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AND LIPOPOLYSACCHARIDE

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By

Richard Thomas Coughlin

A DISSERTATION

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Department of Biophysics

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ABSTRACT

PHYSICAL PROPERTIES OF GRAM NEGATIVE MEMBRANES AND LIPOPOLYSACCHARIDES

By

Richard Thomas Coughlin

The isolated membranes and lipopolysaccharide (LPS) of Escherichia coli were analyzed using the electron spin probes 4-(dodecyl-dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide, CAT_{12}, and 5-doxyl stearate. The position of the head group probe CAT_{12} in the outer membrane was determined to be on the outer monolayer in LPS-protein domains. The results indicate that both the inner and outer monolayer of the outer membrane experience the same cooperative membrane phase transition despite strong transmembrane asymmetry. Using inductively coupled plasma emmission spectroscopy, the outer membrane was found to be enriched in divalent cations when compared to the cytoplasmic membrane. The higher cation affinity was attributed to the presence of anionic LPS in the outer membrane. Elemental analysis of LPS revealed the presence of not only high levels of Mg and Ca but significant levels of Fe, Al, and Zn as well.

A purified sodium salt of LPS isolated from both a rough and deep rough E. coli was prepared. It was relatively free of contaminating cations and had significantly greater head group and acyl chain mobilities when compared to the starting material. Elemental analysis suggests that the sodium salt of the rough LPS was not completely charge neutralized. The binding affinity and capacity of LPS from a variety of

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cations was shown to be quite high yet variable depending upon the cation used. The information obtained with LPS from *Escherichia coli* was then applied to *Yersinia pestis*, *Chromatia vinosum*, and *Thiocapsa roseopersicina* LPS with attention to the potential biological significance of the observed physical phenomenon.

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Finally, I would like to express my thanks to my mother, father, and fiancé, Carol, for their encouragement.

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CHAPTER I

BACKGROUND

Gram Negative Membranes

Electron micrographs of thin sections of gram-negative bacteria show two distinct membranes. The inner or cytoplasmic membrane contains the enzymes for oxidative phosphorylation, electron transport, and active transport. The characteristic shape of the cell is maintained by a two dimensional sheet of peptidoglycan distal to the cytoplasmic membrane. Covalently linked to the peptidoglycan and extending into the outer membrane is the Braun lipoprotein. This protein serves as an attachment site for the outer membrane and is crucial for outer membrane structural integrity. In lipoprotein deficient mutants of Escherichia coli, grown in low Mg media, the association of the outer membrane for the peptidoglycan is weakened (1).

The outer membrane is a highly specialized structure. In comparison with other membranes (e.g., the cytoplasmic membrane) its protein composition is relatively simple. In most enteric bacteria there are four principle proteins, excluding the lipoprotein. Two of these proteins 1a and 1b (Schnaitman nomenclature) (2) form trimeric units in the outer membrane and act as pores (3). The pore proteins (porins) have been referred to as "molecular sieves" because they allow passive

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nonspecific diffusion of low molecular weight (generally <600 daltons) hydrophilic solutes through the outer membrane.

Both proteins and lipids of the outer membrane are asymmetrically distributed. Lipopolysaccharide (LPS) is found exclusively on the outer monolayer of the outer membrane while phospholipids in most gram negative bacteria are found predominantly on the inner monolayer of the outer membrane (4). The association of LPS with outer membrane proteins can be exceptionally strong. In fact, the separation of porin and LPS can only be carried out at high ionic strength (5). In the absence of high salt, detergent solubilization of porin results in a porin complex containing up to three moles of LPS. The interaction of LPS with porin appears to be predominantly hydrophobic in nature since porin can be effectively reconstituted with only the lipid A portion of LPS (6). It is apparent, however, that the polysaccharide region of LPS does interact with the porins. In reconstituted lipid A-porin complexes, a hexagonal lattice was formed as was also detected with LPS-porin complexes, but the lattice constant was significantly less than that of the intact LPS-porin complex.

Whereas most biological membranes are only weakly resistant to the free diffusion of small hydrophobic compounds (7), the outer membrane is quite impermeable to these molecules. Nikaido (8) first noticed that the hydrophobicity of antibodies correlated exceptionally well with their efficacy in inhibiting deep rough (short LPS polysaccharide chain) mutant bacteria. The velocity of diffusion of such molecules across the outer membrane of LPS mutant cells increased as the length of the O-polysaccharide chains decreased. Even deep rough mutants which have only lipid A and the 3-deoxy-D-manno-octulosonic acid (KDO) sugars had

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one tenth the diffusion velocity of the cytoplasmic membrane for small hydrophobic molecules. Thus the porins and LPS form a passive yet selective diffusion barrier to low molecular weight solutes.

The fluidity of the hydrocarbon interior of the outer membrane has recently been shown to be a determinant of the growth limits of *E. coli* (9). Bacteria adapt their outer membrane lipid and protein composition to maintain a mixture of gel and liquid crystalline acyl chain domains at the temperatures of growth. When adaptation is unsuccessful at either temperature extreme and membrane acyl chains are exclusively either gel or liquid crystalline, growth ceases. Nakayama *et al*. (10) have shown in an *E. coli* B smooth strain using X-ray diffraction analysis that isolated native LPS undergoes a phase transition which is relatively independent of the temperature at which the cells had been grown. Moreover, the phospholipids of the outer membrane do adapt to changes in growth temperature by changing the ratio of saturated to unsaturated acyl chains (10). In a fatty acid auxotroph of *E. coli* K12 the outer membrane transition temperature as determined by fluorescent probing and enzyme activity shifted in response to the exogenously supplied fatty acid (11). When the rigid trans Δ $C_{18:1}$ fatty acid was supplied, the membrane phase transition occurred at higher temperatures than with the more fluid cis Δ $C_{18:1}$. Since there are concomitant changes in the outer membrane protein profile as a function of temperature, no complete model exists as yet which explain the adaptation of the membrane physical state.

The interaction of LPS with phospholipid domains is complex. Onji and Liu (12) have shown that LPS will bind to neutral phospholipid (phosphatidylcholine) vesicles and reduce their electrophoretic mobility.

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This effect was potentiated by prior incubation of the phosphatidylcholine vesicles with a cationic detergent. LPS did not interact as well with phosphatidylserine vesicles, however, the addition of 1 mM EDTA which presumably increased the net negative charge of LPS (see Chapter VI) eliminated any LPS-phosphatidylserine interaction. Conversely, 1 mM calcium addition promoted LPS-phosphatidylserine interaction almost to that of phosphatidylcholine vesicles. Thus, LPS undoubtedly can associate with phospholipid domains through strong hydrophobic interaction between lipid A and the phospholipid acyl chains. Head group charge repulsion, however, may prevent the initial association of LPS with the acceptor complex.

The assumption that LPS and phospholipids freely intersperse is far from established. Schindler et al. (13) showed that LPS and phosphatidylethanolamine mixtures have diffusion coefficients which are only slightly different from their pure lipid diffusion coefficients. Phosphatidylethanolamine was thought to have slightly decreased lateral mobility because its motion was hindered by slower moving LPS monomers. Takeuchi and Nikaido (14) challenged this point and suggested instead that LPS and lipids are essentially immiscible. They found that LPS and phospholipids do mix very slowly ($T_{1/2}$ several hours) when magnesium was present and mixing was maximum when sodium, magnesium, and porin were present. The mobility under these conditions was relatively independent of the lipid head group charge since phosphatidylethanolamine and phosphatidylglycerol were roughly equivalent.

An important observation of this work was that very low phospholipid-to-LPS ratios produced an apparently randomly mixed aggregate. An explanation was advanced whereby LPS and phospholipid

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mixtures behave much like water and phenol. When either is in great excess of the other, a single phase develops. As the ratio of the two begins to reach unity, the binary nature of the mixture becomes apparent. Any mixing which occurs is governed by the obligatory thermodynamic equilibrium which exists between immiscible liquid phases.

The facile insertion of rough LPS into phospholipid monolayers is also illustrative of this point. Fried and Rothfield (15) have shown that LPS from Salmonella typhimurium G30A readily inserts into phosphatidylethanolamine monolayers. These monolayers were quite stable and invariant with pressure changes (non compressible) until the LPS surface area fell below 45% of the total area. As the phosphatidylethanolamine content of the monolayer increased, the surface pressure was a function of the surface concentration of phosphatidylethanolamine. The authors interpreted those results to indicate that LPS at low phosphatidylethanolamine/LPS is surrounded by an annulus of "boundary" phospholipids. This explanation may be reworded in light of Takeuchi's theory of LPS miscibility in phospholipids. In monolayers phosphatidylethanolamine miscibility in LPS domains is high but above a critical molar ratio pure phosphatidylethanolamine domains appear which are highly compressible and independent of the presence of LPS in the monolayers. The miscibility of LPS in phospholipid domains may also be dependent upon differences in the LPS core or polysaccharide sugars. Since deep rough mutants of E. coli are thought to have large amounts of phospholipids in the outer monolayer of the outer membrane (16).

Biochemistry of LPS

In the following section and throughout the remainder of this dissertation it will become increasingly clear that LPS is a name attributed to an extraordinarily broad class of lipids. No generalized skeletal diagram can encompass all of the overlapping structural features of LPS nor is any component of its structure common to all bacteria. The recent biochemical characterization by Wexler and Oppenheim (17) of the *Listeria monocytogenes* endotoxin-like component has even broken the exclusive tie between LPS and gram-negative bacteria. Even the cell wall of *Cyanophyta* (blue-green alga) are reported to contain LPS (18). The most completely characterized LPS is that of the enteric bacteria. Conceptually, it has become the point of reference for LPS structural and physiological comparison.

The classic lipid A of enteric bacteria consists of a β 1,6-linked D-glucosamine dissacharide (19) where the amine groups serve as attachment sites for β-hydroxy fatty acids. The hydroxyl group of one of these amide-linked β-hydroxy fatty acids is often esterified with another fatty acid.

The reducing and non-reducing termini of the diglucosamine are usually phosphorylated. They may be pyrophosphorylated, ethanolamine phosphate substituted or even arabinosamine phosphate substituted (e.g. *Salmonella*, 20) depending on strain and culture conditions. An early supposition that LPS is cross-linked via phosphodiester bridges has proven incorrect (21). Nevertheless the strength of LPS self-association is so great that attempts to obtain free monomeric LPS have failed (22).

Lipid A is linked to the core polysaccharide through an acid labile glycosidic bond to KDO. In many LPS molecules there are three molecules

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of KDOs in the inner core which acts as cation binding sites. Phosphorus and ethanolamine phosphate substitution is also reported to occur on KDO sugars (23).

Although the sugars present in the LPS core region are reasonably well conserved from species to species, details of the sugar linkages are confounded by a host of variations and substitutions. The relevant details will be discussed in appropriate sections of the text. The most common sugars of the core are D-glucose, D-galactose and L-glycero-D-manno-heptose (referred to thoughout the text as heptose) and, of course, KDO.

The O-polysaccharide chain of LPS consists of repeating sugar sequences which are the immunological determinants of gram-negative bacteria. Their variability in sequence and sugar composition is overwhelming. While most enteric O-polysaccharides are composed of neutral sugars, some species (e.g. *Chromatia* and *Rhodopseudomonas*, (24)) have a high percentage of cationic sugars while others (e.g. *Bordetella*, *Klebsiella*, *Proteus*, *Brucella*, and *Citrobacter*, (25) have a high percentage of anionic sugars.

The assembly of LPS takes place on the cytoplasmic membrane (26). The core is attached to the completed lipid A by sequential addition of sugars. The synthesis of the O-polysaccharide chain occurs independently on the C_{55} polyisoprenoid lipid carrier. The polymerization of the repeating sugar segments that make up the O-polysaccharide is not uniform. Rather, the distribution of repeating units can range from 19 to 34 units (77 mole percent) in *S. typhimurium* (27). The completed polysaccharide is then attached to the lipid A core complexes. This too occurs on the cytoplasmic membrane but is rapidly

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followed by translocation into the outer monolayer of the outer membrane. The attachment of the O-polysaccharide to the lipid A-core complex is not always complete and in two recent studies of Salmonella (27,28) it was found that two thirds of the LPS which is translocated to the outer membrane has no O-polysaccharide attached at all. Since the cytoplasmic to outer membrane translocation step is essentially irreversible, it appears that in enteric bacteria the majority of LPS in smooth strains is actually rough LPS. This is not true of all LPS. Hurlbert (29) has shown that all of the LPS in Chromatia and Thiocapsa smooth strains has an O-polysaccharide chain attached. The utility of translocating both smooth and rough LPS to the outer membrane in the enterics has not been explained.

Munford (30) and coworkers have compared the size heterogeneity of S. typhimurium LPS isolated from outer membrane and from media supernatant membrane fragments (blebs). As Enterobacteriaceae grow they release membrane fragments which contain protein, phospholipid, and LPS (31). The protein profile is characteristic of the outer membrane and contains few periplasmic or cytoplasmic membrane proteins. The LPS in the membrane fragments is enriched in long chain polysaccharide LPS. Although the enrichment is only slight, it may reflect a greater tendency for more hydrophilic LPS patches of outer membrane to bleb. Since these LPS containing membrane fragments are endotoxins, elucidating the forces responsible for their release into the serum will undoubtedly expand our practical understanding of endotoxin pathology.

Host responses to endotoxins include fever induction, bone marrow necrosis, leukopenia, leukocytosis, depressed blood pressure, mitogenic lymphocyte stimulation, macrophage activation, complement activation,

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Hagemann factor activation, prostaglandin synthesis, interferon production, induction of endotoxin tolerance, nonspecific resistance to infection, tumor necrosis and death (32). Attempts to link structural features of LPS with either endotoxicity or outer membrane function are becoming increasingly successful.

A strong link has been made between the ability of endotoxin to induce shock and the structure of the lipid A moiety of LPS. Lipid A from Salmonella and other enterics has been amply documented to be pyrogenic, toxic, and reactive with complement (32,33). This association, however, is not absolute and numerous exceptions have appeared in the literature as the chemical composition and biological activity of new bacterial LPS are documented. A brief review of some of these unusual lipids may illustrate the complex structure-function relationship.

One of the more structurally complicated lipid A groups is that of Chromobacterium violaceum (34). The diglucosamine backbone has both D- and L- β hydroxy fatty acids attached. The reducing end glucosamine is linked by a phosphodiester to another D-glucosamine while the non-reducing glucosamine is linked by yet another phosphodiester to an arabinosamine. This lipid A will not react with the complement system but is pyrogenic and quite lethal.

An even more complex lipid A is that of Rhodospirillum tenue, a purple non sulfur bacterium (35). The lipid A has a diglucosamine backbone which is like that of enteric LPS with three notable exceptions. The reducing end glucosamine is phosphodiester linked to a D-arabinose; it is also linked to a 4-amino-L-arabinose via the C4 hydroxyl group. As with C. violaceum the non-reducing end glucosamine is phosphodiester linked

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Curiously, lipid A of *R. tenue* is over 100 times more toxic than the intact LPS. Hydrolysis of enteric LPS to lipid A does not usually affect lethality (36). Upon closer examination, it was found that the mild acid treatment used to obtain lipid A from *R. tenue* resulted in the cleavage of the 4-aminoarabinose and arabinose-1-phosphate from the molecule. The lipid A obtained also became immuno-crossreactive with *Salmonella* lipid A. Thus reactive groups on the classic enteric lipid A that are blocked in LPS from *R. tenue* are apparently critical determinants of lipid A toxicity.

Perhaps the least structurally complex lipid A yet characterized is that of *Rhodopsudomonas viridis* (37). Classification of this lipid as a LPS may exceed both the chemical and functional limits of the definition of LPS. There is neither an O-polysaccharide nor any core sugar in this lipid. It is considered a LPS by virtue of its similarity to more recognizable LPS of closely related bacteria (24,36). Instead of diglucosamine, this LPS has a 2,3-diamino-D-glucose backbone. The amino groups are partially substituted by D-3-hydroxymyristic acid. No esterified or nonhydroxylated fatty acids are present. Furthermore, the molecule contains no phosphorus. Although this LPS is nonpyrogenic and only mildly toxic, it compares well with LPS from a *Salmonella* in its ability to interact with complement.

The importance of distinct chemical groups in lipid A are becoming apparent. Lugowski and Ramanowska (38) have shown that the hydroxamate of 3-hydroxymyristic acid alone is a potent inhibitor of the *Shigella sonnei* lipid A/anti lipid A system. A link between the amount of

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phosphorus in lipid A and anticomplement activity has also been made (39). LPS which is rich in phosphorus such as LPS from mutant strain Ra and Rb from _S_. _minnesota_, are not able to inactivate complement, while LPS, low in phosphorus, from such sources as _Anabaena_ _variabilis_, _C_. _vinosum_, and _R._ _viridis_ are able to inactivate complement.

_In_ _vivo_ anticomplement activity measured after injecting LPS into an animal is biphasic (40). An early depression in serum complement level occurs almost instantaneously upon LPS injection while a second decrease is delayed 6 to 9 hours. The early phase is thought to be due to the interaction of large particle size LPS aggregates which also exhibit _in_ _vitro_ anticomplement activity. The delayed interaction occurs only with those LPS's which are also toxic. Thus, LPS from _R_. _viridis_ does not induce the second phase of inactivation despite its anticomplement activity _in_ _vitro_ (41). The delayed phase is not dependent upon particle size. The recent work by Ulevitch _et_ _al_. (42) on the fate of circulating LPS makes it quite clear that LPS remaining in the serum after more than an hour is almost entirely bound to high density lipoproteins. The possible significance of high density lipoproteins acting as mediators in the LPS complement interaction has not as yet attracted attention in the literature.

The serologically diverse family _Chromatiaceae_ produces several other classes of LPS which can provide new insights into endotoxaemia. These purple sulfur bacteria are not recognized human pathogens yet they do produce LPS which retains the capacity to induce several classic endotoxic shock symptoms. Most notably _C_. _vinosum_ has been assayed for its toxicity and anticomplement activity (43). Its lipid A moiety lacks the classic diglucosamine backbone and instead has a glucosamine-mannose group

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group with typically esterified and amide linked fatty acids. Despite these differences C. vinosum is 25% as toxic to mice on a weight basis as S. abortus LPS. Considering the higher average molecular weight of C. vinosum LPS the actual molar toxicity of its LPS is nearly comparable to that of S. abortus. In marked contrast with enteric LPS, however, neither toxicity nor anticomplement activity of this LPS changed after it was washed with NaEDTA. Galanos et al. (44) have shown that the sodium salts of enteric LPS typically have greatly enhanced endotoxic activity (vide infra). Even more startling was the observation that small amounts of either calcium or magnesium actually enhance C. vinosum anticomplementary activity. The explanation for these discrepencies may lie in the differences in the charge character of enteric and Chromatiaceae LPS.

Approximately 90% of the total flora in adult human consists of the species of Bacteriodes (45). The LPS of several Bacteriode species have been shown to have low endotoxin activity (46). Biochemical analysis of the LPS from Bacteriodes fragilis has revealed the complete absence of both KDO and L-glycero-D-mannoheptose (47). More recently, Wollenweber (48) and coworkers have attempted to explain the low endotoxicity of Bacteriodes by their unusual fatty acid composition. Both amide and ester linked fatty acids were found; however, the composition was quite unusual. β-hydroxyl (14:0, 15:0, 16:0, 15-Methyl-16:0, and 17:0) acyl chains were observed as well as significant amounts of iso and anti-iso myristic acid acid. Teleologically, Bacteriodes may have adapted their LPS so that they are less hazardous to their hosts. The acyl chain pattern which they have developed may represent a method of detoxifying their LPS without loss of its function within the outer membrane.

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Haemophilus influenzae provides yet another complication in establishing a clear structure-endotoxin activity relationship with lipid A. (49). Although lipid A from H. influenzae contains glucosamine, phosphorus, β-hydroxy fatty acids as well as other fatty acids, it is not toxic in mice at doses up to 50 mg/Kg. The LD_{50} against mice for enteric LPS is usually about 1 mg/Kg (50). Lipid A from H. influenzae is only mildly pyrogenic but can induce a mitogenic response in polyclonal B-cells in cell culture. Furthermore, when this lipid A was complexed with bovine serum albumin to enhance water solubility, all immunological activity was lost. Thus the proposal by Galanos (51) that a protein carrier such as bovine serum albumin may be essential for full expression of lipid A toxicity, must be weighed against the possibility that reactive groups on Lipid A may be masked in such a complex.

Subtle lipid A modifications are also apparently critical in bacterial resistance to antibiotics. Vaara and coworkers (52) have shown that polymyxin resistant mutants (pmrA) of Salmonella typhimurium have elevated substitution at the non-reducing terminus of the diglucosamine backbone. Sixty to seventy percent of the C4 hydroxyls are phosphodiester linked with 4-amino-4 deoxy-L-arabinose. Only 10 to 15% of non resistant wild type LPS has this substitution. Moreover, this alteration enhances the ability of the pmrA mutant outer membranes to withstand tris-EDTA-lysozyme treatment which is commonly used in the separation of outer membrane and cytoplasmic membrane (53). The pmrA mutant cells were also less sensitive to protamine and polylysine but remained sensitive to several other cationic antibiotics. Thus, the resistance generated by this mutation influences the barrier properties of the outer membrane. It was proposed that the mutation simply lowers

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the binding affinity of LPS for polymyxin by eliminating an anionic attachment site and substituting a repulsive cationic charge.

Although lipid A has been endowed with a reputation as the LPS toxiphore, it is clear that its activity is modulated by the physical properties of the core and O-polysaccharide sugars. The mitogenic activity of LPS demonstrates this principle quite clearly. LPS isolated from a wide variety of gram negative bacteria has been shown to be mitogenic for B-lymphocytes of normal mice. A mouse strain C3H/HeJ was developed which produced defective spleen cells that were unable to divide in response to LPS (54). It was later found that these spleen cells could be induced to divide in response to LPS from several sources including Brucella, Proteus, and Bordetella (55). Further, the polysaccharides but not the lipid A moiety of Brucella pertussis LPS could induce mitogenicity in these cells as well as normal spleen cells. The ability of B. pertussis O-polysaccharide but not common Salmonella or Escherichia O-polysaccharides to elicit the mitogenic response is thought to lie in the anionic character of the Brucella O-polysaccharide. D-Glucuronic acid is present in the repeating O-antigen. Other anionic polysaccharides such as the O-polysaccharide from P. mirabilis, dextran sulfate, and S. typhi Vi polysaccharides have similar mitogenic behavior. The effect is not, however, a general property of anionic polysaccharides. Sulfate, phosphate, and carboxymethyl derivatives of cellulose and heparin sulfate are ineffective mitogens against C3H/HeJ spleen cells. Thus, the enhanced mitogenicity of Brucella, Proteus, and Bordetella LPS can not be explained solely by the presence of repeating negative charges although this property distinguishes them from the neutral or cationic O-polysaccharides of most bacterial LPS.

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The polysaccharide of Bordetella pertussis represents an interesting combination of acidic, basic, and neutral sugars. Recently a trisaccharide was identified into O-antigen which consists of a D-glucose doubly substituted with D-glucosamine and 2-amino-2-deoxy-D-galacturonic acid (56). LPS from Shigella sonnei has also been reported to contain 2-amino-2-deoxy-L-altruronic acid (57). Aminohexuronic acids are relatively uncommon sugars in gram negative bacteria and reports their existence in LPS are quite rare.

Nevertheless, the potential exists for these unusual sugars to coordinate cations, and provide inter and intra molecular cross bridging. In B. pertussis, the unusual trisaccharide confers considerable protections upon its LPS against chemical degradation.

A survey of the sugar and fatty acid content of LPS from 38 strains of Neisseria gonorrhoeae LPS has revealed major differences between virulent and avirulent strains (58). Virulent strains were totally devoid of rhamnose whereas this sugar was common among avirulent strains. Fucose which is found in non-pathogenic N. sicca and N. lactamica was also absent in virulent N. gonorrhoeae. The levels of mannose, galactose and glucose compared to KDO in virulent strains were greater than in the avirulent strains. More subtle variations were also observed in fatty acid content.

The polysaccharides of LPS also contribute significantly to the virulence of plant pathogens. In both temperate and tropical regions, vascular wilt caused by Pseudomonas solanacearum is one of the most important bacterial diseases affecting crops. Whatley et al. (59) have demonstrated that a correlation exists between the presence of O-antigen on LPS and the ability of the bacteria to avoid the hypersensitive

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response (HR). The HR occurs when invading bacteria become attached to host cell wall resulting in a rapid collapse of host cells trapping the bacteria. Virulent cells do not attach to cell walls and multiply freely in intercellular fluid. Leach et al. (50) has found lectins on tobacco and potato mesophyll membranes which bind bacterial LPS. Although LPS alone can not induce the HR, it can block the HR during subsequent challenge by the intact bacterium. It has been proposed that the O-polysaccharide in smooth strains masks the antigenic sites in the LPS core and thus avoids triggering the HR.

The cations associated with LPS have a profound effect on the expression of endotoxic symptoms. Galanos and Luderitz (61) first attempted to generate uniform salts of LPS. Their procedure consisted of electrodialysing crudely isolated native LPS and neutralizing the acidic product with an appropriate base. As we will show in Chapter IV and as Galanos and Luderitz pointed out in their original work, this procedure does not result in a completely uniform salt. It does, nevertheless, result in dramatically different physiochemical and biological properties of the resulting LPS preparation. The triethylamine salt of LPS from Salmonella smooth strains was found to have a very low sedimentation coefficient suggesting a low particle size. It was also highly water soluble. Triethylamine LPS was also highly toxic and pyrogenic in rabbits but did not interact with the complement system. In contrast, the divalent salts of LPS were poorly soluble and had high apparent particle size. They were also less toxic and less pyrogenic than the native LPS but did interact strongly with the complement system (62).

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The principle cation removed during electrodialysis has been shown by us to be magnesium. The capacity of the LPS salts prepared by Galanos and Luderitz to retain the substituted cation was apparently dependent upon the length of the polysaccharide chain. Salts of smooth LPS retained their altered physical properties in the presence of serum. In contrast, triethylamine salts of rough strains such as _S. minnesota_ R3 and R595 became indistinguishable from their native LPS in serum (62).

The binding affinity of LPS anionic sites for polyvalent cations is quite high. Storm _et al_. (63) have demonstrated that LPS has K_d for polymyxin B of between 2.5 and 5 μM. An even higher binding affinity (K_d = 0.3 to 0.5 μM) was suggested by Schindler and Osborn using a fluorescently labeled rough _Salmonella_ LPS (64). They also identified a high affinity binding site for divalent cations using their labeled LPS. The K_d for Mg and Ca were 15 and 6 μM, respectively. Nearly identical values were obtained using LPS from either _S. typhimurium_ G30 (rough strain) or G30A (heptoseless strain). They suggested that the anionic site responsible for this high affinity cation binding is within the KDO trisaccharide complex in the LPS core. We have independently (Chapter III) confirmed that this site does have a very high divalent cation binding affinity. Furthermore, the LPS used by Schindler and Osborn had only been electrodialysed. In studies carried out in cooperation with Dr. Schindler, we have found that electrodialyzed LPS from both strains G30 and G30A retain a considerable number of divalent cations leaving open the possibility that even higher affinity cation binding sites exist within the molecule.

The high affinity cation binding of LPS is not specific for divalent cations. As we will show in Chapter IV, a wide variety of trace elements including

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including iron, aluminum, zinc and lanthanides will bind to LPS with high affinity. The potential significance of this observation has already been demonstrated in the literature.

As early as 1944, A. Schade and L. Caroline (65) reported that conalbumin could inhibit the *in vitro* growth of several gram-negative bacteria by virtue of its profound affinity for ferric iron. Later (66), it was discovered that supplemental iron could offset the serum inhibition of nonpathogenic *Pasteurella septica* strains.

The keen competition between host and pathogen for essential nutrients has caused both organisms to develop multiple mechanisms to acquire and store minerals. The extraordinarily low solubility of ferric iron (less than 10^{-38}M in water) and its irreplaceability in a variety of enzymes has made it the object of particularly fierce evolutionary adaptation.

Neilands (67) has pointed out that *Escherichia* synthesizes several 70-80 K dalton outer membrane proteins when iron levels in the media drop below 1 µM. These proteins are receptors for bacterial enterochelin which in turn have association constants for the ferric ion of up to 10^{50}. Thus in hindsight, it should not have been surprising when it was discovered that serum free iron levels drop dramatically during gram-negative infections (68). The startling observation was that the induced hypoferraemia could be mimicked completely by an intravenous injection of LPS (69).

The association of iron with LPS not only stresses the host but may also be critical for the pathogens survival. Kochan *et al*. (70) have shown that when virulent *E. coli* strains were grown on mammalian sera their growth was inhibited and they eventually died. The bacteriocidal activity

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activity of sera could be overcome in these strains by the addition of exogenous iron or enterochelin. Interestingly, LPS isolated from cells grown in the presence of iron could also act as an iron source for serum inhibited cells.

Althouh LPS can bind trace quantities of iron to the benefit of the parent bacteria, iron binding to LPS can benefit the host. Although free LPS is a potent toxin, the lethality of LPS has been shown to be dependent upon its solubility (44). The solubility of the iron salt of LPS is quite low as Sourek _et al_. have reported (71). Host protection from endotoxins by cations is not limited to iron. Snyder and Walker (72) have shown that injection of zinc chloride prior to challenge by LPS obviated the endotoxin symptoms. Chromium and manganese were even more effective than iron or zinc in detoxifying LPS.

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CHAPTER II

A PROGRAM FOR TRANSITION POINT ANALYSIS OF EXPERIMENTAL DATA*

ABSTRACT

A FORTRAN IV program is presented which determines transition points for data that can be represented by a set of intersecting regression lines. Initial estimates are not required, and no specific spacing of the data is needed. A spline function is used to reduce the large number of computational steps arising from complex linear models while an iterative least squares procedure is used to obtain the final solution.

Examples of experimental and artificially generated data are presented along with an F-statistic for differences in slope at the transition point.

INTRODUCTION

It is often convenient to represent experimental results with a set of connected regression lines. An example can be seen in the sharp

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change in slope of the logarithm of enzyme activity vs. reciprocal temperature. Considerable physical significance is associated with such transitions while the points of transition are frequently difficult to determine accurately. In this paper we are concerned with the cases where 1) the data before and after the transition behave linearly, and 2) the location of the transition is not known. Detailed applications of this analysis to actual experimental data will be given in a separate paper (1). The program has already been used for transition point analysis of membrane fluidity data (2,3).

A number of methods have been developed to estimate transition points (4,5,6). While all of these methods provide line sets, Hudson (6) has pointed out the computational obstacles that this search represents. In determining such transitions there are

$$\sum_{s=0}^{r} 2^s \binom{r}{s} \binom{n-r-2}{s}$$

possible line sets, where r is the number of transitions and n is the number of data points. Clearly, if either n or r becomes large, the number of solutions to be tested becomes prohibitive. This has led Griffiths and Miller (7) to suggest the use of a spline function to model multiphase relationships.

Our model assumes that suitable data can be represented by a set of continuous non-overlapping regression lines. While these lines are not constrained to meet, discontinuous line sets are rejected. In accordance with this model, the variance is assumed to be constant and normally distributed over the entire data range.

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It is expected that the behavior of the data in one phase will not be correlated with that in any other phase. As mentioned above, spline functions are particularly useful approximations under this condition (8). A spline function is defined as a set of polynomial pieces joined smoothly at points called knots. Their piecewise character makes them extremely sensitive to the local properties of the data and they are excellent empirical functions. We have adapted an algorithm using normalized B-spines developed by Dierckx (9) which automatically selects the number and position of the knots. Information obtained from the spline function and its derivatives can then be used to obtain an initial estimate of the transition points. The final solution is determined by an iterative linear regression analysis about the estimated transition points. The efficiency of this method over others stems directly from the curve fitting used to obtain initial estimates of the transitions.

An F-statistic is calculated for each transition which gives an indication of the difference in slope of the two regression lines at the transition point.

PROGRAM

The program package consists of four FORTRAN IV subroutines. It is designed to accept up to 200 weighted data pairs and will search for up to five transition points (6 lines). In the initial steps, data are fitted by a normalized B-spline utilizing the program of Dierckx (9); the spline routines have been slightly modified by the addition of a subroutine from de Boor (10) to speed calculations.

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The user determines the degree of the polynomial pieces used to approximate the data. A few advantages of this particular spline program are the knots need not be specified by the user and that equally spaced data points are not required. Since the spline program does not allow repeated values of the independent variable, data points with the same x value are analyzed as a single point; this point is given increased weight and represents the mean of the combined data pairs. An important aspect of the spline package in our analyses is that the closeness of fit is determined by a smoothing factor which is related to the sum of the residuals squared. The spline parameters which must be assigned by the user are the degree (K), initial smoothing factor (S), and an overestimate of the number of knots (NK).

For our analyses the zeroes of the third derivative of the spline function, i.e., the intervals with the greatest slope change, are used as estimates of the transition points. Note that this requires the degree of the spline to be at least four in order for the third derivative to be a smooth function. Also, polynomials of degree greater than five are not suggested since they may result in overfitting (8). The number of zeroes of the third derivative of the spline function are dependent upon the smoothing factor of the spline calculation; as the smoothing factor decreases the fit becomes tighter. The program varies the smoothing factor from an initial estimate in order to obtain the desired number of transition points; if iterations occur in which the assigned number of transition points is bracketed, a bisection method is used to determine the smoothing factor. Newton-Raphson procedure is utilized to locate zeroes of the third derivative. Starting points for the Newton-Raphson procedure are obtained by inspection of the third derivative of the

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spline. This procedure is completed when the proper number of zeroes and thus approximated transition points are found.

The approximated transition points allow grouping data into linear portions. An interative procedure is then used to consider all possible combinations of data groups which adjoin in an interval about the estimated transition; the size of this interval is determined by the user. Regression lines are calculated for all of these data groups as sets of lines:

$$\begin{array}{l} L_1(X) = a_1\ X_1 + b_1 + e_1 \\ \ \ \vdots \qquad\qquad\quad \vdots \quad\ \vdots \\ L_6(X) = a_6\ X_6 + b_6 + e_6 \end{array}$$

where X_i represents X values on line i and e_i represents the random variable accounting for error of each data point on line i.

The best solution is selected based on the following criteria: (1) the transition point lies between the data groups; and (2) lowest sum of the residuals squared. The intersection of these regression lines are the final transition points and are returned to the user in two arrays which completely describe the lines fitted to the data; thus the first and last points in the array represent the first and last data points.

To aid in the interpretation of the results, an F-statistic for differences in slope of each adjacent pair of lines is calculated; the null hypothesis for this test is that the lines are parallel, i.e. no transition occurred (11). For each transition point analyzed, an F value and its corresponding degrees of freedom are returned.

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given below.

INPUT

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INT -

OUTPUT

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XBRK

The calling sequence for the set of programs described is: CALL BRKANL (X,Y,W,N,NK,KNOT,KN1,COEFF,KD,S,NB,INT,XBRK,YBRK,F,DF,ERRMIN,IER). A description of each parameter and its suggested or restricted values is given below.

INPUT

X - the array of the X (independent) values.
Y - the array of the Y (measured property) values.
W - the array of the weights for each data point.
N - the number of data points ($N<200$).
NK - the maximum number of knots ($3<KD + 1 < NK < N + KD + 1$; usually N/2).
KD - the degree of the spline function ($KD < 4$; usually 4 or 5).
S - the initial smoothing factor (usually $N \pm 2 N$; $S < 0$).
NB - the number of transition points desired ($1 < NB < 5$).
INT - one-half of the interval (in data points) used to check possible solutions after estimation.

OUTPUT

NK - the final number of knots.
KNOT - the array of the knot positions.
NKI - N-KD-1, the dimension of the spline function coefficients.
COEFF - the array of the coefficients of the normalized B-spline function.
S - the final smoothing factor.
XBRK - the array of X values of the transition points (first and last are the first and last data points).

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YBRK - the array of Y values of the transition points (first and last are the Y values at the first and last data points).

F - the array of the F values for the slope comparisons.

DF - the array of the degrees of freedom of the denominator for the F values.

ERRMIN - the lowest square error (for all lines combined).

IER - the error code (described in program).

DISCUSSION

Among the parameters which must be considered to run the program are the least squares search interval term (INT, discussed below), the degree of spline (KD), and the smoothing factor (S). We have obtained good results using spline degrees of 4 and 5. A spline with KD lower than 4 is insufficient to carry out the calculations while very high degrees will usually overfit the data (8). The smoothing factor is adjusted by an iterative process in the program. If the initial estimate by the user is too extreme the method may not converge on a smoothing factor which results in the desired number of transitions. If this occurs the S value and the number of transitions from the last iteration are returned to the calling program. If the number of transitions returned is less than the number desired, the S value must be smaller than the returned value; if the number of transitions is more, S must be greater than the value returned. The degree of the spline and the number of data points also affect the required smoothing factor value, and it may be necessary to adjust the degree in order to obtain a proper fit.

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The solution criteria utilized by the program to determine the best transition points require: (1) that the lines be continuous; and 2) that the final solution have the least square error of other acceptable solutions. The continuity requirement is satisfied by insuring that the transition occurs between the endpoints of each adjacent line, i.e. data group; this may be difficult to satisfy if the data points are too closely spaced near the transitions. The least squares interval term determines how many data points on either side of the transition point estimated by the spline will be included in the least squares search, which checks all possible solutions having transitions in the vicinity of the initial estimate. If INT is too small, a valid solution may be overlooked; if it is too large, computation time and cost increase. The spline greatly increases efficiency of transition point analysis by eliminating many possible solutions that are not reasonable.

Interpretation of the results is facilitated by the statistical test for analyzing differences in slopes between each pair of lines. The least squares portion of the program determines which transition points are best suited to represent the data while the F-test determines if the data show significant transitions at these points. The analysis will fit the data to a given number of transitions, but it cannot determine the correct number of transitions. Since it is often inconvenient to obtain repeated values of the independent variable, it is not possible to confirm linearity of the data groups comprising each line. Information about linearity and the number of transitions can be obtained by plotting the *y* residual at each data point; correlated errors may indicate an improper fit (12).

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Several other approaches have been used to fit segmented lines to experimental data. Some of these techniques require the data to consist of continuous curves (13) while others model the data with a smooth curve such as hyperbola at the transition (6,12,14); a single hyperbolic function, of course, would only be applicable for analysis of a single transition. Quandt (4) presented a solution to piecewise linear regression based on maximum likelihood statistics; however, this method requires all possible solutions to be tested resulting in computational difficulties for large sets of data. As stated above, the advantages of using the spline in the initial steps of the analysis is to reduce the number of possible linear combinations to compute. A potentially promising technique for location of transition points has been reported by McGee and Carleton (15) which consists of regression and cluster analysis methods. This method has the advantage over the one presented here in that the number of breaks need not be specified; but their method's efficiency in handling a large number of data points is questionable.

To evaluate our program two artificially constructed data sets of known variance were generated from a set of lines by reassigning y-values normally distributed about these lines, i.e. each y-value deviated from the predetermined line by an amount that was randomly selected from a normal population. Figure 1 shows the results of an analysis of a two line (one transition) data set of 50 equally weighted points with a standard deviation of 0.25; the estimated transition point occurs at 26.99 which compares favorably with the actual intersection of lines at 27.0. The data was fitted with a fourth degree spline and a smoothing factor of 2.80; the spline required 13 knots. The calculated F value

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indicated that the slopes of the two lines differed significantly ($p<0.05$). Results of the analysis of a simulated six line data set (50 points) is shown in Figure 2. The estimated transitions at 11.0, 19.7, 25.2, 34.2 and 42.3 correspond to actual intersections at 11.0, 20.0, 25.0, 34.0 and 43.0 respectively. The data were fitted with a fourth degree spline (18 knots) and a smoothing factor of 3.485. The F test shows that all transitions are significant ($p<0.05$).

The power of the F test depends on two factors: 1) the level of significance set for the transition points, and 2) the distribution of the data about the lines. Since the position of one transition point is not independent of the position of the other transition points, the possibility of an error is real. Consequently, whenever any part of the model is rejected the entire model must be rejected. The assumption that data are normally distributed usually can not be verified. This point is illustrated by the empirically defined relationship between the hyperfine splitting parameter, $2T_{\parallel}$, of a fatty acid spin labeled membrane sample and temperature.

In electron spin resonance, spin probes have been found to be very sensitive to the physical state of biological membranes. $2T_{\parallel}$ changes inversely with temperature reflecting increased membrane fluidity. Abrupt changes in the slope of $2T_{\parallel}$ versus temperature have been associated with dramatic changes in lipid clustering, enzyme activity, and membrane permeability (16). Despite the importance of this relationship no rigorous mathematical model has been developed which accurately accounts for the experimental results. For this reason, a model consisting of linear segments is often used to represent such data.

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In our laboratory, 5-doxyl stearate has been used to examine the influence of several surface active agents on the physical properties of the human erythrocyte membrane (3). Figure 3 shows the effect of chlorpromazine on erythrocyte membranes. Two abrupt changes in slope can be seen at 11.5 and 33.5°C and both of these transitions are significant at a level of $p<0.05$. Different physical and biochemical techniques have shown transitions in the erythrocyte membrane at temperatures similar to these (3). The absence of curvature in the plot of the residuals of $2T_{\parallel}$ versus temperature support the three line segment fit (Figure 3).

SUMMARY

A FORTRAN IV program is described which allows location of transition points in data that can be represented by a set of continuous regression lines. Spline functions are used to initially represent the data and the third derivative of the spline function is examined to predict the location of the transitions. A more rigorous search is then made in the neighborhood of the estimated transition resulting in a least squares solution. An F-statistic for difference between slopes of the two lines at each transition is calculated to aid in interpretation.

The method offers increased efficiency over simple least squares linear regression methods by reducing the number of computations. The program has been used successfully to analyze membrane fluidity and other types of data (1,2,3).

