL COMPONENTS FOR MAMMALIAN CELL SURFACES

Since 1899 numerous workers have reported the adherence of bacteria or their antigens to mammalian erythrocytes (Madsen, 1899; Ginsberg, Goebel and Horsfall, 1948; Keogh, North and Warburton, 1948; Middlebrook and Dubos, 1948; Boyden, 1951; Neter, 1956). This phenomenon, termed modification by Neter (1956), altered the serological specificity of the erythrocytes (Springer and Horton, 1964), and in general the modifying antigens were polysaccharides or possessed a polysaccharide serological determinant (Neter, 1956). The antigen which adhered to the erythrocytes was referred to by different workers as "erythrocyte-modifying antigen" (Neter, Zalewski and Zak, 1953), "haemosensitin" (Sorkin and Boyden, 1955), "erythrocyte coating antigen" (Rountree and Barbour, 1952).

(Rountree and Barbour, 1952) antigens which coated mammalian cells, included the O-somatic antigens or lipopolysaccharides, the acidic polymers (Vi antigens) and the common (Kunin) antigens of the Gram-negative bacteria (Ginsberg et al., 1948; Keogh et al., 1948; Neter, 1956; Neter, Bertram, Zak, Murdock and Arbesman, 1952). Mycobacterial polysaccharides (Middlebrook and Dubos, 1948; Boyden and Graber, 1954) and various antigens from Gram-positive bacteria, including the teichoic acid-related antigens (Rantz, Randall and Zuckerman, 1956) also coated

erythrocytes. Besides these bacterial antigens, there were others from Rickettsiae (Chang, 1953), Candida (Vogel and Collins, 1955), trypanosomes (Munoz, 1950), schistosomes (Kagan, 1955), Histoplasma (Norden, 1949) and Trichomonas (McEntegart, 1952) with the ability to modify mammalian cells. It is still uncertain whether the various lipopolysaccharides and diverse antigens, attach to the same or different membrane receptors.

The in vivo modification of cells was reported by Skillman, Spurrier, Friedman and Schwartz (1955). They showed that the erythrocytes of the patient were modified by Streptococcal antigen in cases of active rheumatic fever. Also it was suggested that in the presence of specific antibody and complement, whole bacteria attached to red cells prior to engulfment by polymorphs (Robineaux and Nelson, 1955).

Apart from isolated reports on the attachment of various antigenic components to the mammalian cell surfaces the bulk of the literature is concerned with cell modification by bacterial lipopolysaccharide substances.

AFFINITY OF LIPOPOLYSACCHARIDES FOR MAMMALIAN CELL MEMBRANES

The lipopolysaccharide (LPS) of Gram-negative bacteria was found to be a complex in the outer or L-membrane of the organism, composed of polysaccharide, lipid and protein (Luderitz, Staub and Westphal, 1966; Burge and Draper, 1967; De Petris, 1967). After extraction and purification LPS resembled morphologically membrane

fragments of varying shape, including discs, lamellae, ribbons and vesicles (Beer, Braude and Brinton, 1966; Work, Knox and Vesk, 1966; Burge and Draper, 1967; Shands, Graham and Nath, 1967). These LPS fragments were visualized in aqueous solution as a bilayer, each half was covalently linked, with its non-polar hydrophobic lipid buried inside the structure with the polysaccharide moiety exposed to the environment (Shands, 1973). These LPS membranous structures had an affinity for mammalian cell membranes, especially after heat or alkali treatment (Lüderitz, Westphal, Sievers, Kröger, Neter and Braun, 1958; Cižnar and Shands, 1971). This affinity was not confined to erythrocytes as shown in Table 4. The affinity for erythrocytes of different species, was related to the antigen, for example the Vi antigen from Salmonella typhi had an equal affinity for erythrocytes from horses, sheep, rabbits, cows and goats. Likewise erythrocytes from man, dogs, rabbits, sheep, goats and rats were equally modified by Escherichia coli lipopolysaccharide (Corvazier, 1952). However there were instances where the degree of modification was a function of the erythrocyte, for example, human group O blood was more suitable for the attachment of Pasteurella tularease (Franciscella tularensis) antigen than blood from groups A and B (Alexander, Wright and Baldwin, 1950).

The modified erythrocytes were conferred with a new serological specificity, which was dependent on the antigen used, and they were rendered agglutinable by the addition of specific antibody. In the presence of specific antibody and complement, modified erythrocytes lysed and the degree of lysis was partially dependent on the type of erythrocyte used. For example, sheep erythrocytes were more readily

Table 4: Mammalian Cell Modification by Bacterial Antigens

Modifying Antigen	Cell Type or Cellular Component	References
Salmonella antigen	Spleen, liver and kidney cells, leucocytes	De Gregorio (1955)
Tuberculin polysaccharide	leucocytes, lymphocytes	Boyden (1953)
Salmonella typhimurium	Rat heart and liver mitochondria	Greer, Epps and Vail (1973)
E. coli (whole cells)	Rabbit alveston macrophages	Ulrich and Neier (1973)
S. minnesota R from glycolipid	Rat embryo fibroblasts	Bara, Lallier, Brailovsky and Nigam (1973)
E. coli LPS	Rabbit P.M.N. leucocytes	Gimber and Rafter (1969)

lysed than human erythrocytes, although both were equally modified by E. coli lipopolysaccharide (Neter, 1956). Shumway, Bokkenheuser, Pollock and Neter (1963) further suggested that it was conceivable that cellular damage might be caused with cells other than erythrocytes.

Activation of lipopolysaccharide by heat and alkali treatment: Untreated lipopolysaccharide adhered weakly to erythrocytes and for in vitro studies most workers used various treatments to increase the modifying capacity, e.g. heat and alkali treatment (Neter, Bertram and Arbesman, 1952; MacPherson, Wilkinson and Swain, 1953; Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956). Generally the latter increased the modifying capacity to a greater extent, although both were considered to produce the same qualitative effect on the lipopolysaccharide (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956).

Whole bacterial cells were heated at 37°C for several days at pH 10, which released the somatic antigen from the cells and increased the modifying activity (Neter, Gorzynski, Zalewski, Rachman and Gino, 1954). Similarly, extracted LPS was activated by heating at 100°C for 1 - 3 hours with the degree of activation dependent on the LPS used (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956). For example, two Salmonella lipopolysaccharides had optimum modifying capacities at 1000 µg/ml and 6 µg/ml whereas after heat activation the optimum modifying capacities were 1.2 µg/ml and 1.5 µg/ml respectively (Neter, Westphal, Lüderitz and Gorzynski, 1956). It was thought that heating affected either the lipid moiety of the LPS (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956) or that region of the polysaccharide not involved in the antigen:antibody reaction since heated

Shigella dysenteriae LPS had enhanced cellular binding capacity without altered antibody neutralization activity (Neter and Gorzynski, 1954). This effect was reported for other lipopolysaccharides (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956). It was also found that a large portion of the lipid of S. typhi LPS was removed by heating, together with components that interfered with its uptake by cells.

Treatment with 0.25N sodium hydroxide at 56°C for 6 - 60 min (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956), produced the same changes in the LPS as heating, but more efficiently (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956). Neter, Westphal, Lüderitz, Gorzynski and Eichenberger (1956) reported that there was no difference in the cell modifying capacities of E. coli 08 and S. abortus equi lipopolysaccharides treated for 6 or 60 min. They reported that with respect to cell modifying capacity, antibody neutralization, toxicity and pyrogenicity, heating in 0.25N NaOH for 6 min, produced maximum activities compared with heating for 60 min when the pyrogenicity and toxicity of both the lipopolysaccharides was severely reduced (Table 5). Contrariwise, Davies, Crumpton, MacPherson and Hutchison (1958) showed that differences existed in the extent of cell modifying capacity of LPS if different incubation times were used.

The reported increases in modifying capacity after alkali activation were variable, from 2 - 20 fold (Cižnar and Shands, 1971) to a massive 4,000 fold increase (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956) with lipopolysaccharides from E. coli. Quantitatively, 1×10^9 erythrocytes fixed 2 µg of untreated LPS and 30 - 40 µg of alkali activated LPS, the latter representing an average of 10^5 molecules of

Table 5: Summary of the Effects of Heat, NaOH and Periodate on Various Activities of Lipopolysaccharides

Lipopolysaccharide	Treatment	Cell-Modifying Capacity	Antibody Neutralization	Toxicity	Pyrogenicity
E.coli 08 S.abortus equi	None	±	+++	+++	+++
S.abortus equi	100°C, 205 hr (pH 7.2)	+++	+++	+++	+++
E.coli 08 S.abortus equi	NaOH 56°C/6 min	+++	+++	+++	+++
E. coli 08	NaOH 56°C/60 min	+++	+++	++	++
S.abortus equi	NaOH 56°C/60 min	+++	+++	+	+
E.coli 08	Periodate	-	±	+	+++

- to +++ = various degrees of activity

Data from : Neter, Westphal, Lüderitz, Gorzynski and Eichenberger (1956)

LPS per cell (Lüderitz, Westphal, Sievers, Kröger, Neter and Braun, 1958). Although there was this apparent increase in modifying capacity after alkali treatment, Lüderitz, Westphal, Sievers, Kröger, Neter and Braun (1958) found that only 30% of the activated molecules actually had a strong affinity for membranes, the remainder possessed slight affinity even after repeated adsorptions with fresh erythrocytes.

Erythrocytes, modified with alkali-activated LPS, tended to lyse spontaneously. The extent of the haemolysis was dependent on the number of molecules fixed per cell (Cižnar and Shands, 1971). During the lytic process, the shape of the erythrocyte changed from the classical biconcave structure to a more spherical form. Untreated LPS produced little or no haemolysis (Cižnar and Shands, 1971).

In solution, untreated LPS was strongly opalescent and highly aggregated, having a molecular weight of $10 - 20 \times 10^6$. After alkali or heat treatment the solution became clear as a result of irreversible disaggregation of the molecules, which now had an average molecular weight of 2×10^5 (Neter et al., 1956; Lüderitz et al., 1958). This reduction in size was thought to be accompanied by a change in configuration brought about by the action of alkali or heat on the LPS lipids. The alkali was found to cleave ester-linked fatty acids, the amide-linked acids being resistant (Tripodis and Nowotny, 1966). With the lipopolysaccharides from *E. coli* and *S. abortus equi* alkali treatment resulted in lipid degradation with the release of fatty acids and ninhydrin positive material (Neter et al., 1956). However, lipidic groups still remained in the reaction product.

The actual role of the lipid is complex since S. typhi LPS contained approximately 30% phospholipid, most of which was removed by alkali activation (Landy, Trapani and Clark, 1955), and P. pestis LPS contained 50% phospholipid (Davies, 1956) and readily adsorbed to membranes without alkali treatment (Crumpton, Davies and Hutchison, 1958). Aerobacter aerogenes LPS contained no phospholipid but required alkali treatment to activate adherence to membranes (Wilkinson, Dudman and Aspinall, 1955). These findings are inconclusive, but it would seem that the lipids play an important role in activation, since degraded lipid-free polysaccharides, even after heat or alkali activation, failed to modify erythrocytes (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956).

Finally, the involvement of O-acetate and O-acetyl groups has been suggested from data obtained from infra-red spectrophotometry. The spectra revealed that there were no differences between untreated and alkali-treated LPS with the exception of O-acetyl groups; the N-acetyl groups were not affected (Davies et al., 1958). It should also be mentioned that lipopolysaccharides treated with periodate, lose cell modifying capacity. The treatment results in the oxidative cleavage of each fourth sugar unit with the oxidized polysaccharide units remaining bound to the LPS so that the molecular weight remains essentially unchanged (Neter et al., 1956).

Conditions which affect cell modifying activity of lipopolysaccharide. At the present time the conditions required for the optimal uptake of LPS by cells are difficult to define, since there are numerous contra-

dictory reports in the literature. Electrolytes are required for binding since LPS from both E. coli and S. abortus equi failed to modify erythrocytes in 5% glucose or sucrose but attached in isotonic sodium chloride, potassium chloride, sodium citrate and calcium chloride (Neter and Zalewski, 1953). LPS in phosphate buffer (pH 7.3) was far more active than LPS in normal saline (pH 6.3). This could reflect a pH effect since heat activation of LPS in phosphate buffer (pH 4.9) produced less active LPS than that produced in phosphate buffer (pH 7.3) (Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956).

Modification of erythrocytes would also appear to be temperature dependent, with modification occurring rapidly at 37°C and slowly at 4°C (Neter, 1956). Cižnar and Shands (1971) noted that the subsequent lysis of erythrocytes modified at 37°C was temperature dependent, with the optimum at 22°C.

The uptake of E. coli LPS by polymorphonuclear-leucocytes, was temperature independent and inhibited by EDTA and rabbit serum, but was unaffected by iodoacetate and sodium cyanide. It appeared to be pH independent over the range pH 5.8 to pH 8.0 (Gimber and Rafter, 1969).

Pretreatment of the erythrocyte can alter the modifying capacity of the LPS. When erythrocytes were pretreated with papain or trypsin, modification was enhanced (Skillman et al., 1955). However, pretreatment with periodate and receptor destroying enzyme (RDE) produced no such effect (Hayes, 1951; Wright and Feinberg, 1952). Trypsinization has also been reported to enhance the uptake of S. minnesota or form glycolipid by rat embryo fibroblasts (Bara, Lallier, Brailovsky and Nigam, 1973).

Receptor site on cell membranes for bacterial polysaccharide components

Receptor site on cell membranes for bacterial polysaccharide components:

The majority of the work on receptors was done by Springer and his colleagues who have isolated a fraction from the erythrocyte membrane which they have suggested is a lipopolysaccharide receptor (Pavlovski and Springer, 1957; Adye, Springer and Murthy, 1973; Springer, Adye, Bezkorovainy and Murthy, 1973; Springer, Huprikar and Neter, 1970). Observations by other workers provided evidence of the nature of the receptor. Lipopolysaccharide combined significantly with lipoproteins in the presence of alkali, and it was suggested that lipids on the erythrocyte membrane played a part in adsorption (Partridge and Morgan, 1940; Morgan, 1943). Inhibition of adsorption was found when lecithin, cephalin, cholesterol or normal rabbit serum were added to the reaction mixture. In the case of lecithin, it was noted that pretreatment of erythrocytes also caused inhibition (Neter, Westphal and Lüderitz, 1955; Neter, Zak, Zalewski and Bertram, 1952; Neter, Zalewski and Zak, 1953). These inhibitors did not affect the antibody-neutralizing capacity of the LPS.

A receptor from human erythrocytes involved in immuno-adherence haemagglutination (IAH) was released after trypsin treatment of the cells. The human erythrocytes showed a range of IAH reactions, from strongly positive where the receptors were abundant on the cell surface, to weakly positive where only small amounts of the receptor were present. This difference was genetically determined. Other inhibitory fractions were isolated from erythrocytes; an alcohol/ether extract from horse erythrocytes inhibited their modification by a filtrate of *P. mallei* (Boyden, 1950). Similarly, a glycoprotein from human

erythrocytes was inhibitory to subsequent modification by E. coli 086 LPS (Springer, Wang, Nichols and Shear, 1966). Neter has also reported that the A, B and Rh antigens were not blocked on erythrocytes modified by either E. coli or P. tularease LPS (Neter, Bertram, Zak, Murdock and Arbesman, 1952; Wright and Feinberg, 1952).

It is still not known whether there is more than one receptor on the membrane for LPS. It was found that LPS from E. coli 08, 0111 and S. abortus equi bound to the same receptor (Lüderitz, Westphal, Sievers, Kröger, Neter and Braun, 1958) and Springer reported that the "lipopolysaccharide receptor" inhibited the uptake of all LPS preparations by apparently blocking the sites on the LPS molecule that attach to the membrane. However, it was also reported that more than one type of LPS may attach to the same erythrocyte (Neter, Bertram, Zak, Murdock and Arbesman, 1952; Hayes, 1951; Landy, 1954) and that if one LPS was in excess it did not block the uptake of another LPS present in minimal quantities (Neter, Westphal, Luderitz, Gorzynski and Eichenberger, 1956).

Springer's group isolated an LPS receptor by organic solvent treatment of erythrocyte ghosts which inhibited the binding of LPS to erythrocytes (Springer et al., 1973; Adye et al., 1973). The amount of LPS required for optimal coating of erythrocytes and the amount of receptor required to give greater than 95% inhibition of uptake varied depending on the LPS used. This particular receptor also inhibited to a lesser extent cellular modification by the Kunin antigen, while vast amounts of the receptor were required to block Vi, Rantz and Streptococcal group antigens. Results from these inhibition studies generally indicate an homologous area of the membrane to which all LPS attach.

The receptors inhibited the uptake of untreated, treated and alkali-heated S and R forms of the lipopolysaccharides.

When the receptors inhibitory activity was compared to the activity of haemoglobin, gangliosides and phospholipids (Springer, Nagai and Tegtmeyer, 1966; Adye and Springer, 1968; Neter, Zalewski and Zak, 1953), it was found that haemoglobin had 10% of the receptor activity, and the gangliosides and phospholipids had less than 3% of the activity. The attachment of LPS to membranes was freely reversible (Springer, Huprikar and Neter, 1970) and the receptor was able to remove LPS already attached to the membranes, unlike the phospholipids and serum (Neter, 1956; Springer and Horton, 1964).

The receptor was heat labile and inactivated by aldehydes, which indicated protein involvement, since aldehydes form condensation products with amino acids in proteins. Furthermore inactivation was produced by protease action which indicated that proteins were involved in the receptor either as the actual determinant or as a structural protein. It would seem - for maximum activity - that the receptor relies on the protein to give it size and conformation, as in the case of the human erythrocyte MN antigens (Jirgensons and Springer, 1968). The receptor was found to be a lipoglycoprotein (MW 228,000), rich in N-acetyl neuraminic acid (NANA) and galactose. It contained 61% peptide (major amino acids were glutamic acid, aspartic acid, serine and threonine) and 10% non-covalently bound lipid. There was no indication of phospholipids in the receptor. The polysaccharide moiety contained NANA (17%), glucosamine, galactosamine, galactose, glucose, mannose and fucose. The

lipid and NANA were not involved in receptor activity, since 90% of the lipid and 99% of the NANA could be removed before activity was affected.

The linkage between LPS and cell membrane: The interaction between LPS and membrane is open to question since the published work appears to be contradictory. It was reported by Vogel (1957) and Hammerling and Westphal (1967) that lipid A was necessary for the linkage between membrane and LPS and that this interaction was hydrophobic with an intermixing of the LPS fatty acids with membrane lipids. However, Gimber and Rafter (1969) reported that the linkage was mediated by ionic bonds. For A. aerogenes LPS, lipids were not essential for adsorption which seemed to be a function of the size and configuration of the LPS (Davies, Crumpton, MacPherson and Hutchison, 1958). It is important to remember that heat and alkali activation alters the molecular weight of the LPS from 10^6 to 2×10^5 as well as the configuration. The binding was also reversible, which suggested weak bonds and not a covalent hydrophobic bond (Lüderitz, Westphal, Sievers, Kröger, Neter and Braun, 1958).

If lipids are essential for attachment to the membrane, how is this physically achieved if they are situated within a bilayered molecule? Shands (1973) proposed two possible explanations (a) edge attachment occurs and the LPS fatty acids are "solubilized" in the cell membrane, and (b) phase transitions take place in the LPS so that hydrophobic groups become exposed in the proximity of the membrane surface (Figure 10). It was found that stacking of particles (De Pamphilis,

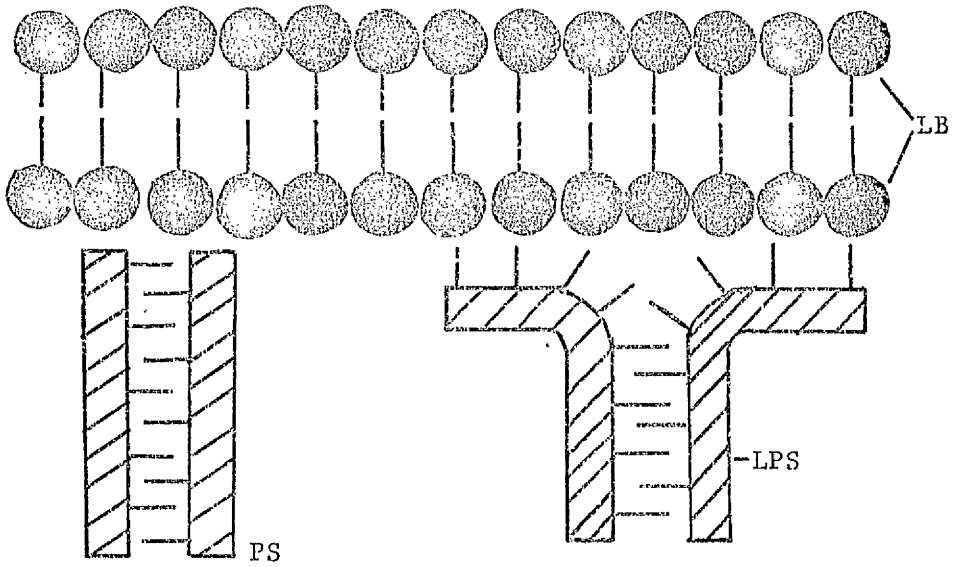
Figure 10: Possible Models for the Interaction of LPS with Mammalian Cell Membranes

Models A and B represent the possible interactions of untreated-LPS with the lipid bilayer. Model C represents the possible interaction of alkali-activated LPS. The data was obtained from Shands (1973) and Benedetto, Shands and Shah (1973).

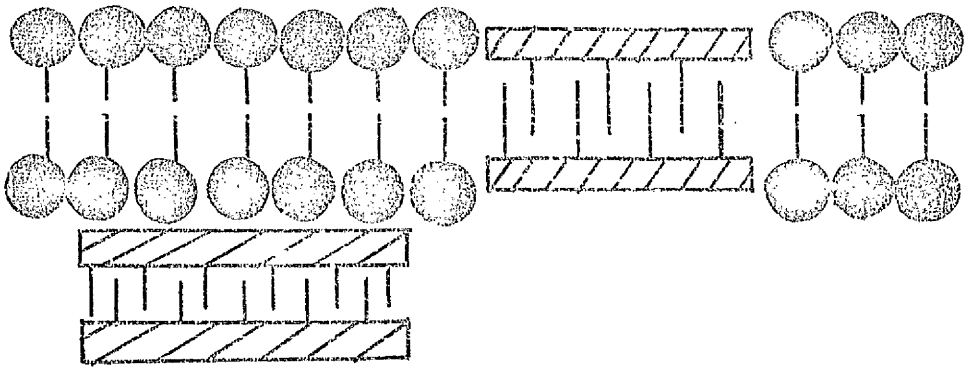
Symbols used :-

LPS	-	LPS molecule
PS	-	Polysaccharide moiety of LPS molecule
LM	-	Lipid moiety of LPS molecule
LB	-	Lipid bilayer of mammalian cell membrane
ALK-LPS	-	Alkali-activated LPS molecule

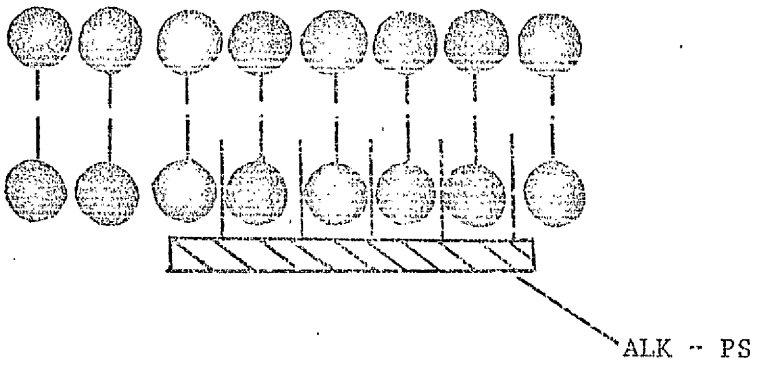
A



B



C



1971.; Schnaitman, 1971) and the morphological change from discs to vesicles (De Pamphilis, 1971) occurred under the appropriate conditions in the presence of lecithin. The linear association of the LPS particles after interaction with membrane involved metallic ions (De Pamphilis, 1971) and the change to the vesicle form could provide a mechanism for incorporation of LPS into membranes. Benedetto, Shands and Shah (1973) recently showed that LPS bound to phospholipid monolayers and decreased their stability. This was characterized by a lipid-lipid linkage between LPS lipid A and membrane lipids (Hammerling and Westphal, 1967). There also appeared to be a surface-adsorptive type of reaction dependent on ionic bonding and inhibited by a net negative charge on the membrane (Benedetto, Shands and Shah, 1973).

AFFINITY OF MYCOBACTERIAL FRACTIONS FOR MAMMALIAN CELL MEMBRANES

In 1948, Middlebrook and Dubos observed that at least one heat-stable component present in a polysaccharide fraction of the tubercle bacillus could be absorbed onto sheep erythrocytes rendering them specifically agglutinable by serum antibodies directed against the polysaccharide fraction of the tubercle bacillus. Middlebrook (1955) found that tuberculin contained two forms of the polysaccharide, one of which (40 per cent) was capable of sensitising sheep erythrocytes. The other polysaccharide was not involved in adsorption to erythrocytes. Sorkin, Boyden and Rhodes (1956) isolated a crude "alpha haemosensitin" by alcohol fractionation of heated culture filtrates of M. tuberculosis var hominis, removing the protein impurities by treatment with ammonium sulphate and TCA. Electrophoretic analyses of this crude preparation revealed that

even the most active preparations were not homogenous probably because of the presence of more than one structurally related type of polysaccharide molecule. This substance can sensitize sheep red cells to antituberculous sera resulting in an haemagglutination reaction. One of these polysaccharides composed of arabinose and mannose was an effective haemosensitin binding to erythrocyte membranes. Sorkin and Boyden (1955) found that saponification of the alpha-haemosensitin reduced the capacity for cellular modification. The material removed was mycolic acid and there was some indication that this lipid moiety of the alpha-haemosensitin was bound to the erythrocytes. Boyden (1951) showed that tannic acid treated erythrocytes adsorbed a protein fraction from tuberculin preparations.

The binding of a polysaccharide fraction from tuberculin to cell membranes was inhibited by cholesterol, lecithin and cephalin (Boyden and Grabar, 1954). These workers also showed that normal serum was not inhibitory to the uptake of mycobacterial polysaccharide. However, a year later Boyden and Andersen (1955) reported that the binding of a polysaccharide from tuberculin to cell membranes was inhibited by serum. It is interesting to recall that serum was a potent inhibitor of LPS binding to cell membranes (Springer, Wang, Nichols and Shear, 1966).

A receptor was isolated that inhibited the adsorption of the polysaccharide to the erythrocyte membrane (Boyden and Grabar, 1954). This receptor differed from the LPS receptor in that it possessed low activity, was insoluble in water, soluble in apolar solvents and heat stable. The receptor had low inhibitory activity when compared to Tween 80, cephalin and lecithin.

THE EFFECT OF BINDING ON MAMMALIAN CELLS

It has already been suggested that the binding of endotoxin (LPS) to receptors on the membranes of host tissue cells is a pre-requisite for most, if not all, of its physiological effects. Hill and Weiss (1964) stated that there was a direct relationship between the lethal effects of endotoxin in various strains of mice and the affinity that the LPS had for erythrocytes from the various strains. It was also reported that the Vi antigen (De Gregorio, 1955) and a poly-saccharide fraction from tuberculin (Boyden, 1953) when injected into guinea-pigs modified the erythrocyte only when large amounts were administered.

The binding of these bacterial components to membranes was more than a surface phenomenon, since membrane instabilities were produced indicating membrane disorganization (Cîznar and Shands, 1971). It is possible that this membrane disorganization acts as a stimulating mechanism, analogous to the effect of phytohaemagglutinin (PHA) binding to carbohydrate structures on the cell membrane and causing a mitogenic effect while still external to the cell (Greaves and Baumminger, 1972). Gimber and Rafter (1969) reported that after the endotoxin was bound to leucocytes, the LPS was partially degraded and Cooper, Cranston and Fessler (1960) showed that new pyrogenic material was released from the cells. The leucocytes had increased glucose metabolism via the hexose monophosphate pathway (Hohnadel and Stjernholm, 1966; Graham, Karnovsky, Shafer, Class and Karnovsky, 1967) and ATP hydrolysis was inhibited in intact cells (Tenney and Rafter, 1968).

With reference to the *in vitro* observation that normal rabbit serum inhibited the uptake of LPS by erythrocytes, it was found that rabbit alveolar macrophages failed to phagocytose *E. coli* in the presence of 30 - 40% serum, and that the inhibition was at the binding stage and not the ingestion stage (Ulrich and Meier, 1973).

MATERIALS AND METHODS

MATERIALS AND METHODS

(I) PREPARATION OF LIPOPOLYSACCHARIDES FROM GRAM-NEGATIVE BACTERIA

Growth of Organisms:

Starter cultures were obtained by inoculating the liquid Starter Medium (Appendix I) with the organism from nutrient agar slopes. The culture was incubated at 37°C for 24 hr. The resultant growth, as a 1% (v/v) inoculum, was used to inoculate the Davis and Mingioli minimal medium (Appendix I). This was incubated at 37°C for 7 hr in an orbital incubator (A. Gallenkamp & Co. Ltd., London) shaking at 150 rev/min. The cells were harvested by centrifugation at 2000 rpm (1300 x g) for 30 min in a MSE 6L Mistral centrifuge (Measuring & Scientific Instruments Ltd., England) and were washed three times in normal saline. The organisms grown under these conditions were Escherichia coli NCTC 8623, Salmonella typhi NCTC 0901 and Salmonella typhimurium NCTC 5710.

Extraction of LPS by the Phenol/Water Method:

LPS was extracted by the method of Westphal, Lüderitz and Bister (1952) as follows: saline-washed cells were resuspended in distilled water to a final concentration of 0.1 grams (wet weight)/ml and 10 volumes of acetone (AnalaR, BDH), precooled to 4°C, added. After standing overnight at 4°C the resulting precipitate was filtered on a Buchner funnel, and washed with a small volume of acetone. The precipitate was dried, ground to a fine powder and resuspended in distilled water to a final concentration of 0.1 gram/ml. This suspension was mixed

with an equal volume of 90% (w/v) phenol (AnalaR, BDH Ltd., Poole, Dorset), and heated at 65-68°C for 20 min. The heated material was chilled to 4°C and centrifuged at 6000 rpm (4400 x g) for 20 min and the aqueous layer removed. The residue was re-extracted by the addition of an equal volume of distilled water, heated, and the aqueous layer separated by centrifugation. The two aqueous extracts were pooled and dialysed against running water for 3 to 4 days and against distilled water for a further 24 hr. The dialysed material was concentrated to a convenient volume by the use of polyethylene glycol (carbowax 4000, BDH Ltd). The concentrated material was centrifuged at 34,000 rpm (100,000 x g) for 4 hr, the pellet was resuspended in distilled water and recentrifuged; this procedure was repeated three or four times until all nucleic acid contamination was removed, as measured by U.V. spectrophotometry. After this purification, the lipopolysaccharide was lyophilized using an Edwards Centrifugal Freeze-Drier (Edwards High Vacuum Ltd., England).

Extraction of LPS by the Aqueous Ether Method:

This extraction was based on the method of Ribi, Milner and Perrins (1959). Washed cells were resuspended in cold distilled water to a final concentration of 0.04 grams (wet weight)/ml and the mixture homogenized for 5 min using a Silverson Homogenizer (Silverson Machines Ltd., London). For every 100 ml of the suspension, 20 ml of diethyl ether (BDH - AnalaR) were added and the mixture shaken for 1 min. The suspension was centrifuged at 7,500 rpm (6,000 x g) for 15 min at 4°C in a MSE 25 centrifuge and the water and ether phases in the supernatant fluid decanted. The extraction was repeated using 20 ml of distilled water and 4 ml of diethyl ether for every 4 grams wet weight of cells. The ether

and aqueous phases from the various extractions were pooled and their volume adjusted with diethyl ether to allow for loss due to evaporation. The organic and aqueous phases were separated in a separating funnel, and the aqueous phase dialysed against distilled water at 4°C for 1 week to remove all traces of ether. The dialysed aqueous extract was centrifuged at 14,000 rpm (25,000 x g) for 30 min in a MSE 25 centrifuge. The sediment contained LPS which was resuspended in distilled water and spun at 36,000 rpm (100,000 x g) for 4 hr; this step was repeated three times and the final sediment lyophilized.

Extraction of LPS by Ethylene diaminetetraacetate (EDTA) Method:

The following modification of Lieve's (1965) method was used: Cells (20 gm wet weight) were stirred in 100 ml of 0.1M Tris/HCl buffer, pH 8.0 and centrifuged at 2000 rpm (1300 x g) for 15 min in a MSE 6L centrifuge. The pellet was resuspended in 200 ml 0.12M Tris/HCl buffer, pH 8, and EDTA added to a final concentration of 5×10^{-4}M. The suspension was incubated at 37°C for 1 hr, after which 98 mg of magnesium chloride was added to give a final concentration of 0.01M. The mixture was cooled to 4°C, centrifuged at 6500 rpm (5,000 x g) for 10 min and the supernatant fluid decanted and centrifuged at 14,000 rpm (23,500 x g) for 30 min. The pellet of LPS was washed twice with distilled water by resuspension and centrifugation at 36,000 rpm (100,000 x g) for 2 hr. The pellet containing LPS was lyophilized.

(II) PREPARATION OF RADIOACTIVELY-LABELLED LPS FROM E. COLI NCTC 8623

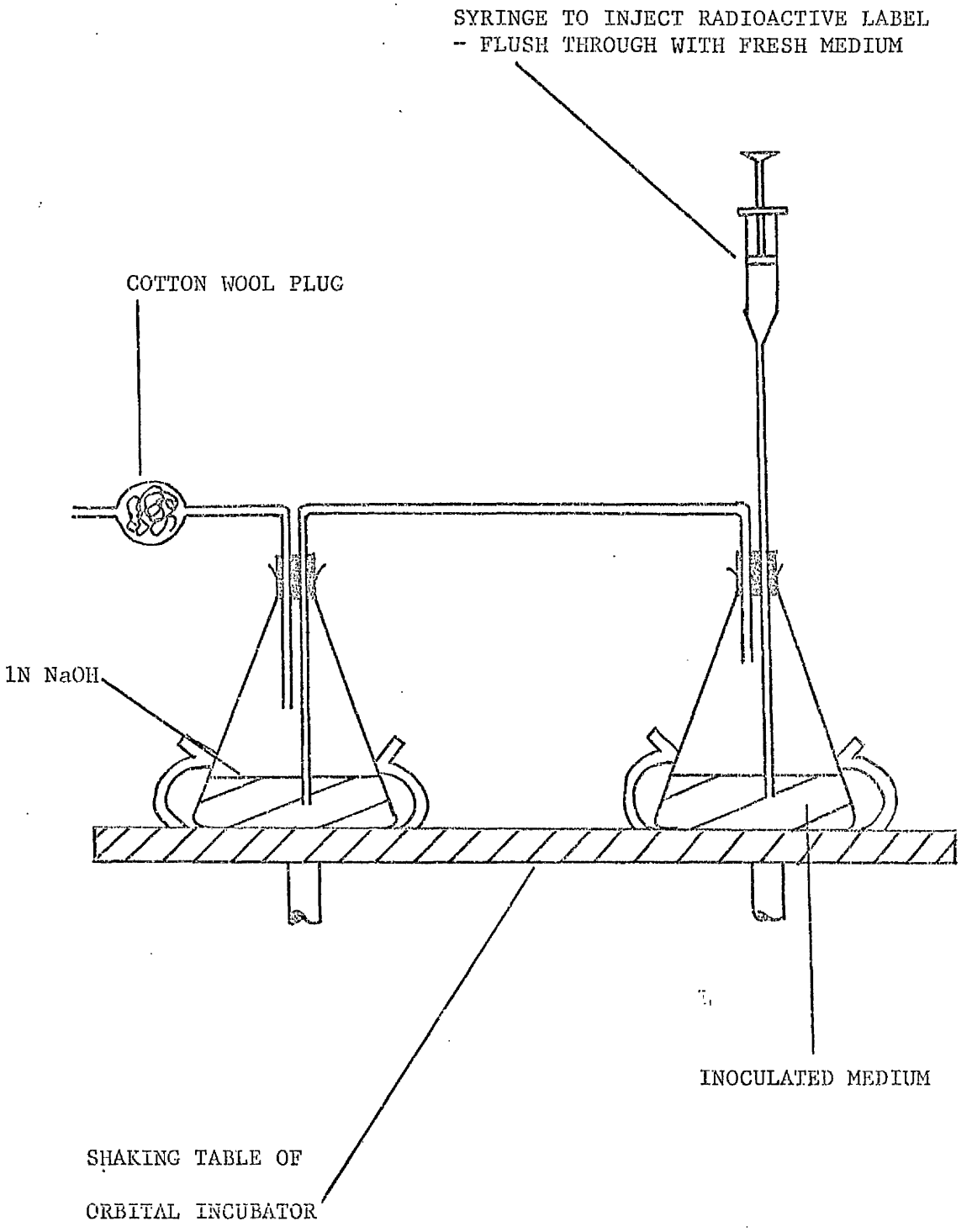
Labelling with ^{14}C-glucose under Diauxic Conditions:

A starter culture of the organism (E. coli NCTC 8623) was grown in Davis and Minglioli minimal medium with glucose as the only carbon source. This culture was used as a 1% (v/v) inoculum to inoculate Davis and Minglioli minimal medium containing lactose (200 mg/100 ml) and ^{14}C-glucose (23 µc/100 ml). The culture was incubated at 37°C for 6 hr in an apparatus designed to absorb evolved $^{14}CO_2$ (Figure 11). This 6 hr incubation enabled the culture to enter the stationary phase, as previously determined from a growth curve with non-radioactive cells (Figure 12). The culture was centrifuged and the LPS extracted by the phenol/water method.

Pulse-labelling with ^{14}C-glucose:

A growth curve was obtained using a 1% inoculum of Escherichia coli NCTC 8623, in Davis and Minglioli minimal medium without added sodium citrate but containing glucose (Figure 13). The stationary phase commenced at 7 hr and it was decided to harvest exponentially growing cells at 6 hr. To prepare ^{14}C-LPS, 2 litres of the inoculated medium containing "cold" glucose were incubated at 37°C for 5 hr in an orbital incubator. These cells were centrifuged at 2000 rpm (1300 x g) for 15 min and washed twice in normal saline. The washed cells were resuspended in 100 ml of the Davis and Minglioli medium (without glucose) and placed in the apparatus designed to absorb evolved $^{14}CO_2$ (Figure 11). Glucose containing 460 µC (Amersham Radiochemicals Ltd., specific activity 230 mC/m.mol of glucose) was added and the culture incubated at 37°C for 63 min. This time

Figure 11 : The Apparatus for the Preparation of ^{14}C-LPS



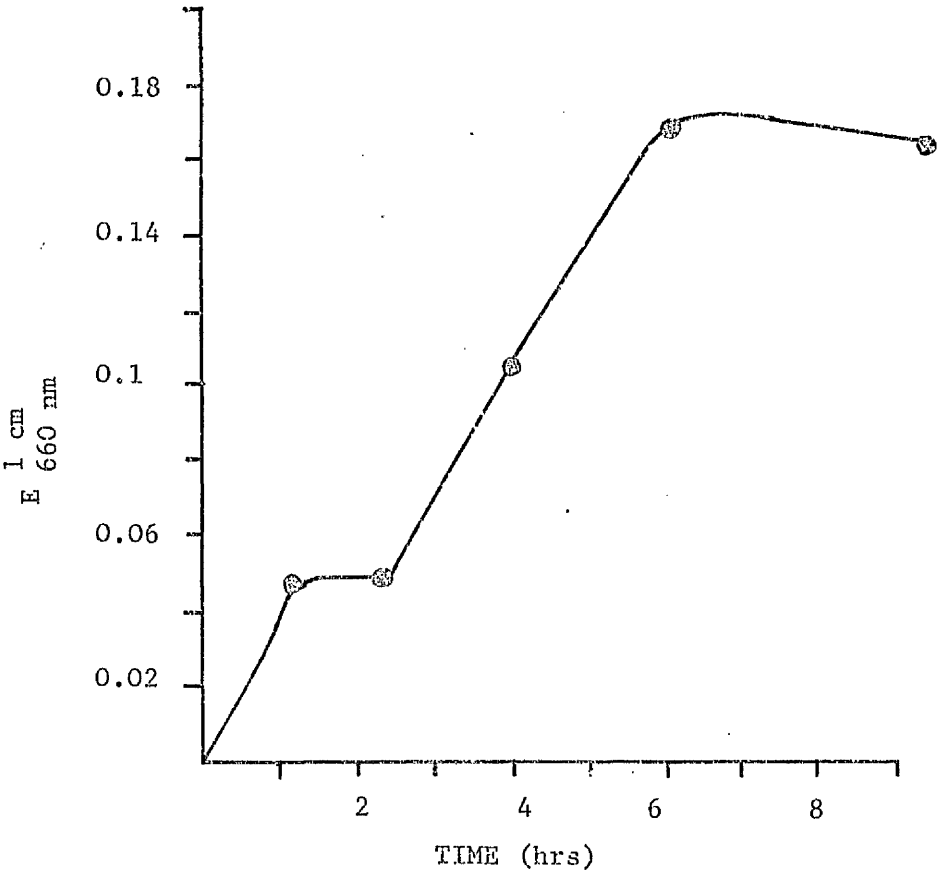
The apparatus is designed to fit onto the shaking table of an orbital incubator and to adsorb $^{14}CO_2$ evolved during incubation.

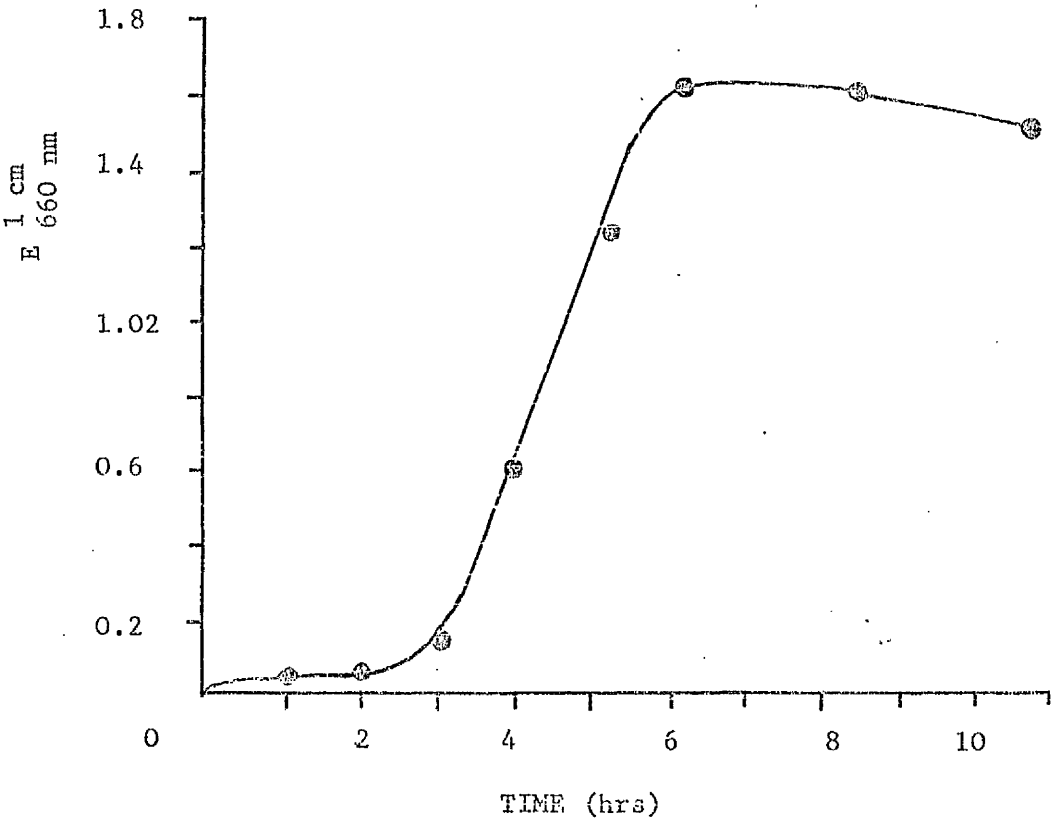
Figure 12: Growth Curve of E. coli NCTC 8623 Grown under Diauxic Growth Conditions

The $E^{\frac{1\ cm}{660\ nm}}$ of aliquots of the culture was plotted against the time of incubation (hours).

Figure 13: Growth Curve of E. coli NCTC 8623 Grown under the Conditions for "Pulse"-Labelling

The $E^{\frac{1\ cm}{660\ nm}}$ of aliquots of the culture was plotted against the time of incubation (hours).


0.18
0.14
0.1
0.06
0.02
E 1 cm 660 nm
2
4
6
8
TIME (hrs)


1.8
1.4
1.02
0.6
0.2
0
E 1 cm 660 nm
2
4
6
8
10
TIME (hrs)

represented 1½ times the doubling time of the original culture. This culture was centrifuged and the LPS extracted by the phenol/water method.

Labelling with ^{32}P-orthophosphate:

A 1% (v/v) inoculum of Escherichia coli NCTC 8623 was used to inoculate the modified Davis and Minglioli medium (Appendix I) to which had been added ^{32}P-orthophosphate (Amersham Radiochemicals Ltd., specific activity 1 mC/ml). The final concentration of the orthophosphate was 2 ml/litre of culture fluid. The culture was incubated at 37°C for 7 hr in an orbital incubator; this yielded a culture entering the stationary phase since a growth curve had previously been obtained. The cells were centrifuged, washed, and the LPS extracted by the phenol/water method.

(III) PREPARATION OF MYCOBACTERIAL POLYSACCHARIDE FRACTIONS

The mycobacterial fractions were obtained from Dr. D.E.S. Stewart-Tull. The following is a summary of the fractions and their methods of preparation.

Fraction	Method of Preparation
ST 80	0.2N TCA extract of M. tuberculosis strain 'C'
ST 81	2.5M Urea extract of M. tuberculosis strain 'C'
ST 82	2% lauryl sulphate extract of M. tuberculosis strain 'C'
ST 83	2% sodium dodecyl sulphate extract of M. tuberculosis strain 'C'
ST 202	Pyridine H_2O (50:50 v/v) extract of M. tuberculosis strain 'DT'
ST 206	Polysaccharide II (Seibert) extract of M. tuberculosis strain 'DT' culture fluid

Fraction	Method of Preparation
ST 208	Glycopeptide extract of M. tuberculosis strain 'DT' culture fluid
ST 209	0.2N TCA extract of M. tuberculosis strain 'DT'
ST 210	Glycopeptide extract of M. tuberculosis strain 'DT' culture fluid
ST 211	Aqueous phase of phenol/water extract of M. tuberculosis strain 'C'.

The detailed methods of extraction presented by Stewart-Tull (1966).

(IV) CHEMICAL TREATMENTS APPLIED TO PURIFIED LPS

Some of the LPS preparations were subjected to chemical treatments to modify their affinity for mammalian cell surfaces. Heat-treated LPS was prepared by dissolving the LPS in 1 ml normal saline and heating in a sealed vial at 100°C for 2-5 hr.

Alkali-treated LPS was prepared by dissolving the bacterial polysaccharide (20 mg) in 1.5 ml 0.25N sodium hydroxide (BDH-AnalaR) and heating at 56°C for 6 min. After cooling the solution was neutralized by adding acetic acid and the bacterial polysaccharide precipitated by the addition of volumes of 100 per cent ethyl alcohol and lyophilized. This method was described by Macpherson, Wilkinson and Swain (1953).

Periodate-treated LPS was prepared as described by Meter, Westphal, Lüderitz, Gorzynski and Eichenberger (1956). The bacterial polysaccharide

(20 mg) was dissolved in 16 ml distilled water and 2 ml (0.2M) sodium acetate buffer, pH 5.0 (Appendix III) and 2.0 ml 0.1N sodium periodate (BDH) added. The mixture was incubated in the dark at room temperature for 6.5 hr, then dialysed against distilled water and lyophilized.

(V) PREPARATION OF MAMMALIAN CELL SUSPENSIONS

Erythrocytes:

Sheep and horse blood was collected asceptically by jugular veni-puncture into an anticoagulant (sterile 3.8% (w/v) sodium citrate). This blood was collected using an intravenous bleeding set (Baxter Division, Travenol Laboratories Ltd., Thetford, England). Rabbit blood was withdrawn from the ear vein of New Zealand White Rabbits (Charles River U.K. Ltd., England) into sodium citrate. Similarly mouse blood was obtained from Charles River Ltd., 'CD' strain mice by anaesthetizing with ether and exsanguinating by cardiac puncture. The blood was collected in sodium citrate. Venous blood from human donors was withdrawn using a syringe from an arm vein into sterile sodium citrate.

The blood collected by these methods was centrifuged and the packed erythrocytes were washed three times in physiological saline; the washed cells were resuspended in saline to provide appropriate concentrations.

Mouse Peritoneal Macrophages and Lymphocytes

Both types of cell were prepared concurrently from mouse peritoneal washings. Mice were injected intraperitoneally with 10 ml of the Cell Growth (CG) Medium (see Appendix II). A few minutes later the

animals were anaesthetized and bled from the heart. Peritoneal washings were removed with a Pasteur pipette, placed in a glass petri-dish and incubated at 37°C for 1 hr to allow macrophage adherence. The supernatant fluid containing the glass non-adherent cells was decanted into a clean petri-dish and re-adsorbed at 37°C for 1 hr. The supernatant fluid from this second adsorption was used as the peritoneal lymphocyte preparation. The glass-adherent cells, considered to be macrophages, were resuspended in fresh CG medium using a rubber policeman, further incubated at 37°C for 1 hr, after which the supernatant fluid was decanted and discarded. The glass-adherent cells were resuspended and this suspension was used as the peritoneal macrophage preparation. To check the relative purity of the two cell preparations, samples were stained with neutral red (0.003% w/v) to determine the percentage of viable cells present; live cells stain with neutral red, dead cells do not (Finter, 1969; Greaves, Potter and McIntegart, 1971). Further samples of the cell preparations were also treated with trypan blue (1% w/v). Although this stain is excluded by viable cells (Harris, Harris and Farber, 1954), it is an intravital stain, being taken up by engulfment, so that it will be seen as discrete granules in the cytoplasm of macrophages (Culling, 1957). Hence the macrophage contamination of the lymphocyte preparation and the lymphocyte contamination of the macrophage preparation could be determined.

