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MEMBRANE STRUCTURAL TRANSITIONS MODULATE GROWTH LIMITS OF ESCHERICHIA COLI

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ABSTRACT

MEMBRANE STRUCTURAL TRANSITIONS MODULATE GROWTH LIMITS OF ESCHERICHIA COLI

By

Andrew Stuart Janoff

Purified cytoplasmic and outer membranes isolated from cells of wild type Escherichia coli K12 grown over a wide range of temperatures were labelled with either the flourescence probe 1,6-diphenyl-1,3,5 hexatriene or fatty acid spin probes. Fluorescence polarization and electron spin resonance spectroscopy revealed broad thermotropic structural transitions. The structural transition in cytoplasmic membranes and lipids extracted from either cytoplasmic or outer membranes did not change with growth temperature. In contrast, the structural transition in the outer membrane occurred at higher temperatures as the growth temperature was increased. As a result, outer membranes derived from cells grown at all incubation temperatures were found to exist within their transition at the temperature of growth. In fact, the temperature range over which the cell could maintain the outer membrane within its transition, in a mixed (gel + liquid crystalline) lipid state, was found to correlate with the temperature range over which growth

occurred. Phenylethanol and procaine decreased the temperature of the transition in the outer membrane without affecting the transition in the cytoplasmic membrane and predictably lowered the maximum temperature for growth. The effect of these local anesthetics on cellular division was minimal or nonexistent at low temperatures consistent with the hypothesis that their mechanism of action derives from the melting of gel phase lipid. Data presented here suggest that adaptive changes which occur in the outer membrane determine the beginning and end of a thermotropic structural transition that defines the temperature range of growth of E. coli. Further, this data suggest that the growth limits of Gram negative bacteria can be altered and restricted by compounds which preferentially alter the structure of the outer membrane.

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INTRODUCTION

The outer membrane of the Gram negative bacteria serves as a first line of defense against the penetration of deleterious materials such as antibiotics and mutagens. It acts as a molecular sieve allowing only certain small molecules into the periplasmic space and keeping periplasmic enzymes and transport proteins from escaping into the surrounding medium. In addition, the outer membrane's polysaccharide chains provide antigenicity. The structure of the outer membrane is, therefore, of critical importance to the cell's functioning and possibly to its survival. However, little is as yet known concerning how this membrane forms "pores" to allow only certain sized molecules into and out of the periplasmic space. In fact, very little is known as to how the membrane is synthesized much less how this synthesis is controlled so that the membrane can function in different environments. Since the outer membrane is the outer most layer of the cell, it is expected that its structure and composition will be markedly affected by changes in the cell's surroundings. Work presented here shows that the growth temperature of the cell influences the fluidity and phase behavior of the phospholipid domains of the outer membrane. In fact it appears that these lipid domains are

altered with growth temperature such that they are always in a mixed state (i.e., within their broad order to disorder transition) at the temperature of growth. The ability of the cell to maintain the outer membrane in this mixed lipid state is what appears to define maximum and minimum growth temperature.

This dissertation is presented in four chapters. Chapter one provides background and a summary of the current literature on the cellular envelope of Gram negative bacteria. Chapters two, three and four detail studies concerning the relationship between growth temperature and the phase behavior of the cellular envelope of Escherichia coli, a typical gram negative bacterium.

Studies pointing to the biological relevance of membrane phase transitions in erythrocytes and bacterial spores are presented in the appendices. While these investigations do not have a direct bearing on the studies comprising the bulk of this dissertation, they are included to lend support to the growing feeling that phase transitions in biological membranes have physiological significance and are not just "phenomena that happen to occur".

CHAPTER I

BACKGROUND

Summary of Current Biochemical Literature

The cellular envelope of the Gram negative organism is a complex structure comprised of two membranes which surround a murein matrix (for a review see Costerton, et. al. (1)). The structure of this envelope and more specifically of the outer membrane appears to be critical for pathogens to be able to enter and multiply in host organisms and to resist host defenses (2). The outer membrane contains the antigenic sites of the cell, receptors for bacteriocins and bacteriophage, and certain critical transport systems (3-9). Furthermore, the outer membrane acts as a screen preventing certain deleterious agents such as antibiotics, fatty acids, bile salts and polycyclic mutagens from entering into the cell. Growth of pathogenic Gram negative organisms *in vivo* has been shown to be much slower than growth *in vitro*, presumably due to the lack of certain required nutrients. Such growth limiting conditions appear to affect determinants of pathogenicity which include alterations in cell structure. Since the surface components are often the major determinant in pathogenicity, the chemistry of these components and the

ability of the pathogens to alter these components due to "phenotypic influences and selection" should be carefully studied. In fact the ability of microorganisms in general to survive and grow in extreme environments has been proposed to be a function of the physical state of the membrane lipids.

Studies of the structure and chemistry of the two membranes in Gram negative bacteria have revealed the inner cytoplasmic membrane to be a typical bilayer containing protein, phospholipid and enzymatic activities typical of procaryotic plasma membranes. In addition, enzymes involved in the synthesis of certain components of the outer membrane are localized in the inner membrane (10).

Distal to the inner membrane, the peptidoglycan matrix can be resolved as a dense line in electron microscopic examinations of thin sections of Gram negative envelopes. Surrounding the cytoplasmic membrane this crosslinked polymer renders the cell osmotically stable. In several Gram negative organisms including E. coli the murein layer contains a small 8000 dalton lipoprotein. However, only one-third of this small protein is covalently attached to the murein while the remaining two-thirds exist in a free form embedded in the outer membrane (4). Inouye (12) has suggested that this lipoprotein embedded in the outer membrane may be involved in pore formation. (For a review on this lipoprotein see Braun (13)).

Between the cytoplasmic and the outer membrane a space

can be visualized in electron micrographs of thin sections of Gram negative bacteria. Within this compartment called the periplasmic space many hydrolytic enzymes are localized which modify molecules that can pass through the pores or transport systems of the outer membrane. These periplasmic enzymes may then prepare substrates for subsequent transport through the cytoplasmic membrane (1). Besides retaining enzymes and other materials within the periplasm, the outer membrane appears to act like a molecular sieve allowing small hydrophilic molecules to enter into this interstitial region.

The outer membrane appears in electron micrographs as a double track structure typical of a lipid bilayer. However, in addition to protein and phospholipid this membrane contains substantial amounts of lipopolysaccharide. Although the amounts of these three components vary with different organisms, outer membrane preparations are usually fairly high in protein, often greater than 50 percent by weight.

The outer membrane contains around 20 to 30 percent phospholipids by weight; the composition of the lipid molecules is similar to that found in the cytoplasmic membrane (14-16). A large portion of this phospholipid appears to be tightly associated with the lipopolysaccharide or protein moieties (14, 17), and may be arranged in specific structures (18, 19).

The lipopolysaccharide present in the outer membrane

usually accounts for 20 percent or more of membrane dry weight and is apparently asymmetrically localized on the outer monolayer of the membrane; the hydrophilic polysaccharide side chains stick out into the medium. The chemical structure of the lipopolysaccharide, which is responsible for the cells' antigenicity, has been determined for several Gram negative species through the use of mutants altered in the synthesis of polysaccharide chains. The polysaccharide structure of the lipopolysaccharide from E. coli K-12 has been under investigation, and the structure of the core polysaccharide has been determined (20-22). Additional studies with lipopolysaccharide mutants have demonstrated that a decrease in the length of the polysaccharide chains will alter the amount of protein and phospholipid incorporated into the outer membrane (18, 19). Osborn and co-workers showed that the core and antigenic side chain of the lipopolysaccharide are synthesized on the cytoplasmic membrane and then subsequently transported to the outer membrane (10). In addition, Mühlradt and co-workers demonstrated that the 0-antigen incorporates into the outer membrane at approximately 220 discrete sites per cell, and these sites appear to be the adhesion sites where the cytoplasmic and outer membranes fuse (23, 24). Mutants with defective lipopolysaccharide synthesis are usually more sensitive to dyes, detergents and antibiotics that are usually impermeable to the outer membrane (10, 25-27). Such results suggest that the structure of the lipopolysaccharide may be

important for maintaining the integrity of the cell envelope.

The protein composition of the outer membrane is rather unique in that it contains few different polypeptide components which comprise the bulk of the membrane protein. In most organisms there are between two and six different major outer membrane proteins whose size range between 32,000 and 42,000 daltons (25, 28-30). These major proteins account for 65 to 75 percent of the total protein and are found in approximately 10^5 copies per cell. Moreover, embedded in the outer membrane, there are about 10^6 copies of the 8000 dalton lipoprotein per cell (19). In addition, as many as 30 minor proteins are associated with the outer membrane.

In K-12 strains of E. coli there are reportedly four distinct major proteins in the outer membrane - 1a, 1b, 3a, 3b (Schnaitman nomenclature, 29-32) or b, c, d, a, (Lugtenberg nomenclature, 33, 34). A fifth protein (2-Schnaitman nomenclature, 31, 35) has been shown to be present in the outer membrane only if the cells are lysogenic for phage PA-2. The amount of major outer membrane protein appears to depend on the structure of the lipopolysaccharide. Mutants with altered lipopolysaccharide, which lack most of the core polysaccharide (heptoseless or deep rough mutants), have much lower quantities of major outer membrane protein (25, 26). Recently, Smit and Nikaido (36) have shown that the major outer membrane proteins are incorporated into the outer membrane of Salmonella typhimurium at the adhesion sites.

The major proteins in the outer membrane may perform two functions. Henning and co-workers have suggested that the protein in the outer membrane may be the shape-determining factor in the cell (37, 38). However, mutants lacking most of the major proteins of the outer membrane have been isolated and these cells have a normal rod shape (39, 40). The proteins of the outer membrane may also associate in a specific manner to form pores or transmembrane channels. Mutant strains of E. coli which are altered in their outer membrane permeability have been described and these cells are defective in proteins 1a and 1b (Schnaitman nomenclature, 41, 42). Since proteins 1a and 1b span the outer membrane (43, 44) and are required for pore formation in reconstituted vesicles (45) they are likely involved in formation of the outer membrane pore. In addition, a set of three or more genes have been shown to exist within the E. coli DNA which can code for different pore proteins but which normally are not expressed (46). Similar outer membrane pore proteins (porins) have been described in S. typhimurium (47).

Recently, attempts have been made to characterize the interactions of the major outer membrane proteins with each other and with other components of the envelope. It is known that the matrix or pore proteins (1a and 1b in E. coli) bind very tightly to the underlying murein layer in a non-covalent fashion. The ultrastructure of this murein-matrix protein complex has been investigated following removal of the lipids and other proteins from outer membranes using

sodium dodecyl sulfate (SDS). The matrix proteins in this complex appear in electron microscopic studies to have three fold symmetry indicative of a trimeric unit (48). Chemical cross-linking experiments using outer membranes from E. coli also suggest that the matrix proteins exist as trimers within the membrane (49, 50). It has been proposed that the lipoprotein may interact with matrix protein perhaps to help stabilize the pore structure (51, 52). The interactions of the other major outer membrane proteins are less well characterized. Protein 3a (Schnaitman nomenclature) apparently exists closer to the cell surface since it is more readily labelled or digested with proteolytic enzymes than are the other major outer membrane proteins (53). Recent evidence suggests that protein 3a may transverse the entire outer membrane and interact with the murein (54). Since mutants missing protein 3a (omp A mutants) have defective growth and nutrient uptake functions (55), this protein may also be involved in stabilizing the pore structure.