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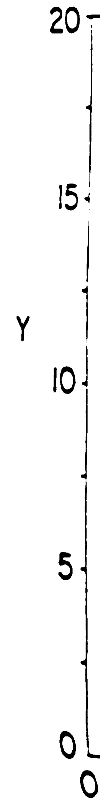

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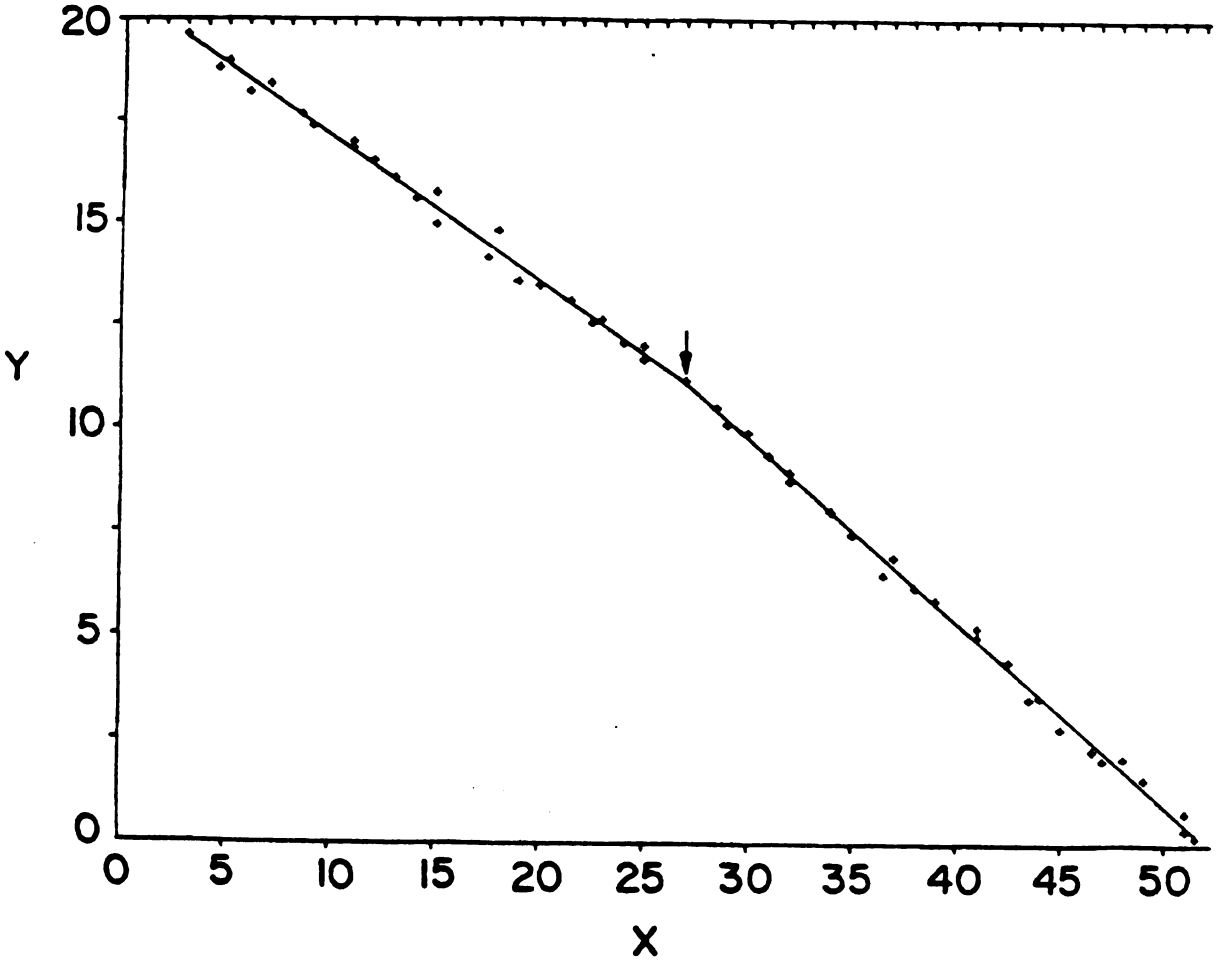

Figure 1. Transition point analysis of data generated from two lines (σ = 0.25) intersecting at x = 27.0. With S = 1.0, KD = 4, NB = 1, and INT = 5 a transition was located at 26.99 (arrow) and accepted at a significance level of $p<0.05$.

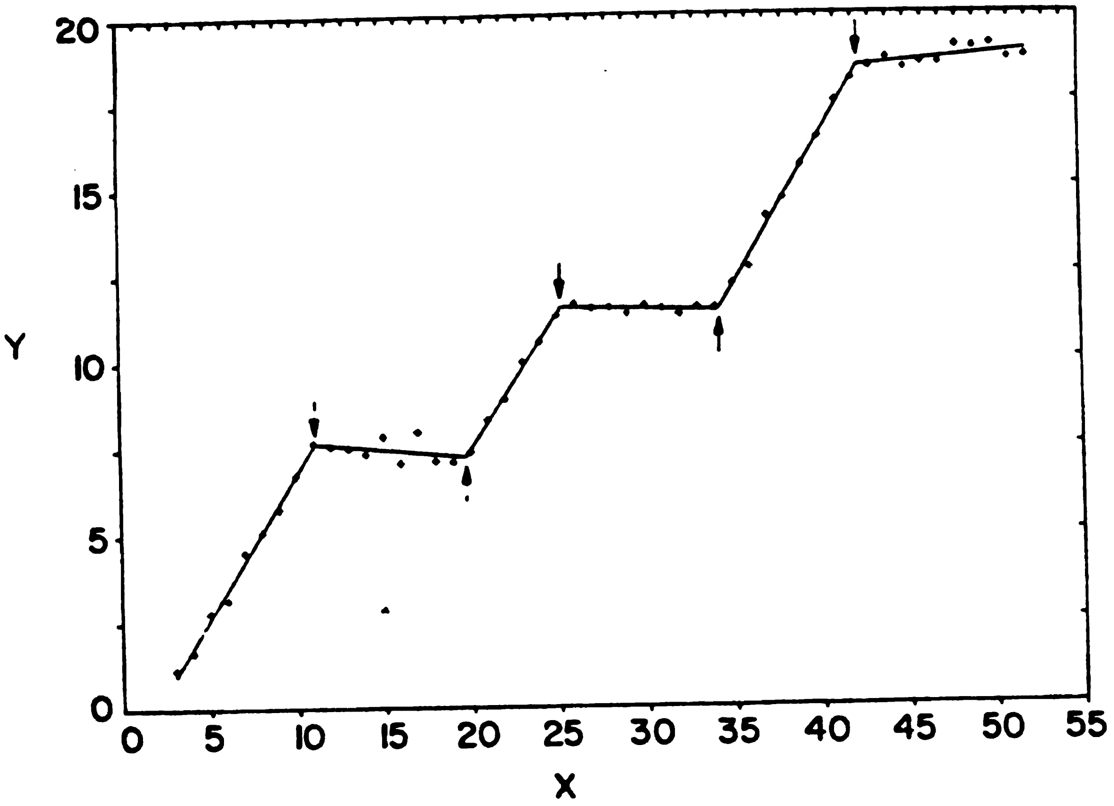

Figure 2. Transition point analysis of six lines (σ = 0.25) intersecting at x = 11.0, 20.0, 25.0, 34.0 and 43.0. With S = 1.0, KD = 4, NB = 5, and INT = 2 transition points were calculated by the program at 11.0, 19.7, 25.5, 32.2, and 42.3. All transitions were significant at $p<0.05$.

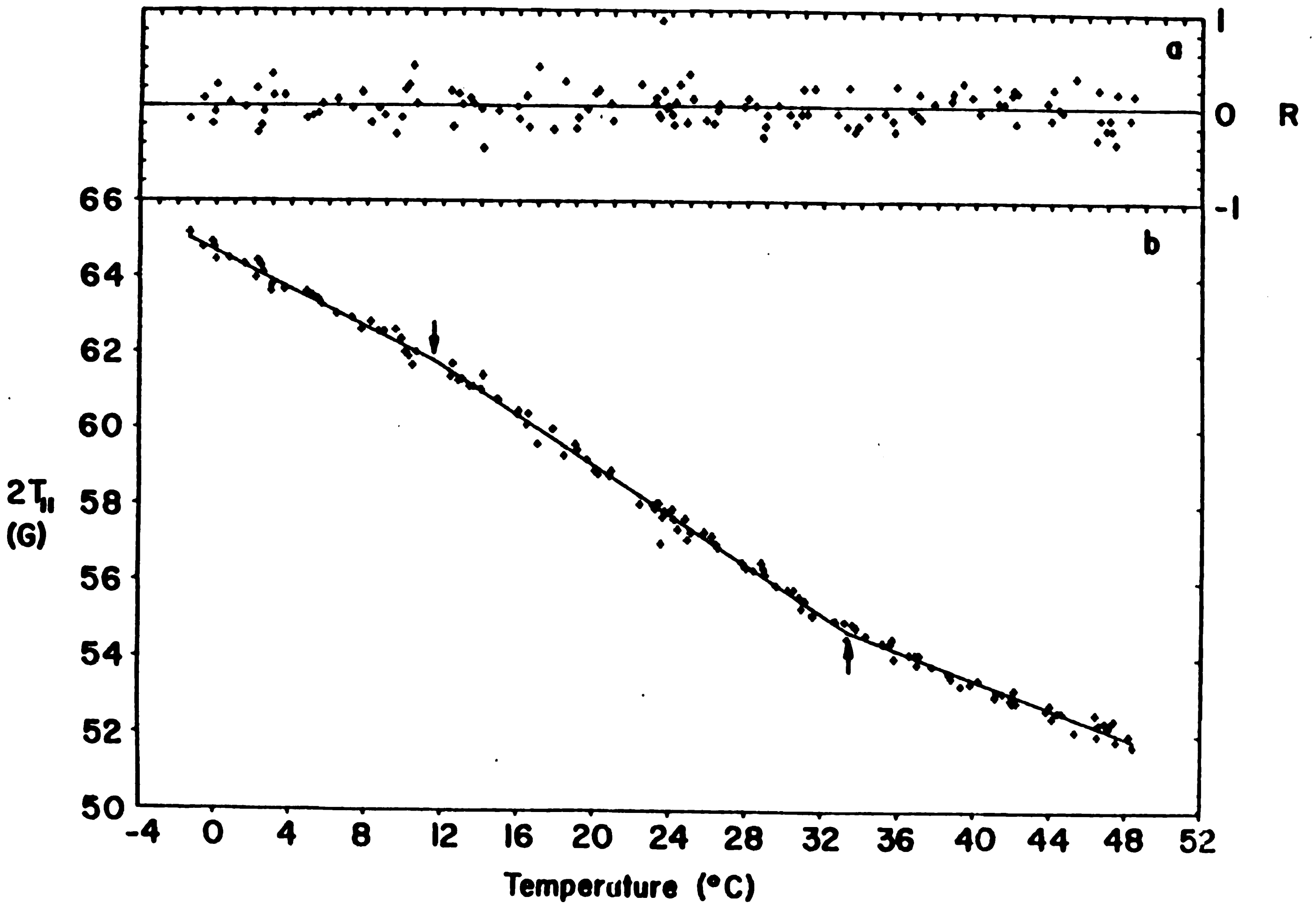

Figure 3. Transition point analysis of human erythrocyte membranes. Membranes were labeled with the electron spin resonance probe 5-doxyl stearate in the presence of 5 x 10^{-5} M chlorpromazine. Transitions in the linear dependence of the spectral parameter $2T_{\parallel}$ versus temperature occurred at 11.5 and 33.5°C. a) A plot of the residuals ($r = Y_{calc} - Y_{obs}$) shows no curvature at these transitions. b) $2T_{\parallel}$ versus temperature of labeled membranes treated with chlorpromazine.

CHAPTER III

A CATION ELECTRON SPIN RESONANCE PROBE USED TO ANALYZE CATION INTERACTIONS WITH LIPOPOLYSACCHARIDE*

SUMMARY

The partitioning of a cationic electron spin resonance probe, 4-(dodecyl dimethyl ammonium)-1-oxy-2,2,6,6,-tetramethyl piperidine bromide, into lipopolysaccharide from Escherichia coli WI485 was shown to increase markedly above approximately 15°C, presumably reflecting a thermal transition. Partitioning was also highly dependent on probe and lipopolysaccharide concentrations, and Scatchard analysis of electrodialyzed lipopolysaccharide revealed a single non-interactive binding site for the probe. Several cations were able to displace probe bound to this site. At concentrations above 30 μM, Ca^{2+} and Mg^{2+} displaced probe bound to electrodialyzed lipopolysaccharide while various polyamines and other cations were less effective. Since this probe is very sensitive to the environment of the lipopolysaccharide, it should prove to be a valuable tool in analyzing lipopolysaccharide structure and interactions with other molecules.

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*Published in Biochem. Biophys. Res. Commun. (1979) 89 (2), 565-570.

INTRODUCTION

The outer membrane of gram negative bacteria consists of a highly asymmetric bilayer. The outer monolayer is comprised of a relatively impermeable lipopolysaccharide (LPS)-protein complex, while the inner monolayer contains phospholipid and protein. The role of LPS or the LPS-protein complex in excluding hydrophobic and amphipathic molecules such as fatty acids, bile salts and certain antibiotics from the intact cell has been studied extensively (1). It has been shown that divalent cations are important in stabilizing the LPS structure. In the intact outer membrane of Escherichia coli two populations of LPS have been described which respond differently to EDTA extraction (2). The LPS population which is readily extracted with EDTA apparently consists of clusters stabilized predominantly by divalent cations.

To further analyze cation-LPS interactions we have utilized the cationic electron spin resonance probe CAT_{12} described by Quintanilha and Packer (3) which partitions into lipid bilayers according to surface charge. We report here that partitioning of CAT_{12} into LPS complexes is sensitive to the LPS thermal transition and to cation-LPS interactions.

MATERIALS AND METHODS

Growth of E. coli strain W1485 was as previously described (4). Cells grown at 37°C were washed three times in distilled water. Hot aqueous phenol was used to extract LPS (5). Extensive dialysis against distilled water yielded the nLPS product. Electrodialysis of the nLPS resulted in an acidic deionized sample which was immediately neutralized to pH 7.0 with NaOH and stored at 4°C. Ketodeoxyoctanoic acid was assayed to quantitate LPS recovery (6). LPS samples were prepared for elemental analysis by wet ashing with concentrated nitric acid (instra-analyzed, Baker Chemical Co.) at 70-75°C for 24 h. Elemental analysis was performed in a Jarrell-Ash 955 Plasma Atomcom plasma emission spectrometer.

The CAT_{12} probe was synthesized according to the procedures of Hubbell, et al. (7). Stock solutions of CAT_{12} were made up at 1 mM concentration in distilled water and were added to LPS samples at room temperature with mild vortexing. All electron spin resonance experiments were carried out with a Varian Century line ESR spectrometer, model E112. An external calibrated thermistor probe monitored the temperature of the sample.

The free probe concentration, [F], was determined by the peak to peak height of the free probe high field signal, corrected with a free probe standard curve. The bound probe concentration, [B], was calculated by subtracting the free probe from the total probe added. The temperature dependent partitioning parameter, Ψ_t, was determined as follows:

$$\Psi_t = - T\log_{10} \frac{[B]/[F]}{C_t}$$

where T is in degrees Kelvin and C_t is the value of [B]/[F] at 310°K. The ionic strength dependent partitioning parameter, Ψ_i, was calculated according to the following equation:

$$\Psi_i = - \log_{10} \frac{[B]/[F]}{C_i}$$

where C_i is the value of [B]/[F] in the absence of added cations. The temperature dependence of CAT_{12} partitioning was analyzed using a linear regression program developed for transition point analysis (8).

RESULTS AND DISCUSSION

Since LPS from E. coli undergoes a broad thermal transition with a midpoint at 22°C (9), it was important to determine whether binding of CAT_{12} to LPS was sensitive to thermally induced structural changes. Figure 1 shows that partitioning of CAT_{12} into nLPS increased dramatically with increasing temperature, apparently reflecting a thermal transition at 15.5 ± 1.5°C. Furthermore, this temperature dependent binding was completely reversible. CAT_{12} binding to edLPS showed a similar temperature dependence but the effect was not as well resolved due to the tighter binding of CAT_{12} to edLPS. Thus, the partitioning of CAT_{12} into LPS is highly dependent on the physical structure of the LPS and is maximal at temperatures above the thermal transition. These results suggest that the barrier function of LPS, i.e., the exclusion of detergent type compounds like the CAT_{12} molecule itself, is greater at temperatures below the thermal transition of the LPS.

To further characterize the CAT_{12} binding site in LPS and to determine the stoichiometry of binding, partitioning was analyzed at 37°C as a function of probe concentration. The Scatchard plot of CAT_{12} binding to edLPS (Figure 2) indicates a single non-cooperative binding site. Saturation occurred at a molar ration of 1:1.5, LPS to CAT_{12}. It has been shown that LPS from E. coli grown under similar conditions as used here contains three negatively charged phosphates attached to the diglucosamine groups in lipid A (10). In addition, the core region of LPS polysaccaride chains contains carboxyl and phosphate groups. The lack of charge neutralization upon binding CAT_{12} may be precluded by the rigidity of the LPS, the presence of tightly bound cations (Table 1), or by other physical constraints.

Table 1. Elemental Analysis of LPS Samples Expressed as Molar Ratios

LPS Preparation	Ca/P	Mg/P	Ca + Mg/LPS[a]
nLPS	0.117	0.434	3.31
edLPS	0.090	0.318	2.45

[a]Assuming 6 moles P/mole LPS.

It has been reported that a high affinity divalent cation binding site exists in edLPS from Salmonella typhimurium (11). This site is reported to occur in equimolar ratios with LPS and is thought to involve carboxyl groups in the LPS core and a phosphate in the lipid A diglucosamine backbone. Table 1 indicates that in E. coli approximately one divalent cation was removed from each LPS molecule upon electrodialysis, consistent with the number of high affinity binding sites reported in edLPS from S. typhimurium. To determine whether

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CAT_{12} is binding near the high affinity cation binding site, Ca^{2+} and Mg^{2+} were titrated at 37°C against edLPS bound with CAT_{12}. Elemental analysis indicated that edLPS initially contained significant levels of these divalent cations (Table 1) consistent with previous reports (12). Figure 3 shows that CAT_{12} was displaced as either ion was added at concentrations above 30 µM. Furthermore, the trivalent cation spermidine was effective in displacing CAT_{12} but only at concentrations above 500 µM. When small cations were added to edLPS at 50 µM concentration, CAT_{12} displacement appeared to depend on the ionic radius (Table 2). Since Yb^{3+} was almost as effective as Ca^{2+}, ionic charge does not appear as critical for binding. When cations were added at high concentrations (1 mM) their efficacy in displacing the probe was

$$\text{Lanthanides}^{3+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$$

consistent with their order in ability to screen charge (13). Neither the trivalent ion spermidine nor the tetravalent ion, spermine, at 1 mM was as effective as Ca^{2+} in displacing probe, and poly-l-arginine and poly-l-lysine each at 1 mM monomeric concentrations were ineffective (data not shown). None of the other cationic molecules tested at 1 mM (l-histidine, l-lysine, l-arginine, l-ornithine, choline and triethanolamine) was able to displace significant levels of CAT_{12} bound to edLPS. The one exception was Tris-HCl, pH 7.5, which, at 1 mM, displaced significant amounts of probe (data not shown).

Table 2.

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Table 2. Displacement of CAT_{12} from edLPS by 50 μM Salt

Salt	Ψ_i	Cation Radius A
Control	0.0	--
$SrCl_2$	-0.043	1.13
$LaCl_3$	0.004	1.15
$MgCl_2$	0.056*	0.65
$YbCl_3$	0.101*	0.94
$CaCl_2$	0.111*	0.99

*Different from control. $p<0.05$.

The results reported here indicate that CAT_{12} interacts with edLPS through a single type of non-cooperative binding site and that this site may involve the high affinity divalent cation site of interaction. Furthermore, binding of the probe is sensitive to the structural change induced by the LPS thermal transition and to competitive ion interactions. In preliminary studies (manuscript in preparation) we have shown that CAT_{12} can partition into outer membranes of E. coli, and it appears that this probe will be useful in further analyzing LPS-protein interactions.

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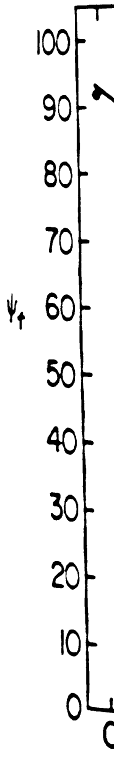

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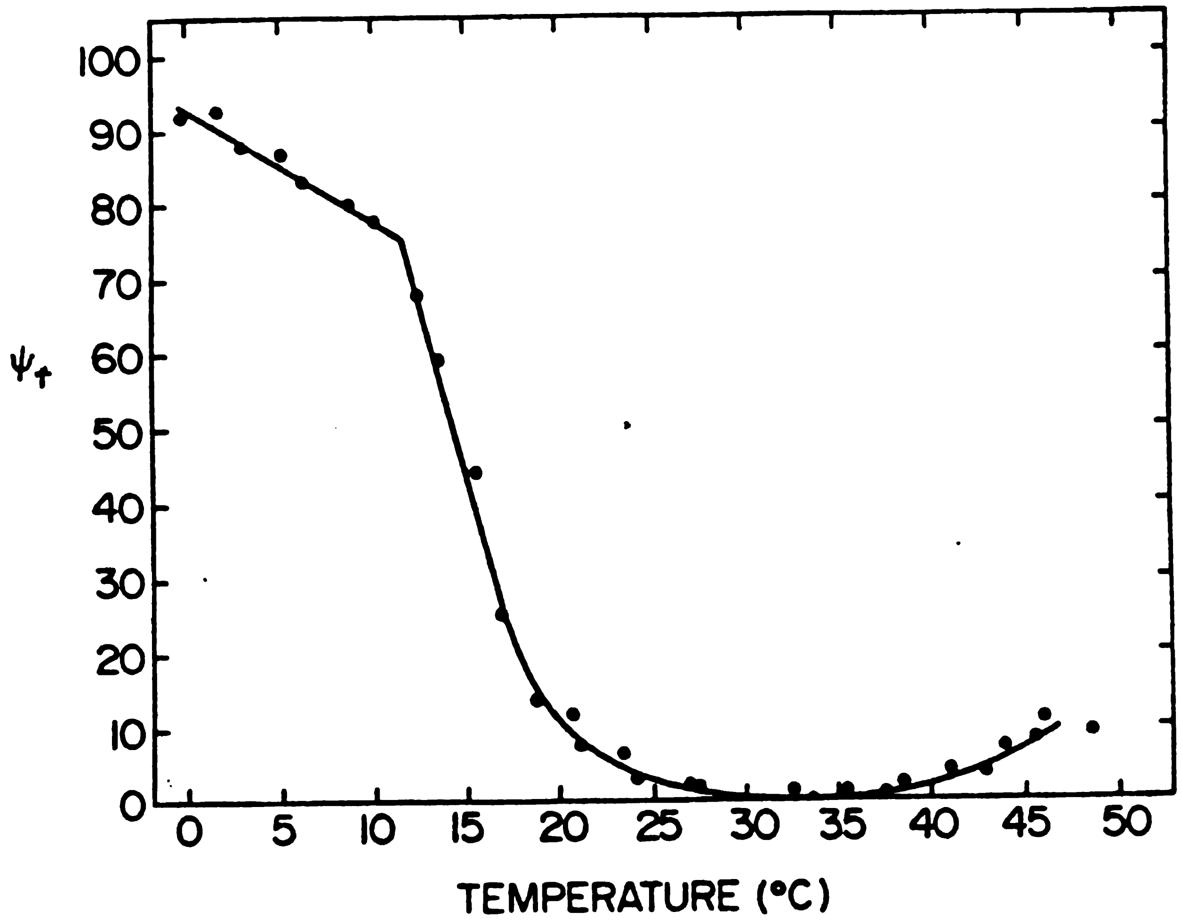

Figure 1. Partitioning of CAT_{12} into nLPS as a function of increasing temperature. The CAT_{12} to nLPS molar ratio was 0.1.

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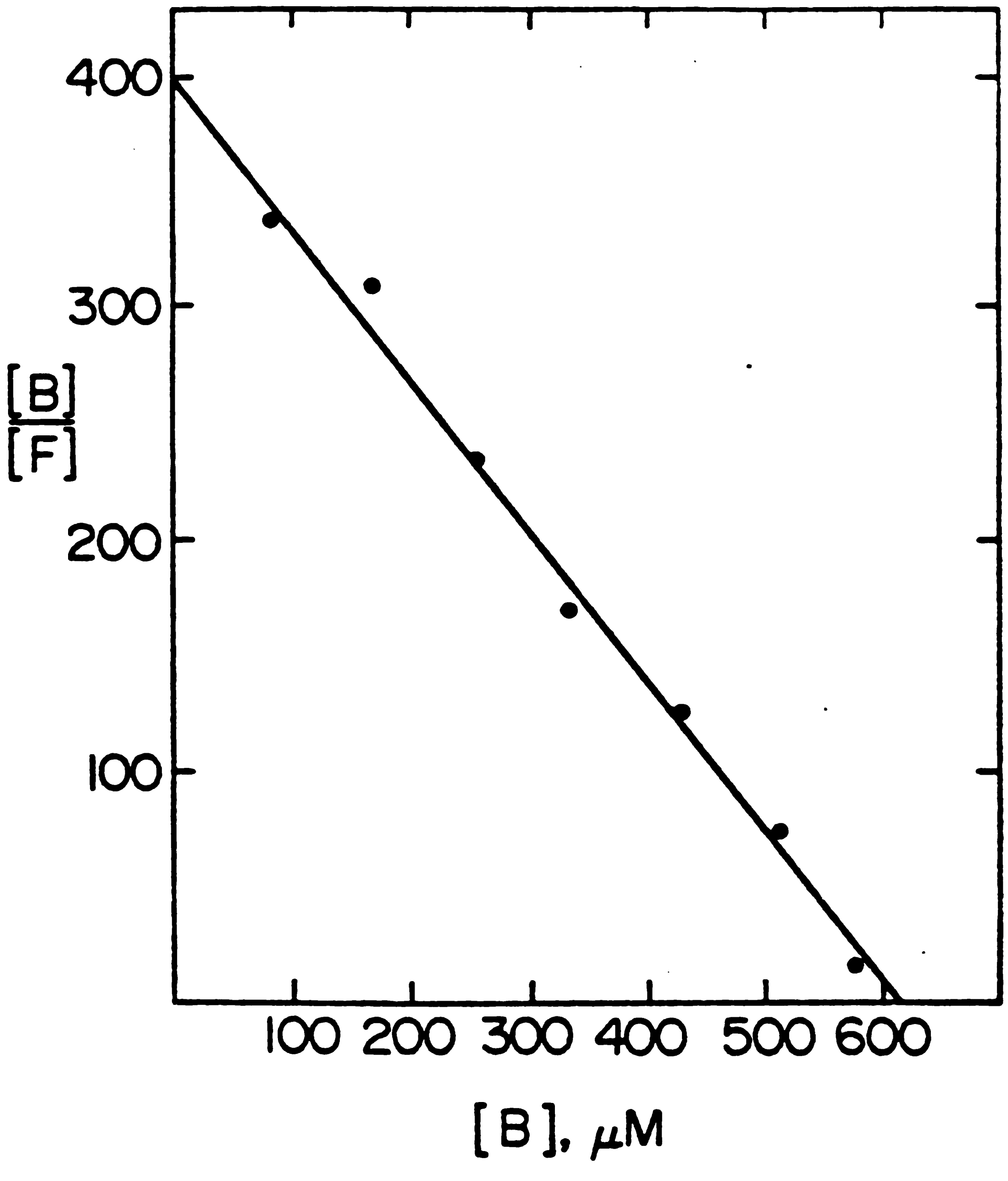
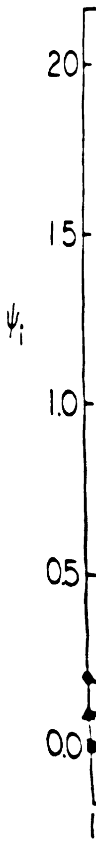


Figure 2. Scatchard analysis of CAT_{12} binding to edLPS. CAT_{12} partitioning was measured at 37°C on samples of 500 μM edLPS as a function of probe concentration.



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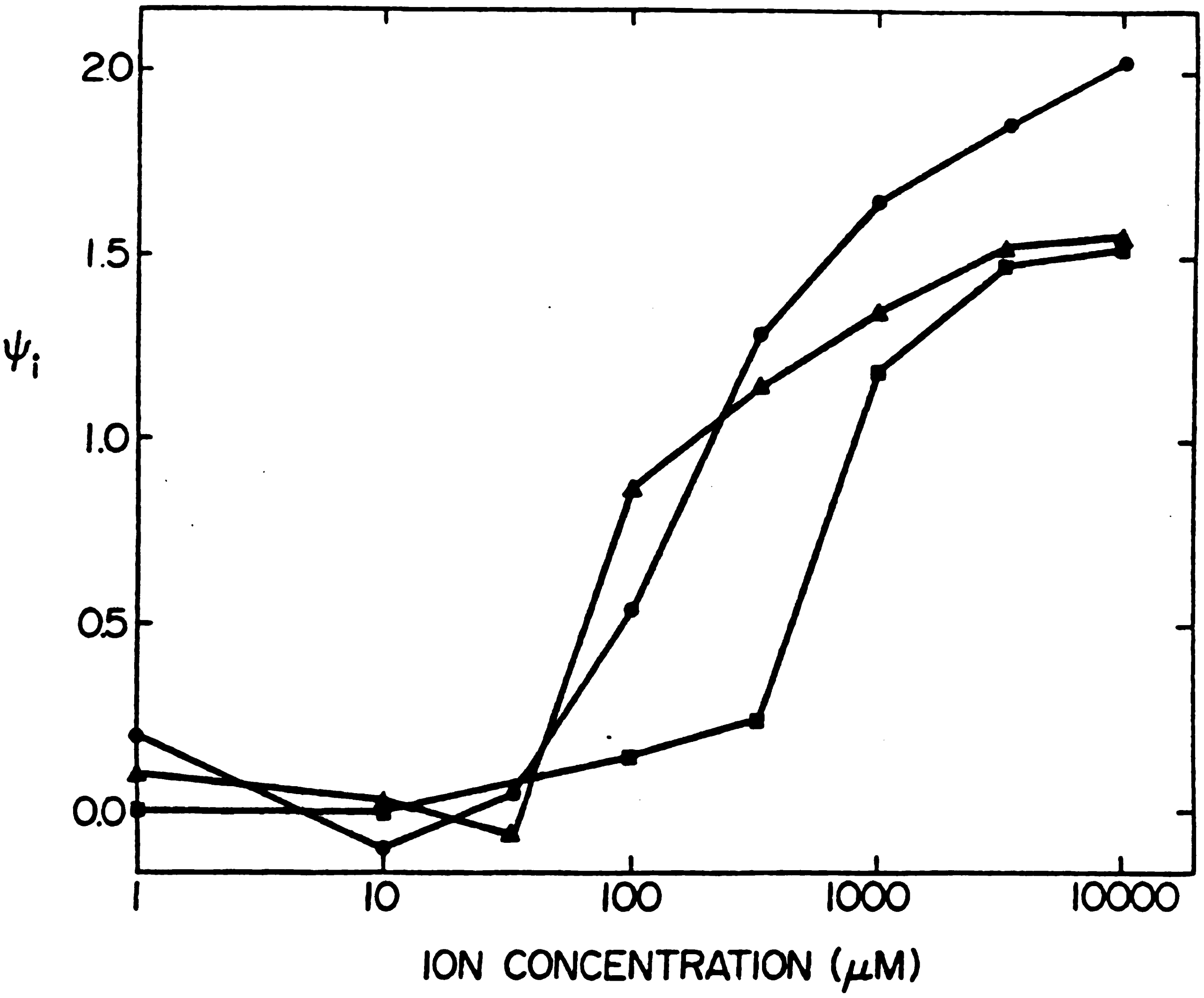

Figure 3. Displacement of CAT_{12} from edLPS was measured at 37°C as a function of Ca^{2+} (●), Mg^{2+}, (▲), and spermidine (■) concentrations. The CAT_{12} to edLPS molar ratio was 0.1.

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CHAPTER IV

QUANTITATION OF METAL CATIONS BOUND TO MEMBRANES AND EXTRACTED LIPOPOLYSACCHARIDE OF ESCHERICHIA COLI

ABSTRACT

Inductively coupled plasma emission spectroscopy was used to quantitate the metal cations bound to outer and cytoplasmic membranes and to extracted lipopolysaccharide from several _Escherichia coli_ k12 strains. The outer membrane was found to be enriched in both calcium and magnesium relative to the cytoplasmic membrane. Both membranes contained significant levels of iron, aluminum, and zinc. The multivalent cation content of lipopolysaccharide resembled that of the intact outer membrane. Lipopolysaccharide extracted from wild type k12 strains contained higher levels of Mg than Ca regardless of the growth medium, but the medium used for growth did effect the relative amounts of bound Mg as well as the levels of the minor cations, iron, aluminum and zinc. In contrast, lipopolysaccharide isolated from a deep rough mutant strain, D21f2, contained more Ca than Mg. Electrodialysis of lipopolysaccharide from wild type k12 strains removed one mole of Mg per mole of lipopolysaccharide but did not significantly affect the level of other bound metal ions. Dialysis of lipopolysaccharide against sodium (ethylenedinitrilo)tetraacetate removed most of the Mg and Ca resulting

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in a sodium salt. The molar content of Na bound to the sodium salt was similar to the level of divalent cations removed, but the monovalent cation neutralized less of the anionic charge on the lipopolysaccharide. The sodium salt was dialyzed against either Tris(hydroxymethyl)aminomethane·HCl, $CaCl_2$, $MgCl_2$, $FeCl_2$, or $TbCl_3$, and the resulting lipopolysaccharide salts were analyzed for their ionic composition. It was shown that Tris(hydroxymethyl)aminomethane and Ca can replace some but not all of the Na bound to the sodium salt, but it appears that all of the other multivalent cations can nearly completely replace Na, resulting in uniform lipopolysaccharide salts. Lipopolysaccharide isolated from the deep rough mutant strain, D21f2, was also converted into a sodium salt. Relative to the wild type LPS, Na was able to neutralize the anionic charge to a greater extent in the mutant lipopolysaccharide. These results suggest that the loss of specific groups in the core region of the lipopolysaccharide from the mutant strain results in a more open structure that allows the binding of larger cations and of more monovalent cations.

INTRODUCTION

Divalent cations play an important role in the stabilization of the outer membrane of gram negative bacteria. In addition to reducing charge repulsion between the highly anionic lipopolysaccharide (LPS) molecules, divalent cations are thought to bridge adjacent LPS molecules and to link LPS with membrane proteins (1). The formation of complexes of outer membrane protein and LPS requires divalent cations such as magnesium

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(2). Chelating agents such as (ethylenedinitrilo)tetraacetic acid (EDTA) effect the release of up to 50% of the LPS from whole cells (3).

The physiochemical and pathophysiological properties of isolated LPS are also influenced by the ions bound. The solubility and morphology of LPS aggregrates vary greatly depending on the salt form (4). As a result, the anticomplement activity of the soluble sodium salt of LPS from Salmonella abortus is much greater than that of the highly aggregated triethylamine salt (5). It has also been observed that mice which have been injected with iron salts prior to LPS administration are resistant to endotoxin stress (6). Iron salts of LPS generally are only slightly soluble and have lower toxicity (7).

The tenacity with which LPS binds cations has been previously noted (8). Even after extensive dialysis LPS remains complexed with a variety of cations. Although monovalent cations and several polyamines may be removed by electrodialysis, most of the tightly associated divalent cations remain bound (4,9). In this study we have quantitated the levels of metal cations bound to isolated membranes and extracted LPS in an attempt to better characterize cation-LPS interactions.

MATERIALS AND METHODS

Growth conditions and procedures for membrane and LPS isolation .

Escherichia coli k12 strains W1485 F$^-$, D21, and D21f2 were grown at 37°C in either M9 minimal medium plus 0.4% glucose or in nutrient broth (1% tryptone, 0.2% yeast extract, 0.4% NaCl). Outer and cytoplasmic membranes were isolated (10), and the purity of the isolates equalled or exceeded that described earlier. LPS was isolated using

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either hot aqueous phenol (11) or chloroform-petroleum ether (12) extraction procedures. Extensive dialysis against double distilled water yielded the native LPS (nLPS) product. Electrodialysis of the nLPS resulted in an acidic product which was analyzed in the acidic form (edLPS). The sodium salt of LPS, NaLPS, was obtained by dialysis of edLPS against three to five changes of 10 mM NaEDTA, pH 7.0 at 4°C, followed by extensive dialysis against double distilled water. Other salt forms were obtained by dialysis of the sodium salt against 10 mM TRIS•HCl, $CaCl_2$, $MgCl_2$, $TbCl_3$, or $FeCl_3$, followed by extensive dialysis against double distilled water.

Porin-LPS-peptidoglycan complexes were isolated from cells grown in nutrient broth and harvested as previously described (10). Cells were washed in 10 mM HEPES, pH 7.5, and lysed using a French pressure cell. Deoxyribonuclease I (Sigma Chemical Co.) was added at 20 µg/ml, and total membranes were pelleted and washed twice with distilled water. The porin-LPS-peptidoglycan complex was isolated by extracting the total membrane fraction with 1% SDS (w/v) twice and washing the complex extensively with distilled water.

Assays

3-deoxy-D-manno-octulosonic acid (KDO) was assayed using the method of Droge, et al. (13) to quantitate LPS recovery. Protein concentrations were determined using the procedure of Lowry et al. (14). Membrane proteins were characterized on SDS polyacrylamide slab gels as previously described (10). Peptidoglycan was quantitated by assaying the levels of muramic acid (15).

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Wet ashing was accomplished by combining equal volumes of sample and nitric acid (Ultra R, Atomergic Chemicals Corp.) in a 15 ml teflon screw cap vial (Tuf-tainer, Pierce Chemical Co.). Samples were incubated at 70 to 75°C for 24 hours. Cobalt was added to give a final concentration of 1 μg/ml, and ashed samples were diluted to give a 15% (v/v) acid solution.

Dry ashing was accomplished by transferring the sample to a quartz crucible, lyophylizing, and then heating in a muffle furnace. The samples were ashed at 500°C for 24 hours. After dilution with water, the samples were filtered through acid washed glass wool and analyzed with cobalt as an internal reference standard.

Samples of nLPS and edLPS were prepared for elemental analysis by both wet and dry ashing procedures. For convenience, most elemental analyses were carried out on wet ashed material, since analyses of dry ashed material yielded virtually identical results.

Elemental analysis

Quantitative elemental analysis was accomplished by plasma emission spectroscopy using a Jarrell-Ash Model 955 Atomcomp Spectrometer with an N + 1 variable wavelength accessory. The observation wavelengths (nm) for the specific elements were set as follows: Ca, 370.6; Mg, 279.0; P, 214.9; Na, 330.3; K, 766.5; Fe, 259.9; Al, 308.2; Zn, 213.8; Co, 228.6; Tb, 350.9. Potassium and terbium were quantitated on the N + 1 channel. The area around each emission line was examined for spectral interference and the background effects by dynamic profiling. Profiles were obtained by manual micrometer adjustment of the entrance slit position. To

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RESULTS

Membrane ionic composition

Since multivalent cations are reported to be critical in stabilizing the structure of the outer membrane in gram negative bacteria, the levels of ions bound to outer and cytoplasmic membranes of E. coli were characterized and compared. The outer membrane contained three times the levels of divalent cations on a per phosphorus basis compared to the cytoplasmic membrane (Table 1). Furthermore, both membranes were shown to contain small but significant levels of iron, aluminum and zinc. The high levels of divalent cations bound to outer membranes presumably result from the presence of anionic LPS in the outer monolayer, and from the underlying anionic peptidoglycan. Note that the multivalent ion composition of nLPS was similar to that of the intact outer membrane (Table 2).

A porin-LPS-peptidoglycan complex was purified and characterized to determine whether certain cations are involved in the interaction of outer membrane proteins with other membrane structures. This complex was

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shown to contain approximately 70% protein, 16% LPS and between 7 and 15% peptidoglycan by weight. SDS polyacrylamide gels showed that the protein in the complex was comprised of approximately equal amounts of porins 1a and 1b with only small amounts of contaminating protein (data not shown). As indicated in Table 3, this complex was enriched in divalent and monovalent cations compared to nLPS. Specifically the Mg content of the complex was nearly twice the amount found in the purified LPS and in the intact outer membrane. However, it could not be determined from these data whether the excess cationic charge was bound to protein, to the anionic mucopeptide layer or between the anionic groups within the complex.

LPS ionic composition

LPS isolated from *E. coli* strain W1485 F^- using either hot aqueous phenol or chloroform-petroleum ether methods resulted in nLPS preparations which had essentially identical ionic compositions. The nLPS isolated under these conditions contained between 7 and 8 moles phosphorus per 3 moles KDO (Table 2) consistent with the findings of Jansson *et al*. (16). Figure 1 indicates the likely position of the phosphate groups in the LPS structure. The ionic composition analysis of nLPS from cells grown in nutrient broth indicated that approximately five metal cations were bound to each LPS molecule with four of these being either calcium or magnesium. In an attempt to remove weakly bound cations including organic amines, the LPS was electrodialyzed, and the ionic composition of the acidic form (pH 3.5 to 4.0) is given in Table 2. Electrodialysis consistently removed one mole of Mg per mole LPS while the levels of all other metal cations were unaffected. The results

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suggest that the native form of LPS from E. coli cultures grown in nutrient medium contains 1 mole calcium and 3 moles magnesium per mole LPS, and one of the magnesium ions is removed with electrodialysis. Furthermore, this LPS contains one mole total of iron, aluminum, and zinc per three moles of LPS, and these ions are not removed with electrodialysis.

Effects of growth media on LPS ionic composition

To assess whether the growth medium influences the ionic content of LPS, cells were also grown in M9 minimal medium containing 0.4% glucose, and the LPS was extracted and analyzed. The levels of metal cations present in the extracted LPS from cells grown in the two media are shown in Table 2. The differences in the ionic content of the two LPS isolates reflect to varying extents the differences in the ionic and phosphorus content of the two media. From elemental analysis, the minimal medium was found to be higher in Mg, Na, K and P content compared to the nutrient broth, whereas the Ca concentrations were similar (data not shown). Thus LPS isolated from cells grown in minimal medium contained higher levels of Mg and is higher in the level of total divalent cations bound per phosphorus compared to LPS isolated from cells grown in nutrient broth. This difference in divalent cation content may reflect the different phosphate content in the two LPS isolates. The phosphorus levels in the two media were dramatically different, and it has been reported that the level of phosphorylation of LPS is sensitive to the level of phosphate in the growth media (17).

LPS salt preparation and characterization

Dialysis of electrodialyzed LPS against NaEDTA, pH 7.0, resulted in replacement of essentially all of the divalent cations with sodium (Table 4). Extensive dialysis of the sodium salt with 10 mM TRIS·HCl, pH 7.0, decreased the level of bound Na from 5 Na/LPS to approximately 1 Na/LPS (mole/mole). Thus it appears that the TRIS cation was unable to replace all of the Na. The Na and TRIS salts contained similar levels of Fe, Al and Zn. They both formed clear pellets upon centrifugation, and the resuspended samples were very soluble and almost water clear.

The calcium and magnesium LPS salts were slightly opalescent in solution and upon sedimentation formed clear to opalescent pellets. Elemental analysis of the CaLPS indicated that although the calcium bound was equivalent in bound cationic charge to that in the nLPS, the CaLPS still contained one to two sodium per LPS even after extensive dialysis (Table 4). In contrast, Mg completely replaced sodium, and the total cationic charge bound as Mg was greater than that seen in the nLPS.