Mouse Spleen Cells:

Spleens were removed from exsanguinated mice and washed in the CG Medium. They were placed in a conical centrifuge tube and broken up, first by mincing with a scalpel and then by expelling the spleen fragments through a narrow-bore syringe needle. Large fragments were allowed to

sediment and the supernatant suspension used as the spleen cell preparation. The viability of the cells was tested by staining with neutral red.

Mouse Thymus Cells:

The thymus was removed by dissection and a cell suspension made by the same technique used for the spleen, except that a more vigorous disintegration was required to produce a suspension of single cells.

Mouse Bone Marrow Cells:

The femurs were removed from exsanguinated mice and muscle and connective tissue removed. The femur heads were cut off and the bone-marrow flushed out with CG Medium to give a suspension of bone-marrow cells.

All cell preparations were used while fresh.

Human White Blood Cells:

Venous blood was collected from healthy donors and 10 ml. of an heparinized sample carefully layered onto 8 ml. Ficoll/hypaque of specific gravity 1.076 in a universal container. The mixture was centrifuged at 400 x g for 20 min, the plasma decanted and the layer containing the white blood cells and platelets removed and resuspended in 5 ml Hanks BSS. This suspension was centrifuged at 70 x g for 10 min and the white blood cell pellet resuspended in Hanks BSS. Before use, the white blood cells were washed twice in CG Medium. These human white blood-cell suspensions were prepared by Mrs. D.L. Davies.

(VI.) PREPARATION AND TESTING OF ANTISERA

Rabbit Anti-mycobacterial Glycopeptide Serum:

A water-in-oil emulsion was prepared by dissolving 10 mg of glycopeptide (ST 208) in 0.5 ml saline and adding 0.5 ml mannide monooleate (Arlacel, Atlas Powder Co., Delaware, U.S.A.). Dried *M. tuberculosis* strain DT (5 mg) obtained from the Ministry of Agriculture, was mixed with 1.5 ml Bayol F (Esso Petroleum Co. Ltd., Purfleet, England) and added to the antigen solution. The mixture was emulsified thoroughly using a syringe and 1 ml was injected intramuscularly into each hind quarter of adult New Zealand white rabbits. A booster injection of the same complete adjuvant mixture was given 4 weeks later. Rabbits were anaesthetized with 0.4 ml/Kg (body weight) of intravenously injected Nembutal (Abbott Laboratories Ltd., England), and bled out by cardiac puncture. Usually between 100-150 ml of blood was obtained. The blood was allowed to clot at 37°C for 30 min, then placed at 4°C for 18 hr and the serum removed and stored at -20°C until required.

Guinea-pig Anti-ovalbumin γ_2-Globulin Fractions:

Albino guinea-pigs were injected with 0.2 ml of an antigen adjuvant mixture into the left hind footpad. The mixture contained 20 mg ovalbumin (Sigma London Chemical Co. Ltd.) in 0.4 ml normal saline to which was added an emulsion containing 0.4 ml Arlacel A, 1.2 ml Bayol 55 and 5 mg of dried *M. tuberculosis* strain DT. The animals were bled on day 21 by cardiac puncture and the serum collected. The γ_1 and γ_2-globulin-containing fractions were obtained by Ampholine column separation using the

technique described by Stewart-Tull and Arbuthnott (1971). These fractions were kindly supplied by Dr. D.E.S. Stewart-Tull.

Rat Anti-mouse Serum:

Rats were injected intramuscularly with 0.2 ml of an antigen adjuvant water-in-oil emulsion consisting of 0.5 ml Arlacel A, 1.5 ml Bayol F, 0.5 ml normal mouse serum and 5 mg dried M. tuberculosis strain DT. The mixture was fully emulsified before injection and a booster injection was given intraperitoneally on day 28. The booster dose had the same ingredients as the primary dose, except that dried Mycobacterial cells were omitted. The animals were bled by cardiac puncture on day 42 and the sera separated.

Sheep Anti-guinea-pig and Sheep Anti-rabbit Sera:

These antisera were prepared by Dr. D.E.S. Stewart-Tull. Scottish mountain ewes were injected intramuscularly with 3 ml of Freund Incomplete Adjuvant containing either 2 ml of rabbit serum or 2 ml of guinea-pig serum. Booster doses were administered intraperitoneally after one month. Blood was obtained periodically from the animal's jugular vein using the technique previously described and the serum separated.

Examination of Sera for Antibodies:

The antibody levels in the antisera were checked by the Ouchterlony double diffusion technique and by immunoelectrophoresis.

Ouchterlony Double Diffusion Technique:

Ionagar (Appendix IV) was distributed into glass petri dishes to a depth of 5 mm and allowed to solidify. Wells were cut in the agar with a cork borer. The arrangement of, and the distance between, the wells depended on the particular antigen:antibody system used to compensate for differing antibody levels. The plates were incubated at room temperature for 24 hr and the precipitin reactions which developed were clearly visible.

Immunoelectrophoresis:

Ionagar was poured onto glass plates (11 cm long) to a depth of 3 mm, solidified, and troughs and wells were cut out. The antiserum was placed in the wells and the plates placed in a Shandon Immunoelectrophoresis apparatus containing barbitone buffer (Appendix III); a constant voltage was applied (10-11 volts per cm of agar). After 2 hr the current was switched off and the antigen solution was placed in the troughs. Diffusion of the components took place and after 24 hr the developed precipitin reactions were observed.

(VII) PREPARATION OF FLUORESCENT ANTIBODY

Fluorescein-labelling was performed on γ-globulins purified by DEAD-cellulose chromatography of ammonium sulphate precipitated globulins as follows :-

Separation of Globulins by Ammonium Sulphate Precipitation

Ammonium sulphate (specially low in heavy metals, Fisons

Scientific Apparatus, England) was added to whole sheep anti-sera to 50% saturation (29.1 grams of $(NH_4)_2SO_4$ for every 100 ml of fluid; obtained from a nomogram described by Dixon, 1953). The mixture was left at 4°C for 18 hr. The resulting precipitate was collected by centrifugation at 3000 rpm (1500 x g) for 10 min and washed with 50% saturated ammonium sulphate to remove albumin and traces of haemoglobin. The washed precipitate was dissolved in 0.02M phosphate buffer, pH 7.5 (see Appendix III) and dialysed in Visking tubing against the same buffer at 4°C for 24 hr to remove ammonium sulphate. The dialysed material was concentrated with polyethylene glycol ('Carbowax 4000' BDH, England) and the concentrate chromatographed on DEAE-cellulose (Whatman Column Chromedia DE11) with a stepwise elution technique described by Stewart-Tull, Wilkinson and White (1965).

DEAE-Cellulose:

DEAE-cellulose (80 grams 'Whatman', Balston Ltd., England) was treated with 2 litres 0.5N hydrochloric acid (BDH-AnalaR) for 30 min. The "fines" and acid were decanted, and the slurry washed with 0.02M phosphate buffer pH 7.5, until the pH was 4.0. It was treated with 2 litres 0.5N NaOH (BDH-AnalaR) for 30 min, the alkali decanted and the treatment repeated. Next, the DEAE-cellulose was washed in 0.02M phosphate buffer, pH 7.5, until the wash liquid was pH 7.0. The cellulose slurry was placed in a pressure vessel, de-gassed on a vacuum pump (700 mm pressure) and poured into a 540 ml Pharmacia glass column (Pharmacia Ltd., London).

Separation of γ-globulin on DEAE Cellulose:

To equilibrate the column, 1.5 - 2.0 litres of 0.02M phosphate

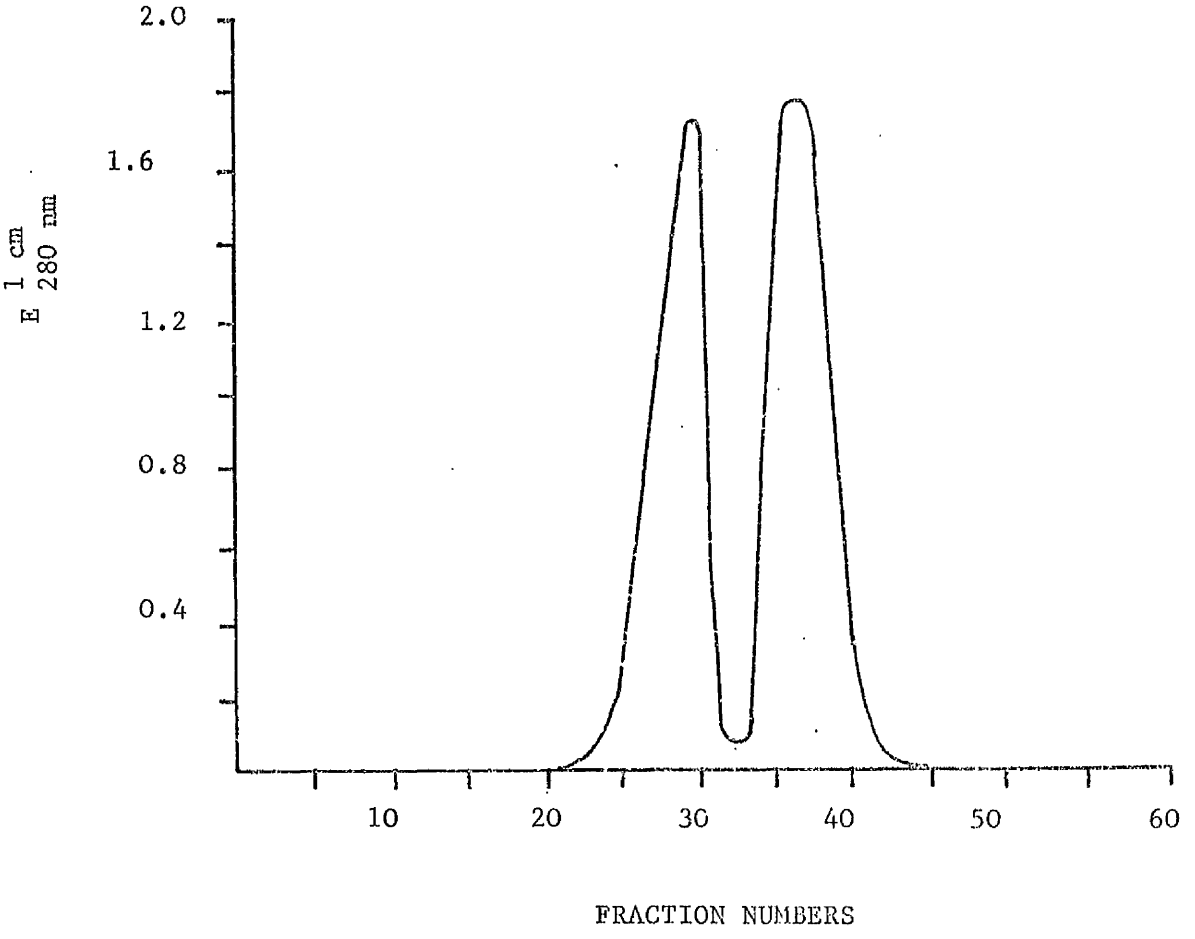
buffer, pH 7.5, was passed through the column. The ammonium sulphate precipitated globulin was applied and the gamma-globulin fraction eluted with 0.02M phosphate buffer, pH 7.5, the effluent being collected on a fraction collector. In order to re-use the column the remaining serum proteins were eluted with 0.3M monosodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$ 46.8 grams/litre). All the fractions from the first peak (Figure 14) were analysed by the Ouchterlony technique against rabbit or guinea-pig sera. The gamma-globulin-containing fractions which showed precipitin arcs were pooled and concentrated to a convenient volume with carbowax.

Preparation of Fluorescein-labelled Sheep Immunoglobulins:

Conjugation of Gamma-globulin with FITC: Sheep gamma-globulin preparations purified on DEAE-cellulose were diluted with normal saline to contain 100 mg protein/8.5 ml; the protein having been estimated by the Lowry method. Carbonate-bicarbonate buffer (1.5 ml of 0.5M, Appendix III) was added to the diluted globulin solution and fluorescein isothiocyanate (5 mg FITC Isomer 1, British Drug Houses) added slowly to the buffered globulin mixture with vigorous stirring. The mixture was stirred without frothing for 6 hr at 25°C and dialysed in Visking tubing (8/32, Scientific Instrument Centre Ltd., London) against normal saline until the dialysate was colourless. The dialysate was filtered through a Sephadex G-25 column to remove remaining unconjugated fluorescein.

Preparation of Sephadex G-25 Column: Sephadex G-25 (15 gm Fine grade, Pharmacia Ltd., London) was added to 200 ml of distilled water with continuous stirring. The slurry was heated for 2 hr in a boiling water

Figure 14: The Elution Profile of the Ammonium Sulphate Precipitated Fraction of Sera on DEAE-Cellulose



The $E^{1\ cm}_{280\ nm}$ of each fraction obtained from the column was plotted against the fraction number. A double peak was always obtained which possessed antibody activity, as determined by the Ouchterlony method.

bath and subsequently allowed to stand for one hour to allow for swelling of the gel. The supernatant liquid and the fines were decanted and the swollen sephadex washed with 200 ml. of 0.01M phosphate buffered saline, pH 7.2 (Appendix III), and allowed to settle. The supernatant fluid and the fines were decanted, the washing repeated several times and the slurry finally resuspended in a convenient volume and poured into a 150 ml glass volumn.

Elution of the FITC Conjugated Sheep Gamma-globulin: The dialysed fluorochrome-globulin conjugate was applied to the column and 0.01M phosphate buffered saline, pH 7.2, used to separate the labelled globulins from the free fluorochrome. The fluorochrome-globulin conjugate was eluted in the void volume of the column as determined by spectrophotometry at 280 and 495 nm (Figure 15). Those fractions having appreciable absorption at both wavelengths were tested for antibody activity by the Ouchterlony method and positive fractions pooled and concentrated to yield the purified FITC conjugated gamma-globulin.

(VIII) MEASURING THE UPTAKE OF BACTERIAL POLYSACCHARIDES TO MAMMALIAN CELL SURFACES

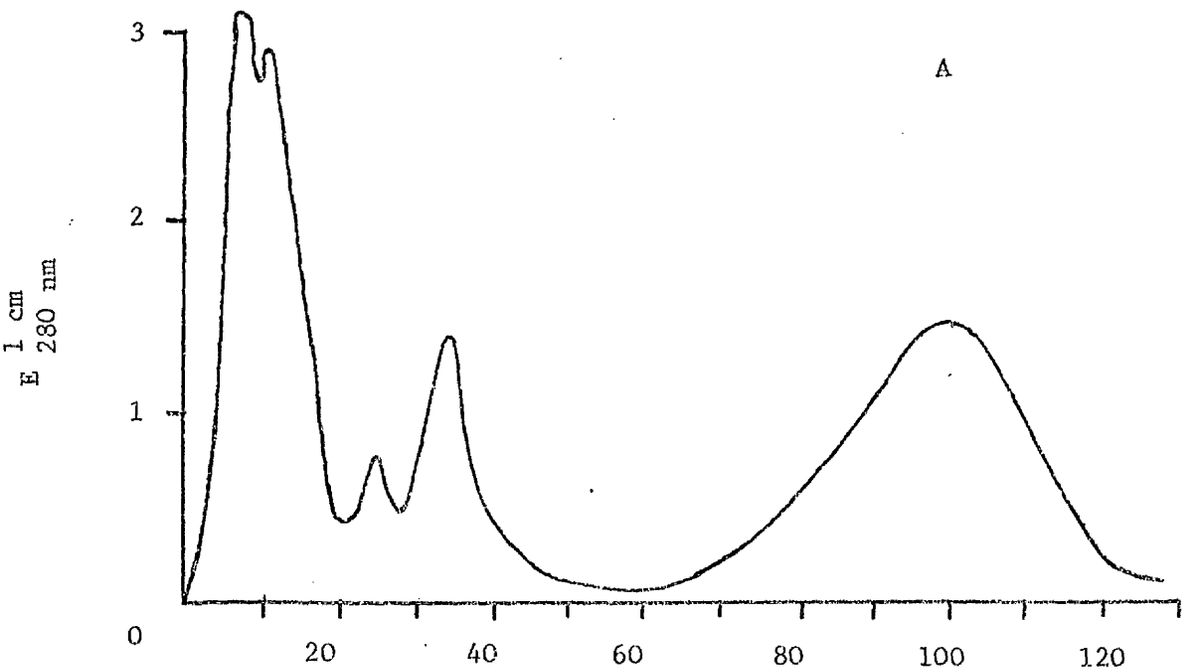
By a Chemical Technique

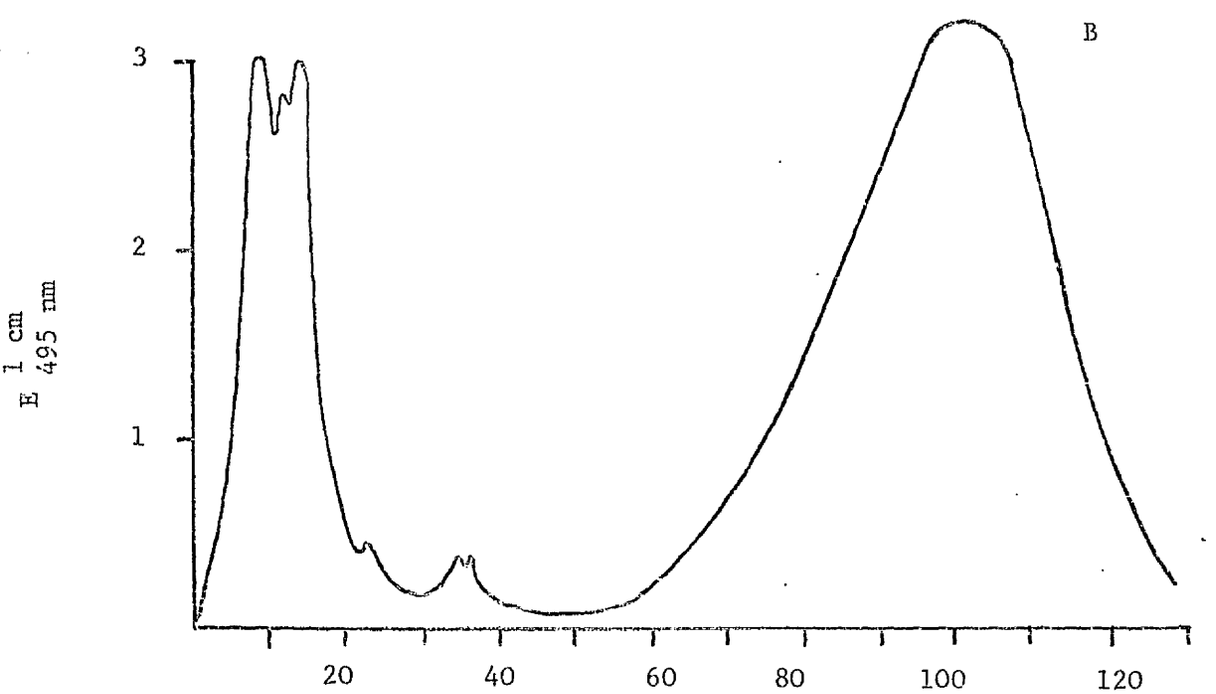
This technique was described by Davies and Stewart-Tull (1973) (Appendix XI). The quantitative measurement of erythrocyte modification by bacterial polysaccharides posed two problems; the accurate standardization of erythrocyte suspensions and a carbohydrate estimation in the presence of haemoglobin. Erythrocyte suspensions can be standardized by

Figure 15: The Elution Profile of FITC-γ-globulin from Sephadex G-25

Figure A represents the $E^{1\ cm}_{280\ nm}$ readings plotted against the fraction number. Figure B represents the $E^{1\ cm}_{495\ nm}$ readings plotted against the fraction number. It should be noted that fluorescein absorbs at both wavelengths of 280 and 495 nm.

 represents the fractions containing antibody activity, as measured by the Ouchterlony and immunoelectrophoretic methods.


A
E 1 cm
280 nm
3
2
1
0
20
40
60
80
100
120
FRACTION NUMBER


B
E 1 cm
495 nm
3
2
1
20
40
60
80
100
120
FRACTION NUMBER

three basic methods. (a) With respect to cell numbers by the use of a haemocytometer, (b) dry weights of cells can be used but this is a time consuming method, (c) a spectrophotometric technique, releasing the haemoglobin from the erythrocytes and measuring the adsorption at 540 nm; the latter method was used. The cells can be lysed in distilled water, but it is difficult to obtain true solutions and to measure all the forms of haemoglobin. Both these factors will affect the preparation of standard curves. The best photometric method is known as the cyan-methaemoglobin method, originally described by King and Gilchrist (1947). This method had several advantages over the other spectrophotometric methods in that (a) all forms of haemoglobin were estimated, (b) a true solution was obtained, (c) it was very accurate (the standard deviations showed a 2% error, Darmady and Davenport (1963)), (d) standard curves were easily prepared using a commercially available solution of cyan-methaemoglobin. In order to correlate one experiment with another the mean corpuscular haemoglobin concentration (MCHC) of each sample was calculated. The MCHC was considered to be an accurately calculated parameter of erythrocyte suspensions (Darmady and Davenport, 1963), and accounted for inherent variations in cell size. Similarly, in order to standardize erythrocyte suspensions on a daily basis, due to variations in haemoglobin content, it was necessary to compare all samples to the Haldane Standard (14.6 grams haemoglobin/100 ml).

The carbohydrate content was measured by the anthrone method. However in the presence of haemoglobin, a brown colouration and an increase in the anthrone OD_{625nm} readings was observed. However, it was found that the anthrone test remained valid in the presence of haemoglobin

(Figure 16a). If the erythrocyte suspension was accurately standardized by the cyanmethaemoglobin method, a precise relationship of OD_{625nm} to haemoglobin content could be estimated (Figure 16b).

Measurement of the Uptake of Bacterial Polysaccharides to Erythrocytes:

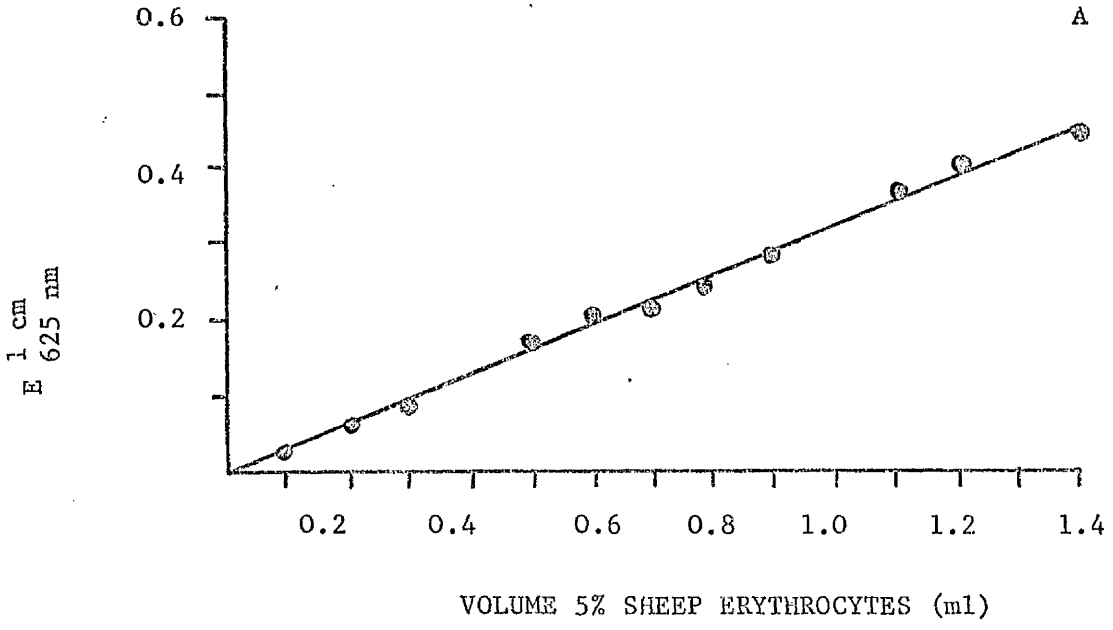
A modification of the method of Middlebrook and Dubos (1948) was used. The bacterial polysaccharide (20 mg) was dissolved in 1.0 ml of 0.85% (w/v) saline solution. To this solution was added 1.0 ml. of a packed erythrocyte suspension: appropriate controls were also set up. The mixture was incubated, with frequent agitation, at 37°C for 1-3 hr. After incubation, the suspension was centrifuged at 2000 rpm (350 x g) for 5 min. The cells were washed three times in normal saline and diluted to 5% with normal saline. The haemoglobin concentration, the PCV and the hexose content were measured as described by Davies and Stewart-Tull (1973, Appendix XI) for both normal erythrocytes and for cells treated with polysaccharide. The carbohydrate content of both treated and untreated erythrocytes was expressed as milligrams of hexose sugar to the Haldane haemoglobin standard of 14.6 grams/100 ml. The calculations were made using the formula :-

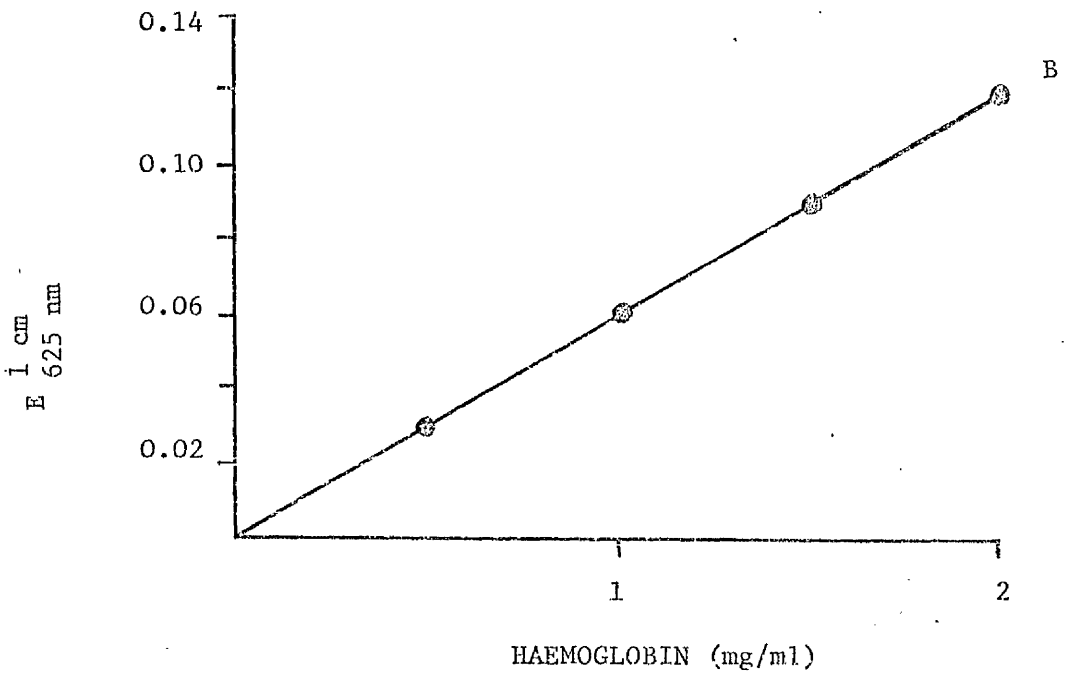
$$\text{Hexose content of erythrocytes} = \frac{MCHC_A}{MCHC_B} \times \text{Hexose mg/100 ml}_B \times \frac{14.6}{\text{Haemoglobin (gram/100 ml)}_B}$$

where A is the value for untreated cells and B the values for the cells treated with a polysaccharide preparation.

Figure 16: The Anthrone Determination of Hexoses in the Presence of Sheep Erythrocytes

The $E^{1\ cm}_{625\ nm}$ obtained by the anthrone method was plotted against (a) the volume of 5% sheep erythrocytes (ml) and (b) the haemoglobin concentration of the erythrocytes (mg/ml).


A
0.6
0.4
0.2
E 1 cm 625 nm
0.2
0.4
0.6
0.8
1.0
1.2
1.4
VOLUME 5% SHEEP ERYTHROCYTES (ml)


B
0.14
0.10
0.06
0.02
E 1 cm 625 nm
1
2
HAEMOGLOBIN (mg/ml)

Calculations Involving Measurements obtained by the Chemical Technique:

From measurements of the carbohydrate content of sheep erythrocytes (mg/100 ml carbohydrate to the Haldane Standard) the various increases in carbohydrate composition of the erythrocytes was calculated using the formulae :-

1 ..... $\frac{(T - N)}{N} \times 100$ = percentage increase in carbohydrate content of erythrocytes (%)

2 ..... $\frac{(T - N)}{100}$ = actual increase in carbohydrate content of erythrocytes (mg/ml)

3 ..... $\frac{(T - N)}{100} \times 2$ = A — the increase in carbohydrate content of erythrocytes per reaction mixture of 2 ml (mg)

4 ..... $\frac{A}{H} \times 100$ = the amount of the polysaccharide fraction adsorbed by the erythrocytes (mg)

Where N is the carbohydrate content of untreated erythrocytes (mg/100 ml carbohydrate to the Haldane Standard)

T is the carbohydrate content of modified erythrocytes (mg/100 ml carbohydrate to the Haldane Standard)

H is the percentage hexose in the modifying polysaccharide fraction calculated by the anthrone method.

By a Fluorescent Antibody Technique

4 ml of FITC-globulin conjugate concentrate was mixed with 1.0 ml of 10% sheep erythrocyte membrane suspension and incubated at 4°C for 24 hr. The membranes were removed by centrifugation at 14,000 rpm (2,500 x g) for 15 min.

Preparation of Sheep Erythrocyte Membranes:

Packed washed sheep erythrocytes were added dropwise to 200 volumes of distilled water with constant agitation. The erythrocyte ghosts were obtained by centrifugation at 20,000 rpm (48,000 x g) for 15 min and were washed twice in 0.02M phosphate buffer (pH 7.5). The erythrocyte ghost membranes were resuspended in buffer to yield approximately a 10% suspension.

Fluorescent Slide Technique:

Erythrocytes treated with bacterial polysaccharide were smeared on grease-free microscope slides and after drying were fixed in absolute alcohol for 10 min and washed thoroughly with 0.02M phosphate buffer (pH 7.5). Slides were treated either with guinea-pig antiovalbumin γ_2-immunoglobulin absorbed with sheep erythrocyte membranes or with rabbit anti-mycobacterial polysaccharide (glycopeptide) serum. The slides were left in moist chambers for 30 min at room temperature then washed in 0.02M phosphate buffer, pH 7.5, to remove excess serum. Either sheep anti-guinea-pig fluorescein conjugate or sheep anti-rabbit fluorescein conjugate was spread over the smears and allowed to react at room temperature for 30 min. After thoroughly washing in buffer, the preparations were mounted in 0.5M carbonate buffered glycerol, pH 9.0

(Appendix III) (Walker, Batty and Thomson, 1971) and the coverslips sealed with nail varnish. Appropriate controls were made at each stage of the procedure, i.e. untreated cells were examined and guinea-pig antiovalbumin γ_1-immunoglobulin replaced the γ_2-immunoglobulin. Similarly, the slides were examined at each stage of preparation, prior to the addition of fluorescent antibody, for natural fluorescence.

The slides were examined in a Leitz Orthoplan microscope (E. Leitz (Instruments) Ltd., London) with fluorescein filters (BG12, 2 x FITC filters) and a Fluorescence Vertical Illuminator according to Ploem (1967) (both obtained from E. Leitz Ltd.). The preparations were photographed using an automatic exposure device. Black and White Kodak film (FP_4; ASA_{125}, DIN 22) was used and developed in Kodak Microdol-X developer and in Ilford Hypam fixer. Colour film (Kodak Daylight; ASA 160; DIN 23) was processed commercially.

By a Radioactive Technique

Measurement of Radioactivity:

The radioactivity of samples containing ^{14}C and ^{32}P was measured using a liquid scintillation method in a Nuclear Enterprises Automatic Beta-Gamma Spectrometer, NE8312 (Nuclear Enterprises Ltd., Edinburgh). Aqueous samples were mixed with 10 volumes of a xylene-based multipurpose liquid scintillant, NE260 (Nuclear Enterprises Ltd., Edinburgh). The counting was carried out over three energy channels and the energy threshold and energy windows were calibrated and set as follows :-

		Channel 1	Channel 2	Channel 3
^{14}C	E	20	20	0
	E	480	980	1000
^{32}P	E	300	300	0
	E	700	700	1000

The efficiency of counting was measured with an automatic external standard device, and by a channels-ratio method.

Measurement of Cell-associated Radioactivity

After incubation of the cells with ^{14}C- or ^{32}P-LPS the cell-associated radioactivity was measured as follows: the cells were centrifuged in a bench centrifuge at 3500 rpm (1750 x g) for 5 min and the pellet of cells washed twice with 0.85% (w/v) sodium chloride. The pellet was resuspended and subjected to wet oxidation (Mahin and Lofberg, 1970) by mixing 0.2 ml with 0.2 ml of 60% (v/v) perchloric acid (BDH - AnalaR) in a scintillation vial. After mixing thoroughly 0.4 ml of 30% (w/v) hydrogen peroxide (BDH - AnalaR) was added and the vial sealed and heated at 70-80°C for 60 min with periodic agitation. After cooling, 8 ml of the liquid scintillant (NE260) was added and the radioactivity measured.

Measurement of the Radioactivity Associated with the Supernatant Fluid from the Reaction Mixtures

The cells incubated with the radioactively-labelled LPS and centrifuged at 2000 rpm (350 x g) for 5 min. Two aliquots (0.5 ml) of the supernatant fluid were removed and the radioactivity in them was measured as previously described.

Measurement of Uptake of Radioactive LPS by Mammalian Cells

Experiments Involving Direct Adsorption:

The mammalian cell suspensions were counted using a haemocytometer, and standardized to contain 1.57×10^8 cells/ml. The radioactively-labelled LPS was stored as a stock suspension. A constant volume (0.1 ml) of radioactively-labelled LPS was added so that the ratio of the amount of LPS added to the number cells remained constant. The radioactivity of the LPS was measured before each experiment to compensate for any radioactive decay. The reaction mixture contained 1 ml of cell suspension of known count and 0.1 ml of ^{14}C- or ^{32}P-LPS. The mixture is incubated at $37^{\circ}C$ for 1 hr unless other conditions are specified. The suspension was centrifuged at 3500 rpm (1750 x g) for 5 min and the radioactivity in the supernatant fluid measured. Since a known amount of radioactivity had been added to the reaction mixture, the difference between this value and that in the supernatant gave a measure of the LPS taken up. In some experiments the radioactivity of the cell pellet was measured after wet oxidation to check that the sum of the measured activities of supernatant and pellet was the same as that of the LPS added.

Experiments Involving Potential Inhibitors of Adsorption:

For these experiments the reaction mixture consisted of 1.0 ml of the standard cell suspension and 0.1 ml of the radioactively-labelled LPS together with 1.0 ml of the inhibitor solution. The potential inhibitors of adsorption, 'cold' homologous and heterologous preparations of LPS, polysaccharides, mono- and di-saccharides were tested at two levels of activity namely 60 µg and 120 µg for ^{32}P-LPS and 30 µg and 60 µg

for ^{14}C-LPS. The control mixtures contained 1.0 ml normal saline instead of the 1.0 ml inhibitor solution. The measurement of radioactivity associated with the cells was measured in the usual way.

Experiments Involving the Partial Determination of the Site of Action of Glucose as an Inhibitor of Erythrocyte Modification

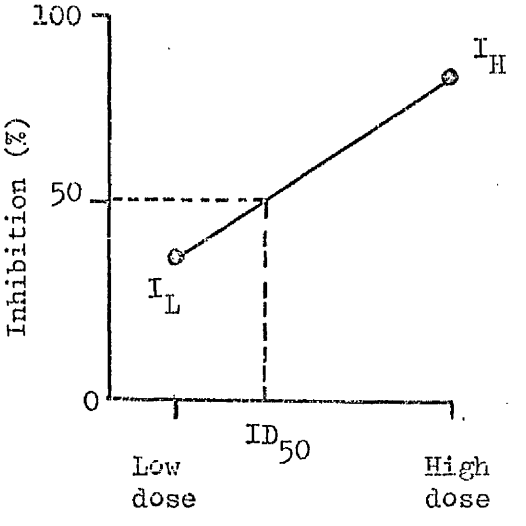
Four different reaction mixtures were set up to investigate this effect. The first reaction mixture (I) consisted of 1.57×10^8 human erythrocytes, together with the ^{14}C-LPS (1615 dpm) and either 36 or 72 µg of glucose in 1.0 ml of normal saline, and incubated at 37°C for one hour. The second reaction mixture (II) consisted of 1.57×10^8 human erythrocytes together with the ^{14}C-LPS (1615 dpm) which was incubated at 37°C for 30 min. After this initial incubation period either 36 or 72 µg of glucose were added to the reaction mixture and the incubation was continued for a further 30 min. The next reaction mixture (III), consisting of ^{14}C-LPS (1615 dpm) and either 36 or 72 µg of glucose in 1.0 ml saline, was incubated at 37°C for 30 min before the addition of 1.57×10^8 erythrocytes and re-incubated for a further 30 min. The final reaction mixture (IV), consisting of 1.57×10^8 erythrocytes and either 36 or 72 µg glucose, was incubated for 30 min at 37°C. After this initial incubation period, the ^{14}C-LPS (1615 dpm) was added and the incubation continued for a further 30 min. Similar reaction mixtures were set up for ^{32}P-LPS using glucose at the 60 and 120 µg level. In each case the per cent inhibition of uptake of the labelled LPS was calculated.

Calculations Involving the Inhibition of the Adsorption of Labelled LPS

If A represented the radioactive label associated with erythrocytes treated with $^{32}P/^{14}C$-LPS alone and B represented the radioactive label associated with erythrocytes treated with $^{32}P-/^{14}C$-LPS in the presence of a potential inhibitor, the percentage inhibition caused by the potential inhibitor was calculated as follows :-

$$\frac{(A - B)}{A} \times 100$$

The percentage inhibition obtained by an inhibitor at a low dose was termed I_L, and the percentage inhibition at a high dose, I_H. By plotting I_L and I_H against the appropriate dose levels, a graph was obtained from which the 50% Inhibition dose (ID_{50}) was calculated.



I_H = Inhibition at the high dose

I_L = Inhibition at the low dose

The relative affinity values were obtained by comparison of the ID_{50} values with the ID_{50} of the standard E. coli NCTC 8623 LPS.

(IX) MEASUREMENT OF ADJUVANT ACTIVITY OF BACTERIAL POLYSACCHARIDES

The adjuvant activity of bacterial polysaccharides was measured by their ability to increase the titre of circulating antibodies to ovalbumin (EA) in mice; antibody titres were determined by passive haemagglutination (HA) of EA-coated formalinized tanned erythrocytes. To permit comparison of relative adjuvant activities of different polysaccharides, all adjuvant tests were set up as 4-point bioassays with two doses of standard LPS and two doses of "unknown" in each experiment.

Determination of Suitable Conditions for Stimulated Immune Responses in Mice

Adult C.D. Mice (Charles Rivers Ltd., London) were divided into groups each containing five animals. The injection mixture was 20 µg ovalbumin (Sigma London Chemical Co. Ltd., England) together with various amounts (30, 60, 90, 120 µg) of lipopolysaccharide extracted from Escherichia coli NCTC 8623 by the phenol/water method of Westphal, Lüderitz and Bister (1952). The ingredients were "solubilized" in 0.1 ml sterile normal saline (0.85% w/v). The injections were administered either intravenously into the peripheral tail veins, or intraperitoneally. There were two immunization schedules; either a single injection or a triple injection of the mixture on days 1, 5 and 10. All the animals were bled out on day 21 by cardiac puncture. Appropriate control animals were set up throughout. Sera were prepared as previously described.

Immunization of Mice

Adjuvant tests were made in adult C.D. Mice (Charles River Ltd.,

London) which were divided into groups of five animals. The injection mixture per mouse consisted of a constant 20 μg ovalbumin and either 30 or 60 μg of bacterial polysaccharide in 0.1 ml normal saline. Injections were given intravenously on days 1, 5 and 10 to groups of 5 mice at each dose level and the animals bled out by cardiac puncture on day 21; the sera were separated and frozen. Each adjuvant test involved a comparison of the "unknown" polysaccharide with LPS of E. coli 8623 which was taken as a standard. In addition, control mice were injected with EA without adjuvant.

Preparation of Indicator EA-coated Erythrocytes:

Group O human erythrocytes were washed three times in normal saline and the washed packed erythrocytes (25 ml) resuspended in 175 ml phosphate buffered saline, pH 7.2. A dialysis sac containing 50 ml of commercial formalin (40% formaldehyde), was submerged in the erythrocyte suspension and the preparation stirred at room temperature for 3 hr. Then the dialysis sac was punctured and the gentle mixing continued for 18 hr. The formalized erythrocytes were washed five times with normal saline and resuspended to a final concentration of 25% (v/v). Formalin (0.2% v/v) was added as a preservative.

To prepare the formalinized cells for coating with EA, tannic acid pretreatment was required. Formalinized cells (2.4 ml) were placed in a universal container and washed twice with phosphate buffered saline (PBS), pH 7.2. The cells were resuspended to a final volume of 10 ml with PBS and 10 ml 0.1 mg/ml tannic acid solution in PBS added. The suspension was incubated at 37°C for 15 min, centrifuged at 2750 rpm

(900 x g) for 5 min and the cells washed once with 20 ml PBS. The cells were resuspended in 10 ml PBS and 10 ml of the antigen solution containing 20 mg EA added. This mixture was incubated at 37°C for 30 min, washed three times with PBS containing 1% (v/v) absorbed rabbit serum and resuspended in the serum-PBS to give a final volume of 1% (v/v). The absorbed rabbit serum consisted of the supernatant fluid obtained after 1 ml normal rabbit serum was incubated with 1 ml 1% (v/v) formalinized tanned erythrocytes for 30 min at room temperature.

Titration of Anti-EA in Mouse Sera by Passive Haemagglutination:

This technique together with an evaluation was reported by Herbert (1967). Before testing, each antiserum was twice-absorbed with equal volumes of formalinized tanned erythrocytes (25% v/v) to remove heterophile antibodies. Serial dilutions of each absorbed antiserum (0.1 ml) were made with PBS-serum in the wells of a haemagglutination tray. To each well was added 0.1 ml of 1% (v/v) tanned erythrocyte EA suspension and the titrations incubated in a moist chamber at room temperature for 18 hr. A positive agglutination reaction was represented by a complete carpet of cells covering the bottom of the well. A negative result was indicated by a small ring or button of cells. The end-point was taken as the highest dilution of serum showing a fully positive reaction; results were expressed as $\log_2$ titre.

Calculation of Relative Adjuvant Activity:

Each adjuvanticity test was set up as a 4-point bioassay with five observations at each of the two doses of standard and unknown poly-

saccharide. A linear relationship between the $\log_{10}$ dose of adjuvant and $\log_2$ HA titre was assumed and the results calculated by standard procedures for the 4-point assay (Finney "Statistical Methods in Biological Assay," 1971) using an Olivetti P101 desk calculator and a programme prepared by Professor A.C. Wardlaw.

This programme provided the following information :-

1) Analysis of variance F-ratios to check for departures from parallelism, for significant slope and for differences between preparations.

2) Log of the relative adjuvant activity.

3) Logs of the lower and upper 95% Confidence Limits of the estimate of the relative adjuvant activity.

(X) HAEMOLYTIC ACTIVITY OF BACTERIAL POLYSACCHARIDES

For the purpose of this investigation, haemolysis was defined as the release (or leakage) of substances into the supernatant fluid which had strong absorptions at 412 nm and 540 nm when measured in a Unicam SP 500 spectrophotometer. The bacterial polysaccharides were bound to the erythrocytes by the modified method of Middlebrook and Dubos (1948) as previously described. The cell suspensions were centrifuged at 2000 rpm (350 x g) for 5 min and the supernatant fluids decanted and read at 412 nm (OD_1). The cells were resuspended in the supernatant fluid and a small amount of saponin (BDH Ltd.) added and the suspension agitated until haemolysis was complete. The resultant fluid was again read at 412 nm

to give values termed OD_2. The per cent haemolysis was calculated using the formula :-

$$\text{Per cent haemolysis} = \frac{OD_1}{OD_2} \times 100$$

To examine the stability of polysaccharide-coated erythrocytes, the method described by Ciznar and Shands (1971) was used. One millilitre of an erythrocyte suspension (5%) was incubated at 37°C for 1 hr with 20 mg of the bacterial polysaccharide in 1 ml normal saline. After incubation the cells were centrifuged at 2000 rpm (350 x *g*) for 5 min. The cells were washed in normal saline and resuspended to a final volume of 2 ml in normal saline. The cells were placed at 4°C and the amount of haemoglobin released into the supernatant fluid was measured for adsorption at 412nm in a Pye Unicam SP 500 spectrophotometer. The per cent haemolysis was based on a comparison with a sample of red cells haemolysed with saponin. Control untreated erythrocytes were incubated and OD_{412nm} read in parallel with the test cells.

RESULTS

SECTION A: UPTAKE OF BACTERIAL POLYSACCHARIDES TO MAMMALIAN CELL SURFACES

During this investigation, the affinity of bacterial polysaccharides for mammalian cell surfaces was studied by several different techniques. Initially a chemical method was devised to measure directly the polysaccharide adsorbed by the cells (Davies and Stewart-Tull, 1973; Appendix XI). Later LPS from E. coli NCTC 8623 was radioactively labelled with either ^{14}C or ^{32}P, and the uptake measured by depletion of the radioactive counts in the supernatant fluids of cells exposed to labelled LPS. The relative affinities of the different bacterial polysaccharides was determined by their ability to displace labelled E. coli LPS in competitive adsorption tests.

The Influence of the Cell

The quantitative variation in polysaccharide adsorption

If the same mycobacterial fraction was tested for its modifying capacity with erythrocytes from the same animal, it was found that the amount of mycobacterial fraction adsorbing to the erythrocytes varied from day to day (Table 6). Under the same conditions, the amount of mycobacterial glycopeptide (ST 208) adsorbed by the erythrocytes varied between 0.44 and 2.4 mg. On subsequent analysis it was found that the carbohydrate concentration of untreated sheep erythrocytes varied considerably from 46 to 77 mg per 100 ml.. By comparing the amount of glycopeptide absorbed by the erythrocytes with the carbohydrate content

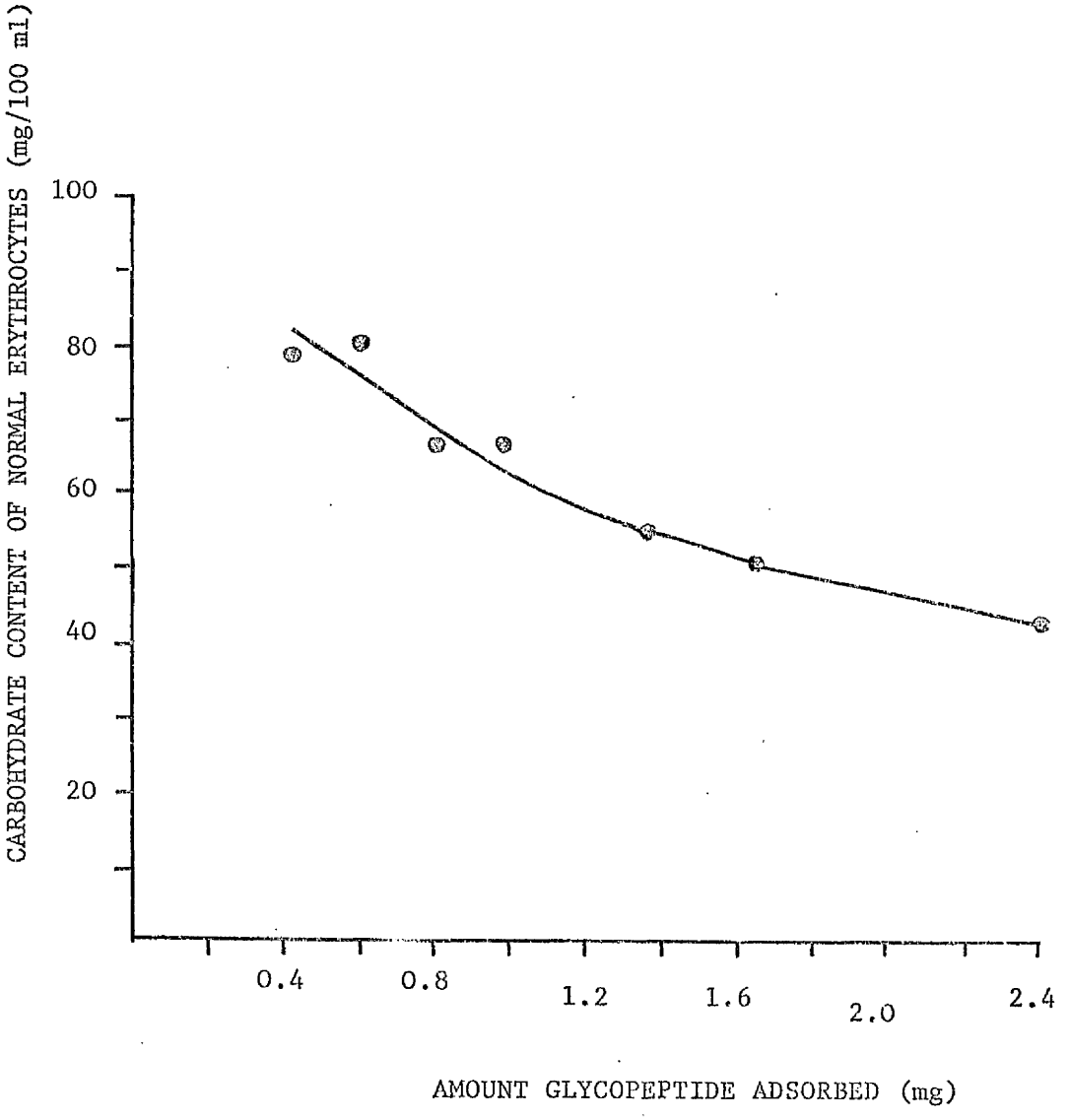
Table 6: THE VARIATION IN THE UPTAKE OF A MYCOBACTERIAL GLYCOPEPTIDE (ST208) BY SHEEP ERYTHROCYTES FROM A SINGLE ANIMAL*

mg carbohydrate /100 ml to the Haldane Standard of the normal untreated erythrocytes	Increase in carbohydrate content of the erythrocytes (%)	Total increase in carbohydrate content (mg)	Mycobacterial Glycopeptide	
			Amount adsorbed (mg)	Amount adsorbed / Amount added x 100
51.13	60.3	0.60	1.66	8.3
54.01	38.7	0.50	1.38	6.9
66.64	27.8	0.37	1.02	5.1
77.87	10.3	0.16	0.44	2.2
66.07	28.1	0.30	0.84	4.2
46.03	52.4	0.86	2.4	12.4
79.32	14.0	0.22	0.61	3.05

* The results are arranged in chronological order.