In addition to the functions described above, the major outer membrane proteins have been shown to function as receptors for bacteriophage, colicins, and in conjugation (31, 56-62). Other minor outer membrane proteins may also be receptors for phage and colicins, but several of these same minor proteins have been shown to be involved in specific transport systems in the outer membrane. For instance, the lambda phage receptor in E. coli appears to be involved in maltose transport (63, 64). In addition, there are at least

three specific iron transport systems (65-67) a vitamin B12 uptake system (68, 69) and a nucleotide uptake system (70) in the outer membrane, and components of these systems act as receptors for certain colicins and bacteriophage (52, 71-75). Recent evidence has also implicated the involvement of outer membrane components in DNA replication and cell division. It has been reported that the origin of replication on the DNA is associated with the outer membrane fraction of the cell (76, 77). Other studies have shown that the outer membrane lipoprotein may be structurally important in the septation process (78).

There is evidence that all three membrane components, protein, phospholipid and lipopolysaccharide, are asymmetrically distributed with respect to the inner and outer monolayer of the outer membrane. For instance, in intact cells of Proteus mirabilis proteins on the outer surface of the outer membrane are not as easily accessible to labelling or proteolytic digestion as those in isolated membranes (79). Similar studies on the accessibility to enzymatic degradation and protein oxidation indicate that proteins in E. coli outer membranes are asymmetrically localized (38, 58) as are proteins in the outer membrane of S. typhimurium (80). Based on chemical analysis of outer membranes from wild type strains and mutants of S. typhimurium altered in lipopolysaccharide biosynthesis, it has been proposed that all of the lipopolysaccharide is localized in the outer monolayer and all of the phospholipid is localized in the inner

monolayer of the outer membrane. The long carbohydrate chain of the lipopolysaccharide on the outer surface is thought to mask and protect the protein from chemical modification by acting as a "picket fence" (18, 79).

As stated previously, mutations in lipopolysaccharide biosynthesis lead to an altered composition of outer membrane constituents as well as to changes in other membrane properties (18, 25, 26). Such lipopolysaccharide mutants are usually more permeable to dyes, and more readily cleaved in freeze-etch studies reflecting a higher content of phospholipid in the outer membrane. The presence of Mg^{2+} in the growth medium appears to alter the structure of outer membranes in lipopolysaccharide mutant strains and in wild type cells apparently due to specific interactions. Addition of Mg^{2+} to cells mutant in lipopolysaccharide synthesis causes the mutant membrane to look and function similar to that of the wild type cell (19). Fuil and Brandtin have also reported that magnesium starvation of wild type cells will alter the membrane surface structure (81). Such studies point to the importance of the physical architecture of the outer membrane.

Summary of Current Biophysical Literature

Few studies have addressed the question of the relationship of the fluid properties of the outer membrane to its function. In contrast, the fluid properties of the cytoplasmic membrane of *E. coli* and other bacteria have been

studied and correlated with the functioning of various membrane associated enzymes. Phospholipid bilayers as well as biomembranes have been shown to undergo a phase-like transition from a gel to a fluid state (82) with increases in temperature. In the case of heterogeneous lipid bilayers and biomembranes these transitions are usually broad and phase separation of different lipids probably occurs in certain temperature ranges. Techniques to follow such phase changes include X-ray diffraction, dilatometry, differential scanning calorimetry, fluorescent spectroscopy and electron spin resonance spectroscopy (82). Studies of phase transitions of cytoplasmic membranes have been carried out with mutant strains aberrant in fatty acid synthesis supplemented with specific fatty acids (83-85). The temperature range for the lipid phase transition of the cytoplasmic membrane of such fatty acid auxotrophs of E. coli has been shown to be rather broad and to correlate with changes in the activation energies for membrane transport systems and membrane enzyme activities (85-88). It has been reported that the lipid phase transition temperature of the cytoplasmic membranes of Acholeplasma laidlawii (89) and Bacillus stearothermophilus (90) is critical in determining the upper and lower limits of growth. Thus the fluid nature of the cytoplasmic membrane appears to be critical for its ability to function physiologically, and perhaps in the case of Gram positive organisms for defining growth limits.

Studies of the fluid nature of the outer membrane of

various Gram negative organisms have shown that the outer membrane is much more rigid than the cytoplasmic membrane (17, 91-93). However, some of these studies report that the range of temperature in which the outer membrane undergoes a phase change is approximately the same as that of the cytoplasmic membrane (17, 91). A large amount of the lipid in the outer membrane is reported not to be available to take part in the phase transition (17). In these studies, mutant strains aberrant in membrane synthesis were analyzed. In the report by Overath et. al. (17) an unsaturated fatty acid auxotrophic strain of E. coli was investigated. These cells were cultured in the presence of non-ionic detergent, and during the membrane purification the murein was degraded and the membranes were treated with ethylenediaminetetraacetic acid (EDTA), sonicated and frozen. Such manipulations could have altered or rearranged components within the outer membrane. Recent studies have indicated that the outer membrane's structural transition begins and ends at temperatures higher than that of the cytoplasmic membrane (82, 94, 95). Further new evidence has suggested that alterations in the fluidity of the membranes in E. coli can significantly alter the synthesis and assembly of outer membrane components (96-99). These results suggest that all growth may require a specific outer membrane lipid state.

Little is known with respect to how the cell controls the synthesis of the outer membrane so that it can adapt itself to different environments. It is reported that cells

of E. coli (100) and Acholeplasma (101) have the capacity of homeoviscous adaptation; i.e. in response to changes in the growth temperature the cells are able to maintain a constant lipid fluidity in the membrane within a narrow range. Lugtenberg and Peters (16) have shown that the ratio of unsaturated and cyclic fatty acids to saturated fatty acids in the phospholipids decreases in both cytoplasmic and outer membranes from cells of E. coli grown at increasing temperatures. Similar changes with growth temperature in the fatty acid composition of the phospholipid fraction from the two membranes of Proteus mirabilis have been reported (102). The latter study also showed decreases in the amounts of unsaturated fatty acids in the lipid A component of the outer membrane as the growth temperature increased. The increase in the unsaturated fatty acid content of the two membranes which occurs with increasing growth temperature is reported to be the biochemical change responsible for homeoviscous adaptation. Rottem et. al. using P. mirabilis, have shown that the intrinsic order of the phospholipid regions of the outer and cytoplasmic membranes increases in membranes from cells grown at elevated temperatures (102).

Several mutant strains of Gram negative bacteria which have aberrant outer membrane structure and cannot grow at elevated temperatures have been described. Mutant strains of several species which have heptose-deficient lipopolysaccharide are unable to grow at elevated temperatures (19,

103).

The addition of monovalent or divalent ions to the medium allows for growth at the previously restrictive high temperatures. The phospholipid domains in the outer membranes of these deep rough mutant strains have been shown to be more fluid compared to that from the wild type strain (92). The inability of deep rough mutants to grow at elevated temperatures may be due to the lack of ordered structure in the outer membrane which is essential for this membrane to act as a permeability barrier. In the absence of stabilizing ions at the elevated temperature these mutant cells are reported to leak periplasmic enzymes (104).

A second mutation involving an outer membrane structure has also been reported to render cells of E. coli temperature sensitive. Strains containing mutations in the omp A gene coding for the major outer membrane protein 3a are unable to grow above 42°C (55).

A third mutation which apparently alters the structure of the murein lipoprotein is reported to result in restriction of growth at elevated temperatures (105). Strains of E. coli mutant in the gene mlp A which lack the lipoprotein reportedly are unable to grow well at elevated temperatures and are reported to leak periplasmic enzymes.

A final type of mutant strain that is of interest includes the unsaturated fatty acid auxotrophic strains whose growth limits can be altered by altering the unsaturated fatty acid added to the medium (106).

In summary, it appears that the structure of the phospholipid regions of the cell membranes, especially the outer membrane in Gram negative bacteria, may be crucial in adapting the cells to different growth temperatures. Other components of the outer membrane also change in response to changes in the growth conditions. Fatty acids in the lipopolysaccharide domains of the outer membranes are altered in response to changes in growth temperature (102). Furthermore, it has been reported that the protein composition of the outer membrane of E. coli is different in cells grown at different temperatures (41, 107) or in different ionic environments (108). The mechanisms by which the structure of the outer membrane is altered in response to changes in the environment are not known. But an understanding of the importance of the structure of the outer membrane in modulating growth limits in Gram negative bacteria and an analysis of the specific components that are critical in maintaining such outer membrane structure might suggest methodologies for inhibiting microbial growth during host infections or in conditions of pollution induced microbial blooms. It is hoped that the studies described in the body of this dissertation will provide insight into the development of such methodologies.

Choice of Organism

Escherichia coli K12 was used in the investigations described herein since the structure and biochemical

composition of the cytoplasmic and outer membranes of this organism has been studied extensively. In addition, this organism is an attractive choice because of its genetic manipulative flexibility and the availability of many mutant strains with altered outer membrane synthesis and structure. Furthermore, this organism is important as a potential pathogen and the structure of the outer membrane may be a critical determinant in its pathogenicity.

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CHAPTER II

RELATIONSHIP OF GROWTH TEMPERATURE AND THERMOTROPIC LIPID PHASE CHANGES IN CYTOPLASMIC AND OUTER MEMBRANES

The cell envelope of Gram negative bacteria is a complex multilayered structure comprised of two membranes with a peptidoglycan matrix sandwiched in between (1). The cytoplasmic membrane has been shown to be a typical bilayer containing protein and phospholipid (2). The outer membrane, which lies distal to the peptidoglycan matrix, contains, in addition, substantial amounts of lipopolysaccharide (3). The relative fluidity of both of these membranes has been described (4-7). Moreover, the effect of different growth temperatures on the fluidity and composition of the unseparated envelope has been studied (8, 9). Surprisingly, however, the extent to which the fluid properties of each membrane are altered with changes in growth temperature has not been fully investigated. Furthermore, the lipid state of each membrane at the growth temperature is not known. These parameters may be critical with respect to the cell's ability to survive environmental stress. It has been suggested that the freezing out of membrane lipids may define the lower temperature limit for cell growth (10).

In this study, purified cytoplasmic and outer membranes isolated from cells of wild type Escherichia coli K12 grown at various temperatures were labelled with the fatty acid spin probe 5-doxyl stearate. Stearic acid spin probes have been shown to partition only into phospholipid domains in the outer membrane (5, 6). Electron spin resonance (ESR) spectroscopy revealed broad thermotropic lipid phase changes. The temperature of the phase change in the outer but not the cytoplasmic membrane was shifted dramatically with growth temperature. Data presented here indicate that the temperature range over which the cell can maintain the outer membrane phospholipids in a mixed (presumably get + liquid crystalline) state correlates with the temperature range over which growth occurs.

Materials and Methods

Growth of cells

Escherichia coli strain W1485F$^-$ was grown for 15 to 20 generations at 12, 20, 37, and 43°C in M9 minimal medium supplemented with 0.4% glucose (final concentration). The optical density of the cultures was monitored at 560 nm using a Beckman DB spectrophotometer. Growth was stopped late in logarithmic phase by the addition of chloramphenicol (20 µg/ml, final concentration).

Preparation of Membranes

Cells were washed twice in 10mM HEPES (n-2-hydroxyethyl-piperazine-N -2-ethanesulfonic acid), pH 7.5, and then lysed with a French pressure cell. Membranes were isolated according to the procedure of Schnaitman (11) except that the discontinuous sucrose gradient contained 12 ml of 0.77 M, 16.8 ml of 1.44 M, and 4.8 ml of 2.12 M sucrose in 10 mM HEPES. The gradient was centrifuged at 25,000 rpm in a Spinco SW27 rotor for 18 hr. Following fractionation the outer and cytoplasmic membrane bands were washed twice in 10 mM HEPES, pH 7.5.