The TbLPS appeared as a white gel upon sedimentation but readily resuspended in water. As shown in Table 4, Tb replaced nearly all the Na as well as a significant amount of the Fe. The amount of cationic charge bound in the terbium salt was somewhat less than that bound to the MgLPS but similar to that detected in the nLPS. The ferric salt of LPS formed an orange gel which was not as soluble as the other salts but which could be dispersed by sonication. Elemental analysis of FeLPS indicated that Fe replaced all of the sodium as well as most of the other trace cations (Table 4). The level of Fe recovered in this salt, however, was exceedingly high, probably due to the formation of insoluble iron

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Ionic content of LPS from a heptoseless mutant

LPS was isolated from *E. coli* strain D21f2. LPS from this mutant strain lacks the carbohydrate groups distal to the KDO units (see Figure 1). Extraction of the LPS with chloroform-petroleum ether, followed by electrodialysis of the sample resulted in an acidic edLPS which was analyzed for the levels of metal cations bound. As shown in Table 5, the mutant LPS contained more Ca than Mg in the electrodialyzed form. Treatment of this heptoseless LPS with 10 mM NaEDTA, pH 7.0, resulted in nearly total replacement of the divalent cations with Na. Analysis of the levels of KDO/P in the LPS of this mutant indicated that this molecule contained approximately 4 phosphate groups. These results imply that the sodium salt of the mutant LPS has bound approximately 8 metal cationic charges, approximately 1.5 more cationic charges than were detected bound to the acidic electrodialyzed form. NaLPS from the mutant strain D21f2 also retained significant levels of iron, aluminum, and zinc.

DISCUSSION

The outer and cytoplasmic membranes of gram negative bacteria differ significantly in their composition and function. The results presented here indicate that within the envelope of the gram negative bacteria, the outer membrane is enriched in divalent cations (Table 1). The anionic LPS on the outer monolayer of this membrane and the underlying anionic

peptidoglycan layer provide multiple sites for cation interaction. Previous evidence suggests that porin proteins in the outer membrane associate with the peptidoglycan through divalent cation bridges (19). We found that the porin-LPS-peptidoglycan complex is enriched in Mg compared to LPS or to the intact outer membrane (Tables 1 and 2). Presumably either porin-LPS or porin-peptidoglycan complexes or the porin proteins themselves contain Mg binding sites.

Divalent cations also appear to be critical in stabilizing the structure of pure LPS domains within the outer membrane. The major metal cations detected in nLPS were Mg and Ca (Table 2). The level of ions recovered in extracted LPS was dependent on the medium in which the cells were grown. The different levels of magnesium recovered in nLPS isolated from cells grown in the two media used in this study could be the result of differences in the LPS structure. Conversely, the varying levels of bound metal cations may result from differences in the levels of organic polyamines bound to LPS. Polyamines such as spermidine and spermine may be binding to and neutralizing part of the anionic charge on nLPS, and the levels of polyamines present may be dependent on the growth medium.

Regardless of the growth medium used, electrodialysis of the extracted nLPS resulted in the removal of approximately one Mg per LPS (Table 2). This Mg binding site in the LPS is likely to reside within the KDO groups in the core polysaccharide region (see Figure 1). Electrodialysis results in a drop of the pH of the sample to approximately 4. The partial neutralization of the carboxyl group of the KDO would then allow release of the bound cation. Previous studies have shown that either Ca or Mg can readily bind to this site after neutralizing the edLPS to pH 7.0 with NaOH (19). Electrodialysis may also remove spermine and spermidine bound to nLPS (4) although the removal of polyamines cannot be detected directly by elemental analysis.

Our elemental analysis of metal cations associated with LPS differs from that reported by Galanos and Luderitz (4) for LPS isolated from several Salmonella strains. First, we detected relatively low levels of monovalent cations in nLPS from E. coli whereas Galanos and Luderitz detected relatively high levels. This discrepancy may reflect differences in the structure of the cation binding sites in the LPS of these two organisms. Otherwise, the low monovalent ion content in our nLPS may be the result of extensive dialysis against double distilled water prior to elemental analysis. It should be noted that Galanos and Luderitz described uniform salts prepared from edLPS which was neutralized with an appropriately chosen base. Although LPS prepared in this manner was shown to have dramatically different endotoxin activity (5), it is clear from our results that these LPS samples were not uniform salts but were heterogeneous with respect to their associated cations. Secondly, we found significant levels of iron, aluminum and zinc tightly bound to nLPS and edLPS. The presence of these ions represents a major unreported component in LPS. We believe the presence of these ions is not the result of contamination of LPS during isolation since the intact outer membrane isolated using entirely different procedures contained similar levels.

Considering the profound influence iron has on the pathology of LPS, the detection of iron bound to LPS may be of considerable importance. It has been proposed that for some gram negative organisms, the ability of cells to compete for iron within a host correlates with the virulence of the strain (20). In addition, it has been shown that LPS when injected

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into animals can induce hypoferremia as if the LPS were removing iron from the serum (21,22).

Since the ferric ion has an exceedingly low solubility in water, bacteria such as E. coli produce and excrete several low molecular weight chelators called siderophores which can complex with Fe (III) ions. The iron-siderophore complex is then taken up by the cell utilizing specific membrane receptor proteins (23). These siderophores have very high affinities for ferric ions; enterochelin, for instance, has an affinity constant for Fe^{+3} of $10^{52}M^{-1}$. With such potent ferric iron uptake systems, it is somewhat surprising to find high levels of iron bound to LPS. It has been observed, however, that when iron starved cultures of E. coli were given LPS isolated from cells grown in complete medium, growth resumed, suggesting that iron was associated with the added LPS (24). The ferric iron bound to LPS may thus serve as an iron reservoir accessible to the siderophore-receptor system.

The presence of aluminum in LPS and outer membranes may be fortuitous. However, at neutral pH, aluminum hydroxylate complexes present in the growth medium would contain a net positive charge. Such complexes have been shown to bind tenaciously to negatively charged surfaces (25) and can dramatically alter membrane physical properties (26). Aluminum is potentially very toxic for the cell (27) and has been shown to inhibit enzymes such as Na,K-ATPase by binding to the substrate, ATP. Binding of aluminum to LPS on the cell surface may serve to sequester the toxic ion and prevent it from entering the cell.

The LPS binding capacity for zinc may also be critical in the pathology of this endotoxin. Zinc has been shown to reduce the lethality of endotoxin-challenged mice (28). Protection was maximized when mice

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were pretreated with zinc. It has also been observed that intraperitoneally administered zinc chloride prevented endotoxin-induced hyperaminoaridemia and the elevation of plasma transaminase levels (29). Thus, the ability of LPS to bind these transition metals may be important in pathogen-host reactions as well as for the bacterial cell physiology. The only other metal reported to be bound to LPS is copper. Sourek (30) found that Shigella dysenteriae LPS isolated using the hot aqueous phenol method was contaminated with copper. We have found no significant levels of copper or of manganese, molybdenum, arsenic, cadmium, chromium, mercury, lead, selenium, thallium or cobalt in any of our LPS isolates.

We have shown that the divalent cations bound to LPS can be removed and defined salts can be formed. The NaLPS contained lower cationic charges bound per phosphorus than were detected in nLPS (Tables 2 and 4). The lack of equivalent charge neutralization with Na likely results in a LPS complex with a higher net negative charge compared to nLPS. This conclusion is supported by studies of the LPS head group mobility, as discussed in the following paper. Upon formation of the TRIS salt, all but approximately one Na per LPS molecule was displaced. The number of TRIS cations bound could not be determined in this study, but the overall anionic charge of the TRIS salt of LPS is likely to be equal to or greater than that of the sodium salt. Presumably the larger TRIS cation cannot associate with more anionic sites than can Na.

The calcium salt also contained between one and two Na. The reason that Ca was unable to completely displace Na in the LPS is not clear. The ionic radii of Ca^{2+} and Na^{+} are nearly the same. Perhaps dialysis was not extensive enough for complete exchange. However, Mg which is substantially smaller, was able to replace all of the Na

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following dialysis of NaLPS with $MgCl_2$ (Table 4). Calcium and magnesium may interact with the anionic sites differently since their ability of form chelation complexes is different. Both the magnesium and calcium salts of LPS from the k12 strain had higher levels of cationic charge bound than was detected in nLPS (Table 2 and 4). This difference may result from the presence of undetected polyamines in nLPS which were removed upon formation of the defined salts.

Of the trivalent salts which we formed, the terbium salt contained bound cationic charge at levels significantly lower than that in the divalent salt forms (Table 4). The Tb^{3+} ion is close in ionic radius to Ca^{2+} and may be excluded from one or more sites which are specific for small ions. In contrast, the smaller ferric cation replaced essentially all of the other detectable cations bound to NaLPS. The FeLPS complex was relatively insoluble, due perhaps to the presence of insoluble ferric hydroxylates trapped within the FeLPS aggregates. The exceedingly high levels of iron detected in FeLPS is consistent with this interpretation.

Thus, the nLPS from k12 wild type strains preferentially binds cations of small ionic radius. Furthermore, at least some of the cation binding sites in this LPS are unable to accomodate two monovalent cations to neutralize the charge originally balanced by one divalent ion. In contrast, LPS from the heptoseless mutant strain, D21f2, appeared to have a more "open" structure which could accommodate the binding of larger cations. We have shown that the edLPS from this strain contains approximately two Ca and only one Mg per LPS. Furthermore, the Na salt of this mutant LPS appeared to neutralize more of its anionic charge with Na than was seen in the NaLPS from the parent D21 strain (Tables 4 and

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One structural difference in the mutant LPS which may account for the binding of larger cations and more monovalent cations is the absence of ethanolamine groups (Figure 1). The presence of covalently bound amines in the LPS of the k12 strain could allow crossbridging to anionic groups within and between LPS molecules, rigidifying and restricting other cationic interactions.

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Table

Element/Ph (Molar
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Ca + Mg/P
Fe/P
Al/P
Zn/P

Table 1 Elemental Composition of Membranes from *E. coli* Strain W1485F Grown in Minimal Media

Element/Phosphorous (Molar Ratio)	Cytoplasmic Membrane (4 Isolates)	Outer Membrane (4 Isolates)
Ca/P	0.03 ± 0.01	0.09 ± 0.03
Mg/P	0.15 ± 0.04	0.45 ± 0.04
Ca + Mg/P	0.18 ± 0.05	0.54 ± 0.07
Fe/P	0.006 ± 0.003	0.009 ± 0.005
Al/P	0.006 ± 0.004	0.009 ± 0.002
Zn/P	0.0014 ± 0.0005	0.0006 ± 0.0002

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Table 1 Elemental Composition of Membranes from *E. coli* Strain W1485F Grown in Minimal Media

Element/Phosphorous (Molar Ratio)	Cytoplasmic Membrane (4 Isolates)	Outer Membrane (4 Isolates)
Ca/P	0.03 ± 0.01	0.09 ± 0.03
Mg/P	0.15 ± 0.04	0.45 ± 0.04
Ca + Mg/P	0.18 ± 0.05	0.54 ± 0.07
Fe/P	0.006 ± 0.003	0.009 ± 0.005
Al/P	0.006 ± 0.004	0.009 ± 0.002
Zn/P	0.0014 ± 0.0005	0.0006 ± 0.0002

Table 2 Elemental Composition of LPS from _E. coli_ Strain W1485F
Grown in Either Nutrient or Minimal Medium

Table 2 Elemental Composition of LPS from *E. coli* Strain W1485F Grown in Either Nutrient or Minimal Medium

Element/Phosphorous	Nutrient Medium		M9 Glucose Minimal Medium	
(Molar Ratio)	Native LPS	Electrodialyzed LPS	Native LPS	Electrodialyzed LPS
Ca/P	0.116 ± 0.003	0.135 ± 0.004	0.12	0.12 ± 0.02
Mg/P	0.33 ± 0.01	0.21 ± 0.03	0.43	0.30 ± 0.02
Ca+Mg/P	0.45 ± 0.01	0.35 ± 0.03	0.55	0.42 ± 0.04
Na/P	0.09 ± 0.05	0.04 ± 0.04	0.085	0.06 ± 0.02
Fe/P	0.04 ± 0.01	0.02 ± 0.01	0.01	0.02 ± 0.01
Al/P	0.01 ± 0.01	0.012 ± 0.001	0.02	0.01 ± 0.01
Zn/P	0.01 ± 0.00	0.012 ± 0.001	0.002	0.004 ± 0.001
+/P	1.11	0.85	1.28	1.00
P/3 KDP	7.3 ± 0.7	7.7 ± 0.4	N.D.	N.D.

Table 3 Elemental Composition of LPS and of Porin-LPS-Peptidoglycan Complexes from *E. coli* Strain D21 Grown in Nutrient Broth

Element/Phosphorous (Molar Ratio)	Porin-LPS-Peptidoglycan Complex	Native LPS
Ca/P	0.12 ± 0.01	0.116 ± 0.003
Mg/P	0.61 ± 0.04	0.33 ± 0.01
Ca+Mg/P	0.73 ± 0.05	0.45 ± 0.01
Na/P	0.65 ± 0.08	0.09 ± 0.05
Fe/P	0.07 ± 0.05	0.04 ± 0.01
Al/P	0.02 ± 0.01	0.01 ± 0.01
Zn/P	0.010 ± 0.004	0.01 ± 0.00

Table 4 Elemental Composition of Specific Salts of LPS from *E. coli* Strain D21 Grown in Nutrient Media

Element/Phosphorous (Molar Ratio)	Na-LPS (3 Isolates)	Tris-LPS (3 Isolates)	Ca-LPS (3 Isolates)	Mg-LPS (3 Isolates)	Tb-LPS (3 Isolates)	Fe-LPS (3 Isolates)
Ca/P	0.0055 ± 0.0006	0.03 ± 0.01	0.56 ± 0.04	0.002 ± 0.001	0.0005 ± 0.0007	0.008 ± 0.001
Mg/P	0.054 ± 0.008	0.05 ± 0.011	0.009 ± 0.005	0.78 ± 0.01	0.015 ± 0.004	0
Ca+Mg/P	0.060 ± 0.009	0.08 ± 0.02	0.57 ± 0.05	0.78 ± 0.01	0.015 ± 0.005	0.008 ± 0.001
Na/P	0.54 ± 0.03	0.17 ± 0.05	0.2 ± 0.1	0.07 ± 0.04	0.06 ± 0.04	0
Fe/P	0.015 ± 0.003	0.05 ± 0.04	0.027 ± 0.006	0.003 ± 0.001	0.008 ± 0.004	2.5 ± 0.3
Al/P	0.011 ± 0.005	0.01 ± 0.01	0.015 ± 0.005	0.002 ± 0.001	0.09 ± 0.02	0.097 ± 0.004
Zn/P	0.0003 ± 0.0003	0.001 ± 0.001	0.005 ± 0.003	0	0.001 ± 0.001	0.0011 ± 0.0001
Tb/P	N.D.	N.D.	N.D.	N.D.	0.28 ± 0.01	N.D.
Total + /P	0.74	0.52	1.50	1.66	1.24	N.D.
P/3 KDO	9.9 ± 1.1	N.D.	N.D.	8.7 ± 0.5	N.D.	N.D.

Table 5 Elemental Composition of LPS from *E. coli* Strain D21f2 Grown in Nutrient Media

Element/Phosphorous (Molar Ratio	Electrodialyzed-LPS pH 4.0 (3 Isolates)	Na-LPS (3 Isolates)
Ca/P	0.46 ± 0.05	0.019 ± 0.004
Mg/P	0.18 ± 0.02	0.014 ± 0.001
Ca+Mg/P	0.64 ± 0.07	0.032 ± 0.005
Na/P	0.2 ± 0.1	1.5 ± 0.1
Fe/P	0.032 ± 0.003	0.021 ± 0.005
Al/P	0.03 ± 0.02	0.08 ± 0.02
Zn/P	0.02 ± 0.01	0.0007 ± 0.0002
Total + /P	1.51	1.84
P/3 KDO	N.D.	4.3 ± 0.2

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CHAPTER V

PHYSICAL PROPERTIES OF DEFINED LIPOPOLYSACCHARIDE SALTS

ABSTRACT

The electron spin resonance probes 5-doxyl stearate and 4-(dodecyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperdine bromide were used to characterize the fluidity of the acyl chain and head group regions, respectively, of defined salts of lipopolysaccharide from E. coli K12 (LPS). The removal of the weakly bound divalent cations from native LPS by electrodialysis and their replacement by sodium had little effect on the mid-point of the lipid phase transition or on head group mobility. In contrast, lipopolysaccharide acyl chain mobility increased following electrodialysis. The replacement of most of the remaining cations with sodium resulted in a further dramatic increase in mobility in both the polar and nonpolar regions of LPS. Head group mobility of the sodium salt of LPS was shown to be reduced with the addition of divalent cations. Furthermore, evidence is presented which suggests that low magnesium concentrations may actually induce phase separations in NaLPS. The magnesium salt of LPS closely resembled the native form of LPS in both head group and acyl chain mobility although the cation charge to phosphorous ratio in the magnesium salt was greater than that detected in nLPS. Additional analysis of other LPS salts support our hypothesis that

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many of the observed differences in the physical and pathological properties of LPS salts may simply be explained by the degree of LPS charge neutralization.

INTRODUCTION

The physical state of LPS has been reported to have a profound impact on its endotoxicity. A strong correlation seems to exist between the particle size of enteric LPS aggregates and their ability to inactivate serum complement (1). LPS salts which have low particle size (e.g. triethylamine) do not interact with the complement system, whereas high particle size salts (e.g. sodium) are potent inactivators of complement.

This pattern may not be common to all LPS. For example, the native and sodium salts of *Chromatia vinosum* LPS are equally effective activators of complement (2).

Another indication of the importance of the physical state of LPS on endotoxicity is the effect that mild alkaline treatment has on the ability of LPS to act as a mitogen (3). Although high pH has been reported to cleave esterified fatty chains from LPS, mild treatment simply lowers particle size without apparent chemical denaturation. In this case, alkaline treated LPS of low particle size had enhanced B cell mitogenicity.

Attempts to characterize LPS aggregates by sedimentation coefficients or particle sizes can be complicated by gross changes in the morphology of LPS as it is purified and transformed into defined salts. Furthermore, correlations between LPS aggregate size or shape and

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endotoxicity do not give attention to subtle differences in the intermolecular interactions between LPS within these aggregates.

We have attempted to characterize the differences between various LPS salts in their head group and acyl chain mobilities. This was done in the hope that such differences may point toward a better molecular understanding of the interaction of cations with this complex anionic lipid, and its involvement in bacterial pathology.

MATERIALS AND METHODS

Cell growth and LPS isolation, computer analysis, and ESR were carried out as in Chapter III. All ESR results were reversible in the temperature range indicated. We observed that LPS solutions which had been frozen in solution and then thawed did not resuspend well. Consequently, all ESR experiments were performed on fresh or lypholized and resuspended LPS samples stored in solution under nitrogen at 4°C.

RESULTS

ESR probing of D21 defined LPS salts

Defined salts of D21 LPS were probed with either CAT_{12} or 5DS to measure head group and acyl chain mobility, respectively. Spectral parameters were measured as a function of temperature. We found that nLPS was only slightly more restricted in head group mobility than edLPS (Figure 2). Even this small difference diminished with increasing temperature. In contrast, acyl chain mobility of these two samples probed with 5DS was significantly different as determined when either

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$2T_{\parallel}$ (Figure 3) or order parameter S, (Figure 4) was plotted as a function of temperature. Both probes indicated that a broad temperature dependent transition occurs in native and electrodialyzed LPS with T_m at 29 ± 9°C and 24 ± 5°C, respectively.

Mg-LPS closely resembled nLPS particularly with respect to acyl chain order (Figure 4). Head group mobility appeared to be intermediate between native and electrodialyzed LPS (Figure 2).

The hydrophobic acyl chain region of NaLPS probed with 5DS was shown to be more fluid than that of edLPS up to 30°C (Figure 3). Above 30°C the difference in acyl chain mobility between NaLPS and edLPS was slight. In contrast, head group mobility detected with CAT_{12} in NaLPS was dramatically greater than that of edLPS over the entire temperature range examined. Again a broad acyl chain transition was observed with a T_m at 15 ± 12°C. Tris-LPS appeared to be similarly to NaLPS in its physical properties (Figures 3 and 4).

The terbium salt of LPS from strain D21 represented something of an anomally. Acyl chain mobility of this salt was more severely restricted than that of nLPS. The spectra at all temperatures examined was like that of a rigid glass (4). In contrast, the head group mobility of TbLPS represented the most fluid of the LPS salts. Even at -5°C CAT_{12} reported an unusually fluid environment.

Scatchard analysis of CAT_{12} binding to NaLPS

The above results suggested that the interaction of CAT_{12} with NaLPS may be complex. Thus we examined the affinity of CAT_{12} for NaLPS by Scatchard analysis. Figure 5 indicates that CAT_{12} has 4.4 binding sites on NaLPS isolates from strain D21. In addition some of

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these sites show strong positive cooperativity (5). this is in contrast to our previous results with edLPS which indicates that CAT_{12} bound to a single site in a noncooperative fashion. The CAT_{12} to LPS weight ratio used throughout was 0.074. This is equivalent to one mole of probe per 50 moles of LPS or 300 moles of LPS fatty acids.

<u>Magnesium titration of NaLPS from strain D21</u>

The sodium salt of LPS from strain D21 probed with CAT_{12} at 37°C, was mixed with increasing concentrations of magnesium chloride (Figure 6). The low field line width, Γ_l , was used as an indicator of the formation of multiple spin probe domains. At 37°C, Γ_l the sodium salt is already rather broad, presumably due to lifetime broadening (6). As the magnesium concentration approached that of the NaLPS, Γ_l increased dramatically. The observed broadening could be explained by either of two mechanisms 1) as the level of Mg increased CAT_{12} was forced into increasingly smaller "pure" NaLPS domains resulting in spin-spin exchange broadening; or 2) CAT_{12} partitioned randomly between both "pure" NaLPS domains and domains bound with Mg. Figure 6 provides evidence which strongly supports the latter interpretation. At least two distinct low field peaks could be resolved when the concentration of Mg equalled that of NaLPS. As the Mg to NaLPS molar ratio approached 2, the line width of the rigid population reached a value of about 4 Gauss and did not change even at very high Mg concentrations. In the rigid spin probe population, the hyperfine splitting parameter, $2T_{\parallel}$ could be measured. CAT_{12} mobility was greatly reduced in this population and

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was nearly equal to that of CAT_{12} in the defined salt of MgLPS (vide infra).

The observed decrease in Γ_{ℓ} of the rigid population accompanied by an increase in $2T_{\parallel}$ is taken as evidence that the head group mobility of NaLPS has decreased upon addition of Mg. These changes can not be explained by altered polarity of the spin probe environment since Γ_{ℓ} has been shown to be insensitive to such changes (7).

Electron micrographs of D21 LPS

The structure of various LPS salts was characterized in the electron microscope with samples negatively stained by sodium phosphotungstate, pH 7.0. NaLPS of D21 was characterized as long tubular structures approximately 9 nm in diameter. These tubes are frequently swollen to twice this diameter (Figure 7). There were also a number of irregularly shaped vesicles present. The Tris salt had a nearly identical structure (Figure 8).

The addition of one mole of calcium per mole of NaLPS resulted in the complete conversion of the tubes to vesicles (Figure 9). Tubes can be seen at several places on most of the vesicles. The tubes appear to loop back onto the vesicles. The diameter of these tubes is equal to the minimum tube diameter seen in NaLPS before the addition of calcium. Although most vesicles were approximately 60 nm in diameter, several very large vesicles were also present.

Magnesium was less effective than calcium in inducing the vesicularization of NaLPS. In fact, four moles of magnesium per mole of NaLPS were required to completely convert tubes into vesicles (Figure 10). Again, in virtually every case, there were one or more looping tubes present on each vesicle. In contrast to the round shape of calcium-induced vesicles, the magnesium induced vesicles appeared irregular.

Both terbium (data not shown) and ferric chloride (Figure 11) when added to NaLPS produce similar results. When either is added at molar ratio of one, the tubular structure of NaLPS appeared to stabilize. Furthermore, there appeared to be fewer regions of swelling within tubes of either the terbium or iron treated NaLPS. The minimum tube diameter remained 9 nm.

ESR probing of a heptoseless mutant of strain D21

The sodium salt of LPS from strain D21f2, a deep rough mutant of strain D21, was probed with CAT_{12} and 5DS. Figure 12 indicates that the heptoseless mutant produced LPS which in the sodium salt was substantially more restricted in head group mobility than the head group of NaLPS from the parent strain. Above 45°C, however, head group mobility of NaLPS from strain D21f2 increased dramatically. In contrast, 5DS probing showed the acyl chain mobility and order to be virtually identical in the sodium salts of D21 and D21f2 (Figure 13). Furthermore, both sodium salts showed an abrupt change in the temperature dependence of order parameter around 35°C (Figure 14).

DISCUSSION

In the preceding paper we have shown that *E. coli* LPS extracted and purified according to standard protocol remains heavily contaminated with a variety of cations (8). With further treatment we were able to obtain

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a sodium salt of LPS which was relatively free of these contaminants. NaLPS was then used to obtain various chemically defined salts of LPS. We report here that these specific salts are very distinct in their physical properties. These differences are interesting in light of the fact that certain cations are able to modulate the endotoxic activity of LPS.

When acidic edLPS is neutralized with an appropriate base, the different salts had greatly altered ability to inactivate serum complement (9). A strong correlation has been observed between endotoxin activity and the apparent aggregate weight of these salts. One important difference between our defined LPS salts and those of Galanos _et_ _al_. is that our preparations were converted to a relatively pure sodium salt with low levels of contaminating ions. Thus, our edLPS neutralized with NaOH which may be analogous to the sodium salt of _Salmonella_ (10).

The ESR spin probes CAT_{12} and 5DS were used to monitor changes in head group and acyl chain mobilities of the LPS salts. Our initial reservations concerning cationic "detergents" as a spin probe of a polyanionic lipid system were confirmed. NaLPS has multiple CAT_{12} binding sites; some of which display strong cooperativity. This probe could induce vesicularization of NaLPS tubes at high CAT_{12}- to NaLPS-molar ratios (data not shown). Nevertheless, CAT_{12} apparently did not alter either the local LPS environment or the morphology of LPS aggregates when applied at or below a probe-to-LPS weight ratio of 0.074.

The relatively high degree of head group mobility in the NaLPS compared to edLPS, pH 7, may simply result from the low cation charge-to-phosphorus ratio as noted in the preceding paper (8). Incomplete charge neutralization may also be responsible for the tube-like structures seen

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in the electron micrographs of NaLPS. The high degree of surface curvature afforded by this conformation would result in less densely packed core sugars. This is consistent with the high degree of head group mobility observed with CAT_{12}.

The head group structure of the NaLPS from strain D21 may be partially stabilized by proton transfer complexes between phosphate or carboxyl groups and ethanolamines. Proton transfer complexes are now thought to exist at low ionic strength in virtually all phospholipids with amine containing head groups. These non covalent bonds act as a two dimensional polymeric network in phosphatidylethanolamine monolayers and are largely responsible for the observed rigidity of the head group detected with deuterium NMR (11).

The observation that NaLPS of the D21 strain may not be fully charge neutralized leaves open the potential for hydrogen bonding between and within NaLPS molecules. An early model which attempted to explain the high apparent molecular weights of LPS proposed that the phosphate groups in Lipid A were diester linked (12). The existence of such LPS multimers has since been disproven using both biochemical and ^{31}P NMR techniques (13). Nevertheless, no explanation for the high apparent molecular weight of LPS is apparent. Divalent cation cross bridging between adjacent LPS is certainly reasonable in native salts. Recently though, Schands et al. (14) have reported analytical centrifugation analyses of NaLPS which indicate that its minimum aggregate weight is that of a dimer.

We suggest that hydrogen bonding between ethanolamine groups and the carboxyl groups of the KDO units or the phosphate groups in NaLPS may contribute to the stability of NaLPS aggregates. The presence of such bonds in the presence of divalent cations is, however, uncertain.

Systematic studies of the association of cations with anionic lipids have only recently been reported (15,16,17). Mixtures of acidic and neutral phospholipids can form multilamellar tubes which are visible under a light microscope (18). Upon exposure to calcium, these tubes can twist back upon themselves into helices. We found that equimolar calcium converted NaLPS tubes into vesicles as did magnesium, albeit at higher magnesium concentration. Calcium has been reported to converted normally bilayer vesicles of sodium phosphatidic acid into H_{II} phase (19,20). Furthermore, phosphatidic acid can form a calcium ionophore across lipid bilayers by forming an inverted micelle in the presence of calcium (21).

In the pure sodium salt of LPS, the tube structure is thought to be H_I whereby the polysaccharide chains are extended radially into the aqueous phase. The possibility remains that at low divalent cation concentrations, restricted region of inverted H_{II} phase exist. This may be what we have identified as loop on vesicles (Figures 9 and 10). In fact, both nLPS and NaOH neutralized edLPS consist of bilayer sheets which are regularly and heavily pitted (Figure 15 and 16). This sort of structure has also been recognized by Burnell and coworkers (22). In nLPS the pits are arranged in both random, cubic, and hexagonal arrays. The hexagonal packing of the pits was most commonly observed. The repeat distance for the hexagonal array was 144 A. Comparison of Figure 15 and 16 indicates that the loss of one magnesium through electrodialysis and its replacement with sodium results in the formation of tubes similar to what had been seen in the sodium salt of LPS. This morphological change as mentioned earlier is accompanied by a dramatic increase in LPS toxicity (1). Surprisingly, head group mobility of nLPS and edLPS were very similar (Figure 2). Unfortunately, electron micrographs are often misleading and can lead to erroneous interpretations with regard to molecular organization. These may be particularly true when analyzing what are thought to be lipidic particles and cubic phase lipids (23).

CAT_{12} proved to be very sensitive to the binding of cations to NaLPS. The addition of Mg to NaLPS produced a dramatic decrease in the mobility of bound CAT_{12}. This decrease in head group mobility was not the result of a change in the surface curvature of LPS aggregates. Electron micrographs showed that Mg could not induce complete vesicularization of NaLPS tubes at molar ratios below 4 (Mg:LPS). Furthermore, examination of the low field line width provided strong evidence that magnesium can promote the formation of separate domains within NaLPS aggregates when the molar ratio of Mg to NaLPS is below one.

Apparently, not all cations altered the mobility of the LPS head group in the same way. The similarity between native and electrodialyzed LPS of strain D21 detected with CAT_{12} suggested that the removal of one weakly bound divalent cation and its replacement by sodium does not significantly alter the packing of the core sugars. Experiments described in the preceding paper suggest that the most weakly bound divalent cation in nLPS from strain D21 is magnesium which is presumably located in the KDO core.

Curiously, the divalent cations induced vesicles of NaLPS almost without exception but the vesicles retained regions of tubular LPS which loop back onto the vesicles. The minimum diameter of these tubular regions was about 9 nm. The bilayer thickness of LPS is 10 nm detected by X-ray diffraction analysis (25). For this reason, we believe that the tubes and loops are regions of LPS that are in a typical H_I conformation (24).

Ferric and terbium chloride seemed to relax the twisted tubes of NaLPS into long smooth tubes. This may partially explain the exceedingly high head group mobility detected by CAT_{12} and the very low acyl chain mobility detected with 5DS in TbLPS. Conversely, the tight packing of the acyl chains of TbLPS may not allow CAT_{12} to completely partition into TbLPS.

The broad phase transition observed in nLPS with both CAT_{12} and 5DS was shifted down 5°C after electrodialysis and the temperature over which the transition occurred, narrowed substantially. As evidenced by fluorescent probing and X-ray diffraction analysis, a broad order-disorder transition occurred at 22 ± 5°C in nLPS of the E. coli strain B/r (25). Neither the midpoint nor width of the observed transition in this smooth strain was altered by electrodialysis.

X-ray diffraction experiments demonstrated that the phase transition temperature of nLPS from E. coli B grown at 37°C to be 25°C (26). Below 25°C the diffraction data suggested that the LPS acyl chains were still more disordered than acyl chains of phospholipids. In contrast, a comparison of the order parameter calculated for 5DS in extracted phospholipid, (Figure 4) and nLPS showed just the opposite results. nLPS acyl chains are reported to be substantially more ordered than phospholipids. This apparent discrepany may be explained by the difference in time scales of detection for the two techniques rely. X-ray diffraction analysis may be considered a static measurement of the hydrocarbon chain order. Order determined by electron spin resonance probes is also a static parameter; however, the technique measures the distribution of acyl chain conformations in the time range of 10^{-9} to 10^{-7} sec. Thus, our order parameter measurements indicate that the motion of the hydrocarbon interior of nLPS aggregates is greatly decreased relative to that of phospholipids.

In this context, it is interesting to note that lateral diffusion of LPS determined by the technique of flourescence recovery after photobleaching (27) indicated that phospholipids diffuse much faster relative to one another than does LPS ($D_{LPS}/D_{PL} = 0.6$). Thus, LPS domains in the outer membrane are most accurately thought of as more restricted than phospholipid domains regardless of the conformation of the acyl chains.

Our laboratory has previously shown that outer membranes extracted from *E. coli* grown at different temperatures have altered physical properties (28). Most notably the beginning and end of the membrane lipid transition seen with 5DS correlates well with the limits of the growth temperature. The acyl chains of both phospholipids (26) and LPS (29) have been reported to change in response to growth temperature. We have found no detectable changes in either acyl chain mobility or transition temperature (data not shown) in response to changes in growth temperature in the range of 12 to 43°C. The similarity between the midpoints of the phase transitions of nLPS and outer membrane from *E. coli* grown at 37°C, therefore, may be coincidental. Emmerling *et al.* (25) reached a similar conclusion in their smooth *E. coli* strain.

Finally, a comparison of NaLPS of strain D21 and the heptoseless mutant strain D21f2 shows that the two have virtually identical acyl chain mobility. The Lipid A moiety of the two LPS isolated from bacteria grown under the same condition has been reported to be the same (30). Consistent with the elemental analysis presented in the preceding paper, NaLPS from strain D21f2 has less head group mobility than the NaLPS from the parent strain. Furthermore, electron micrographs (data not shown) indicate that NaLPS from strain D21f2 is vesicular.

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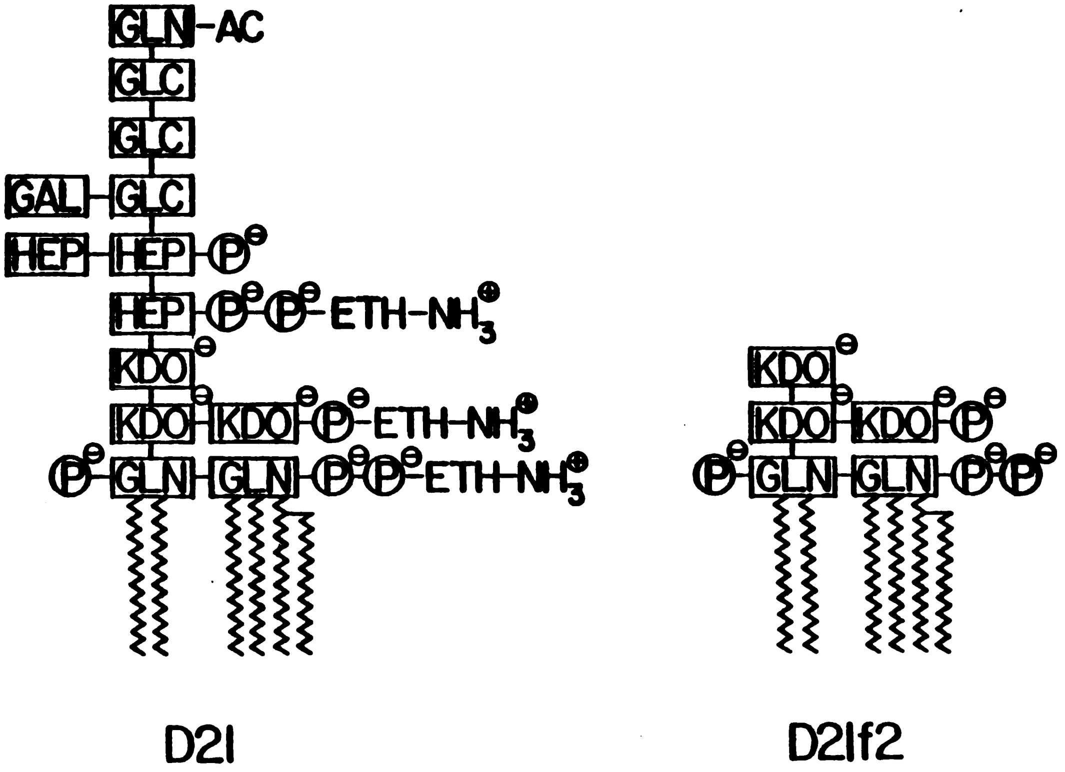

Figure 1. Structure of E. coli K12 strains D21 (rough) and D21f2 (deep rough) LPS.

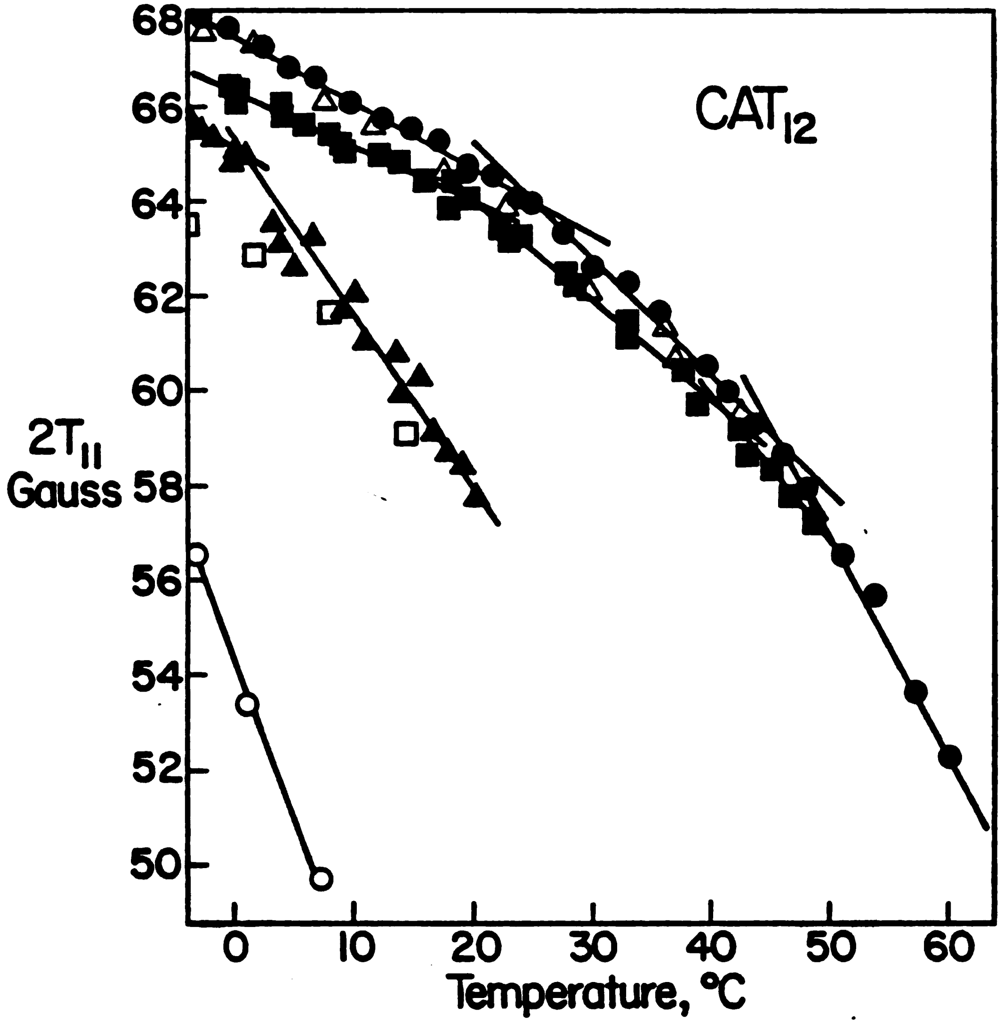

Figure 2. The temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$, of CAT_{12} bound to defined salts of LPS from *E. coli* strain D21. The LPS salts included native LPS (●), electrodialyzed LPS neutralized with NaOH (■), NaLPS (▲), TRIS-LPS (□), MgLPS (△), and TbLPS (○). All LPS salts were resuspended in double distilled water at an approximate concentration of 0.5 mM and mixed with approximately 10 μM CAT_{12} (final concentration). The hyperfine splitting parameter of CAT_{12} is inversely proportional to the head group mobility.

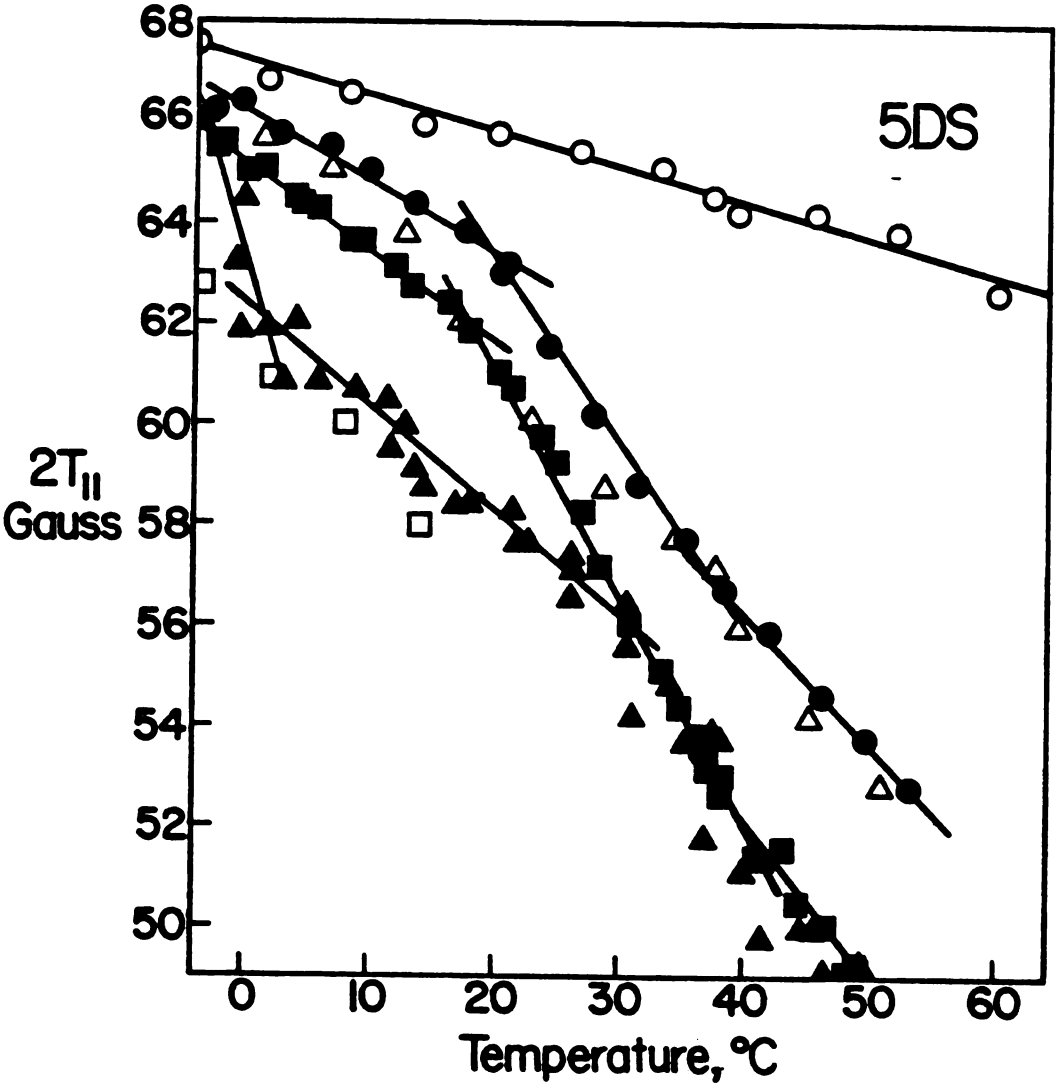

Figure 3. The temperature dependence of the hyperfine splitting parameter $2T_{\parallel}$, of 5DS bound to defined salts of LPS from E. coli strain D21. The LPS salts studies were native LPS (●), electrodialyzed LPS neutralized with NaOH (■), NaLPS (▲), TRIS-LPS (□), MgLPS (△), and TbLPS (○). All LPS salts were suspended in double distilled water at an approximate concentration of 0.5 mM and mixed with approximately 5 µM 5DS (final concentration). The hyperfine splitting parameter of 5DS is inversely proportional to the lipid acyl chain fluidity.