The carbohydrate content of the untreated sheep erythrocytes (mg carbohydrate/100 ml) were plotted against the amount of glycopeptide adsorbed (mg) (Figure 17).

Figure 17: The Variation in the Uptake of Glycopeptide with Carbohydrate Content of the Erythrocytes



of the erythrocytes (Figure 17) it was observed that an inverse relationship existed. Hence erythrocytes showing high carbohydrate content were able to bind only relatively small quantities of the mycobacterial glycopeptide. The actual reason for the fluctuation of the carbohydrate content of the erythrocytes was not determined but there are numerous reports showing that fluctuations in erythrocyte membrane composition can arise from variations in diet (McBride & Jacob, 1970; Westerman, Wiggans & Mao, 1970; Sardet, Hansma & Ostwald, 1972).

Effect of different erythrocyte species

A range of mycobacterial fractions were tested for their ability to adsorb to mouse, horse, rabbit, human and sheep erythrocytes. The mycobacterial fraction (20 mg) was incubated with the erythrocytes at $37^{\circ}C$ for one hour. The amount of each fraction adsorbed by erythrocytes from each species was calculated by the chemical method (Table 7). It was evident that the mycobacterial fraction, ST210, possessed the greatest affinity for erythrocyte membranes and that fraction ST206 possessed the least affinity. Generally, the fractions could be arranged in order of increasing affinity as ST206, < ST209, < ST202, < ST208, < ST210. There were exceptions to this order in the sheep erythrocytes with fractions ST208 and ST209 and in the horse erythrocytes with the mycobacterial fractions ST202 and ST206.

Effect of erythrocyte concentration

The effect of erythrocyte concentration on the uptake of bacterial polysaccharides was studied using ^{32}P- and ^{14}C-labelled *E. coli*

Table 7: THE UPTAKE OF MYCOBACTERIAL POLYSACCHARIDES BY MAMMALIAN ERYTHROCYTES

Mycobacterial fraction	Hexose content of poly-saccharide (%)	Increase in carbohydrate content of cells		Amount polysaccharide adsorbed (mg)	% Fraction adsorbed
		%	mg/ml		
Mouse Erythrocytes					
ST202	37.6	70.2	0.56	2.96	14.8
ST206	50.0	76.2	0.61	2.44	12.2
ST208	36.0	70.2	0.56	3.10	15.5
ST209	27.6	42.6	0.34	2.46	12.3
ST210	12.0	38.1	0.30	4.98	24.9
Horse Erythrocytes					
ST202	37.6	27.4	0.29	1.53	7.6
ST206	50.0	56.7	0.61	2.44	12.2
ST208	36.0	63.8	0.68	3.76	18.8
ST209	27.6	22.9	0.24	1.73	8.6
ST210	12.0	54.7	0.58	9.62	48.1
Rabbit Erythrocytes					
ST202	37.6	8.8	0.20	1.06	5.3
ST206	50.0	0.68	0.016	0.064	0.32
ST208	36.0	23.5	0.55	3.06	15.3
ST209	27.6	5.1	0.12	0.86	4.3
ST210	12.0	10.3	0.24	4.02	20.1
Human Erythrocytes					
ST202	37.6	49.3	0.78	4.13	20.6
ST206	50.0	17.6	0.28	1.12	5.6
ST208	36.0	51.8	0.82	4.5	22.6
ST209	27.6	17.6	0.28	2.02	10.1
ST210	12.0	20.8	0.33	5.47	27.3
Sheep Erythrocytes					
ST202	37.6	74.7	0.27	1.46	7.3
ST206	50.0	54.6	0.28	1.14	5.7
ST208	36.0	58.3	0.20	1.16	5.8
ST209	27.6	40.2	0.35	2.58	12.9
ST210	12.0	35.5	0.16	2.82	14.1

NCTC 8623 LPS and rabbit erythrocytes. The numbers of erythrocytes used ranged from 0.812 x 10^7 to 1.56 x 10^9 and the reaction mixtures were incubated at 37°C for 1 hr. The results in Figure 19 show that the amount of LPS adsorbed to the rabbit erythrocytes was dependent on the number of cells available up to a certain level - about 10^9 cells per test mixture. If the number of cells present was increased above 10^9, no further adsorption of LPS to the cells could be detected. Hence the maximum amount of LPS adsorbed was 45% of the available LPS. The apparent inability to bind the remaining 66% could be a characteristic of this system. From investigations using ^{14}C-LPS (Figure 18) and ^{32}P-LPS (Figure 19) it was found that the uptake of LPS as a function of erythrocyte concentration was not linear, but followed three distinct phases. During this process with ^{32}P-LPS in the initial uptake stage (I), there was an uptake of 2.8% of the LPS, in the second phase (II) there was the uptake of a further 31.2% of the LPS. The remainder of the LPS (66%), as previously explained, was not adsorbed by the erythrocytes (phase III). A similar effect was noted with ^{14}C-LPS.

Effect of different types of mouse cell

Cell suspensions of peritoneal lymphocytes and macrophages, spleen cells, thymus cells, bone marrow cells and erythrocytes were prepared from the same mouse. It was considered essential that the different cells should come from the same animal to facilitate a comparison of the results without the complication of a between-animal variation. The cell suspensions (5.0 and 2.5 x 10^8 cells per test) were incubated with ^{32}P-LPS (6837 dpm), and the amount of LPS adsorbed by each cell type was calculated in terms of the radioactivity (dpm) of the cell pellet

Figure 18: The Uptake of ^{14}C-LPS from E. coli NCTC 8623 by Different Concentrations of Rabbit Erythrocytes

The amount of LPS adsorbed by the cells was calculated in terms of the radioactivity (dpm) associated with the cell pellet and plotted against the number of rabbit erythrocytes (cell number x 10^8). Graph A represents an added amount of LPS of 4803 dpm and Graph B an added amount of LPS of 2104 dpm.

I represents the standard error associated with each observation.

The full results are presented in Appendix VII.

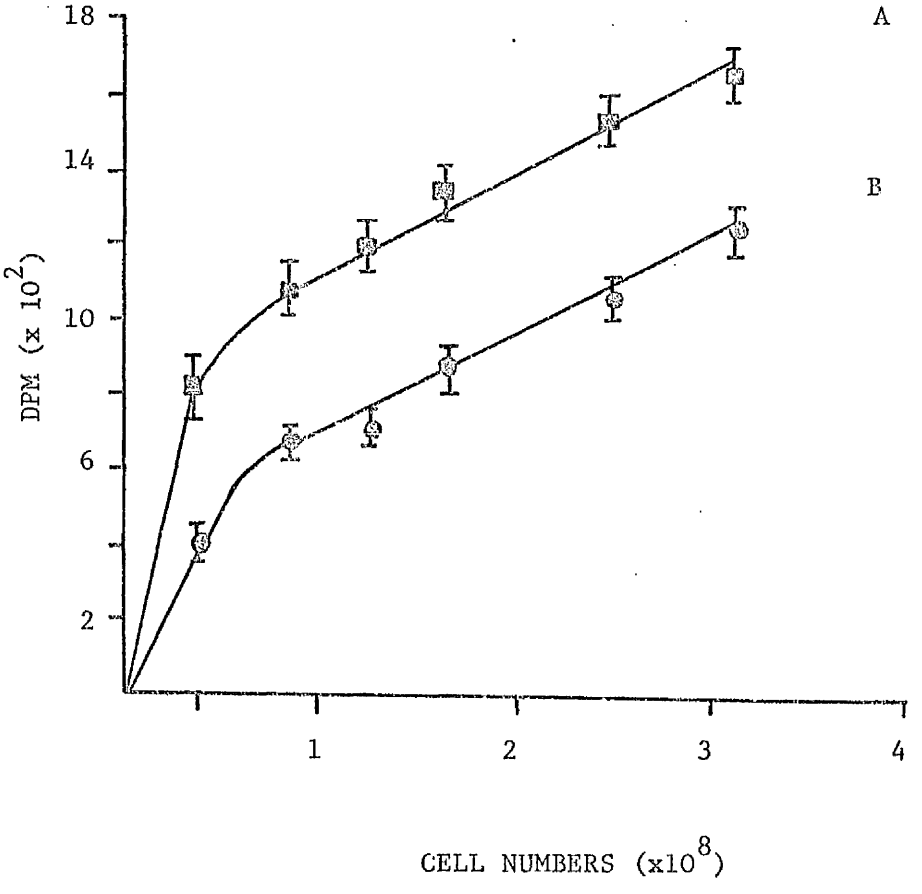

18
14
10
6
2
DPM (x 10^2)
A
B
1
2
3
4
CELL NUMBERS (x10^8)

Figure 19: The Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Different Concentrations of Rabbit Erythrocytes

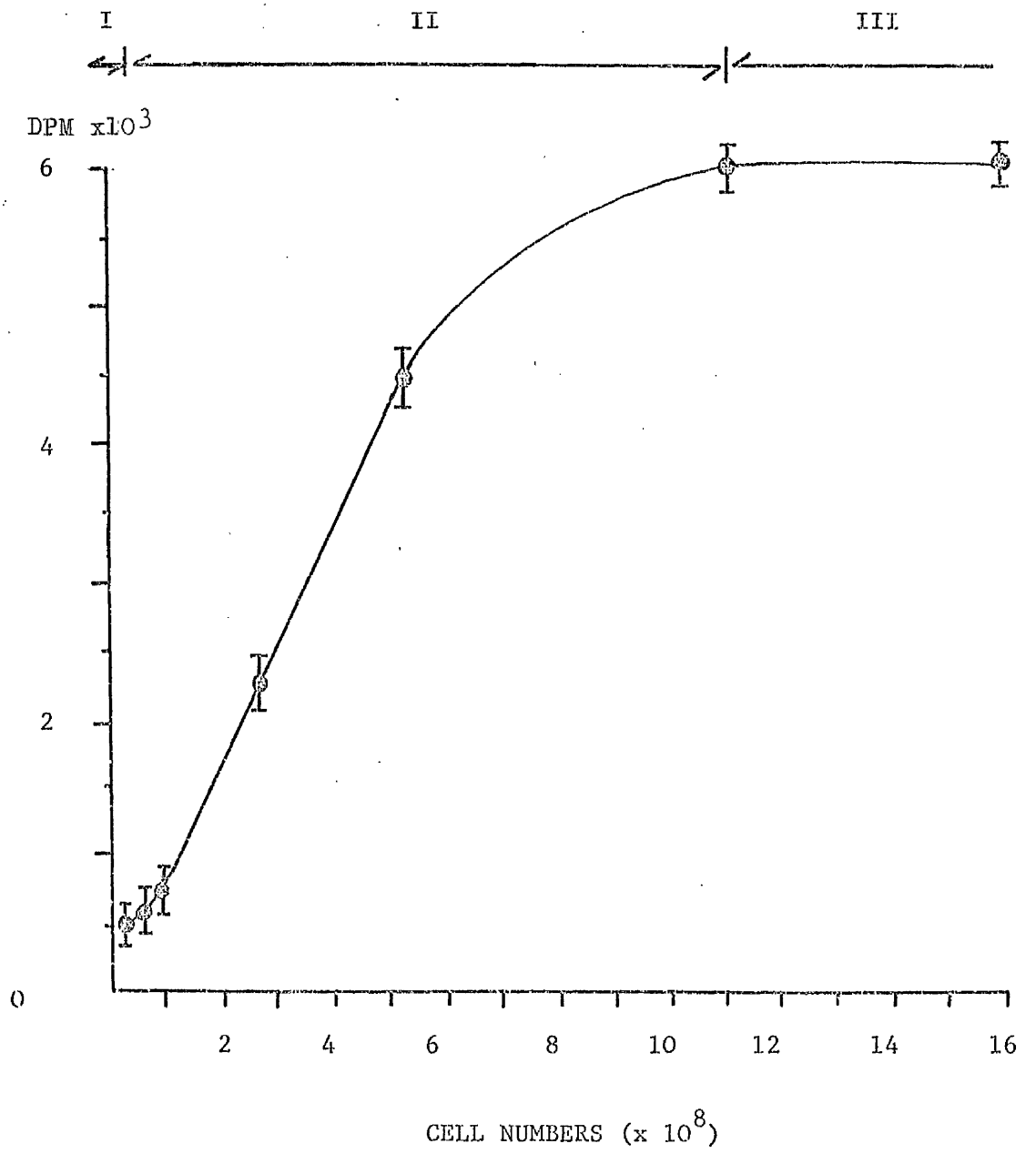
The amount of LPS adsorbed by the cells was calculated in terms of the radioactivity (dpm) associated with the cell pellet and plotted against the number of rabbit erythrocytes used. (Cell number x 10^8).

Symbols

I, II and III represented the three phases of LPS adsorption (page 93).

I represents the standard error associated with the observation.

The full results are presented in Appendix VII.


I
II
III
DPM x10^3
6
4
2
0
2
4
6
8
10
12
14
16
CELL NUMBERS (x 10^8)

(Table 8). An analysis of variance was carried out using the erythrocyte result as the standard and the relative affinity of LPS for the different cell types was calculated (Table 9). The results from the F ratios and subsequent probability values showed that the uptake of ^{32}P-LPS was dependent on the number of cells present. ~~The ^{32}P-LPS was dependent on the number of cells present.~~ The ^{32}P-LPS was most efficiently adsorbed by macrophages (relative affinity 11.51) compared with the erythrocytes (relative affinity 1.0) which presumably reflected the phagocytic activity of these reticuloendothelial cells. The affinity of the LPS was approximately equal for spleen, thymus and bone marrow cells (relative affinity 5.63, 6.50 and 6.49 respectively); the lymphocytes were poor at absorbing LPS.

The Effect of Polysaccharide Concentration

Variable amounts of the polysaccharide (0.03 mg to 18 mg) were incubated with either 4.8 x 10^8 human or rabbit erythrocytes for one hour at 37^{o}C. When the erythrocytes were incubated with the mycobacterial glycopeptide (ST208), the results (Figure 20) showed that the amount of polysaccharide adsorbed was dependent on the amount of the polysaccharide in the reaction mixture. The relationship obtained was linear for both rabbit and human erythrocytes. However, there was a limit to the amount of glycopeptide that could be absorbed by either of the erythrocyte species. From Figure 20 the maximum average amount of glycopeptide adsorbed by a single rabbit erythrocyte was 1.85 pgram. Similarly the maximum average amount of glycopeptide adsorbed by a human erythrocyte was 1.89 pgram. Presumably at such concentrations all the available sites for the adsorption

Table 8: THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY DIFFERENT CELLS FROM ADULT CD MICE

Cells	Number of cells ($\times 10^7$)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (25 dpm)	Total dpm associated with cell pellet	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$
Peritoneal lymphocytes	5.0	86.70	6101	736	0.107
	2.5	87.15	6276	571	0.083
Peritoneal macrophages	5.0	86.65	5606	1185	0.173
	2.5	87.75	5856	980	0.143
Erythrocytes	5.0	86.60	6284	552	0.080
	2.5	87.25	6420	417	0.061
Spleen cells	5.0	86.75	5876	961	0.140
	2.5	87.35	6050	787	0.114
Thymus cells	5.0	87.00	5866	971	0.142
	2.5	86.80	6034	803	0.117
Bone marrow cells	5.0	86.85	5785	1052	0.153
	2.5	87.55	5993	844	0.123

Table 9: ANALYSIS OF VARIANCE ON UPTAKE OF ^{32}P-LPS BY VARIOUS MOUSE CELL TYPES

All preparations and cell lines are compared to the erythrocyte result

Cell type	Probability Values for the Null Hypothesis (%)			Relative Affinity for LPS	95% Confidence Limits	
	Cell types (preparations)	Slope	Parallelism		Upper limit	Lower limit
Erythrocyte	-	-	-	1.0	-	-
Lymphocyte	< 1	< 1	> 10	2.19	3.08	1.75
Macrophage	< 1	< 1	< 1	11.51	13.32	9.54
Spleen	< 1	< 1	> 10	5.63	7.78	4.41
Thymus	< 1	< 1	> 10	6.50	8.23	4.77
Bone marrow	< 1	< 1	> 10	6.49	8.32	5.31

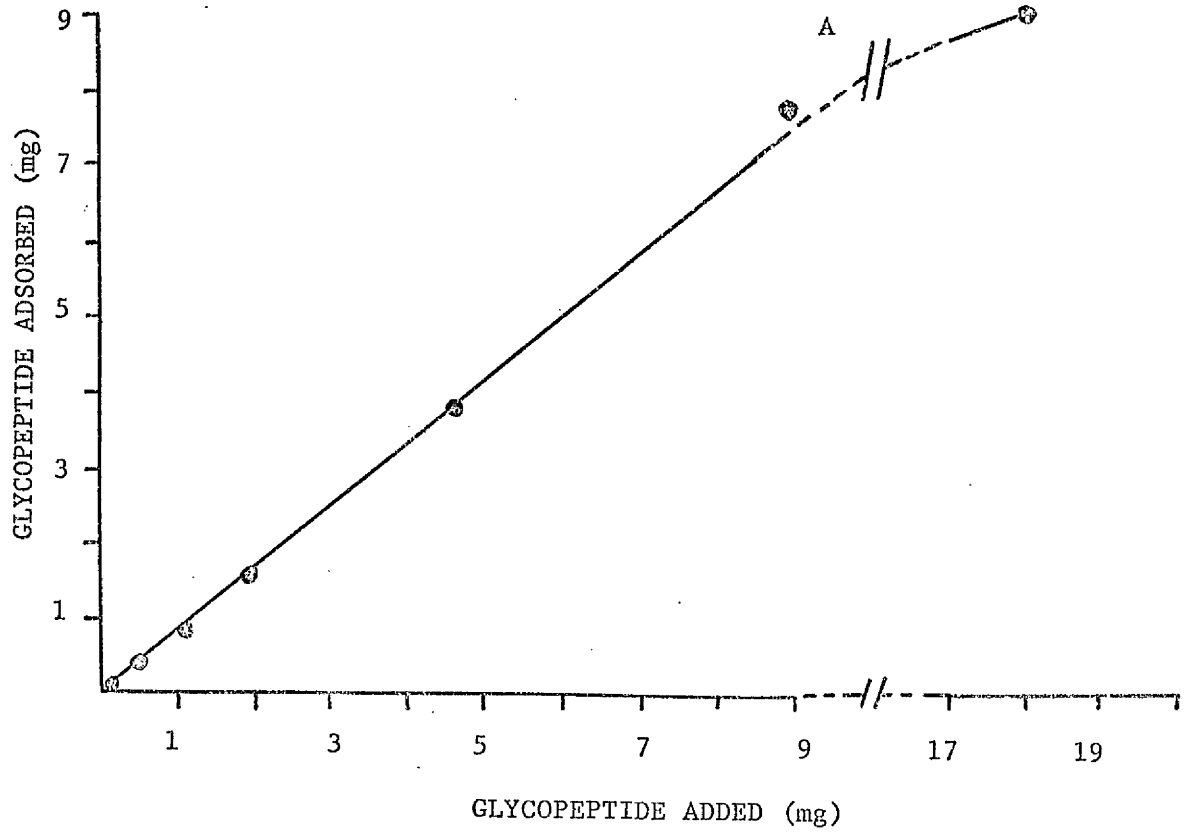
The analysis of variance results compare the uptake of ^{32}P-LPS by different mouse cell lines to the uptake of ^{32}P-LPS by mouse erythrocytes. The probability values for the cell preparations indicate that at a probability value of less than one per cent the adsorption of LPS by the various cell lines is different than the adsorption obtained using the erythrocytes. If the probability value for the cell preparations was greater than 10 per cent, it could be concluded that the uptake of LPS by any cell line was similar to the uptake obtained using erythrocytes. From the results it was concluded that all the cell lines adsorbed LPS to a different extent than erythrocytes.

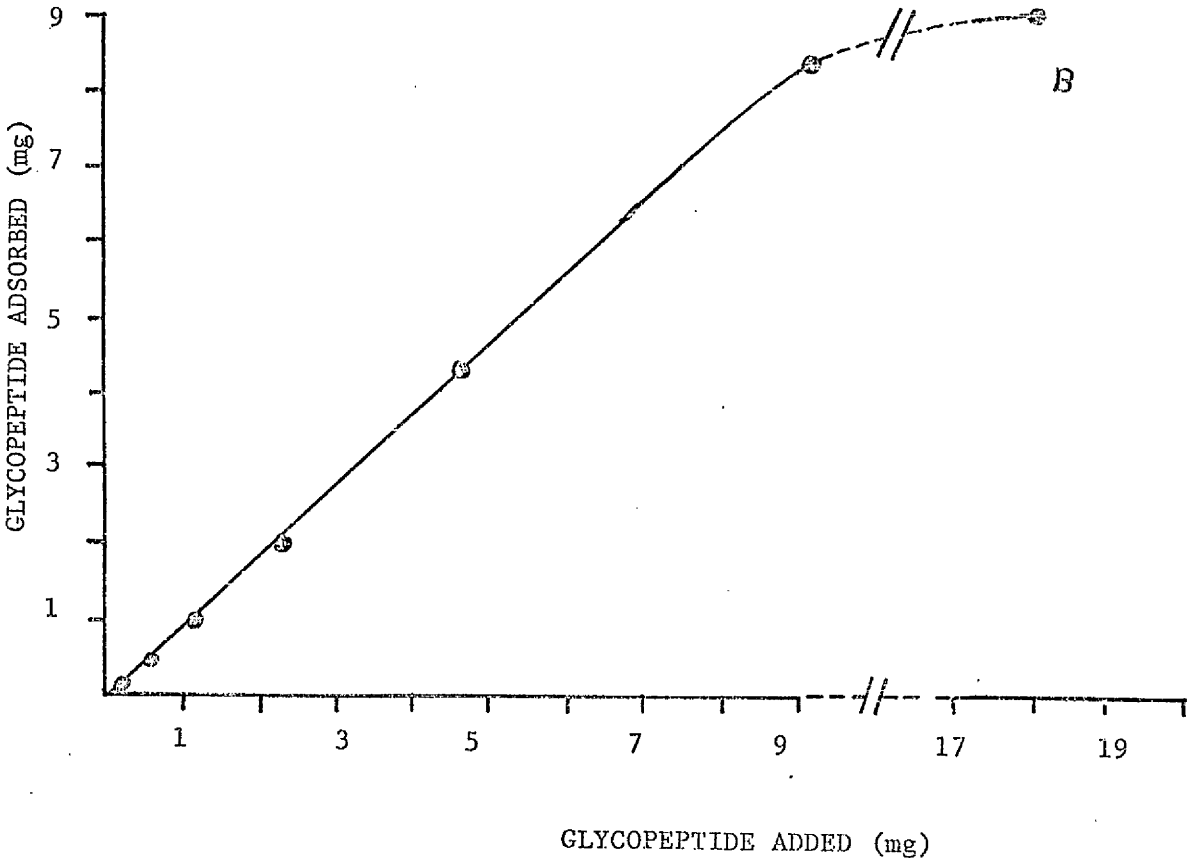
The cell lines were tested for the adsorption of LPS at 5.0×10^7 and 2.5×10^7 cells. The probability values of the slope indicate whether the adsorption at the high cell concentration is greater than at the low cell concentration. If the probability value is less than one per cent then there is a difference in the adsorption of LPS at the two cell concentrations. A probability value of greater than 10 per cent would indicate that the LPS adsorption was independent of the cell concentration. From the results which have probability values of less than one per cent, it can be concluded that over the cell concentration range used, the adsorption of LPS is dependent on the cell numbers present.

The probability values concerned with parallelism indicate whether the comparative levels of adsorption of LPS by the different cells are the same at both the low and high cell concentrations. If the probability values are greater than 10 per cent, the comparative levels of adsorption are the same at both the low and high cell concentrations. A probability value of less than one per cent indicates a difference in the comparative level of adsorption at either the low or high cell concentration. The results indicate that lymphocytes, spleen, thymus and bone marrow cells have similar comparative adsorption levels, but the macrophages do not have similar comparative adsorptions which could affect the accuracy of the relative affinity value.

Figure 20: The uptake of Variable Amounts of Mycobacterial Glycopeptide (ST208) by (a) Rabbit (b) Human Erythrocytes

The amount of glycopeptide (mg) adsorbed by the erythrocytes was plotted against the amount of glycopeptide (mg) added to the system. Figure A represents the result using rabbit erythrocytes and Figure B the result with human erythrocytes. The full results are presented in Appendix VI.


A
GLYCOPEPTIDE ADSORBED (mg)
9
7
5
3
1
1
3
5
7
9
17
19
GLYCOPEPTIDE ADDED (mg)


B
GLYCOPEPTIDE ADSORBED (mg)
9
7
5
3
1
1
3
5
7
9
17
19
GLYCOPEPTIDE ADDED (mg)

of glycopeptide were filled. In similar experiments using LPS from E. coli NCTC 8623 it was found that the amount of LPS adsorbed by the cells was dependent on the amount of LPS in the reaction mixture. From Figure 21 the maximum average amount of LPS adsorbed by a single rabbit erythrocyte was 14.1 pgram and by each human erythrocyte 16 pgram. From this it was concluded that human erythrocytes were able to adsorb greater quantities of LPS and glycopeptide than the rabbit erythrocytes.

A similar series of experiments to the above were performed in which different concentrations of ^{14}C-LPS were incubated with 1.57×10^8 human erythrocytes for one hour at 37°C. The degree of uptake was determined by the decrease in radioactivity in the supernatant fluid. The amount of LPS adsorbed (dpm) was plotted against the amount of LPS added (dpm) (Figure 22). The amount of LPS adsorbed by the erythrocytes was proportional to the amount of LPS added until a plateau was reached where further increases in the amount of LPS added did not produce corresponding increases in cell-associated LPS. It was assumed that the plateau represented the stage at which all the available sites for the adsorption of LPS were filled.

The Effect of Temperature and Time of Incubation

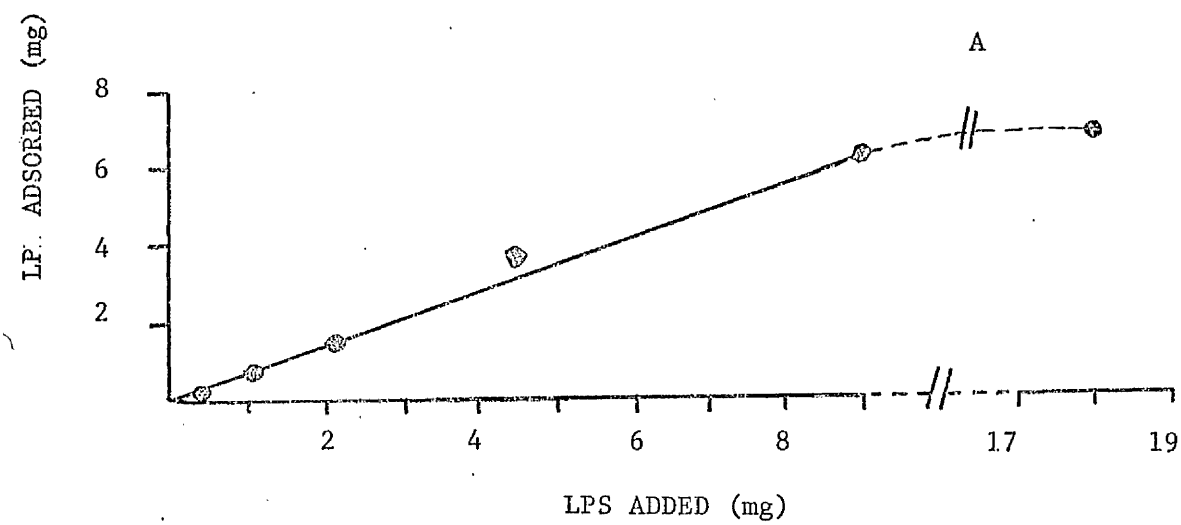
Temperature of incubation

A constant amount of ^{14}C-LPS (containing 1600 dpm) was incubated with 1.57×10^8 human erythrocytes for one hour at temperatures of incubation from 0°C to 50°C. It was found that the uptake of LPS by human erythrocytes was temperature dependent (Figure 23). The optimum

Figure 21: The Uptake of Variable Amounts of LPS from E. coli NCTC 8623 by (a) Rabbit (b) Human Erythrocytes

The amount of LPS (mg) adsorbed by the erythrocytes was plotted against the amount of LPS (mg) added to the system. Figure A represents the result using rabbit erythrocytes and Figure B the result with human erythrocytes.

The full results are presented in Appendix VI.


A
LP. ADSORBED (mg)
8
6
4
2
2
4
6
8
17
19
LPS ADDED (mg)

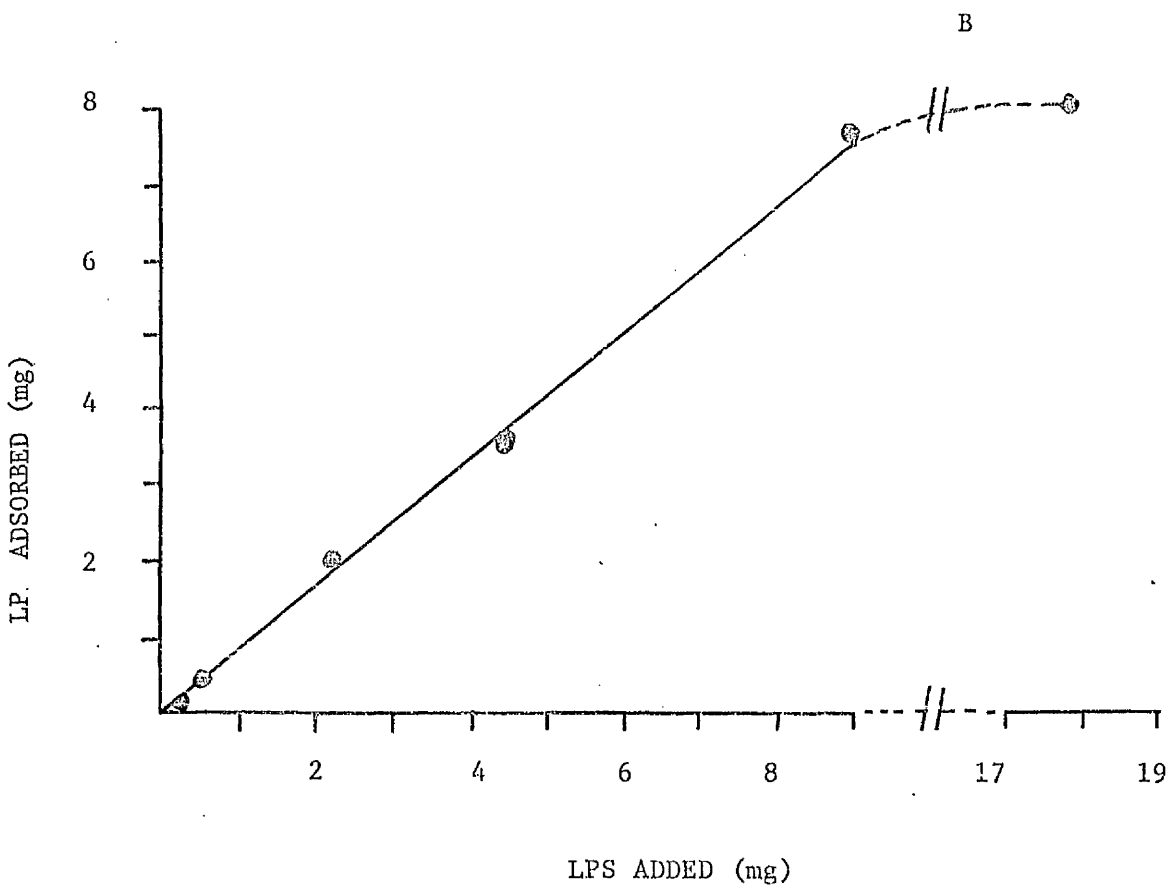

B
LP. ADSORBED (mg)
8
6
4
2
2
4
6
8
17
19
LPS ADDED (mg)

Figure 22: The Uptake of Variable Amounts of ^{14}C-LPS from *E. coli* NCTC 8623 by Human Erythrocytes

The amount of LPS adsorbed by the erythrocytes, in terms of the cell-associated radioactivity (dpm) was plotted against the amount of ^{32}P-LPS added (dpm).

Figure A represents the result in the presence of 3.14×10^8 erythrocytes and Figure B with 1.57×10^8 erythrocytes.

I represents the standard error associated with each observation.

The full results are presented in Appendix VII.

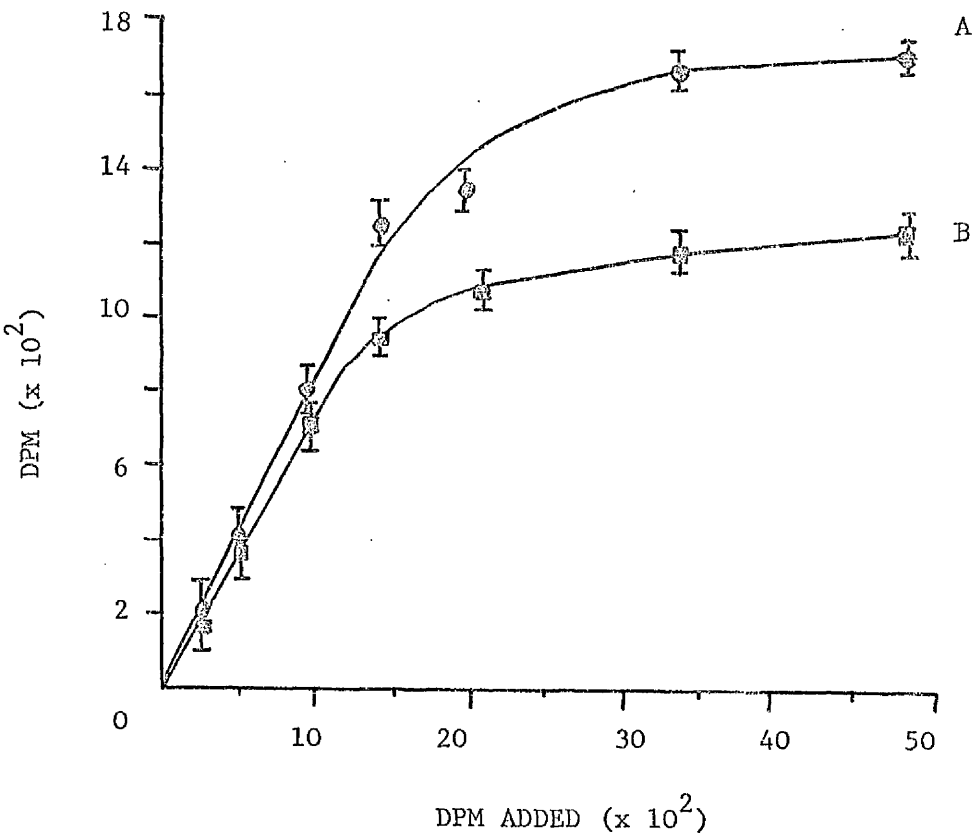
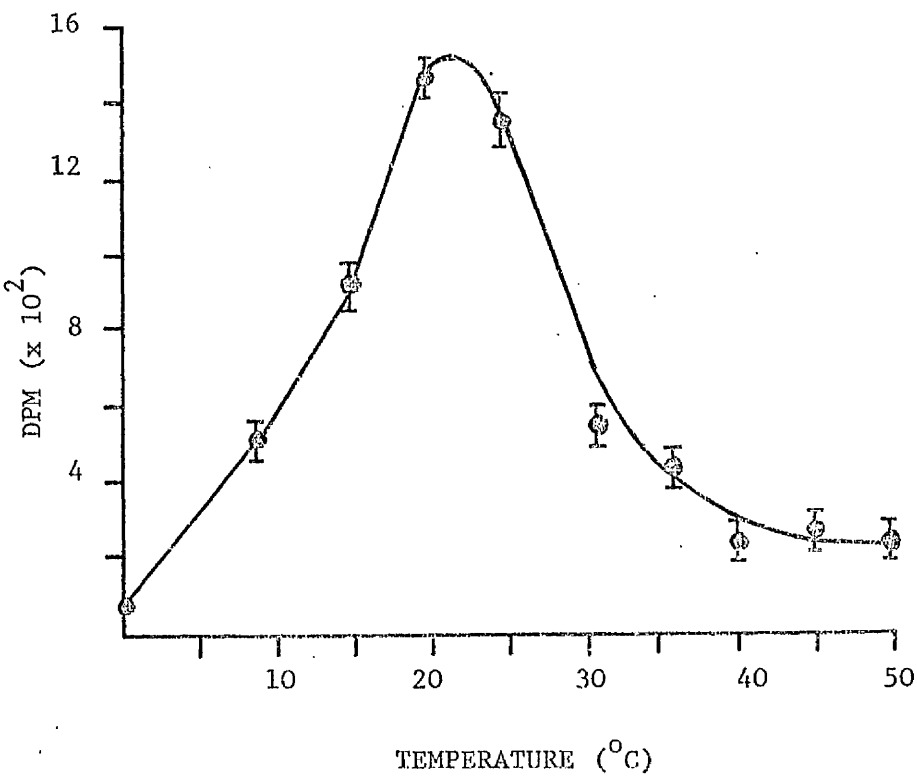

18
14
10
6
2
0
10
20
30
40
50
A
B
DPM (x 10^2)
DPM ADDED (x 10^2)

Figure 23: The Variation with Temperature of Incubation in the Uptake of ^{14}C-LPS by Human Erythrocytes



The amount of LPS adsorbed, in terms of the cell-associated radioactivity (dpm), was plotted against the temperature of incubation (^{o}C). The full results are presented in Appendix VII.

I represents the standard error associated with each observation.

temperature for this system was 22.5°C. Above 40°C, there was little change in the amount of LPS adsorbed, although the stability of the erythrocytes was probably affected.

All the experiments in this investigation were done at 37°C to allow a comparison with the results of other workers, and also this represented the temperature of the biological assays. The fact that all the binding studies were not carried out at the optimum temperature and its implications, will be discussed later.

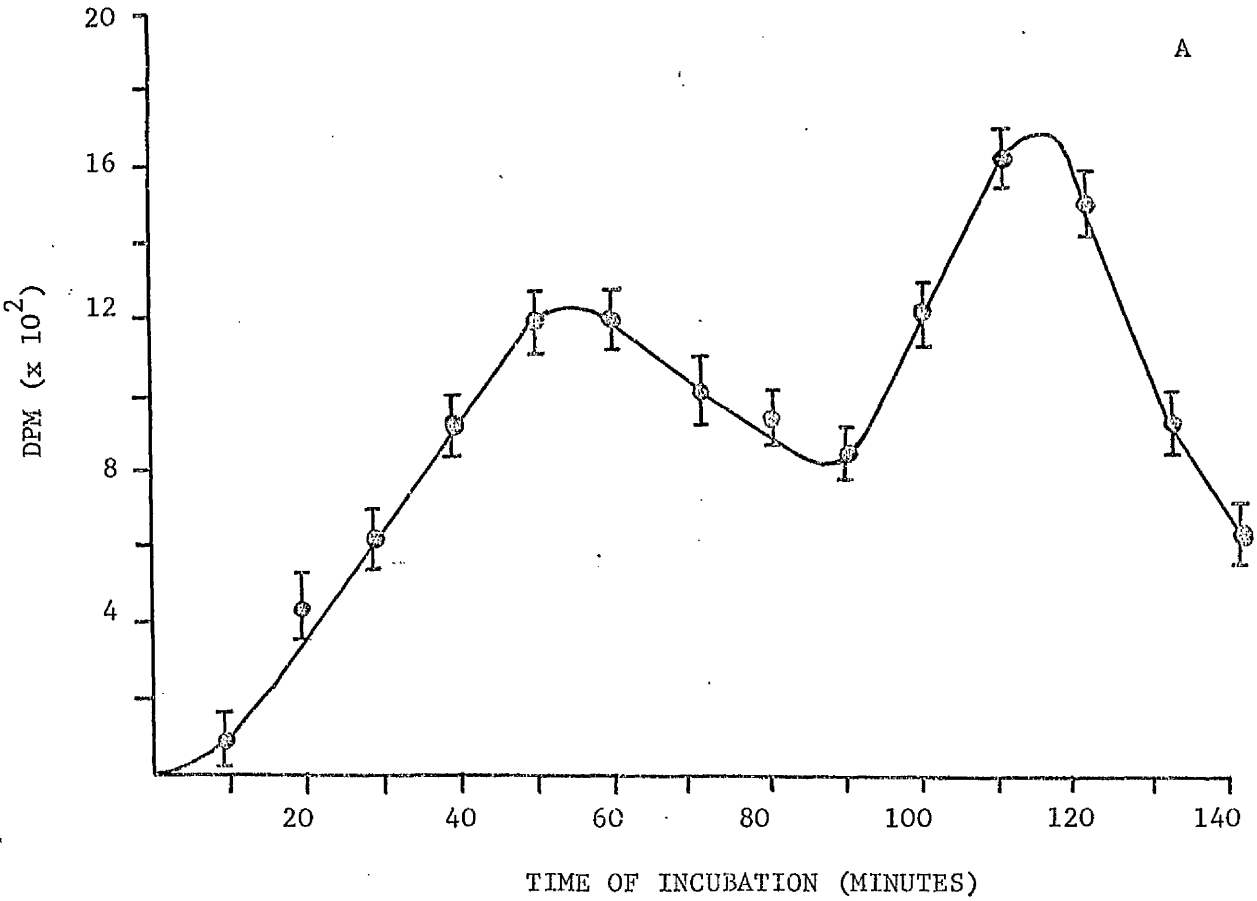
Time of incubation

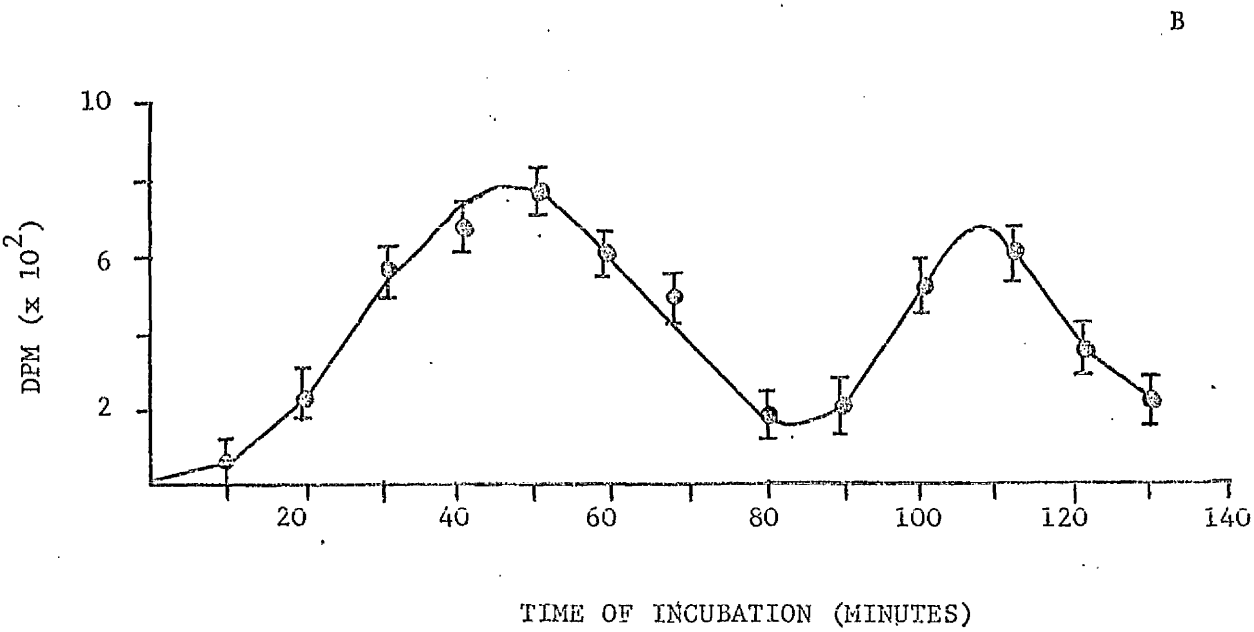
The variation in the uptake of ^{32}P-LPS by mammalian cells at 37°C was measured at different incubation times, from 10 to 180 min. The amount of LPS adsorbed by the cells was calculated in terms of the radioactivity associated with the cell pellet and plotted against the time of incubation. In all instances a cyclic fluctuation in the adsorption of LPS to the cells was observed. In each experiment the same LPS concentration and the same number of cells (1.57×10^8) were used. The human cells came from the same donor, with the erythrocyte and white blood cell suspensions prepared from the same sample of venous blood. From the results (Figure 24) the erythrocytes appeared to have a greater affinity for the LPS than the white blood cells. This difference in uptake could either be due to a greater abundance of LPS-receptor sites on erythrocyte surfaces or the greater avidity of erythrocyte receptors for the LPS molecules. The LPS uptake by human erythrocytes showed two peaks at 60 and 114 min (Figure 24). The white blood cells exhibited a similar response with peaks at 50 and 110 min. The rabbit erythrocytes showed

Figure 24: The Variation with Time of Incubation in the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Human Erythrocytes and White Blood Cells

The amount of LPS adsorbed by the cells, in terms of the cell-associated radioactivity (dpm) was plotted against the time of incubation (minutes). Figure A represents the result obtained with human erythrocytes and Figure B the result with human white blood cells. The full results, together with Chi-square analysis of the fluctuations, are presented in Appendix VII.

I represents the standard error associated with each observation.


A
20
16
12
8
4
DPM (x 10^2)
20
40
60
80
100
120
140
TIME OF INCUBATION (MINUTES)

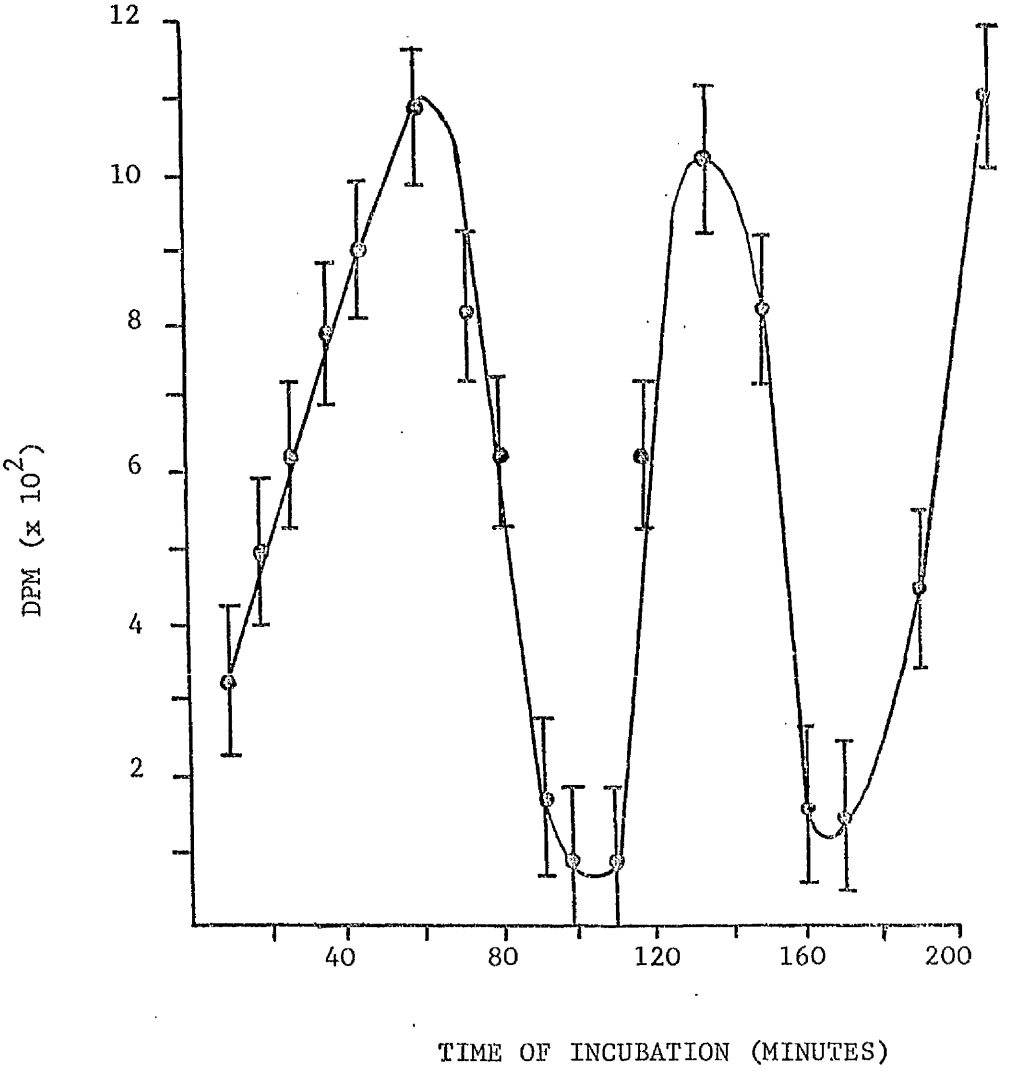

B
10
6
2
DPM (x 10^2)
20
40
60
80
100
120
140
TIME OF INCUBATION (MINUTES)

similar patterns of activity (~~Table~~ Fig 25), with peaks at 60 min and 130 min. Similarly mouse peritoneal exudate cells showed the same pattern of uptake (~~Table~~ Fig 26). The peritoneal macrophage uptake response was greater than the peritoneal lymphocyte response, which is in accordance with the functional activity of macrophages. The macrophage response was biphasic over 140 min, with peaks of uptake activity occurring at 54 and 89 min. However, the peritoneal lymphocytes showed an atypical biphasic pattern (Figure 26); there were peaks at 42 and 70 min.