Assays

Succinate dehydrogenase was determined by the method of Osborn et. al. (12). Ketodeoxyoctanoic (KDO) acid was assayed using the method of Dröge et. al. (13). Protein concentrations were determined using the procedure of Lowry et. al. (14). Membrane proteins were characterized on sodium dodecyl sulfate (SDS) polyacrylamide slab gels (15). Prior to dry weight determination, membrane isolates were washed twice in 0.1 mM HEPES pH 7.5.

Spin Labelling

The fatty acid spin probes 5-doxyl stearate or 12-doxyl stearate (5-DS, 12-DS Synvar Corp., Palo Alto, CA) were dissolved as 15 mM stock solutions in absolute ethanol. Depending on protein concentration, between 3-10 µl aliquots

of stock solution were added to between 0.4 ml and 0.7 ml of fresh, refrigerated membranes in 10mM HEPES. The mixture was then placed in a bath sonicator (125 watts) for 10 minutes at room temperature. The concentration of spin label approximated 0.1% of the lipid weight of the membranes. All electron spin resonance (ESR) experiments 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., Standord, CT.) was used to monitor the temperature of the sample. Control experiments (not shown) indicated that the ethanol added during labelling did not alter the ESR spectra.

The order parameter (S) was calculated as described elsewhere (16). The experimental curves (hyperfine splitting parameter vs. temperature and S vs. temperature) were analyzed in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. This method allows the determination of break points (18, 19). For each growth temperature membranes were isolated two to four times, and each isolate was fully characterized.

Results

Membrane Isolation

The degree of purity of cytoplasmic and outer membrane isolates derived from cells grown at 12, 20, 37 and 43°C is

shown in Table 1. Succinate dehydrogenase activity was between 7 and 20 times greater in cytoplasmic membrane fractions while ketodeoxyoctanoic acid, a component of lipopolysaccharide, was between 5 and 8 times more concentrated in outer membrane fractions. The percentage of protein in either membrane fraction was not appreciably influenced by the growth temperature. Outer membrane protein electrophoretic patterns on SDS polyacrylamide gels indicated that outer membrane protein 1a (Schnaitman's nomenclature) decreased and protein 3b increased with increasing temperatures (data not shown) as has been previously reported (19, 20). Growth temperature also affected the concentrations of several cytoplasmic membrane proteins (not shown).

Membrane Spin Labelling

Typical ESR spectra of 5-DS labelled cytoplasmic membranes are shown in Figure 1. Little or no unbound probe was present. The single symmetric low field peak was taken as evidence that the majority of label was present in a single lipid environment. The low field peak remained symmetric over the temperature interval examined. Similar data were obtained with 5-DS and 12-DS labelled outer membrane samples. The hyperfine splitting parameter, $2T_{\parallel}$ (Figure 1), is related to the rotational mobility of the spin label and therefore reports the local fluidity of the membrane lipids (21). High valves of $2T_{\parallel}$ reflect low fluidity.

Table 1. Biochemical characterization of isolated cytoplasmic and outer membrane fractions.

	Cytoplasmic Membrane				Outer Membrane			
Growth Temperature (°C):	12	20	37	43	12	20	37	43
$\frac{\text{mg Protein}}{\text{mg Membrane}}$	0.49	0.57	0.49	0.52	0.52	0.50	0.49	0.52
$\frac{\mu\text{M KDO}^a}{\text{mg Membrane}}$	0.03	0.03	0.02	0.03	0.17	0.14	0.16	0.14
$\frac{\text{Rel SDH}^b}{\text{mg Membrane}}$	1.8	3.2	4.9	3.6	0.12	0.16	0.32	0.50

a. ketodeoxyoctanoic acid
b. relative succinate dehydrogenase activity

Figure 1. Electron paramagnetic resonance spectra of E. coli cytoplasmic membranes isolated from cells grown at 37°C and labelled with 5-doxyl stearate. The spectra were taken at the temperatures indicated. Scan range was 100 Gauss.

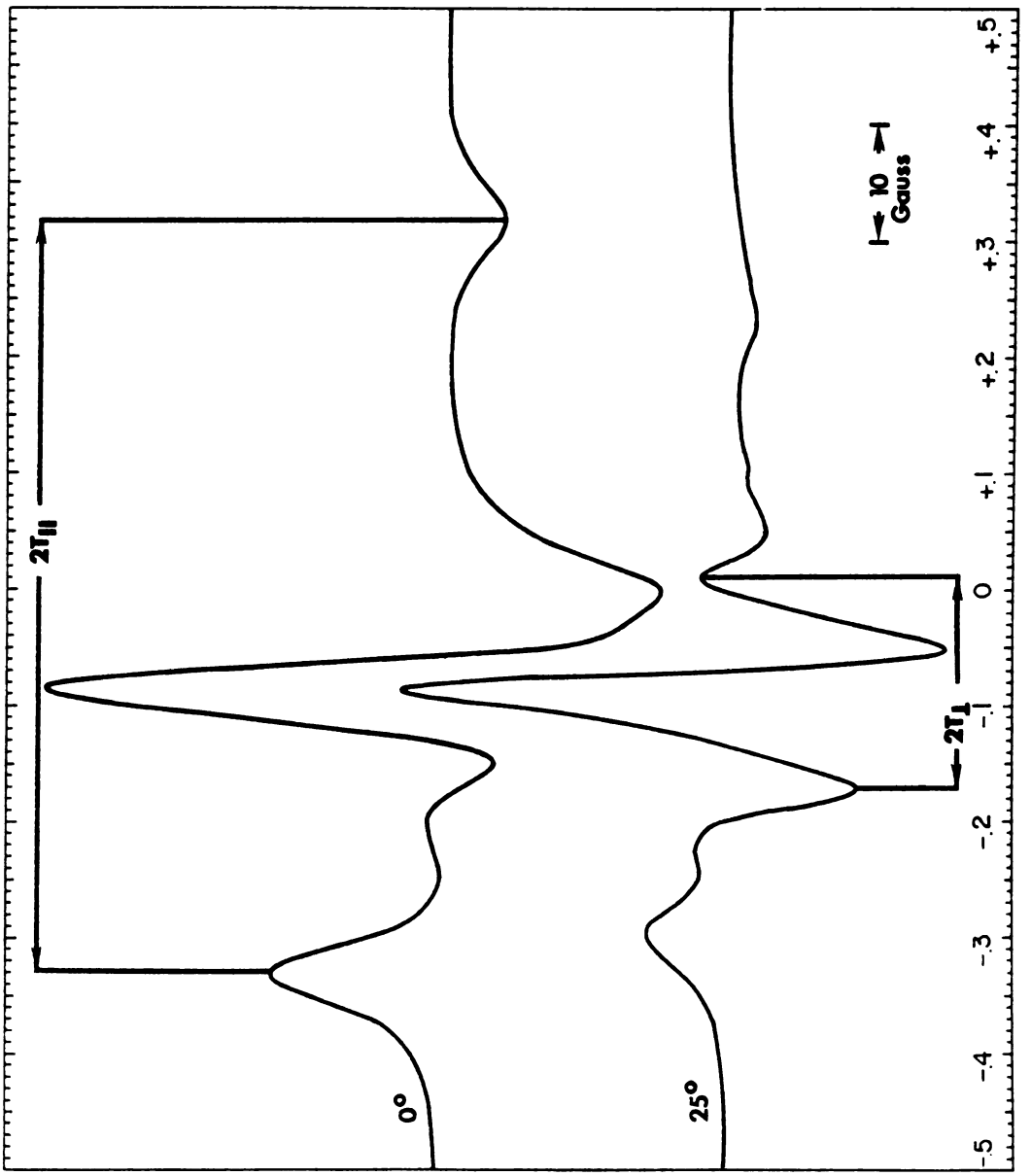

Figure 1.

The temperature dependence of $2T_{\parallel}$ in cytoplasmic and outer membranes isolated from cells of E. coli grown at the temperatures indicated and labelled with 5-DS is illustrated in Figures 2-5. Cytoplasmic membranes exhibited lower $2T_{\parallel}$ values at midrange temperatures than did outer membrane isolates. This suggests a greater overall fluidity in the cytoplasmic membrane fraction. In all fractions $2T_{\parallel}$ decreased with increasing temperatures and discontinuities in slope were inferred. Such breaks in the temperature dependence of spin label parameters have been correlated with lipid phase separations or lipid phase transitions from gel → gel + liquid crystalline, and from gel + liquid crystalline → liquid crystalline lipid states (17, 18, 22-27). Figure 6 shows the temperature dependence of $2T_{\parallel}$ in outer membranes isolated from cells grown at 43°C and labelled with 12-DS. In these studies high temperature spectra could not be properly analyzed due to a large free probe signal. A low temperature break identical to those determined in similar preparations labelled with 5-DS could however be identified. In addition, 12-DS, which contains a doxyl group that would be expected to partition further into the membrane than that of 5-DS, revealed a greater fluidity than revealed by 5-DS. Thus the outer membrane preparations possess qualities generally expected for typical lipid bilayers. The data presented in Figures 2-5 suggest that all growth temperatures the outer membrane phospholipids existed in a mixed (presumably gel + liquid crystalline)

Figure 2. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in E. coli outer (open circles) and cytoplasmic (closed circles) membranes labelled with 5-doxyl stearate. Membranes were isolated from cells grown at 12°C. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

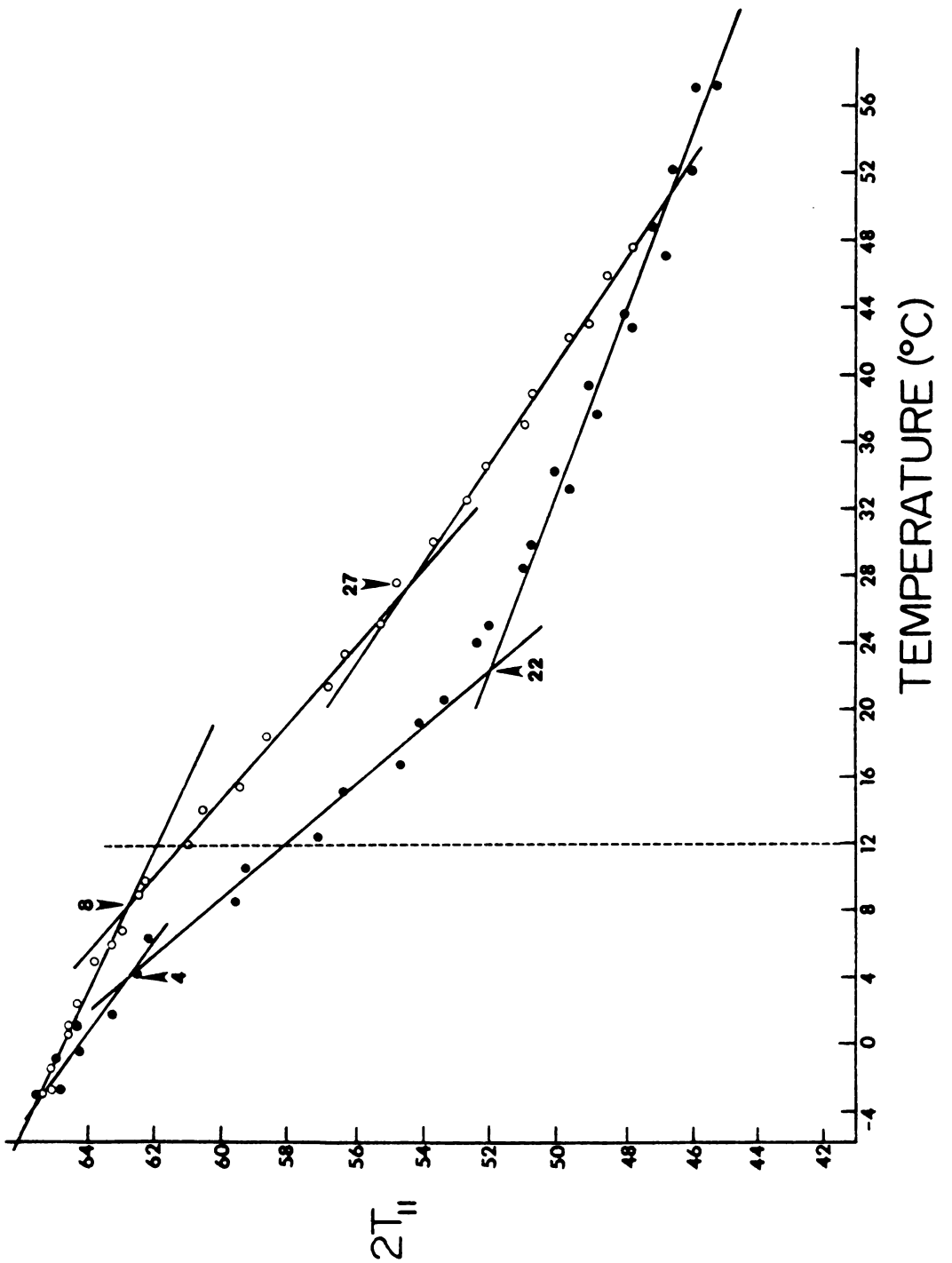

Figure 2.