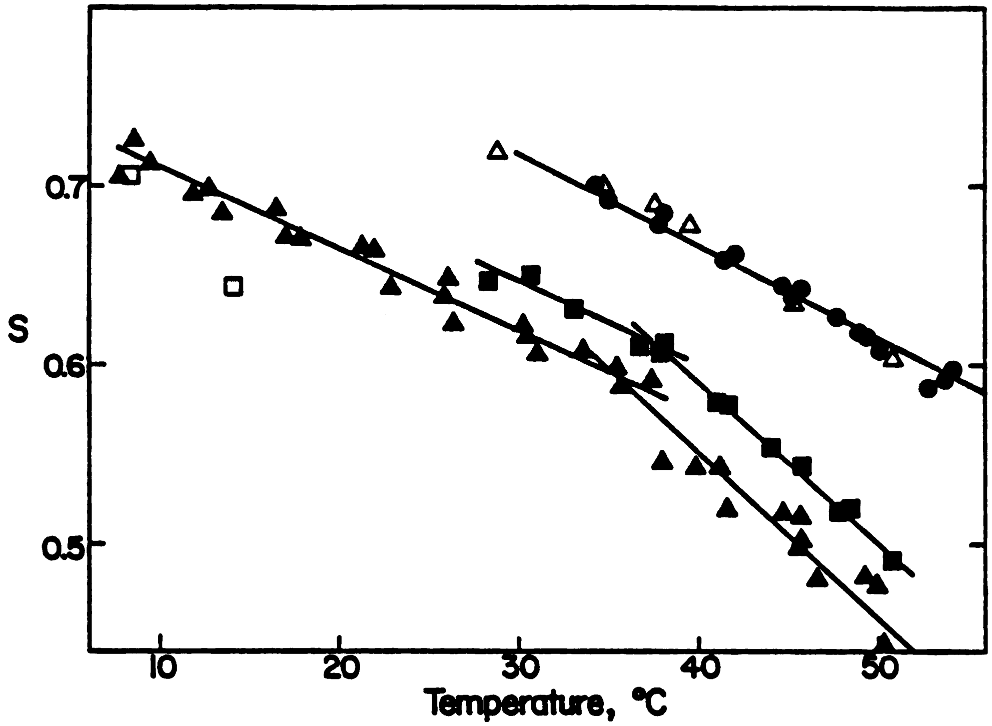

Figure 4. Temperature dependence of 5DS order paramater in native (●), electrodialyzed (■), sodium (▲), magnesium (△), and TRIS (□) LPS. LPS to 5DS molar ratio was 50.

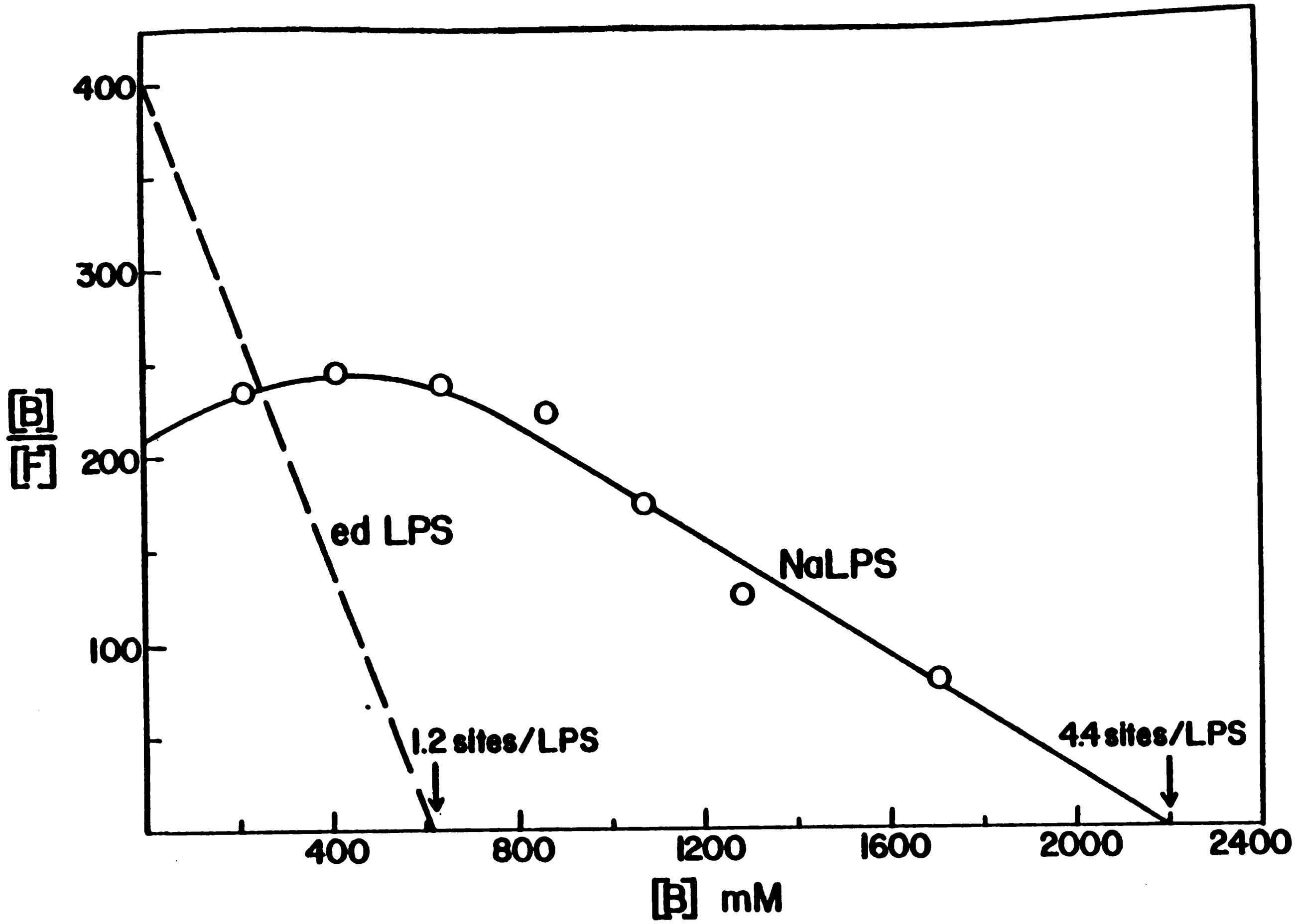

Figure 5. Scatchard analysis of CAT_{12} binding to electrodialyzed LPS (dashed line) and NaLPS (○) from <u>E. coli</u> K12 grown at 37°C. CAT_{12} partitioning was measured at 37°C on samples with an LPS concentration of 500 µM as a function of probe concentration.

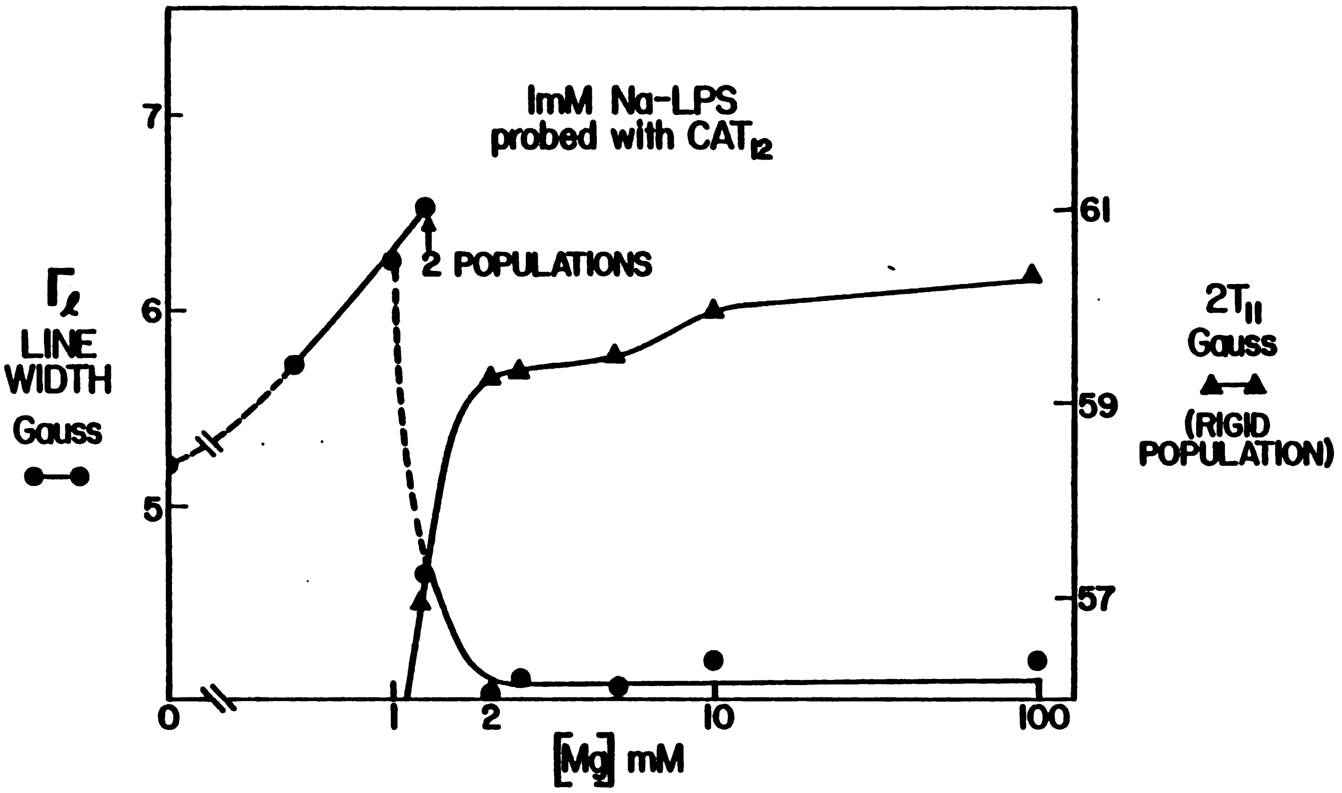

Figure 6. Titration of NaLPS from E. coli strain D21 with $MgCl_2$. A 1 mM solution of NaLPS suspended in double distilled water and mixed with approximately 20 µM CAT_{12} (final concentration) was titrated with increasing amounts of $MgCl_2$. The line width of the low field peak indicates increasing heterogeneity of the probe environment as $MgCl_2$ is added. At approximately equimolar Mg^{+2} and LPS two peaks can be resolved in the spectra and the rigid population appears to saturate at a ratio of 2:1, Mg^{+2}:LPS.

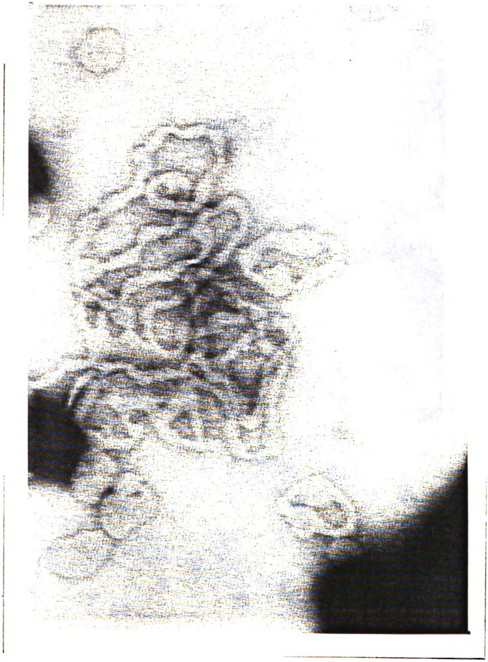

Figure 7. Electron micrograph of the sodium salt of LPS isolated from *E. coli* strain D21, stained with sodium phosphotungstate, pH 7.0.

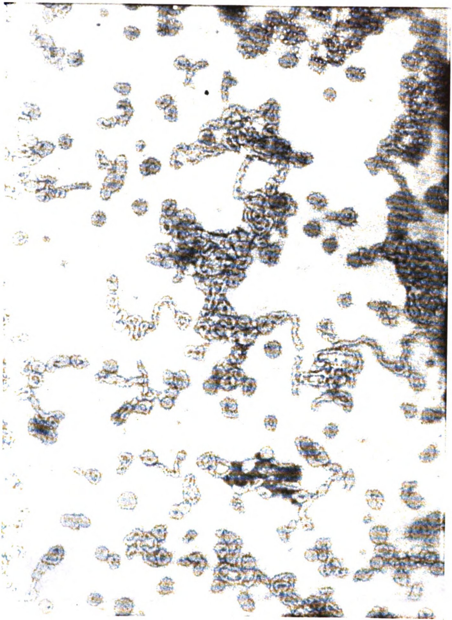

Figure 8. Electron micrograph of the TRIS salt of LPS isolated from *E. coli* strain D21, stained with sodium phosphotungstate, pH 7.0.

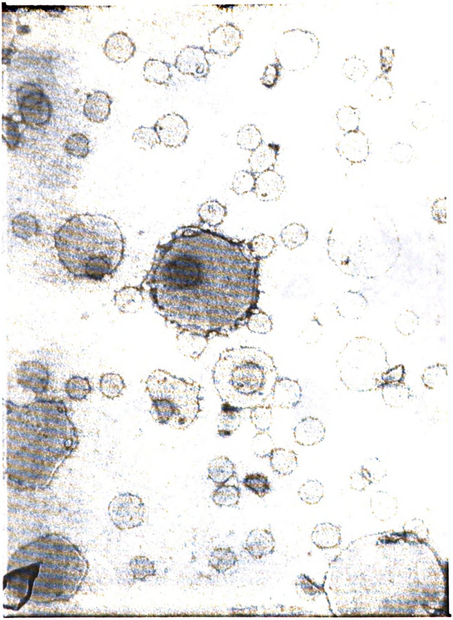

Figure 9. Electron micrograph of NaLPS with $CaCl_2$ added (Ca/LPS, 1) isolated from E. coli strain D21, stained with sodium phosphotungstate, pH 7.0.

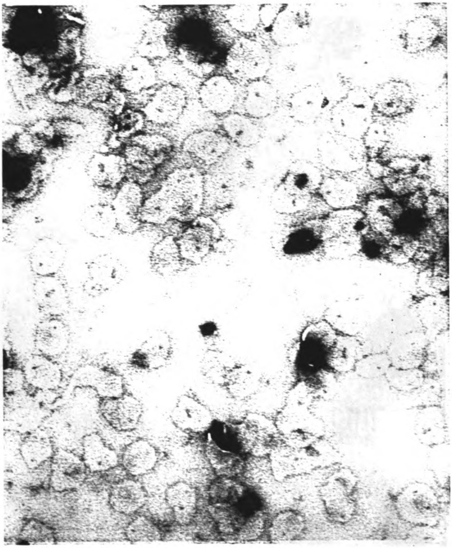

Figure 10. Electron micrograph of NaLPS with $MgCl_2$ added isolated from *E. coli* strain D21 (Mg/LPS, 4), stained with sodium phosphotungstate, pH 7.0.

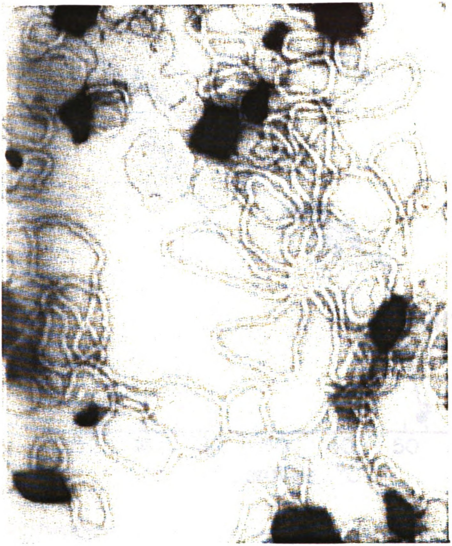

Figure 11. Electron micrograph of NaLPS with $FeCl_3$ added (Fe/LPS, 1) isolated from E. coli strain D21, stained with sodium phosphotungstate, pH 7.0.

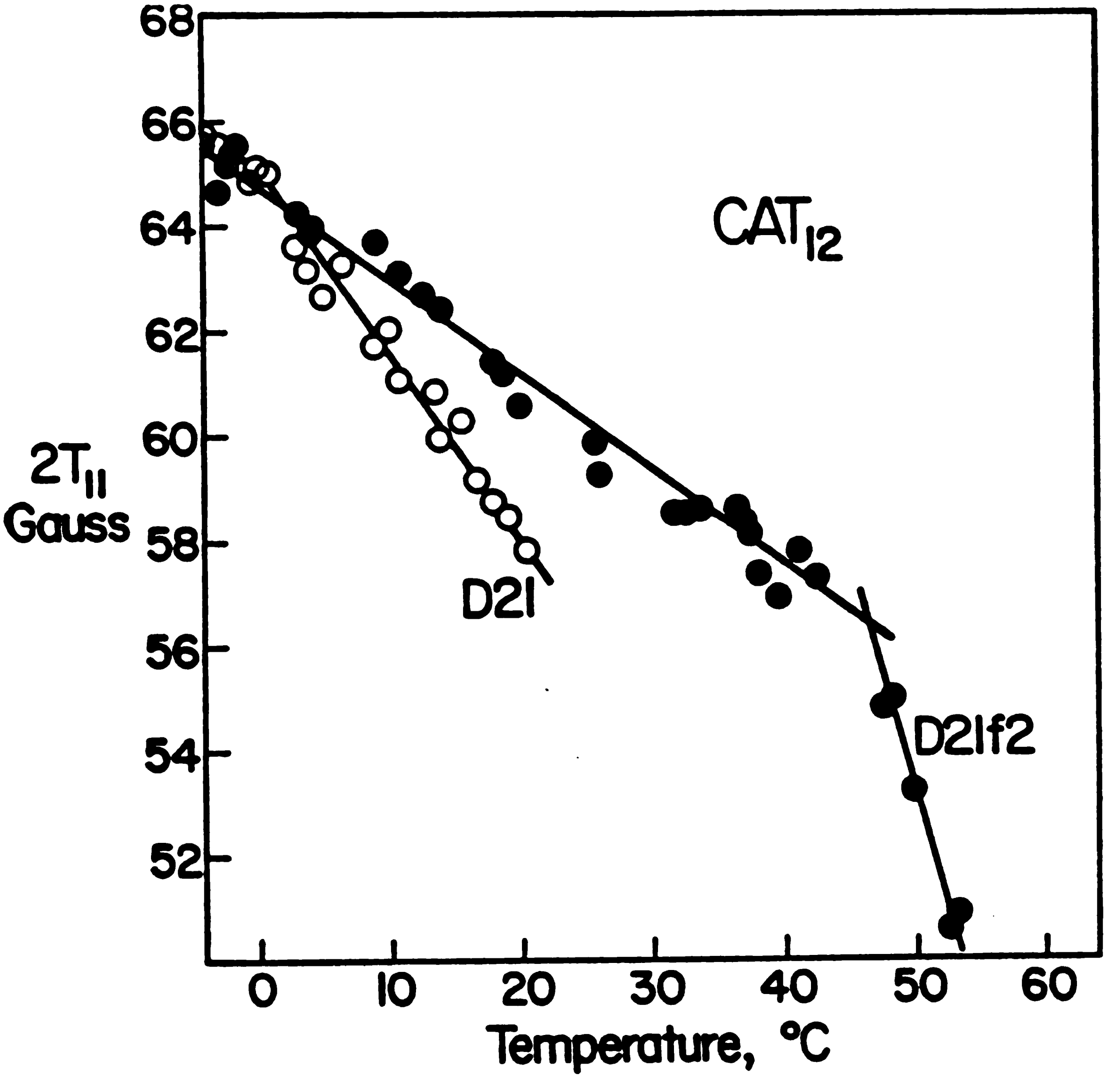

Figure 12. Temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$, of CAT_{12} in the sodium LPS salts of D21 (○) and D21f2 (●). The probe to LPS weight ratio was 0.074.

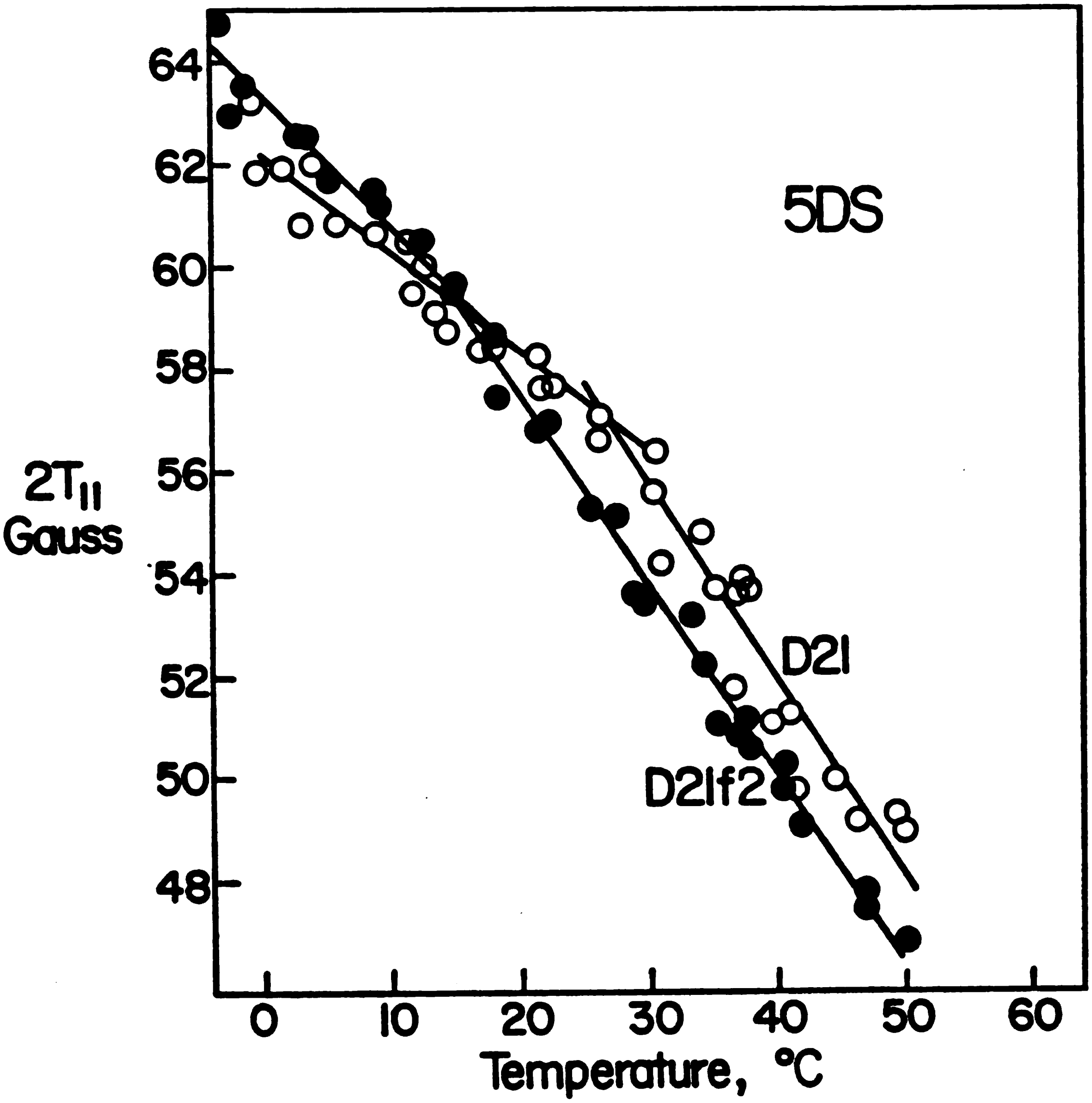

Figure 13. Temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$, of 5DS in the sodium LPS salts of D21 (○) and D21f2 (●). The probe to weight ratio was 0.074.

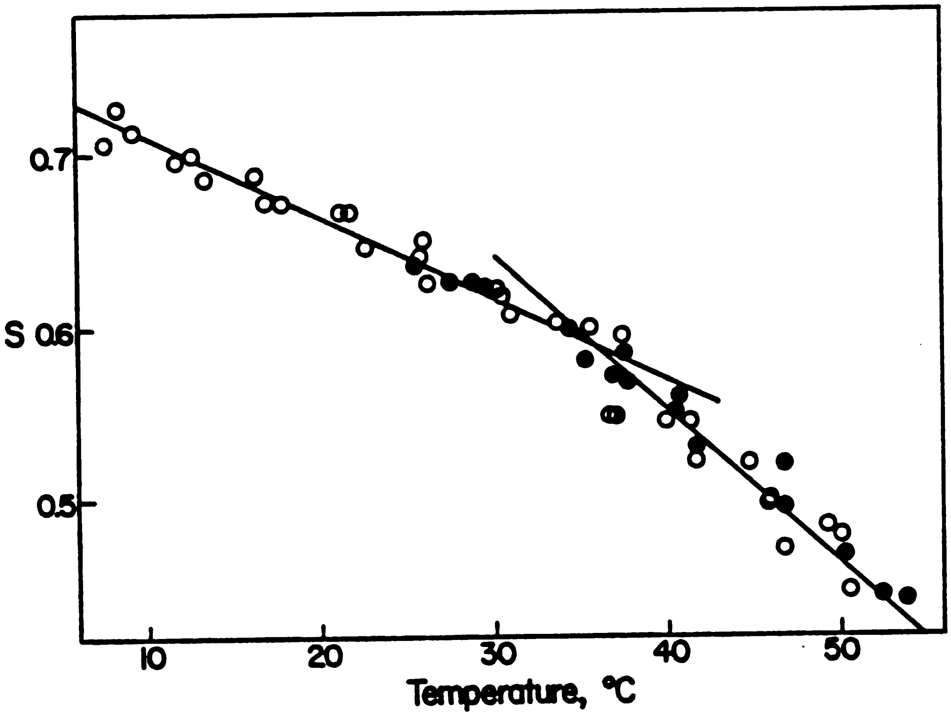

Figure 14. Temperature dependence of 5DS order parameter, 5, in the sodium LPS salts of D21 (○) and D21f2 (●). The probe to weight ratio was 0.074.

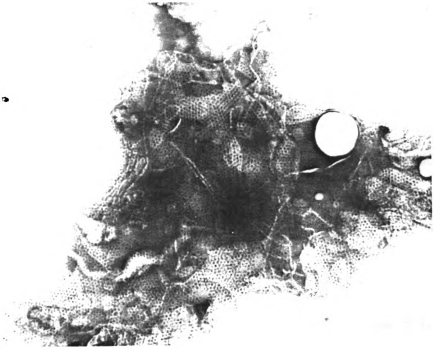

Figure 15. Electron micrograph of nLPS from E. coli strain D21, stained with sodium phosphotungstate, pH 7.0.

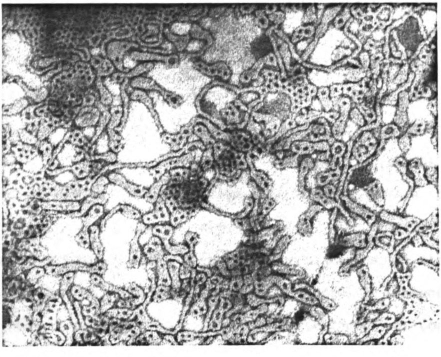

Figure 16. Electron micrographs of edLPS from E. coli strain D21, stained with sodium phosphotungstate, pH 7.0.

CHAPTER VI

ELECTRON SPIN RESONANCE PROBING OF LIPOPOLYSACCHARIDE DOMAINS IN THE OUTER MEMBRANE OF ESCHERICHIA COLI

SUMMARY

Two electron spin resonance probes, an anionic probe 5-doxyl stearate and a cationic probe 4-(dodecyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperdine bromide, were used to analyze the physical properties of the phospholipid and lipopolysaccharide domains in the outer membrane of *Escherichia coli*. Scatchard analysis of the binding of the cationic probe to lipopolysaccharide and to phospholipid indicated that this probe has a 5 fold greater affinity for anionic lipopolysaccharide than for phospholipid. In the intact outer membrane this cationic probe likely associates with the lipopolysaccharide containing outer monolayer. The temperature dependence of the mobility of the cationic probe in the outer membrane indicates that a structural transition occurs at 9°C in the outer monolayer. A similar 9°C transition was detected in the outer membrane using 5-doxyl stearate. This anionic probe has been shown to partition into the phospholipid enriched domains. A porin-lipopolysaccharide-peptidoglycan complex probed with the cationic probe also was shown to undergo a thermally induced structural change at 9°C. In contrast, purified lipopolysaccharide was shown to have structural transitions at 20°C and 40°C. It is proposed that a structural rearrangement of the intact outer membrane occurs at approximately 9°C in both the lipopolysaccharide and phospholipid domains of the outer membrane. Furthermore, this structural transition appears to be highly dependent on lipid-protein interactions. A second thermotropic transition that occurs in the outer membrane at approximately 40°C to 42°C appears to result mainly from changes in the lipopolysaccharide domain structure.

INTRODUCTION

The outer membrane of gram negative bacteria is a highly asymmetric structure. The outer monolayer is composed of anionic lipopolysaccharide (LPS) and protein, whereas the inner monolayer contains phospholipid and protein. Moreover, phospholipids and LPS isolated from *Escherichia coli* upon reconstitution are reportedly unable to coexist in a single phase (1). Within the intact outer membrane of *Salmonella*, spin labeled fatty acids have been shown to preferentially partition into the phospholipid domains of the inner monolayer (2) perhaps as a result of the greater fluidity of these domains compared to that of the LPS domains, and to charge repulsion between the anionic head groups of the fatty acid probe and LPS. Previous analysis of extracted phospholipid from the outer membrane of 37°C grown *E. coli* indicated that the phospholipid undergoes a structural transition at approximately 4° and 24°C (3). Results reported here demonstrate that the structural transition of purified LPS begins at 20°C and ends at 40°C. We also found that the cationic electron spin resonance (ESR) probe 4-(dodecyl dimethyl ammonium)-1-oxy-

2,2,6,6-tetramethyl piperdine bromide (CAT_{12}) preferentially partitions into LPS compared to phospholipid domains. Applying this cationic probe and the acyl chain probe 5-doxyl stearate (5DS) we have evidence to suggest that both lipid domains located on either side of the outer membrane undergo a cooperative temperature dependent rearrangement. The onset of this rearrangement at 9°C in both lipid domains of the intact membrane appears to be determined mainly by protein-lipid interactions. In contrast, the end of the thermotropic phase change in the intact outer membrane at around 42°C may be determined by rearrangements within the LPS domains.

MATERIALS AND METHODS

Cell growth and membrane isolation

Cultures of *E. coli* strain W1485 F^- were grown at 37°C in M9 minimal medium supplemented with 0.4% glucose (final concentration). Cultures were harvested, and the cytoplasmic and outer membranes were isolated as described previously (4). The degree of purity of the separated membranes equalled or exceeded that previously described (5).

Lipid isolation

LPS was extracted from whole cells with aqueous phenol (5). The extracted LPS was sedimented at 78,000 g for 60 min and washed twice with double distilled water. The resuspended sample was dialyzed extensively against double distilled water yielding the native LPS (nLPS) product. Electrodialysis of nLPS resulted in an acidic sample (pH approximately 4) which was neutralized with NaOH to pH 7 (edLPS). Phospholipid was extracted from isolated outer membranes with chloroform/methanol (2/1) as previously indicated (6).

Porin-LPS-peptidoglycan isolation

A porin-LPS-peptidoglycan complex was isolated from cells grown and harvested as described above. Cells were washed in 10 mM N-2-hydroxyethylpiperiazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, and lysed using a French pressure cell at 12,000 psi. Total membranes were treated with 20 µg/ml deoxyribonuclease I (Sigma Chemical Co.), pelleted and washed twice with double distilled water. The porin-LPS-peptidoglycan complex was extracted with 1% sodium dodecyl sulfate (SDS). The protein composition of the complex was characterized on SDS polyacrylamide slab gels (7).

Assays

Succinate dehydrogenase activity was determined by the method of Osborn et al. (8) to quantitate cytoplasmic membrane levels. Lipopolysaccharide levels were quantitated by analysis of 3-deoxy-D-manno-octulosonic acid (KDO) content (9). Protein concentration was assayed using the procedure of Lowry et al. (10). Peptidoglycan levels were quantitated by measuring the muramic acid content of acid hydrolyzed peptidoglycan (11).

Spin labeling

The fatty acid spin probe 5DS was dissolved as a 30 mM solution in absolute ethanol. An aliquot of the probe was dried onto the bottom of a clean test tube under a stream of N_2, and the sample was added at room temperature with mild vortexing. The spin label comprised less than 0.1% of the lipid weight. The CAT_{12} was always less than 0.3% of that of the lipid acyl chains present in the sample. The partitioning of CAT_{12} into lipid was calculated as previously described (12). All electron spin resonance experiments were carried out as described earlier using a Varian Century Line electron spin resonance spectrometerm X-band, model E112 (13). The temperature dependence of the order parameter, S, and the hyperfine splitting parameter, $2T_{\parallel}$, was analyzed in terms of linear components using a linear regression program developed for transition point analysis (14). Discontinuities in plots of the temperature dependence of $2T_{\parallel}$ and S are interpreted to reflect structural rearrangements of the lipid domains in which the probe is bound (15).

RESULTS

Membrane spin labeling

Two electron spin resonance probes, CAT_{12} and 5DS (Figure 1), were used to monitor intact outer membranes, isolated LPS and porin-LPS-peptidoglycan complexes. Negatively charged 5DS likely partitions into those lipid domains which contain lower levels of anionic groups. The nitroxide group probes acyl chain mobility. In contrast, the positively charged CAT_{12} is thought to partition into more anionic lipid domains and probe the motion within the lipid head group region. ESR spectra indicate that the spin label movement in the head group region is sensitive to temperature (Figure 2). At higher temperatures broadening of the low field peak suggests that the probe

probe partitions into more than one environment. Figure 3 shows the temperature dependence of $2T_{\parallel}$ reported by CAT_{12} and by 5DS (Figure 4) bound to intact outer and cytoplasmic membranes. The $2T_{\parallel}$ parameter can be calculated from spectra of CAT_{12} bound to outer membranes only at temperatures below 30°C. At low temperatures, the CAT_{12} in the outer membrane appears to experience a slightly more rigid environment than does 5DS. In contrast, the CAT_{12} probe in the cytoplasmic membrane appear to reside in a similar or slightly more fluid environment.

Lipid spin labeling

The partitioning of CAT_{12} into purified phospholipid and LPS was analyzed at 37°C as a function of probe concentration in order to assess the preferential association of CAT_{12} with specific domains in the outer membrane. From Scatchard analysis of CAT_{12} binding (Figure 4) it can be concluded that this cationic probe has approximately 5 fold greater binding affinity for LPS than for phospholipid. The binding of this probe to both types of lipids appears to be non-cooperative and at a single type of site. Similar Scatchard analysis of 5DS binding is not possible since micelles form at high probe concentrations.

Plots of the temperature dependence of the spectral parameters, $2T_{\parallel}$ and S, for 5DS and CAT_{12} bound to purified LPS, indicate discontinuities at 20°C and 40°C (Figure 5). These results suggest that structural transitions occur at these temperatures which can be detected within both the head group and the acyl chain regions. Comparison of ESR spectra of CAT_{12} bound to isolated LPS and intact outer membrane measured at 37°C indicates that the head group region in the pure LPS appears to be more rigid than that of LPS in the intact membrane (data not shown).

Porin-LPS-mucopeptide labeling

Isolated porin-LPS-mucopeptide complexes were shown to contain approximately 70% protein, 15% LPS and between 5 and 15% peptidoglycan by weight. SDS polyacrylamide gels indicated that the protein was comprised of approximately equal amounts of porins 1a and 1b with only minor contamination from other membrane proteins (data not shown). These complexes were probed with CAT_{12}, and the plots of the temperature dependence of $2T_{\parallel}$ indicated a discontinuity at 9°C (Figure 6). The mobility of the probe within this complex was similar to that of the probe bound to intact outer membranes when compared at temperatures above 9°C. At temperatures below 9°C, the LPS head group appeared to be more rigid in the isolated porin-LPS-mucopeptide complex than LPS within the intact outer membrane.

DISCUSSION

Thermotropic structural rearrangements have been shown to occur in the lipid domains of biological membranes, and these transitions usually occur over a broad temperature range (15). These changes often affect membrane enzyme activities and other membrane functions (16). It is also known that the presence of membrane proteins and the composition of the lipid's acyl chains dramatically affect the temperature of the membrane's thermotropic rearrangement of the lipid components and the lipid acyl chain mobility. In this study we set out to determine whether specific membrane components modulate the lipid domain structure in the outer membrane of E. coli. Previously we had shown that 5DS labeled outer membranes undergo thermotropic structural transitions at temperatures different from those of the extracted phospholipids (3). Outer membranes from cells grown at 37°C labeled with 5DS appear to undergo transitions at 9° and 42°C whereas the phospholipid extracted from the same membrane undergoes structural transitions at 4° and 24°C (3). Since 5DS is reported to probe the phospholipid containing domains of the outer membrane (2), we believe that other components within the outer membrane are modulating the temperature of this transition.

We report here the use of a cationic electron spin resonance probe, CAT_{12}, to analyze the structure of the outer membrane. We have shown that CAT_{12} preferentially partitions into LPS more readily than into phospholipids, and in the intact outer membrane this probe likely partitions with the same specificity. Using this probe to analyze the structure of LPS in the intact membrane we have found that LPS associated with protein in the intact membrane and in the isolated porin-LPS-peptidoglycan complex has a thermotropic transition beginning at 9°C. In contrast extracted LPS probed with either CAT_{12} or 5DS undergoes a thermotropic transition starting at 20°C and ending at 40°C. Since the LPS domains in the intact outer membrane, probed with CAT_{12}, appear more fluid compared with pure LPS, it is proposed that proteins when associated with LPS disorder the head group region. Such disordering of the LPS may also cause the decrease in the temperature of the beginning of the thermal transition phospholipids have been reported when the lipid is mixed with membrane proteins (17). The end of the thermal transaction in the intact outer membrane can only be detected using the anionic probe 5DS which presumably probes the phospholipid domains (2). The temperature of the end of the transition of the intact membrane detected with 5DS is 42°C, very similar to the end of the transition of the isolated LPS (approximately 40°C). Perhaps temperature dependent structural rearrangements which occur in the LPS domains of the intact membrane affect the structure of the phospholipid domain structure as well. Since in the intact outer membrane both 5DS and CAT_{12} detect a 9°C structural transition, both lipid domains may be coupled in their structural rearrangements, perhaps through the structure or packing of transmembrane proteins.

In the porin-LPS-peptidoglycan complexes, CAT_{12} detected a more rigid environment below 9°C than was detected with the same probe in the intact outer membrane. The domains being probed in these two structures are likely to be similar in composition. However, in the porin-LPS-peptidoglycan complex the LPS present is probably tightly bound to the protein. We have shown that this porin-LPS complex is enriched in divalent cations compared to the intact outer membrane (Chapter IV), and these cations may be involved in tightly binding LPS to the porin proteins.

The cationic probe CAT_{12} detected a slightly more rigid environment in the outer membrane than did 5DS (Figure 3). These differences are more difficult to interpret. First, the nitroxide free radical in the CAT_{12} probe is located in the head group region of the probe and probes the mobility of the head group of the lipid into which it partitions. In contrast, the nitroxide free radical in 5DS is located five carbons into the acyl chain of the probe and is likely probing the mobility of the lipid acyl chains at this level. Secondly, as previously suggested, these two probes are probably probing different lipid domains within the outer membrane which may have dramatically different head group and/or acyl chain mobility.

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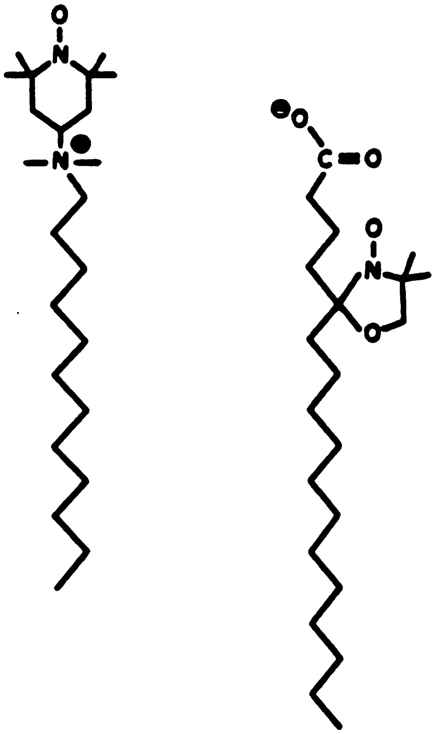

CAT_{12}

5DS

Figure 1. The chemical structure of the two electron spin resonance probes CAT_{12} and 5DS.