When ^{14}C was used as the radioactive label, the uptake of LPS by human erythrocytes still showed the characteristic response, with peaks at 60 and 130 min. From these results the cyclic fluctuation in LPS adsorption is independent of the radioactive-label and is outside the limits of gross experimental error (Statistical Analysis - Appendix VII). The mode of adsorption and its implications in the biological effects exerted by LPS will be discussed later.

Under the same conditions, using the chemical technique to estimate adsorbed polysaccharide, similar results were obtained. The amount of mycobacterial glycopeptide (ST208) adsorbed by sheep erythrocytes showed an initial increase until 100 min when 5.5 mg of the glycopeptide was cell associated (Figure 27a). As the time progressed the amount of the cell-associated glycopeptide decreased. This apparent increase then decrease in the cell-associated glycopeptide tended to infer that the actual bond between the cell membrane and the glycopeptide was a relatively weak one. However, not all of the cell-associated glycopeptides became detached from the erythrocyte membrane since the fluorescent antibody technique indicated the continued presence of cell-associated glycopeptide throughout the experiment.

Figure 25: The Variation with Time of Incubation in the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes



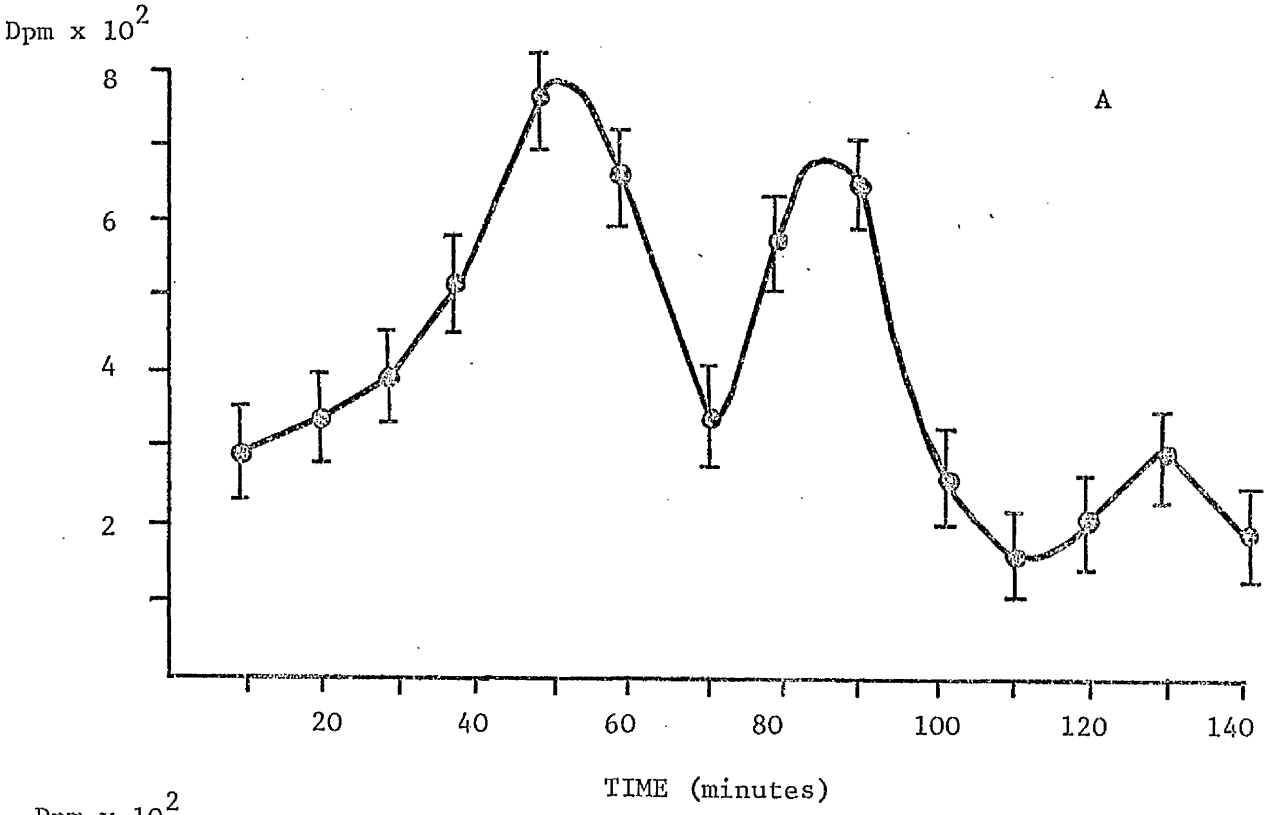
The amount of LPS adsorbed by the cells, in terms of the cell-associated radioactivity (dpm) was plotted against the time of incubation (minutes). The full results, together with Chi-square analysis of the fluctuations, are presented in Appendix VII.

I represents the standard error associated with each observation.

Figure 26: The Variation with Time of Incubation in the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Mouse Peritoneal Cells

The amount of LPS adsorbed by the cells in terms of the cell-associated radioactivity (dpm) was plotted against the time of incubation (minutes). Figure A represents the results with mouse peritoneal macrophages and Figure B with mouse peritoneal lymphocytes. The full results together with a Chi-square analysis of the fluctuations are presented in Appendix VII.

I represents the standard error associated with each observation.


Dpm x 10^2
8
6
4
2
A
20
40
60
80
100
120
140
TIME (minutes)

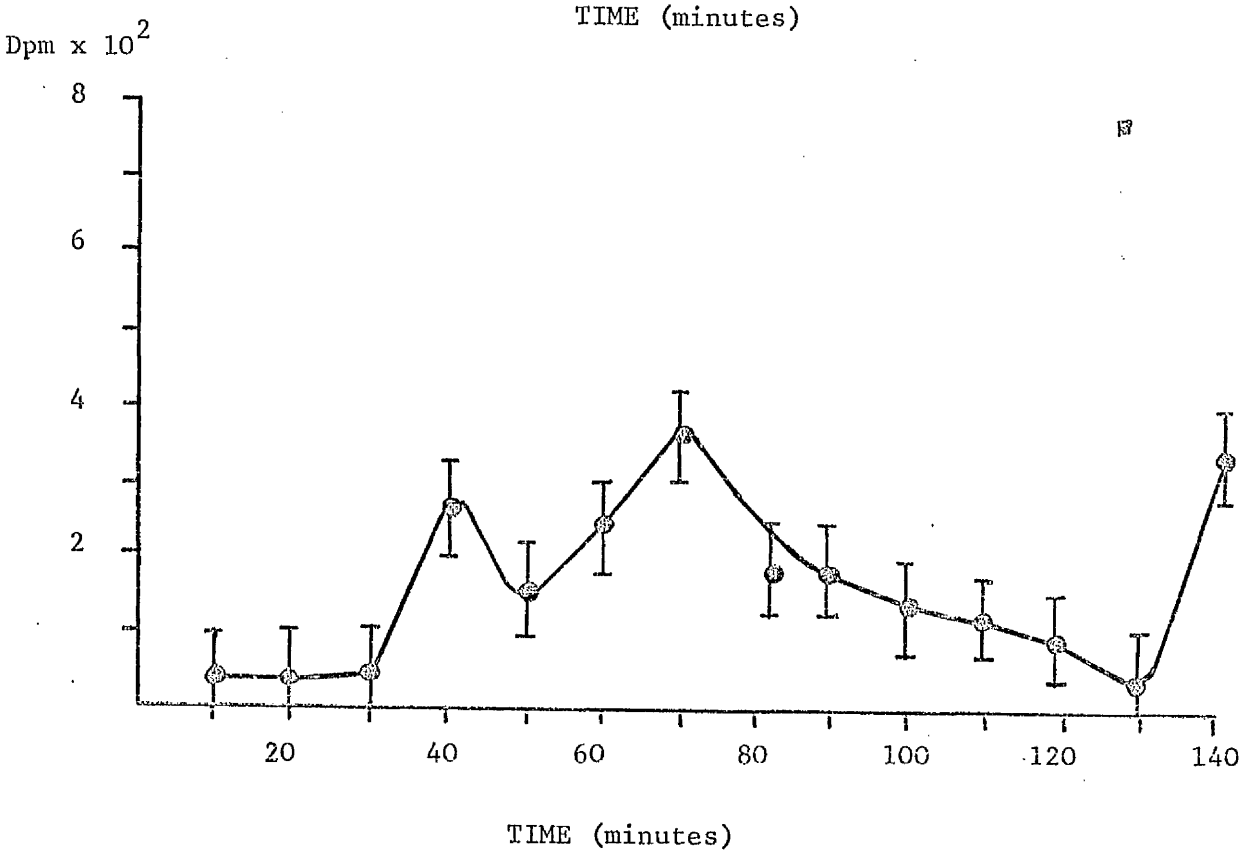
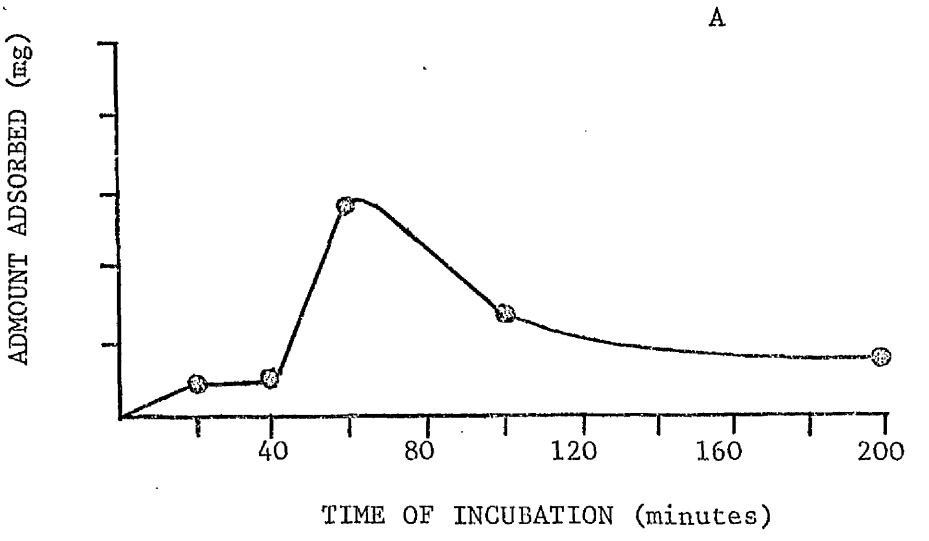

Dpm x 10^2
8
6
4
2
B
20
40
60
80
100
120
140
TIME (minutes)

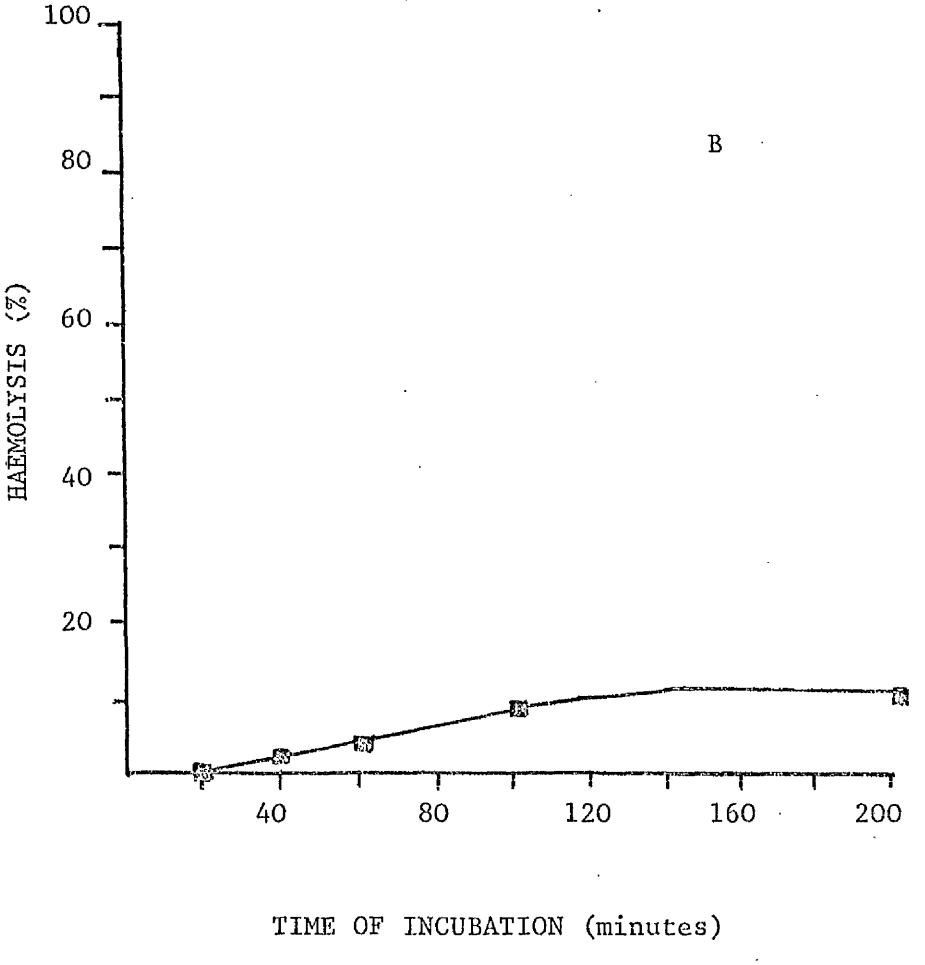
Figure 27: The Variation with Time of Incubation in the Uptake of Mycobacterial Glycopeptide (ST208) by Sheep Erythrocytes

Figure A : The amount of glycopeptide (mg) adsorbed by the erythrocytes was plotted against the time of incubation (minutes).

Figure B : The haemolysis (%) of the erythrocytes produced during the incubation was plotted against the time of incubation (minutes).

The full results are presented in Appendix VI.


A
ADMOUNT ADSORBED (mg)
40
80
120
160
200
TIME OF INCUBATION (minutes)


B
HAEMOLYSIS (%)
100
80
60
40
20
40
80
120
160
200
TIME OF INCUBATION (minutes)

During the incubation period, it was observed that the cells became slightly haemolysed with the leakage of haemoglobin into the supernatant fluid. This haemolysis reached a maximum level after 60 min of 11%, when compared to the haemolysis observed in untreated erythrocyte suspensions (Figure 27b).

Similarly sheep erythrocytes incubated with 20 mg of a LPS preparation from E. coli NCTC 8623 followed a similar adsorption response to the mycobacterial polysaccharide-incubated cells (Figure 28a). The maximum amount of LPS adsorbed by the cells occurred after 60 min, followed by a decrease in the cell-associated LPS. This fluctuation in the amount of cell-associated LPS tended to favour a weak bond between the cell membrane and the LPS molecule, although not all LPS molecules were removed from the cell surface, being demonstrable by the fluorescent antibody technique throughout the experiment. The maximum amount of LPS adsorbed corresponded to 11.35% of the material added. A leakage of the haemoglobin from the erythrocytes was observed during the incubation period (Figure 28b).

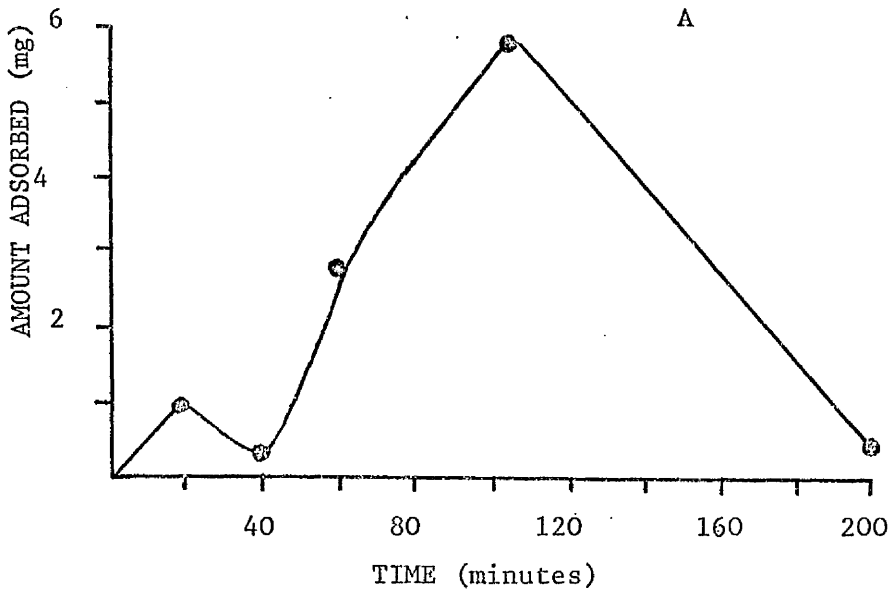
If rabbit erythrocytes were incubated for 200 min at 37°C with ^{32}P-LPS, peaks of uptake activity occurred at 60, 130 and 200 min; these peaks were termed I, II and III respectively (Figure 29a). The erythrocytes incubated for 60 min at 37°C were placed at 4°C and the supernatant fluid removed. The cells (termed t_{60} cells) were washed in saline, fresh ^{32}P-LPS added and incubation at 37°C continued. It was observed that peaks of uptake activity occurred after 80 and 140 min (Figure 29b). These peaks corresponded to peaks II and III of the original incubation mixture, and occurred at 140 and 200 min if the total time at 37°C of these cells was taken into account. The supernatant fluid from the t_{60} cells was added to an untreated suspension of rabbit

Figure 28: The Variation with Time of Incubation in the Uptake of LPS from E. coli NCTC 8623 by Sheep Erythrocytes

Figure A : The amount of LPS (mg) adsorbed by the erythrocytes was plotted against the time of incubation (minutes).

Figure B : The haemolysis (%) of the erythrocytes produced during incubation was plotted against the time of incubation (minutes).

The full results are presented in Appendix VI.


A
AMOUNT ADSORBED (mg)
6
4
2
40
80
120
160
200
TIME (minutes)

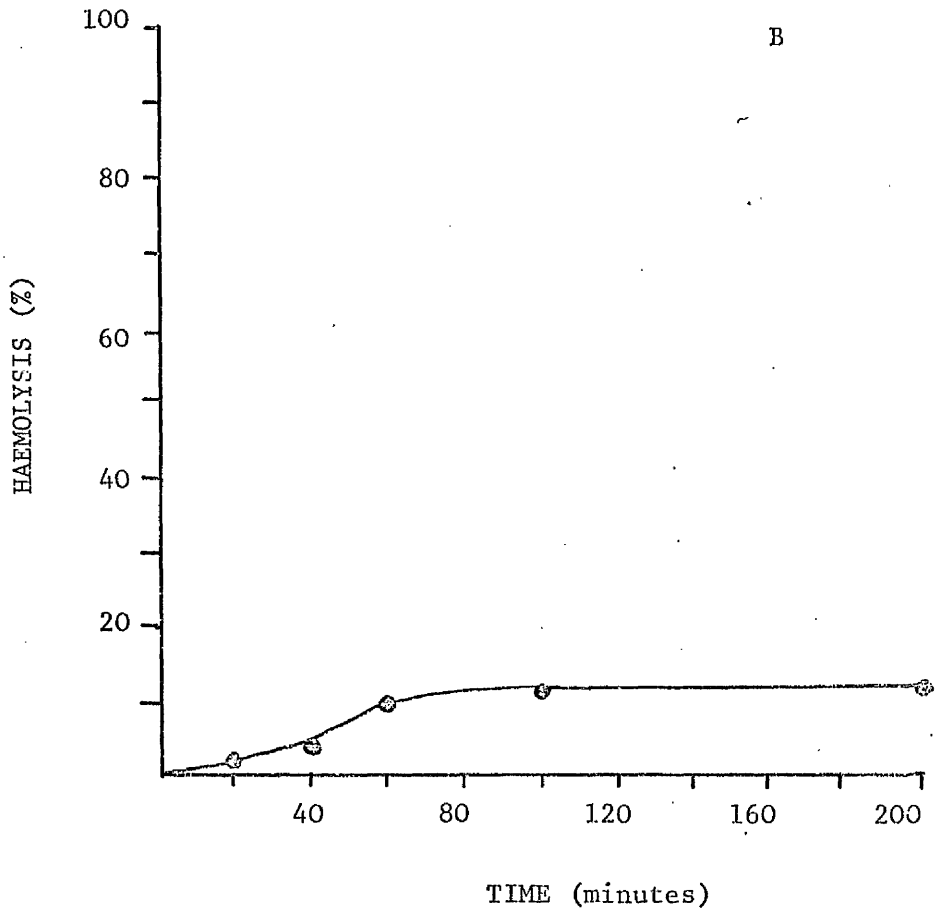
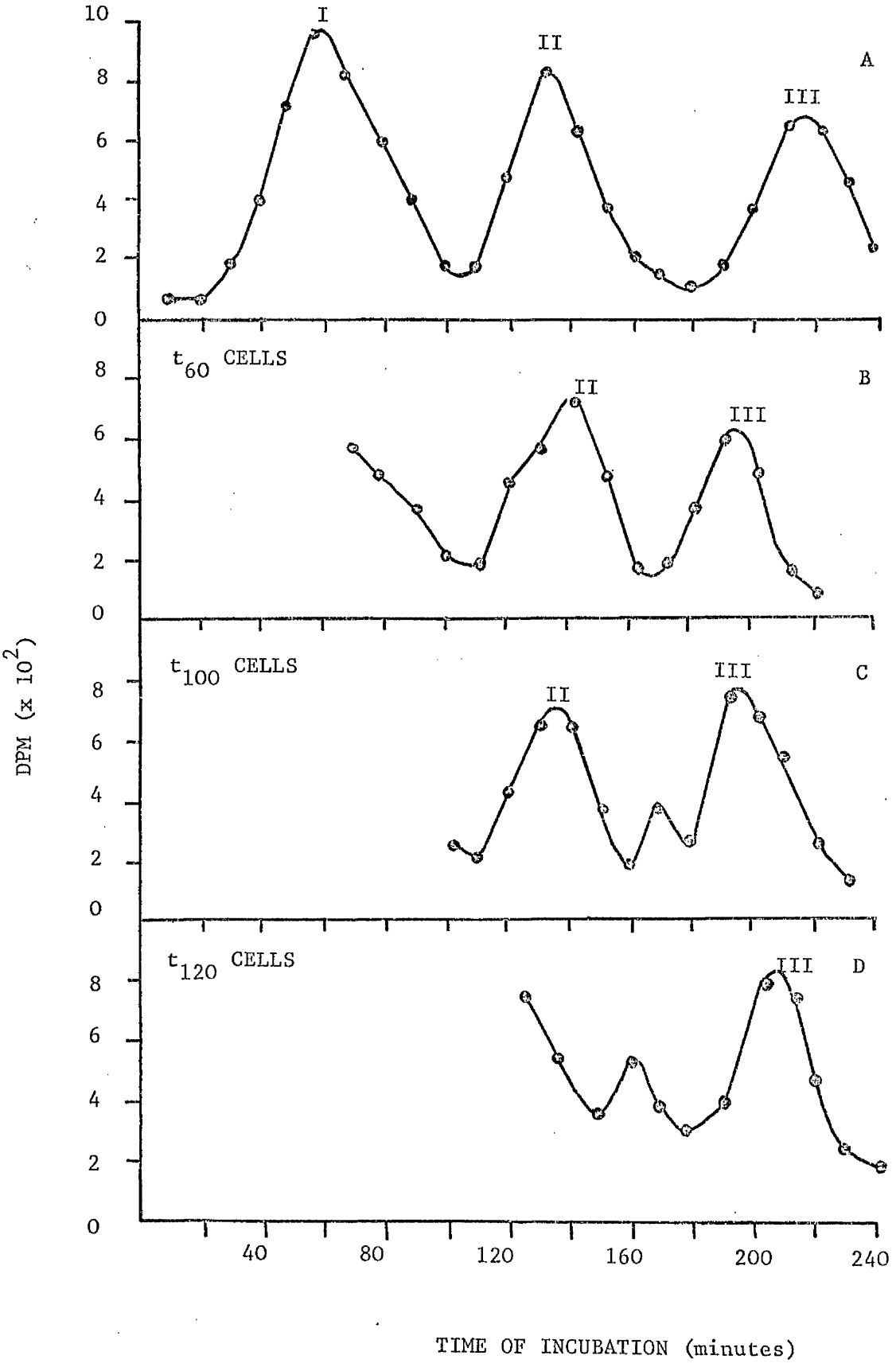

B
HAEMOLYSIS (%)
100
80
60
40
20
40
80
120
160
200
TIME (minutes)

Figure 29: The Variation with Time of Incubation in the Uptake of ^{32}P-LPS by Rabbit Erythrocytes. The Effect of Preincubating the Cells with LPS

The amount of LPS adsorbed by the cells in terms of the cell-associated radioactivity (dpm) was plotted against the time of incubation (minutes). Figure A represents a complete incubation. Figure B represents cells (t_{60}) incubated for 60 minutes, washed and reincubated with fresh ^{32}P-LPS. Figure C represents cells (t_{100}) initially incubated for 100 minutes with LPS, washed and reincubated with fresh LPS. Similarly, Figure D represents cells (t_{120}) that were incubated for 120 minutes, washed and reincubated. Figures B, C and D represent the results obtained on reincubation. I, II and III represent the major peaks of uptake activity.


I
II
III
A
t_{60} CELLS
B
t_{100} CELLS
C
t_{120} CELLS
D
DPM (x 10^2)
TIME OF INCUBATION (minutes)

erythrocytes which behaved characteristically upon subsequent incubation with peaks of uptake similar to those in Figure 29a. A similar pattern of results emerged for cells taken after 100 and 130 min (Figures 29c and 29d respectively. The t_{100} cells were cells taken from a trough of uptake activity. These cells, on further incubation, showed peaks II and III. The t_{130} cells produced only peak III on subsequent incubation at 37°C. The t_{100} and t_{130} cells revealed an additional minor peak of uptake between peaks II and III. Hence it was concluded that cells incubated with ^{32}P-LPS did not alter their adsorption patterns on subsequent incubation with a fresh sample of ^{32}P-LPS.

During the initial incubation period with LPS, a small degree of haemolysis was observed. On subsequent incubation with fresh LPS no haemolysis was recorded. Therefore, although the cells behaved normally with respect to their adsorption characteristics, it would seem that membrane surface changes occurred during the initial incubation, which were not observed upon further incubation with fresh LPS.

The Relative Affinities of Bacterial Polysaccharides for Mammalian Cell Membranes

The inhibition of uptake of labelled LPS by erythrocytes by variable amounts of unlabelled homologous LPS

Human erythrocytes (1.57×10^8/test) and ^{14}C-LPS (987 dpm) from *E. coli* NCTC 8623 were incubated in the presence of variable amounts of unlabelled LPS from *E. coli* NCTC 8623. Both LPS preparations were obtained from the same cells and competed for the same membrane receptor site. Hence, by increasing the amount of unlabelled LPS, the ^{14}C-label

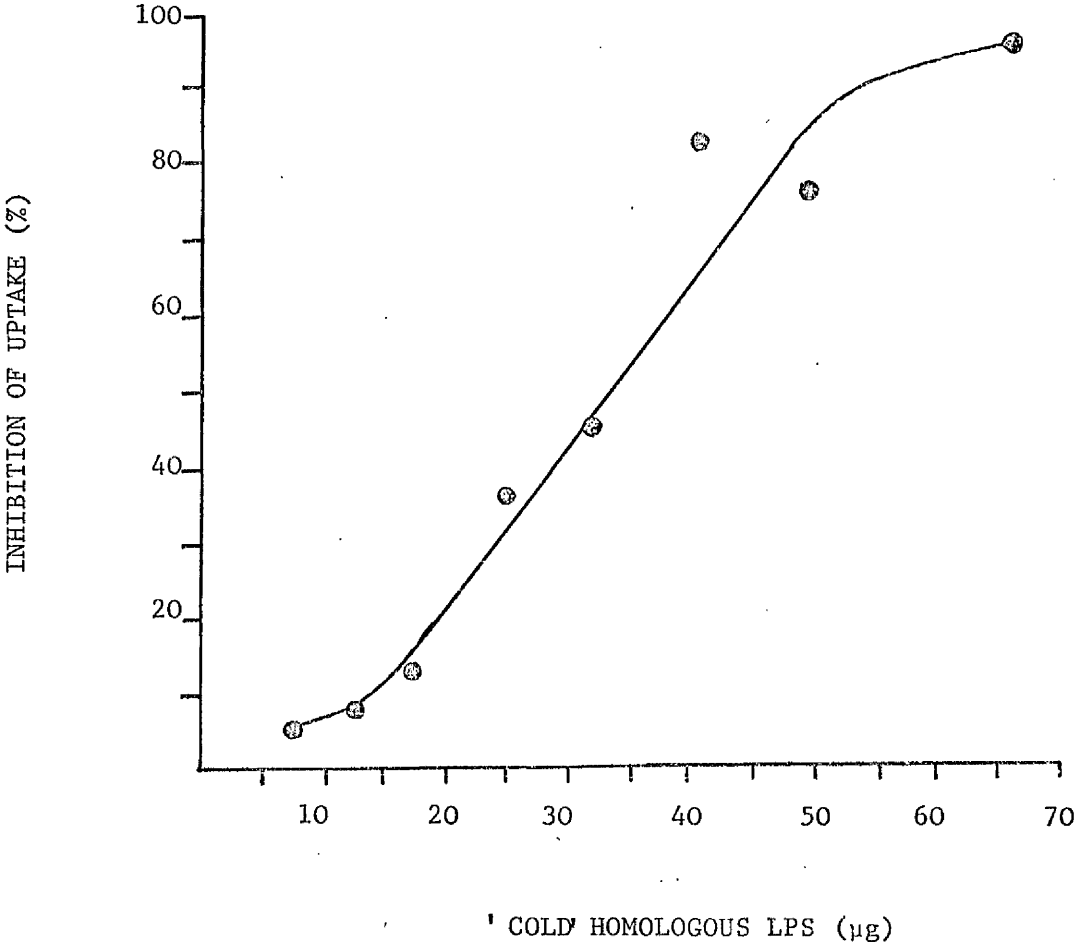
associated with the cell pellet after incubation gradually decreased. By comparing results obtained in the presence and absence of unlabelled LPS, the degree of inhibition of uptake was plotted against the amount of unlabelled homologous LPS (Figure 30). From these results the degree of inhibition appeared to be linear over the central range tested. The later experiments to determine the inhibitory effect of other LPS preparations were carried out at the 30 μg and 60 μg levels of the LPS preparations.

Similarly, the uptake of ^{32}P-LPS by rabbit erythrocytes was inhibited by the addition of variable amounts of unlabelled homologous LPS, ranging from 6 to 220 μg. The degree of inhibition was calculated and plotted against the amount of 'cold' homologous LPS (Figure 31). The results showed a similar pattern to the ^{14}C-LPS results, and the inhibitory effects of other LPS preparations on the ^{32}P-LPS adsorption were tested at the 60 μg and 120 μg level of the LPS preparations.

The inhibition of uptake of labelled LPS by erythrocytes by the addition of unlabelled homologous and heterologous LPS

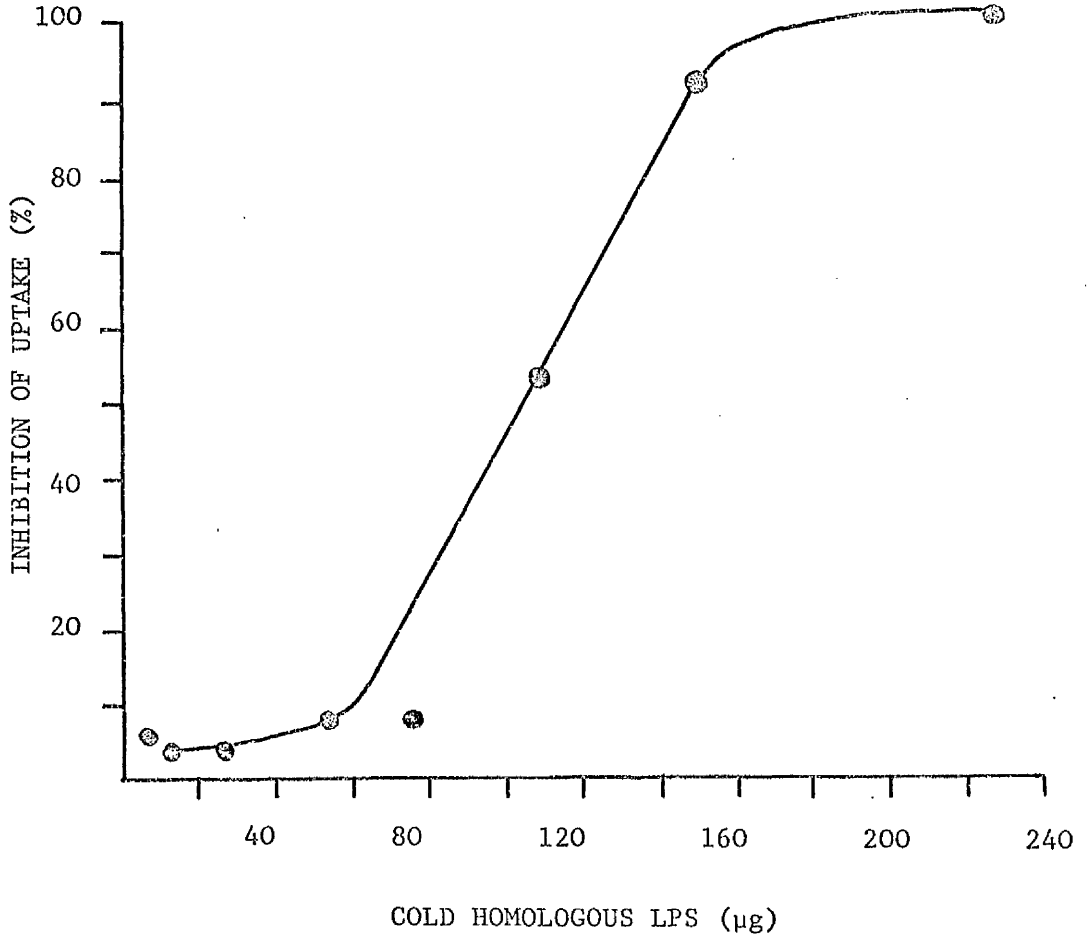
Erythrocytes (1.57 x 10^8/test) and ^{14}C-LPS were incubated at 37°C for one hour in the presence of either 30 μg or 60 μg of a 'cold' heterologous LPS preparation. By comparing results obtained under these conditions with results obtained in the absence of a competing LPS, the degree of inhibition caused by the addition of a cold LPS was determined for each dose. In order to eliminate errors caused by variation in the adsorption of LPS, a standard test-LPS was included in each experiment, namely, the homologous unlabelled LPS from E. coli NCTC 8623. The degree of inhibition caused by the different lipopolysaccharides was compared

Figure 30: The Uptake of ^{14}C-LPS from E. coli NCTC 8623 by Human Erythrocytes. Inhibition of Uptake by the Addition of "cold" Homologous LPS



The inhibition (%) was plotted against the amount of "cold" homologous LPS (µg) added to the test system. The full results are presented in Appendix VII.

Figure 31: The Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes. Inhibition of Uptake by the Addition of "cold" Homologous LPS



The inhibition (%) was plotted against the amount of "cold" homologous LPS (µg) added to the test system. The full results are presented in Appendix VII.

with the inhibition caused by the standard-test LPS for each cell preparation. From the results the amount of LPS causing 50% inhibition ($I.D._{50}$) was calculated. By comparing the ID_{50} dose level of the test-LPS preparations to the standard test-LPS, a relative affinity (RA) value was calculated; the standard homologous E. coli LPS had an RA value of 1.0. If a test-LPS showed a greater affinity for membranes, the ID_{50} level was less than the ID_{50} level of the standard test-LPS and the RA value was greater than 1.0. The relative affinities of four different lipopolysaccharides were also calculated using the ^{14}C system for human erythrocytes (Table 10).

When ^{32}P-LPS was used the test-LPS were tested at the 60 µg and 120 µg levels. The results of inhibition by cold LPS of the adsorption of ^{32}P-LPS to human, rabbit and mouse erythrocytes are presented in Tables 11, 12 and 13. Generally, it appeared that the affinity of an LPS for the erythrocyte was dependent on the type of LPS and not on the erythrocyte species. For example, LPS from Fr. tularensis had relative affinities values for rabbit, mouse and human erythrocytes of 0.61, 0.58 and 0.60 respectively. Similarly LPS from S. typhi NCTC 0901 had relative affinities of 1.26, 1.24 and 1.20 for the erythrocytes from rabbits, mice and human beings. There were exceptions to this; LPS from Sh. flexneri had relative affinities of 0.78, 0.58 and 0.80 for rabbit, mouse and human erythrocytes. The Sh. flexneri LPS had less of an affinity for mouse erythrocytes.

Using the same LPS system, the RA values of Mycobacterial polysaccharides were also obtained (Tables 11, 12 and 13). The Mycobacterial polysaccharides appeared to inhibit the uptake of ^{32}P-LPS

Table 10:

THE UPTAKE OF ^{14}C-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of Uptake of labelled LPS (%)		Inhibition Dose at 50% Inhibition ($I.D_{50}$) (μg)	Relative Affinity
	Dose Level 30 μg	Dose Level 60 μg		
S. typhi NCTC 0901	49.1	81.1	30.6	1.31
S. typhimurium NCTC 5710	27.4	61.2	50.8	0.78
E. coli B (Ether Extraction)	11.0	34.0	80.2*	0.50
E. coli B (EDTA Extraction)	44.3	71.4	36.5	1.10

Table 11:

THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES - INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS POLYSACCHARIDES

Lipopolysaccharide species	Inhibition of uptake of labelled LPS (%)		Inhibition dose at 50% inhibition ($I.D._{50}$) (µg)	Relative Affinity
	Dose level 60 µg	Dose level 120 µg		
Bd. pertussis NCTC 18334	33.1	52.5	115	0.65
Serratia marcescens	28.3	51.2	117	0.64
F. tularensis	28.1	48.7	125*	0.60
Sh. flexneri	40.0	57.7	94	0.80
E. coli 055:B5	36.5	57.1	101	0.74
S. typhi NCTC 0901	49.1	76.3	62	1.20
S. typhimurium NCTC 5710	36.8	57.1	100	0.75
E. coli B (EDTA Extraction)	48.2	72.5	64	1.20
E. coli B (Ether Extraction)	20.0	44.3	136*	0.55
Mycobacterial Fractions				
ST82	47.6	64.8	69	1.09
ST208	25.2	48.0	127*	0.59
ST210	50.0	71.2	60	1.25
ST211	24.4	41.2	150*	0.50
PPD	32.0	54.3	108	0.69

Table 12:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY RABBIT ERYTHROCYTES. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharides Species	Inhibition of Uptake of labelled LPS (%) Dose level 60 µg	Dose level 120 µg	Inhibition dose at 50% inhibition ($I.D._{50}$) (µg)	Relative Affinity
Bd. pertussis NCTC 18334	18.2	54.1	113	0.73
Serratia marcescens	7.4	45.8	127*	0.66
F. tularensis	4.7	40.7	135*	0.61
Sh. flexneri	24.8	56.3	107	0.78
E. coli 05S:B5	9.7	53.6	116	0.71
S. typhi NCTC 0901	47.7	78.5	66	1.26
S. typhimurium NCTC 5710	7.3	55.9	113	0.73
E. coli B (EDTA Extraction)	43.9	74.8	72	1.15
E. coli B (Ether Extraction)	35.10	43.6	148*(NP)	0.56
Mycobacterial Fractions				
ST 82	42.2	78.2	73	1.14
ST 208	34.4	47.8	135*(NP)	0.61
ST 210	48.8	81.0	62	1.34
ST 211	11.45	39.2	142	0.51
PPD	29.9	51.1	118	0.70

Table 13:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY MOUSE ERYTHROCYTES. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of Uptake of labelled LPS (%)		Inhibition Dose at 50% Inhibition ($I.D_{50}$) (μg)	Relative Affinity
	Dose level 60 μg	Dose level 120 μg		
Bd. pertussis NCTC 18334	27.5	50.3	118	0.60
F. tularensis	22.8	49.1	122*	0.58
Sh. flexneri	20.1	49.2	123*	0.58
S. typhi NCTC 0901	51.2	80.0	57	1.24
S. typhimurium NCTC 5710	36.9	64.0	89	0.79
E. coli B (EDTA Extraction)	48.2	71.8	65	1.09
E. coli B (Ether Extraction)	14.6	42.5	131*	0.54
Mycobacterial Fractions				
ST 82	48.3	71.6	64	1.10
ST 208	25.4	53.7	113	0.63
ST 210	51.2	82.1	59*	1.20
ST 211	6.9	39.1	140*	0.50
PPD	27.0	58.3	105	0.67

from E. coli NCTC 8623 by the erythrocytes. The relative affinity that a Mycobacterial fraction possessed for an erythrocyte was not species specific. For example PPD showed relative affinities of 0.70, 0.67 and 0.69 for rabbit, mouse and human erythrocytes.

Since the mycobacterial polysaccharides were able to inhibit the adsorption of ^{32}P-LPS from E. coli by all the erythrocyte species tested, it was concluded that either the LPS and the mycobacterial polysaccharides shared a common membrane receptor or that the membrane receptor for the LPS was in close proximity to the mycobacterial polysaccharide receptor, so that inhibition of adsorption was due to steric hindrance.

The inhibition of uptake of ^{32}P-LPS by various mouse cell lines by the addition of unlabelled homologous and heterologous bacterial polysaccharides

The system obtained for the measurement of RA values for different polysaccharides with respect to erythrocytes was extended to lymphocytes, macrophages, spleen cells, thymus cells and bone marrow cells. The results, together with the RA values are presented in Tables 14 to 18 . The RA values are dependent on the cell species used, for example, the LPS from S. typhimurium NCTC 5710 had RA values of 0.79, 1.63, 0.49, 0.82, 1.42 and 0.87 for erythrocytes, thymus cells, bone marrow cells, lymphocytes, macrophages and spleen cells respectively. Hence a lipopolysaccharide can have a high RA for one cell type and a low RA value for another cell type. The RA values were also dependent on the lipopolysaccharide species. The LPS from S. typhimurium NCTC 5710 had the maximum RA value of 1.63 for thymus cells, while LPS from E. coli B

Table 14:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY MOUSE PERITONEAL LYMPHOCYTES. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of uptake of labelled LPS (%)		Inhibition dose at 50% inhibition ($I.D._{50}$) (μg)	Relative Affinity
	Dose level 60 μg	Dose level 120 μg		
Bd. pertussis NCTC 18334	27.1	43.8	140*	0.53
F. tularensis	10.6	42.4	134*	0.52
Sh. flexneri	22.6	50.1	120	0.59
E. coli 055:B5	21.8	71.8	94	0.75
S. typhi NCTC 0901	48.3	76.8	63	1.13
S. typhimurium NCTC 5710	39.4	66.0	87	0.82
E. coli B (EDTA Extraction)	28.7	51.0	118	0.63
E. coli B (Ether Extraction)	39.6	60.2	91	0.82
Mycobacterial Fractions				
ST82	40.1	77.4	75	1.0
ST208	41.2	68.1	84	0.85
ST210	38.2	75.7	77	0.98
ST211	31.2	77.0 (N.P.)	82	0.92
PPD	40.1	68.5 (N.P.)	80	0.93

Table 15:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY MOUSE PERITONEAL MACROPHAGES. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of uptake of labelled LPS (%) Dose level 60 µg	Dose level 120 µg	Inhibition dose at 50% inhibition ($I.D._{50}$) (µg)	Relative Affinity
Bd. pertussis NCTC 18334	20.6	61.2	103	0.72
F. tularensis	35.1	47.2	136*	1.02
Sh. flexneri	44.5	56.0	88	1.59
E. coli 055:B5	39.2	51.0	112	1.25
S. typhi NCTC 0901	7.0	57.3	112 (N.P.)	0.67
S. typhimurium NCTC 5710	53.0	80.0	53*	1.42
E. coli B (EDTA Extraction)	43.9	56.1	88	1.60
E. coli B (Ether Extraction)	41.6	60.1	94	1.48
Mycobacterial fractions				
ST82	35.9	46.7	140*	1.0
ST208	38.6	52.7	110	1.27
ST210	35.4	48.1	135*	1.03
ST211	37.3	51.4	116	1.20
PPD	31.2	49.1	123* (N.P.)	1.13

Table 16:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY MOUSE SPLEEN CELLS. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide species	Inhibition of uptake of labelled LPS (%)		Inhibition dose at 50% inhibition ($I.D._{50}$) (μg)	Relative Affinity
	Dose level 60 μg	Dose level 120 μg		
Bd. pertussis NCTC 18334	28.3	42.4	150* (N.P.)	0.50
F. tularensis	21.2	56.4	108	0.69
Sh. flexneri	26.6	59.0	105	0.72
E. coli 055:B5	37.1	72.0	83	0.90
S. typhi NCTC 0901	44.2	76.2	74	1.04
S. typhimurium NCTC 5710	34.5	72.5	88 (N.P.)	0.87
E. coli B (EDTA Extraction)	33.5	62.5	97	0.79
E. coli B (Ether Extraction)	40.0	66.3	86	0.88
Mycobacterial fractions				
ST82	36.7	73.1	82	0.91
ST208	41.0	71.1	81	0.94
ST210	43.0	80.7	71	1.05
ST211	33.1	61.9	95	0.79
PPD	33.0	70.1	87	0.86

Table 17:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM *E. COLI* NCTC 8623 BY MOUSE THYMUS CELLS. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of uptake of labelled LPS (%)		Inhibition Dose at 50% inhibition ($I.D._{50}$) (μg)	Relative Affinity
	Dose level 60 μg	Dose level 120 μg		
Bd. pertussis NCTC 18334	25.1	60.1	113 (N.P.)	0.72
F. tularensis	35.7	71.4	88	0.93
Sh. flexneri	25.5	63.8	98	0.84
E. coli 055:B5	31.3	66.3	95	0.86
S. typhi NCTC 0901	46.3	77.2	67	1.22
S. typhimurium NCTC 5710	55.3	86.3	51*	1.63
E. coli B (EDTA Extraction)	29.1	55.4	110	0.74
E. coli B (Ether Extraction)			118	0.69
Mycobacterial Fractions				
ST82	29.4	50.9		1.10
ST208	41.9	76.2	74	1.11
ST210	40.1	79.2	74	1.30
ST211	30.1	41.9	164* (N.P.)	0.50
PPD	32.5	59.4	101	0.81

Table 18:

THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM *E. COLI* NCTC 8623 BY MOUSE BONE MARROW CELLS. INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HETEROLOGOUS LPS

Lipopolysaccharide Species	Inhibition of uptake of labelled LPS (%)		Inhibition dose at 50% inhibition ($I.D._{50}$) (μg)	Relative Affinity
	Dose level 60 μg	Dose level 120 μg		
Bd. pertussis NCTC 18334	29.1	41.8	164*	0.50
F. tularensis	25.5	40.1	167*	0.49
Sh. flexneri	33.4	57.0	101	0.81
E. coli 055:BS	37.1	51.4	117	0.70
S. typhi NCTC 0901	27.4	57.4	104 (N.P.)	0.62
S. typhimurium NCTC 5710	24.7	46.2	130*	0.49
E. coli B (EDTA extraction)	17.9	53.7	115	0.71
E. coli B (Ether extraction)	35.1	53.6	110	0.74
Mycobacterial Fractions				
ST82	43.1	62.1	82	1.0
ST208	37.0	60.5	93	0.89
ST210	22.7	34.1	205*	0.40
ST211	38.1	53.4	107	0.76
PPO	40.1	61.4	90	0.91

extracted with EDTA had the least affinity having a RA value of 0.69. However, for bone marrow cells, the LPS from E. coli NCTC 8623 exhibited the greatest affinity, with an RA value of 1.0; the LPS from F. tularensis showed the least affinity for the bone marrow cells, with an RA value of 0.49. Hence the LPS showing the greatest affinity varied depending on the cell type. The same held true for the LPS exhibiting the least affinity. Therefore the amount of LPS adsorbed by a cell type is a function of both the LPS species and the cell species.