Figure 3. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in E. coli outer (open circles) and cytoplasmic (closed circles) membranes labelled with 5-doxyl stearate. Membranes were isolated from cells grown at 20°C. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

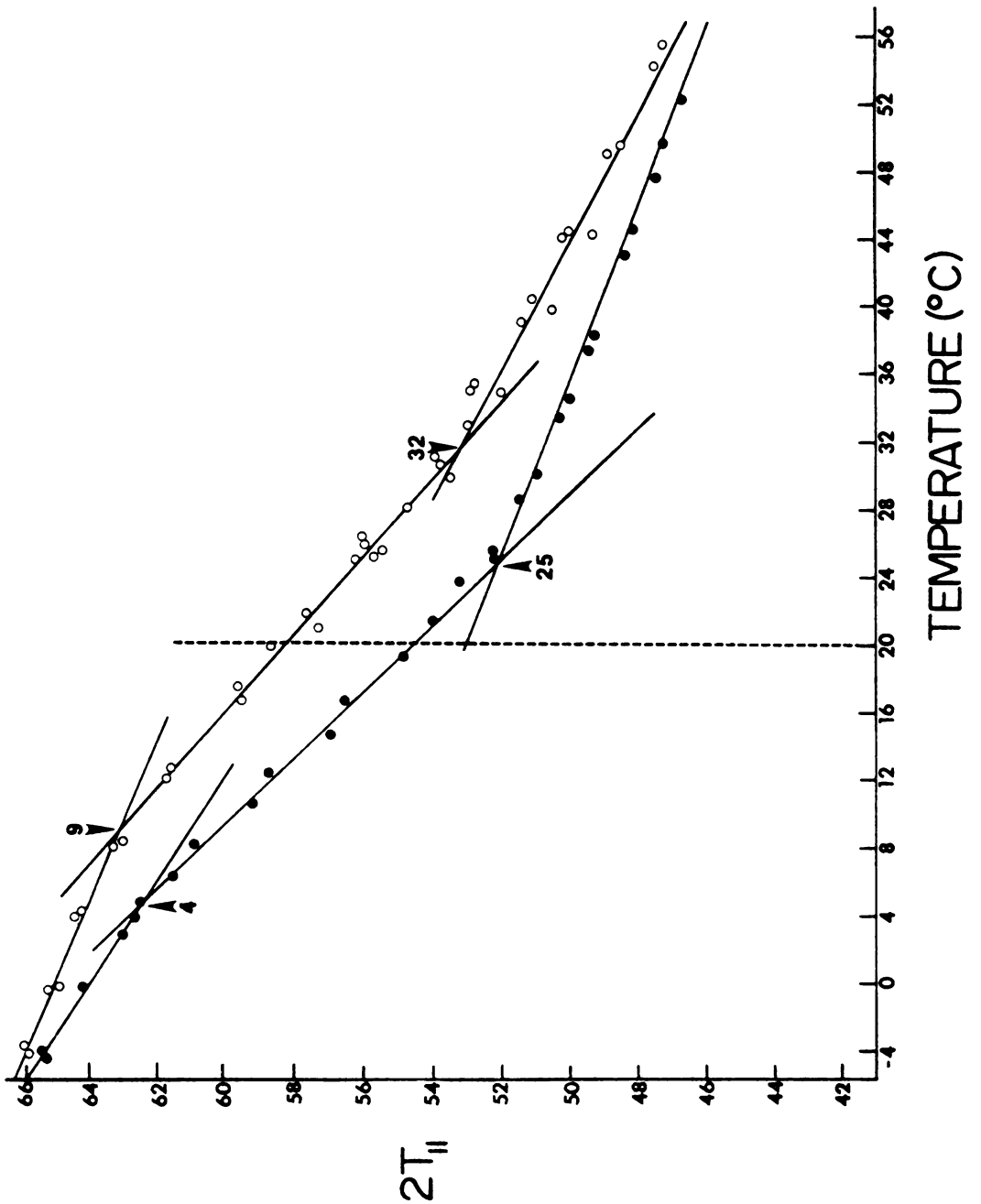

Figure 3.

Figure 4. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in E. coli outer (open circles) and cytoplasmic (closed circles) membranes labelled with 5-doxyl stearate. Membranes were isolated from cells grown at 37°C. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

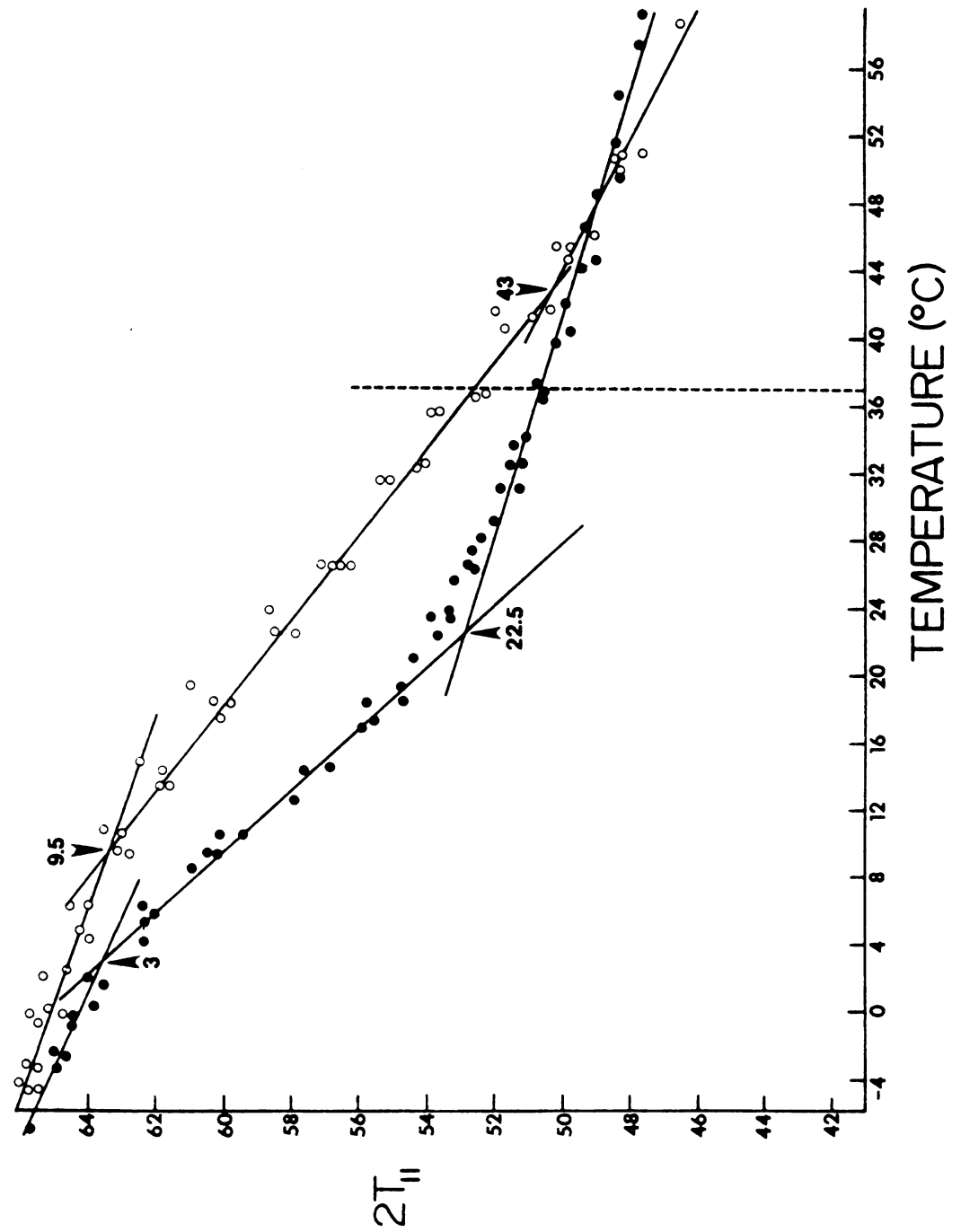

Figure 4.

Figure 5. Hyperfine splitting parameter, $2T_{||}$ (Gauss), as a function of temperature in E. coli outer (open circles) and cytoplasmic (closed circles) membranes labelled with 5-doxyl stearate. Membranes were isolated from cells grown at 43°C. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

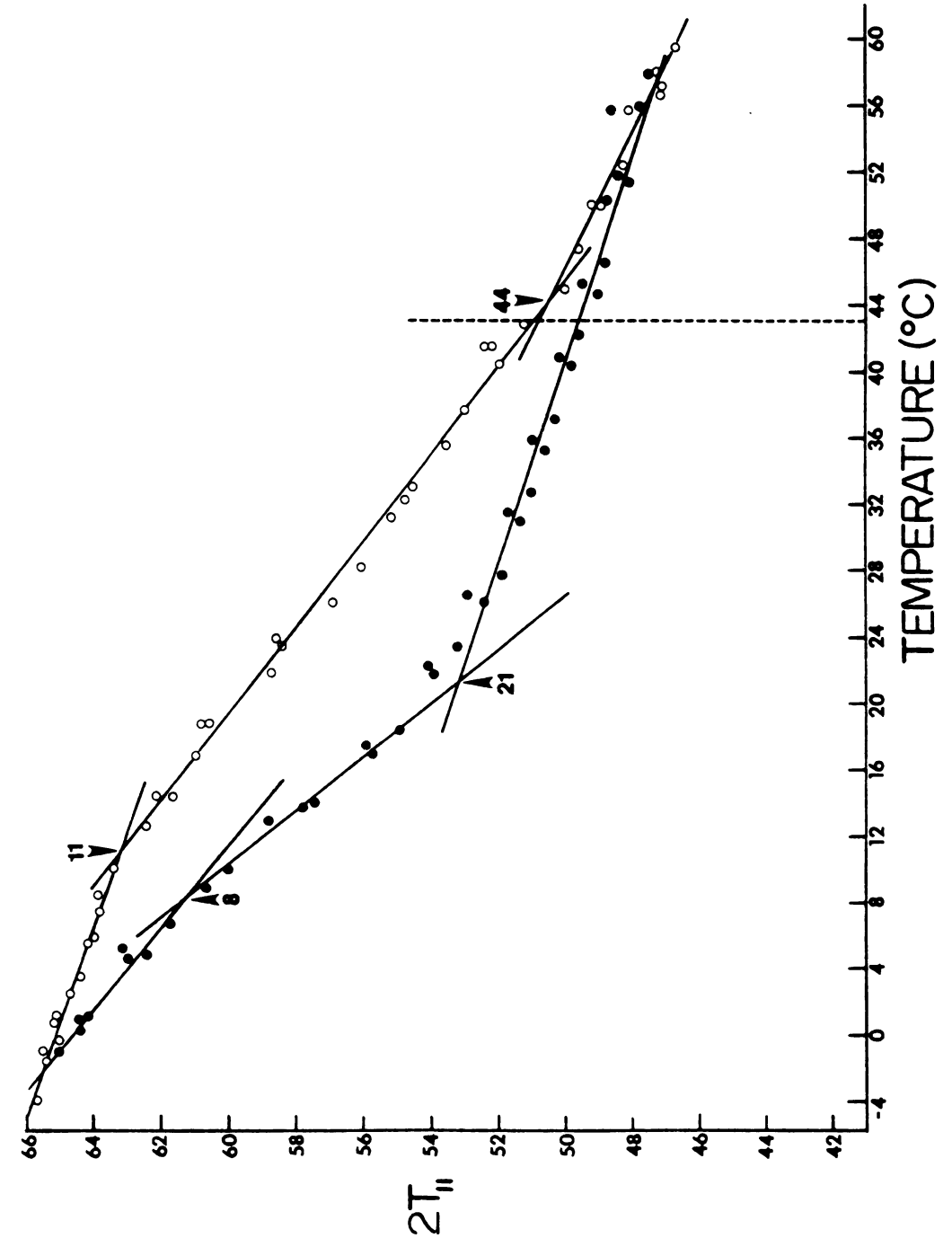
66
64
62
60
58
56
54
52
50
48
46
44
42
$2T_{11}$
-4
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
TEMPERATURE (°C)
11
8
44
21