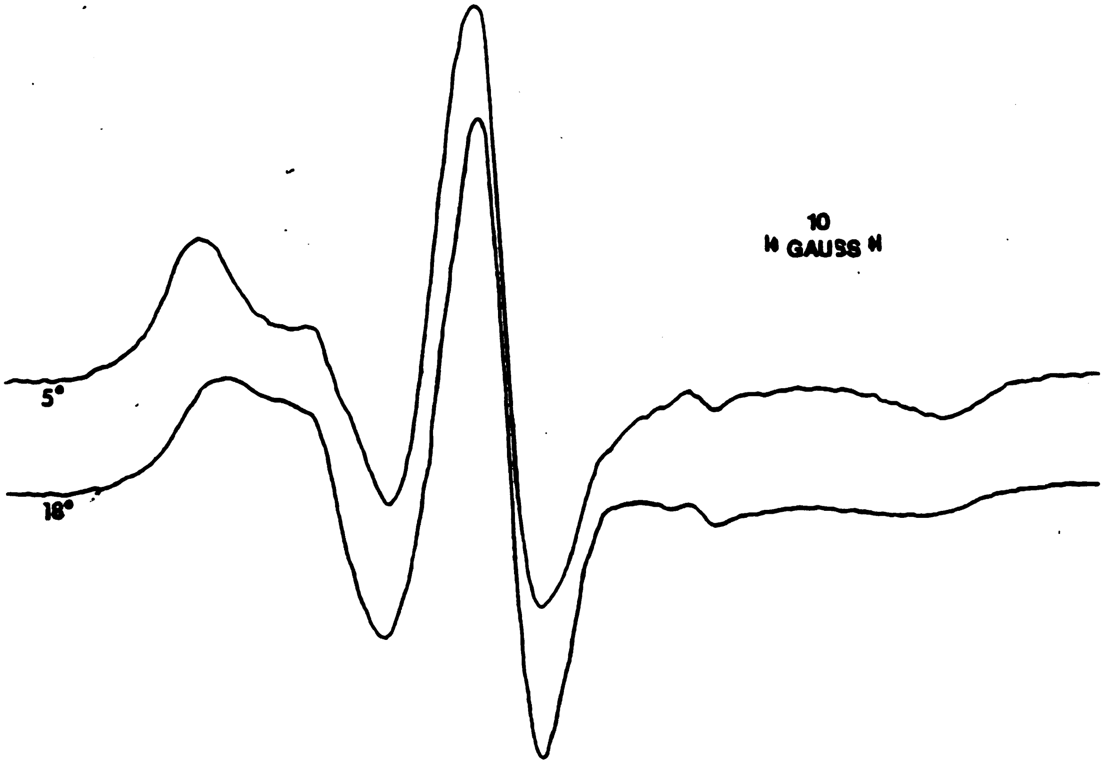

Figure 2. Electron spin resonance spectra of CAT_{12} bound to intact outer membranes from cells grown at 37°C in 10 mM Hepes, pH 7.5. The membrane concentration was approximately 20 mg/ml protein and probe was added at a 1:300 (probe; lipid acyl chain) dilution. The spectra were recorded at 5° and 18°C.

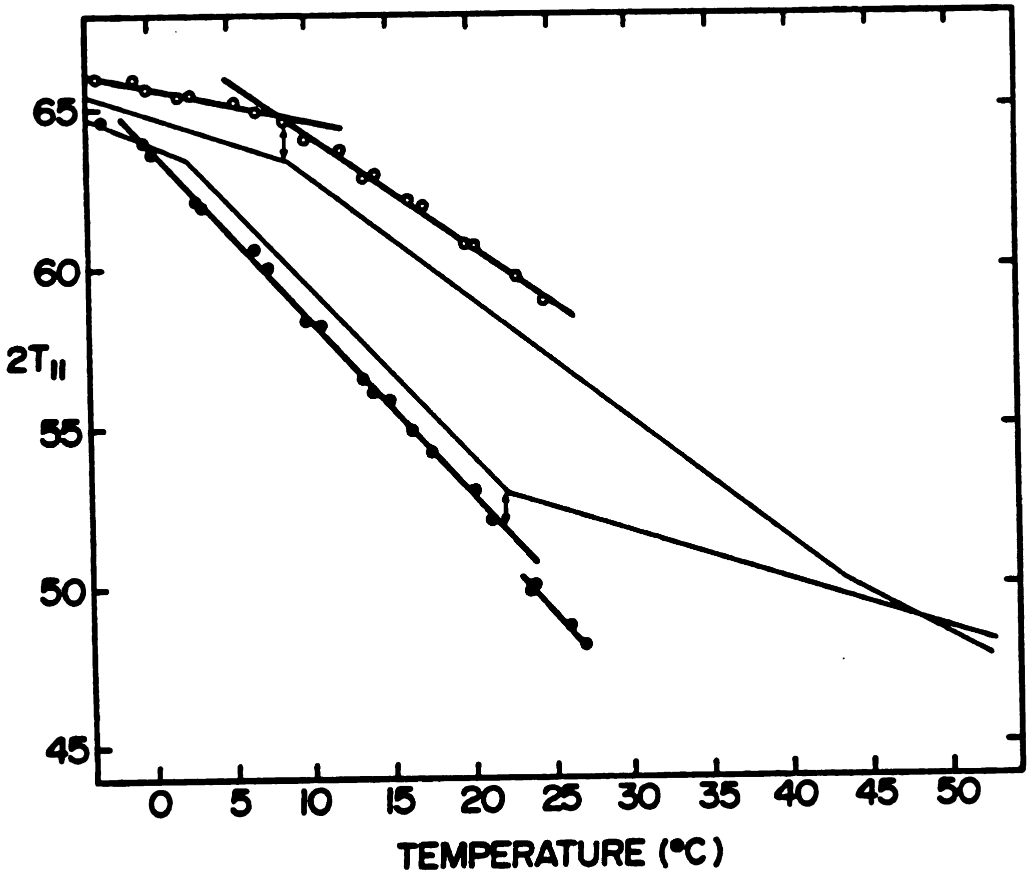

Figure 3. The temperature dependence of the hyperfine splitting parameter, $2T_{11}$, of CAT_{12} bound to outer membranes (○) and cytoplasmic membranes (●) from E. coli W1485F grown at 37°C. 5DS results (solid line) are for outer membrane (top) and cytoplasmic membrane (bottom) (ref. 4).

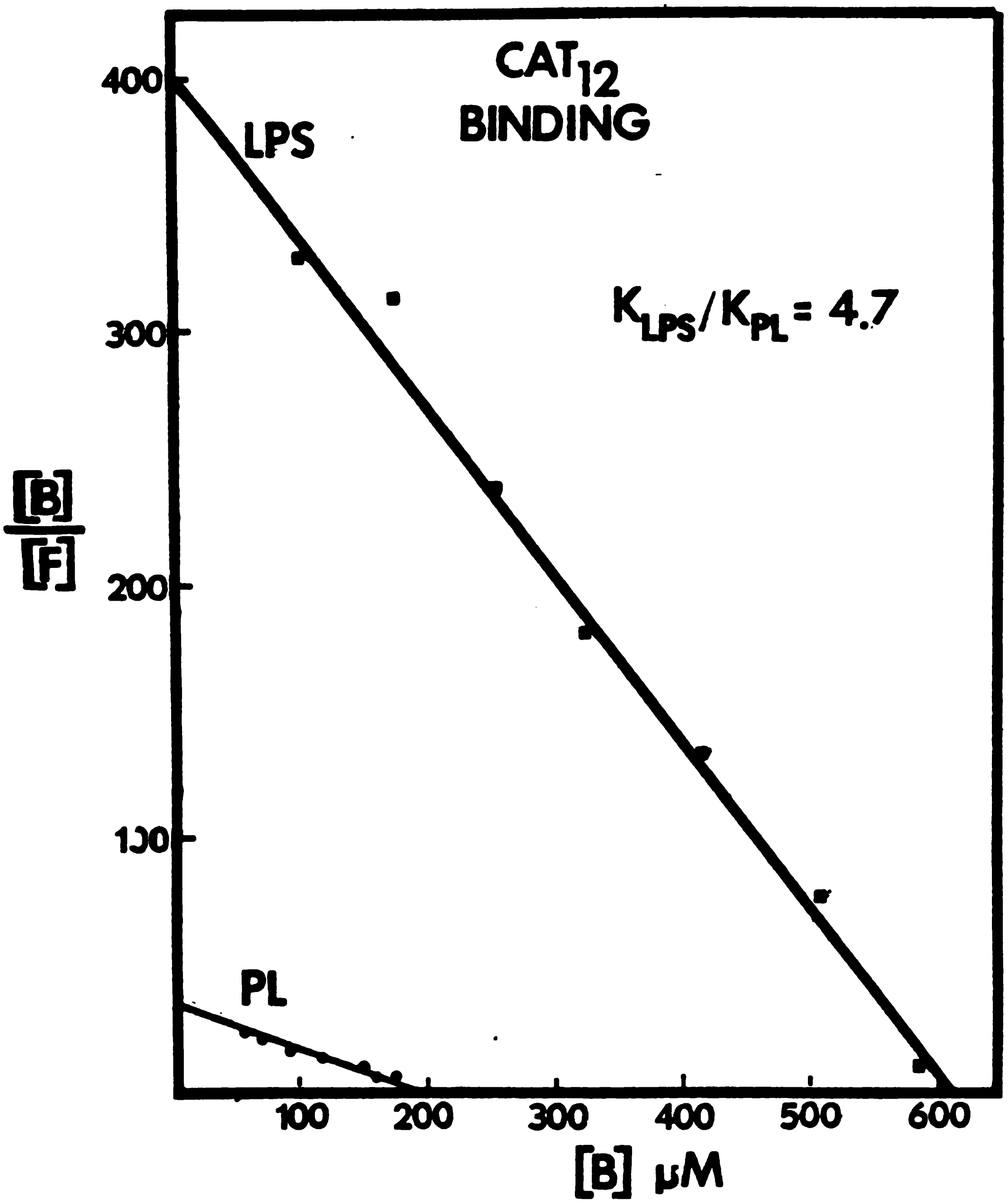

Figure 4. Scatchard analysis of CAT_{12} binding to electrodialyzed LPS (■) and to phospholipid (●) from the outer membrane of E. coli W1485F grown at 37°C. CAT_{12} partitioning was measured at 37°C on samples of LPS and phospholipid at 500 µM concentration as a function of probe concentration.

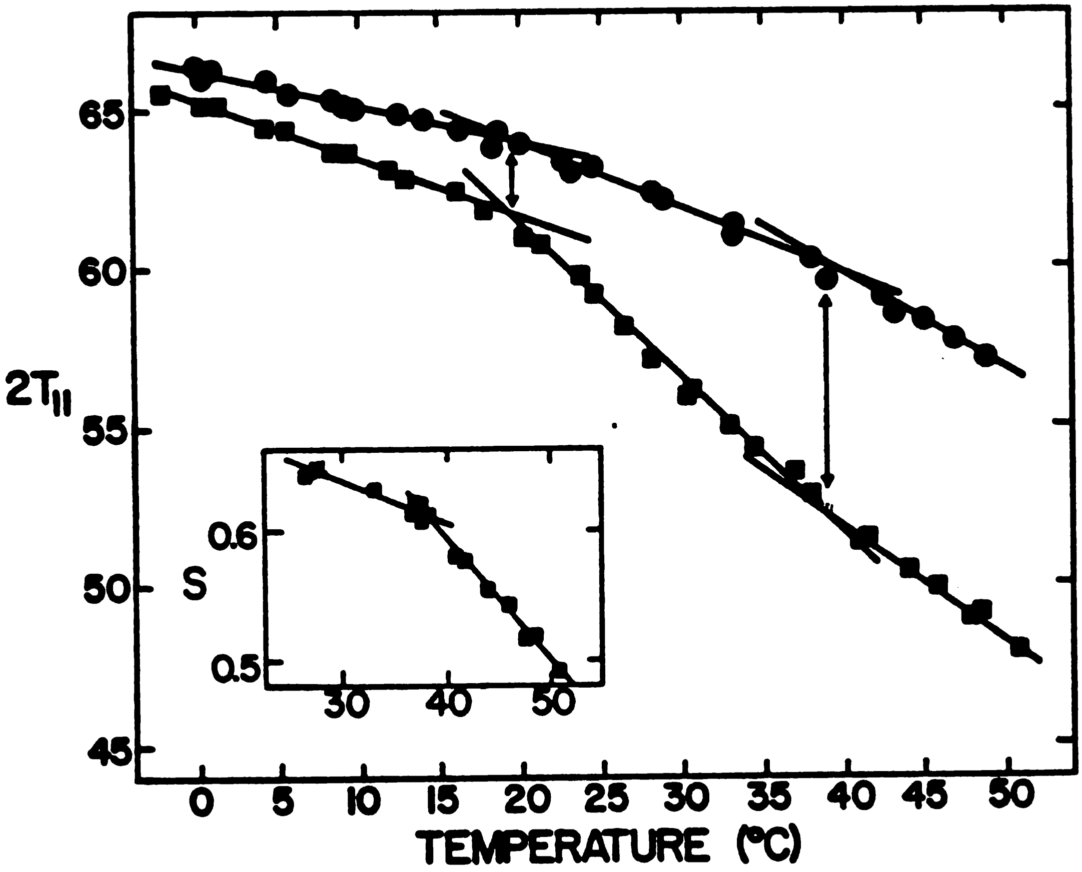

Figure 5. The temperature dependence of the hyperfine splitting parameter, 2T$_{11}$, and the order parameter, S, (Insert) of CAT$_{12}$ (●) and 5DS (■) bound to electrodialyzed LPS.

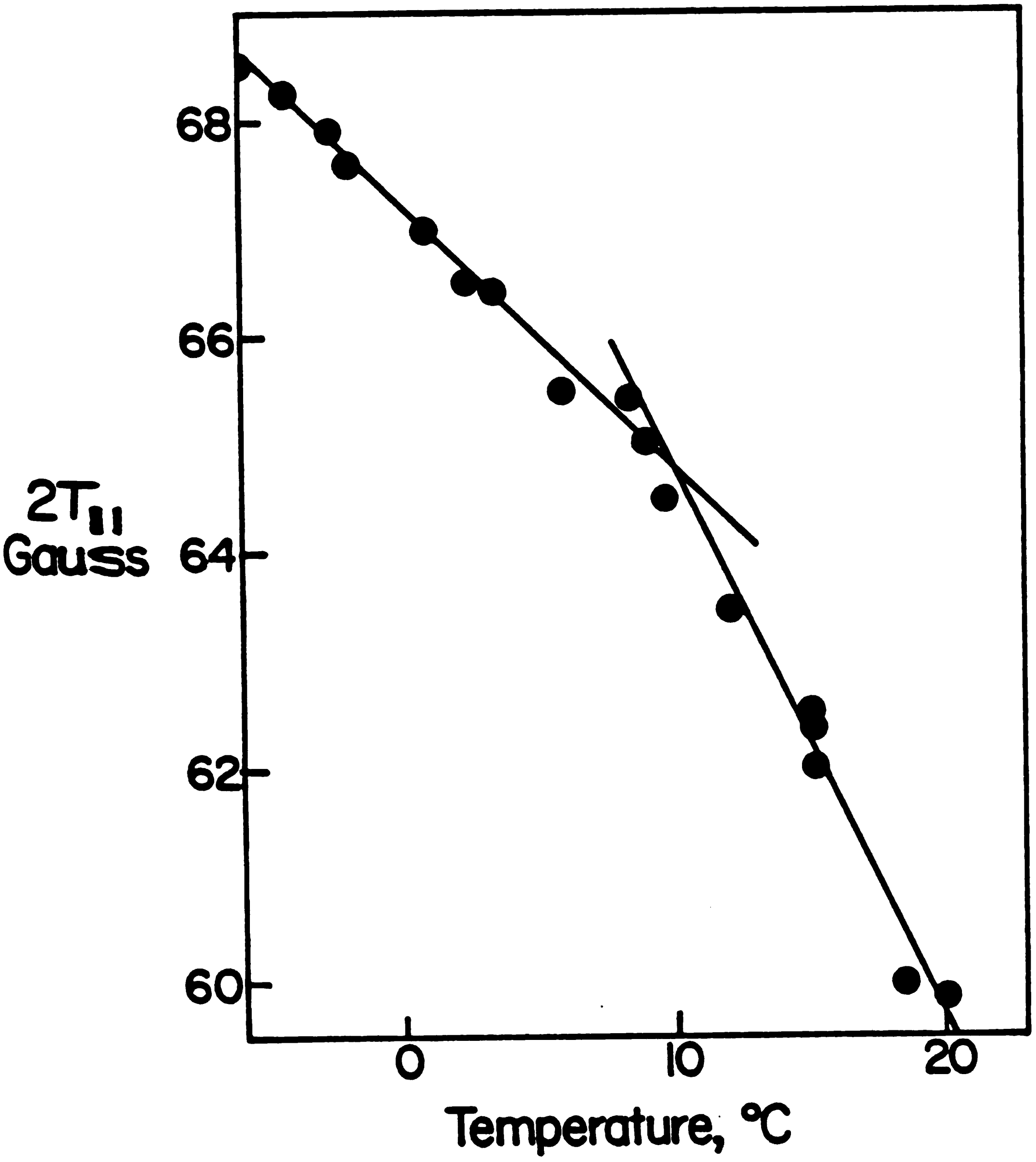

Figure 6. The temperature dependence of the hyperfine splitting parameter, $2T_{11}$ of CAT_{12} bound to porin-LPS-peptidoglycan complexes isolated from *E. coli* W1485F grown at 37°C.

CHAPTER VII

IONIC BRIDGING WITHIN LIPOPOLYSACCHARIDE AGGREGATES. AN ELECTRON SPIN RESONANCE, pH TITRATION STUDY

ABSTRACT

The head group packing of lipopolysaccharide (LPS) from *Escherichia coli* D21 and D21f2 was examined under conditions of varying hydroxide ion concentration. It was concluded that fixed charges in the core region contribute significantly to the surface potential of LPS aggregates, and that the polysaccharide core is a region of high polarity where the pK of ionizable groups may be altered substantially.

Using the electron spin resonance probe CAT_{12} as a reporter of the head group mobility, we analyzed the structure of sodium salts of LPS from both strain D21 and its heptoseless mutant strain D21f2. At neutral pH, the head group mobility of the magnesium salt of LPS from strain D21 was substantially less than that of the sodium salt. However, at high pH (pH 12) the two salts were approximately equally fluid. The changes at high pH were completely reversible precluding the possibility that alkaline hydrolysis of LPS had occurred. We have interpreted these results as an indication that ionic bridging stabilizes pure LPS aggregates and LPS domains in membranes.

INTRODUCTION

The charged nature of enteric LPS has already been amply discussed here (1) and in the literature (2). Ionizable groups such as phospho mono and diesters, carboxyl and amine groups as well as polar sugars all contribute to produce a complex three dimensional zone of high charge density. The capacity of this zone to bind counterions (e.g., metals, antibiotics) and form ionic bridges for long range cooperative interactions is unquestionably defined by the mutual interaction of charges within the zone.

In general, the pK of an ionizable group is strongly influenced by the polarity of its environment. When the ionizable group is fixed to a surface, it alters the environment of its neighbors by generating a surface potential Ψ_0. When the charges on a surface are uniformly and closely packed in a low ionic strength solution, they change the surface pH, pH_s (3). The relationship described by equation 1 assumes that protons behave as a diffuse double layer of counterions.

equation 1 $$pH_s = pH_{bulk} + \frac{e\Psi_0}{2.3\ kT}$$

The constant e is the electronic charge (-1.61×10^{-19} coul.), pH_{bulk} is the bulk pH, K is Boltzmann's constant (1.38×10^{-23} Joules/°K) and T is the temperature in degrees Kelvin (310 for our purposes).

Although this simple relationship is not fully applicable to a three dimensional zone of charge as is expected in the core region of LPS, the usefulness of more complex models are limited by the uncertain position of charges. Overall, the relationship described by equation 1 does provide a useful insight into the balance of electrostatic forces in the LPS core.

Romeo and coworkers (4) have given the cross sectional area of Salmonella typhimurium LPS in a monomolecular film at an air-water interface to be 232 ± 50Å^2. At the time it was thought that LPS was a polymer having 13.5 acyl chains. The corrected cross sectional area (assuming 6 acyl chains per LPS) is 103Å^2. From this we can compute the surface potential contribution from the three phosphorus of lipid A and the three carboxyl groups of the inner core. The surface potential is:

equation 2 $$\Psi_o = - \frac{0.0506 \text{ volts}}{Z} \ln (272\sigma)$$

where $Z = 1$ when we assign a single charge to each group and σ is the surface charge density (6/103Å^2). The contribution of just these inner core groups to the surface potential is -140 millivolts. From this we can calculate the change in pHs. Substituting Ψ_o into equation 1 yields an increase of about 2.3 pH units at the hydrophobic-hydrophilic interface of LPS. Thus, all ionizable groups distal to this interface may be expected to have their pK's shifted up by as much as 2.3 units. If the interface is reasonably planar, the surface potential influence on ionizable groups should fall off as 1/r.

Although this effect may seem large, it is actually very typical of the field influence of fixed charges on monolayers. The pK_a of acetic acid in aqueous solution is 4.75, while the apparent pK_a of arachidic acid micelles is greater than 9 (5). In this case, the close packing of fatty acids produced a Ψ_0 of more than -200 mV. Similarly, the phosphodiester group of phosphatidylinositol has a higher pK_a in monolayers than a phosphodiester free in bulk solution (6).

The simple relationship described above can be greatly complicated by the presence of neighboring polar groups and counterions. The influence of the carboxyl group of phosphatidylserine on its amine group is quite strong. Although the pK_a of the phosphodiester group of both phosphatidylethanolamine and phosphatidylserine are the same (pKa=4.2), the pKa of the amine group in phosphatidylserine is 1.6 units higher than that of phosphatidylethanolamine [pK_{NH_2} 7.8 and 9.4, respectively, (7)].

The influence of counterions depends strongly on the nature of their binding. Neither sodium nor potassium alter the pKs of most ionizable groups on phospholipids by much more than half a pH unit (6). In contrast, monovalent detergents such as sodium deoxycholate can greatly influence surface pH by virtue of their ability to intercalate between lipids and thus reduce their overall surface charge density. More important in the case of LPS are the effects of tightly bound polyvalent cations. McLaughlin and coworkers (3) found that when bimolecular lipid membranes were formed out of acidic lipids (e.g., phosphatidylserine) the observed decrease in the surface potential due to the binding of Sr^{2+} and Ba^{2+} was well described by the diffuse double layer model. When either Ca^{2+} or Mg^{2+} were used, however, it was necessary to consider some specific lipid-ligand interactions of the Stern type. Both Ca^{2+} and Mg^{2+} were able to effect a decrease in membrane

potential at a much lower concentration than either Sr^{2+} or Ba^{2+} by coordinating with anionic sites rather than simply screening those sites.

Emmerling *et al*. (8) observed a single pH titratable group on the native LPS of a smooth strain of *E. coli* B/r. The pH of the neutral product was approximately 8.0 and the lone titratable group in the alkaline range had an estimated pK of about 8.5. They assumed that the group they were titrating was an ethanolamine of the inner core of lipid A. The use of native LPS which undoubtedly contained a variety of polyvalent organic (amine containing) and inorganic cations certainly complicates the interpretation of their results. Furthermore, most published accounts of the chemical composition of enteric LPS agree that either phosphomono esters or diesters are the most prevalent ionizable groups. In addition three carboxyl groups are also present in the core of enteric LPS (9). Thus, it would be difficult to rationalize the pH of a neutral LPS suspension being just below the pK of an amine residue.

Olins and Warner (10) working with *Azotobacter vinelandii* have reported the pH titration of extracted LPS. Unfortunately, during isolation they treated their LPS with 0.1 M HCl (pH 2.2) which likely hydrolysed their LPS to lipid A and the polysaccharide chains were probably removed in subsequent purification. Interestingly though, they observed three titratable groups in their NaEDTA washed sample ($pK_1 = 1.3$; $pK_2 = 2.5$; $pK_3 = 6.5$). With what is known now of the chemical structure of lipid A, the first two ionizable groups might well have been phosphomono or phosphodiester and the last ionizable group a phosphomonoester.

The cation binding results presented in chapter III suggest that divalent cations may lower the surface potential of LPS from E. coli. This, in turn, may dramatically influence the electrostatic forces governing LPS-LPS self association. We report here evidence for the existence of multiple pH titratable groups above pH 7 in a suspension of NaLPS from E. coli D21 and of a single titratable group in the NaLPS from the heptoseless mutant D21f2.

RESULTS AND DISCUSSION

Our assumption is that at neutral pH the NaLPS from both strains D21 and D21f2 is close to but below the second pK of phosphomonoester groups. Although an accurate measurement of the pH of a highly viscous sample such as NaLPS is not possible, we have estimated by dilution that the pH of both D21 and D21f2 sodium LPS is close to 7. Figure 1 shows the change in the rotational correlation time τ_c of the ESR spin probe CAT_{12} bound to NaLPS from E. coli D21 LPS as a function of increasing hydroxide ion concentration. It is apparent that even below the actual LPS concentration, the addition of hydroxide ions has measurably increased head group mobility. We interpret this as an indication that at pH 7 D21 NaLPS is very close to the pK of one of its ionizable groups. As the added hydroxide ion concentration exceeded the LPS concentration, the rotational correlation time did not increase with further hydroxide ion addition. Apparently, no change in the net charge of the LPS core occurred until pH 11 (calculated) where head group mobility again increased dramatically. Further increases in head group mobility are evident at pH 12 and 12.5 (calculated).

The reported pK of ethanolamine in solution is 9.4 (11). Taking the surface potential contribution into account, amine group titrations in the pH range 11 to 12 are quite reasonable. The more distal the amine group is from the interface, the lower its apparent pK_a. The existence of up to three different ethanolamine groups on D21 LPS is supported by biochemical characterization performed by Prehm *et al*. (9) and our own ^{31}P NMR analysis (Figure 3).

Our belief that at pH 7 the NaLPS is below the pK of the second ionization of a phosphomonoester is supported by the work of Abramson *et al*. (12). They found that the pK of the second ionizable group of phosphatidic acid is about 8.6. In the presence of sodium, this pK is shifted down to 8.0.

Furthermore, Rosner *et al*. (13) have determined that the pK_a's for phosphomonoesters of LPS from a heptoseless mutant of an *E. coli* K12 strain using ^{31}P NMR ranged from 6.75 to 8.10. The pK_a of the monoester of the lipid A linked pyrophosphate group was 7.27. If the pH of our NaLPS is close to 7, it would seem quite reasonable that the first observed increase in head group mobility with increasing pH is the result of the double ionization of phosphomonoesters on LPS.

Although the influence of the surface potential within the core is likely to be unequally felt even by chemically equivalent groups, some simple assumptions about the charged state of NaLPS of D21 should be possible. At pH 7.0:

1) All ethanolamine groups are fully charged.
2) All KDO carboxyl groups are fully charged.
3) All phosphodiester and most phosphomonoesters are singly charged.

Analysis of the effect of pH in the magnesium salt of LPS from strain D21 was complicated by its low head group mobility at neutral pH. Thus the parameter 1/2 W (the low to mid field peak splitting) was used to compare the different salts. Calculation of the rotational correlation time for comparison with the sodium salt was not possible except at very high pH. As Table 1 indicates, at high pH 1/2 W is nearly identical in both the sodium and magnesium D21 LPS salts. The effect of high pH on the MgLPS was completely reversible upon neutralization with HCl, indicating that the increased head group mobility was not due to hydrolysis of acyl chains. The slight increase in head group mobility of Mg•LPS after neutralization can be accounted for by the presence of sodium ions in solution. Thus the quintessential factor governing LPS self-association at high pH is charge repulsion within the LPS core regardless of the counterions present.

Our results with the NaLPS of D21f2, a heptoseless deep rough mutant, were even more obvious. We found that head group packing of NaLPS from strain D21f2 increased abruptly above its isoelectric point and did not increase further. Since D21f2 LPS lacks ethanolamine groups (Figure 4) (2), we suggest that the lone titratable group seen in Figure 2 is that of the second pK of phosphomonoester groups.

The relatively broad (4.3 Gauss) low field linewidth of CAT_{12} in D21f2 NaLPS decreased to a minimum of 3.8 Gauss with added hydroxide ion concentration. Once the hydroxide ion concentration exceeded the LPS concentration, the low field linewidth increased dramatically. At least two models may explain this behavior. 1) At neutrality not all phosphomonoesters are singly charged. The substoichiometric addition of hydroxide ions reduced the heterogeneity of spin probe environments thus reducing Γ_{ℓ}. The subsequent increase in linewidth is the result of our inability to determine linewidths accurately on such fluid samples. 2) The dramatic increase in linewidth at the same hydrogen ion concentration that produces an equally dramatic increase in head group mobility are distinct events which both describe the partial double ionization of phosphomonoesters. Accordingly, the 6.0 Gauss linewidth just above the apparent pK_a of the phosphomonoester would be the result of spectral addition of rigid and fluid spin probe populations.

The preliminary nature of these experiments has prevented us from supporting either model. We were, however, encouraged by the precipitous drop in $2T_{\parallel}$ occuring at three times the actual LPS concentration. This is completely consistent with our expectation that the three phosphomonoesters of D21f2 carry a single charge at neutral pH.

Browning (14) has recently presented deuterium NMR results which substantiates the existence of proton transfer complexes between adjacent lipids having both anionic and cationic groups. The hydrogen-bonded ion pair suggested was of the type:

equation 3 $-PO_2 \cdot \cdot \cdot H - NH_2 -$

although other donor and acceptor combinations are possible in phospholipid dispersions. The existence of such a complex in phosphatidylethanolamine and phosphatidylcholine has previously been suggested on the basis of infrared spectroscopy measurements (15). The emerging model is that phospholipids with amine containing head groups are free to form "polymeric" two dimensional complexes with other adjacent amine containing lipids. Moreover, there is an additional

possibility for hydrogen bond formation with the carboxyl group of phosphatidylserine with its amine. Browning (14) has shown that the methyl ester of a phosphatidylserine may also form a proton transfer complex between its amine and an adjacent carbonyl group.

Increasing the intermolecular spacing of either phosphatidylcholine or phosphatidylethanolamine by addition of cholesterol, increased the rate of head group motion as detected by deuterium NMR (14). Since the addition of phosphatidylcholine to phosphatidylethanolamine did not increase head group mobility, it appears that proton transfer complexes between these lipids are sterically possible. Phosphatidylserine, however, was shown to be unaffected by either cholesterol or phosphatidylcholine addition. Thus, intramolecular hydrogen bond formation may also exist.

The functional role of a network of noncovalent, proton transfer complexes in LPS is only speculative. It is important to remember that native LPS typically contains many polyvalent cations which undoubtedly are more effective in competing for anionic groups than are the ethanolamine moieties of LPS. Furthermore, it has long been recognized that the addition of EDTA to gram negative membranes promotes the release of a large percentage of the LPS (16). Although the importance of divalent cations in stabilizing LPS aggregates is not in doubt, proton transfer complexes in LPS may have considerable biological importance. Recent biochemical characterization of polymyxin (a pentavalent amine containing antibiotic) resistant mutants of gram negative bacteria indicate that resistance may occur via alteration in the extent of amine substitution (sugar amines or ethanolamines) on LPS (17) modulating the amount of intermolecular complex stability.

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Table 1 Hyperfine Splitting of CAT_{12} in LPS Salts at 37°C

Preparation	1/2 W, Low to Mid Field Peak Splitting (Gauss)
NaLPS, pH 7	18.90
NaLPS, pH 12	18.65
MgLPS, pH 7	25.12
MgLPS, pH 12	18.95
MgLPS, pH 7 pH 12 pH 7	24.70
MgLPS, pH 7 + 58 mM NaCl	24.85

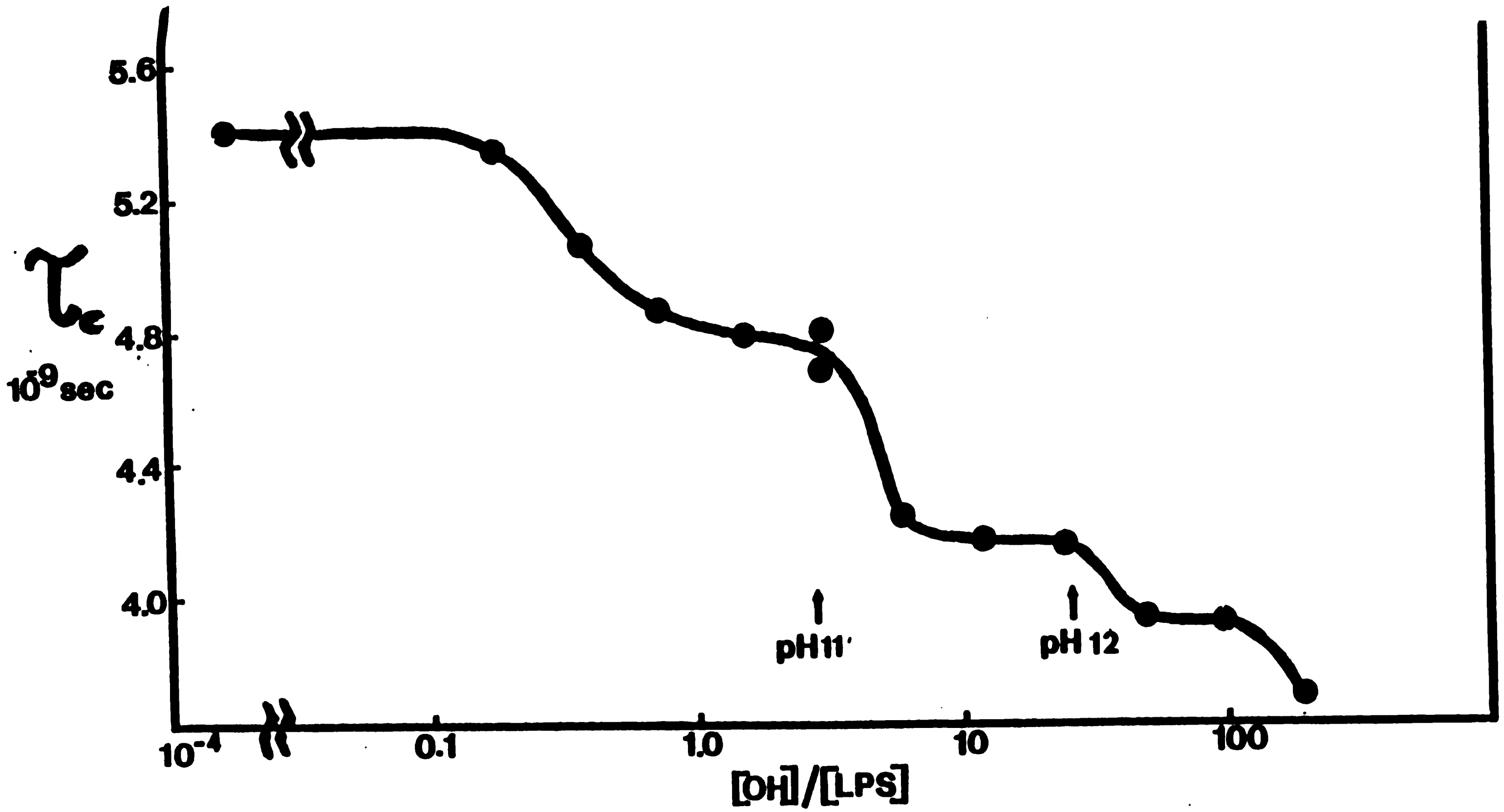

Figure 1. The pH dependence of the rotational correlation time (●) of CAT_{12} bound to NaLPS from E. coli strain D21. A 553 μM sample of NaLPS suspended in double distilled water was mixed with approximately 10 μM CAT_{12} (final concentration). The rotational correlation time of the probe was measured after the addition of increasing amounts of NaOH. The rotational correlation time of CAT_{12} is directly proportional to the mobility of the head group region of the LPS.

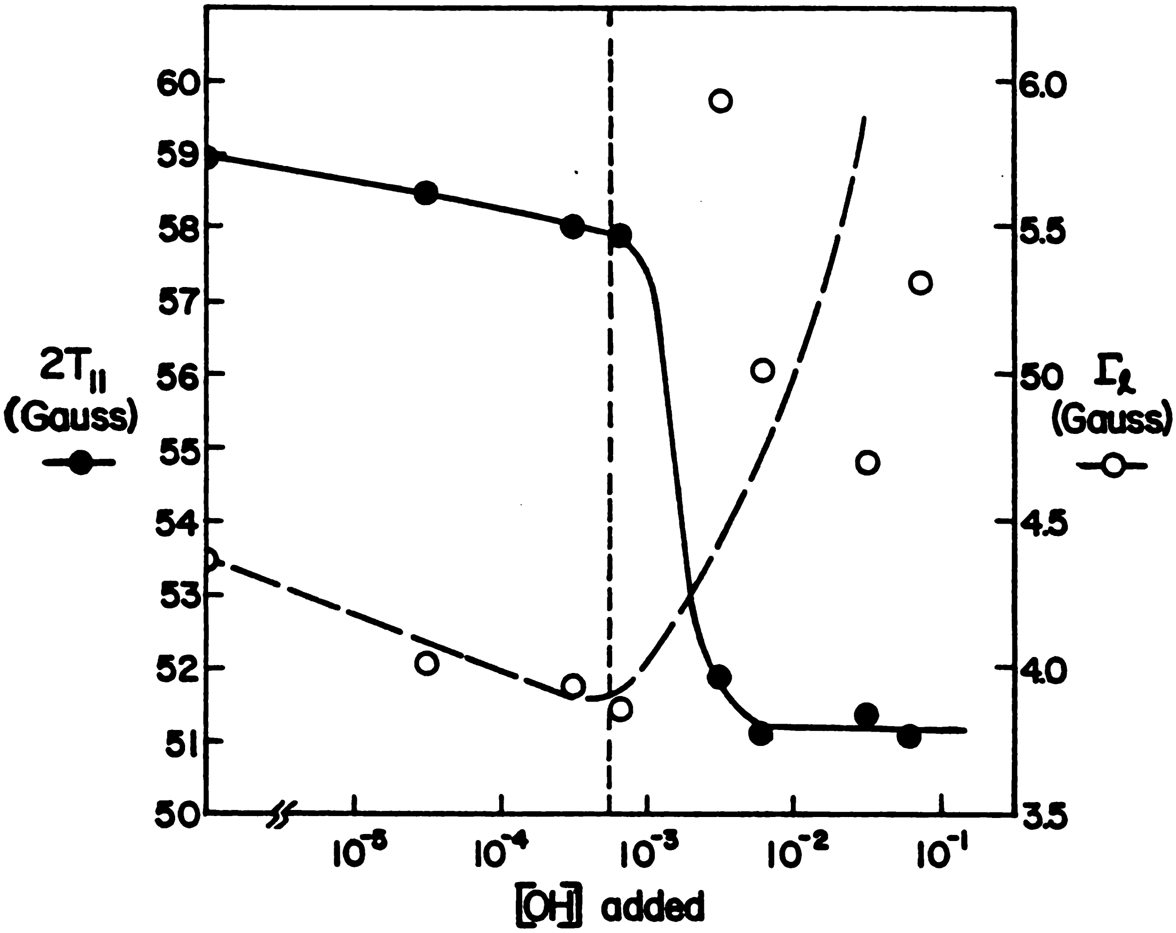

Figure 2. The pH dependence of the hyperfine splitting parameter, $2T_{\parallel}$, (●); and low field line width, Γ_ℓ, (○), of CAT_{12} bound to NaLPS from E. coli strain D21f2. A 553 µM sample of NaLPS suspended in double distilled water was mixed with approximately 10 µM CAT_{12} (final concentration).

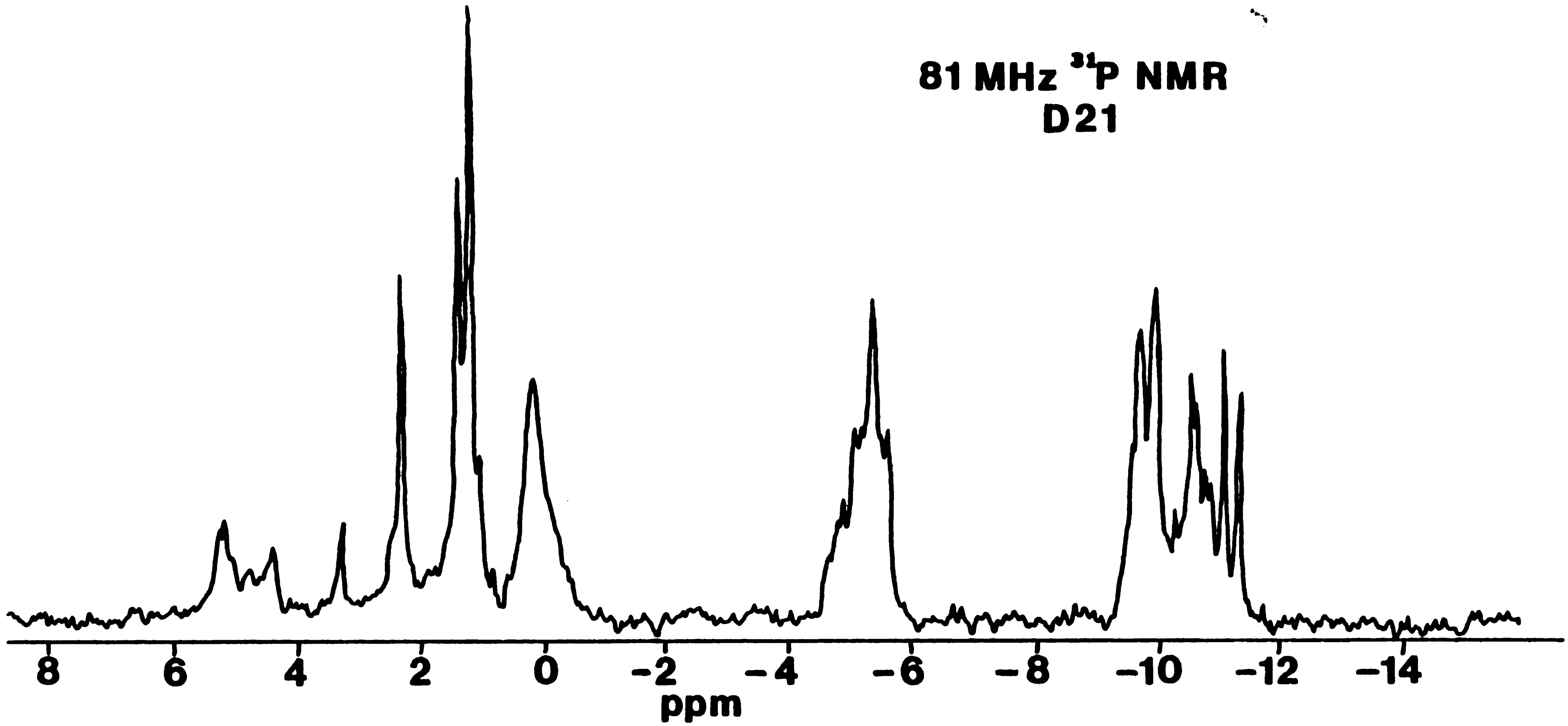

Figure 3. ^{31}P NMR spectra of the NaLPS of *E. coli* strain D21 taken at sodium deoxycholate at pH 8.0. The two high field extrema are from pyrophosphate groups which both mono (70%) and diester (30%). The spectra was taken by Dr. Redmond, School of Chemistry, Macquarie University, North Ryde, N.S.W., Australia.