The same considerations were true for polysaccharides from mycobacterial cells. Hence the amount of mycobacterial polysaccharide adsorbed was dependent on both the polysaccharide and the cell species. From the results, it was observed that the uptake of mycobacterial fractions by the various cell lines was comparable with the uptake of LPS by the same cells on a weight to weight basis. Hence macrophage adsorbed comparable amounts of ST211 (RA value of 1.20) and LPS from E. coli 055:B5 (RA value of 1.25). Generally the mycobacterial polysaccharides were taken up to a greater extent than the lipopolysaccharides. For example, the RA values of LPS for spleen cells ranged from 0.5 to 1.04. The range of RA values of LPS for spleen cells ranged from 0.5 to 1.04. The range of RA values of the mycobacterial polysaccharides for the same cells was 0.79 to 1.05. Similarly the range of RA values of mycobacterial polysaccharides for lymphocytes was 0.85 to 1.0, while the equivalent values for LPS were 0.52 to 1.13. This range of values could reflect the wider range of the LPS species.

The significance of these results and the influence of affinity on the biological effects of the bacterial polysaccharides will be discussed later.

The Effect of Chemical Treatment on Polysaccharide Uptake

Both a mycobacterial glycopeptide (ST208) and a LPS from E. coli NCTC 8623 were tested for their ability to modify sheep erythrocytes after the polysaccharide fractions were treated with heat, alkali and periodate. From the data obtained (Table 19) it was concluded that heat and alkali treatments were beneficial to the uptake of the bacterial polysaccharides by sheep erythrocytes. The periodate treatment of the polysaccharides was detrimental to their adsorption by the erythrocytes.

In the case of the mycobacterial glycopeptide (ST208) the amount of glycopeptide absorbed by 2.5×10^8 sheep erythrocytes, after heat treatment, increased from 0.61 to 1.22 mg. Similarly alkali treatment of the glycopeptide increased the amount adsorbed to 1.05 mg. However, periodate-treated glycopeptide showed a decreased ability to adsorb to the erythrocytes; only 0.4 mg adsorbed to the erythrocytes.

By similar treatments of the LPS, the amount adsorbed by the erythrocytes increased from 0.51 mg to 1.24 mg after heat treatment and to 0.63 mg after alkali treatment. The periodate-treatment resulted in only 0.2 mg of the LPS being adsorbed by the erythrocytes.

These same effects were also demonstrated by the radioactive technique.

The effect of the various treatments was observed on LPS extracted by the phenol/water method from E. coli NCTC 8623, S. typhi NCTC 0901, S. typhimurium NCTC 5710. Human erythrocytes (1.57×10^8) were incubated with a constant amount of ^{32}P-LPS and 120 µg of a treated

Table 19: THE EFFECT OF HEAT, ALKALI AND PERIODATE TREATMENT ON THE ADSORPTION OF BACTERIAL POLYSACCHARIDES BY SHEEP ERYTHROCYTES

Polysaccharide	Carbohydrate content of cells mg/100 ml to Haldane Standard	Increase in carbohydrate content of cells (mg)	Polysaccharide Fraction	
			Amount adsorbed (mg)	$\frac{\text{Amount Adsorbed}}{\text{Amount Added}} \times 100$
Untreated Erythrocytes	79.32	-	-	[f]
Normal Glycopeptide (ST208)	96.82	0.35	0.61	3.05
Heat-treated " "	114.82	0.71	1.22	6.10
Alkali-treated " "	110.12	0.61	1.05	5.25
Periodate-treated " "	91.06	0.23	0.40	2.0
Normal LPS from *E. coli* NCTC 8623	90.32	0.22	0.51	2.55
Heat-treated " "	106.06	0.53	1.24	6.20
Alkali-treated " "	92.90	0.27	0.63	3.15
Periodate-treated " "	83.63	0.08	0.20	1.0

Number of sheep erythrocytes used per test = 2.5×10^8

LPS. The ability of the treated LPS to inhibit the binding of the ^{32}P-LPS was measured and expressed as a percentage of the total bound LPS. Hence 100 per cent represented the case where no labelled-LPS was adsorbed onto the erythrocytes, and zero per cent represented total adsorption of all the available ^{32}P-LPS. Since it was found that LPS from different organisms inhibited the adsorption of the E. coli ^{32}P-LPS by mammalian cells, a comparison of the different treatments on the various lipopolysaccharides was possible.

The results (Table 20a) showed that 120 μg of untreated 'cold' homologous LPS from E. coli NCTC 8623 caused a 24.5 per cent inhibition of the adsorption of ^{32}P-LPS. When the LPS was heat or alkali-treated the inhibition of uptake of labelled LPS increased to 33 and 27.1 per cent respectively. It was concluded that heat or alkali-treatment increased the affinity of the LPS for the cell membrane. However if the 'cold' LPS was treated with periodate, the inhibition decreased to 7.8 per cent, which indicated a decrease in the affinity of LPS for mammalian cell membranes. Using LPS from S. typhi and S. typhimurium a similar pattern of results emerged (Table 20a). Hence on heat treatment the inhibition of adsorption produced by S. typhi LPS at the 120 μg level increased from 29.3 per cent to 44.9 per cent. Similarly alkali treatment of the LPS increased the inhibition to 37.8 per cent. The periodate treatment caused a decrease in inhibition from 29.3 per cent to 16.0 per cent. Similarly normal LPS from S. typhimurium caused a 18.8 per cent inhibition of adsorption of the ^{32}P-LPS, but after heat or alkali treatment the inhibition increased to 41.5 and 47.1 per cent respectively.

Table 20 a:

THE EFFECT OF ALKALI, PERIODATE AND HEAT TREATMENT ON THE ADSORPTION OF LPS BY HUMAN ERYTHROCYTES

Lipopolysaccharide species	LPS treatment	Inhibition at 120 µg level (%)	Relative increase of decrease in affinity
Escherichia coli NCTC 8623	Untreated	24.5	1.0
	Heat	33.0	1.34
	Alkali	27.1	1.106
	Periodate	7.8	0.31
Salmonella typhi NCTC 0901	Untreated	29.3	1.0
	Heat	44.9	1.53
	Alkali	37.8	1.29
	Periodate	16.0	0.54
Salmonella typhimurium NCTC 5710	Untreated	18.8	1.0
	Heat	41.5	2.20
	Alkali	47.1	2.50
	Periodate	6.7	0.35

Table 20 b:

RELATIVE AFFINITIES OF TREATED AND UNTREATED LIPOPOLYSACCHARIDE PREPARATIONS

LPS treatment	E.coli NCTC 8623	S.typhi NCTC 0901	S.typhimurium NCTC 5710
Untreated	1.0	1.19	0.76
Heat	1.0	1.36	1.25
Alkali	1.0	1.39	1.73
Periodate	1.0	2.05	0.85

The extent to which the various treatments affected the affinity was dependent on the lipopolysaccharide species. Hence heat and alkali treatment affected the LPS from S. typhimurium to a greater extent than the LPS from E. coli or S. typhi. Similarly, S. typhi LPS was not as susceptible to periodate treatment as the other lipopolysaccharides.

If the Salmonella LPS preparations were compared with E. coli it was observed that the untreated S. typhi LPS possessed a greater affinity than the E. coli LPS for cell membranes (Table 20b). Similarly, the untreated S. typhimurium LPS possessed less affinity for membranes than the E. coli LPS. However both heat and alkali treatment increased the affinity for membranes of the Salmonella LPS to a greater extent than the E. coli LPS. Both heat and alkali-treated S. typhi and S. typhimurium lipopolysaccharides possessed greater affinity for cell membranes than the alkali or heat-treated E. coli LPS. Periodate treatment revealed that the S. typhiLPS was the least susceptible of the three lipopolysaccharides.

Variation with time of incubation in the uptake of untreated and heat-treated ^{32}P-LPS from E. coli NCTC 8623 by rabbit erythrocytes

Untreated and heat-treated ^{32}P-LPS (4408 dpm) was incubated with 1.57×10^8 rabbit erythrocytes at 37°C for time periods ranging from 35 to 155 min. The amount of LPS adsorbed was calculated in both cases, in terms of the radioactivity (dpm) associated with the cell pellet, and plotted against the time of incubation (Figure 32). The heat-treated LPS was adsorbed to a greater extent by the cells than untreated LPS as previously determined. The untreated LPS showed the 'characteristic'

Figure 32: The Variation with Time of Incubation in the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes. The Effect of Heat-Treatment of the LPS

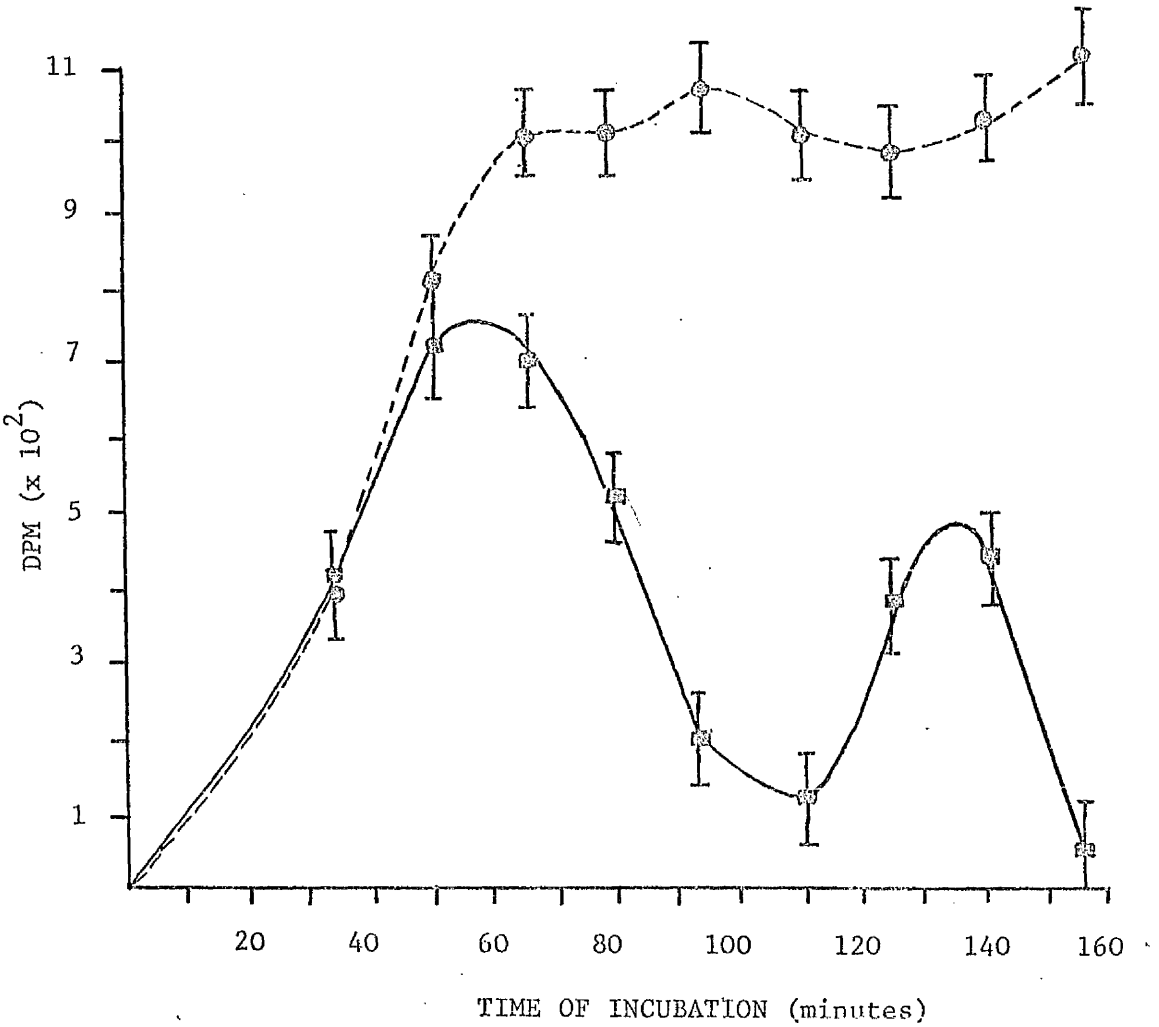
The amount of LPS adsorbed by the cells was calculated in terms of the cell-associated radioactivity (dpm) and plotted against the time of incubation (minutes).

—⊜— represents the response with untreated LPS

- - -⊜- - - represents the response with heat-activated LPS

I represents the standard error associated with each observation.

The full results, together with Chi-square analysis of the fluctuations are presented in Appendix VII.


11
9
7
5
3
1
DPM (x 10^2)
20
40
60
80
100
120
140
160
TIME OF INCUBATION (minutes)

uptake pattern with peaks of activity at 52 and 135 min. However, the heat-treated LPS did not show the usual cyclic fluctuation of uptake activity but maintained a steady level within the limits of experimental error (Statistical Analysis - Appendix VII). It was concluded from these results that the bond between the heat-treated LPS and erythrocyte membrane was stronger than the bond between untreated LPS and membrane. This finding will be discussed later with reference to the altered biological activity of heat-treated LPS.

Inhibition of Polysaccharide Uptake by Normal Serum and Simple Organic Substance

The effects of normal serum on polysaccharide uptake

The uptake of ^{32}P-LPS by 1.57×10^8 rabbit erythrocytes was observed in the presence of various dilutions of normal rabbit serum. The amount of LPS adsorbed by the erythrocytes in the presence and absence of the serum was compared and the degree of inhibition of LPS adsorption in the presence of serum was calculated. The degree of inhibition of adsorption of the LPS by the erythrocytes, was plotted against the serum dilution (Figure 33). Normal untreated LPS and heat-treated LPS were compared by this method; at low dilutions the serum caused inhibition of adsorption of both types of LPS. Normal rabbit serum, neat and $\frac{1}{2}$, caused 54 per cent and 52 per cent inhibition respectively of the adsorption of the untreated ^{32}P-LPS. The same serum dilutions caused 50 per cent and 10 per cent inhibition of the adsorption of heat-treated LPS. From this it appeared that the untreated LPS was more susceptible to the inhibitory action of the serum components. If the erythrocytes were

Figure 33: The Effect of Normal Rabbit Serum on the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes

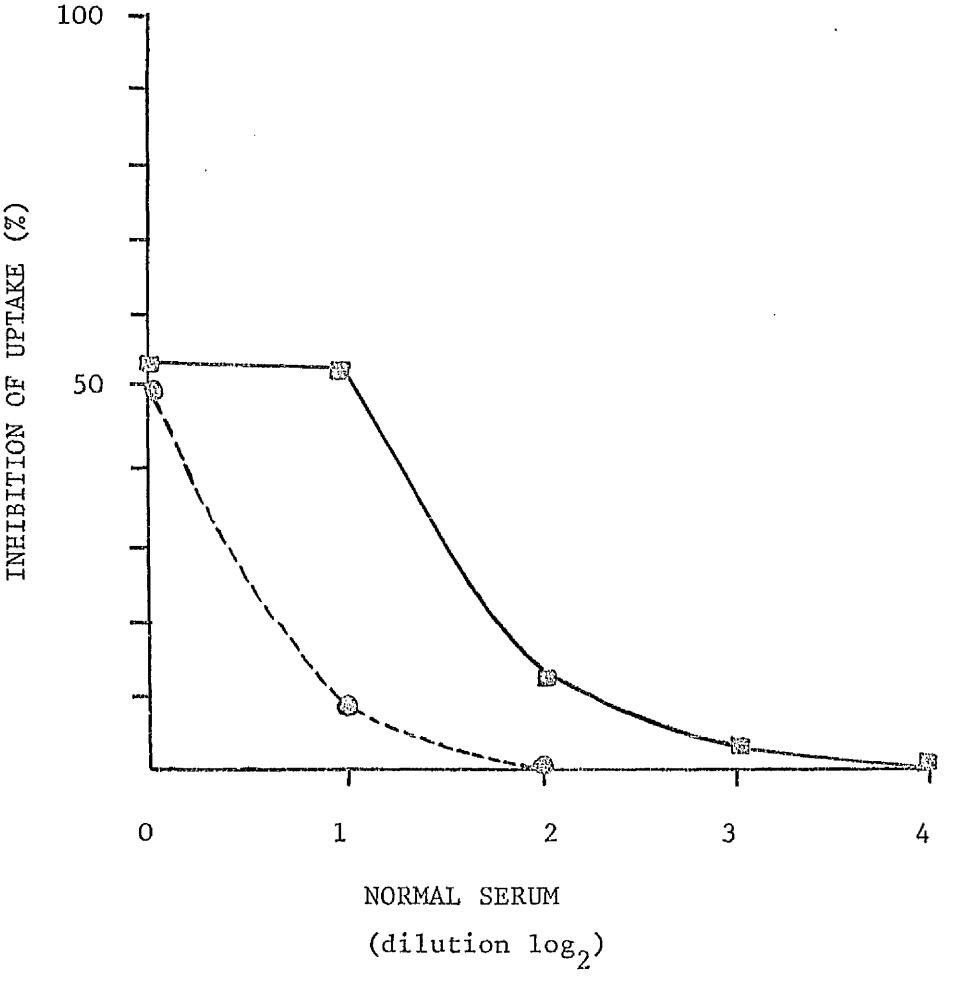
The degree of inhibition (%) of uptake of the labelled LPS was plotted against dilution (log_2) of the rabbit serum.

Symbols

—⊠— represents the effect observed when untreated LPS was used to modify the erythrocytes.

- -⊙- - represents the effect observed when heat activated LPS was used to modify the erythrocytes.

The full results are presented in Appendix VII.


100
50
INHIBITION OF UPTAKE (%)
0
1
2
3
4
NORMAL SERUM
(dilution log2)

incubated with undiluted serum, washed and incubated with ^{32}P-LPS, a different effect was observed. The inhibition of uptake of untreated LPS remained at approximately the same level. With pretreatment of the erythrocytes the degree of inhibition decreased from 54.0% to 45.1%. However, the degree of inhibition of uptake of heat-treated LPS decreased markedly from 50.4% to 10.6%. In the case of untreated LPS it appeared that serum components attached to sites on the erythrocyte membrane which blocked the uptake either by direct competition for the membrane site or by steric hindrance. However the opposite seemed to be true for the heat-treated LPS. Pretreatment of the erythrocytes with serum did not apparently block membrane receptors for heat-treated LPS to the same extent. The significance of this form of inhibition on the in vivo biological activity of the lipopolysaccharides will be discussed later.

The effects of cholesterol on polysaccharide uptake

The adsorption of ^{32}P-LPS from E. coli NCTC 8623 by rabbit erythrocytes was observed in the presence of variable amounts of cholesterol. It was observed that cholesterol had an inhibitory effect on the adsorption of the LPS by the erythrocytes. The degree of inhibition was plotted against the concentration of cholesterol (Figure 34). It was apparent that cholesterol was an equally effective inhibitor against either untreated or heat-treated LPS causing 72.2 per cent and 68.3 per cent inhibition at the 150 μg level.

If the erythrocytes were preincubated with 150 μg cholesterol, washed, and then incubated with the LPS, the degree of inhibition was markedly reduced. The reduction in inhibition was slightly greater for

Figure 34: The Effect of Cholesterol on the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes

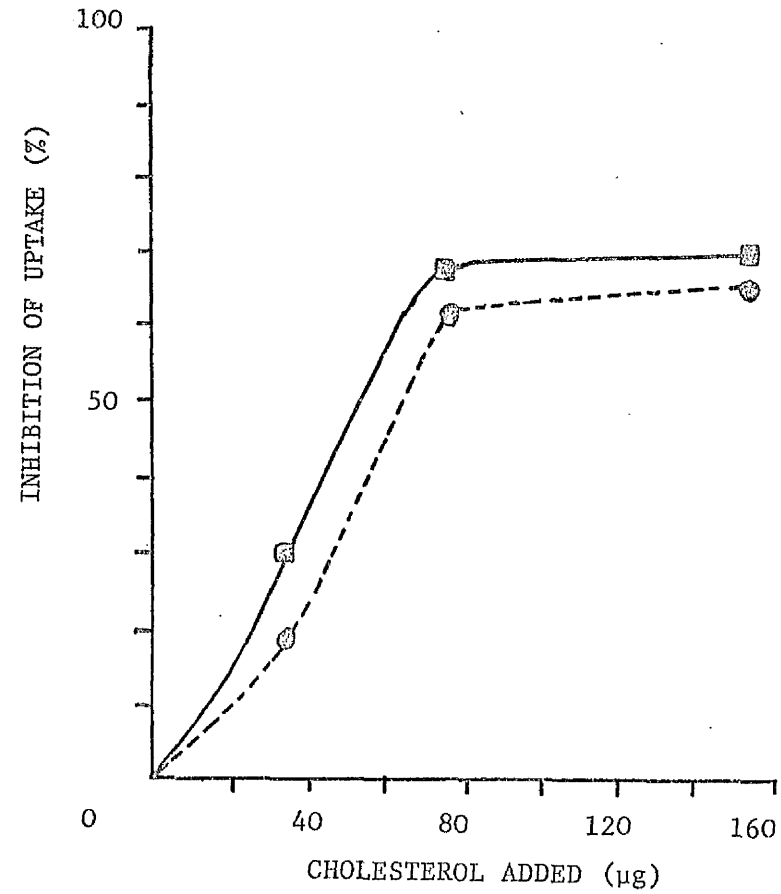
The degree of inhibition (%) of uptake was plotted against the amount of cholesterol (μg) added to the test system. The full results are presented in Appendix VII.

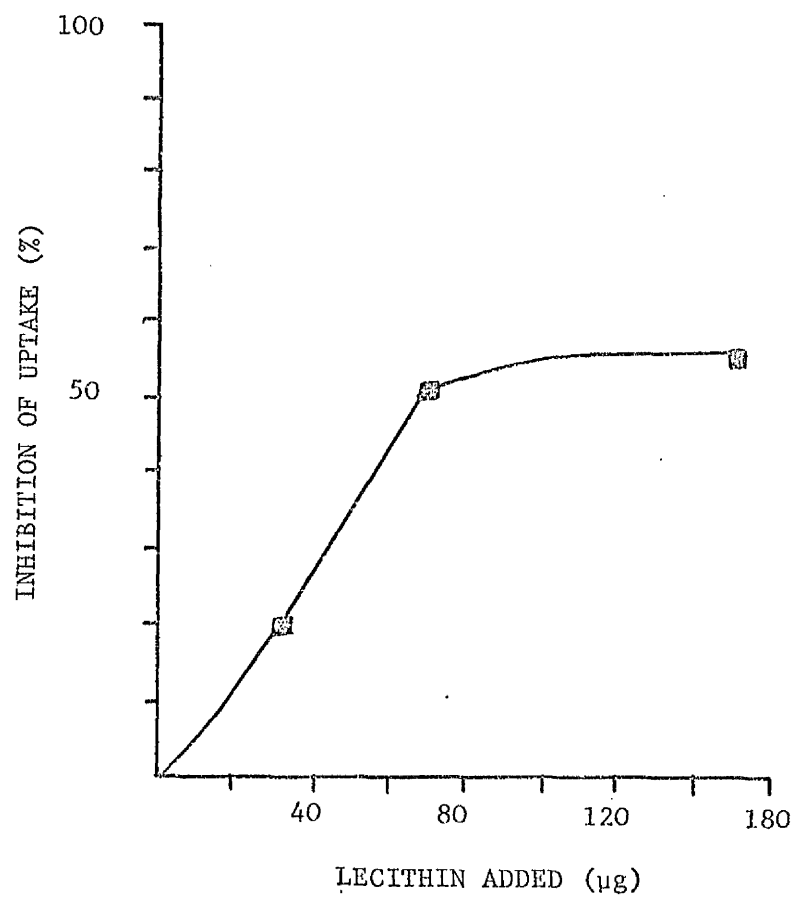
—◎— represents the results obtained with untreated LPS

--◎-- represents the results obtained with heat-activated LPS.

Figure 35: The Effect of Lecithin on the Uptake of ^{32}P-LPS from E. coli NCTC 8623 by Rabbit Erythrocytes

The degree of inhibition (%) of the uptake of untreated LPS was plotted against the amount of lecithin (μg) added per test system. The full results are in Appendix VII.


100
50
0
INHIBITION OF UPTAKE (%)
40
80
120
160
CHOLESTEROL ADDED (µg)


100
50
INHIBITION OF UPTAKE (%)
40
80
120
180
LECITHIN ADDED (µg)

the heat-treated LPS. By pretreatment the degree of inhibition decreased from 72.2 per cent to 16.3 per cent for the untreated LPS, and from 68.3 per cent to 1.4 per cent for the heat-treated LPS. These results indicate that cholesterol, in its inhibitory capacity, bound preferentially to the LPS, rather than to the erythrocyte membrane. Earlier workers (Neter et al., 1958) suggested that cholesterol was the membrane site of LPS attachment.

The effects of lecithin on polysaccharide uptake

The inhibitory effect of lecithin upon the adsorption of LPS by rabbit erythrocytes was observed by incubating the cells and the LPS together with variable amounts of lecithin for one hour at 37°C. The degree of inhibition was plotted against the concentration of lecithin (Figure 35). Lecithin appeared to be slightly less effective than cholesterol at causing inhibition of the LPS adsorption. Cholesterol at the 150 μg level caused 72.2 per cent inhibition of adsorption while lecithin caused 54.9 per cent inhibition. If the erythrocytes were preincubated with 150 μg lecithin for one hour, washed, and then incubated with the LPS, the degree of inhibition remained approximately at the same level, i.e. 52.3 per cent as compared to 54.9 per cent. This indicated that lecithin acted by blocking sites on the erythrocyte membrane, rather than attaching preferentially to the LPS.

The effect of sugars on polysaccharide uptake

The uptake of LPS by erythrocytes was observed in the presence of various monosaccharides. The uptake of ^{14}C-LPS by 1.57×10^8 human

erythrocytes was found to be inhibited by glucose, galactose, rhamnose, mannose, fucose, arabinose and glucosamine (Table 21). The comparative level of inhibition was dependent on the type of monosaccharide used; glucose was the best inhibitor and galactose the worst. At the 72 μg level, glucose inhibited the uptake of 89 per cent of the ^{32}P-LPS that normally adsorbed to the erythrocytes. Similarly 72 μg of galactose inhibited the uptake of 60 per cent of the normally adsorbed LPS. The other monosaccharides gave levels of inhibition between these two extremes. The degree of inhibition was also dependent on the amount of the monosaccharide used. Hence 36 μg of glucose inhibited the uptake of 76 per cent of the ^{32}P-LPS, while 72 μg of glucose inhibited 89% of the LPS adsorbed. This dose-dependence of glucose on the degree of inhibition attained was found to be true for all the monosaccharides tested.

To determine whether the inhibitory effect was directed at the LPS or at the erythrocyte membrane, further experiments were carried out. Four reaction mixtures were set up in the presence of glucose and the amount of LPS adsorbed by the erythrocytes was calculated and compared with the amount of LPS adsorbed by erythrocytes under similar conditions in the absence of glucose. The results using both ^{14}C-LPS (Table 22) and ^{32}P-LPS (Table 23) showed that the greatest inhibition of LPS adsorption occurred when the LPS was incubated with the glucose prior to exposure to the erythrocytes (reaction mixture III). Under these conditions from the ^{14}C-LPS results 72 μg of glucose caused 89 per cent inhibition of uptake of the normally adsorbed LPS. This indicated that the glucose inhibited LPS adsorption by combining with sites on the LPS molecule. Hence if the LPS and glucose were incubated simultaneously

Table 21:

THE EFFECT OF SUGARS ON THE UPTAKE OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES

Sugar added	Amount sugar added (µg)	Total dpm of cell pellet corrected for background (88 dpm)	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$	Inhibition of uptake of LPS (%)
none	-	900	0.35	-
Glucose	72	95	0.03	89.4
	36	215	0.08	76.2
Galactose	72	361	0.14	60.0
	36	678	0.26	25.0
Rhamnose	72	121	0.04	86.6
	36	274	0.107	69.6
Mannose	72	187	0.07	79.3
	36	433	0.17	52.0
Fucose	72	81	0.03	91.0
	36	395	0.15	56.3
Arabinose	72	99	0.03	89.0
	36	259	0.101	71.3
Glucosamine	72	324	0.12	64.1
	36	592	0.23	34.5

Table 22:

THE UPTAKE OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES --

THE EFFECT OF THE ADDITION OF GLUCOSE

Reaction Mixture	Amount of Glucose Added (µg)	Efficiency of Counting (%)	Total dpm of cell pellet corrected for background (77 dpm)	Ratio: $\frac{\text{dpm cell}}{\text{dpm total}}$	Inhibition of uptake of LPS (%)
I	36	86.65	307	0.19	8.6
	72	85.95	279	0.17	16.8
	none	86.00	336	0.21	-
II	36	87.00	149	0.09	46.6
	72	86.65	95	0.05	65.8
	none	86.65	279	0.17	-
III	36	85.45	75	0.04	70.3
	72	86.05	25	0.01	89.8
	none	86.10	253	0.15	-
IV	36	86.30	235	0.14	10.0
	72	86.35	153	0.09	41.5
	none	86.25	262	0.16	-

Key to Reaction mixtures:

I LPS + Glucose + Erythrocytes incubated for one hour at 37°C.

II LPS + Erythrocytes incubated 30 min at 37°C. Glucose added, and incubation for a further 30 min.

III LPS + Glucose incubated for 30 min at 37°C. Erythrocytes added, and incubation for a further 30 min.

IV Glucose + erythrocytes incubated for 30 min at 37°C. LPS added and incubation for a further 30 min.

Table 23: THE EFFECT OF GLUCOSE ON THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM *E. COLI* NCTC 8623 BY RABBIT ERYTHROCYTES

Total dpm of ^{32}P-lipopolysaccharide per test = 3114

Reaction Mixture	Amount of Glucose Added (µg)	Efficiency of Counting (%)	Total dpm of supernatant liquid corrected for background (28 dpm)	Total dpm of cell pellet	Ratio: $\frac{\text{dpm cell}}{\text{dpm total}}$	Inhibition of uptake of LPS (%)
I	240	86.65	2850	264	0.084	53.6
	120	86.35	2597	517	0.166	8.3
	None	86.40	2550	564	0.181	-
II	240	86.35	2956	158	0.051	68.0
	120	86.40	2728	386	.124	21.9
	None	86.35	2619	495	0.159	-
III	240	86.55	3141	0	0	100
	120	86.45	2977	137	0.044	27.9
	None	86.25	2925	129	0.061	-
IV	240	86.65	3002	112	0.036	57.3
	120	86.65	2897	217	0.070	17.6
	None	86.40	2847	267	0.086	-

Key to Reaction Mixtures: see Table 22

with the erythrocytes (reaction mixture I) it was assumed that the LPS competed for both the glucose determinants and the erythrocyte membrane receptors. This competition resulted in an inhibition of uptake of the LPS by the erythrocyte membranes. However the results from reaction mixtures II and IV indicated that other factors should be considered. If the erythrocytes were preincubated with 72 μg of glucose (reaction mixture IV) the inhibition of ^{14}C-LPS adsorption was increased to 41 per cent. This indicated that it was possible that the glucose could combine with the erythrocyte membrane. If the erythrocytes were preincubated with LPS, prior to the addition of glucose (reaction mixture II), the ^{14}C-LPS adsorption was further inhibited, i.e. 65 per cent at the 72 μg level. This result indicated that although the LPS possessed an affinity for both the membrane receptor and the monosaccharide, the latter was able to remove LPS already bound to the erythrocyte membrane. This tended to indicate that the bond between the LPS molecule and membrane receptor was a relatively weak one. The significance of these results will be discussed later.

The leakage of haemoglobin from erythrocytes modified by bacterial polysaccharides

During the modification of erythrocytes by bacterial polysaccharides it was observed that the supernatant fluid from the reaction mixtures developed a visible red colour during the incubation period. It was observed that the supernatant fluid had maximum adsorptions at 412 nm and 540 nm which indicated that haemoglobin was released from the erythrocytes during incubation with the polysaccharide.

Initially it was observed that when 2.5×10^8 sheep erythrocytes were incubated with 20 mg of a mycobacterial glycopeptide, a maximum degree of haemoglobin leakage, 11 per cent, was attained after 60 min incubation (Figure 27b). Under similar conditions, 20 mg of LPS from E. coli NCTC 8623 produced 11 per cent leakage (Figure 28b). When 5×10^9 sheep erythrocytes were incubated with either 1 mg or 2 mg of the mycobacterial glycopeptide (ST208) for various periods of time (Figure 36a), it was observed that the level of leakage was dependent on the amount of polysaccharide present. Hence after 7 hours incubation 1 mg of ST208 produced 3.4 per cent haemoglobin leakage whereas 2 mg of the glycopeptide produced 8.7 per cent leakage. Similar results were obtained with 5×10^9 horse erythrocytes (Figure 36b). At the one milligram level, ST208 produced 4 per cent leakage and 2 mg of the glycopeptide produced 9.5 per cent leakage.

A similar pattern of results was obtained with LPS from E. coli NCTC 8623. One milligram of the LPS produced 4.2 per cent leakage and 2 mg produced 9 per cent leakage with 5×10^9 sheep erythrocytes (Figure 37a). When the LPS was incubated with horse erythrocytes (Figure 37b), 1 mg of the LPS produced 4.4 per cent leakage and 2 mg, 9.5 per cent leakage.

Erythrocytes from other species were tested with a variety of bacterial polysaccharides (Table 24). Haemoglobin leakage was observed with all the polysaccharides tested. Horse erythrocytes seemed the most susceptible to the haemolytic action of the polysaccharides while human erythrocytes the most resistant. For example, the mycobacterial preparation ST206 produced 33.5 per cent leakage in horse erythrocytes

Figure 36: The Haemolysis of Erythrocytes on Incubation with Mycobacterial Glycopeptide (ST208). The Effect of Time of Incubation

The degree of haemolysis (%) of the erythrocytes was plotted against the time of incubation (minutes) with the glycopeptide. Figure A represents the result with sheep erythrocytes and Figure B with horse erythrocytes.

---●--- represents haemolysis with a glycopeptide concentration of 1 mg/ml.

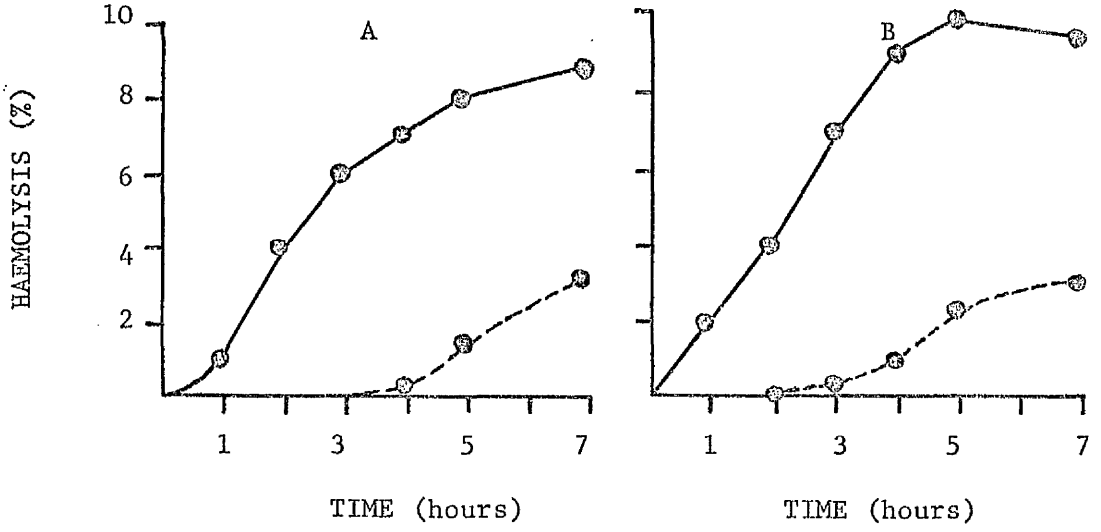
——●—— represents haemolysis with a glycopeptide concentration of 2 mg/ml.

Figure 37: The Haemolysis of Erythrocytes on Incubation with LPS from E. coli NCTC 8623. The Effect of Time of Incubation

The degree of haemolysis (%) of the erythrocytes was plotted against the time of incubation (minutes) with the LPS. Figure A represents the result with sheep erythrocytes and Figure B with horse erythrocytes.

- - ●- - - represents haemolysis with a LPS concentration of 1 mg/ml.

——●—— represents haemolysis with a LPS concentration of 2 mg/ml.


HAEMOLYSIS (%)
10
8
6
4
2
A
B
1
3
5
7
TIME (hours)
TIME (hours)

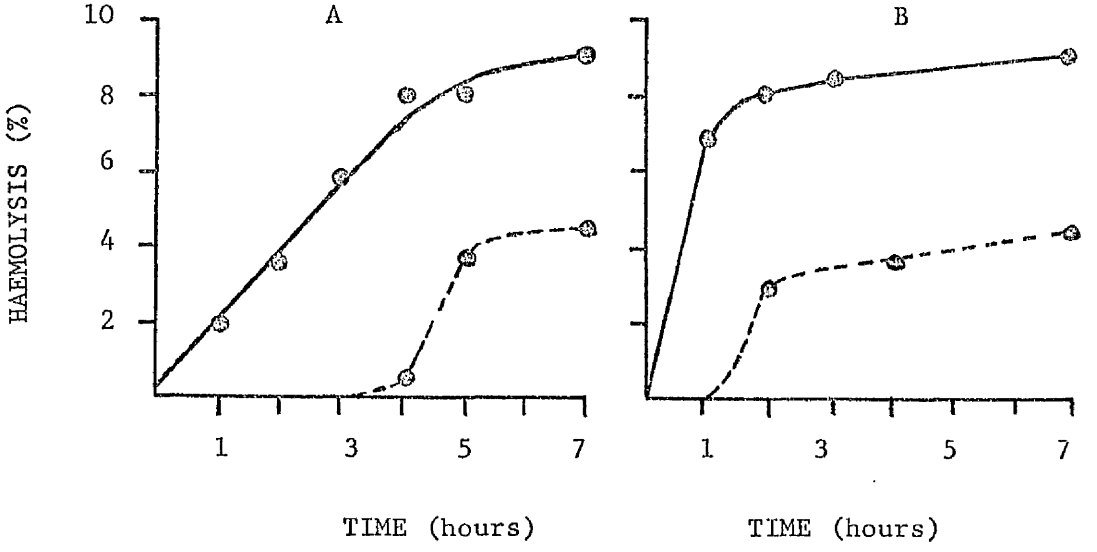

HAEMOLYSIS (%)
10
8
6
4
2
A
B
1
3
5
7
TIME (hours)
TIME (hours)

Table 24: THE DEGREE OF HAEMOLYSIS PRODUCED BY THE ADSORPTION OF BACTERIAL POLYSACCHARIDES TO MAMMALIAN ERYTHROCYTES

	Percent Haemolysis			
Bacterial	Erythrocyte Species			
Polysaccharide	Mouse	Horse	Human	Rabbit
ST202	23.3	38.6	15.4	15.8
ST206	12.3	33.5	0.73	1.6
ST208	14.0	31.6	1.15	2.4
ST209	20.6	30.9	0.45	5.3
ST210	11.2	30.9	1.0	1.7
E. coli NCTC 8623 LPS	7.9	31.4	0.62	2.7
S. typhimurium NCTC 5710 LPS	9.1	30.9	0.47	1.5

Table 25: THE DEGREE OF HAEMOLYSIS PRODUCED BY THE ADSORPTION OF UNTREATED AND ACTIVATED LPS TO SHEEP ERYTHROCYTES

Lipopolysaccharide from *E. coli* NCTC 8623	Degree of Haemolysis (%)*
Untreated-LPS	4.4
Heat-activated-LPS	13.8
Alkali-activated-LPS	20.9

*5×10^9 SRBC used

but only 0.73 per cent in the human erythrocytes. The age of the erythrocytes might be an important factor in this result. The degree of haemoglobin leakage was also dependent on the bacterial polysaccharide. For example, the degree of leakage from human erythrocytes ranged from 15.4 per cent with the mycobacterial preparation ST202 to 0.45 per cent with the mycobacterial preparation ST209.

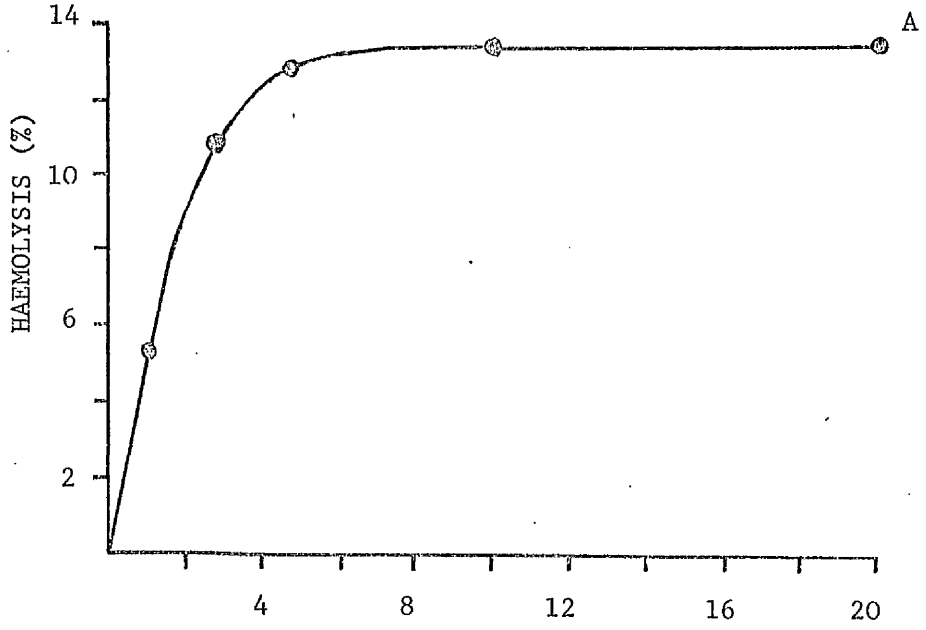
Since the degree of leakage was dependent on the amount of polysaccharide used, dose-response curves were set up for sheep erythrocytes (5×10^9) with variable concentrations of the polysaccharide (Figure 38). When the glycopeptide (ST208) was tested, an initial sharp increase in the degree of haemoglobin leakage was observed with only a relatively small increase in the amount of glycopeptide. Hence, the maximum degree of leakage (per cent) was produced by 10 mg of the glycopeptide, so that increasing the amount of glycopeptide did not significantly increase the degree of leakage. When LPS from E. coli NCTC 8623 was used a linear relationship over 0.3 to 20 mg range was observed; a per cent increase in leakage was produced for each milligram of the LPS added.

The stability of modified sheep erythrocytes

The stability of modified sheep erythrocytes was measured over 6 days and compared to untreated erythrocytes (Figure 39). It was observed that normal untreated erythrocytes gradually lysed over several days. After 6 days an approximate 30 per cent increase in haemolysis was observed in these cells. However, modified erythrocyte lysis was in

Figure 38: The Dose-Response Relationship between the Degree of Haemolysis and the Amount of Polysaccharide

The haemolysis (%) of sheep erythrocytes was plotted against the amount of polysaccharide (mg) used per test. Figure A represents the relationship observed with the Mycobacterial glycopeptide (ST208) and Figure B the relationship with *E. coli* NCTC 8623 LPS.


14
10
6
2
HAEMOLYSIS (%)
A
4
8
12
16
20
POLYSACCHARIDE (mg)

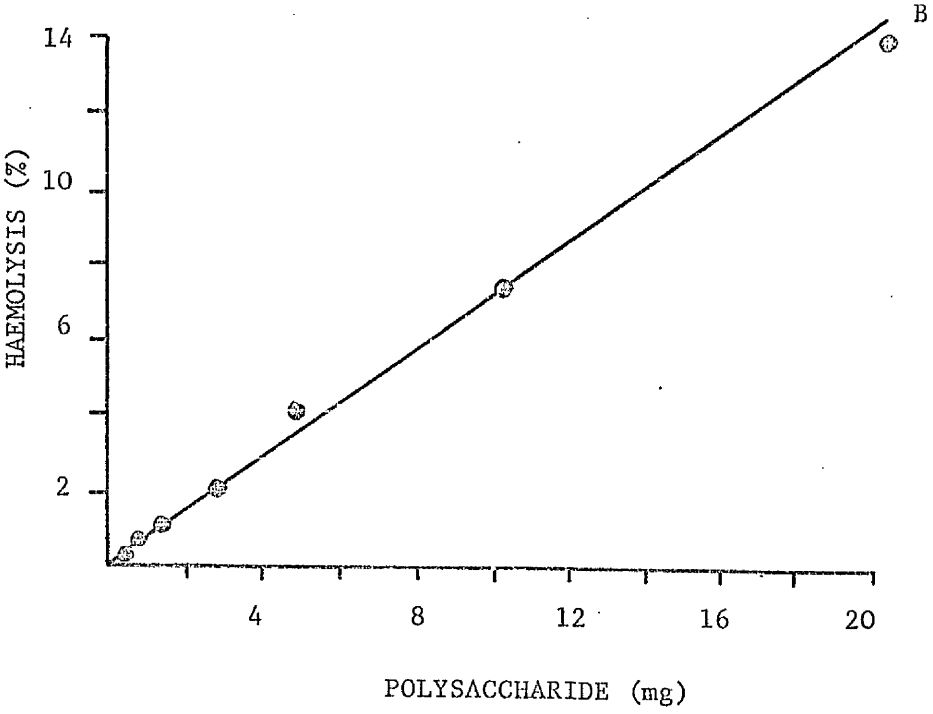

14
10
6
2
HAEMOLYSIS (%)
B
4
8
12
16
20
POLYSACCHARIDE (mg)

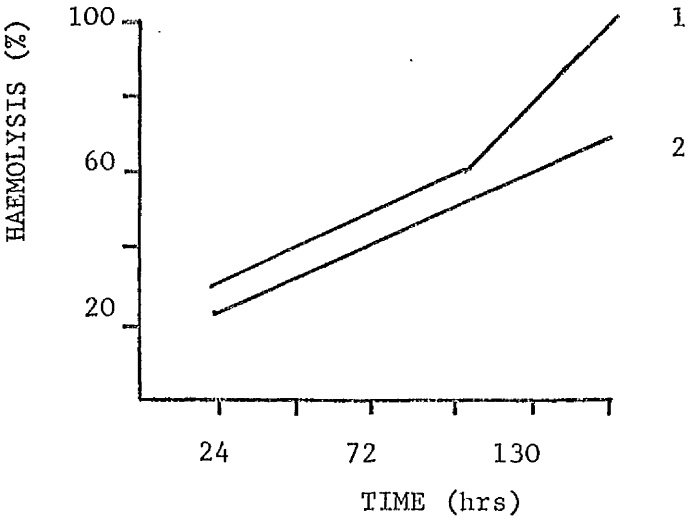
Figure 39: The Stability of Sheep Erythrocytes treated with Mycobacterial Glycopeptide (ST208)

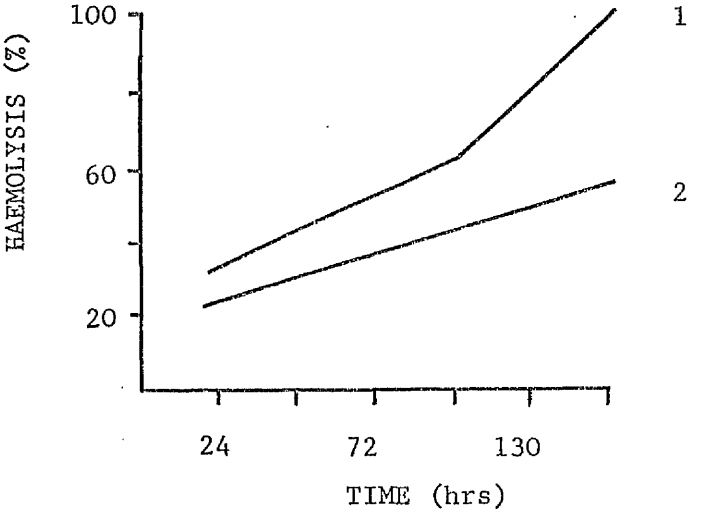
The haemolysin (%) of the erythrocytes was plotted against the time of incubation (hours) at $4^{\circ}C$, after modification at $37^{\circ}C$. Each graph is an observation of the same phenomenon using different batches of sheep erythrocytes.

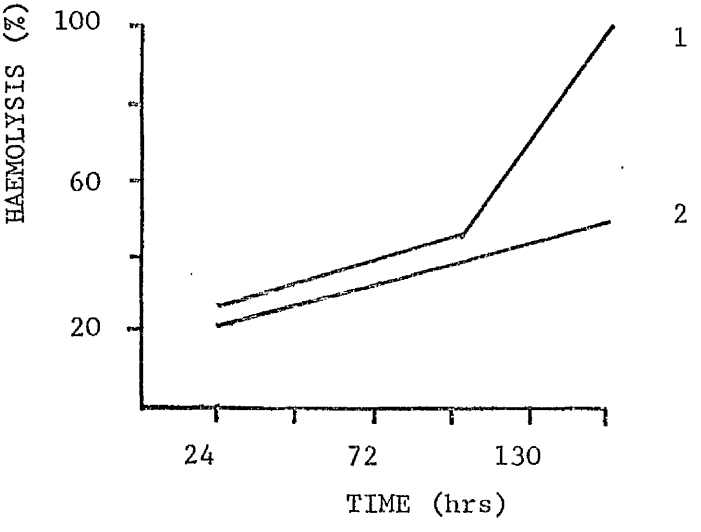
Symbols

1 represents the haemolysis observed with erythrocytes treated with glycopeptide.

2 represents the haemolysin observed with untreated erythrocytes.


HAEMOLYSIS (%)
100
60
20
1
2
24
72
130
TIME (hrs)


HAEMOLYSIS (%)
100
60
20
1
2
24
72
130
TIME (hrs)


HAEMOLYSIS (%)
100
60
20
1
2
24
72
130
TIME (hrs)

two stages; an initial stage of gradual lysis over 4 days, followed by an increased rate of haemolysis, to give 100 per cent haemolysis after 6 days. From these results it was concluded that the stability of the modified erythrocytes was altered, either due directly to the action of the polysaccharide or to the release of the polysaccharide from the cell membrane into the supernatant fluid causing an osmotic imbalance which consequently affected erythrocyte stability.