Figure 5.

Figure 6. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in E. coli outer membranes labelled with 12-doxyl stearate. The membrane sample was isolated from cells grown at 43°C.

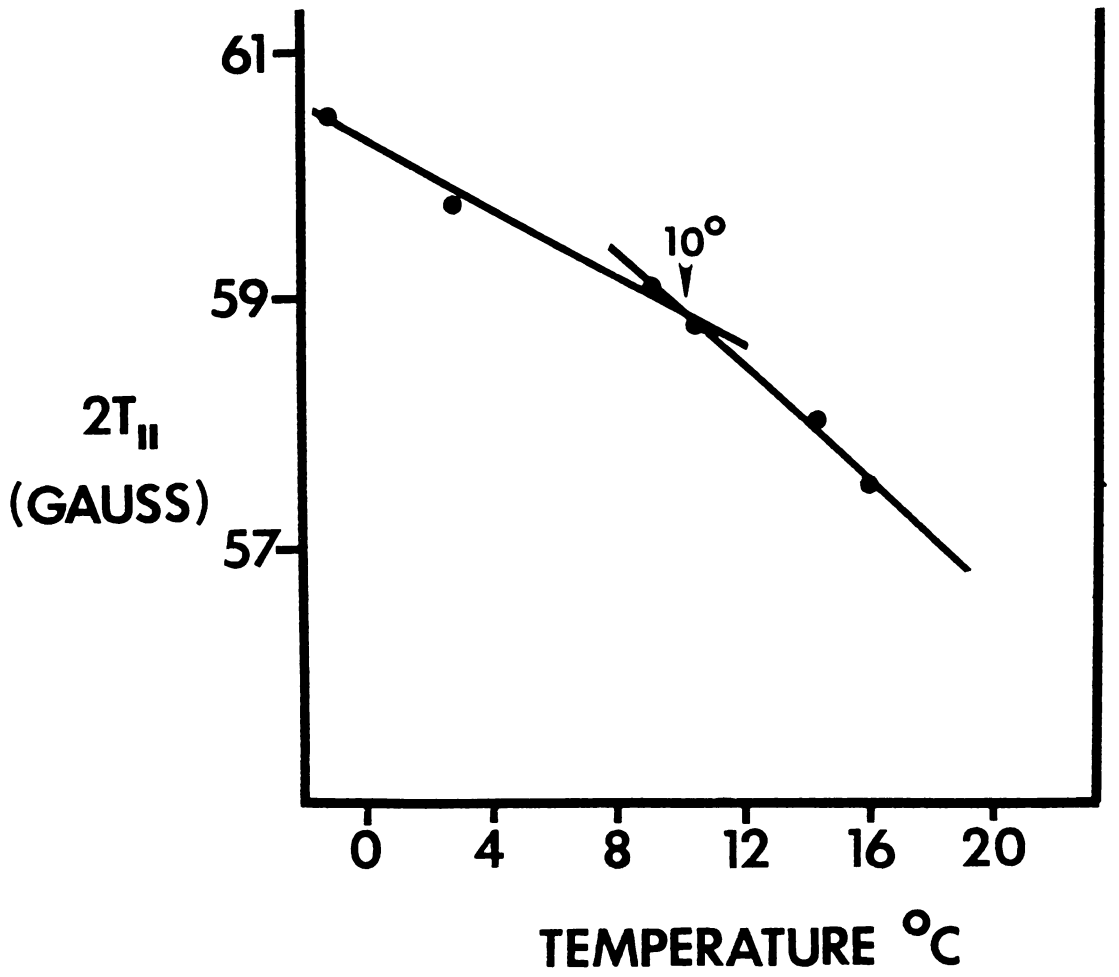

Figure 6.

lipid state. Cytoplasmic membranes, however, appeared to exist in a liquid crystalline state at the growth temperature when derived from cells grown at 37 and 43°C, but in a mixed lipid state at the growth temperature when derived from cells grown at 20 and 12°C.

From electron spin resonance spectra which were recorded at temperatures higher than about 12°C the order parameters, S, could also be determined. The order parameter, which is dependent on $2T_{\perp}$ (Figure 1) as well as $2T_{\parallel}$, 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.

When the order parameter was plotted as a function of sample temperature, changes in slope occurred corresponding to presumed lipid phase change temperatures. These break points agreed quite well with those determined by the hyperfine splitting parameter. Figure 7 shows, for example, the temperature dependence of the order parameter in cytoplasmic membranes isolated from cells grown at 37°C.

Table 2 summarizes the lower (T_L) and upper (T_U) transition temperatures and transition midpoint temperatures (T_M) found in cytoplasmic and outer membrane isolates. The lower transition temperatures were determined using the hyperfine splitting parameter. The upper transition temperatures represent an average of values determined using the hyperfine splitting parameter, and the order parameter. These data show that T_U in outer membrane isolates changed

Figure 7. The temperature dependence of the order parameter in E. coli cytoplasmic membranes labelled with 5-doxyl stearate. Cells were grown at 37°C. The arrow indicates the phase change.

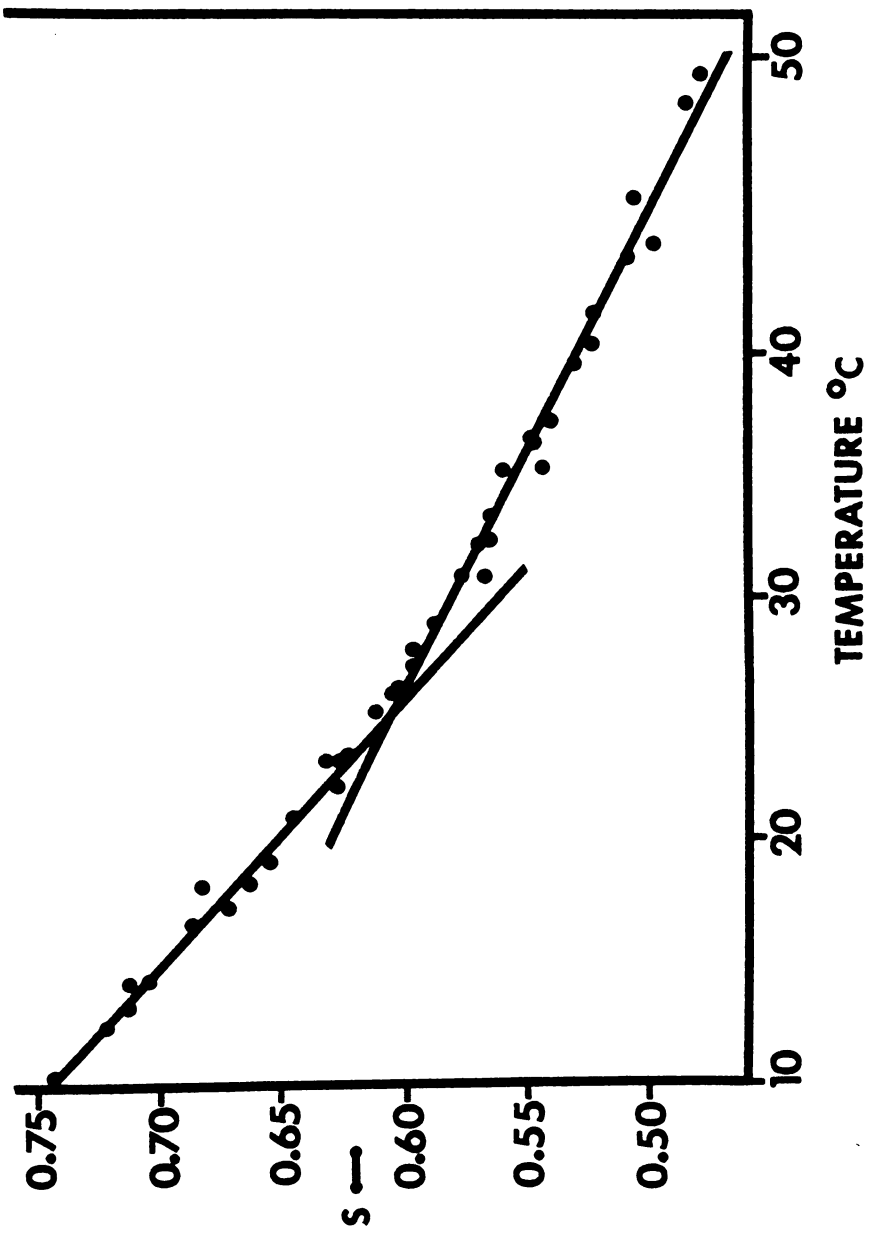

Figure 7.

Table 2. Lipid phase transition temperatures (°C) of isolated cytoplasmic and outer membrane fractions as determined by the hyperfine splitting parameter and the order parameter.

	Cytoplasmic Membrane				Outer Membrane			
Growth Temperature (°C):	12	20	37	43	12	20	37	43
T_L[a]	4.0	4.3	3.0	7.8	8.3	8.8	9.5	10.7
T_U[b]	24.5	25.4	23.7	21.2	28.0	31.8	43.0	41.5
T_M[c]	14.2	14.8	13.4	14.5	18.2	20.3	26.3	27.6

a. lower transition
b. upper transition
c. transition midpoint

markedly such that T_U was always above the cell growth temperature. Indeed the T_M values found in outer membranes increased with increasing incubation temperatures. In contrast, T_U and T_M in cytoplasmic membrane isolates did not change appreciably in response to changes in the growth temperature. Data presented in Table 3 indicate however, that both cytoplasmic and outer membrane isolates exhibited increased order when isolated from cells grown at increasing incubation temperatures.