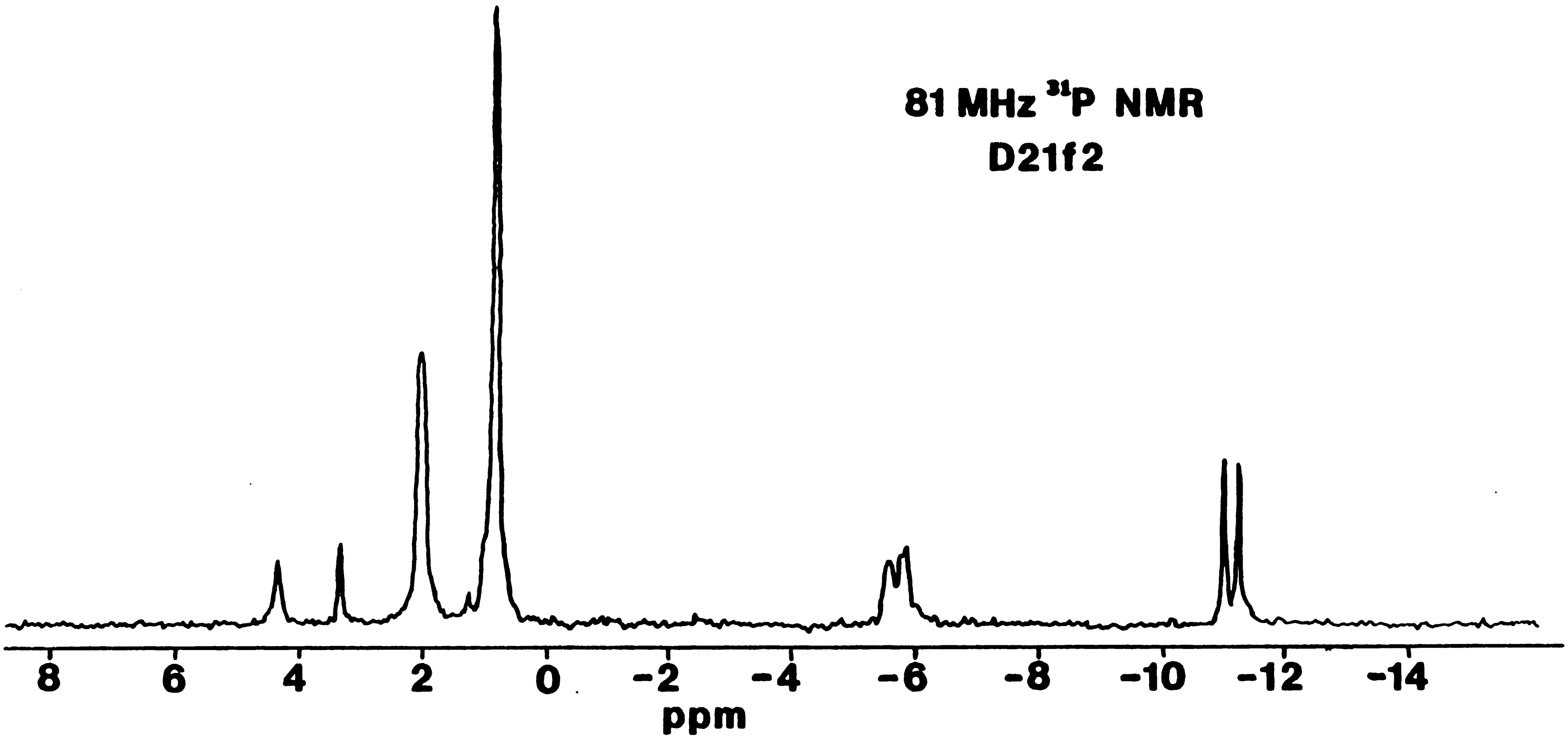

Figure 4. ^{31}P NMR spectra of the NaLPS of E. coli strain D21f2 taken at 81 MHz. NaLPS was dispersed by sodium deoxycholate pH 8.0. The pyrophosphates are completely unsubstituted. The spectra was taken by Dr. Redmond, School of Chemistry, Macquarie University, North Ryde, N.S.W., Australia.

CHAPTER VIII

PHYSICAL PROPERTIES OF _YERSINIA PESTIS_ LIPOPOLYSACCHARIDE MAY DETERMINE VIRULENCE

INTRODUCTION

It is difficult to begin a discussion of the physical properties of _Yersinia pestis_ lipopolysaccharide (LPS) without at least touching upon the historical importance of this pathogen.

> The initial shock, 1346-50, was severe. Die-offs varied widely. Some small communities experienced total extinction; others, e.g., Milan, seem to have escaped entirely. The lethal effect of the plague may have been enhanced by the fact that it was propagated not solely by flea bites, but also person to person, as a result of inhaling droplets carrying bacilli that had been put into circulation by coughing or sneezing on the part of an infected individual. Infections of the lungs contracted in this fashion were 100 per cent lethal in Maudiaria in 1921, and since this is the only time that modern medical men have been able to observe plague communicated in this manner, it is tempting to assume a similar mortality for pneumonic plague in fourteenth-century Europe.
>
> Whether or not pneumonic plague affected Europeans in the fourteenth century, die-off remained very high. In recent times, mortality rates for sufferers from bubonic infection transmitted by flea bites has varied between 30 and 90 per cent. Before antibiotics reduced the disease to triviality in 1943, it is sobering to realize that in spite of all that modern hospital care could accomplish, the average mortality remained between 60 and 70 per cent of those affected (1).

The role that LPS plays in the pathogenicity of _Y. pestis_ has only recently been addressed. Virulence has been linked to a 42 md plasmid which confers calcium dependence and the ability for cells to autoagglutinate (2). Virulent and avirulent strains grow normally at 25°C with or without calcium in their growth media. At 37°C, however, virulent strains are unable to grow in the absence of added calcium (2.5 mM).

Changes in the outer membrane protein profile have been reported to occur as a function of growth temperature in a virulent strain of Y. pestis (3). Nonetheless, these changes were not affected by the presence or absence of calcium.

More recently, temperature and calcium dependent changes in outer membrane proteins have been found in cal$^+$ (virulent) and cal$^-$ (avirulent) strains of Y. pestis (Dr. W. T. Charnetzky, personnel communication). The expression of these proteins on SDS-PAGE gels was found to be dependent upon the bound LPS. That is, LPS from calcium deprived cal$^+$ cells grown at 37°C did not influence protein migration whereas LPS from 37°C grown calcium supplemented cal$^+$ cells or cal$^-$ cells with or without calcium did alter the migration of the proteins. The new positions of the proteins and LPS cofactors were identical with the position of the proteins isolated from cells grown under the same conditions as the LPS cofactors themselves. Thus, the apparent differences in migration of these outer membrane proteins was a consequence of altered LPS and not altered proteins.

MATERIALS AND METHODS

Y. pestis nLPS was a gift of Dr. Willard T. Charnetzky of the Department of Bacteriology and Public Health, Washington State University, Pullman, Washington 99164. The bacteria were grown in broth consisting of: N-Z amine (3%), lactic acid (10 mM), $MgCl_2$ (2 mM), K_2HPO_4 (25 mM), citric acid (10 mM), $MnCl_2$ (0.01 mM), and $FeCl_2$

(1 mM). After autoclaving, xylose (10 mM), $Na_2S_2O_3$ (25 mM), and potassium gluconate (10 mM) were added. Calcium was removed from N-Z amine by treatment with sodium oxalate. Where specified, $CaCl_2$ (2.5 mM) was added. The medium was adjusted to pH 7.0 with 5.5 N NaOH.

Cells were grown aerobically at either 26 or 37°C as specified in shaken water baths. Cells were harvested at mid exponential phase. The hot aqueous phenol extraction procedure was used to isolate LPS. Electrodialysed and sodium salts of LPS were obtained as described in Chapter III. ICP analysis of LPS isolated was carried out as indicated in Chapter III. ESR spectra of probed samples were analyzed as in Chapter III with a probe to LPS weight ratio of 0.074.

RESULTS AND DISCUSSION

Although the endotoxin activity of *Yersinia* LPS is not thought to be of critical importance in the pathology of the bacteria, LPS may be a critical determinant of virulence. We considered LPS to be a logical candidate for the cell component responsible for the strong calcium requirement in virulent strains.

Indeed, the calcium levels in native LPS from cells supposedly grown in low calcium media are comparable to that of cells grown in calcium supplemented media (Table 1). This was true of LPS from cal^+ and cal^- cells. In fact, LPS from cal^+ cells generally bound calcium at levels equal to or greater than magnesium regardless of growth conditions. nLPS from cal^- cells, like nLPS of *E. coli* D21, consistently bound more magnesium than calcium. Calcium starved, 37°C grown cal^+ cells produced nLPS which was conspicuously low in its cation charge to phosphorous ratio. This was the direct result of exceptionally low sodium content.

The results of the elemental analysis of Y. pestis Na-LPS are summarized in Table 2. Unfortunately, the ashed samples of Na-LPS from cal$^+$ 37°C grown cells with added calcium and cal$^-$ 37°C grown cells without added calcium were heavily contaminated with sodium and aluminum from an unknown source.

The data do not indicate any major differences metals bound to in the Na-LPS correlated with the strain or growth conditions. The Na/P ratio of NaLPS from Y. pestis is, however, four times that from E. coli D21. Y. pestis LPS has been reported to have only three phosphorus per LPS whereas our analysis indicates E. coli D21 LPS has between seven and nine (4).

Figure 1 clearly shows that Na-LPS of Y. pestis grown at 37° and 25°C are distinctly different in their head group mobility. This type of temperature adaptation is opposite to that observed in the hydrocarbon interior of both prokaryotic and eukaryotic membranes (5). Typically, when cells are grown at lower temperatures, membrane lipids are altered so that their acyl chains become more fluid (6). Homeoviscous adaptation is still a controversial topic and the argument has never been proposed to extend beyond the membrane hydrocarbon interior into the polar head group region.

The significance of head group mobility adaptation in Y. pestis LPS may lie in the single departure from the pattern. Na-LPS from cal$^+$ Y. pestis grown at 37°C in the absence of calcium appears to have head group mobility intermediate between that of Na-LPS from 37 and 25°C grown cells. It is apparent that the critical adaptation of LPS characterized by increased head group mobility is incomplete in cal$^+$ cells shifted up from 26 to 37°C in the absence of calcium.

The nature of the differences in the chemical modification(s) between LPS from *Y. pestis* grown at 26°C and at 37°C may be quite subtle. We could not discern a pattern to the phosphorus to KDO ratio which could account for the observed pattern of LPS head group mobility. There were no perceptible differences in the migration pattern of any of the Na-LPS on 12% SDS PAGE gels (data not shown). There was, however, one additional ESR spectral parameter which correlated extremely well with growth temperature and may provide some insight as to the nature of the LPS modification.

Table 3 Low Field Line Width of CAT$_{12}$ in Na-LPS of *Yersinia pestis*

	26°				37°			
	Cal$^+$		Cal$^-$		Cal$^+$		Cal$^-$	
	-Ca^{2+}	+Ca^{2+}	-Ca^{2+}	+Ca^{2+}	-Ca^{2+}	+Ca^{2+}	-Ca^{2+}	+Ca^{2+}
Gauss	4.15	4.00	3.90	4.06	4.63	4.50	4.40	4.85

Mean	4.02 Gauss	Mean	4.60 Gauss
SD	± 0.10 Gauss	SD	± 0.19 Gauss
var	± 0.01	var	± 0.03

The low field linewidth, Γ_l, of CAT$_{12}$ bound to LPS, was measured for each NaLPS sample at 37°C. The results are summarized in Table 3. Simply, Γ_l of CAT$_{12}$ bound to LPS obtained from 26°C grown cells was significantly smaller than Γ_l of LPS from 37°C grown cells. No differences were observed which were correlated with the strain or the presence of calcium in the media. The narrow linewidth of Na-LPS from 26°C cells (ave. 4.0 G) was very typical of spectra characterized by classic lifetime broadening (7). The broader signal produced by Na-LPS from 37°C grown cells (ave. 4.6 G) may be indicative of a inhomogenous spin probe environment.

The CAT_{12} hyperfine splitting parameter of Na-LPS from 26 and 37°C was 4 Gauss. Although the observed line broadening is quite large, it is clearly not as large as would be expected by simple spectral addition of 26°C NaLPS and the rigid component of the 37°C NaLPS. Moreover for magnetically dilute spins, two spin probe populations which differ in their hyperfine splitting parameter by 4 Gauss would be resolved as separate low field peaks rather than as a broadened single peak.

Our conclusion from these results is that the head group structure of LPS from 37°C grown *Y. pestis* is more heterogeneous than LPS from 25°C grown *Y. pestis*, but the source of that heterogeneity is not known.

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Table 1. Elemental Analysis of *Yersinia pestis* Native LPS

	cal$^+$, calcium dependent				cal$^-$, calcium independent			
	26°C		37°C		26°C		37°C	
	no Ca^{2+}	$+Ca^{2+}$	no Ca^{2+}	$+Ca^{2+}$	no Ca^{2+}	$+Ca^{2+}$	no Ca^{2+}	$+Ca^{2+}$
Ca/P	0.28	0.31	0.29	0.32	0.20	0.04	0.10	0.17
Mg/P	0.22	0.25	0.20	0.33	0.25	0.31	0.38	0.40
Ca+Mg/P	0.50	0.55	0.49	0.65	0.45	0.35	0.48	0.57
Na/P	0.46	0.30	0.00	1.56	1.29	0.81	0.55	0.64
Fe/P	0.01	0.04	0.02	0.00	0.01	0.01	0.02	0.02
Al/P	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.02
Zn/P	0.01	0.00	0.02	0.07	0.09	0.08	0.03	0.03
mMP/g LPS	0.58	0.62	0.52	0.62	0.79	0.97	0.68	0.63
charge/P	1.56	1.59	1.11	3.07	2.42	1.72	1.66	1.95

Table 2. Elemental Analysis of *Yersinia pestis* Sodium LPS

	cal^+, calcium dependent				cal^-, calcium independent			
	26°C		37°C		26°C		37°C	
	no Ca^{2+}	Ca^{2+}	no Ca^{2+}	Ca^{2+}	no Ca^{2+}	Ca^{2+}	no Ca^{2+}	Ca^{2+}
Ca/P	0.01	0.07	0.04	0.11	0.04	0.02	0.09	0.03
Mg/P	0.01	0.06	0.00	0.05	0.03	0.02	0.05	0.03
Ca+Mg/P	0.02	0.13	0.04	0.16	0.07	0.04	0.14	0.06
Na/P	2.33	2.40	2.90	7.08	1.89	1.66	4.67	1.92
Fe/P	0.01	0.04	0.04	0.02	0.16	0.01	0.02	0.02
Al/P	0.03	0.06	0.03	0.81	0.03	0.02	0.60	0.02
Zn/P	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
mMP/g LPS	0.54	0.61	0.58	0.65	0.55	0.72	0.56	0.56
charge/P	2.46	2.96	3.20	9.89	2.60	1.80	6.81	2.16

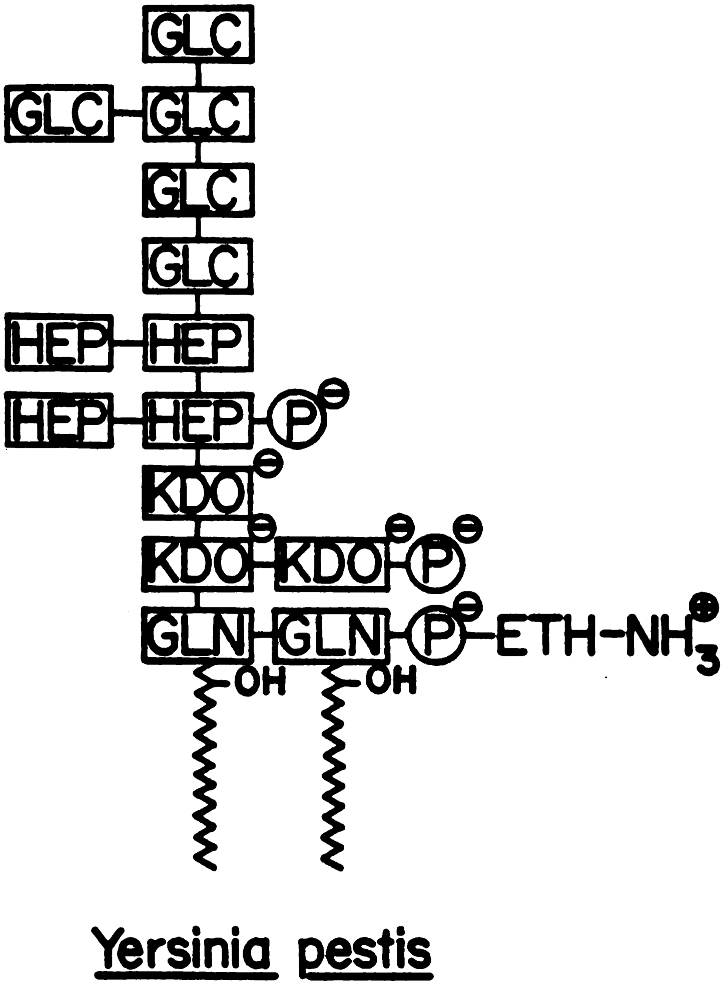


Figure 1. Chemical structure of Yersinia pestis LPS based on data obtained from reference 8.

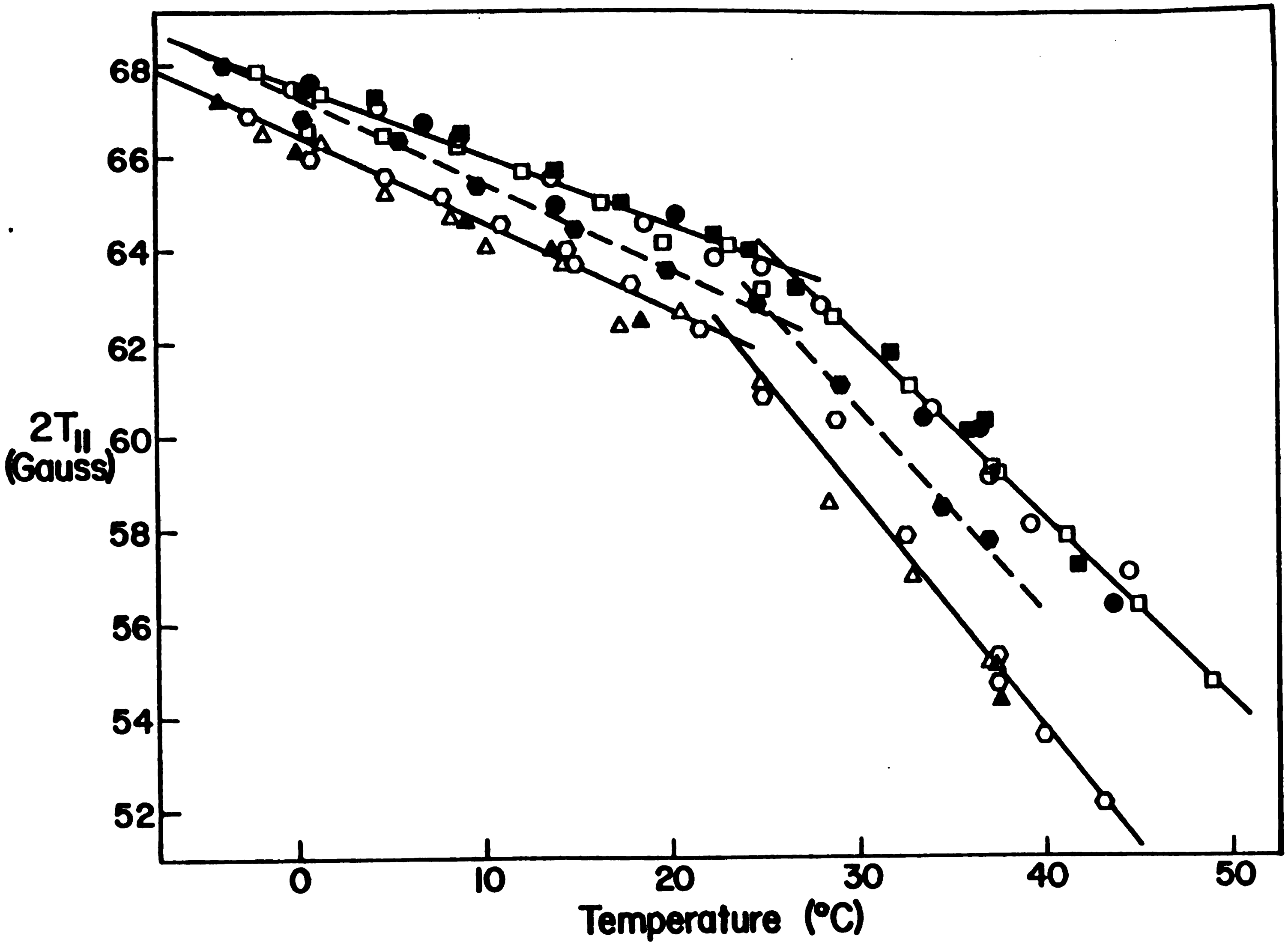


Figure 2. Temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$, of CAT_{12} in NaLPS from 37°C grown *Yersinia pestis* cal$^-$ with (△) and without (▲) supplemented calcium; and cal$^+$ cells with (☆) and without (★) supplemented calcium; and 26°C grown *Yersinia pestis* cal$^-$ with (□) and without (■) supplemented calcium; and cal$^+$ cells with (⬡) and without (⬢) supplemented calcium.

CHAPTER IX

CHARACTERIZATION OF THE PHYSICAL PROPERTIES OF CHROMATIACEAE LIPOPOLYSACCHARIDES

ABSTRACT

Lipopolysaccharides (LPS) isolated from Chromatrum vinosum and Thiocapsa roseopersicina were analyzed by inductively coupled plasma emission spectroscopy and electron spin resonance (ESR) spectroscopy. The LPS ionic compositions and fluidities were compared with those of rough LPS from enteric bacteria. LPS from both C. vinosum and T. roseopersicina contained a rather large number of cations on LPS weight basis despite low levels of phosphorus and acidic sugars. Calcium and sodium dominated all other metal cations in the elemental profile of C. vinosum native LPS (nLPS). LPS from T. roseopersicina had a higher phosphorus to weight ratio than LPS from C. vinosum and contained nearly equal amounts of magnesium and calcium. The total cation binding capacity of T. roseopersicina nLPS greatly exceeded that of enteric rough nLPS.

The ESR head group spin probe CAT_{12} indicated that the polysaccharide core region of Chromatiaceae nLPS was less restricted or rigid than D21 nLPS from E. coli. Treatment of Chromatiaceae nLPS with NaEDTA did not dramatically alter LPS head group mobility.

Surprisingly, the acyl chain mobility of _C_. _vinosum_ and _T_. _roseopersicina_ nLPS were identical over the temperature range -5°C to 50°C and an acyl chain structure transition was observed at 24 ± 9°C in both preparations.

Several mechanisms are presented which may account for the high cation binding capacity of _Chromatiaceae_ LPS. The significance of the physical properties of the lipopolysaccharide from these photosynthetic bacterial are discussed with regard to their low endotoxic activity.

INTRODUCTION

The recent biochemical analysis of LPS from the family _Chromatiaceae_ has greatly broadened the definition of LPS as a lipid class. These photosynthetic purple sulfur bacteria are composed of ten genera; the LPS of only two, _Chromatia_ and _Thiocapsa_, have been investigated extensively (1). Their LPS are characterized by a lipid A which has a D-glucosamine-D-mannose rather than the "classic" diglucosamine sugar backbone and are totally devoid of phosphorus. The fatty acid composition, however, is very much like that of enteric LPS. The polysaccharide chain is low in KDO content and certainly lacks the KDO trisaccharide cage common to enteric bacteria.

In addition, the core sugar L-glycero-D-mannoheptose found in enteric LPS is substituted with D-glycero-D-mannoheptose. This unusual sugar has also been detected in LPS from some _Yersinia_ and _Salmonella_ strains. In those strains, however, when the D isomer is present, the O-polysaccharide is synthesized but cannot be attached to the LPS core. _Chromatiaceae_ have apparently evolved the appropriate O-polysaccharide translocase and are able to assemble a complete LPS on to a core containing the D-heptose isomer. Recently analysis of LPS from smooth strains of Salmonella has revealed that approximately 60 mole percent of the total LPS is actually rough LPS (2,3). SDS-PAGE gels of both C. vinosum and T. roseopersicina indicates that both are composed of purely smooth LPS (4) and there is no indication of significant levels of a rough component.

The polysaccharide chains of LPS from both Chromatiaceae species also contain several amine containing sugars. C. vinosum LPS contains mannosamine and quinovosamine while LPS from T. roseopersicina has glucosamine, mannosamine, and 3-amino-3,6,-dideoxy-D-galactose (5). LPS from C. vinosum also contains only the D isomer of rhamnose while T. roseopersicina LPS has only the L isomer. Finally, LPS from C. vinosum is unique in that its O-polysaccharide contains the pentose sugars D-ribose (pyranosidic when internal, furanosidic when terminal), L-arabinose and 3-O-methyl-D-ribose. None of these sugars are present in T. roseopersicina or in enteric LPS.

The anticomplement activity of C. vinosum LPS has been reported to be only 25% that of S. abortusequi LPS (4). Like enteric LPS, alkaline hydrolysis of C. vinosum LPS significantly decreased anticomplement activity. In contrast, treatment with NaEDTA resulted in no change in endotoxic activity. Conversion of enteric nLPS to a sodium salt is usually accompanied by a sharp increase in endotoxic activity (6).

Although Chromatiaceae are not recognized pathogens, the unique biochemical composition of their LPS alone makes their further biophysical characterization worth while. It is easy to become transfixed by the volumous reports on enteric LPS and ignore the potential insight other LPS may supply. The results presented here show that this family of photosynthetic bacteria can greatly broaden our definition of bacterial endotoxins and perhaps provide new perspectives for understanding the pathogenicity of more conventional gram-negative species.

MATERIALS AND METHODS

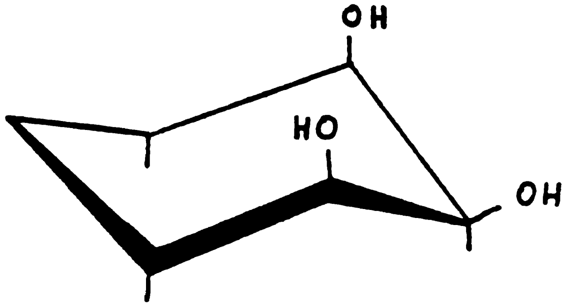
C. vinosum and *T. roseopersicina* were the generous gift of Dr. Hurlbert, Department of Biochemistry, Washington State University. Inductively coupled plasma emission spectroscopy and electron spin resonance techniques were performed as before (Chapter III).

RESULTS AND DISCUSSION

Despite the low phosphorus and acidic sugar content of *C. vinosum* LPS, a considerable number of cations were detected bound to the native LPS (Table 1). We can not rule out the possibility that many of these cations simply carried over during the LPS isolation through some undetermined mechanism (e.g., intrapment within LPS aggregates). There is, however, at least one nontrivial explanation for this behavior.

Neutral sugars can form stable complexes with polyvalent cations when two or three hydroxyl groups of a pyran ring are stereochemically arranged to fit into the coordination sphere of the cation. Sugars possessing the *ax*-*eq*-*ax* sequence of oxygen atoms on the six-membered ring (Figure 1) have this ability (7).

Figure 1



Pyran ring containing the ax-eq-ax sequence.

The coordination geometry for neutral sugars is much less flexible than that of acidic sugars such as KDO. Although KDO has a slightly higher affinity for calcium than magnesium (8), the specificity of neutral sugars is much greater. A marked preference for cations of 1Å ionic radius (La^{3+}, Ca^{2+}, Na^{+}) has been demonstrated (9). For example, α-D-allopyranose has a stability constant for calcium of 6.2/mole and only 0.19/mole for magnesium. It is interesting to note that C. vinosum nLPS retained substantially more calcium and sodium than magnesium (Table 1).

Calcium and sodium were both present in ten fold excess over magnesium in nLPS from C. vinosum. As Table 1 indicates, this is quite uncommon in other gram negative nLPS. It should be pointed out though, that the enterics listed have exclusively rough LPS while the Chromatiaceae LPS is exclusively smooth. Together, the bound cationic charge to LPS weight ratio of C. vinosum nLPS was comparable to that of enteric nLPS.

An analysis of the reported O-polysaccharide sugars of C. vinosum shows that β-D-rhamnose and β-ribopyranose have the required ligand geometry which would preferentially bind Ca^{2+} and Na^{+}.

The elemental analysis of C. vinosum LPS also confirmed the early observation by Hurlbert et al. (5) that the phosphorus content is quite low (Table 1). In fact, we have calculated the minimum molecular weight of monomeric LPS required for 1:1, phosphorus to LPS molar ratio, to be 41,000. Although this is not an entirely unreasonable value in light of the smooth nature of C. vinosum LPS, it almost certainly rules out the possibility of more than one phosphorus per LPS.

Although LPS from T. roseopersicina was shown to have a much higher phosphorus to LPS weight ratio than C. vinosum LPS, it was lower in phosphorus than enteric LPS (Table 1). This may actually be a reflection of smooth character and high molecular weight of T. roseopersicina LPS, compared to the LPS of the enteric species. Furthermore, T. roseopersicina retained an exceedingly large number of cations bound to its nLPS. The total cationic charge to LPS weight ratio was almost twice that of the rough E. coli strain D21. This becomes even more significant when it is remembered that the contribution of the polysaccharide chain in the LPS of T. roseopersicina to its total weight may be as much as 90%. Unlike LPS from C. vinosum, T. roseoperisicina LPS contained about as much magnesium as calcium. Thus, neutral sugars can not totally account for the high cation binding capacity. It is suggested that the bound Mg may be interacted with the phosphate groups in nLPS from T. roseoperisicina whereas nLPS from C. vinosum lacking such phosphate, also lacks Mg binding sites.

Our electron spin resonance experiments produced equally surprising results. In Chapter III we showed that the lipid head group spin probe CAT_{12} has a rather high affinity for specific sites in E. coli LPS suspensions. We suggested that likely sites for CAT_{12} binding were the phosphorus of lipid A. Melhorn et al. (10) have shown that CAT_{12} has high water solubility and very low affinity for neutral phospholipids regardless of the fluidity of their acyl chains. Nevertheless, the binding affinity of CAT_{12} for LPS of the family Chromatiaceae was quite high. At the same CAT_{12} to LPS weight ratio used with enteric LPS from E. coli, little free probe signal was evident in the LPS from Chromatiaceae in the temperature range -5 to 37°C. Since the lipid A moiety of LPS from both C. vinosum and T. roseopersicina has been reported to be devoid of phosphorus, the high CAT_{12} affinity was unexpected.

The hyperfine splitting parameters, $2T_{\parallel}$, of bound CAT_{12} indicated that the nLPS of both C. vinosum and T. roseopersicina were less restricted in head group mobility than nLPS from E. coli D21. In addition, the head group of LPS from T. roseopersicina was less restricted than that of C. vinosum (see Figures 2 and 3). Interestingly, electrodialysis and NaEDTA treatment of C. vinosum and T. roseopersicina nLPS did not alter head group mobility. This observation is inconsistent with the usual rigidifying effect of polyvalent cations on anionic lipids (11). It is possible that divalent cations are intramolecularly bound and thus have a minimal effect on LPS-LPS interactions of these species or at least have a minimal effect on the CAT_{12} binding site at the polar-nonpolar interface. Our explanation for the location of calcium in C. vinosum is consistant with either of these hypothesis.

Hurlbert has also observed that the lethality of C. vinosum nLPS is unaffected by treatment with NaEDTA (4). This is in marked contrast the with finding that the sodium salt of enteric LPS has increased endotoxic activity when compared to nLPS (6).

It is apparent from Figure 4 that the hydrocarbon interior of nLPS from Chromatiaceae are more disordered than that of nLPS from E. coli

D21. The nLPS from both species of Chromatiaceae appear to undergo an acyl chain melt at 24 ± 9°C. The similarity of both the 5DS hyperfine splitting parameter and order parameter data for C. vinosum and T. roseopersicina nLPS is, however, somewhat surprising. The two species do have very similar Lipid A moieties, differing only in the presence of oleic acid and β-hydroxycapric acid in T. roseopersicina. Nevertheless, the large difference in polysaccharide composition, phosphorus content, metal ion profile and head group mobility make the similarity in acyl chain mobility difficult to explain. We have observed identical acyl chain mobilities in the sodium salts of E. coli D21 and D21f2 LPS (12). Here, too, substantial differences exist between these two lipids in both head group mobility and polysaccharide composition. Furthermore, the relative insensitivity of head group mobility in either C. vinosum or T. roseopersicina to the loss of divalent cations suggests that the differences in cation composition are inconsequential in the packing of either the polysaccharide or acyl chains. We are left with the conclusion that head group and acyl chain mobilities may often be regarded as separate phenomena.

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12. Chapter V.

Table 1 Elemental Analysis of Native Lipopolysaccharide from Several Gram Negative Bacteria

mM Cation/g LPS	Chromatia vinosum	Thiocapsa roseopersicina	Escherichia coli D21	Salmonella* G30	Yersinia pestis cal
Ca/g LPS	0.25	0.56	0.15	0.04	0.11
Mg/g LPS	0.02	0.49	0.40	0.56	0.25
Ca+Mg/g LPS	0.27	1.05	0.55	0.60	0.35
Na/g LPS	0.32	0.26	0.11	0.51	0.40
Fe/g LPS	0.01	0.00	0.05	0.01	0.01
Al/g LPS	0.09	0.01	0.01	---	0.01
Zn/g LPS	0.00	0.00	0.00	---	0.02
P/g LPS	0.0245	0.397	1.21	1.17	0.62
cation charges/g LPS	1.07	2.39	1.39	1.75	1.01

*Assume 7 P/LPS and 6000 M.W.

Salmonella G30 LPS had been electrodialyzed. It was the gift of Dr. M. Schindler, Biochemistry Department, Michigan State University.

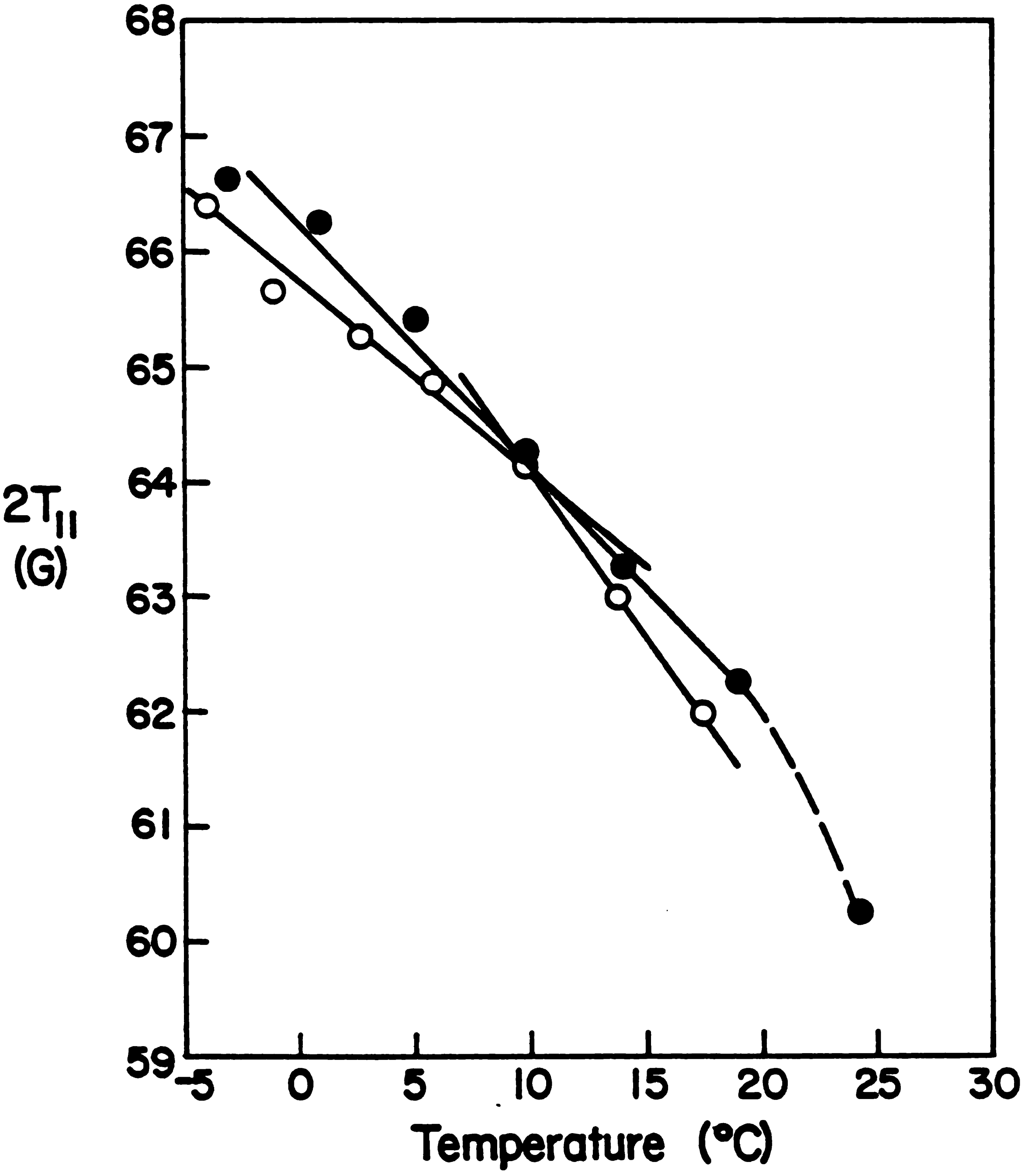

Figure 2. Temperature dependence of the hyperfine splitting parameter $2T_{\parallel}$ of CAT_{12} in *Chromatia vinosum* native LPS (○) and sodium LPS (●). The probe to LPS weight ratio was 0.074.

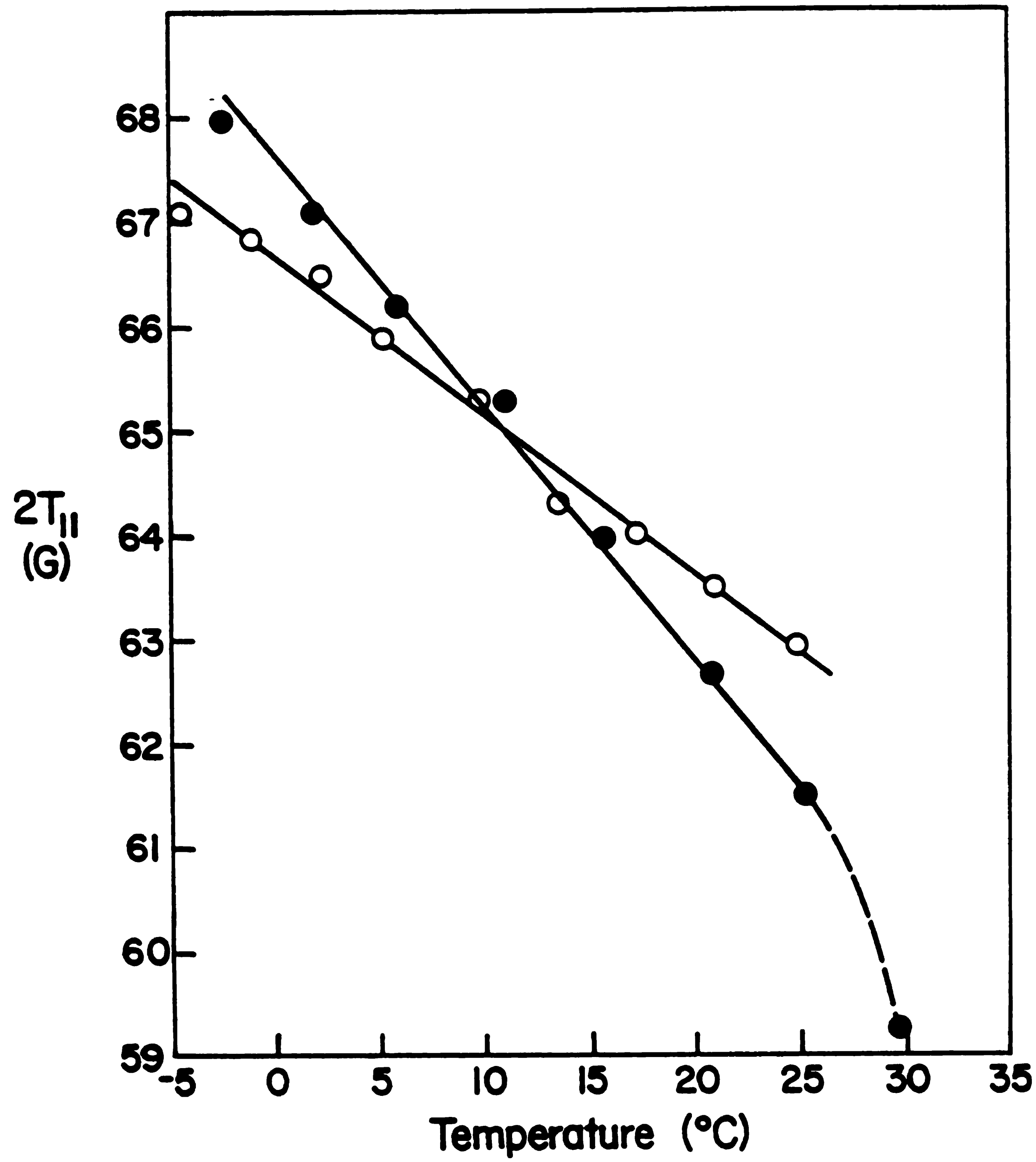

Figure 3. Temperature dependence of the hyperfine splitting parameter $2T_{\parallel}$ of CAT_{12} in Thiocapsa roseopersicina native LPS (○) and sodium LPS (●). The probe to LPS weight ratio was 0.074.