The Detection of Cell-Associated Polysaccharide

Cell-associated bacterial polysaccharide was detected qualitatively either by fluorescent antibody or by a haemagglutination technique.

Sheep erythrocytes modified either with *E. coli* NCTC 8623 LPS or with the mycobacterial glycopeptide (ST208) were treated with rabbit anti-polysaccharide serum, followed by treatment with sheep anti-rabbit fluorescent antibody. From the results (Table 26) only modified cells exhibited strong positive flucrescence as an even layer over the whole cell surface (Plate 1a, Appendix XI).

Further, the reported dual affinity of the glycopeptide for mammalian cell membranes and guinea-pig γ_2-immunoglobulin was investigated (Appendix XI) and extended to *E. coli* NCTC 8623 LPS. Both polysaccharides exhibited the same pattern of results (Table 26). The polysaccharides showed a dual affinity for the sheep erythrocytes and guinea-pig γ_2-immunoglobulin, but not for γ_1-immunoglobulin. This dual affinity was demonstrated by fluorescent antibody and the fluorescence obtained was in the nature of discrete sectors over the cell surface (Plate 1c , Appendix XI).

Table 26: THE DETECTION OF CELL-ASSOCIATED POLYSACCHARIDE

Reaction Mixture	
SRBC + PS + R/PS + S/R*	Fluorescence
SRBC + R/PS + S/R*	no fluorescence
SRBC + PS + S/R*	" "
SRBC + S/R*	" "
SRBC + PS + γ_2 + S/G-p*	Fluorescence
SRBC + PS + γ_1 + S/G-p*	no fluorescence
SRBC + γ_2 + S/G-p*	" "
SRBC + γ_1 + S/G-p*	" "
SRBC + PS + S/G-p*	" "
SRBC + S/G-p*	" "
SRBC + PS + γ_2 + EA	Haemagglutination
SRBC + PS + γ_1 + EA	no haemagglutination
SRBC + γ_2 + EA	" "
SRBC + γ_1 + EA	" "
SRBC + PS + EA	" "
SRBC + EA	" "

Abbreviations : SRBC - sheep erythrocytes; PS - polysaccharide fraction either glycopeptide (ST208) or *E. coli* NCTC 8623 LPS; γ_1 - guinea-pig anti-ovalbumin-γ_1-immunoglobulin; γ_2 - guinea-pig anti-ovalbumin-γ_2-immunoglobulin; EA - ovalbumin; S/G-p* - sheep anti-guinea-pig fluorescent antibody; S/R* - sheep anti-rabbit fluorescent antibody; R/PS - rabbit anti-polysaccharide serum.

Similarly if the modified erythrocytes were treated with γ_2-immunoglobulin as before, and subsequently with ovalbumin (1 mg/ml) haemagglutination occurred. This also was evidence for the dual affinity of the bacterial polysaccharides.

From the fluorescent antibody and haemagglutination results, it was concluded that the γ_2-immunoglobulin, after fixation to the cell surface by the bacterial polysaccharide, still retained exposed antigenic determinants, and was still functional as an antibody.

SECTION B : ADJUVANT ACTIVITY OF BACTERIAL POLYSACCHARIDES

Determination of Suitable Conditions for Immunization of Adult CD Mice

Adult CD mice in groups of five, were injected with 20 μg of ovalbumin in a saline solution with either 30, 60, 90 or 120 μg of LPS from E. coli NCTC 8623. Control mice were injected with the antigen alone. The mice were either given a single injection or three injections which were administered either intravenously or intraperitoneally. The animals were bled out after 21 days. The mean haemagglutinating anti-ovalbumin titres were calculated (Table 27) and plotted against the amount of lipopolysaccharide (Figure 40). It was evident that the levels of circulating antibody depended on at least three parameters in this system. The LPS was important; in the absence of the LPS only comparatively low levels of antibody were detected. However if the amount of LPS present was increased the anti-ovalbumin antibody levels also increased, until a limit was reached when further increases in LPS caused a relative suppression of detectable anti-ovalbumin antibody levels in the serum. In the case of single injections, 90 μg of LPS produced the highest antibody levels; an increase in LPS beyond 90 μg. caused a decrease in the anti-ovalbumin antibody levels. Similarly, 60 μg LPS in the triple injection method produced the highest antibody levels. The antibody level was also dependent on the route of administration. Intravenous injections stimulated higher antibody levels than intraperitoneal injections. For example, intravenously 60 μg LPS produced an anti-ovalbumin antibody titre of 7.5, but intraperitoneally, the antibody titre was 1.3. The third factor affecting antibody titres

Table 27: DETERMINATION OF SUITABLE CONDITIONS FOR IMMUNIZATION OF ADULT CD MICE USING 20 μg OVALBUMIN AS ANTIGEN AND E. COLI NCTC 8623 LIPOPOLYSACCHARIDE AS ADJUVANT

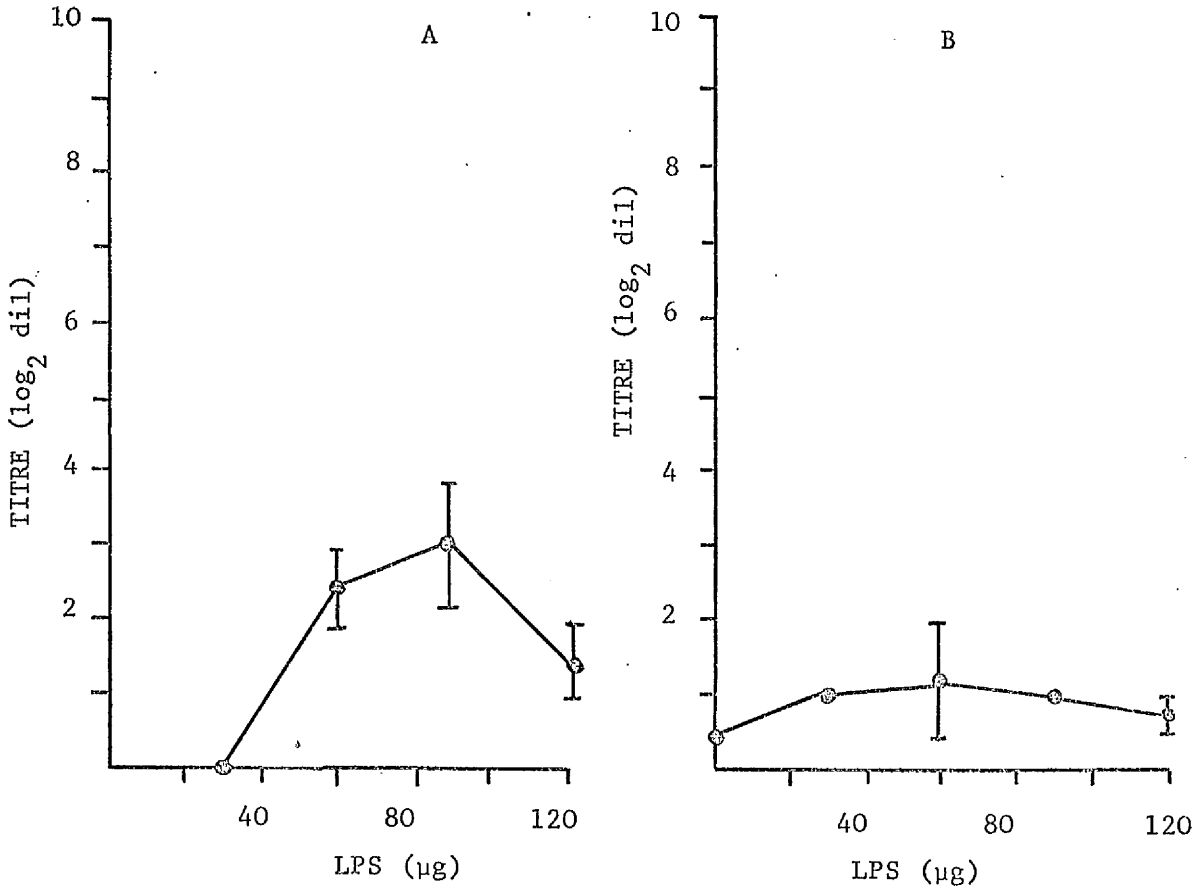
Immunization Procedure		Mean Haemagglutination antibody titre (log_2 dilution)	Standard deviation in log_2 dilution values
Injection course and site	Amount of lipopoly-saccharide per injection (μg)		
Single injection intraperitoneally	0	0	0
	30	0	0
	60	2.5	0.57
	90	3.0	0.81
	120	1.5	0.57
Single injection intravenously	0	0	0
	30	1.5	0.57
	60	2.5	0.57
	90	3.5	1.28
	120	3.0	0.81
Triple injection intraperitoneally	0	0.5	0.57
	30	1.0	0
	60	1.3	0.88
	90	1.0	0
	120	0.8	0.4
Triple injection intravenously	0	1.0	0
	30	4.0	0.81
	60	7.5	0.57
	90	5.0	0.81
	120	4.5	0.57

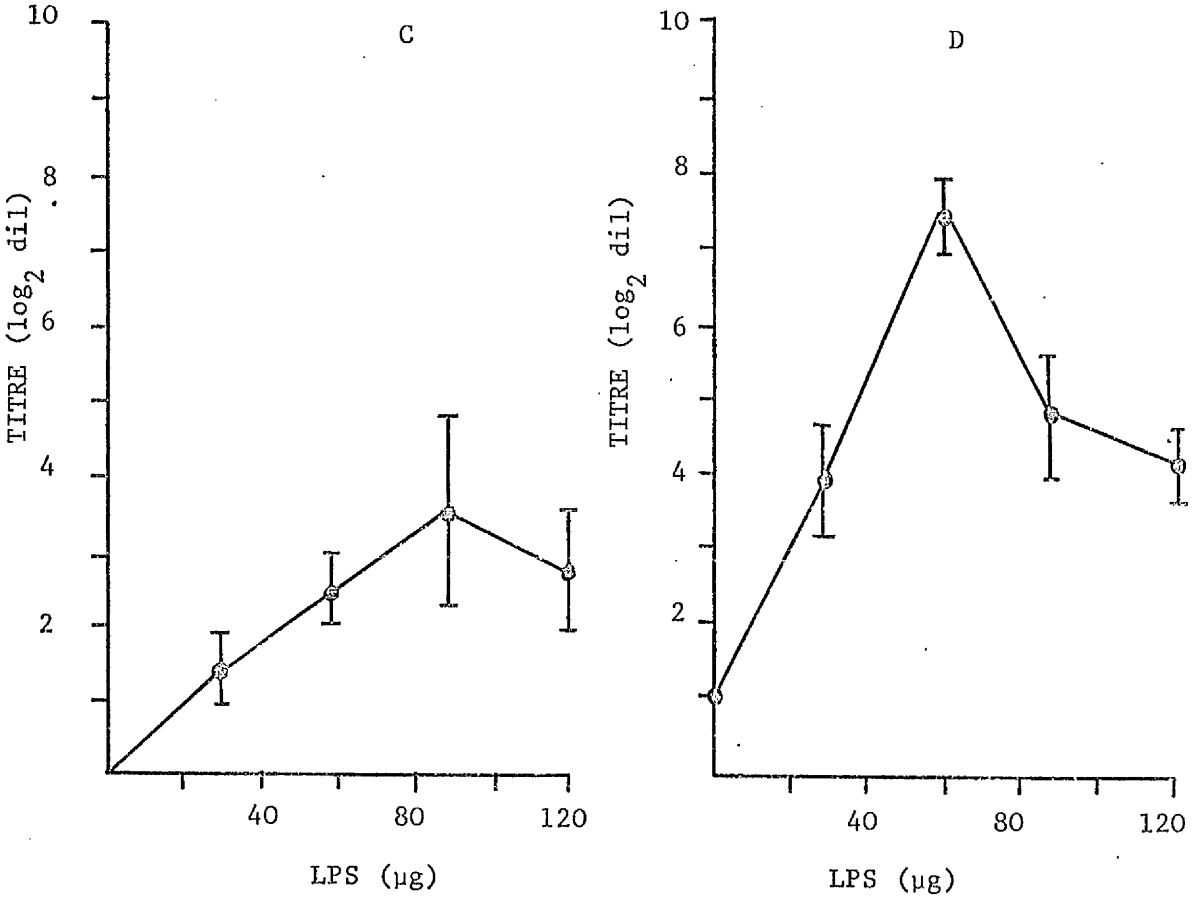
These results are presented graphically in Figure 40

Figure 40: Determination of Suitable Conditions for the Immunization of Mice

The antibody titre ($\log_2$ dilution) was plotted against the amount of LPS (μg) in the antigen:adjuvant injection mixture. Figure A represents the titres obtained by a single intraperitoneal injection and Figure B by triple intraperitoneal injections. Figure C represents titres obtained by a single intravenous injection, Figure D by triple intravenous injections.

I represents the standard deviation associated with the plotted mean value of antibody titre.


A
B
10
8
6
4
2
TITRE (log_2 dil)
40
80
120
LPS (µg)


C
D
10
8
6
4
2
TITRE (log_2 dil)
40
80
120
LPS (µg)

was the number of injections. Three intravenous injections stimulated higher antibody levels than a single injection. However, intraperitoneally, a single injection produced higher antibody levels.

A Comparison of the Adjuvant Effects of LPS from E. coli NCTC 8623 and S. typhi NCTC 0901

The mean haemagglutinating anti-ovalbumin antibody titres were calculated (Table 28) in sera from mice immunized with 20 μg of ovalbumin and either 30, 60 or 90 μg of the lipopolysaccharide. The mice were injected intravenously with either a single or a triple injection. By plotting the anti-ovalbumin antibody levels against the amount of lipopolysaccharide used (Figure 41) it was observed that the LPS from S. typhi stimulated higher anti-ovalbumin antibody levels than E. coli LPS. With a single injection, 60 μg of S. typhi LPS stimulated an antibody titre of 3.5, while the equivalent titre for E. coli LPS was 2.8. Similarly with a triple injection the comparable antibody titres were 10 and 8.2 for S. typhi LPS and E. coli LPS respectively. From the graphs it was observed that approximately half the amount of S. typhi LPS was required to stimulate a certain antibody level when compared to E. coli LPS. For example, an anti-ovalbumin antibody titre of 8 was stimulated by 27 μg of S. typhi LPS and by 55 μg of E. coli LPS. By this method E. coli NCTC 8623 LPS was given a relative adjuvancy value of 1.0, and S. typhi NCTC 0901 LPS had a relative adjuvancy value of 2.0.

Determination of the Relative Adjuvancies of Bacterial Polysaccharides

The anti-ovalbumin antibody titres were calculated for sera

Table 28: A COMPARISON OF THE ADJUVANT EFFECTS ON ANTIBODY PRODUCTION OF LPS FROM *E. COLI* NCTC 8623 AND *S. TYPHI* NCTC 0901

Immunization Procedure			Mean Haemagglutinating Antibody titre (log_2 dilution)	Standard Deviation
Injection course and site	LPS	Amount of LPS per injection (μg)		
Single injection, intravenously	*E. coli* NCTC 8623	0	0	0
	"	30	0.8	1.09
	"	60	2.8	0.8
	"	90	1.0	0
	S. typhi NCTC 0901	0	0	0
	"	30	1.0	0.81
	"	60	3.5	0.57
	"	90	2.5	0.57
Triple injection,	*E. coli* NCTC 8623	0	1.0	0.89
	"	30	6.6	0.54
	"	60	8.2	1.48
	"	90	6.0	0.57
	S. typhi NCTC 0901	0	1.0	0.89
	"	30	8.2	0.83
	"	60	10	0.81
	"	90	8.0	1.0

These results are represented graphically in Figure 41

Figure 41: A Comparison of the adjuvant Effects on Antibody Production of LPS from E. coli NCTC 8623 and S. typhi NCTC 0901

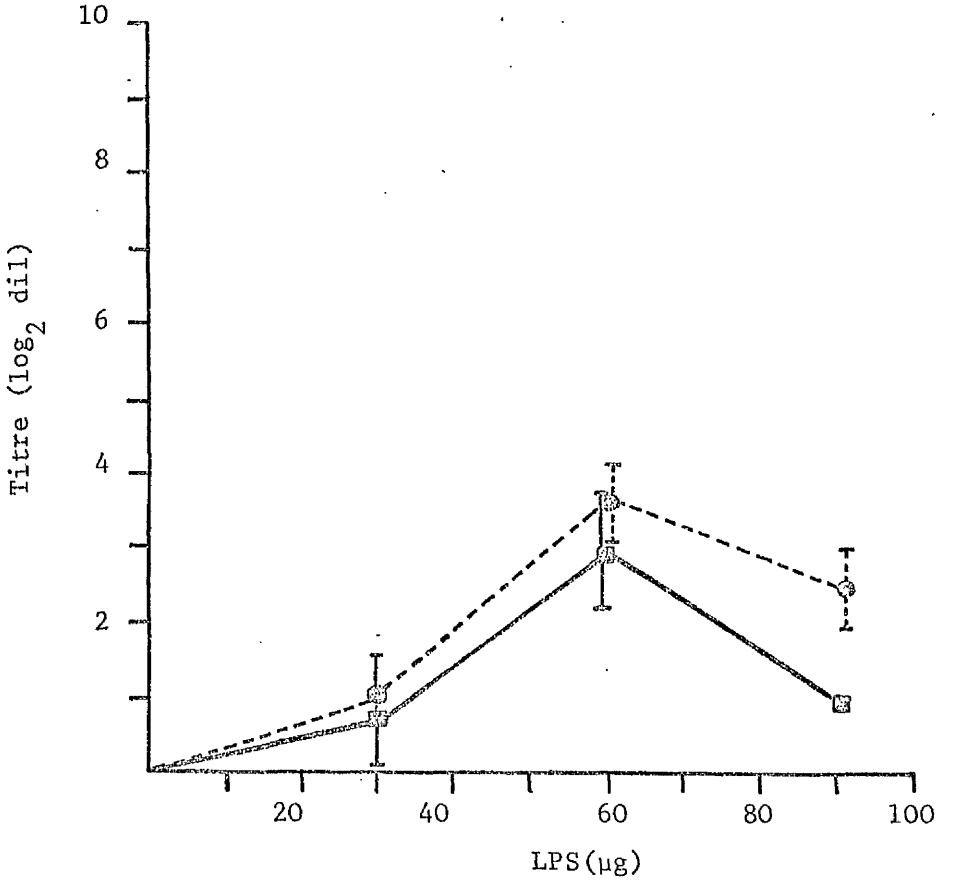
The antibody titre ($\log_2$ dilution) was plotted against the amount (μg) of LPS used per injection. Figure a represents the results from a single injection and Figure b the results from a triple injection.

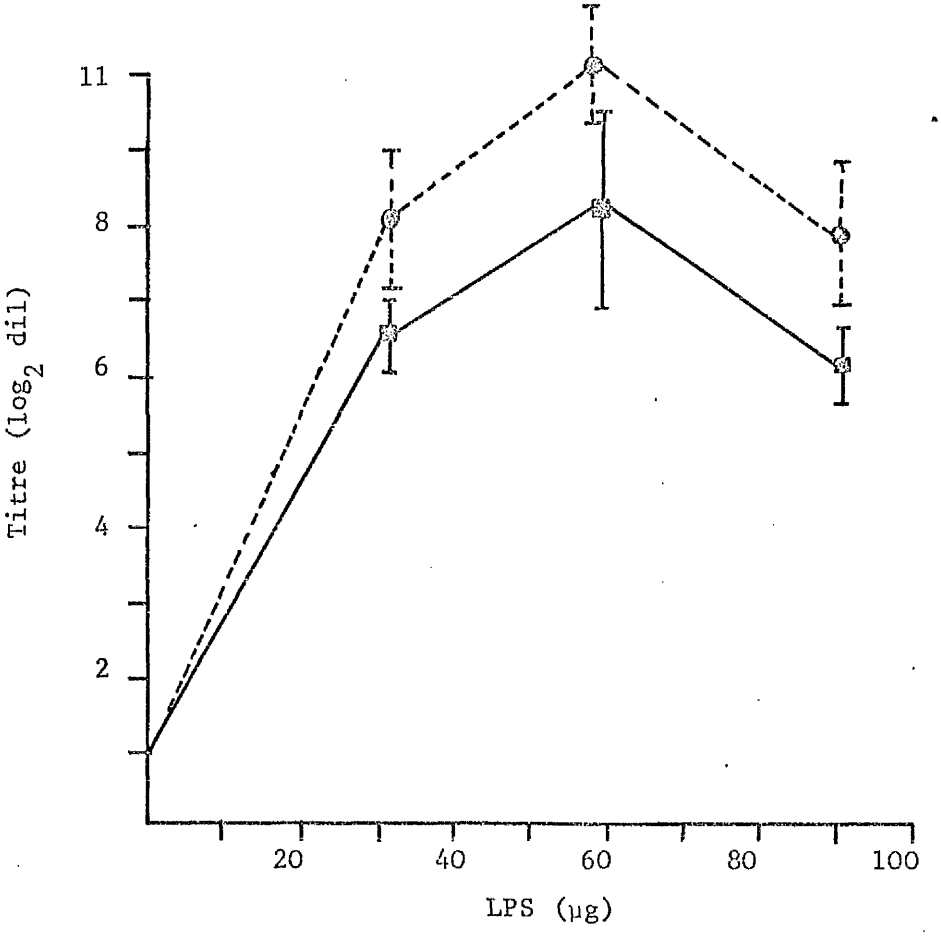
Symbols

—○— represents the response using E. coli NCTC 8623 LPS.

---○--- represents the response using S. typhi NCTC 0901 LPS.

I represents the standard deviation associated with each mean observation.


10
8
6
4
2
Titre (log_2 dil)
20
40
60
80
100
LPS(µg)


11
8
6
4
2
Titre (log_2 dil)
20
40
60
80
100
LPS (µg)

from mice given three intravenous injections of 20 μg of ovalbumin and either 30 or 60 μg of the bacterial polysaccharide. The relative adjuvancy values were calculated using the E. coli NCTC 8623 LPS as a standard preparation, with an adjuvancy value of 1.0 (Table 29a and 29b). The LPS preparation which stimulated the highest antibody levels was from S. typhi NCTC 0901, having a relative adjuvancy value of 1.9. The least effective LPS preparation was extracted from E. coli B by EDTA having a relative adjuvancy value of 0.18. The statistical analysis of these results is presented in Appendix VIII.

The mycobacterial polysaccharide containing fractions were also tested by the same method. The relative adjuvancy values, again compared to E. coli NCTC 8623 LPS, ranged from 0.58 for ST208 to 0.95 for ST210. Throughout, the antibody levels produced by the mycobacterial fractions were comparable to those produced by the lipopolysaccharides (Table 30).

The Effect of Alkali and Periodate Treatment on the Adjuvant Activity of Bacterial Polysaccharides

A comparison was made of the antibody levels stimulated by untreated, alkali-treated and periodate-treated polysaccharides. The mice were given three intravenous injections containing 20 μg of ovalbumin and 60 μg of the appropriate bacterial polysaccharide preparation. The lipopolysaccharides from E. coli NCTC 5710 were tested together with the mycobacterial glycopeptide (ST208). Alkali and periodate treatment caused a sharp decrease in the adjuvant activity of these polysaccharides (Table 31). For example, the anti-ovalbumin antibody titre stimulated

Table 29a:

THE DETERMINATION OF THE RELATIVE ADJUVANCIES OF LIPOPOLYSACCHARIDES FROM GRAM-NEGATIVE BACTERIA

Lipopolysaccharide	Mean Haemagglutinating anti-ovalbumin antibody titre (log_2 dilution)		Relative[2] adjuvancy	95% Confidence limits	
	High dose/ 60 µg LPS	Low dose/ 30 µg LPS		Lower limit	Upper limit
E. coli NCTC 8623	8.2 (1.48)[1]	6.6 (0.54)			
S. typhi NCTC 0901	10 (0.81)	8.2 (0.83)	1.99	1.34	2.65
E. coli NCTC 8623	10 (0.81)	7.6 (1.34)			
E. coli B (Ether extraction)	7.0 (0.81)	4.3 (2.3)	0.50	0.18	0.84
E. coli NCTC 8623	11.0 (0.81)	6.8 (0.7)			
Sh. flexneri	3.8 (1.09)	1.4 (0.57)	0.24	0.12	0.35
E. coli NCTC 8623	11.0 (0.81)	6.8 (0.7)			
F. tularensis	9.0 (1.0)	8.6 (0.57)	0.96	0.72	1.2
E. coli NCTC 8623	8.4 (0.89)	7.6 (0.89)			
S. typhimurium NCTC 5710	7.2 (1.14)	6.2 (1.22)	0.44	0.15	0.83
E. coli NCTC 8623	11.0 (0.81)	6.8 (0.7)			
Bd. pertussis NCTC 18334	8.5 (1.00)	4.25 (0.25)	0.64	0.52	0.75

1. Figures in brackets represent the standard deviation of the mean anti-ovalbumin antibody titre.

2. The adjuvancy values are all compared to the LPS from *E. coli* NCTC 8623 which was given a relative adjuvancy value of 1.0.

Table 29b: THE DETERMINATION OF THE RELATIVE ADJUVANCIES OF LIPOPOLYSACCHARIDES FROM GRAM-NEGATIVE BACTERIA

Lipopolysaccharide	Mean Haemagglutinating anti-ovalbumin Antibody Titres (log_2 dilution)		Relative Adjuvancy[2]	95% Confidence Limits	
	High dose/ 60 µg LPS	Low dose/ 30 µg LPS		Lower limit	Upper limit
E. coli NCTC 8623	11.0 (0.84)[1]	6.8 (0.70)			
E. coli 055:B5	6.4 (0.54)	2.6 (0.54)	0.46	0.38	0.54
E. coli NCTC 8623	11.0 (0.81)	6.8 (0.70)			
E. coli B (EDTA extraction)	4.0 (1.64)	1.4 (0.89)	0.18	0.06	0.30

1. Figures in brackets represent the standard deviation of the mean antibody titre.

2. The adjuvancy values are all compared to LPS from *E. coli* NCTC 8623 which was given a relative adjuvancy value of 1.0.

Table 30:

THE DETERMINATION OF THE RELATIVE ADJUVANCIES OF MYCOBACTERIAL POLYSACCHARIDE CONTAINING FRACTIONS

Mycobacterial Fraction	Mean Haemagglutinating anti-ovalbumin antibody titre (log_2 dilution)		Relative[2] adjuvancy	95% Confidence limits	
	High dose/ 60 µg fraction	Low dose/ 30 µg fraction		Lower limit	Upper limit
E. coli NCTC 8623 ST82	11.0 (0.81)[1] 9.8 (0.44)	6.8 (0.70) 6.0 (1.0)	0.90	0.84	0.95
E. coli NCTC 8623 ST208	11.0 (0.81) 7.6 (1.0)	6.8 (0.70) 7.0 (1.4)	0.58	0.25	0.86
E. coli NCTC 8623 ST210	10.0 (0.81) 9.6 (0.57)	7.6 (1.34) 7.3 (0.57)	0.95	0.60	1.4
E. coli NCTC 8623 ST211	11.0 (0.81) 8.0 (1.22)	6.8 (0.70) 4.4 (0.54)	0.62	0.50	0.72
E. coli NCTC 8623 PPD	10.0 (0.81) 9.0 (0.57)	7.6 (1.34) 6.25 (1.25)	0.79	0.62	0.83

1. Figures in brackets represent the standard deviation of the mean antibody titre.

2. The adjuvancy values are all compared to LPS from *E. coli* NCTC 8623 which was given a relative adjuvancy value of 1.0.

by S. typhi NCTC 0901 decreased from 10.2 to 2.5 and 3.8 after alkali and periodate treatment respectively. Similarly the antibody titre of 6.2 stimulated by the mycobacterial glycopeptide was lowered to 1.5 and 1.0 after alkali and periodate treatment. It should be mentioned that the antibody levels stimulated by alkali and periodate treated polysaccharides were comparable to those stimulated by the antigen in a saline solution without an adjuvant.

Table 31:

THE EFFECTS ON STIMULATED ANTIBODY PRODUCTION OF ALKALI AND PERIODATE TREATMENT OF THE POLYSACCHARIDES

Bacterial Lipopolysaccharide (60 μg)	Mean Haemagglutinating Antibody Titres (log_2 dilution)		
	Unheated	Alkali-treated	Periodate-treated
E. coli NCTC 8623	8.0 (1.0)*	2.8 (0.57)	3.6 (0.80)
S. typhi NCTC 0901	10.2 (0.8)	2.5 (0.3)	3.8 (0.57)
S. typhimurium NCTC 5710	7.2 (1.0)	2.3 (0.57)	1.5 (0.80)
Mycobacterial glycopeptide (ST208)	6.2 (1.2)	1.5 (0.80)	1.0 (0.80)

Mean antibody titre in absence of bacterial polysaccharide = 1.0 (0.80)

* The figures in brackets represent the standard deviations of the mean antibody titres

DISCUSSION

A: THE UPTAKE OF BACTERIAL POLYSACCHARIDES TO MAMMALIAN CELL SURFACES

1. The Influence of the Cell

The quantitative variation in polysaccharide adsorption

The preliminary experiments using the chemical method revealed that it was necessary to use accurate methods for standardizing erythrocyte suspensions, in order to detect the adsorption of small amounts of bacterial polysaccharides to the cell surface. This necessity for accurate standardization was due to the inherent variability in carbohydrate composition of the erythrocyte membranes. Throughout this investigation it was apparent that the uptake of a particular bacterial polysaccharide by sheep erythrocytes was quantitatively variable. Upon closer analysis, it was found that the fluctuation in the amount of bacterial polysaccharide adsorbed was inversely related to the carbohydrate content of the untreated erythrocyte membranes (Figure 17). It was noted that the carbohydrate content of untreated erythrocytes varied quite considerably. It is possible that this variation could be related to the diet of the sheep, although there are numerous other factors which could affect erythrocyte membrane composition.

However, it should be mentioned that erythrocytes from non-pregnant sheep behaved normally with respect to cellular modification by the mycobacterial glycopeptide (ST208); the expected amount of glycopeptide adsorbed was 1.7 mg. After the sheep had lambed, the erythrocytes adsorbed less than 0.2 mg of the fraction. This inability

to modify the erythrocytes after lambing did not appear to be related to the carbohydrate composition of the erythrocyte membrane. It is possible that factor(s) produced during pregnancy, present in the serum, either blocked membrane receptor sites or altered the membrane so that modification was prevented. Stimson (personal communication) found that an α-macroglobulin produced during human pregnancy, bound to erythrocyte membranes and persisted for some time in the post-partum parental serum. It is interesting to speculate that such a component might block the binding of bacterial polysaccharides to the erythrocyte membrane. Further investigation of this phenomenon is warranted on the basis of susceptibility to infections from endotoxin-containing organisms.

Effect of erythrocyte concentration

In the presence of variable numbers of rabbit erythrocytes, the adsorption of LPS appeared to be dependent on the number of cells present up to a certain limit (Figure 19). Beyond this level any increase in cell numbers did not result in a corresponding increase in the amount of LPS adsorbed. As shown in Table A8 (Appendix VII), 34% of available LPS was the maximum amount that could be adsorbed by the cell numbers used. Further experiments revealed that the uptake of this 34% of LPS occurred in two phases; during the initial phase (I) there was an uptake of a small proportion of the LPS (2.8%) followed by the second phase (II) in which 31.2% of the LPS was taken up by the cells. Proportionately less cells were required to adsorb 2.8% than 31.2% of the LPS. It is possible that the phenol/water extraction produced a heterogeneous preparation of LPS consisting of at least three "populations"

of molecules. These were termed LPS_I, LPS_{II} and LPS_{III} based on the uptake phase during which the molecules were adsorbed by the erythrocytes (Figure 19). Shands (1971) stated that LPS molecules in an aqueous environment were held in several different configurations, e.g. discs, lamellae, vesicles and ribbons. It is interesting to speculate that each of these configurations could have a different affinity for cell membranes or that cells could adsorb more molecules of one type of configuration than another due to steric hindrance. Therefore, one could conclude that the $LPS_{I, II}$ and $_{III}$ molecules represent different configurations of the same LPS molecule. LPS_I molecules have a greater affinity for membranes than LPS_{II} molecules, which in turn have a greater affinity for membranes than LPS_{III} molecules which possess little or no affinity. Hence the observed differences in uptake could reflect the different avidities of the LPS molecules for the membrane receptor. However, the receptor sites on the membrane could also exert an influence on LPS adsorption. If all the LPS molecules compete for the same receptor sites, the latter could have different avidities for the different LPS molecular configurations. Another possibility is that there are different receptors for the different LPS molecular populations. These receptors are situated permanently on the cell surface so that there is always a situation where $LPS_I > LPS_{II} > LPS_{III}$ receptors. On the other hand, there could be the same numbers of receptors for $LPS_{I, II}$ and $_{III}$ but due to the fluid and mobile nature of the membrane the proportion of receptors exposed to the environment at any one time varies in such a manner that there are always more LPS_I receptors than LPS_{II} receptors on the "external" membrane surface. The possibility of two populations of LPS molecule

possessing different affinities for the membrane was reflected in both the ^{14}C- and ^{32}P-LPS results. Heat and alkali treatment affected the polysaccharides in such a way as to increase their affinity for cell membranes. The activation process will be considered in more detail later (see page 130), but during LPS extraction by the phenol/water method, the bacterial cells were heated at 65°C for 20 min and it is possible that partial activation of the LPS molecules occurred. Since 65°C is below the optimum temperature for heat activation only a few molecules would be heat-activated and these might represent the LPS_I molecular population; these molecules are termed "autoactivated" LPS molecules.

Effect of different types of mouse cell

Besides the adsorption of LPS by mammalian erythrocytes it was found that other cell types could also adsorb LPS (Table 8). A comparison of the adsorption of a standard amount of LPS from E. coli NCTC 8623 by the various cells, showed that the greatest adsorption was exhibited by mouse peritoneal macrophages, while mouse erythrocytes exhibited the least adsorption. On a quantitative basis the relative affinity of the LPS from the different cell types was calculated. The relative affinities for peritoneal macrophages was 11.5, for thymus and bone marrow cells 6.5, for spleen cells 5.6, for peritoneal lymphocytes 2.2 and for erythrocytes 1.0.

The adsorption of large quantities of LPS by the peritoneal macrophages presumably reflects the functional phagocytic activity of the reticuloendothelial system. The bone marrow cells represent

haematopoietic stem cells at different stages of differentiation and maturation, and these have equal LPS-adsorptive properties to the thymus cells which represent a population of T-lymphocytes (thymocytes) derived indirectly from the bone-marrow stem cells. The spleen cells, adsorbing relatively less LPS, consist of two basic cell populations, the T and B lymphocytes, of which the latter form the majority.

Although the majority of the published work was carried out using erythrocytes, it appears that such cells have relatively insignificant LPS-absorptive properties when compared to other cell types. The differences in the amount of LPS adsorbed by the various cells might indicate either the number of LPS-receptor sites present on each cell membrane or the different avidities of the receptor sites. Another possibility is that the surface topography of each cell type is such that when LPS molecules cluster around the membrane steric hindrance is accentuated.

2. The Effect of Polysaccharide Concentration

By varying the number of LPS molecules in contact with a constant number of erythrocytes it was observed that the number of LPS molecules adsorbed was dependent on the number of molecules available. However, a limit was reached when a further increase in the number of available LPS molecules did not result in a corresponding increase in adsorbed LPS. This state presumably reflected a situation where either all the available receptor sites on the erythrocyte membrane were filled, or due to steric hindrance no further LPS molecules could be adsorbed

onto the membrane. In Figure 22 the maximum amount of LPS adsorbed by the erythrocytes, in terms of detectable radioactive counts, was 1600 dpm. However, if LPS was added in amounts below 1600 dpm approximately 80% was adsorbed by the erythrocytes. The remaining 20% remained in the supernatant fluid although adsorption sites were available on the erythrocyte membrane.

When an identical experiment was performed using the chemical method to detect adsorbed polysaccharide a similar result was obtained. A limit was reached beyond which it was found that increased amounts of polysaccharide available for adsorption did not result in further increases in cell-associated polysaccharide. This was an indication that all the available sites for polysaccharide adsorption were filled. Although rabbit erythrocytes were able to adsorb a comparative level of 9.0 mg of the Mycobacterial glycopeptide (ST208) (Figure 20), when it was tested in quantities below this level, only 84% of the available material was adsorbed to the cells; the residue remained in the supernatant fluid. This phenomenon appeared to be reproducible since it was also found with the LPS preparation from E. coli NCTC 8623 (Figure 21). It indicated that a fraction of the polysaccharide preparations lacked the capacity to bind to the cell membranes.

3. The Effect of Time of Incubation

From observations involving the time of exposure of polysaccharides to cells during the cellular modification process, it was observed that the uptake of untreated bacterial polysaccharide by all the cell types tested, exhibited a cyclic fluctuation (Figures 24 to 29).

This fluctuation seemed to follow a regular cycle, irregardless of the cell type. With blood cells (i.e. erythrocytes and white blood cells) it was observed that those from both human beings and rabbits showed peaks of maximum uptake at approximately 60 and 120 min from the beginning of the incubation period. Peritoneal exudate cells also showed similar response patterns with the uptake peaks occurring slightly earlier than found with the blood cells. If the lipopolysaccharide was heat-activated, a different response was observed (Figure 32). The erythrocytes adsorbed the activated LPS to a greater extent than the untreated LPS, but the pattern of adsorption failed to show significant cyclic fluctuation, which was characteristic of the untreated LPS.

From the shapes of the curves obtained with untreated LPS it was observed that the decrease in the amount of cell-associated LPS was gradual. This gradual decline in cell-associated LPS was possibly either related to a slow release of LPS molecules from the cell surface of an asynchronous rapid release by all the modified cells. Since the adsorption of bacterial polysaccharides onto cell membranes is considered to be a passive process, the slow release mechanism seems less likely than a mechanism whereby the adsorbed molecules are suddenly released due to changes in the cell surface; these changes occurring asynchronously throughout the cell suspension.

There appeared to be a dissociation of the majority of the adsorbed LPS molecules from the cell membrane with a small amount of the LPS remaining cell-associated. It is interesting to observe that there appears, once again, to be two different LPS molecular populations adsorbing onto the cell membrane and exhibiting different affinities.

The minor population of LPS molecules with a strong affinity remained cell-associated throughout the experiment, while the major population exhibiting weak bond formation appeared to show the cyclic fluctuation in the adsorption patterns.

The nature of the bond between the bacterial polysaccharide and the cell membrane is still open to debate. Various workers (Vogel, 1957; Hammerling and Westphal, 1967) subscribed to the idea of lipid: lipid covalent hydrophobic interactions between the lipopolysaccharides and the membranes with strong bond formation. Other work from Luderitz et al., (1958), Springer et al., (1973) and Adye et al., (1973) indicated that the bond between the LPS and the membrane was weak in nature and reversible. The findings from this investigation involving the time sequence indicate that possibly two types of bonds existed between the LPS molecules and the cell membrane. One bond type is relatively strong, possibly a lipid:lipid interaction, while the other bond is relatively weak and possibly suggests that the bacterial polysaccharide is held at the surface by weak charge effects e.g. coulombic forces. Recently, Springer and his colleagues during their investigation of the cell-receptor site, have confirmed that relatively weak bonds exist between the LPS molecule and the cell membrane (Adye et al., 1973; Springer et al., 1973). Similarly, Benedetto, Shands and Shah (1973) reported that two bond types could be observed during LPS interactions with artificial phospholipid bilayers. One of the bonds was of the strong covalent type, while the other involved ionic bridges. It was stated earlier that it was thought possible that the minor, stronger affinity population (LPS_I molecules) arose due to partial heat activation during the extraction process, and it is tempting to speculate that published

results indicating strong bond formation were obtained with an increased proportion of "auto-activated" LPS molecules in the preparations due to the particular extraction procedure utilised. The uptake pattern observed with activated-LPS (Figure 32) showed that once activated molecules became adsorbed to the membrane they remained firmly bound. Hence, activation of LPS strengthens the bond formed between the LPS molecule and the membrane. The evidence for weak bond formation could be further strengthened when it is considered that normal untreated LPS had an optimum temperature of adsorption of 22.5°C and that the whole adsorption process was temperature dependent. All the experiments performed during the present investigation were carried out at 37°C to enable a comparison of results with the published findings and to compare *in vitro* findings with the *in vivo* observations. Therefore 22.5°C favours the optimum bond formation between the LPS molecules and the cell membrane, so that at 37°C the bond formation takes place under adverse conditions.

It was found that the uptake of both mycobacterial glycopeptide (ST208) and LPS from *E. coli* NCTC 8623 followed the same pattern if the chemical method was used to measure the cell-associated polysaccharide. The LPS showed a gradual increase in the amount which was cell-associated until a maximum level representing 11% of the available material was reached after 60 min (Figure 28). It was found that with continued incubation there was a gradual decline in the quantity of cell-associated LPS. This same response was observed with the mycobacterial fraction (ST208) which showed a maximum uptake at 100 min of 27% of the available glycopeptide. This dissociation of the erythrocytes and polysaccharide

again tend to indicate that a relatively weak bond was involved in the link between the polysaccharide molecules and the cell-membrane. However, it was noticed that not all of the material became dissociated from the cell surface indicating that another type of bond linked a smaller number of polysaccharide molecules to the cell membrane.

4. The Relative Affinities of Bacterial Polysaccharides for Mammalian Cell Membranes

The relative affinity (R.A.) values of the bacterial lipopolysaccharides and the mycobacterial polysaccharide-containing fractions were calculated from their relative abilities to inhibit the uptake of ^{32}P- and ^{14}C-LPS, by different cell types. It was observed with each type of cell that all the LPS preparations and mycobacterial fractions inhibited uptake of the labelled LPS, although the degree of inhibition was dependent upon the amount and the type of polysaccharide preparation used. Thus inhibition was possibly due to either competition for a common membrane receptor site or binding to different closely situated receptors so that steric hindrance occurred. In addition, the different LPS preparations may share the same membrane receptor and the mycobacterial polysaccharide-containing fractions may have a different receptor which is situated in the region of the LPS receptor. If a common receptor is present the different degrees of inhibition produced by the polysaccharides are related to the differing avidities of these polysaccharides for the receptor site. Although Springer et al., (1973) tested only a small number of LPS preparations with erythrocyte membranes they have confirmed the postulate that the

LPS preparations share a common receptor site. In conclusion from the results of the present investigation it appears that the LPS preparations do share common receptor sites on each particular cell type.

On close analysis of the relative affinity values (Table 32a) no distinct pattern emerges. However, all the polysaccharide preparations generally follow the trend expressed for LPS from E. coli NCTC 8623, in that macrophages adsorb the greatest amount of the polysaccharides and erythrocytes the least amount. The relative affinity values of the polysaccharides will be considered later with respect to their relative adjuvancy values.

5. The Effect of Chemical Treatment on Polysaccharide Uptake

By subjecting the LPS preparations to either heat or alkali treatment it was found that the affinity for membranes could be increased. Earlier reports (Ciznar and Shands, 1971; Neter, Westphal, Luderitz, Gorzynski and Eichenberger, 1956) suggested that 20-fold and 4000-fold increases in the adsorption of LPS could be achieved after such activation. In this investigation increases in the order of 2-3-fold were produced. It would seem that the specificity of heat and alkali treatment in the activation of LPS is a questionable phenomenon. The heat and alkali treatments were thought to affect the LPS lipids with the cleavage of ester-linked fatty acids (Tripodic and Nowotny, 1966) so that there was a change in the configuration of LPS molecules together with a corresponding change in the molecular weight, from 1×10^6 to 2×10^5. The reduction in molecular weight obviously removes some of the

factors contributing to the steric hindrance phenomenon through either reduction in size of the molecule or removal of some inactive components. However, it seems hard to accept that activation could produce the vast increases in LPS adsorption reported above especially as there must be a limit to the number of receptor sites available on the membrane.

The interaction of the activated LPS with the membrane is completely different from the interaction of untreated LPS. Activated LPS was found by Ciznar and Shands (1971) to produce spontaneous haemolysis of the modified erythrocytes. However during this study it was considered that this was not true haemolysis but the leakage of haemoglobin. The degree of leakage produced by activated LPS was approximately 3-5 times greater than that produced by untreated LPS (Table 25). The activated LPS seemed to reorganize erythrocyte membranes to a greater extent than untreated LPS, which indicated a more physical interaction between the activated LPS and the membrane. The binding capacity of polysaccharides was susceptible to periodate treatment which caused reduction in the amount of LPS adsorbed onto the membrane. Periodate treatment is generally considered to affect the polysaccharide moiety of lipopolysaccharides (Neter et al., 1956). This portion of the molecule might be involved with the weak binding forces while the lipid portion is involved with the strong binding forces. It is possible that LPS_{II} molecules which bind by relatively weak forces are prevented from interacting with membranes after periodate treatment. The LPS_{I} molecules however are able to exhibit lipid:lipid interactions and as such are relatively resistant to periodate treatment. The activation by heat and alkali-treatment and the inactivation by periodate treatment was confirmed both by the radioactive and chemical methods.

6. Inhibition of Polysaccharide Uptake by Normal Serum and Simple Organic Compounds

It was found that various agents inhibited the binding of both untreated and heat-activated lipopolysaccharides to erythrocyte membranes.

a) Normal rabbit serum:

In an undiluted form this inhibited the uptake of the untreated and activated LPS (Figure 33). However if the serum was diluted ($\frac{1}{2}$), the degree of inhibition remained at the same level for the untreated LPS but decreased by 80% for activated-LPS. It appeared that the activated-LPS was less susceptible to the action of the inhibitory activity of the serum, and this could be due to the increased affinity of the activated-LPS for the membrane. However if the erythrocytes were pretreated with neat serum, and subsequently washed, the inhibition of uptake remained at the same level for the untreated-LPS which indicated that the serum components bound to sites on the erythrocyte membrane. Pretreatment caused 45% inhibition of uptake and this could indicate that there was an insufficient quantity of the inhibitor in the serum to fill all the available receptor sites on the erythrocyte membrane. In the presence of serum it would be expected that the LPS molecules would compete with the serum inhibitors for the available receptor sites, but under these conditions it was observed that the inhibition was at the 50% level. This indicates that competition does not take place and that the serum components preferentially block the receptor sites; the LPS molecules adsorbing to the remaining available receptor sites.

The results from the pretreatment studies using activated-LPS indicated that the serum inhibitors did not preferentially bind to membrane receptors. If the activated-LPS was incubated together with the erythrocytes in the presence of neat serum the inhibition was at the 50% level, but if the erythrocytes were pretreated with the serum and subsequently washed, the level of inhibition decreased to 10 per cent. This suggests that the serum inhibitors bind to the activated LPS molecules although there was some binding to the erythrocyte membrane. It is possible in the original experiments that the erythrocyte receptor sites and activated LPS molecules might compete for sites on the serum inhibitors or vice versa. Since serum components do attach to the membrane during pretreatment, it is also possible that activated-LPS molecules are able to displace serum inhibitors attached to the erythrocyte membrane; due to their lower adsorptive activity, untreated LPS molecules are unable to do this.

b) Cholesterol:

This was found to be equally effective in inhibiting the uptake of both untreated and heat-activated LPS by erythrocytes (72.0 and 68.0 per cent respectively, Figure 34). If the erythrocytes were treated with 150 µg cholesterol prior to their exposure to the LPS preparations, the levels of inhibition decreased from 72 to 10 per cent for the untreated LPS and from 68 to 1.4 per cent for the activated LPS. This indicated that cholesterol inhibited the uptake of both the LPS preparations by combining with sites on the molecule. Neter, Luderitz and Westphal (1955) suggested that cholesterol could be the membrane receptor site for the attachment of activated LPS. The results of the

present study tend to indicate that cholesterol could act as the site of attachment for both untreated and activated LPS. However, Springer et al., (1973) have isolated a membrane fraction, which does not contain cholesterol (or a phospholipid) but inhibits the uptake of LPS to a greater extent than cholesterol. The possibility that cholesterol acts through a non-specific mechanism requires further investigation.

c) Lecithin:

A phospholipid (lecithin) was found to inhibit the uptake of untreated LPS (Figure 35). If the erythrocytes were pretreated, the inhibition remained at the same level (52%) as the inhibition produced in the presence of both lecithin and LPS. This indicated that the lecithin attached to sites on the erythrocyte membrane and this attachment was preferential to the adsorption of the LPS molecules.

d) Monosaccharides:

The adsorption of the lipopolysaccharides by erythrocytes was inhibited in the presence of monosaccharides at concentrations which would not alter the osmotic balance of the reaction mixture. The degree of inhibition produced by the monosaccharides was dependent on both the amount and the type of monosaccharide (Table 21). The monosaccharides tested could be arranged in order of their ability to produce increasing degrees of inhibition as galactose glucosamine (Arabinose, Rhamnose, Fucose, Mannose) glucose. In order to determine the possible mode of inhibitory action of the glucose, several test systems were devised (Tables 22 and 23). From these experiments it was observed that the greatest degree of inhibition was produced when the lipopoly-

saccharide was incubated with the glucose, prior to the addition of the erythrocytes. This indicated that the glucose acted by attaching to groups on the LPS molecule. If the erythrocytes were incubated with the LPS prior to exposure to the glucose a relatively high degree of inhibition occurred. This showed that the glucose could affect the removal of LPS already bound to the erythrocyte membrane and substantiates weak bond formation. The evidence from experiments with both ^{32}P- and ^{14}C-LPS indicated that the glucose acted as an inhibitor by binding to sites on the LPS molecule. However if the glucose and the erythrocytes were incubated together before the addition of the LPS, the degree of inhibition was greater than if all three constituents had been incubated together. From this, it was concluded that although the major inhibitory role of glucose was the combination of the monosaccharide with the LPS molecule, inhibition could also occur through a blockage of membrane receptor sites by glucose residues. In the latter case the glucose residues either attached to the same receptor site as the LPS molecule or attached to a site close to the LPS-receptor, causing blockage of LPS adsorption, or affected the membrane in such a way as to render LPS adsorption impossible.