Discussion

The evidence presented here indicates that of the two membrane components in the intact cellular envelope of E. coli, the outer membrane represents an area of greater order and microviscosity at physiological temperatures. In addition, the inherent order of both cytoplasmic and outer membrane fractions was found to increase as a function of elevated growth temperatures. These findings are consistent with those of Lugtenberg and Peters (28) who have shown that the outer membrane is enriched in saturated fatty acids, and the saturated fatty acid content of both membranes increases with increasing growth temperatures. Saturated fatty acids are known to increase membrane lipid order (29, 30). Moreover, while doxyl stearate spin probes have been shown to situate only in phospholipid domains in the outer membrane (5, 6) their mobility is reportedly significantly

Table 3. Order parameter, S, as a function of growth temperature in cytoplasmic and outer membrane isolates.

	Cytoplasmic Membrane				Outer Membrane			
Growth Temperature (°C):	12	20	37	43	12	20	37	43
Cuvette Temperature (°C)								
12	.70	.72	.73	.74	.76	.77	.80	.80
20	.63	.64	.65	.65	.69	.71	.73	.74
37	.51	.52	.54	.55	.56	.57	.59	.60
43	.48	.49	.51	.52	.51	.53	.54	.56

reduced by the presence of lipopolysaccharide in this membrane fraction (31).

In multicomponent systems, such as biological membranes, it is more likely that inflections in the temperature dependence of ESR spectral parameters reflect phase separations rather than phase transitions (18). It is possible, however, that spin probes could report changes in miscibilities rather than solid to fluid transitions or separations. Nevertheless at temperatures between the upper and lower inflections, lipids can be assumed to exist in two phases (a mixed state) either because of gel and liquid crystalline separation or partial lipid immiscibilities.

In these studies, over a wide range of growth temperatures (12-43°C), the phospholipids in isolated outer membranes were found to exist within their broad phase change in a mixed (presumably gel + liquid crystalline) state, at the temperature of growth. This occurred because T_U in this membrane fraction changed dramatically so that it always existed above the temperature of growth. In contrast, T_U in the cytoplasmic membrane did not change appreciably with growth temperature. As a consequence, the lipids in this inner membrane apparently existed in a mixed state during growth at low temperatures, but in a disordered state during growth at elevated temperatures.

The temperatures of the phase changes observed here in isolated outer membranes are similar to those reported by Emmerling et. al. (32). Using fluorescence techniques,

they reported phase transition mid-points at between 25 and 29°C in outer membrane-murein complexes isolated from cells grown at 30°C. Further, Melchior and Steim (10) reported that endothermic transitions attributed to the outer membrane occurred at higher temperatures than those attributed to the cytoplasmic membrane. In contrast, Overath and coworkers (7) have reported that the two membranes from an unsaturated fatty acid auxotrophic mutant of E. coli have similar transition temperatures. The discrepancy between their data and that presented here may be due to differences in their experimental procedure: cells aberrant in lipid synthesis were cultured in the presence of fatty acids and a nonionic detergent. During the membrane purification the murein was degraded and the membranes were treated with ethylenediaminetetraacetic acid, sonicated and frozen. Such manipulations could have altered or rearranged membrane components.

Esser and Souza (33) and McElhaney (34) have proposed that the existence of two lipid phases in bacterial membranes at the growth temperature may be necessary for survival and division. It is suggested here that in E. coli the cell must keep the outer but not the cytoplasmic membrane in a mixed lipid state to be able to grow.

Figure 8 shows that as the growth temperature was raised, the difference between T_U in the outer membrane and the temperature of growth diminished in a linear fashion. Thus, at a hypothetical growth temperature of 47°C, T_U would

Figure 8. The difference between the growth temperature, T_G, and the upper transition temperature, T_U, (——, $T_U - T_G$) or between T_G and the lower transition temperature, T_L, (----, $T_G - T_L$) in outer membrane isolates as a function of growth temperature. The area between 8 and 47°C indicates the temperature range over which the outer membrane can exist in a heterogenous (presumably gel + liquid crystalline) lipid state.

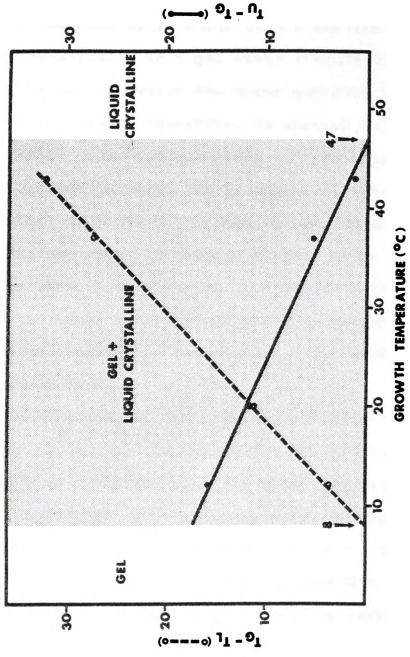
LIQUID CRYSTALLINE
GEL + LIQUID CRYSTALLINE
GEL
47
8
GROWTH TEMPERATURE (°C)
10
20
30
40
50
-10
-20
-30
$T_G - T_L$ (o---o)
$T_U - T_G$ (•——•)

Figure 8.

be expected to occur at 47°C and the membrane would exist essentially in a liquid crystalline lipid state. Similarly, it can be predicted that at a growth temperature of 8°C, T_L in the outer membrane would occur at 8°C and the membrane would exist essentially in a gel state (Figure 8), The temperature range over which the outer membrane lipids can exist in a mixed state, therefore, is assumed to be from about 8 to 47°C. Shaw and coworkers (35) have shown that the lower temperature limit for E. coli cell growth in a glucose minimal media is 7.8°C, and we have found that the upper temperature limit in glucose minimal media is about 45°C. It is therefore suggested that the temperature range over which the outer membrane can exist in heterogeneous lipid state may define the temperature range over which cell growth can occur.

It is understandable that the adaptability of the outer membrane may be crucial for the cell's ability to grow over a broad range of temperatures. The outer membrane appears to act as a molecular sieve allowing small hydrophilic molecules to enter into the periplasm of the cell. This is thought to occur because outer membrane proteins are associated in a specific manner to form pores or transmembrane channels (36, 37). The maintenance of a pore structure may require a specific bulk lipid state, specific phospholipid domains or a specific physical state of the boundary lipids. Thus at high temperatures where a mixed lipid state can no longer be maintained, the resulting state might be too

disordered to allow proper association of the pore forming proteins or proper lipid-protein interactions. The mediation of strong protein - protein interactions by the lipid matrix is consistent with theoretical models (38). Mutants defective in outer membrane synthesis are known to be temperature sensitive. Yet growth at elevated temperatures has been demonstrated in at least some of these mutants, when divalent cations, which rigidify membranes, were added to the medium (39,40).

Several investigators have suggested that the inability of E. coli to grow at temperatures below 8°C is linked to an inhibition of protein synthesis (41-43). Low temperatures, however, have been shown to inhibit in vitro protein synthesis to a lesser extent than whole cell protein synthesis and growth in E. coli (44). This implies that the primary cause of growth inhibition at low temperatures could be a membrane mediated phenomenon. It seems likely that at low temperatures the highly ordered state found in the outer membrane restricts the incorporation of membrane components and in turn restricts growth.

Summary

Purified cytoplasmic and outer membranes isolated from cells of wild type Escherichia coli grown at 12, 20, 37 and 43°C were labelled with either the fatty acid spin probe 5-doxyl stearate or 12-doxyl stearate. Electron spin resonance spectroscopy revealed broad thermotropic phase

changes. The inherent viscosity of both membranes was found to increase as a function of elevated growth temperature. The lipid order to disorder transition in the outer membrane but not the cytoplasmic membrane was dramatically affected by the temperature of growth. As a result, the cytoplasmic membrane presumably existed in a gel + liquid crystalline state during cellular growth at 12 and 20°C, but in a liquid crystalline state when cells were grown at 37 and 43°C. In contrast, the outer membrane apparently existed in a gel + liquid crystalline state at all incubation temperatures. Data presented in this chapter indicate that the temperature range over which the cell can maintain the outer membrane phospholipids in a mixed (presumedly gel + liquid crystalline) state correlates with the temperature range over which growth occurs.

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CHAPTER III

CORRELATION BETWEEN TEMPERATURE RANGE OF GROWTH AND STRUCTURAL TRANSITIONS IN MEMBRANES AND EXTRACTED LIPIDS

The factors which limit bacterial growth at extreme temperatures are not known. In the case of Gram positive thermophiles, it has been suggested that the existence of two lipid phases in the membrane may be necessary for survival and cellular division. These organisms adapt to different environmental temperatures presumably by changing their lipid composition. Maximal and minimal growth temperatures are thus thought to be defined by the beginning and end of the membrane lipid phase separation of the lipid composition present (1, 2).

Although the structure and function of the Gram negative cell envelope has recently been under intensive investigation the situation remains complex due to the existence of both an outer and cytoplasmic membrane. The cytoplasmic membrane has been shown to be a typical bilayer containing protein and phospholipid (3) while the outer membrane contains, in addition, substantial amounts of lipopolysaccharide (4). In this chapter it is reported that for Escherichia coli K12, a typical Gram negative mesophile,

adaptive changes which occur in the outer not cytoplasmic membrane appear to determine the temperature range of growth and these adaptive changes apparently require alterations in outer membrane components other than phospholipids. This represents the first evidence to suggest that the temperature dependence of a membrane structural transition can be modulated in response to growth temperature by components other than phospholipids.

Materials and Methods

Preparation of Membranes and Lipids

Membranes from cultures of E. coli strain W1485F$^-$ were isolated and characterized as described in Chapter II. The degree of purity of cytoplasmic and outer membrane isolates equalled or exceeded that described in Chapter II. Lipids were extracted from the purified membranes by the method of Folch et. al. (5) and resuspended in 10mM N-2-hydroxyethylpiperazine-N'-2'-ethane sulfonic acid (HEPES) pH 7.5. The extracts were assayed for protein (6), ketodeoxyoctanoic acid (KDO) (7) a component of lipopolysaccharide, and phosphate (8) and were shown to contain less than 4.0% of the protein and less than 1.0% of the KDO found in the isolated membranes. Prior to incorporation of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), lipid extracts were dispersed in a bath sonicator. The dispersions cleared upon sonication indicating the formation of stable structures.

Optical densities of lipid dispersions or membranes were matched to insure equal light scattering.

Labelling Procedures

1,6-diphenyl-1,3,5-hexatriene (Sigma Chemical Corp.) was incorporated into membranes and lipids by methods to be described elsewhere (Rouslin, W., MacGee, J. and Gupte, S., unpublished). The molar ratio of probe:lipid was less than 1:100. Fluorescence polarization of the samples (membranes at approximately 1 mg/ml protein, extracted lipids at approximately 10 mg/ml phospholipid) as a function of temperature was measured using a Perkin-Elmer MPF 44A fluorescence spectrometer in the ratio mode with the polarizer accessory. The probe was excited at 358 nm and its fluorescence was detected at 428 nm. In all preparations the fluorescence intensity was nearly equal. For each growth temperature two independently isolated membrane and lipid samples were characterized. Inflection points in the temperature dependence of fluorescence polarization were determined as described in Chapter II. The figures presented represent typical experiments.

Spin labelling procedures were identical to those described in Chapter II.

Results and Discussion

Data presented in Figure 1 show the temperature dependence of label fluorescence polarization (which reflects

Figure 1. Temperature dependence of 1,6,diphenyl-1,3,5-hexatriene polarization in outer membranes isolated from cells of E. coli grown at 20°C (open circles) and 37°C (closed circles). Arrows indicate transition temperatures.