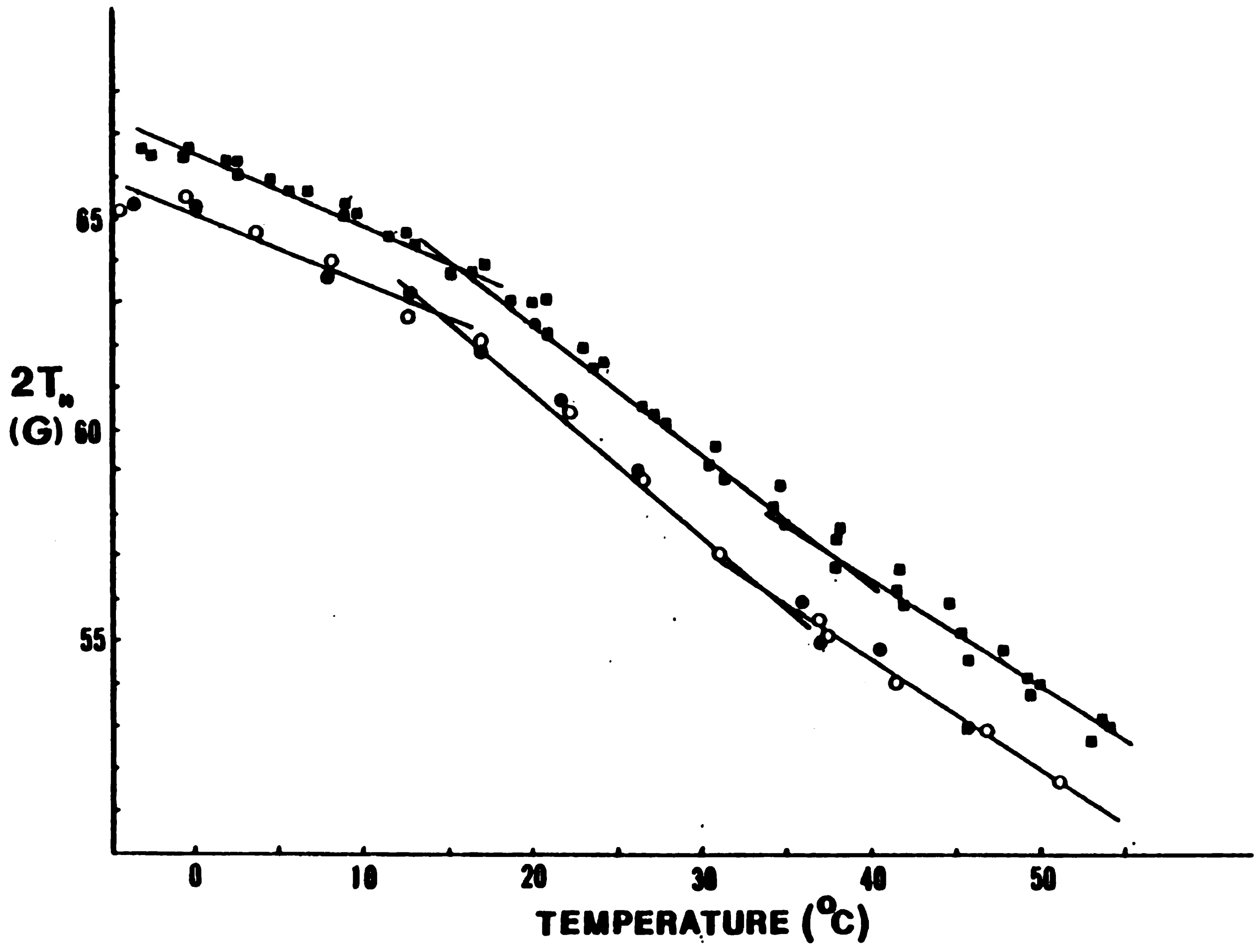

Figure 4. Temperature dependence of the hyperfine splitting parameter $2T_{\parallel}$ of 5DS in *Chromatia vinosum* (●) and *Thiocapsa roseopersicina* (○) nLPS. The probe to LPS weight ratio was 0.074. Data for *E. coli* D21 nLPS (■) is included for reference.

APPENDIX A

USE OF ELECTRON SPIN RESONANCE TO STUDY BACILLUS MEGATERIUM SPORE MEMBRANES*

SUMMARY

Membranes from dormant and heat-activated spores were labeled with the fatty acid spin probe 5-doxyl stearate and analyzed using electron spin resonance spectroscopy. Membranes from dormant spores were slightly less fluid above 23° than membranes from heat-activated spores. Also L-proline caused a much larger increase in the upper transition temperature than did D-proline when added to membranes from heat-activated spores. Thus a compound known to trigger germination in this strain may interact stereospecifically to alter the biophysical properties of the spore membranes.

INTRODUCTION

The mechanism of breaking the dormant state of bacterial spores is not known. In *B. megaterium* QM B1551, rapid germination occurs if the spores are first heat-activated followed by the addition of a

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*Published in Biochem. Biophys. Res. Commun. (1981) 100 (3), 1137-1142.

stereospecific compound like L-proline (1). One model to explain these processes suggests the spore membrane(s) may be involved (2), and to study this possibility we have used electron spin resonance (ESR) spectroscopy. We report here what appears to be the first demonstration of biophysical changes in spore membranes that result from heat-activation and L-proline.

MATERIALS AND METHODS

B. megaterium spores were grown in supplemented nutrient broth, harvested and stored as previously described (3). All references to spore weights are on a dry weight basis. Spores (50 mg/ml) were extracted with sodium dodecyl sulfate-dithiothreitol and then washed as previously described (4). The details of the isolation and characterization of spore membranes will be described elsewhere (Racine, F.M. and Vary, J.C., in preparation) but briefly the methods were as follows. Sodium dodecyl sulfate-dithiothreitol extracted spores (4) were lysed in 0.1 M HEPES (pH 7.5) containing lysozyme (0.5 mg/ml), RNase (2.5 µg/ml) and DNase I (2.5 µg/ml) at 30° for 12 min followed by sonication (8 times for 30 s each) at 0° as previously described (4). Spore membranes were isolated by methods similar to those used for the isolation of E. coli cytoplasmic membranes (5). Membranes from heat-activated spores were obtained by first heating spores (50 mg/ml) at 60° for 10 min, followed by centrifugation at 5,000 x g for 10 min and then lysis as described above. The final membrane preparation in 10 mM HEPES (pH 7.5) contained 10-20 mg of protein/ml as determined by the method of Lowry et al. (6) and was stored on ice for further analysis.

All ESR studies were done with freshly prepared membranes (0.4 ml) to which 4-5 μl of 30 mM 5DS was added. The labeling techniques that were used have been previously described (7). ESR spectra of such spin labeled preparations allow the determination of the hyperfine splitting parameter, $2T_{\parallel}$, which reports the local fluidity of the membrane lipids (8). High values of $2T_{\parallel}$ reflect low fluidity. All ESR studies were carried out with a Varian Century Line ESR spectrometer, model E-112, equipped with a variable temperature controller. An external calibrated thermistor probe (Omega Engineering, Inc., Stamford, CT) was used to monitor the temperature of the sample. The data ($2T_{\parallel}$ vs. temperature) were analyzed by an iterative least squares program to be described elsewhere (Coughlin, R.T., Brunder, D.G., and McGroarty, E.J., in preparation). Briefly, a B-spline (9) was used to provide a smooth fit for the ESR data and points of inflection were used to group data. Regressions lines were calculated for each group and then plotted. This analysis allowed the determination of break points. Such breaks in the temperature dependence of $2T_{\parallel}$ have been correlated with lipid phase separations or lipid phase transitions from gel to liquid crystalline lipid states (7). All temperature dependent ESR parameters were shown to change reversibly up to 46°. For each set of data, at least 3 independently isolated membrane preparations were used.

DISCUSSION

Membranes isolated from either dormant or heat-activated spores had the same phospholipids and in the same ratios as whole spores, similar to previously published data for total phospholipids in this strain (10).

The membranes contained no peptidoglycan, a distribution of about 20 proteins ranging from 13,000 to 130,000 daltons, several respiratory associated enzyme activities and a unique carotenoid (unpublished).

The temperature dependence of $2T_{\parallel}$ in membranes from dormant spores is illustrated in Figure 1. Of particular importance are the transition temperatures where the slopes of the lines change at 6° and 26° suggesting a change in the relative ratio of gel and liquid crystalline lipid. When the same experiment was done with membranes from heat-activated spores (Figure 2a) the transition temperatures were slightly different, 7° and 23°. The more noticeable difference, however, is that above the upper transition temperature membranes from heat-activated spores exhibit a greater slope (-0.29 gauss/°C) than do dormant spore membranes (-0.25 gauss/°C). This implies that above the upper transition, membranes from heat-activated spores are more fluid than those from dormant spores. We feel that this change in fluidity should be interpreted with caution with respect to any possible functional role. But it is apparent that there is a physical difference between membranes from dormant and heat-activated spores which to date has never been reported.

Finally, we tested the effect of adding <u>in vitro</u> a known trigger reagent, L-proline, to membranes from heat-activated spores. The results (Figure 2) show a dramatic change in the upper transition temperature from 23° before to 31° after the addition of L-proline. When the same experiment was done with D-proline (30 mM) which cannot trigger germination (1), a much smaller shift occurred in the upper transition temperature (from 23° to 27°). Preliminary experiments suggest that the addition of L-proline to dormant spore membranes caused no dramatic changes in the temperature dependence of $2T_{\parallel}$. In all of these experiments the low transition temperature did not change significantly. From these results, it is apparent that L-proline interacts with the membrane and causes a change in the supramolecular structure. We have no evidence that the transition temperature shift to 31° is fortuitous or significant with respect to 30° being optimal for triggering germination. The important point is that L-proline may interact with membranes from heat-activated spores in a stereospecific manner to cause a biophysical change in the spore membrane.

While these data do not explain the mechanism of triggering germination, techniques described here provide us with a useful tool to further analyze other trigger compounds, membranes isolated at different times during sporogenesis and to determine the role of spore membranes in development. Of particular interest are the recent data with a proline affinity analog (Rossignol, D.P. and Vary, J.C., submitted) which indicates a possible method to isolate the proline trigger site. Using these present and other biophysical techniques, we hope to study the interactions of L-proline with the trigger site both _in vitro_ and _in vivo_.

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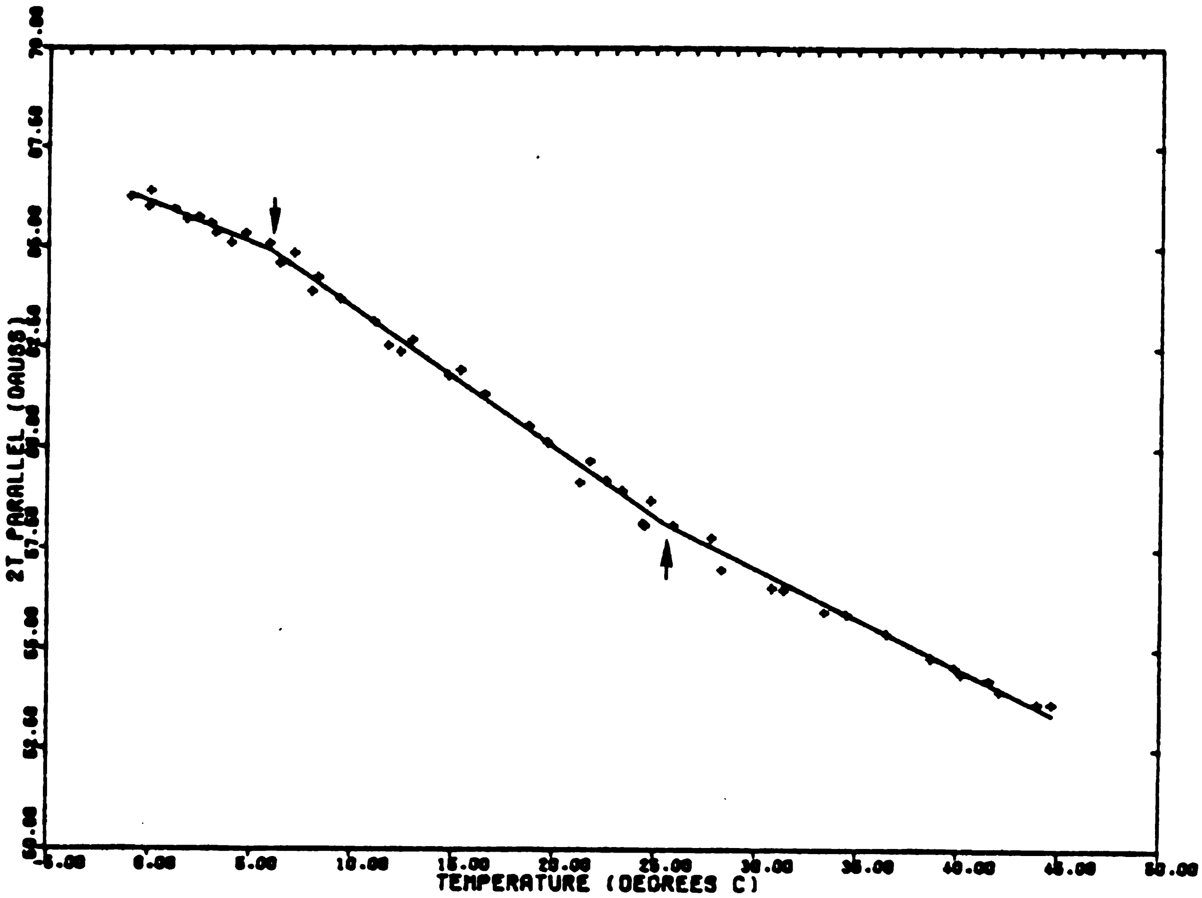
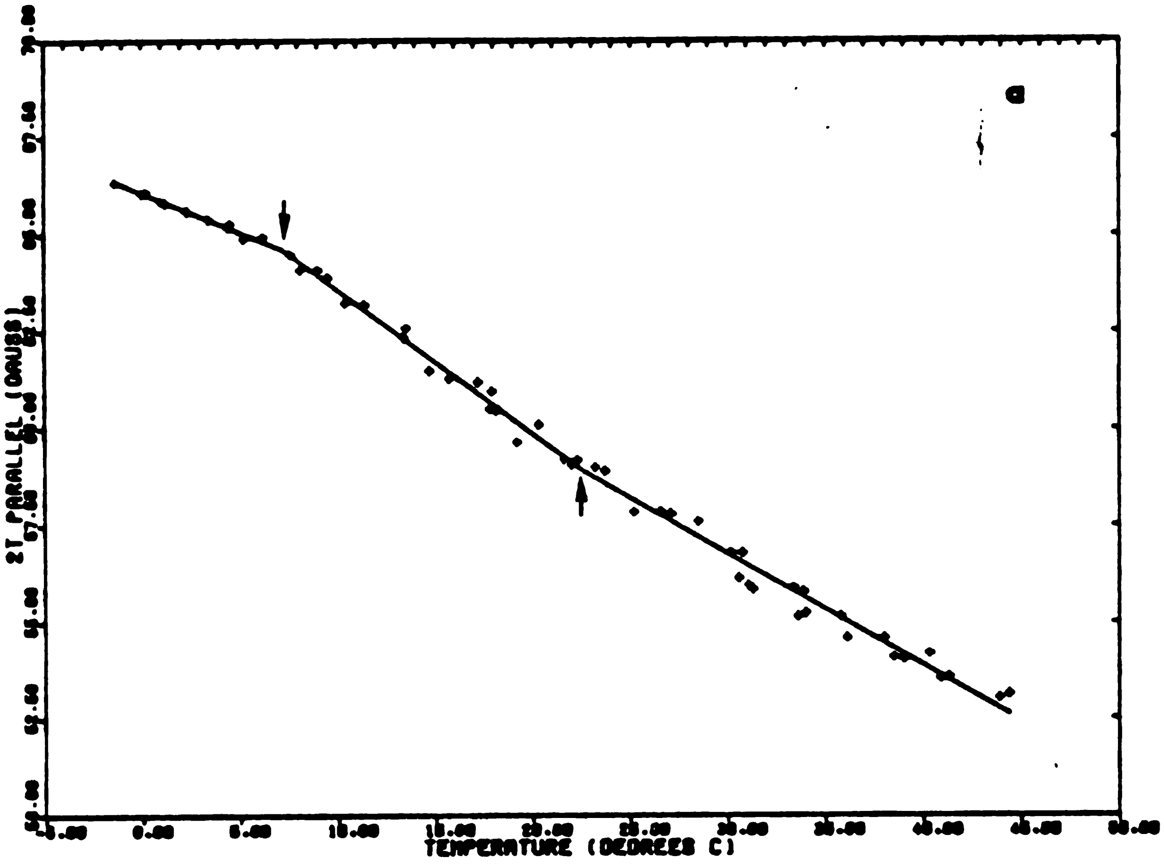


Figure 1. Temperature dependence on $2T_{\parallel}$ in dormant spore membranes. Membranes were isolated and ESR spectra recorded at the indicated temperatures in the presence of 5 DS as described in Materials and Methods. Arrows indicate transition temperatures.

Figure 2. Effect of L-proline. Membranes from heat-activated spores were isolated and analyzed as described in the legend of Figure 1 in the absence (a) and presence (b) of 30 mM L-proline.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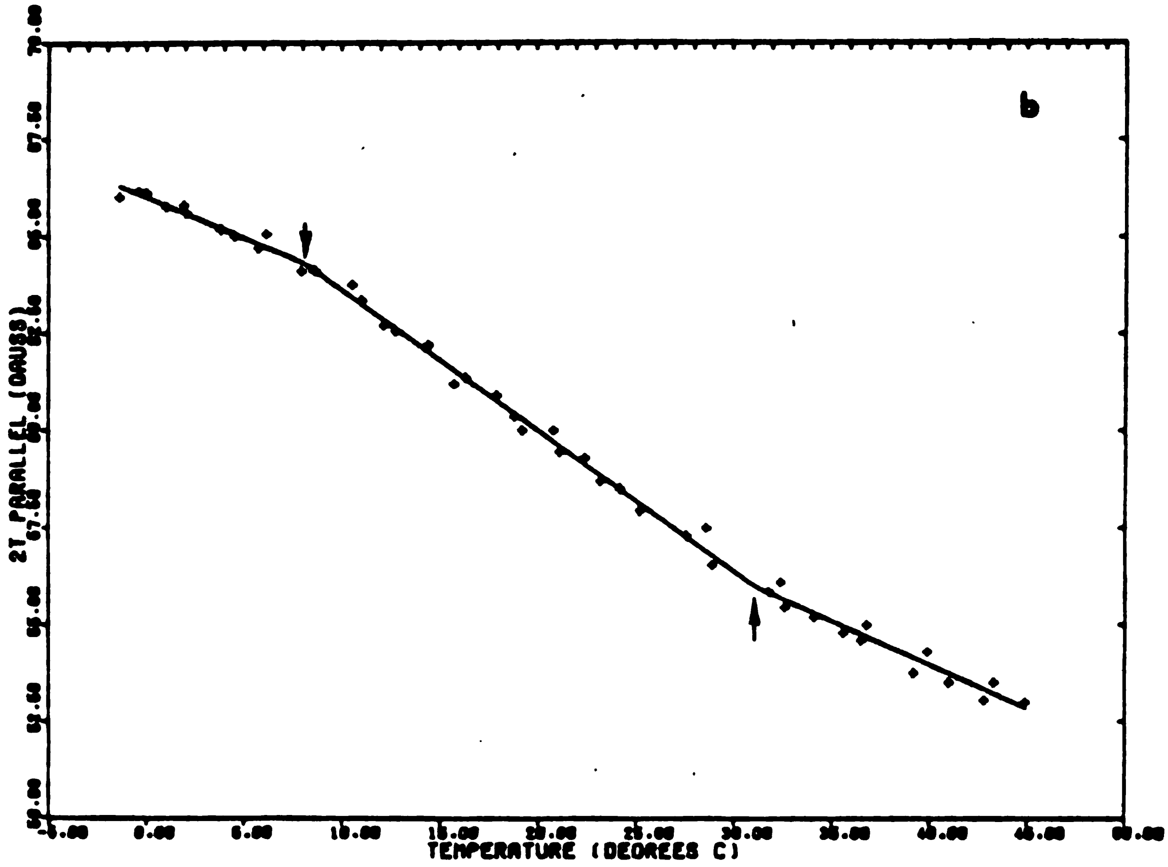
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APPENDIX B

THE MODIFICATION OF HUMAN ERYTHROCYTE MEMBRANE STRUCTURE BY MEMBRANE STABILIZERS: AN ELECTRON-SPIN RESONANCE STUDY*

ABSTRACT

Membrane structure in intact human erythrocytes was analyzed by electron-spin resonance (ESR) spectroscopy. The spin probes 5-doxyl stearate and 5-doxyl stearate methyl ester revealed thermally-induced structural transitions in the membrane at 37°C and 15°C. The addition of propranolol, diazepam, chlorpromazine, or Pluronic F68 all caused a decrease in temperature of the upper transition, but did not markedly alter the temperature of the lower transition. In addition, diazepam caused a significant decrease in the ordering or packing of the membrane-lipid acyl chains. It is proposed here that the protection from hypotonic hemolysis that has been reported in the presence of these drugs is mediated by a structural rearrangement in the erythrocyte membrane involving a change in protein-lipid interactions.

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*Published in American Journal of Hematology (1981) <u>10</u>, 171-179.

INTRODUCTION

Present approaches to the treatment of hemolytic disorders are limited to both in therapeutic principle and in effectiveness. They depend mainly on the use of agents such as corticosteroids, for which the mechanism of action is uncertain, or on the prolongation of survival of damaged cells by splenectomy. It is axiomatic that hemolysis results ultimately from structural failure in the erythrocyte membrane: attempts by Brewer (1) to diminish irreversible sickling by divalent ion substitution suggest that erythrocyte resistance to hemolysis may be modified therapeutically. The present study examines the hypothesis that there are topographical sites in the membrane that are especially critical with respect to hemolysis and that might be modifiable with therapeutic effect.

The rapid removal from the circulation of cells showing prehemolytic damage mandates an indirect approach to the problem; consequently, we have done an initial investigation *in vitro* on the structural perturbations of the erythrocyte membrane induced by several agents affecting the mechanical and osmotic resistance of red cells. It is anticipated that this may ultimately lead to the identification of susceptible membrane structures that are affected in common in diverse hemolytic processes.

Osmotic fragility and mechanical deformability of human erythrocytes are reportedly altered by low concentrations of a variety of membrane-stabilizing drugs (2). Such drugs have the potential to protect erythrocytes from hemolysis and to increase deformability in the microcirculation, provided that ancillary effects in other systems can be held within acceptable limits. There is at present insufficient understanding of the action of these compounds to define the optimal molecular form for erythrocyte-related effects. Although protein-lipid interactions may be affected, alterations in membrane architecture have not been characterized in detail. One technique useful in studying membrane architecture involves incorporating electron-spin resonance (ESR) probes such as 5-doxyl stearate (5DS) into the lipid bilayer. The unpaired electron of the doxyl group absorbs microwave energy when the sample is inserted into a magnetic field. The spectrum of the absorption reflects the structure and fluidity of the membrane in the region of the probe. Since 5DS reportedly localizes in a lipid region that is closely associated with proteins and devoid of cholesterol in the human erythrocyte membrane (3,4), electron-spin resonance spectroscopy of erythrocytes labeled with 5DS should reveal structural information specifically related to protein-lipid interactions.

In this paper, we report that 5DS- and 5-doxyl stearate methyl ester (5DS-ME)-labeled human erythrocytes exhibit discontinuities in the temperature dependence of ESR spectral parameters. These temperature-induced changes in membrane structure correlate with other reported temperature-dependent phenomena of erythrocytes that are presumed to reflect membrane structural changes (5-9). We report that a variety of membrane stabilizing agents affect the temperature dependence of these measured spectral parameters. Thus, membrane stabilization may be the result of a structural rearrangement in the erythrocyte membrane predominantly involving a change in protein-lipid interactions.

MATERIALS AND METHODS

Preparation of Cells

Blood was obtained from healthy individuals by venipuncture; informed written consent was obtained from each donor. Anticoagulation was effected by defibrination. Using standard procedures, the buffy coat was removed and the cells were washed three times in 300 mOsm NaCl. To remove serum albumin and other potential binding substances of the spin probe, the reserve serum was filtered using an Amicon ultrafiltration apparatus and Diaflo ultrafiltration membrane Um10 (molecular exclusion 10,000 daltons). Cells were washed once in serum ultrafiltrate and packed in fresh ultrafiltrate to a hematocrit of 70 ± 2 ml/dl. The cells were analyzed within 18 hours of collection.

Electron-Spin Resonance Spectroscopy

The spin probes 5DS and 5DS-ME (Syva Corp., Palo Alto, California) were dissolved as a 30 mM solution in absolute ethanol. The labeling procedure used was described previously (10), except that the ethanol was evaporated prior to the addition of erythrocytes. Drugs were added following labeling. To standardize drug concentrations with respect to the stabilizing effect, concentrations were selected that caused approximately equivalent degrees of submaximal protection against hypotonic hemolysis (11,12). The final probe concentration was approximately 0.4 μmoles/mg of membrane protein. At this concentration, 5DS has been reported to minimally perturb erythrocytes (13) and to localize in lipid domains in close association with proteins (3,4).

All ESR studies were performed by standard methods as described previously (10). The spin labels used incorporate into the membrane in such a manner that the unpaired electron of the doxyl radical is situated close to, but shielded from, the aqueous phase. A typical spectrum of erythrocytes labeled with 5DS is shown in Figure 1. The distance between the low-field and high-field microwave absorption peaks, $2T_{\parallel}$ (the hyperfine splitting parameter) reports the rotational mobility of the probe and therefore the viscosity of the surrounding environment. With increasing temperatures, intermolecular influences upon the unpaired electron of the probe result in lower values of $2T_{\parallel}$ which reflect a more fluid environment. The hyperfine splitting parameter $2T_{\parallel}$ is therefore directly related to the viscosity of the environment from which the probe is reporting. High values of $2T_{\parallel}$ indicate rigid environments, while low values of $2T_{\parallel}$ indicate more flexible environments (14).

In studies reported here, the directly measured parameter, $2T_{\perp}$, could be determined in 5DS-labeled preparations above about 12°C. This parameter is used along with $2T_{\parallel}$ to calculate the order parameter S (14). The order parameter measures the deviation of the observed ESR signal from the case of a completely uniform orientation of the probe. For a uniformly oriented sample, $S = 1$; for a random sample, $S = 0$ (14).

The hyperfine coupling constant also calculated from the directly measured $2T_{\parallel}$ and $2T_{\perp}$ parameters is considered to reflect local polarity (15) and thus reflects the position of the probe within the membrane.

Data Analysis

The data ($2T_{\parallel}$ vs. temperature, S vs. temperature) were analyzed in terms of linear components by an iterative least-squares program to be described elsewhere (Brunder, D.G., Coughlin, R.T., McGroarty, E., in press). Briefly, a B-spline (a piece-wise set of polynomials that are smooth at the points of connection) was used to provide a fit for the ESR data and points of inflection were used to group data. Regression lines were calculated for each group and break points were determined. This analysis has been shown in other membrane systems to permit characterization of subtle changes (16). All ESR spectral parameters changed with temperature reversibly up to 48°C. For each set of data, blood samples were examined from at least two healthy, nonsmoking individuals.

RESULTS

Control Erythrocytes

ESR spectra of 5DS-labeled, intact erythrocytes showed little or no free probe in the supernatant, indicating that its site is predominantly in the cellular phase (Figure 1). The shape of the spectra indicated that the majority of the fatty acid label was in a single environment over the temperature interval examined (0-48°C). The hyperfine splitting parameter, $2T_{\parallel}$, decreased in a discontinuous fashion as a function of temperature, and a break point was determined to occur at 37°C (Figure 2a). Spectra recorded at cuvette temperatures above approximately 12°C allowed the determination of the order parameter, S. When S was plotted as a function of cuvette temperature, a similar discontinuity was observed (Figure 3). The transition temperature determined with the order parameter agreed quite well with that determined using $2T_{\parallel}$ and presumptively indicates a structural change in the erythrocyte membrane.

A second spin probe 5DS-ME was also used to analyze erythrocyte membranes. This uncharged probe causes less perturbation of the cells. Spectra of 5DS-ME-labeled cells allowed determination of the hyperfine coupling constant, which indicated that the probe was in an environment similar to that of 5DS-labeled cells at temperatures up to approximately 28°C. Above that temperature, the spectra changed in such a manner that the spectral parameters were difficult to measure.

In spectra of 5DS-ME-labeled cells recorded between 0°C and 28°C, $2T_{\parallel}$ was shown to decrease discontinuously with a break at approximately 15°C (data not shown). This discontinuity is presumed to reflect a second structural change in the erythrocyte membrane that occurs at lower temperatures than the transition detected with 5DS. Therefore, it appears that erythrocyte membranes exhibit two thermotropic transitions, one at 15°C and a second at about 37°C (Table 1).

Effects of Membrane Stabilizing Agents on Membrane Structure

To further characterize erythrocyte membrane-mediated phenomena, we analyzed the changes in membrane structure induced by compounds known to protect human erythrocytes from osmotic hemolysis, propranolol, diazepam, chlorpromazine (11), and pluronic polyols (polyoxypropylene-poly-oxy-ethylene condensates) (12). The addition of any of these drugs did not appreciably alter the position of the spin probes as determined by the hyperfine coupling constant. Propranolol (Sigma Chemical Corp.) when added to 5DS-labeled erythrocytes caused a slight decrease in the upper transition temperature as measured using $2T_{\parallel}$ and S (Table 1). In addition, a low temperature transition could be detected with 5DS when propranolol was added (Figure 2b). The temperature of the lower transition detected with 5DS-ME was not significantly affected by the addition of propranolol (Table 1).

Similar studies were carried out with labeled cells in the presence of diazepam, chlorpromazine, or Pluronic F68. All of these agents reduced the upper transition temperatures but did not appreciably alter the lower transition temperature (Table 1). Furthermore, the addition of diazepam or chlorpromazine (but not Pluronic F68) permitted the detection of the low-temperature transition in 5DS-labeled cells. In addition, the presence of diazepam was shown to significantly alter the order parameter as indicated in Figure 3. The other perturbants induced only slight changes in the order parameter. The addition of diazepam caused a decrease in S by as much as 2% at low temperatures; changes greater than 1% in S are regarded as significant (17,18).

DISCUSSION

Numerous investigations employing a variety of techniques have reported temperature-dependent changes in erythrocyte membranes. Considering the diversity of approaches utilized, the temperatures at which changes were found to occur are remarkably similar to those reported here. Thus quenching of intrinsic tryptophan fluorescence by spin labels showed discontinuities at 15°C and 35°C (4). Laser-raman spectroscopy (5) and viscosimetry (6) revealed a discontinuity at approximately 18°C. A discontinuity in the susceptibility of human at 37°C (8). Finally, the preservation of membrane lipid asymmetry by Mg^{+2} upon lysis has been shown to diminish above 18°C and disappear at about 40°C (9). These data support the contention that temperature-induced structural transitions occur in erythrocyte membranes and can be detected using spin-labeling techniques. Apparently, these transitions occur in local areas not influenced by cholesterol.

Data presented here indicate that there are two thermally induced structural transitions in human erythrocyte membranes that can be detected using the ESR probes described above; one of these is found at 15°C, and the other at 37°C. These transitions are presumed to be associated with changes in the conformation of either membrane proteins, phospholipids, or both. Two independent studies, one involving quenching of intrinsic tryptophan fluorescence (2,3), the other involving the determination of binding affinities (19), support the contention that the spin probe 5DS is closely associated with erythrocyte membrane protein. The high-temperature transition demonstrated in this study, therefore, appears to be associated with protein-lipid interaction.

In all cases, the addition of the membrane stabilizing drugs to the spin-labeled erythrocytes predominantly affected the high but not the low transition temperature. Since 5DS reportedly localizes in close proximity with membrane proteins and since lipids interact more strongly with proteins at elevated temperatures (20), it appears highly probable that these agents perturb protein-lipid interactions. In fact, stabilization of the erythrocytes by other drugs has been reported to require an intact membrane protein structure (21).

Addition of propranolol, chlorpromazine, and diazepam (but not Pluronic F68) to erythrocytes allowed for the determination of a erythrocytes to benzyl-lysolecithin has been reported to occur at about 15°C (7). Transfer of phospholipid from hemagglutinating virus of Japan to erythrocyte membrane was reported to begin at about 19°C and saturate low-temperature transition using 5DS and caused hemolysis at high concentrations in isotonic saline (data not shown). Since Pluronic F68 did not cause hemolysis at high concentrations, it is probable that its structural interaction with the membrane has unique features that may be especially important to its effectiveness as an antihemolytic agent. Diazepam was shown to significantly alter membrane order. We are aware of at least one instance in which a drug-induced decrease in erythrocyte membrane order (17) can be correlated with a protein conformational change (22).

Propranolol, diazepam, chlorpromazine, and Pluronic F68 have been reported to protect erythrocytes against hypotonic hemolysis. Preliminary evidence (manuscript in preparation) indicates that membrane structural changes induced by some of these drugs are maximal at concentrations that cause maximal protection to hypotonic hemolysis. Thus, it is probable that these compounds affect specific membrane domains such as sites of lipid-protein interaction and that these domains are especially significant to cell fragility. The marked reduction of intact cell osmotic fragility and the increase in mechanical deformability that is brought about by an elevation in temperature, or by the presence of perturbants such as the drugs used in this study (17,23,24), might therefore be mediated by specific membrane structural changes. If the relevant changes, as suggested here, are related to lipid-protein interactions in the erythrocyte membrane, there is the possibility of developing more highly specific agents to modify these structures in a controlled fashion. Such agents might find wide application in a variety of disease states in which red cell destruction or erythrocyte perfusion in the microcirculation are critical factors.

CONCLUSIONS

The human erythrocyte membrane was shown by biophysical probing techniques to undergo structural changes at 15°C and 37°C. Addition of membrane stabilizing drugs caused a decrease in the high-temperature transition but did not appreciably alter the structural change detected at lower temperatures. Furthermore, one of these drugs, diazepam, caused a significant disordering of the membrane lipids. It is proposed that these drugs are altering the membrane structure by changing the lipid-protein interactions within the membrane.

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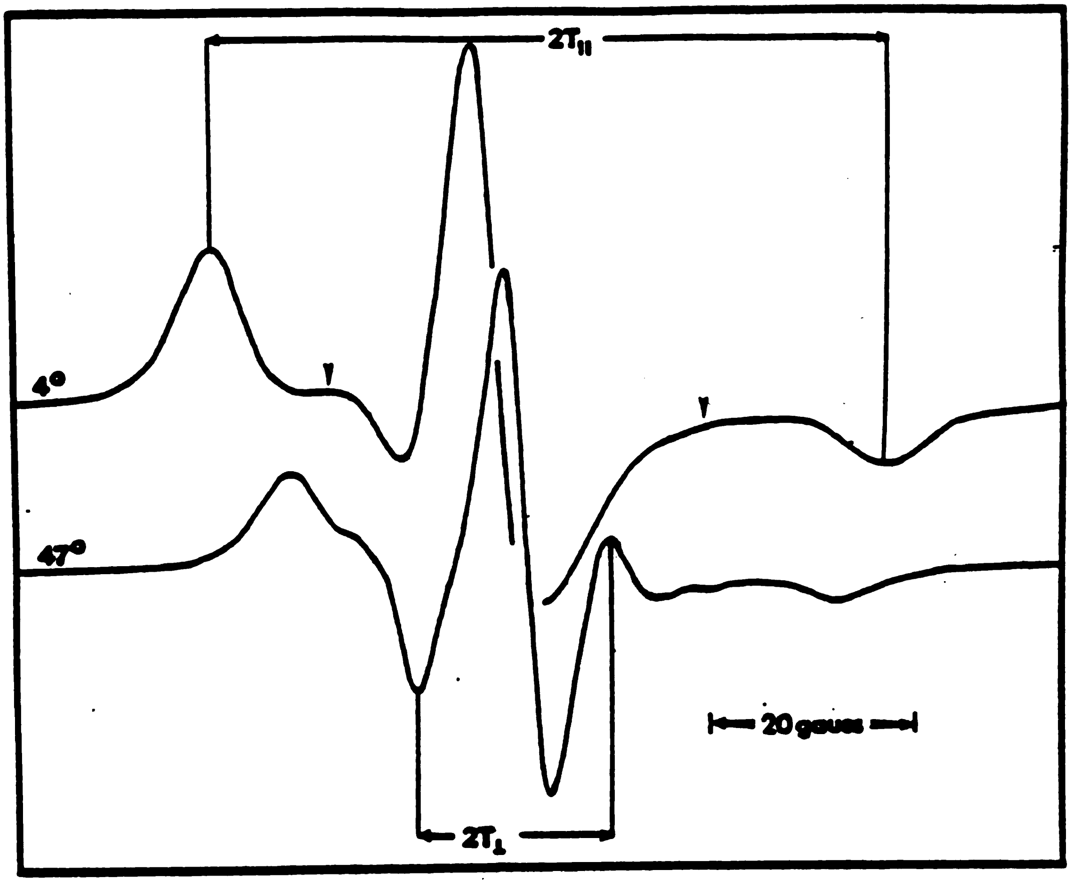


Figure 1. Electron-spin resonance spectra of human erythrocytes labeled with 5-doxyl stearate. The spectra were taken at the temperatures indicated. Scan range was 100 gauss. Absence of free probe is indicated by the lack of a major absorbtion signal at points indicated by arrows. Symmetry of high and low field peaks reveals that the probe is present in a single environment in the membrane.

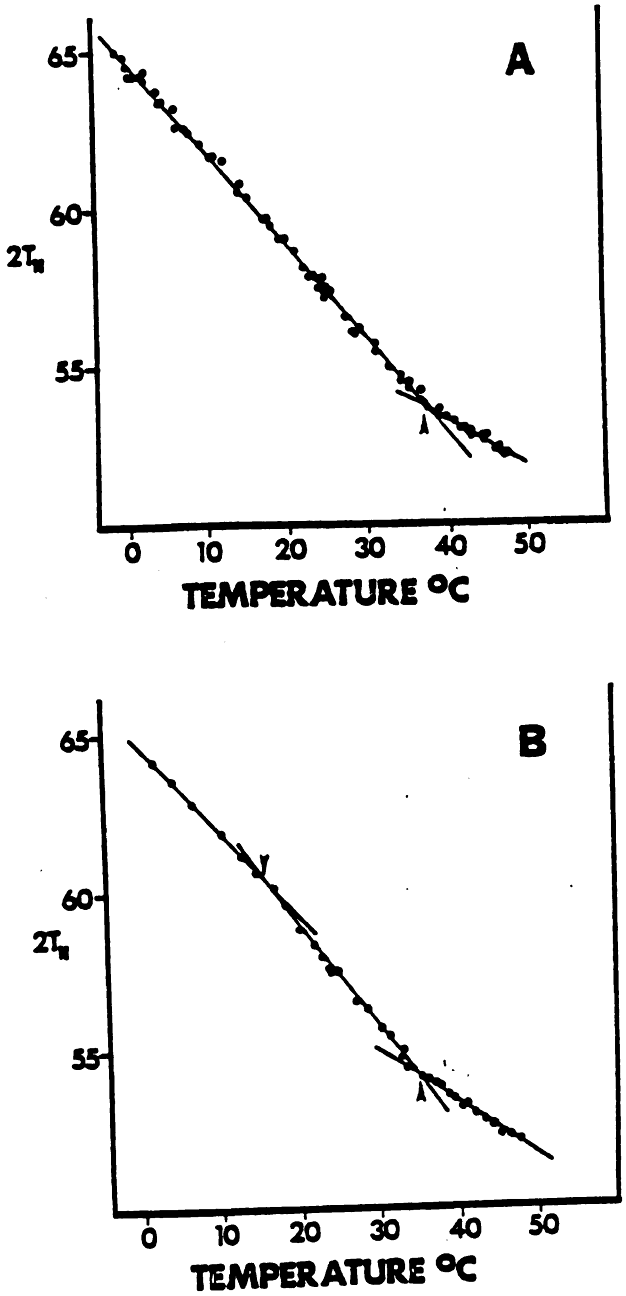
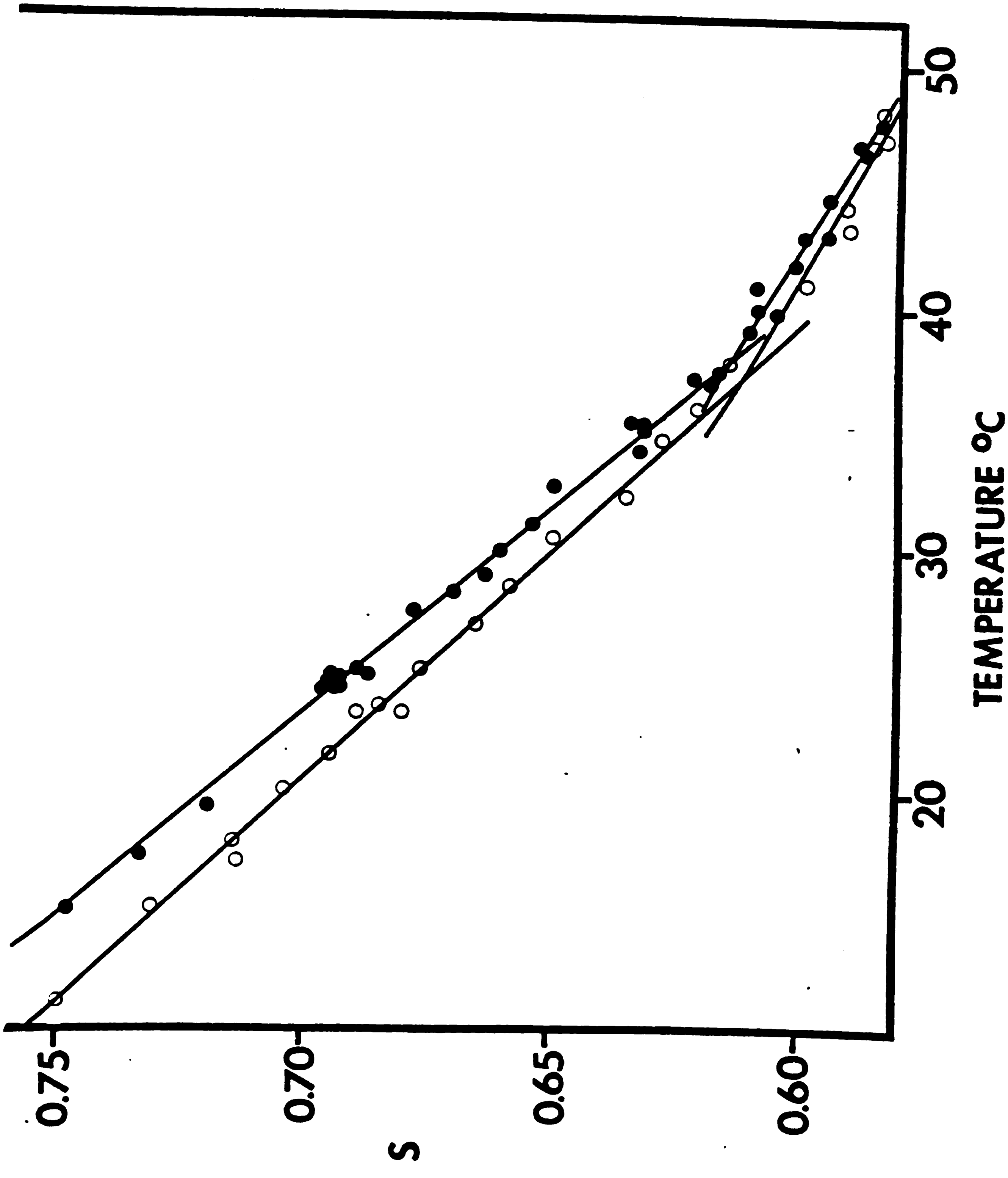


Figure 2. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in erythrocytes labeled with 5-doxyl stearate in the absence of perturbants (A), and in the presence of 5×10^{-4} M propranolol (B). Arrows indicate the transition temperatures.