With respect to the glucose residue-LPS binding, it is interesting to speculate that the glucose is unable to compete with the lipid:lipid interactions which possibly characterize the primary binding of "autoactivated" LPS molecules. However, it is possible that the glucose could compete with the weaker interactions involved in the secondary binding; hence glucose could block the additional LPS:LPS interactions (see page 138).

7. Leakage of Haemoglobin from Modified Erythrocytes

Throughout this investigation, it was observed that the interaction of bacterial polysaccharide molecules with erythrocyte membranes caused a slight leakage of haemoglobin from the cell. The degree of haemoglobin leakage was dependent on a number of factors :- i) the sample of erythrocytes prepared from different species of animal, ii) the amount of polysaccharide added to the system, iii) the duration of contact between the polysaccharide molecules and the erythrocyte membrane, and iv) the activation-status of the polysaccharide molecule.

If LPS from E. coli NCTC 8623 was incubated with rabbit erythrocytes, a small amount of haemoglobin was observed in the supernatant fluid. However if these cells were washed and treated with a fresh batch of LPS, no further haemoglobin leakage was observed. Hence, this leakage of haemoglobin from erythrocytes could possibly result from partial "membrane reorganization" caused by the initial contact with the bacterial polysaccharide. Once the "reorganization" occurred no further haemoglobin leakage could be induced.

Ciznar and Shands (1971) reported that endotoxin caused haemolysis of sheep erythrocytes. This effect must not be confused with the observation of haemoglobin leakage. It was concluded that the latter effect was a function of the combined inference of changes in the ionic and osmotic environment within the test system.

B: GENERAL DISCUSSION OF POLYSACCHARIDE UPTAKE

The published findings on cellular modification by bacterial polysaccharides do not seem to agree on a wide number of aspects, including the optimum conditions for uptake, the nature of the receptor site, the nature of the linkage between the bacterial polysaccharide and the membrane and the significance of the results with respect to the biological activity of the polysaccharides. Whether these contradictions have arisen due to differences in the polysaccharide preparations or differences in mammalian cell cultures is not absolutely clear. Consequently, a comparison between the results of this investigation and those of other workers was approached with caution since the published results were obtained by procedures not defined using the same parameters as described in this investigation. Throughout the present discussion, it is generally assumed that the bacterial polysaccharides have an affinity for cell membranes, but it should be mentioned that the membranes could have the affinity for the bacterial component. Results obtained by the chemical method used to detect polysaccharide adsorption are in general agreement with those obtained by the radioactive technique. The cyclic fluctuation in the adsorption of LPS by mammalian cells over a period of time using the radioactive technique was not immediately apparent using the chemical method. Recent experiments have shown the same cyclic fluctuation when the time of incubation was extended using the chemical method.

Possible models for the binding of LPS molecules to erythrocyte membranes to explain cyclic fluctuation

Several models for the binding of LPS molecules to erythrocyte

membranes are proposed in order to account for the cyclic fluctuation in the amount of cell-associated LPS; these models are represented in Figure 42. The models presented assume that the LPS preparation consists of a mixture of non-activated and "autoactivated" molecules. Model A consists of a situation where the autoactivated-LPS molecules (A-LPS) attach preferentially onto the cell surface (primary binding). The untreated molecules bind either onto the remaining receptor sites or bind to the already adsorbed A-LPS molecules to form a linear arrangement (secondary binding). De Pamphilis (1971) showed that such a linear association of LPS molecules occurred under appropriate conditions in the presence of lecithin. The A-LPS molecules are eventually incorporated ("interiorization") into the erythrocyte membrane, which causes the release of molecules that were secondarily bound. Model B consists of a situation whereby all the available erythrocyte receptors are filled either by non-activated or "autoactivated" molecules. However, only one or a limited number of LPS molecules can be incorporated into the membrane structure and these would generally be the "autoactivated" molecules. After incorporation, membrane changes occur which reverses the LPS affinity for membranes so that the remaining molecules become detached from the cell surface. Model C is identical to model B but the incorporation process is random so that either non-activated or "autoactivated" molecules are incorporated. Once incorporation has taken place the cycle becomes repetitive and results in the observed cyclic fluctuations. The relationship between these hypothetical binding models and the experimentally observed adsorption data is shown in the miniaturized graphs showing the extent of binding with time (I, II and III). In each case the upper graph represents the first cycle and the lower graph the second cycle.

Figure 42: Possible Models for the Binding of LPS Molecules to Mammalian Cell Membranes

A possible model is presented to explain the observed cyclic fluctuation in the uptake of LPS molecules (see Figures

The small graphs are representations of the experimental results; the amount of cell-associated LPS is plotted against the time of incubation.

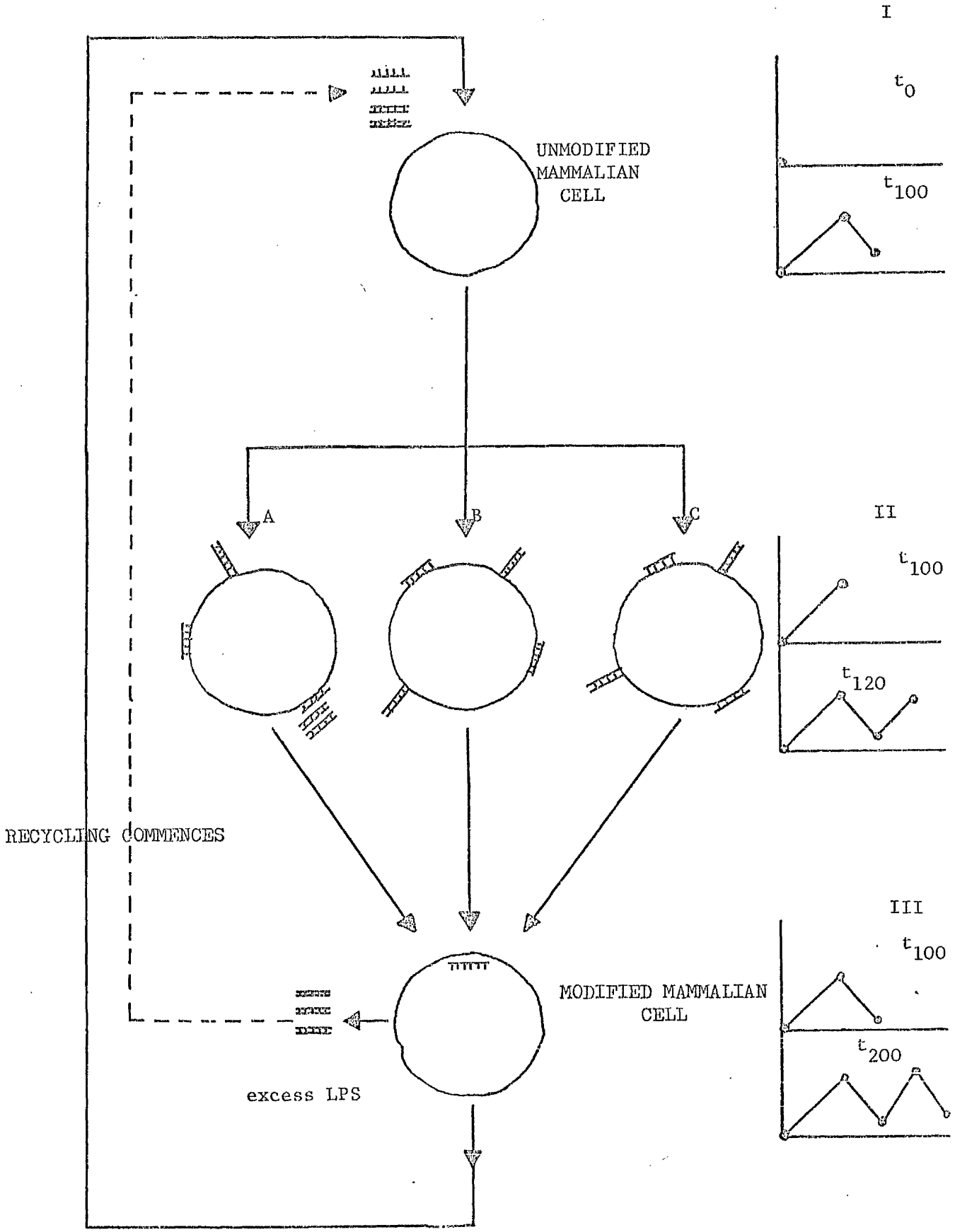
Symbols used:

IIIII represent "autoactivated" LPS molecules

IIIII represent non-activated LPS molecules

t_0, t_{60} ... t_{200} represent the time of incubation, i.e. 0, 60, ... 200 minutes.

A, B, C represent the different ways of cellular modification.

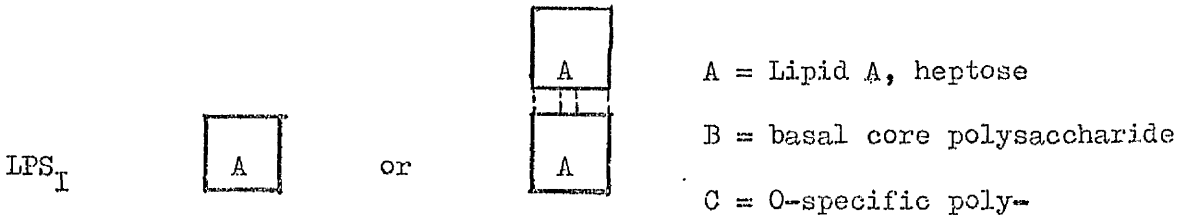

I
t_0
t_{100}
UNMODIFIED MAMMALIAN CELL
A
B
C
II
t_{100}
t_{120}
RECYCLING COMMENCES
III
t_{100}
t_{200}
MODIFIED MAMMALIAN CELL
excess LPS

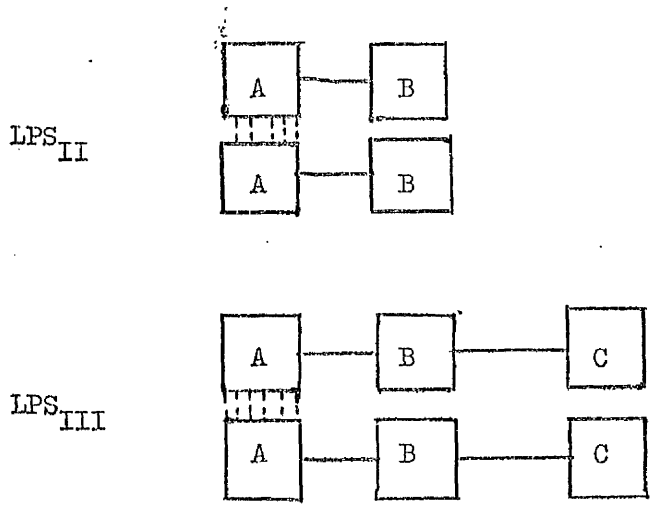
Possible models to explain the treatment of LPS by heat, alkali and periodate, with respect to their effect on cell modification

A hypothetical model is proposed to account for the observed differences in the LPS preparations upon heat, alkali and periodate treatment. This hypothesis could be tested by the use of the R-type mutants of the Salmonella species.

It has been proposed that LPS extracted from the gram-negative organisms by the phenol/water method consists of three molecular populations, designated LPS_I, LPS_{II} and LPS_{III} (see page 122). The LPS_I molecules are "autoactivated" (page 123) and might consist of lipid A - heptose and have the greatest affinity for the cell membrane.

The LPS_{II} molecules might consist of lipid A - heptose - basal core polysaccharide . The majority of the LPS molecules consist of lipid A - heptose - basal core polysaccharide - D-specific polysaccharide and these were termed the LPS_{III} molecules which have little or no affinity for cell membranes. This interpretation is possible when it is considered that Luderitz et al., (1958) found that only approximately 30% of the LPS molecules, even after alkali-treatment, had a strong affinity for cell membranes; the remainder possessed only slight affinity. Hence the three LPS molecular populations could be represented in an aqueous environment as :-





Each molecular population has a different configuration in an aqueous environment, e.g. discs, lamellae and ribbons. Alkali and heat treatment could cause LPS_{II} molecules to be converted into LPS_{I} molecules (Figure 43). It was reported that heat and alkali treatment affected LPS lipids (Tripodic and Nowotny, 1966). However, it is also a well-known fact that isolated polysaccharides are affected by heat and alkali which cause contraction and aggregation of the chains (Read and Gunstone, 1958; Rees, 1967). Hence LPS_{II} molecules could be converted into LPS_{I} molecules by possibly disrupting the lipid:lipid interactions and dissociating and aggregating the polysaccharide moiety. The affinity of the polysaccharide moiety is lost due to conformational changes caused by heat and conversion of hexoses into isomers both of which block or destroy active groups. The LPS_{III} molecules which have low affinity values remain unaffected by heat and alkali treatment, since the amount of polysaccharide in the denatured or aggregated state is sufficient to block the active LPS_{I} component by steric hindrance.

The periodate treatment affects the polysaccharide moieties so that LPS_{II} and LPS_{III} molecules are susceptible to the oxidation, resulting in a loss of binding capacity (Figure 43). The treatment

Figure 43: Hypothetical Models to Explain the Effect of Heat, Alkali and Periodate Treatment on LPS Preparations

Symbols used:

A : Lipid A - heptose

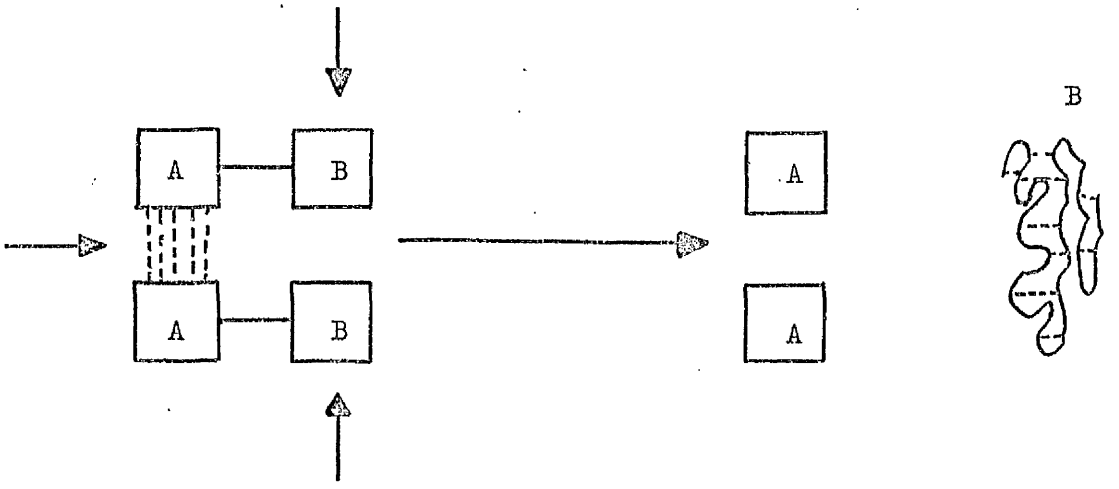
B : Basal Core polysaccharide

C ; O-specific polysaccharide

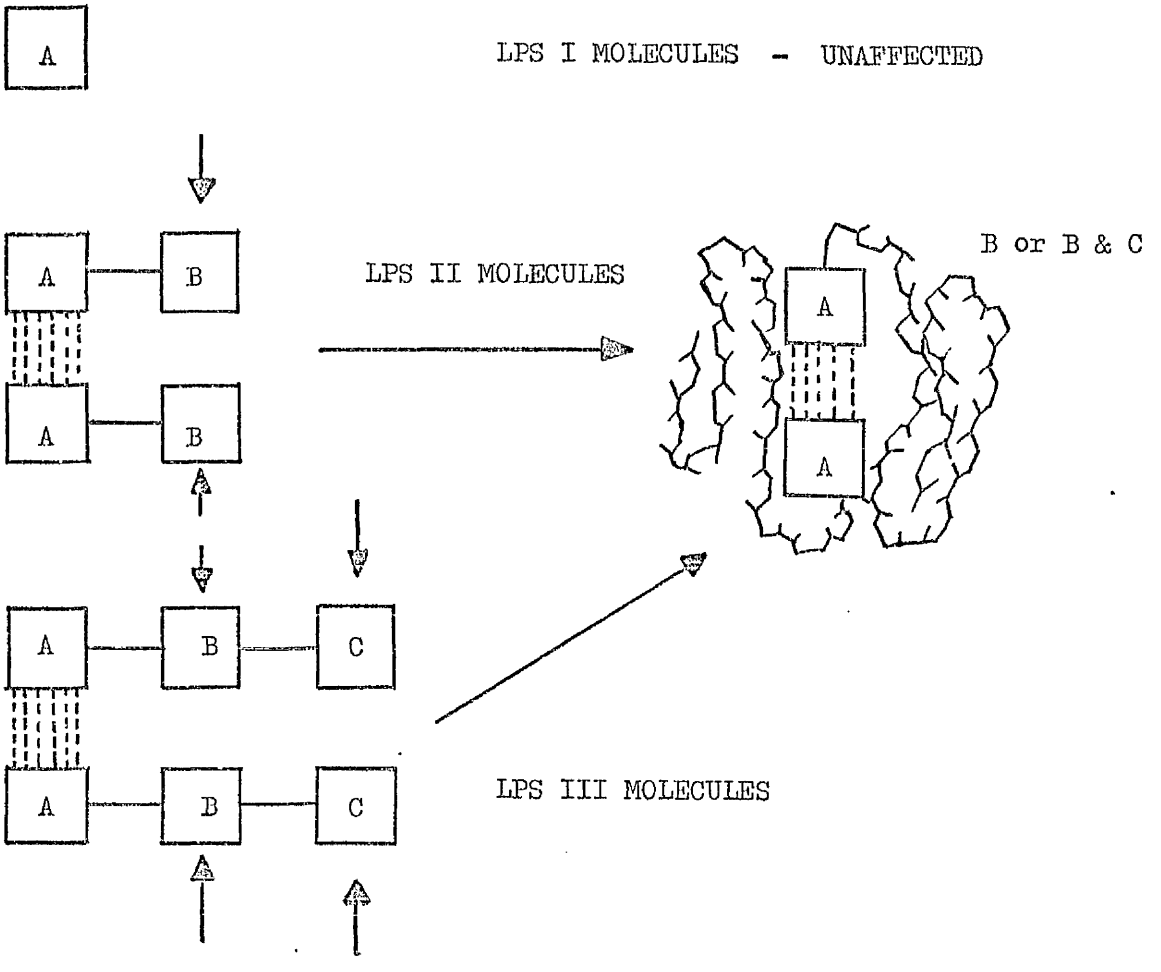
 : represents the sites of action of heat, alkali and periodate

 : represents lipid:lipid interactions

(a) HEAT AND ALKALI TREATMENT



(b) PERIODATE TREATMENT



with periodate would oxidise the 1,2-diol groups in the monosaccharide residues causing a disruption of the bond between C_2 and C_3. However, the glycosidic linkages remain intact and it is possible that the conformational changes due to the action of periodate would destroy active binding groups of LPS_{II} and cause steric hindrance so that LPS_I component is unable to bind to cell surfaces.

C: THE ADJUVANT ACTIVITY OF THE BACTERIAL POLYSACCHARIDES

In order to measure the biological activity of the polysaccharides, the mouse was used as the experimental animal, and although it is generally regarded as a poor antibody producer it was economically suitable to use in large numbers so that a statistical comparison of the results was possible. The biological effect of the polysaccharides, namely their adjuvant activity, was defined as the ability to stimulate increased levels of circulating antibody after exposure to a protein antigen, ovalbumin extracted from chicken eggs. Ovalbumin was chosen as the antigen because highly pure preparations were available, and the chicken was phylogenetically distinct from the mouse. A particulate antigen, such as sheep erythrocytes, would have given greater antibody titres, but would have been unsuitable for the passive haemagglutination assays. Also adsorption of the bacterial polysaccharide onto the erythrocyte membrane would have taken place in the injection mixture, so confusing the results obtained.

The anti-ovalbumin antibody levels produced in the mouse were controlled by several parameters (Figure 40). The antibody levels

were dependent on the presence of a LPS preparation or mycobacterial fraction and furthermore on the amount and type of polysaccharide preparation used as the adjuvant. The levels of anti-ovalbumin antibody in the serum were directly related to the dose level of polysaccharide adjuvant. However, a limit was reached beyond which further increases in the amount of polysaccharide caused either a plateau effect or even suppression of the anti-ovalbumin antibody production. This suppression of the anti-ovalbumin antibody production might be due to antigenic competition between the ovalbumin and the bacterial polysaccharide. It is also possible that since LPS is a T-independent antigen that the increased doses of LPS cause an increase in macrophage activity which triggers T-cell function, which in turn suppresses B-cell activity (see page 28). Since it was shown that the higher dose levels of LPS suppressed the immune response, published results should be treated with caution unless the correct dose-response parameters are observed. For example, Johnson, Gaines and Landy (1956) investigated the effect of alkali-treatment on the adjuvancy of S. typhosa LPS in rabbits. They reported that alkali treatment decreased the adjuvancy of the LPS, but the alkali-treated LPS was used at 20 μg and 200 μg levels, while the untreated LPS was used at a 5 μg level. Hence the relatively decreased antibody levels with alkali-treated LPS could be due to the suppressive effect of excess LPS.

The anti-ovalbumin antibody levels were also dependent on the route used for immunization so that in general intravenous injections produced higher levels of antibody than intraperitoneal injections. Although the macrophage is required for a well-developed immune response,

they also catabolize a large proportion of the injected antigen; the undestroyed antigen being "presented" by the macrophage to the immunocompetent cells. Despite the abundance of macrophages in the peritoneal cavity, anatomically they are poorly situated with respect to the main lymphoid tissues, so that only weak antigenic stimuli reach the lymphoid tissues after an intraperitoneal injection and a relatively poor antibody response elicited.

The anti-ovalbumin response was also found to be dependent on the number of injections given. Intraperitoneally a single injection appeared to induce higher antibody titres than a triple injection. This could possibly reflect the state of activation of the peritoneal macrophages. On the first injection the numbers of macrophages in the peritoneal cavity will be relatively low and they will be in a resting state, so that some of the antigen will not be eliminated and will be presented to the immunocompetent cells. However this initial injection could act as a stimulus so upon further injection of antigen not only are the macrophages more abundant but some of them are activated and elimination of the antigen is virtually complete. Intravenously, it was observed that a triple injection appeared to stimulate higher antibody levels than a single injection. It is reasonable for one to conclude that the difference between these antibody levels reflects the difference between a primary and secondary immune response. In both cases the LPS acted as an adjuvant. Although a secondary response is possible it should be remembered that the interval between the injections was only four days and it is possible that the repeated injections of antigen were acting synergistically on the immunocompetent cells in a single response.

The antibody levels were measured by the passive haemagglutination technique using tanned erythrocytes coated with ovalbumin. Since it is impossible to control the amount of ovalbumin adsorbed by the tanned cells, an "internal" standard of LPS from E. coli NCTC 8623 was used in each animal experiment. Similarly in order to check the "durability" of the tanned erythrocytes an "external" standard of rabbit anti-ovalbumin antiserum was incorporated into each haemagglutination assay. Hence the antibody titres measured by this technique cannot be compared directly with similar published observations due to the inability to quantitate ovalbumin adsorption by tanned erythrocytes, an inherent fault of the passive haemagglutination technique.

For each LPS and mycobacterial polysaccharide-containing fraction, the relative adjuvancy was calculated with respect to the standard LPS preparation from E. coli NCTC 8623 which was given an arbitrary relative adjuvancy value of 1.0. From the statistical analyses it was found that all the preparations tested had different relative adjuvancy values (Table 29). It should be mentioned that both alkali and periodate treatment decreased the relative adjuvancy of the LPS preparations.

D: THE RELATIONSHIP BETWEEN RELATIVE AFFINITY AND RELATIVE ADJUVANCY VALUES FOR BACTERIAL POLYSACCHARIDES

A comparison was made of the relative affinity values of the various bacterial polysaccharides with the relative adjuvancy values obtained for the same polysaccharides (Table 32 ; Figure 44). Generally, no apparent relationship existed between the two parameters for mouse erythrocytes, thymus cells and bone-marrow cells (Figure and c). Although it appears that a direct relationship exists between the relative affinity and adjuvancy values for the mycobacterial preparations.

The spleen cells and especially the peritoneal lymphocytes showed the direct relationship between affinity and adjuvancy for most of the polysaccharide preparations (Figure 44f and d). The spleen cell results were less precise than the results from the peritoneal lymphocytes and emphasises the heterogeneity of the cell preparations. The lymphocyte results showed two exceptions, the lipopolysaccharides extracted from Franciscella tularensis (4) and Bordetella pertussis NCTC 18334 (2). These do not show a direct relationship having relatively low affinity values and higher adjuvancy values. The spleen cell findings show a wider scatter of results and once again the LPS extracted from F. tularensis and B. pertussis do not show the direct relationship (Figure 44f).

The mouse-peritoneal macrophage results show a different pattern with an inverse relationship existing between the relative affinity and relative adjuvancy values (Figure 44e). However the LPS

Table 32 a :

THE RELATIVE AFFINITIES AND ADJUVANCIES OF BACTERIAL POLYSACCHARIDES

	Lipopolysaccharide species	Erythrocytes			Mouse cells			Macrophages	Spleen	Relative Adjuvancy
		Rabbit	Mouse	Human	Thymus	Bone marrow	Lymphocytes			
1.	*E. coli* NCTC 8623	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2.	*Bd. pertussis* NCTC 18334	0.73	0.60	0.65	0.72	0.50	0.53	0.72	0.50	0.64
3.	*Ser. Marcescens*	0.66	N.T.	0.64	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
4.	*F. tularensis*	0.61	0.58	0.60	0.93	0.49	0.52	1.02	0.69	0.96
5.	*Sh. flexneri*	0.78	0.58	0.80	0.84	0.81	0.59	1.59	0.72	0.24
6.	*E. coli* 055:B5	0.71	N.T.	0.74	0.86	0.70	0.75	1.25	0.90	0.42
7.	*S. typhi* NCTC 0901	1.26	1.24	1.20	1.22	0.62	1.13	0.67	1.04	1.99
8.	*S. typhimurium* NCTC 5710	0.73	0.79	0.75	1.63	0.49	0.82	1.42	0.87	0.44
9.	*E. coli* B (Ether)	0.56	0.54	0.55	0.74	0.74	0.82	1.48	0.88	0.50
10.	*E. coli* B (EDTA)	1.15	1.09	1.20	0.69	0.71	0.65	1.60	0.79	0.18
	Mycobacterial Fractions									
11.	ST82	1.14	1.10	1.09	1.10	1.0	1.0	1.01	0.91	0.90
12.	ST208	0.61	0.63	0.59	1.11	0.89	0.85	1.27	0.94	0.58
13.	ST210	1.34	1.20	1.25	1.30	0.40	0.98	1.03	1.05	0.95
14.	ST211	0.51	0.50	0.50	0.50	0.76	0.92	1.20	0.79	0.60
15.	PPD	0.70	0.67	0.69	0.81	0.91	0.93	1.13	0.86	0.79

Table 32b:

THE RELATIVE AFFINITIES AND ADJUVANCIES OF BACTERIAL POLYSACCHARIDES

All figures related to the affinity of *E. coli* NCTC 8623 LPS for mouse erythrocytes

Lipopolysaccharide Preparation	Relative Affinity for Mouse Cells						Relative Adjuvancy
	Erythrocytes	Thymus cells	Bone marrow cells	Peritoneal lymphocytes	Peritoneal macrophages	Spleen cells	
E. coli NCTC 8623	1.0	6.5	6.49	2.19	11.50	5.63	1.0
Bd. pertussis NCTC 18334	0.60	4.68	3.24	1.16	8.28	2.81	0.64
F. tularensis	0.58	6.04	3.18	1.13	11.73	3.88	0.96
Sh. flexneri	0.58	5.46	5.25	1.29	18.20	4.05	0.24
E. coli 055:B5	N.T.	5.59	4.54	1.64	14.30	5.06	0.42
S. typhi NCTC 0901	1.24	7.93	4.02	2.47	7.70	5.85	1.99
S. typhimurium NCTC 5710	0.79	10.59	3.18	1.79	16.33	4.89	0.44
E. coli B (Ether extraction)	0.54	4.81	4.80	1.79	17.02	4.95	0.50
E. coli B (EDTA extraction)	1.09	5.85	4.60	1.42	18.40	4.44	0.18
Mycobacterial Fractions							
ST82	1.10	7.15	6.49	2.19	11.50	5.12	0.90
ST208	0.63	7.21	5.77	1.86	14.60	5.29	0.58
ST210	1.20	8.45	2.59	2.14	11.84	5.91	0.95
ST211	0.50	3.25	4.93	2.01	13.80	4.44	0.60
PPD	0.67	5.26	5.90	2.03	12.99	4.84	0.79

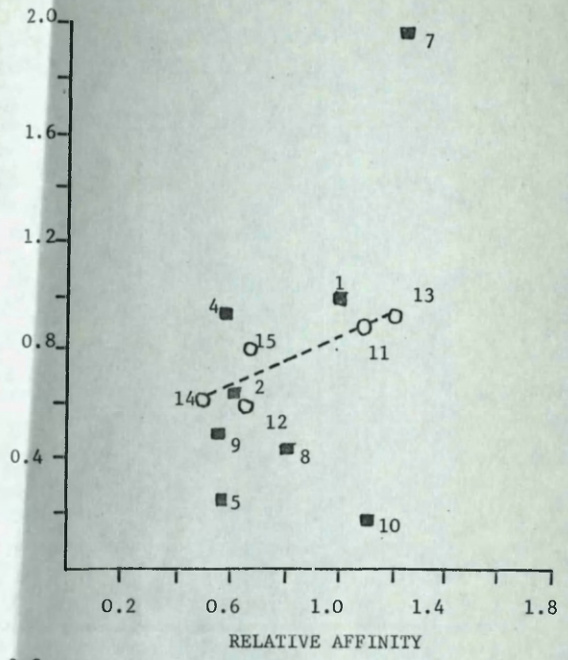
Figure 44: The Relationship between the Relative Affinity of Bacterial Polysaccharides for Cell Surfaces and Relative Adjuvancy

The relative adjuvancy of the bacterial polysaccharides was plotted against their relative affinity for cell surfaces.

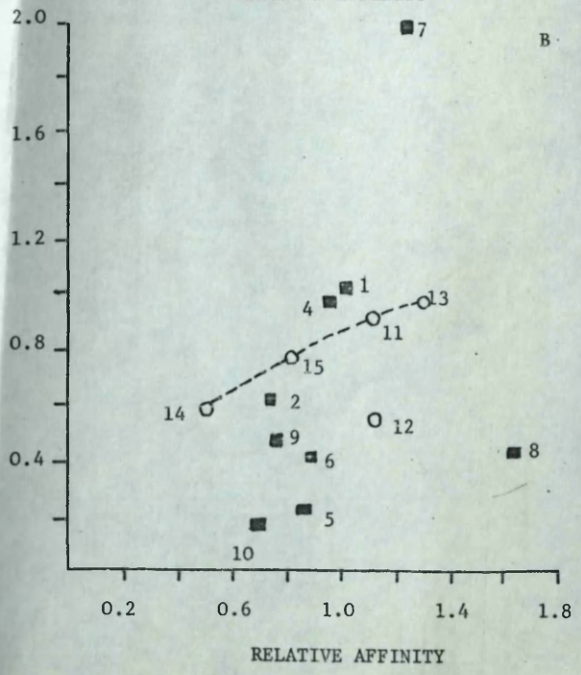
A Mouse erythrocytes
B " Thymus cells
C " Bone marrow cells
D " Peritoneal lymphocytes
E " Peritoneal macrophages
F " Spleen cells

The numbers on the graphs refer to the bacterial polysaccharides in Table 32a.

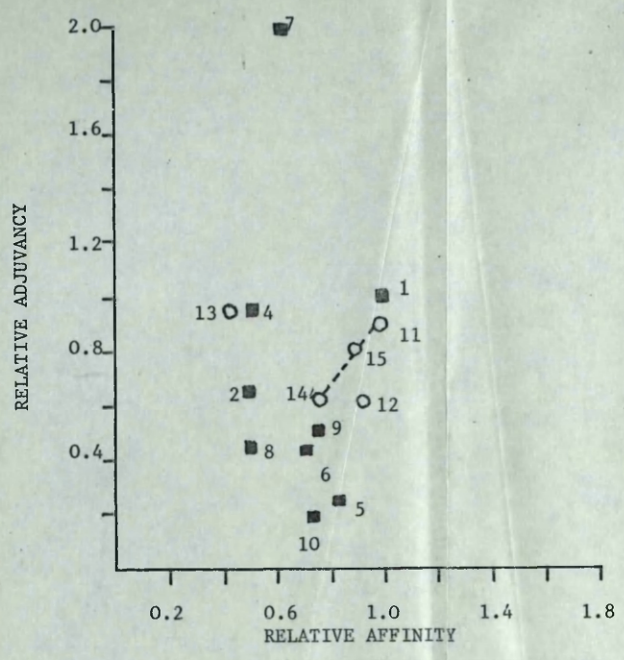
A MOUSE ERYTHROCYTES



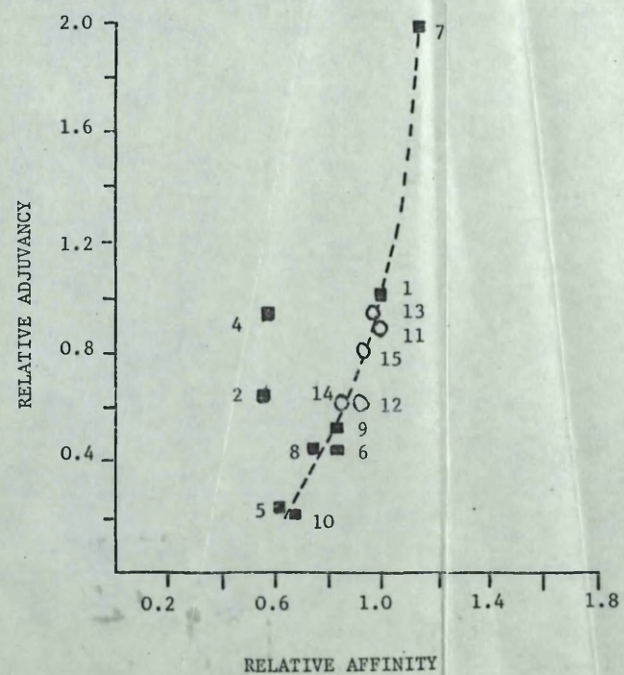
B MOUSE THYMUS CELLS

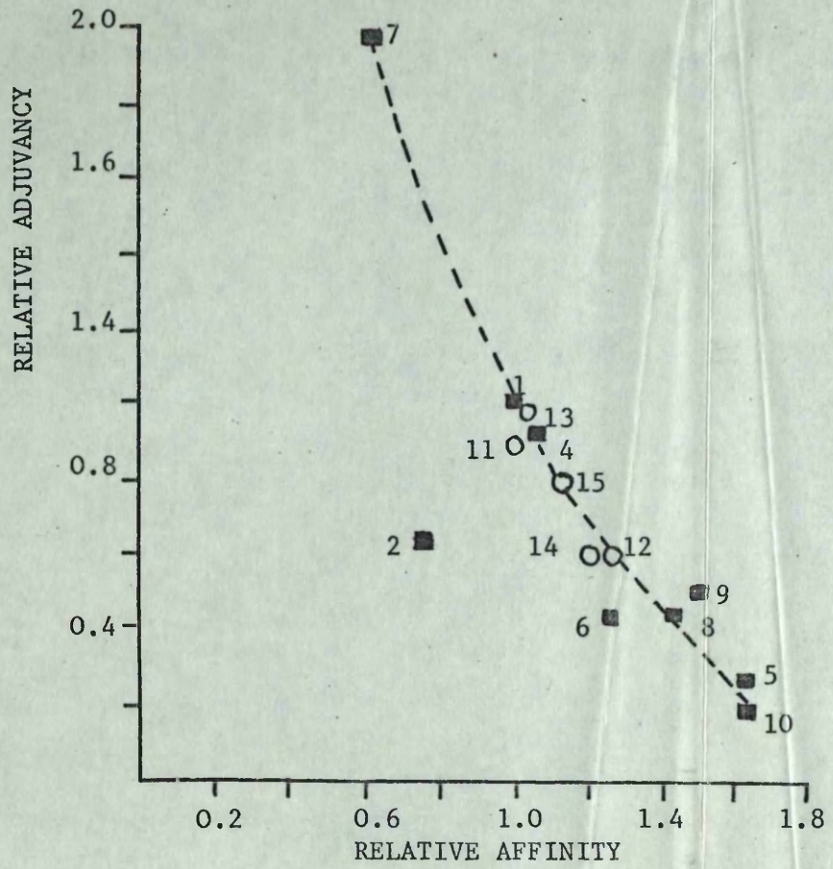


C MOUSE BONE MARROW CELLS

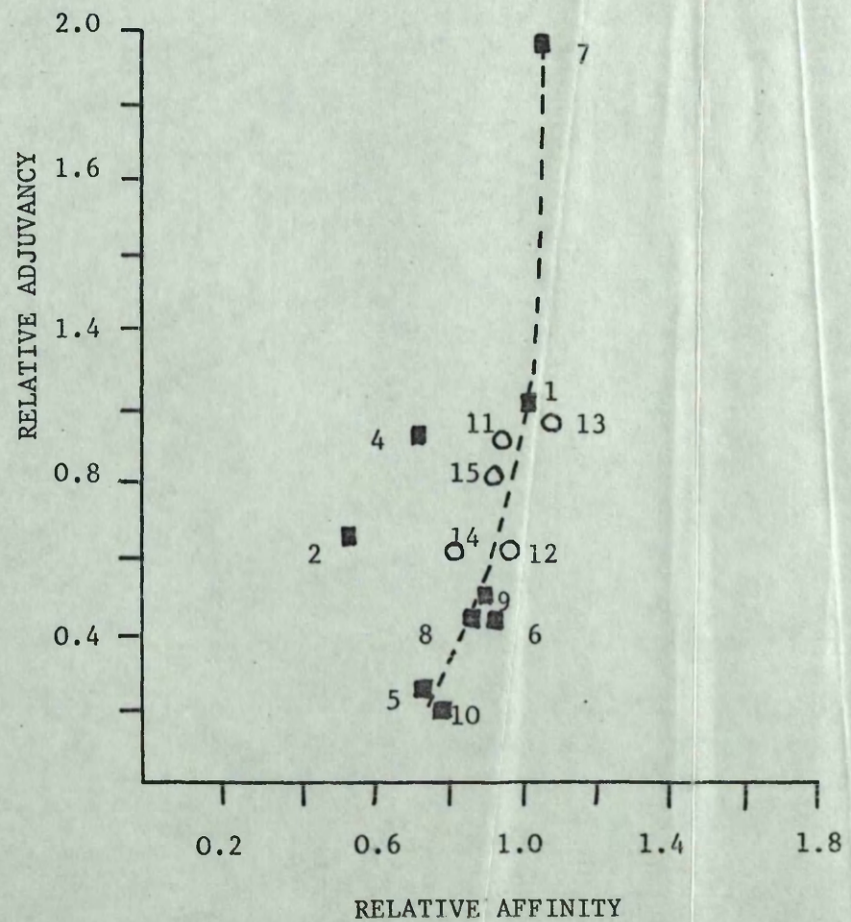


D MOUSE PERITONEAL LYMPHOCYTES





E MOUSE PERITONEAL MACROPHAGES



F MOUSE SPLEEN CELLS

from B. pertussis NCTC 18334 does not adhere to this inverse relationship.

From these findings it is likely that the adjuvancy of LPS from B. pertussis and F. tularensis is not related to the affinity for cell membranes. However, the adjuvancy of other lipopolysaccharides and the mycobacterial polysaccharide-containing fractions seems to be related to the affinity of these polysaccharides for the membranes of peritoneal lymphocytes, macrophages and spleen cells.

Since lipopolysaccharides are directed in vivo against B-lymphocytes (Andersson, Möller and Sjöberg, 1972), one could propose that in the case of mouse peritoneal lymphocytes and spleen cells a relationship exists between adjuvancy and affinity for B-cell membrane receptor sites. Some slight differences could be due to T-cell "contamination" of the cell suspensions, e.g. Figure 44f numbers 2 and 4.

The relative affinity values are based on the affinity of E. coli NCTC 8623 LPS for each cell membrane. Since it was shown that this affinity varies depending on the cell type, the relative affinities were corrected to allow the comparison between a particular polysaccharide preparation and the different cell types. This comparison is shown in Table 32b, which compares the different affinities of bacterial polysaccharides for the different cell types to the affinity of E. coli NCTC 8623 LPS for the erythrocyte membrane.

A Tentative Model for an in vivo Role of Lipopolysaccharide Affinity for Membranes in the Adjuvant Response in the Mouse

LPS extracted from Gram-negative bacteria probably does not

exert an adjuvant effect through any one single mechanism. It was shown that LPS molecules were adsorbed not only to a wide variety of mouse cells (page 93) but also to immunoglobulins under the appropriate conditions (page 114). These observations, coupled with the fact that membrane "reorganization" takes place on cellular modification (page 111), indicate that LPS could exert an adjuvant effect through the proposed adjuvant mechanisms of feedback inhibition, alteration of intracellular processes and the release of nucleic acid breakdown products. The latter mechanism is probably of minor importance with respect to LPS's since only limited membrane "reorganizations" are produced. These alterations in membrane integrity appear to be insufficient to allow the release of oligodeoxyribonucleotides.

The model presented here is based on the following observations :-

(i) the affinity of LPS for macrophage membranes is inversely related to LPS adjuvancy (page 145)

(ii) the affinity of LPS for peritoneal lymphocyte and spleen cell membranes is directly related to LPS adjuvancy (page 145)

(iii) the degree of LPS adsorption is dependent on the cell species (page 93)

(iv) various substances, e.g. serum, cholesterol &c cause inhibition of LPS adsorption by mammalian cells (page 107)

(v) the adsorption of LPS to cell membranes follows a cyclic fluctuation with time of exposure (page 95)

(vi) the majority of the bonds formed between the LPS molecule and the cell membrane are relatively weak (page 127)

(vii) partial membrane "reorganization" is caused by LPS interacting with cell membranes (page 111)

(viii) LPS adsorbs to membrane glycoprotein receptors (Adye et al., Springer et al., 1973)

(ix) lymphocyte recirculation depends on the integrity of membrane surface glycoproteins (Gesner and Ginsberg, 1964)

(x) LPS is a thymus-independent antigen (Andersson and Blomgren, 1971)

(xi) LPS is mitogenic for B-cells (Andersson, Sjöberg and Möller, 1972)

(xii) LPS causes hyperplasia in lymphoid tissue (Farthing and Holt, 1962) with an increase in the number of AFC (Freedman, Nakano and Braun, 1966)

(xiii) LPS facilitates the cooperation between ARC (T) and ASC (B) (Nakano, Uhiyama and Saito, 1973)

(xiv) B-cells require two signals for the induction of antibody synthesis (Watson, Trenkner and Cohn, 1973)

(xv) activated LPS has increased affinity for mammalian cell membranes (page 105) and a decreased adjuvancy (page 118).

When lipopolysaccharide is injected into the bloodstream it tends to localize in the B-cell areas of the lymphoid tissue. However, due to the affinity of the LPS for various cell membranes "sidetracking" occurs which diverts the LPS molecules away from the B-cell areas by adsorbing the LPS molecules onto the various cell membranes. The amount of LPS reaching the B-cell area is very small; a result not presented in this thesis showed that only 1-2% of injected ^{32}P-LPS reached the spleen, the majority (80%) being adsorbed by the liver. For the sake of simplicity, the antigen is not represented in this model. It will arrive independently in the lymphoid B-cell areas, probably by means of

macrophage transportation, since it was shown that for adjuvant action of the LPS, the antigen could be administered independently (Luecke and Sibal, 1962).

Since the majority of injected LPS molecules are adsorbed by the liver, it is essential that the maximum amount of LPS possible reaches the lymphoid B-cell areas. The amount of LPS available for adsorption by the B-cells is decreased by a phenomenon known as "sidetracking". "Sidetracking" is defined (by the author) as the adsorption of LPS adjuvant to mammalian cells other than B-lymphocytes.

The main "sidetracking" hazard is the macrophage and this is reflected by the macrophage membrane having the greatest relative affinity for LPS molecules. This macrophage effect is countered by the existence of an inverse relationship between the affinity for the macrophage membrane and the adjuvancy of the LPS molecule. The involvement of the macrophage in the immune response will probably trigger T-cell reactions. However LPS has been shown to be a thymus independent antigen and the effect of the involvement of T-cells in an anti-LPS response is to suppress the specific antibody synthesis. Therefore, it is possible that LPS is also a thymus-independent adjuvant. This hypothesis is supported by the findings of Campbell, Kind and Rowlands (1965) who showed that under the appropriate conditions, LPS acted as an adjuvant in thymectomized mice. Similarly Andersson, Möller and Sjöberg (1972) showed that LPS was unable to stimulate DNA synthesis in pure T-cell cultures. However, findings presented in this thesis show that LPS molecules are adsorbed by thymus cells. If it is assumed that LPS adsorbs to surface glycoproteins (as in erythrocytes) and that the

integrity of these structures is required for lymphocyte circulation, then it is possible that the adsorption of LPS to thymus cells has the effect of "removing" them from circulation. The ~~relative~~ affinity of LPS molecules ~~onto~~ for the thymus cells could be an essential step in the LPS-adjuvant response. The relative affinity of LPS for erythrocytes is very low and so prevents adsorption of LPS to the most common cell the LPS will encounter before reaching the "target areas".

Although "sidetracking" could be important in the case of thymus cells, to prevent excess adsorption taking place, numerous factors are present which may or may not interfere with this process, namely serum components especially albumin, α_1-globulin and β-lipoprotein (Nass, Matijevitch and Springer, 1964), phospholipids and metabolites e.g. monosaccharides. Also the nature of the bond between the LPS molecule and the cell membrane is relatively weak so that dissociation takes place thus releasing the LPS molecule from the membrane.

Once in the B-cell area, an asynchronous adsorption of LPS molecules to the B-cells takes place. This adsorption could activate the induction of B-cell mitogenesis which could lower the threshold of antigenic concentration required to stimulate a specific B-cell response. This activation could take place through a number of mechanisms (Figure 45) which initially concern the attachment of numerous LPS molecules to a few cells; as the cells are activated the excess LPS molecules become dissociated from the activated cell and are available for attachment to non-activated immunocompetent cells. It is also conceivable that the adsorption of LPS to B-cells facilitates lymphocyte:lymphocyte and/or lymphocyte:macrophage cooperation. This cooperation could be

Figure 45 : Possible Models for the Activation of Immunocompetent Cells

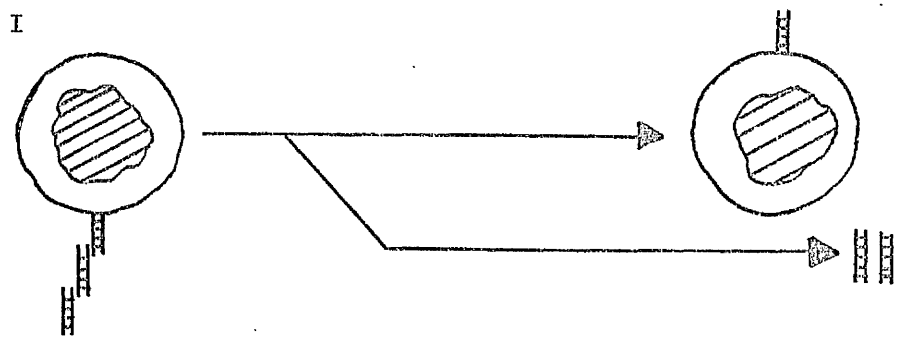
Models I, II and III represent the activation of immunocompetent cells by untreated LPS preparations. The LPS molecules bind to the cell surface and "activate" the cell. The majority of the adsorbed molecules become detached and affect other immunocompetent cells. A few of the molecules are "interiorized" into the cell membrane, so "activating" the cell.

Model IV represents the activation of immunocompetent cells by alkali and heat-activated LPS molecules. Once they are adsorbed to the cell surface they are unable to affect other cells.

IMMUNOCOMPETENT CELLS

"ACTIVATED" IMMUNOCOMPETENT CELLS

I



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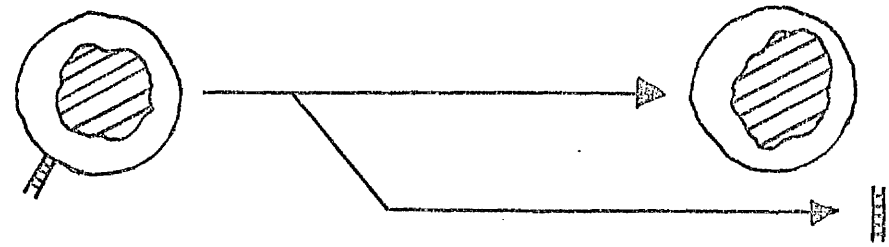

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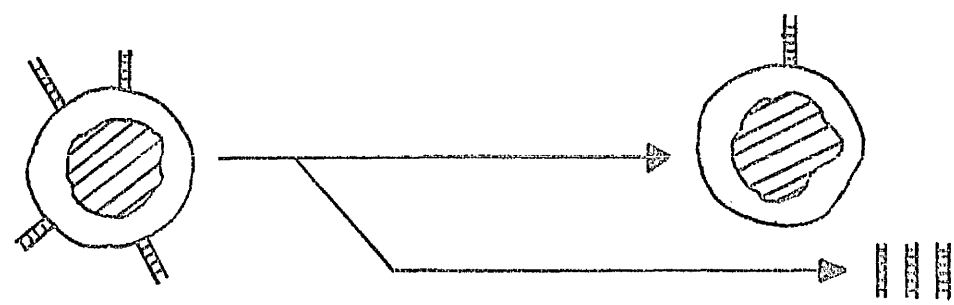
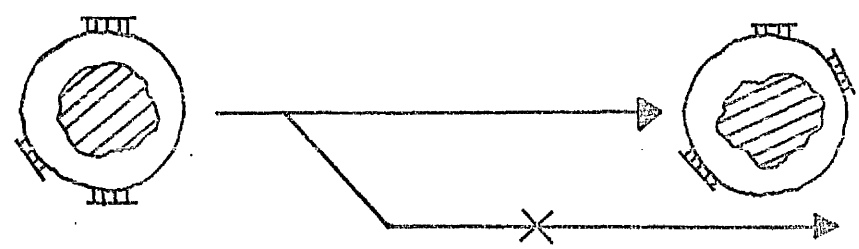


IV



brought about either by bridge formation between the cells or an alteration of local surface conditions, such as membrane charge.