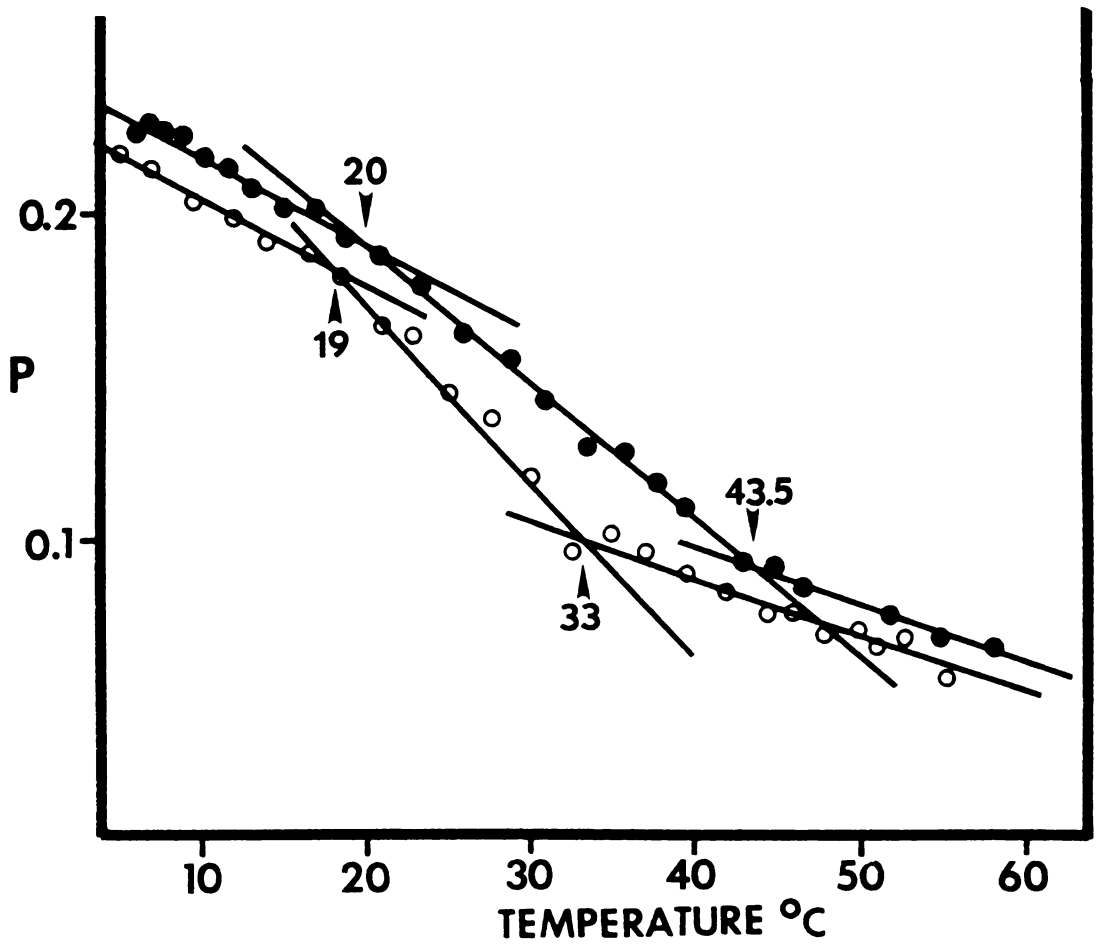

Figure 1.

membrane fluidity) in outer membrane fractions isolated from cells grown at 20 and 37°C. Inflection points represent the beginning and the end of a broad phase separation or gel to liquid crystalline phase change based on studies in well defined model systems (9, 10). As can be seen, the end of the phase change shifted dramatically as a function of growth temperature so that the outer membrane always existed within its broad phase change, in a heterogeneous lipid state, at the temperature of growth. These data support electron spin resonance (ESR) experiments which suggested that the temperature range over which the outer membrane can maintain a mixed lipid state correlates with the temperature range over which growth occurs (Chapter II). In fact identical high temperature transitions were observed in experiments using fatty acid spin probes and polarization of 1,6-diphenyl-1,3,5-hexatriene. While ESR probing indicated that the phase change began at 9°C, fluorescence polarization shows a phase change begins at 19°C. The discrepancy may arise from the possible partitioning of the fluorescence probe into both monolayers of the outer membrane. The spin label used in the previous ESR experiments partitions only into the phospholipid inner monolayer and not the lipopolysaccharide outer monolayer (11-13).

As shown in Figure 2, lipids extracted from outer membranes did not exhibit transition points similar to outer membranes nor did transition points change as a function of growth temperature. Cytoplasmic membranes (Figure 3) and

Figure 2. Temperature dependence of 1,6,diphenyl-1,3,5-hexatriene polarization in lipids extracted from outer membranes isolated from cells of E. coli grown at 20°C (open circles) and 37°C (closed circles). Arrows indicate transition temperatures. The lower transition apparently occurs below ∿4°C and could not be detected.

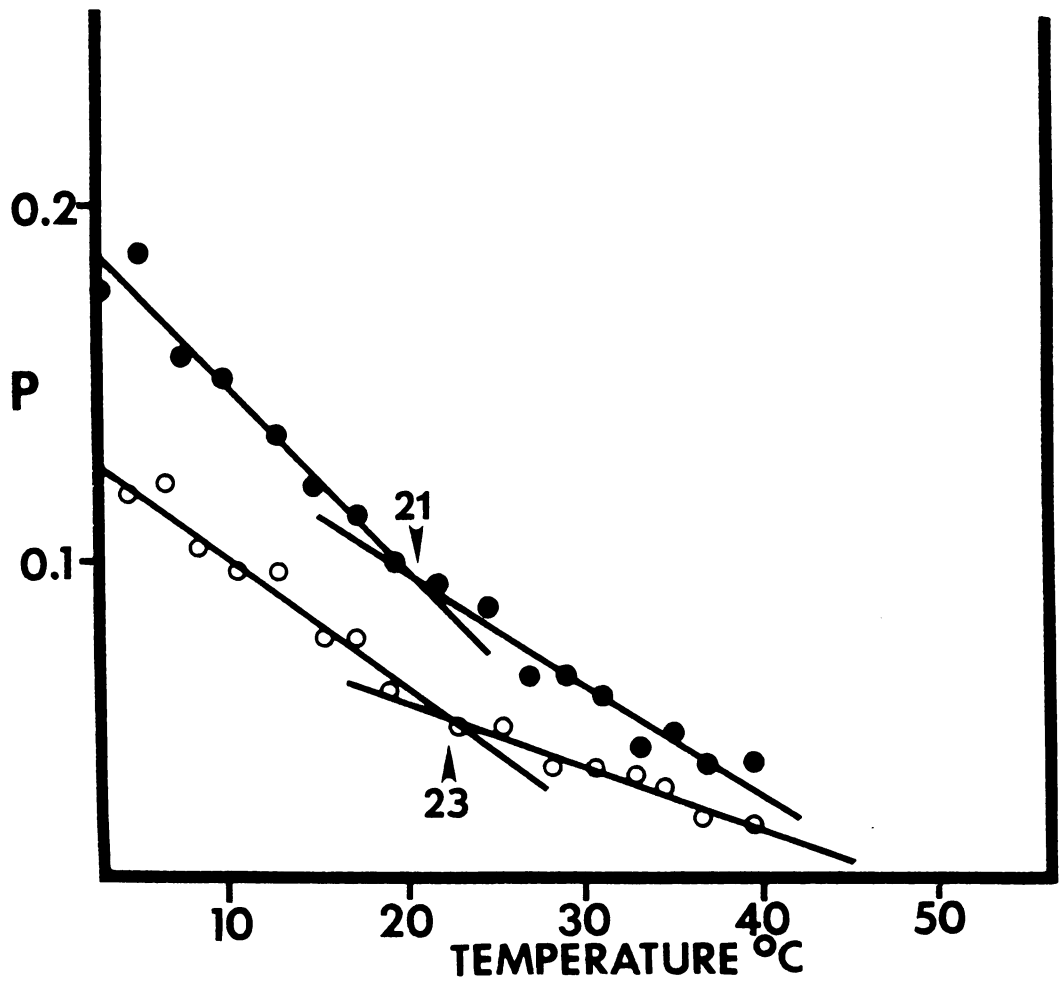

Figure 2.

Figure 3. Temperature dependence of 1,6,diphenyl-1,3,5-hexatriene in cytoplasmic membranes isolated from cells of E. coli grown at 20°C (open circles) and 37°C (closed circles). Arrows indicate transition temperatures. The lower transition apparently occurs below ~4°C and could not be detected.

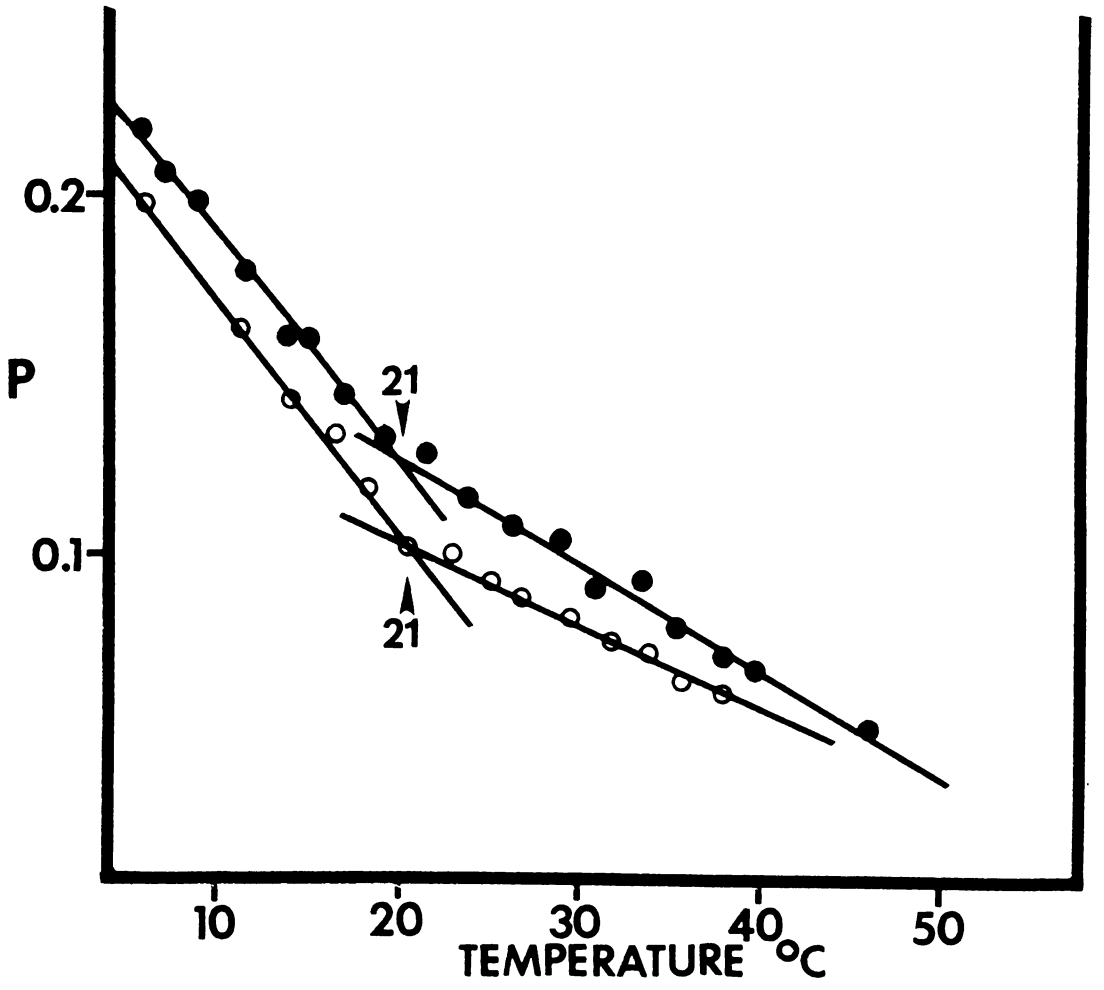

Figure 3.

their extracted lipids (Figure 4) from cells grown at 20 and 37°C all exhibited transitions at 21-22°C. Thus, lipids extracted from both outer membranes and from cytoplasmic membranes exhibited break points identical to those exhibited by intact cytoplasmic membranes regardless of growth temperature. Similar data were obtained using the spin label 5-doxyl stearate (Figure 5). Previous ESR experiments also indicated that the cytoplasmic membrane did not shift its (∿20°C) phase transition as a function of growth temperature (Chapter II). Thus the structural transition of the outer not the cytoplasmic membrane changes as a function of growth temperature and this change apparently requires components other than phospholipids. While it is known that the fatty acid composition of the phospholipids in the outer membrane changes as a function of growth temperature (14, 15) these changes do not appear to alter the structural transition shown here (Figure 2). This is perhaps the first demonstration that changes in membrane architecture induced by non-phospholipid components may provide a mechanism for environmental adaptation. The possibility that changes in membrane structure could arise in connection with lipid changes due to altered interactions of phospholipid with other membrane components however, is not ruled out.