Figure 3. The temperature dependence of the order parameter, S, in erythrocytes labeled with 5-doxyl stearate in the absence of perturbants (closed circles) and in the presence of 3 x 10^{-4} M diazepam (open circles).


S
0.75
0.70
0.65
0.60
TEMPERATURE °C
20
30
40
50

APPENDIX C

LIGAND EFFECTS ON MEMBRANE LIPIDS ASSOCIATED WITH Na^+,K^+-ATPase: COMPARATIVE SPIN PROBE STUDIES WITH RAT BRAIN AND HEART ENZYME PREPARATION

SUMMARY

(1) The lipid order of rat brain and heart (Na^+,K^+)ATPase was studied with the electron spin resonance probe 5-doxyl stearate.

(2) The degree of acyl chain order was greater for rat brain enzyme preparations than for rat heart preparations.

(3) In the absence of added ligands (e.g. Na, K, Mg, ATP), neither rat brain nor rat heart enzyme lipids appear to undergo a lipid phase transition.

(4) A change in the temperature dependence of lipid order was detected in both enzyme preparations in the presence of those ligands required to phosphorylate the enzyme (Na, Mg, and ATP). The presence of these ligands also increased lipid order above the lipid phase transition but not below.

(5) The addition of potassium to the phosphorylated enzyme significantly lowered the transition temperature of rat brain enzyme (30°C to 19°C) but only slightly effected the rat heart enzyme (35°C to

31°C). In either case, however, the actual value of order parameter was not significantly changed by the addition of potassium.

(6) The addition of 1 μM ouabain to the phosphorylated rat brain enzyme did not affect either the transition temperature or the value of lipid order. The potassium induced decrease in the transition temperature was blocked by 1 μM ouabain.

(7) 100 μM ouabain added to the phosphorylated rat heart enzyme destroyed the lipid melt but did not change the value of order parameter. No further change was seen upon addition of potassium.

(8) The differences in lipid order between the brain and heart enzyme do not appear to be species dependent.

(9) The effect of high concentrations of ouabain on lipid order suggest that enzyme inhibition under these conditions is the result of nonspecific lipid perturbation.

INTRODUCTION

The physical state of membrane lipids is now widely recognized as playing a role in modifying the activity of many membrane-bound enzymes (1). In particular, the fluidity of the membrane lipids has been reported to be an important determinant of Na^+,K^+-ATPase activity (2). The logarithm of the specific activity of Na^+,K^+-ATPase obtained from Chinese hamster cell line (CHO-KI) is found to be linearly related to the order parameter of membrane lipids; the activity decreased as the ordering of the membrane lipids increased (3). A partial removal of membrane lipids results in a reversible inactivation of the enzyme associated with a slowing of the conformational change in phosphoenzyme from an ATP-sensitive to a K^+-sensitive form (4). Although the presence of either phosphatidylserine or phosphatidylinositol has been implicated by many as being important for maximal enzyme activity, a complete enzyme conversion of phosphatidylserine into phosphatidylethanolamine or the complete hydrolysis of phosphatidylinositol has no effect on enzyme activity (5). These results are consistent with earlier observations that Arrhenius plots of the enzyme reaction (i.e., hydrolysis of ATP observed in the presence of Na^+, K^+, Mg^{2+}, and ATP) have a break point at about 20°C, presumably corresponding to the phase transition of membrane lipids (6), and suggest that fluidity or the physical state of the membrane lipids, rather than a specific lipid, is important for the turnover of Na^+,K^+-ATPase.

Membrane lipids associated with Na^+,K^+-ATPase also appear to influence the sensitivity of the enzyme to cardiac glycosides, such as ouabain. Exposure of dog brain or dog heart enzyme to high concentrations of deoxycholate or glycerol results in a loss in the ability of the enzyme to form a stable complex with ouabain (7). Although differences in the protein structure have been suggested as the primary cause of the species-dependent variation in the glycoside sensitivity (8,9), the difference in membrane lipids associated with Na^+,K^+-ATPase seem at least partially to account for the lower affinity of rat heart enzyme for ouabain compared to the affinity of the rat brain enzyme (10).

Thus, possible differences in properties of membrane lipids associated with Na^+,K^+-ATPase preparations obtained from rat brain and rat heart were examined in the present study using an electron spin probe, 5-doxyl stearate.

METHODS

Crude preparations of brain and heart Na^+,K^+-ATPase were obtained from male Sprague-Dawley rats weighing 250-300 grams as described previously for brain tissue (11). Rats were decapitated and brains and hearts were rapidly removed. Twelve brains or 24 hearts were pooled and homogenized. The enzyme preparations were prepared with deoxycholate and NaI treatment of microsomal fractions. The same procedure was used for both tissues, so that the results are comparable. Brain Na^+,K^+-ATPase had a specific activity of 311 ± 21 μmol Pi/mg protein/hr (mean ± S.E. of 6 preparations) at 37°C. Mg^{2+}-ATPase activity, assayed in the presence of 5 mM $MgCl_2$, 5 mM Tris-ATP, 50 mM Tris-HCl buffer (pH 7.5) and 0.1 mM ouabain, accounted for 6.4 ± 0.4 percent of the total ATPase activity, assayed in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer. Na^+,K^+-ATPase activity is the difference between total and Mg^{2+}-ATPase activity. The specific Na^+,K^+-ATPase activity of 3 rat heart enzyme preparations used in the present study was in the range of 12.7 to 22.0 μmol Pi/mg protein/hr, with Mg^{2+}-ATPase activity accounting for 39 to 44% of the total ATPase activity.

The fatty acid spin probe, 5-doxyl stearate (5DS), was used to assess the physical state of the membrane lipids. A 30 mM solution of 5DS in absolute ethanol was prepared. An aliquot was transferred to an acid-washed test tube, and dried under a stream of air at the room temperature. Subsequently, 0.2 ml aliquot of the Tris-HCl buffer solution (pH 7.5) containing appropriate ligands was added and mixed vigorously using a Vortex mixer (A. H. Thomas Company, Philadelphia, Pennsylvania) to resuspend 5DS attached to the test tube walls. The enzyme preparation was then added and mixed by gently swirling the sample.

The final protein concentration was typically 2 and 5 mg/ml for brain and heart preparations, respectively. The concentration of 5DS was adjusted to produce a final probe to protein weight ratio of less than 0.005.

Samples were preincubated at 37°C for 60 min in the sample chamber of an electron spin resonance (ESR) spectrometer. Subsequently, the samples were cooled at a rate of approximately 0.5°C/min until the temperature reached 10°C. ESR spectra were obtained after the sample was then elevated at a rate of 0.5°C/min, and spectra recorded at approximately 5°C intervals. Experiments were repeated at least three times using three separate enzyme preparations. All ESR data reported in this paper represent the pooled data from these experiments.

A Varian Century Line ESR spectrometer (Model E-112), equipped with a variable temperature controller was used for all ESR recordings. The temperature of the samples was monitored continuously using an externally calibrated thermistor probe. The order parameter was calculated as described elsewhere (12). ESR data were analyzed using a computer curve-fitting program which has been described previously (13). Briefly, the transition points that can be represented by an intersection of two regression lines, were determined for each set of data. A fourth degree spline was used to simplify the transition search process, whereas a more rigorous interactive least squares procedure was used to obtain the final solution. Statistical significance of the difference in slopes of the regression line above and below each transition point was estimated using the F-test for parallelism. Confidence limits were set at $\alpha = 0.05$. The F-test as well as an examination of the residuals around the transition point indicated that many of the order parameter data sets were well represented by two intersecting line segments. When the difference in slopes of two regression lines were not statistically significant, a single line was fit to the set of the data.

The metal composition of the Na^+,K^+-ATPase preparations was assayed by plasma emission spectroscopy using a Jarrell-Ash Model 955 Atomcomp spectrometer. Preparations of rat brain and heart enzyme were wet ashed for the elemental analysis: equal volumes of concentrated nitric acid and sample (approximately 2 mg protein/ml) were mixed and incubated at 70-75°C for 24 hr. The product was then diluted such that the final concentration of the acid was less than 15 percent (V/V). The N+1 channel (wavelength set at 766.5 nm) of the spectrometer was used to determine the potassium concentration.

The fatty acid spin probe, 5-doxyl stearate, was purchased from Syva Corp., Palo Alto, California. Ouabain octahydrate and Tris-ATP were purchased from Sigma Chemical Company, St. Louis, Missouri. Other chemicals were of reagent grade, except for the nitric acid (Ultra^R grade, Atomergic Chemetals Corp., Plainview, NY) which was used in wet ashing.

RESULTS

ESR spectra in the absence of ligands

Rat brain and rat heart Na^+,K^+-ATPase preparations were labeled with the fatty acid spin probe, 5DS. Schreier et al. (12) have shown previously that this probe can be used to measure lipid order, which can be quantified as the order parameter. A typical ESR spectrum obtained with a rat brain enzyme preparation suspended in a 50 mM Tris-HCl buffer (pH 7.5) at 37°C is shown in Figure 1. Virtually, all of the spin label was membrane-bound under these conditions as indicated by the absence of sharp spectral lines which are characteristic of free probe. The absence of excessive broadening of the low field extreme suggests that there is a relatively homogeneous spin probe environment (14). Similar results were obtained with rat heart enzyme preparations (data not shown). Elemental analysis of both brain and heart enzyme preparations indicates that they were relatively free of sodium, potassium, and magnesium (Table 1). Therefore, the spectrum shown in Figure 1, and those obtained in the following studies, may be regarded as those of ligand-free enzyme, unless these ions were added to the incubation medium.

Table 1 Background Level of Ions in Samples

Element	Rat Brain Enzyme (μM)*	Rat Heart Enzyme (μM)*
Na	934	482
Mg	16	23
Ca	24	42
K	8	34
Al	<1	<1

*Adjusted to 2 mg protein/ml.

As indicated in the Methods section, all samples were incubated at 37°C for 60 min in the sample chamber of the ESR spectrometer. ESR spectra taken at 15 min intervals during this period indicated that there was no appreciable time-dependent change in order parameter at 37°C (data not shown). Furthermore, comparison of the mid-field peak height at 37°C at the beginning and at the end of a temperature scan indicated that the loss of the spin probe signal was less than 5% during the entire experimental period which lasted approximately 5 hr.

Gradual elevation of the sample temperature caused a decrease in the order parameter when either brain or heart enzyme preparation probed with 5DS was suspended in a 50 mM Tris-Buffer solution in the absence of Na^+, K^+, Mg^{2+} and ATP (Figures 2a and 2b). A linear relationship was observed between the temperature and the order parameter. There was no indication of an abrupt change in temperature-dependence of the membrane order parameter at temperatures ranging from 10 to 47°C with either brain or heart enzyme preparations.

Samples exposed to temperatures above 47°C and then cooled to 37°C gave values of order parameter which were significantly greater than corresponding value observed before heating (data not shown). If the sample temperature did not exceed 47°C, changes in the order parameter with temperature were completely reversible.

Order parameter measurements for brain and heart enzyme preparations observed at 37°C were 0.54 and 0.52, respectively. The order parameter for rat heart enzyme preparations was significantly lower than that for the rat brain enzyme preparations at all temperatures examined (compare Figures 2a and 2b).

These results indicate that there are some differences in the lipid order in rat brain and heart enzyme preparations. In the absence of added ligands, no abrupt change in the order parameter was observed with either of these enzyme preparations.

ESR spectra in the presence of ligands

The addition of K^+ to the sample (in 50 mM Tris-HCl buffer containing neither Mg^{2+}, Na^+, nor ATP) resulted in a large increase in the membrane order parameter of cardiac enzyme preparations at high temperatures (Figure 2b). This effect of K^+ was much smaller in the enzyme preparations obtained from the brain. There was no indication of an abrupt change in the temperature dependence of the membrane order parameter in the presence of K^+ in the temperature range of 10 to 47°C in the brain enzyme preparations, however, the heart enzyme had an apparent transition at 35.5°C.

When the Na^+,K^+-ATPase preparations were incubated in the presence of 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl and 5 mM Tris-ATP, the temperature dependence of order parameter did not follow a simple linear function in either brain or heart enzyme preparations (Figure 3b). As can be seen in this figure, a transition in the temperature dependence of order parameters occurred at 19 and 31°C for the brain and heart enzyme preparations, respectively.

In the presence of Na^+, Mg^{2+}, and ATP, but not K^+, nearly 100% of the enzyme molecules exist in the phosphorylated form (15). The order parameter observed under this condition at temperatures below 30°C (Figure 3a) was not significantly different from that observed at the corresponding temperature in the absence of any added ligands (Figures 2a and 2b). In the presence of 100 mM Na^+, 5 mM Mg^{2+}, and 5 mM Tris-ATP, however, an abrupt change in the temperature dependence of the order parameter occurred at 30 and 35°C for the brain and heart enzyme preparations, respectively. Therefore, above these temperatures, the order parameter was higher in the presence of ligands than in the absence.

ESR spectra in the presence of ouabain

Several investigators (16,17) proposed that cardiac glycosides, such as ouabian, alter certain properties of membrane lipids associated with Na^+,K^+-ATPase by combining with the enzyme and indirectly affecting a large number of lipid molecules associated with it. Therefore, the effect of ouabain on the order parameter was studied in an attempt to determine whether ouabain induces a change in the physical state of membrane lipids. The addition of 1 μM ouabain (final concentration) to the incubation medium containing Na^+, Mg^{2+}, and ATP failed to cause appreciable changes in membrane order parameter (Figure 4a). A slight increase (3°C) in the calculated membrane transition temperature for the brain enzyme was noted; however, it was not possible to determine if ouabain significantly affected the transition temperature as confidence limits could not be assigned to the position of the transition. The addition of 15 mM K^+ (final concentration) to the above incubation mixture did not measurably affect the order parameter (Figure 4b). The transition temperature of the brain enzyme preparation decreased slightly (30°C); however, the significance of this change could not be evaluated.

Rat heart Na^+,K^+-ATPase has a substantially lower affinity for ouabain compared to rat brain enzyme, primarily owing to the inability of the former enzyme to form a stable complex with the glycoside (18,19,10). For this reason, 100 μM ouabain (final concentration) was added to the rat heart Na^+,K^+-ATPase preparations in the presence of 100 mM Na^+, 5 mM Mg^{2+}, and 5 mM Tris-ATP. The addition of ouabain resulted in a disappearance of the membrane transition without markedly affecting the order parameters for membrane lipids (Figure 4a). The results obtained in a medium containing 15 mM K^+, in addition to Na^+, Mg^{2+}, and ATP, were virtually the same as those obtained in the medium without K^+. These effects of ouabain, i.e., elimination of phase transition with little alteration in the membrane order parameter, were observed also with rat brain Na^+,K^+-ATPase preparations and with dog heart or brain enzyme preparations when the concentration of the glycoside was 100 μM (Figure 5a and 5b). These results suggest that ouabain at high concentrations is capable of altering the cooperative association of membrane lipids.

DISCUSSION

The primary aims of the present study were to examine whether there are differences in membrane lipids associated with highly ouabain-sensitive and less ouabain-sensitive Na^+,K^+-ATPases, whether ouabain is capable of inducing changes in membrane lipids, and to determine whether such an effects of ouabain, if observed, are related to the specific interaction of the glycoside with Na^+,K^+-ATPase. In order to examine possible indirect effects of the ouabain on membrane lipids associated with the enzyme, relatively crude enzyme preparations were used. While highly purified enzyme preparations are capable of binding ouabain, extensive detergent treatments needed to obtain such preparations may impair the ability of the glycoside to affect the membrane lipids only secondarily affecting the enzyme.

The ESR spectra obtained with a spin probe, 5-doxyl stearate (5DS), and either brain or heart Na^+,K^+-ATPase preparations, were free from both spin-spin broadening and free probe contributions. Moreover, the environment of the spin probe appeared to be relatively homogenous, indicating that the condition of the present study is adequate to assess changes in the ordering of the membrane lipids from calculated order parameters. The order parameter, obtained from 5DS spectra, represents the deviation of the observed ESR signal from that which would occur in a uniformily oriented solid. This value is equal to unity for a rigid environment, and approaches zero for a highly fluid environment. Because the spin label is located at the 5 position of stearic acid, the order parameter is sensitive to the physical state of the hydrophobic interior of the membrane (12). Generally, changes in the order parameter greater than 1% are regarded as significant with respect to the physical state of membrane lipids (20). As expected, the order parameter indicated a decreased membrane lipid order at elevated temperatures. In the absence of ligands or in the presence of K^+ alone, no abrupt change (break point) in the temperature dependence of the order parameter was observed in rat brain enzyme preparations in the temperature range between 10 and 47°C, although a transition at 35.5°C was induced in the heart enzyme preparation by K^+. Above 47°C, apparently irreversible change in the membrane lipid order occurred. This finding is consistent with earlier reports in which heat denaturation of the enzyme has been shown to occur at about 50°C (21).

In the presence of Mg^{2+}, Na^+ and ATP, i.e., under the condition which favor phosphoenzyme formation (15), a transition in the temperature dependence of order parameter was observed at about 30°C in both brain and heart enzyme preparations. Addition of K^+ to this preparation inducing dephosphorylation of the phosphoenzyme and a conformational change in enzyme protein (15), lowered the transition temperature for the brain enzyme to 19°C. Moreover, addition of ligands which are known to affect Na^+,K^+-ATPase reaction significantly altered the order parameter at higher temperatures (e.g., above 30°C), although their effects were minimal at low temperatures. The sensitivity of the order parameter to the additions of ligands which alter the state of membrane-bound Na^+,K^+-ATPase strongly suggests that the observed parameter represents the state of membrane lipids tightly associated with the enzyme, although the possibility that this parameter represents the properties of bulk membrane lipids in these crude enzyme preparations cannot be ruled out.

Na^+,K^+-ATPase preparations obtained from various sources have been shown to undergo a phase transition at about 19°C (22,23). In contrast, no abrupt change in the temperature dependence of the membrane order parameter was observed in the present study in the absence of added ligands. The addition of Mg^{2+}, Na^+, K^+ and ATP, however, caused a transition to occur at about 19°C with the rat brain enzyme preparation. Since the phase transition was estimated from the assay of enzyme activity performed in the presence of various ligands in earlier studies, the present results may not be incompatible with the reported data. Barnett and Parazotti (24) observed no abrupt change in temperature dependence of the [^{3}H]ouabain binding reaction assayed in the presence of Mg^{2+}, Na^+ and ATP. Under similar ligand conditions, our results indicated that both brain and heart enzyme from rat show a transition in the temperature dependence of order parameter at about 30°C. The transition at this relatively high temperature might have been overlooked in the above study (24) due to the limited temperature range examined. Alternatively, the temperature and magnitude of the phase transition of membrane lipids may be dependent on the source of enzyme and ligand conditions.

The lack of a transition in the temperature dependence of order parameter observed in the absence of added ligands may not be surprising, since the enzyme preparations used in the present study has a high cholesterol to phospholipid molar ratio (approximately 0.5), which is likely to destroy any apparent "phase transition" (25). Gupte _et al_. (26) have shown that Mg^{2+} interacts primarily with the polar head groups of phospholipids in highly purified Na^+,K^+-ATPase obtained from outer medulla of lamb kidney. Thus, the cooperative phase behavior of lipids in Na^+,K^+-ATPase preparations may be a cation-induced phenomenon which does not normally exist in the absence of ligands.

The ESR results indicate that at 37°C the lipids in both rat brain and heart enzyme preparations are in a relatively disordered state. Moreover, the order parameter data demonstrates that lipids associated with rat heart enzymes are less rigid than those associated with the rat brain enzyme at all corresponding temperatures. Similarly, dog heart enzyme was more disordered at all temperatures examined than dog brain enzyme. The order parameter in the higher temperature ranges was significantly increased by K^+ in the absence of other ligands, and the effect of K^+ was substantially greater with the rat heart enzyme than with the brain enzyme preparations. The lipid order transition temperature was significantly lower in the rat heart enzyme than in rat brain enzyme preparations. These results clearly indicate that membrane lipids found in rat heart Na^+,K^+-ATPase preparations have different physical properties compared to those contained in brain enzyme preparations. The tissue dependent differences in lipid order do not seem to be species dependent. The order parameter calculated for dog brain lipids was also greater than for dog heart enzyme lipids over the entire temperature range examined. Moreover, the species differences in lipid order for any given tissue were slight.

A concentration of 100 μM oubain, necessary to markedly bind to and inhibit rat heart Na^+,K^+-ATPase, caused a disappearance of the transition observed in the presence of Na^+, Mg^{2+}, and ATP. Addition of K^+ to this sample should inhibit the glycoside binding, but was shown to have no affect on the above action of the glycoside. Moreover, lower concentrations of ouabain (1 μM) which should cause a similar degree of enzyme inhibition in the brain enzyme failed to significantly change the transition temperature or the physical state of membrane lipids associated with the brain enzyme. In the heart enzyme preparation, a high concentration of ouabain (100 μM) is required to eliminate the transition, indicating that the ouabain-induced changes are not specifically related to the ability of the glycoside to combine with the glycoside binding sites on Na^+,K^+-ATPase. The only action of ouabain observed at a low enzyme inhibiting concentration was the elimination K^+ induced lowering of the transition temperature of brain enzyme preparations. Whether this action of ouabain is related to ouabain-induced "conformational change" in Na^+,K^+-AtPase proposed to be the mechanism of the positive inotropic action of the glycoside (16,17) has yet to be examined. Unfortunately, similar effects of ouabain could not be observed with the rat heart enzyme preparation, because K^+ did not cause a marked change in the transition temperature and because a high concentration of ouabain required to cause enzyme inhibition abolished the transition.

Kimelberg and Papahadjopoulis (27) suggested that cholesterol may interfere with Na^+,K^+-ATPase activity through general disordering of membrane lipids. More recently, Davis et al. (28) reported that the synthetic estrogen, ethynyl estradiol, inhibits hepatic Na^+,K^+-ATPase activity presumably by a similar mechanism. Therefore, the absence of an apparent transition in the Na^+,K^+-ATPase observed in the presence of various ligands and a high concentration of ouabain is likely due to the general disruptive properties that various steroids seem to have on membrane lipids order.

In conclusion, there are substantial differences in physical properties of membrane lipids associated with rat brain and rat heart Na^+,K^+-ATPase preparations. These differences seem to be more dependent upon the type of tissue than the donor species. The binding of small amounts of ouabain to Na^+,K^+-ATPase seems to have minimal effect on the physical state of the bound lipids. In high concentrations, ouabain causes a nonspecific change in membrane lipids.

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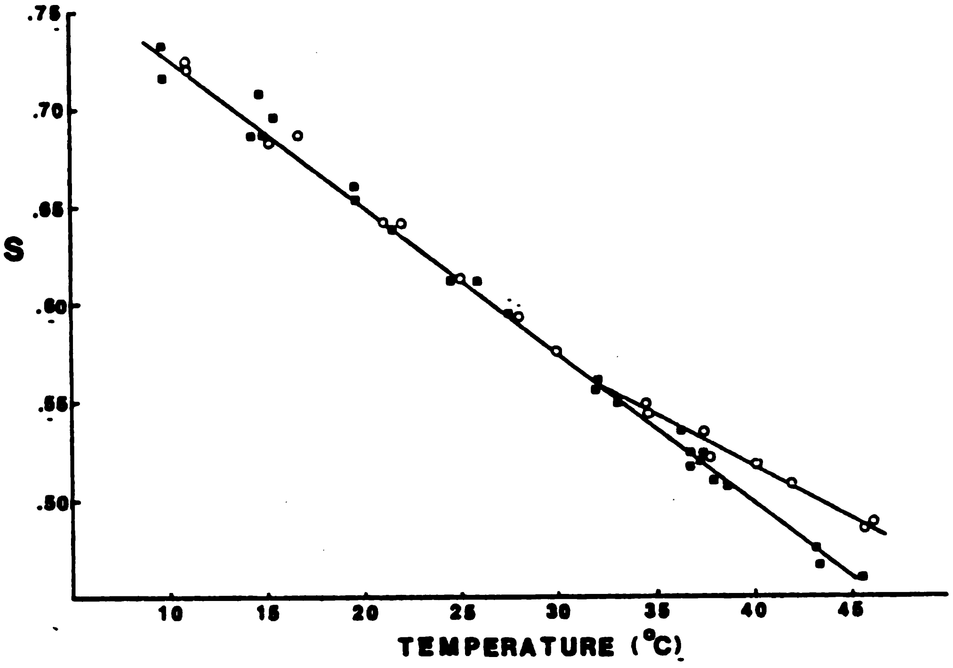
Figure 1. Typical ESR spectrum of rat brain (Na^+,K^+)-ATPase preparations at 37°C. The enzyme preparation was suspended in 50 mM Tris-HCl buffer (pH 7.5) yielding a final protein concentration of 2 mg/ml. In this and in all following ESR experiments, the probe (5-doxyl stearate) to protein weight ratio was 0.005.

Figure 2. Temperature dependence of the order parameter of 5DS bound to A) rat brain enzyme in the presence (O), and absence (■) of 15 mM KCl; B) rat heart enzyme in the presence (O) and absence (■) of 15 mM KCl.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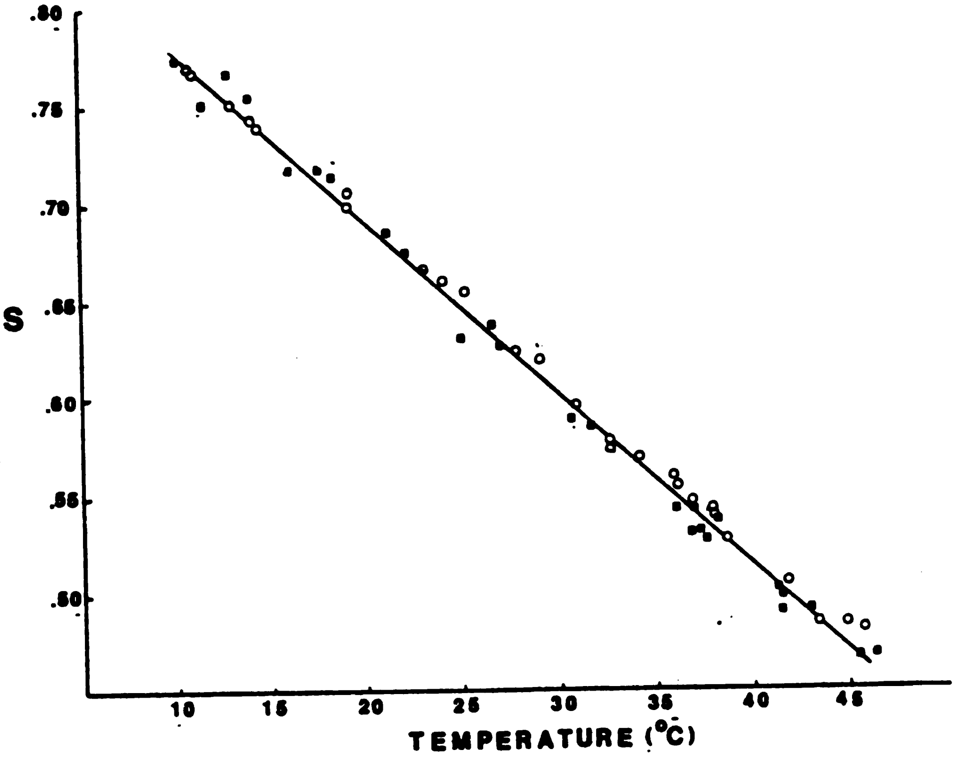
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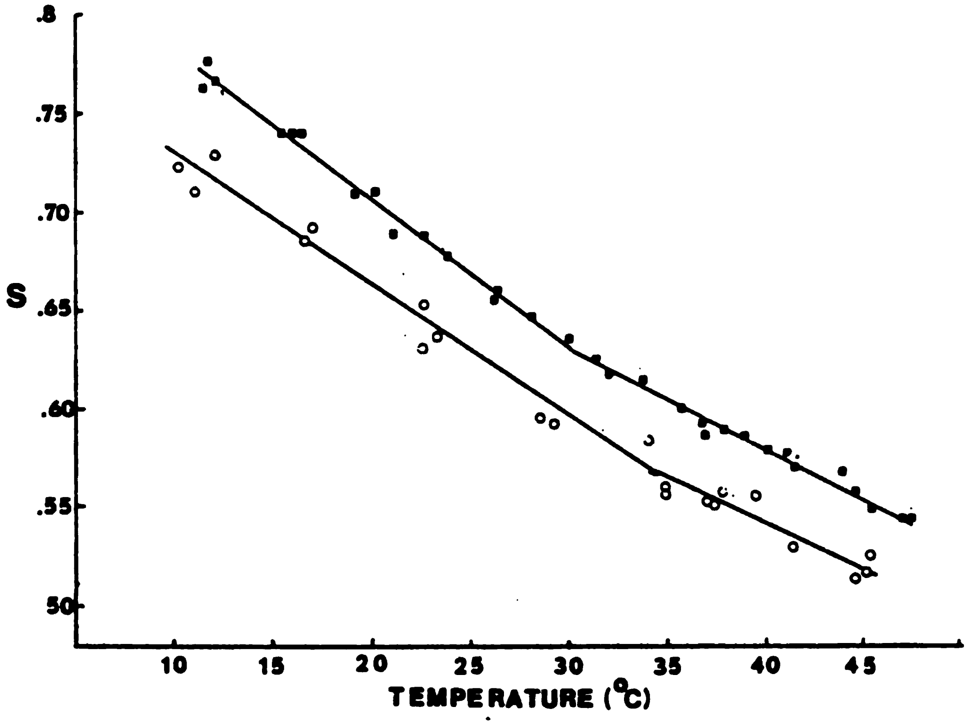
Figure 3. Temperature dependence of the order parameter of 5DS bound to A) rat brain (■) or heart (O) enzyme suspended in 5 mM $MgCl_2$, 100 mM NaCl and 5 mM Tris-ATP (phosphorylated enzyme); B) rat brain (■) or heart (O) enzyme suspended in 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP, and 15 mM KCl (dephosphorylated enzyme).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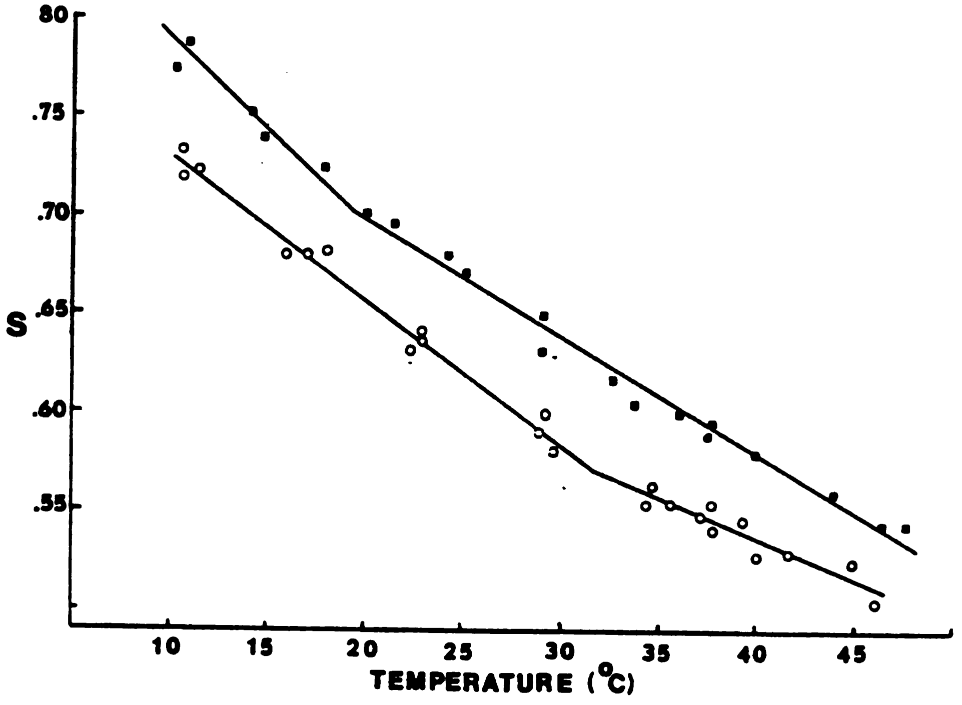
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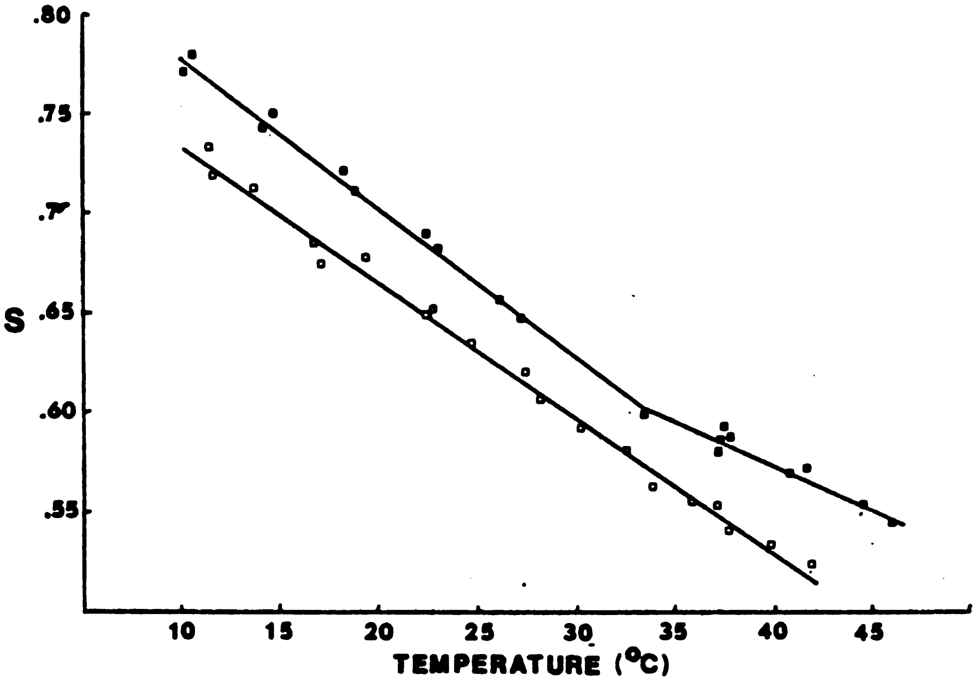
Figure 4. Temperature dependence of the order parameter of 5DS bound to A) rat brain (■) or heart (O) enzyme in 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP and either 1 μM (rat brain) or 100 μM (rat heart) ouabain; B) rat brain (■) or heart (O) enzyme in 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP, 15 mM KCl and either 1 μM (rat brain) or 100 μM (rat heart) ouabain.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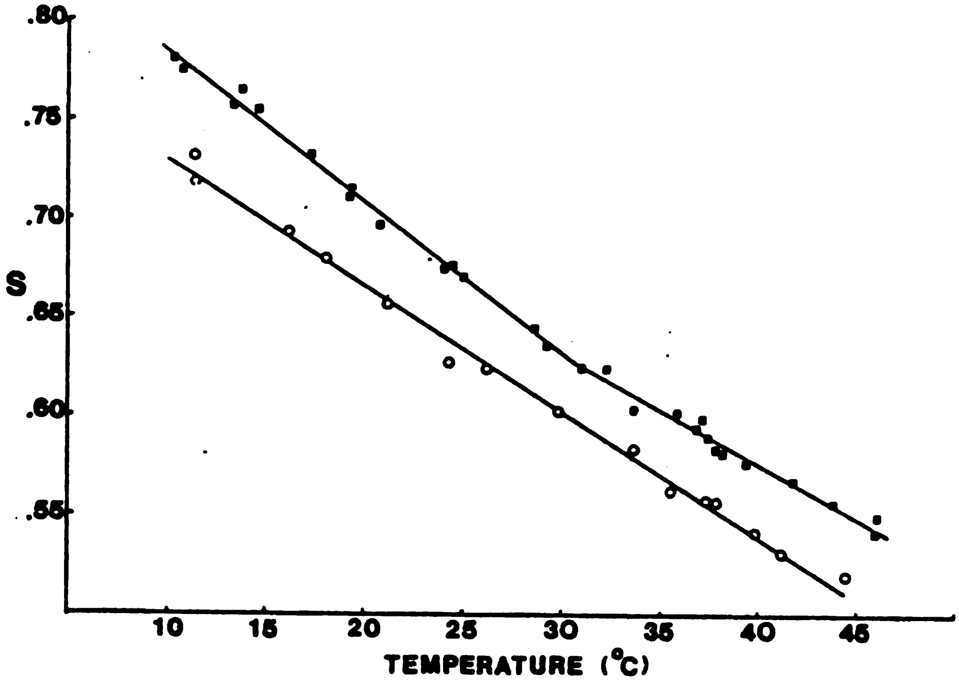
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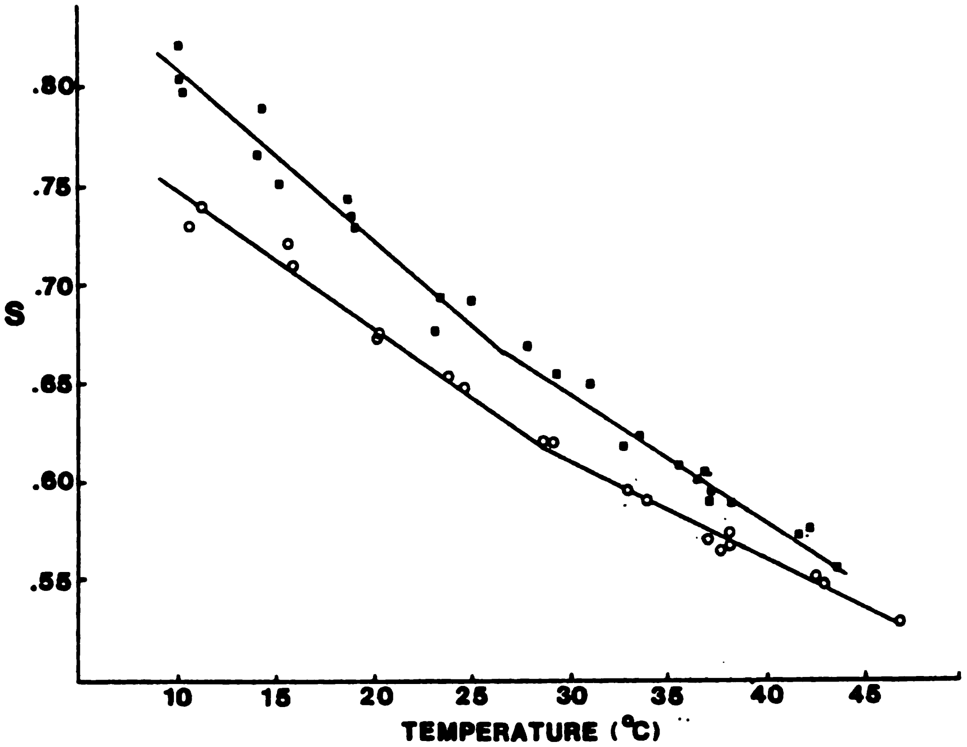
Figure 5. Temperature dependence of the order parameter of 5DS bound to A) dog brain (■) or heart (O) enzyme in 5 mM $MgCl_2$, 100 mM NaCl, and 5 mM Tris-ATP; B) dog brain (■) or heart (O) enzyme in 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP, and 100 μM ouabain.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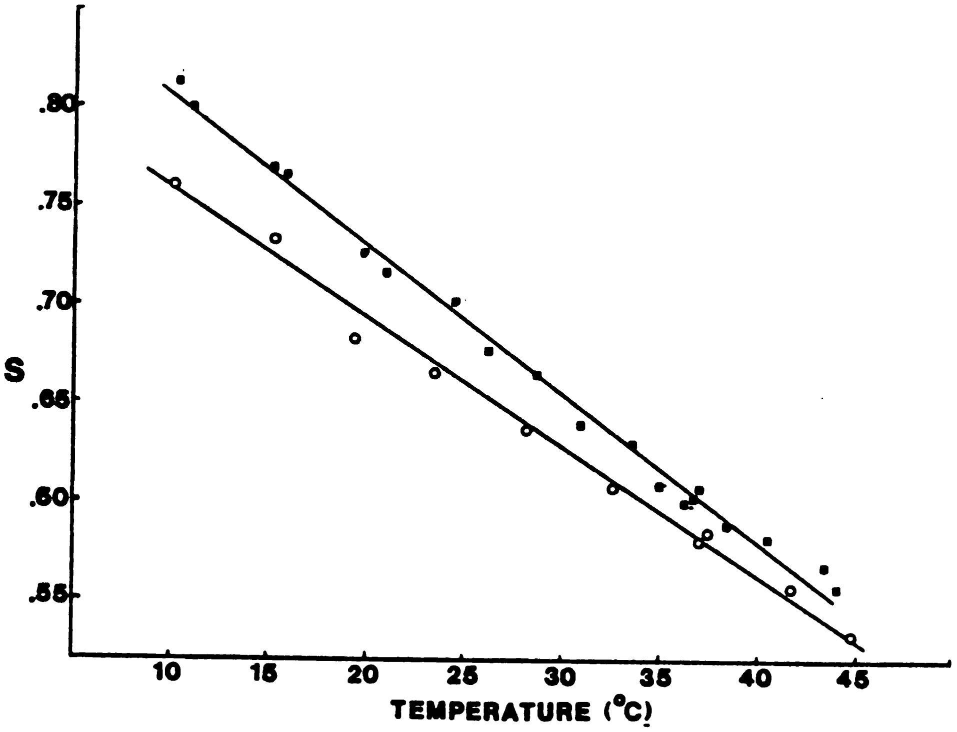
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