This present investigation has shown that the activation of the LPS molecules by heat or alkali increases their affinity for cell membranes but decreases their biological activity. This could be accounted for in the proposed model by assuming that, since the activated-LPS molecules have a greater affinity for cell membranes and are less susceptible to the inhibitory activity of serum, they are unable to escape "sidetracking". Also those molecules reaching the B-cell area will only activate a limited number of B-cells due to the apparent inability of the activated LPS molecules to become dissociated from the cell membrane once they have been adsorbed (Figure 45).

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SUMMARY

The object of this investigation was to examine the possible relationship between the affinity of bacterial polysaccharides for mammalian cell surfaces and their activity as immunological adjuvants. The uptake of the bacterial polysaccharides by the mammalian cells was measured by two independent methods. The first was a chemical method devised during this present investigation and based on the accurate standardization of erythrocyte suspensions by the cyanmethaemoglobin method. The second method based on labelling LPS from E. coli NCTC 8623 with ^{14}C or ^{32}P enabled the study to be extended beyond erythrocytes to other mammalian cell types. The results from both methods supported each other.

Over a limited range, the uptake of untreated LPS from E. coli NCTC 8623 by mammalian cells was found to be dependent on (i) the mammalian cell type e.g. macrophages adsorb comparatively more LPS than erythrocytes, (ii) the number of cells present, (iii) the amount of LPS available for adsorption, (iv) the temperature of incubation; the System has an optimum at 22°C, (v) the duration of the incubation period; a cyclic fluctuation in cell-associated LPS was observed over a period of time, (vi) the presence of various agents. Inhibition of uptake was observed in the presence of (a) normal serum (b) cholesterol (c) lecithin (d) monosaccharides (e) other LPS preparations (f) mycobacterial polysaccharide-containing fractions, and (vii) the activation-status of the LPS. Heat and alkali treatment potentiated LPS adsorption; periodate treatment inhibited it.

In order to account for some of the observed reactions, it was proposed that the LPS extracted from E. coli NCTC 8623 by the phenol/water method consisted of three distinct molecular populations. The LPS_I molecular population (3%) exhibited a strong affinity for cell membranes, the LPS_{II} molecules (30%) a moderate affinity and the LPS_{III} molecules

little or no affinity. Molecular models for each population were proposed.

It was also observed that the amount of untreated LPS adsorbed by mammalian cells followed a cyclic fluctuation with the time of incubation; no such fluctuation was observed with heat-activated LPS molecules. A model was proposed to account for this cyclic fluctuation in adsorption. Similarly, models were presented to account for the observed reactions and the molecular changes caused by heat, alkali and periodate treatment of the LPS molecule.

A comparison of the amounts of the various bacterial polysaccharides adsorbed by different cells was made and these differences expressed mathematically as relative affinity (RA) values.

The adjuvant effect of the bacterial polysaccharides in mice was studied. The adjuvant effect was defined for the purposes of this investigation as the ability of the bacterial polysaccharides to stimulate increased circulating antibody levels to the protein antigen, ovalbumin. The levels of circulating anti-ovalbumin antibody were measured by a passive haemagglutination technique and the antibody level was found to be dependent on (i) the presence of a bacterial polysaccharide adjuvant, (ii) the amount of the bacterial polysaccharide present, (iii) the type of polysaccharide adjuvant, (iv) the route of immunization either intravenously or intraperitoneally and (v) the injection schedule either single or triple injection. The increased

levels of anti-ovalbumin antibody stimulated by each bacterial polysaccharide was compared to the levels stimulated by E. coli NCTC 8623 LPS. The comparative adjuvant activities of the bacterial polysaccharides were expressed mathematically as relative adjuvancy values.

By comparing the relative affinity values of the various polysaccharides for the different mammalian cells with the relative adjuvancy values, several relationships became apparent. The relative affinity of the polysaccharides for spleen cells and peritoneal lymphocytes showed a direct relationship to the relative adjuvancy values. An inverse relationship existed between the relative affinity for peritoneal macrophages and relative adjuvancy.

Finally a hypothesis was proposed to explain the role of the affinity of bacterial polysaccharides for cell surfaces with respect to their observed adjuvant activity.

APPENDIX I - GROWTH MEDIA FOR GRAM-NEGATIVE BACTERIA

(i) Medium for Starter Cultures

3% (w/v) Casamino acids (Difco Ltd.)

1% (w/v) Yeast extract (Oxoid Ltd.)

1% (w/v) Glucose (BDH Ltd.)

The casamino acids and the yeast extract were sterilized at 121°C (15 lb/sq in) for 15 min. The glucose was sterilized by filtration and added aseptically to the other ingredients.

(ii) Davis and Mingioli Minimal Medium (Davis and Mingioli, 1950)

Basal Medium: K_2HPO_4 (7 grams)
KH_2PO_4 (3 grams)
$MgSO_4.7H_2O$ (0.1 gram)
$(NH_4)_2SO_4$ (1 gram)
$Na_3C_6H_5O_7.2H_2O$ (0.5 grams)

These ingredients were dissolved in 980 ml of distilled water and sterilized at 121°C for 15 min. 20 ml of 10% (w/v) sterile glucose were added to this to complete the basal medium.

Trace Compound Solution: $FeSO_4.7H_2O$ (0.5 grams)
$ZnSO_4.7H_2O$ (0.5 grams)
$MnSO_4.3H_2O$ (0.5 grams)
0.1N Sulphuric acid (10 ml)
Distilled water (990 ml)

5 ml of the trace compound solution were added to the basal medium.

(iii) Modified Davis and Minglioli Minimal Medium

This medium was used to grow organisms for labelling with ^{32}P.

$MgSO_4 \cdot 7H_2O$ (0.1 grams)
$(NH_4)_2SO_4$ (1 gram)
$Na_3C_6H_5O_7 \cdot 2H_2O$ (0.5 grams)
Trace solution (5 ml)

These ingredients were dissolved in 980 ml of distilled water and sterilized at 121°C for 15 min. 20 ml of 10% (w/v) sterile glucose were added asceptically to the basal medium. Since this medium was phosphate-free 1% (w/v) casamino acids were added as suggested by Rowley, Howard and Jenkin (1956).

APPENDIX II - MEDIA FOR MAMMALIAN CELL CULTURE

(i) Hanks Balanced Salt Solution (Cruickshank, 1969)

Stock Solution A: (1) NaCl (160 grams)
KCl (8 grams)
$MgSO_4 \cdot 7H_2O$ (2 grams)
$MgCl_2 \cdot 6H_2O$ (2 grams)
Distilled Water (800 ml)

(2) $CaCl_2$ (2.8 grams)
Distilled Water (100 ml)

The two solutions were mixed and the volume adjusted to 1 litre with distilled water. 2 ml of chloroform were added as a preservative and the solution was stored at 4°C.

Stock Solution B: $Na_2HPO_4.12H_2O$ (3.04 grams)
KH_2PO_4 (1.2 grams)
Glucose (20 grams)
Distilled Water (800 ml)

To this solution was added 100 ml of 0.4% (w/v) phenol red solution. The final volume of the stock solution was adjusted to 1 litre with distilled water and 2 ml of chloroform added.

Stock Solution C: $NaH\ CO_3$ (1.4 grams)
Distilled Water (100 ml)

This solution was sterilized at 115°C (10 lbs/sq in) for 10 min.

Hanks Balanced salt solution was prepared by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of distilled water. This was sterilized by placing in a Koch steamer for 1½ hours. To each 20 ml of this sterile solution was added, asceptically, 0.5 ml of sterile stock solution C.

(ii) Medium for the Maintenance and Growth of Mammalian Cells - Cell Growth Medium (CGM)

5% (v/v) filtered rabbit serum
0.5% (w/v) lactalbumin hydrolysate
Heparin (30 units/100 ml) (Activity 100,000 I.U/amp, Koch-Light Laboratories Ltd., London)
1% (v/v) Cristamycin (1 ml contained 10,000 u. penicillin and 10 mg Streptomycin; Glaxo Laboratories Ltd., England).

The above ingredients were added asceptically to Hanks balanced salt solution.

APPENDIX III - BUFFERS AND DILUENTS

(i) 0.02M Phosphate buffer, pH 7.5

Solution A:	$NaH_2PO_4.2H_2O$	3.12 grams/litre
Solution B:	$Na_2HPO_4.12H_2O$	7.17 grams/litre

95 ml of solution A and 405 ml of solution B were mixed and the volume made up to 1 litre with distilled water.

(ii) 0.5M Carbonate-bicarbonate buffer

37 gm $NaHCO_3$ (BDH-AnalaR)

6 gm Na_2CO_3 (anhydrous, BDH-AnalaR)

1 litre distilled water

This buffer has a pH = 9.0.

(iii) 0.01M Phosphate-buffered saline (PBS) pH 7.2

0.01M phosphate buffer (pH 7.2)

Solution A:	$NaH_2PO_4.2H_2O$	1.56 grams/litre
Solution B:	$Na_2HPO_4.12H_2O$	3.58 grams/litre

140 ml of Solution A and 360 ml of Solution B were mixed and the volume adjusted to 1 litre with distilled water.

PBS (pH 7.2) consisted of 8.5 grams/litre sodium chloride (BDH-AnalaR) using the 0.01M phosphate buffer as diluent.

(iv) 0.5M Carbonate-buffered glycerol (pH 9.0)

10 ml 0.5M Carbonate-bicarbonate buffer (pH 9.0)

90 ml Glycerol.

(v) 0.1M Tris (hydroxymethyl) aminomethane HCl (Trid HCl) buffer pH 8.0

12.1 grams Tris (BDH-AnalaR) were dissolved in 1 litre distilled water and the pH adjusted to 8 with concentrated hydrochloric acid.

(vi) 0.05M barbitone buffer, pH 8.5

10.3 grams sodium barbitone (BDH-AnalaR)

1.84 grams diethyl barbituric acid (barbitone) (BDH-AnalaR)

The above ingredients were dissolved in 1 litre of distilled water.

(vii) 0.2M Sodium acetate buffer, pH 5.0

Solution A : 0.2M solution of acetic acid (11.55 ml in 1000 ml)

Solution B : 0.2M sodium acetate (16.4 g $C_2H_3O_2Na$ in 1000 ml)

14.8 ml of solution A was mixed with 35.2 ml of solution B and the volume adjusted to 1 litre with distilled water.

APPENDIX IV - MEDIUM FOR DETECTION OF ANTIBODY:ANTIGEN REACTIONS

Ion agar No. 2 (Oxoid)	10 grams
0.05M barbitone buffer, pH 8.5	1 litre

The ion agar was dissolved in the barbitone buffer and autoclaved at 121°C (15 lb/sq in) for 15 min. The molten agar was cooled to 50°C and Thiomersal (1/10,000 dilution w/v, BDH-AnalaR) was added to give a final concentration of 0.01% (v/v). This agar was used for both Ouchterlony double diffusion method and immunoelectrophoresis.

APPENDIX V - A COMPARISON OF THE METHODS USED TO PREPARE RADIOACTIVELY-LABELLED ^{14}C-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623

Escherichia coli NCTC 8623 was grown in Davis and Minglioli minimal medium with 'cold' glucose, and subsequently grown a) under diauxic growth conditions using medium containing ^{14}C-glucose and lactose and b) using fresh minimal medium containing ^{14}C-glucose. The resultant growth from both methods was dried and weighed. From the results (Table A1) it appeared that the diauxic growth method did not give such a high yield of cells; 24-26 mg/100 ml as opposed to the 'doubling-time' method, 31.77 mg/100 ml. By monitoring the radioactivity throughout the experiment, it was concluded that the 'doubling-time' method produced more efficiently labelled LPS than the diauxic growth method. The amount of label incorporated into the LPS was very low, 0.11% by the diauxic growth method and 0.49% by the 'doubling-time' technique (Table A1). These differences could reflect the different cell yields, although calculations, assuming equal cell yields, still revealed a more efficiently labelled LPS by the 'doubling-time' method. These results also showed that relatively more of the radioactive-label was incorporated into the LPS by the 'doubling-time' method. In the diauxic method, 0.92% of the radioactive-label associated with the cells, was incorporated into the LPS, whereas 1.32% of the cell-associated label was incorporated into LPS by the 'doubling-time' method. These differences might reflect the stage in the growth cycle when the radioactively-labelled glucose was utilized. Under diauxic growth conditions, the ^{14}C-glucose was utilized at the beginning of the logarithmic phase, but in the 'doubling-time' method, the ^{14}C-glucose was not utilized until the end of the logarithmic phase.

Table A1: A COMPARISON OF THE METHODS FOR PREPARING ^{14}C-LPS FROM E. COLI NCTC 8623

	Cells grown under diauxic growth conditions	Cells grown under conditions of short exposure to ^{14}C glucose (Doubling-time method)
Amount of ^{14}C-glucose added/100 ml culture medium (dpm)	5.1×10^7	5.1×10^7
Total dry weight of cells obtained	24.26	31.77
Specific activity of the dried cells (dpm/mg)	0.02×10^7	0.06×10^7
Total activity of cells (dpm)	0.65×10^7	1.9×10^7
Efficiency of labelling cells (%) $\left(\frac{\text{Total dpm cells}}{\text{Total dpm added}} \times 100\right)$	12.7	37.2
Total activity of LPS extracted by phenol/water method	0.60×10^5	2.5×10^5
Efficiency of labelling LPS (%) $\left(\frac{\text{Total dpm LPS}}{\text{Total dpm added}} \times 100\right)$	0.11	0.49
Proportion of label incorporated into LPS (%) $\left(\frac{\text{Total dpm LPS}}{\text{Total dpm cells}} \times 100\right)$	0.92	1.32
Calculate activity of LPS (dpm), assuming both methods yielded 31.77 mg dry weight of cells	0.78×10^5	2.5×10^5

APPENDIX VI : RESULTS OBTAINED BY CHEMICAL METHOD

Table A2: THE UPTAKE OF VARIOUS AMOUNTS OF MYCOBACTERIAL GLYCOPEPTIDE (ST208) BY A CONSTANT NUMBER OF RABBIT ERYTHROCYTES

Amount of Glycopeptide added (mg)	Carbohydrate content of cells mg/100 ml to Haldane Standard	Increase in cell carbohydrate content (mg/100 ml)	Increase in cell carbohydrate content (mg)	Mycobacterial Fraction: Amount adsorbed	Mycobacterial Fraction: Amount adsorbed / Amount added x 100
18	381.25	194.6	5.85	9.98	55.4
9	346.60	160.0	4.81	8.20	91.1
4.5	259.96	73.3	2.20	3.75	83.3
2.25	215.95	29.3	0.88	1.50	66.6
1.125	204.66	18.0	0.54	0.92	81.7
0.56	196.25	9.6	0.29	0.50	89.2
0.28	189.65	3.0	0.09	0.17	60.7
0.14	185.39	0	0	0	0
nil	186.65	-	-	-	-

Table A3: THE UPTAKE OF VARIOUS AMOUNTS OF MYCOBACTERIAL GLYCOPEPTIDE (ST208) BY A CONSTANT NUMBER OF HUMAN ERYTHROCYTES

Amount of Glycopeptide added (mg/100 ml)	Carbohydrate content of cells mg/100 ml to Haldane Standard	Increase in cell carbohydrate content (mg/100 ml)	(mg)	Mycobacterial Fraction: Amount adsorbed (mg)	Mycobacterial Fraction: Amount adsorbed / Amount added x 100
18	277.76	176.0	5.28	9.01	50.0
9	266.76	165.0	4.95	8.30	92.2
4.5	189.42	87.66	2.63	4.42	98.2
2.25	141.42	39.66	1.19	2.01	89.3
1.125	122.08	20.32	0.61	1.01	89.7
0.56	110.1	8.34	0.25	0.42	75.0
0.28	103.73	2.0	0.06	0.10	35.7
0.14	101.97	0.21	0.006	0.01	7.1
nil	101.76	-	-	-	-

Table A4: THE UPTAKE OF VARIABLE AMOUNTS OF LPS FROM E. COLI NCTC 8623 BY A CONSTANT NUMBER OF RABBIT ERYTHROCYTES

Amount of LPS added (mg)	Carbohydrate content of cells mg/100 ml to Haldane Standard	Increase in cell carbohydrate content		LPS Fraction	
		(mg/100 ml)	(mg)	Amount adsorbed (mg)	$\frac{\text{Amount adsorbed}}{\text{Amount added}} \times 100$
18	280.98	94.33	2.83	6.57	36.5
9	279.84	93.28	2.79	6.46	71.7
4.5	242.28	55.72	1.65	3.82	84.8
2.25	212.00	25.44	0.75	1.73	76.8
1.125	197.86	11.30	0.33	0.76	65.5
0.56	192.72	6.16	0.18	0.41	73.2
0.28	212.0	25.44	0.75	1.73	-
0.14	187.49	0.84	0.024	0.055	39.28
nil	186.65	-	-	-	-

Table A5: THE UPTAKE OF VARIABLE AMOUNTS OF LPS FROM E. COLI NCTC 8623 BY A CONSTANT NUMBER OF HUMAN ERYTHROCYTES

Amount of LPS added (mg)	Carbohydrate content of cells mg/100 ml to Haldane Standard	Increase in cell carbohydrate content		LPS Fraction	
		(mg/100 ml)	(mg)	Amount adsorbed (mg)	$\frac{\text{Amount adsorbed}}{\text{Amount added}} \times 100$
18	212.76	111.01	3.33	7.72	42.8
9	209.42	107.66	3.23	7.50	83.3
4.5	146.76	45.00	1.35	3.14	69.7
2.25	130.42	28.66	0.86	2.01	89.3
1.125	111.06	9.30	0.28	0.66	58.6
0.56	104.42	2.66	0.08	0.20	35.7
0.28	103.09	1.33	0.04	0.10	35.7
0.14	94.22	0	0	0	0
0.070	102.37	0.61	0.001	0	0
0.035	101.76	0	0	0	0
nil	101.76	-	-	-	-

Table A6a: THE VARIATION IN THE UPTAKE OF A MYCOBACTERIAL GLYCOPEPTIDE (ST208) BY SHEEP ERYTHROCYTES WITH TIME OF INCUBATION

Time of incubation (minutes)	mgs carbohydrate/ 100 ml compared to Haldane Standard		Increase in cell carbohydrate content of cells (%)	Increase in carbohydrate content of cells (mg/ml)	Total carbohydrate increase per reaction mixture (mg)	Mycobacterial Fraction		Hae lys (%
	Untreated cells	Treated cells				Amount adsorbed	Amount adsorbed / Amount added x 100	
20	132.63	151.42	14.16	0.18	0.36	1.0	5.0	2.
40	106.60	109.84	3.03	0.03	0.06	0.16	0.8	3.
60	80.94	134.90	66.60	0.53	1.06	2.9	14.5	11.
100	75.01	169.60	126.00	0.94	1.98	5.5	27.5	11.
200	78.01	87.87	12.4	0.09	0.18	0.5	2.5	11.

Table A6b: THE VARIATION IN THE UPTAKE OF A LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY SHEEP ERYTHROCYTES WITH TIME OF INCUBATION

Time of incubation (minutes)	Carbohydrate mg/100 ml compared to Haldane Standard		Increase in carbohydrate content of cells		Total increase in carbohydrate per reaction mixture	LPS Fraction		
	Untreated cells	Treated cells	(%)	(mg/ml)		Amount adsorbed (mg)	$\frac{\text{Amount adsorbed}}{\text{Amount added}} \times 100$	Haemolysis
20	132.63	144.12	8.67	0.11	0.22	0.50	2.5	0
40	106.60	118.68	11.36	0.12	0.24	0.55	2.7	2.3
60	80.94	130.2	60.85	0.49	0.98	2.27	11.35	4.24
100	75.01	112.59	50.09	0.37	0.74	1.71	8.55	6.37
200	78.01	104.58	34.05	0.26	0.52	1.20	6.00	11.42

APPENDIX VII : RESULTS AND STATISTICAL ANALYSES OF RADIOACTIVE EXPERIMENTS

Table A7: THE UPTAKE OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY DIFFERENT CONCENTRATIONS OF RABBIT ERYTHROCYTES

Concentration of erythrocytes (numbers of cells x 10^8)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (90 dpm)	Total dpm associated with cell pellet	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$	Standard Error
Total dpm of ^{14}C-LPS per test = 4803					
3.14	83.90	3189	1614	0.33	63.2
2.5	85.75	3299	1504	0.31	63.7
1.57	87.70	3469	1334	0.27	64.3
1.25	87.70	3663	1140	0.23	65.1
0.78	86.65	3767	1036	0.21	65.5
0.39	86.60	3997	806	0.16	66.3
Total dpm of ^{14}C-LPS per test = 2104					
3.14	84.10	852	1252	0.59	38.5
2.5	84.15	1084	1020	0.48	40.0
1.57	85.75	1230	874	0.41	40.8
1.25	85.75	1369	735	0.34	41.7
0.78	87.00	1394	710	0.33	41.8
0.39	86.65	1694	410	0.19	43.6

The total radioactivity (dpm) associated with the cell pellet at the end of the incubation period was plotted against the concentration of erythrocytes (number of cells x 10^8) (Figure 18).

Table A9: THE UPTAKE OF VARIABLE AMOUNTS OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES

Lipopolysaccharide added (dpm)	Efficiency of counting	Total dpm of cell pellet corrected for background (72 dpm)	Standard error	Ratio: $\frac{\text{dpm cells}}{\text{dpm added}}$
Number of erythrocytes used per test = 1.57×10^8				
4606	86.15	1260	63.5	0.27
3548	86.10	1190	53.3	0.33
2034	85.95	1106	38.5	0.54
1316	85.90	946	29.0	0.71
1024	85.75	708	25.9	0.69
507	85.85	364	18.0	0.71
208	85.90	190	10.6	0.91
Number of erythrocytes used per test = 3.14×10^8				
4606	87.75	1614	61.6	0.35
3548	86.65	1582	52.5	0.44
2034	86.60	1252	37.5	0.61
1316	85.65	1127	27.4	0.85
1024	86.60	804	24.9	0.78
507	87.00	420	17.2	0.82
208	86.55	202	10.3	0.97

The radioactivity (dpm) associated with the cell pellet at the end of the incubation period was plotted against the amount of radioactivity (dpm) added to each test suspension (Figure 22)

Table A10: THE VARIATION IN THE UPTAKE OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES WITH TEMPERATURE OF INCUBATION

Total dpm ^{14}C-LPS per test = 1600					
Temperature of incubation (°C)	Efficiency of counting (%)	Total dpm of supernatant fluid corrected for background (73 dpm)	Total dpm of cell pellet	Standard Error	Ratio: dpm cell / dpm total
0	85.70	1517	83	39.4	0.05
8.5	85.85	1072	528	36.5	0.33
15	85.65	688	912	33.8	0.57
20	85.80	144	1456	29.5	0.91
25	85.40	256	1344	30.5	0.84
31.5	85.50	1072	528	36.5	0.33
37	85.45	1192	408	37.4	0.25
40	85.65	1388	212	38.7	0.13
45	85.25	1360	240	38.5	0.15
50	85.00	1390	210	38.7	0.13

These results are represented in Figure 23

Table A11: THE VARIATION IN UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES WITH TIME OF INCUBATION

Total dpm of ^{32}P-LPS per test = 4906

Incubation time (minutes)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (22 dpm)	Total dpm of cell pellet	Standard error	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$
10	87.20	4804	102	69.6	0.020
20	87.05	4570	436	68.8	0.089
30	86.95	4308	598	67.8	0.122
40	86.70	3993	913	66.7	0.186
50	86.70	3862	1044	66.2	0.213
60	86.80	3754	1152	65.8	0.235
70	86.90	3869	1037	66.2	0.211
80	86.95	3931	975	66.5	0.198
90	86.95	4100	806	67.1	0.164
100	86.95	3599	1347	65.2	0.266
110	86.90	3262	1644	63.9	0.335
120	86.95	3350	1556	64.3	0.317
130	86.90	3911	995	66.4	0.202
140	86.65	4263	643	67.7	0.131

The total radioactivity (dpm) associated with the cell pellet was plotted against the time of incubation (minutes) (Figure 24)

Table A8: THE UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE (LPS) FROM E. COLI NCTC 8623 BY DIFFERENT CONCENTRATIONS OF RABBIT ERYTHROCYTES

Total dpm of ^{32}P-LPS per test = 17477

Concentration of erythrocytes (numbers of cells x 10^7)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (25 dpm)	Total dpm associated with cell pellet	Standard error	Ratio $\frac{\text{dpm cells}}{\text{dpm total}}$
156	85.40	11467	6010	120.3	0.34
104	83.90	11535	5942	120.3	0.34
52	85.45	12905	4572	123.4	0.26
26	86.00	15219	2258	127.9	0.129
13	86.60	16814	663	131.0	0.037
6.5	86.60	16713	764	130.8	0.043
3.25	87.05	16877	600	131.0	0.034
1.625	87.05	16957	520	131.2	0.029
0.812	87.15	16977	500	131.3	0.028

Different concentrations of rabbit erythrocytes were incubated with a constant amount of lipopolysaccharide, for one hour at 37°C. The final volume of the reaction mixture was 1.1 ml. The total radioactivity (dpm) associated with the cell pellet at the end of the incubation period was estimated, and plotted against the concentration of erythrocytes. (Number of cells x 10^8) (Figure 19).

The Variation in the Uptake of ^{32}P-LPS by Human Erythrocytes with Time of Incubation

Chi-square Analysis

Assume that at 60 min incubation a plateau is reached and a Null Hypothesis (NH) is set up which states that "the apparent fluctuations in the cell-associated radioactive counts are no greater than can reasonably be accounted for by experimental error." i.e. The counts observed between 60 and 140 min are random fluctuations about the mean plateau value of 1129 dpm.

Time (minutes)	Observed (O) Result (dpm)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
60	1152	23	529	0.46
70	1037	92	8464	7.49
80	975	154	23716	21.00
90	806	323	104329	92.40
100	1347	218	47524	42.09
110	1644	515	265225	234.92
120	1556	427	182329	161.49
130	995	134	17956	15.09
140	643	486	236196	209.20
	Σx = 10155			Σx = 784.14

n = 9

$\bar{x}$ (E) = 1129

p = 0.1%

The probability associated with a Chi-square value 784.14 and 8 degrees of freedom is 0.1%. Therefore the NH is rejected so that the fluctuations can not be accounted for by experimental error. Similar Chi-square analyses were set up for other cells.

Table 12A: THE VARIATION IN UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY HUMAN WHITE BLOOD CELLS WITH TIME OF INCUBATION

Total dpm of ^{32}P-LPS per test = 4906

Incubation time (minutes)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (22 dpm)	Total dpm of cell pellet	Standard Error	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$
10	86.80	4862	44	69.9	0.008
20	86.80	4622	284	69.0	0.058
30	86.95	4382	524	68.2	0.107
40	86.70	4318	588	68.0	0.120
50	86.65	4264	642	67.7	0.131
60	86.70	4386	520	68.2	0.105
70	86.65	4416	490	68.3	0.090
80	86.70	4686	220	69.3	0.044
90	86.80	4672	234	69.2	0.049
100	86.75	4380	526	68.2	0.107
120	86.45	4615	291	69.0	0.059
130	86.70	4677	229	69.2	0.046
140	86.65	4485	420	68.5	0.085

The radioactivity (dpm) associated with the cell pellet was plotted against the time of incubation (minutes) (Figure 24)

The Variation in the Uptake of ^{32}P-LPS by Human White Blood Cells with Time of Incubation

Chi-square Analysis

Time (minutes)	Observed Results (O)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
40	588	172	29584	71.11
50	642	226	51076	122.77
60	520	104	10816	26.00
70	490	74	5476	13.16
80	220	196	38416	92.34
90	234	182	33124	79.62
100	526	110	12100	29.08
120	291	125	15625	37.56
130	229	187	34969	86.46
140	420	4	16	0.03
	$\Sigma x = 4160$			$\Sigma x = 558.13$
	$n = 10$			$p = 0.1\%$
	$\bar{x}$ (E) = 416			

The Null Hypothesis states that the apparent fluctuations in the cell-associated radioactive counts are no greater than can reasonably be accounted for by experimental error. From the probability value obtained the Null hypothesis is rejected and the fluctuations are real and are not due to chance.

Table 13A: THE VARIATION IN UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY RABBIT ERYTHROCYTES WITH TIME OF INCUBATION

Time of Incubation (min)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (33 dpm)	Total dpm associated with cell pellet	Standard Error	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$
Total dpm of ^{32}P-LPS per test = 12131					
10	85.30	11787	344	109.4	0.028
20	84.80	11634	497	109.0	0.040
30	85.12	11501	630	108.7	0.052
40	84.95	11330	801	108.3	0.066
50	85.20	11230	901	108.0	0.074
60	85.10	11046	1085	107.6	0.089
70	85.20	11305	826	108.2	0.068
80	84.65	11514	617	108.7	0.050
90	84.70	11950	181	109.7	0.015
100	84.90	12010	121	110.0	0.010
110	84.95	12022	109	109.9	0.009
120	85.00	11484	647	108.7	0.053
130	84.45	11081	1050	107.7	0.086
140	84.40	11279	852	108.2	0.070
150	84.40	11951	180	109.7	0.010
160	84.35	11931	200	109.7	0.016
170	84.50	11646	485	109.0	0.040
180	84.30	10874	1257	107.3	0.103

The radioactivity (dpm) associated with the cell pellet was plotted against the time of incubation (minutes) (Figure 25).

The Variation in the Uptake of ^{32}P-LPS by Rabbit Erythrocytes with Time of Incubation

Chi-square Analysis

Time (minutes)	Observed Result (O)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
60	1085	503	253009	434.7
70	826	244	59536	102.3
80	617	35	1225	2.1
90	181	401	160801	276.3
100	121	461	212521	365.2
110	109	473	223729	384.4
120	647	65	4225	7.3
130	1050	468	219024	376.3
140	852	270	72900	125.3
150	180	402	161604	277.7
160	200	382	145924	250.7
170	485	97	9409	16.2
180	1257	675	455625	782.9
	$\Sigma x = 7560$			$\Sigma x = 3400.9$
	$n = 13$			$p = 0.1\%$
	$\bar{x}$ (E) = 582			

The Null Hypothesis states that the apparent fluctuations in the cell-associated radioactivity are no greater than can be reasonably accounted for by experimental error. From the probability value obtained the Null hypothesis is rejected and the fluctuations are real and are not due to chance.

Table 14A: THE VARIATION IN THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY MOUSE PERITONEAL LYMPHOCYTES WITH TIME OF INCUBATION

Time of incubation (minutes)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (22 dpm)	Total dpm associated with cell pellet	Standard error	Ratio: dpm cells / dpm total
Total dpm of ^{32}P-LPS per test = 4906					
10	86.90	4871	45	69.9	0.009
20	86.95	4868	38	69.9	0.008
30	86.80	4882	24	69.9	0.005
40	86.70	4622	284	69.0	0.057
50	86.80	4752	154	69.5	0.031
60	86.70	4635	271	69.0	0.055
70	86.95	4572	374	68.8	0.068
80	86.95	4679	227	69.2	0.046
90	86.70	4662	244	69.2	0.049
100	86.70	4721	184	69.4	0.037
110	86.70	4719	187	69.4	0.038
120	86.55	4756	150	69.4	0.030
130	86.55	4875	31	69.9	0.006
140	86.45	4572	334	68.8	0.068
after 48 hours from time 0 minutes*					
0	86.90	4529	21		0.004
20	86.90	4436	124		0.027
40	86.75	4327	223		0.049

* Figures corrected for radioactive decay

Results represented in Figure 26

The Variation in the Uptake of ^{32}P-LPS by Mouse Peritoneal Lymphocytes with Time of Incubation

Chi-square Analysis

Time (minutes)	Observed Result (O)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
40	284	62	3844	17.3
50	154	68	4624	20.8
60	271	49	2401	10.8
70	374	152	23104	104.0
80	227	5	25	0.10
90	244	22	484	2.1
100	184	38	1444	6.5
110	187	35	1225	5.5
120	150	72	5184	23.4
130	31	191	36481	164.3
140	334	112	12544	56.5
	$\sum x = 2440$			$\sum x = 411.4$

$n = 11$

$\bar{x}$ (E) = 222

p = 0.1%

The Null Hypothesis states that the apparent fluctuations in the cell-associated radioactive counts are no greater than can reasonably be accounted for by experimental error. From the probability value obtained the Null Hypothesis is rejected and the fluctuations are real and are not due to chance.

Table 15A: THE VARIATION IN UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY MOUSE PERITONEAL MACROPHAGE WITH TIME OF INCUBATION

Time of incubation (min)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (22 dpm)	Total dpm associated with cell pellet	Standard error	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$
Total dpm of ^{32}P-LPS per test = 4906					
10	87.05	4587	319	68.9	0.065
20	87.05	4571	335	68.8	0.068
30	86.80	4512	394	68.6	0.080
40	86.70	4378	528	68.1	0.107
50	86.65	4147	759	67.3	0.154
60	86.30	4257	649	67.7	0.132
70	86.55	4567	339	68.8	0.069
80	86.35	4263	643	67.7	0.131
90	86.40	4323	584	67.9	0.119
100	86.35	4598	308	68.9	0.062
110	86.35	4725	181	69.4	0.036
120	86.30	4573	333	68.9	0.067
130	86.35	4532	374	68.7	0.076
140	86.15	4655	251	69.1	0.051
after 48 hours from time 0 minutes*					
0	86.95	4540	10		0
20	86.75	4320	230		0.050
40	87.00	3907	643		0.141

* Figures corrected for radioactive decay.
Results represented in Figure 26

The Variation in the Uptake of ^{32}P-LPS by Mouse Peritoneal Macrophages With Time of Incubation

Chi-square Analysis of Results

Time (minutes)	Observed Results (O)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
50	759	317	100489	227.3
60	649	207	42849	96.9
70	339	103	10609	24.0
80	643	201	40401	91.4
90	584	142	20164	45.6
100	308	134	17956	40.6
110	181	261	68121	154.1
120	333	109	11881	26.8
130	374	68	4624	10.4
140	251	191	36481	82.5
	Σx = 4421			$\Sigma_4 x$ = 799.9
	n = 10			p = 0.1%
	$\bar{x}$ (E) = 442			

The Null Hypothesis states that the apparent fluctuations in the cell-associated radioactive counts are no greater than can reasonably be accounted for by experimental error. From the probability value obtained the Null hypothesis is rejected so that the fluctuations are real and are not due to chance.

Table 16A: THE UPTAKE OF ^{14}C-LPS FROM E. COLI NCTC 8623 BY HUMAN ERYTHROCYTES - INHIBITION OF UPTAKE OF LABELLED LPS BY THE ADDITION OF COLD HOMOLOGOUS LPS

Amount of cold LPS added (µg)	Efficiency of counting (%)	Total dpm of cell pellet corrected for background (88 dpm)	Ratio $\frac{\text{dpm cells}}{\text{dpm total}}$	Inhibition of uptake by cold LPS (%)
66.6	85.75	33	0.03	94.6
50.0	85.80	144	0.145	76.4
41.6	85.85	98	0.09	83.9
33.3	85.85	346	0.35	43.3
25.0	85.90	386	0.39	36.8
16.60	85.55	541	0.54	11.4
12.50	85.50	565	0.57	7.4
8.30	85.75	585	0.59	4.1
0	85.95	611	0.62	0

The inhibition of uptake (%) was plotted against the amount of cold homologous LPS (µg) added per test (Figure 30).

Table 17A: THE INHIBITION OF UPTAKE OF ^{32}P-LIPOPOLYSACCHARIDE FROM E. COLI NCTC 8623 BY COLD HOMOLOGOUS LPS OBSERVED WITH RABBIT ERYTHROCYTES

Total dpm of ^{32}P-LPS per test = 15076

Amount of cold homologous LPS added (µg)	Efficiency of counting (%)	Total dpm of supernatant liquid corrected for background (22 dpm)	Total dpm associated with cell pellet	Ratio: dpm cells / dpm total	Inhibition of uptake (%)
220	85.55	15229	0	0	100
150	85.60	14839	237	0.015	90.7
110	85.70	13971	1105	0.073	54.4
75	85.65	12803	2273	0.150	6.30
55	85.65	12860	2216	0.147	8.20
27.5	85.60	12713	2363	0.156	2.5
13.7	85.50	12759	2317	0.153	4.4
6.8	85.65	12830	2246	0.149	6.9
0	85.625	12666	2410	0.160	-

The inhibition of uptake (%) was plotted against the amount of cold homologous LPS (µg) added per test (Figure 31)

The variation in the Uptake of ^{32}P-LPS by Rabbit Erythrocytes, with Time of Incubation :

The Effect of Heat Treatment of the LPS

Chi-square Analysis

	Untreated LPS				Heat-treated LPS			
Time (minutes)	Observed Results (O)	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$	O	(O - E)	$(O - E)^2$	$\frac{(O - E)^2}{E}$
65	602	288	82944	264.2	998	25	625	0.6
80	432	118	13924	44.3	1000	23	529	0.5
95	196	118	13924	44.3	1060	37	1369	1.3
110	116	198	39204	124.9	1028	5	25	0.02
125	388	74	5476	17.4	980	43	1849	1.8
140	416	102	10404	33.1	1004	19	361	0.4
155	56	258	66564	212.0	1092	69	4761	4.7
	$\Sigma x = 2207$			$\Sigma = 740.2$	$\Sigma x = 7162$			$\Sigma = 9.27$
	$n = 7$			$p = < 0.1\%$	$n = 7$			$p = < 50720\%$
	$\bar{x}$ ($\bar{E}$) = 314				$\bar{x}$ ($\bar{E}$) = 1023			

The Null Hypothesis states that the apparent fluctuations in the cell-associated radioactivity are no greater than can be reasonably accounted for by experimental error. From the probability value obtained for the untreated LPS, the Null hypothesis is rejected and the fluctuations are real and are not due to chance. For the heat-treated LPS, the Null hypothesis cannot be rejected and the observed fluctuations are due to chance.

Table 18A: VARIATION WITH TIME OF INCUBATION IN THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY RABBIT ERYTHROCYTES. THE EFFECT OF HEAT TREATMENT OF THE LPS

Total dpm of ^{32}P-LPS per test = 4408

Time of incubation (minutes)	Efficiency of counting (%)	Total dpm of supernatant fluid corrected for background (22 dpm)	Total dpm of cell pellet	Standard error	Ratio: dpm cells / dpm total
Untreated LPS					
35	84.40	4012	396	68.9	0.08
50	84.20	3676	732	63.6	0.16
65	84.25	3806	602	64.1	0.13
80	84.20	3976	432	64.7	0.09
95	84.05	4212	196	65.6	0.04
110	84.10	4292	116	66.0	0.02
125	84.20	4020	388	64.9	0.08
140	83.90	3992	416	64.8	0.09
155	83.60	4352	56	66.2	0.01
Heat Treated LPS					
35	84.40	4132	376	65.4	0.08
50	84.20	3608	800	63.3	0.18
65	84.40	3410	998	62.5	0.22
80	84.20	3408	1000	62.5	0.22
95	83.70	3348	1060	62.3	0.24
110	83.95	3380	1028	62.4	0.23
125	83.95	3428	980	62.6	0.22
140	83.95	3404	1004	62.5	0.22
155	83.60	3316	1092	62.2	0.24

These results are represented graphically in Figure 32

Table 19A:

THE EFFECTS OF NORMAL RABBIT SERUM ON THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY RABBIT ERYTHROCYTES

Normal rabbit serum dilution	Efficiency of counting (%)	Total dpm associated with cell pellet corrected for background (22 dpm)	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$	Inhibition of uptake (%)
Untreated ^{32}P-LPS				
Neat	87.75	240	0.14	54.0
1/2	87.65	245	0.15	52.9
1/4	86.50	455	0.27	12.7
1/8	86.60	506	0.31	3.0
1/16	85.85	516	0.32	0
-	85.95	522	0.32	0
Pretreatment with neat serum	86.70	287	0.17	45.1
Heat-treated ^{32}P-LPS				
Neat	87.75	323	0.19	50.4
1/2	86.95	585	0.35	10.2
1/4	86.85	670	0.41	0
1/8	85.35	635	0.38	2.3
1/16	85.40	650	0.40	0
-	85.50	652	0.40	0
Pretreatment with neat serum	87.50	582	0.35	10.6

These results are represented graphically in Figure 33

Table 20A:

THE EFFECTS OF CHOLESTEROL ON THE UPTAKE OF ^{32}P-LPS FROM E. COLI NCTC 8623 BY RABBIT ERYTHROCYTES

Cholesterol added (µg)	Efficiency of counting (%)	Total dpm associated with cell pellet - corrected for background (22 dpm)	Ratio: $\frac{\text{dpm cells}}{\text{dpm total}}$	Inhibition of uptake (%)
Untreated ^{32}P-LPS				
150	86.50	145	0.08	72.2
75	87.00	155	0.09	70.2
37.5	86.75	353	0.21	32.3
0	85.95	522	0.32	0
Pretreatment with 150 µg cholesterol	86.00	436	0.26	16.3
Heated-treated ^{32}P-LPS				
150	87.05	206	0.12	68.3
75	86.95	230	0.14	64.7
37.5	86.75	520	0.31	20.1
0	85.50	652	0.40	0
Pretreatment with 150 µg cholesterol	86.75	642	0.39	1.4

These results are represented graphically in Figure 34

Table 21A:

THE EFFECTS OF LECITHIN ON THE UPTAKE OF UNTREATED ^{32}P-LPS FROM *E. COLI* NCTC 8623 BY RABBIT ERYTHROCYTES

Lecithin added (µg)	Efficiency of counting (%)	Total dpm associated with cell pellet corrected for background (22 dpm)	Ratio: dpm cells / dpm total	Inhibition of uptake (%)
150	87.70	235	0.14	54.9
75	86.95	259	0.15	50.2
37.5	86.85	416	0.25	20.3
0	85.95	522	0.32	0
Pretreatment with 150 µg lecithin	86.05	248	0.15	52.3

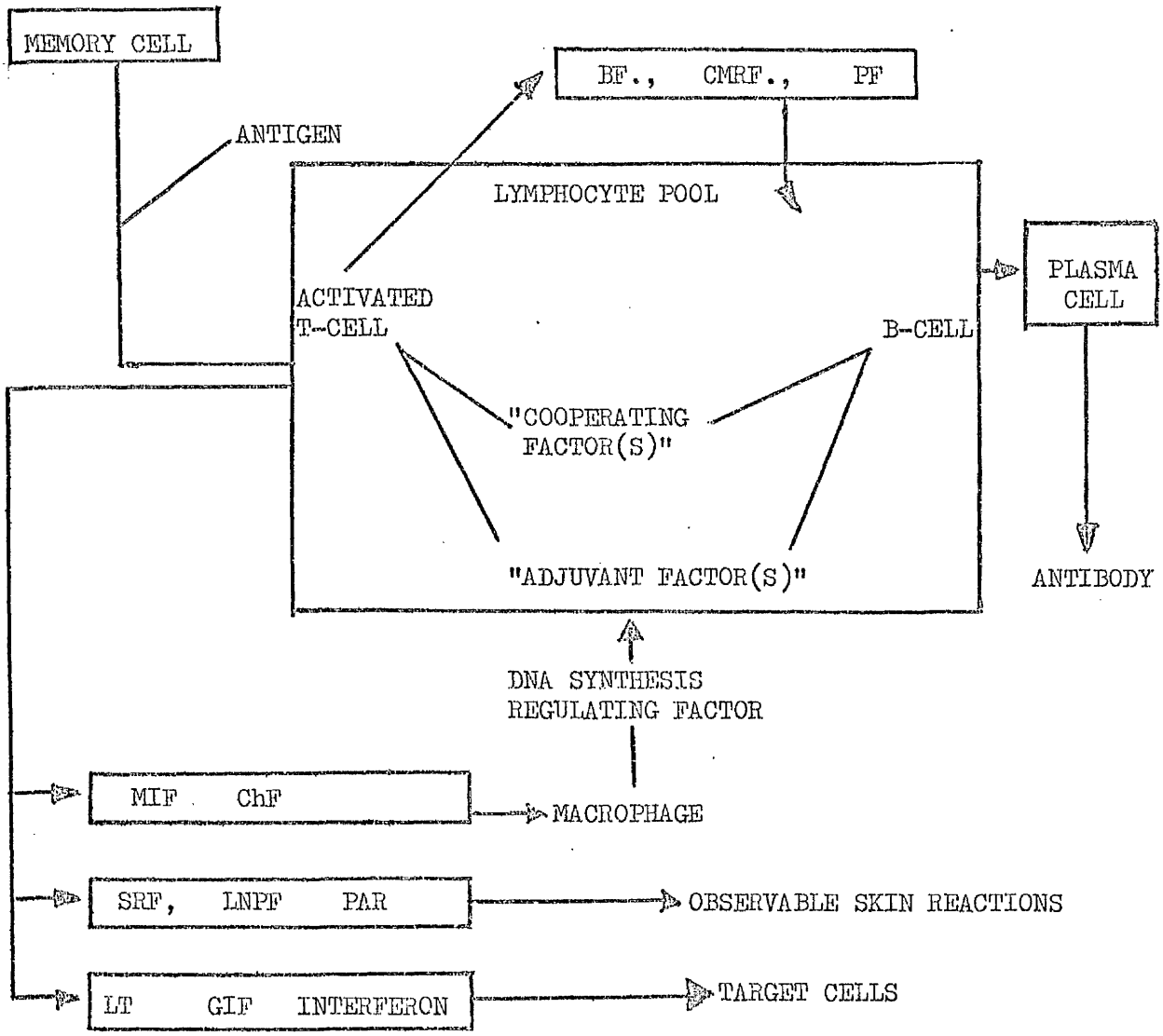
These results are represented graphically in Figure 35

APPENDIX VIII - ANALYSIS OF VARIANCE OF THE RELATIVE ADJUVANCY ASSAYS OF VARIOUS BACTERIAL POLYSACCHARIDES

LPS	Individual antibody titres (log_2 dilution)		Analysis of Variance probability values (%)		
	low dose	high dose	Preparation	Slope	Parallelism
S. typhi NCTC 0901	8,10,8,8,8	10,9,9,9,10	< 5> 1	< 5> 1	>10
S. typhimurium NCTC 5710	8,6,6,5,5	6,7,7,8,9	< 5 >1	< 5 >1	>10
E. coli B (ether)	7,3,3	8,6,8	< 5 >1	< 5 >1	>10
E. coli B (EDTA)	2,1,2,2,0	4,4,3,3,0	<1	<1	>10
E. coli 055:B5	2,3,2,2,3	6,6,7,7,6	<1	<1	>10
Sh. flexneri	2,1,1,2	2,4,5,4	<1	<1	>5 > 10
F. tularensis	9,8,9,9	10,8,9,9	<1	<1	>10
Bd. pertussis NCTC 18334	4,4,4,5	8,8,8,10	<1	<1	>10
Mycobacterial Fractions					
ST82	6,7,7,5,5	9,10,10,10,10	<5 >1	<1	>10
ST208	9,6,8,6	8,6,8,8	<1	<1	< 1
ST210	8,7,7	10,10,9	>10	<1	>10
ST211	4,4,5,4,5	7,8,7,8,10	< 1	<1	>10
PPD	6,5,7	9,10,9	< 1	<1	>10

The analysis of variance results compare the adjuvancy of the different polysaccharide preparations to the adjuvancy of E. coli NCTC 8623 LPS.

APPENDIX IX - THE POSSIBLE ROLE OF SOLUBLE FACTORS IN HUMORAL AND CELL MEDIATED RESPONSES



MIF - Macrophage Migration Inhibition Factor
Ch.F. - Chemotactic Factor
SRF - Skin Reactive Factor
LNPF - Lymph Node Permeability Factor
PAR - Production of Antigen Recognition
LT - Lymphotoxin
GIF - Growth Inhibitory Factor
BF - Blastogenic Factor
CMRF - Conditioned Medium Reconstituting Factor
PF - Potentiating Factor

APPENDIX X - GLOSSARY OF TERMS USED IN IMMUNOLOGY REVIEW

Bone Marrow derived Cells (B lymphocytes): Lymphocytes found in the peripheral lymphoid organs, which are derived from the bone marrow but not processed by the thymus.

Thymus derived cells (T lymphocytes): Lymphoid cells, arising in the bone marrow and subsequently"processed" by the thymus before migrating to the secondary lymphoid organs.

Antigen reactive cells (ARC): Thymus derived cells which react by rapid division in response to an antigen but do not give rise to antibody-forming cells (AFC).

Antigen sensitive cells (ASC): Bone marrow derived cells which divide in response to an antigen, giving rise to an antibody-forming cell (AFC).

Antibody-forming cell (AFC): A plasmacell synthesizing antibody. These cells are formed by transformation of antigen sensitive cells in response to an antigen.

Extended definitions of the above and other immunologic terms used in the review can be found in "A Dictionary of Immunology" 1971, edited by W.J. Herbert and P.C. Wilkinson. (Blackwell Scientific Publications, Oxford and Edinburgh).

APPENDIX XI