It is understandable that the adaptability of the outer membrane may be crucial for the cell's ability to grow over a broad range of temperatures. Outer membrane proteins presumably associate in a specific manner to form

Figure 4. Temperature dependence of 1,6, diphenyl-1,3,5-hexatriene polarization in lipids extracted from cytoplasmic membranes isolated from cells of E. coli grown at 20°C (open circles) and 37°C (closed circles). Arrows indicate transition temperatures. The lower transition apparently occurs below ∿4°C and could not be detected.

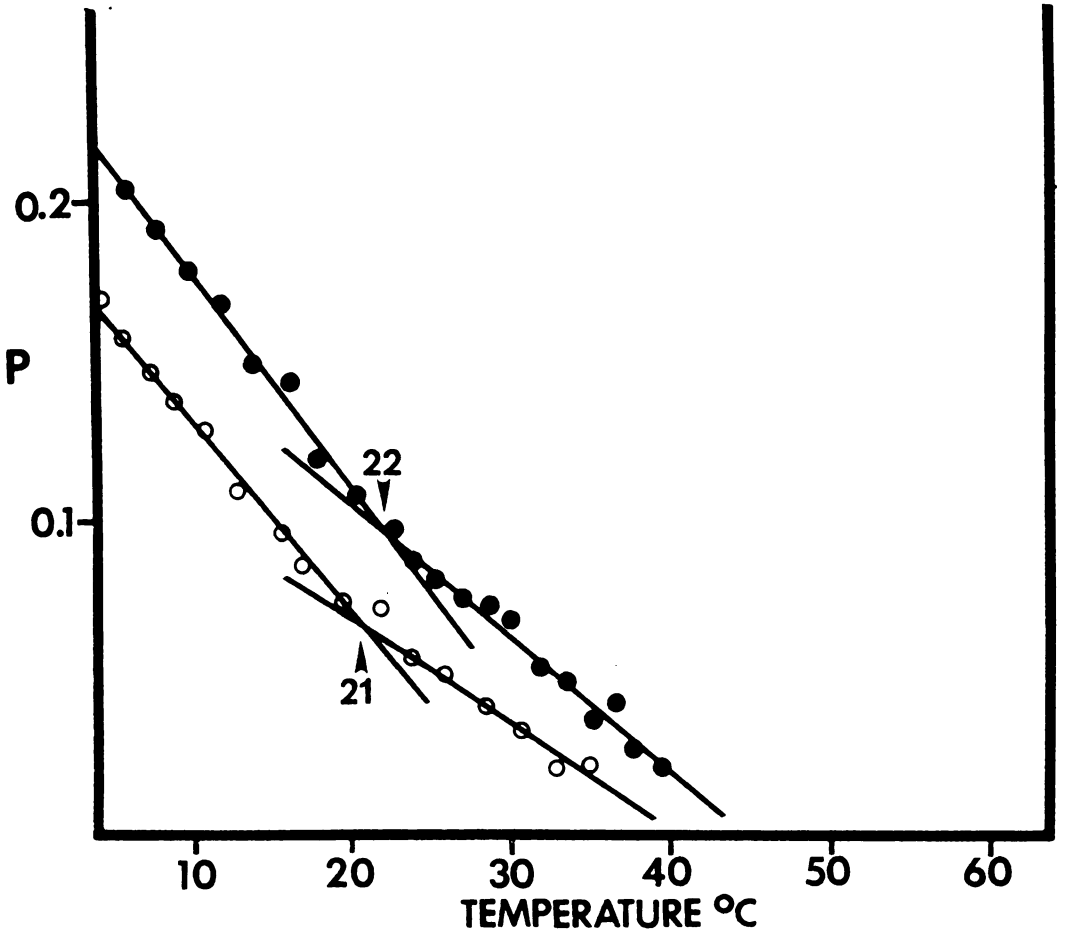

Figure 4.

Figure 5. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss) as a function of temperature in E. coli outer membranes (open circles), cytoplasmic membranes (closed circles), lipids extracted from outer membranes (open squares) and lipids extracted from cytoplasmic membranes (closed squares). The membranes and extracted lipids were isolated from cells grown at 37°C in M9 minimal medium supplemented with 0.4% glucose. The membranes and vesicles were labelled with 5-doxyl stearate. The arrows indicate phase transitions.

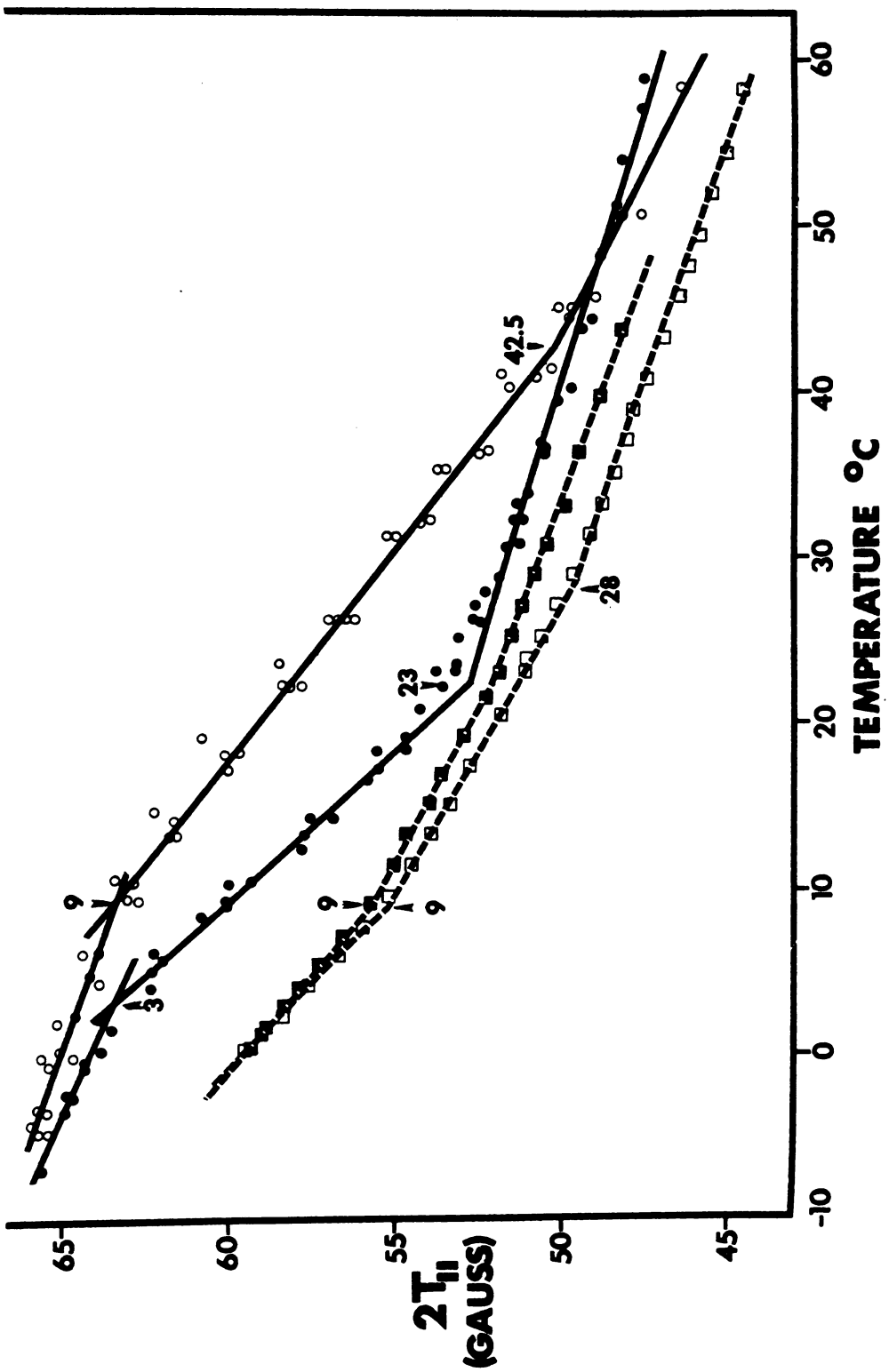
65
60
55
50
45
$2T_{\perp}$ (GAUSS)
-10
0
10
20
30
40
50
60
TEMPERATURE °C
42.5
23
28
9
9
9
3

Figure 5.

pores or transmembrane channels (16, 17). Such pore structure may require a specific lipid state. In this regard the recent evidence that induced increases or decreases in the fluidity of the outer membrane affect the processing and assembly of outer membrane proteins (18-20) is intriguing.

Lastly, it should be noted that experiments reported here confirm ESR observations (Chapter II) which suggested that the phase transition in the outer membrane of E. coli always occurs at higher temperatures than in the cytoplasmic membrane. Similar differences in the phase transition of cytoplasmic and outer membranes have recently been detected by deuterium magnetic resonance quadrupolar echo spectroscopy (21).

Summary

Purified cytoplasmic and outer membranes isolated from cells of wild type Escherichia coli grown at different temperatures were labelled with 1,6-diphenyl-1,3,5-hexatriene and analyzed using fluorescence polarization techniques. Lipids extracted from the membranes were similarly analyzed using fluorescence polarization. The thermotropic order to disorder transition in outer membranes changed as a function of growth temperature. The order to disorder transition in cytoplasmic membranes and lipids from either cytoplasmic or outer membranes did not change with growth temperature. These data are interpreted to indicate that adaptive changes which occur in the outer membrane may determine the

temperature range of growth of E. coli. These changes apparently require alterations in outer membrane components other than phospholipids.

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CHAPTER IV

ANESTHETICS ALTER OUTER MEMBRANE ARCHITECTURE AND TEMPERATURE RANGE OF GROWTH.

In recent years the functional significance of biological membrane structure has been intensively investigated. Attention has focused on membrane architecture as a basis by which the temperature range of microbial growth is determined (1, 2). Although definitive evidence is lacking, a relationship seems to exist between the physical state of membrane lipids and the upper and lower temperature limits of growth in various bacteria (3, 4, 5). Studies presented in Chapter II and Chapter III concern investigations of spin labelled and fluorescently labelled membranes and lipids isolated from Escherichia coli. These studies and ^{2}H-NMR studies of Davis et. al. (6) and Nichol et. al. (7) have indicated that: 1) both the outer and cytoplasmic membranes undergo broad thermotropic order-to-disorder transitions over which gel and liquid crystalline lipid domains clearly exist, and 2) the midpoint of the phase transition of the outer membrane occurs at significantly higher temperatures than that of the cytoplasmic membrane. Moreover, data presented in Chapters II and III indicate that the

thermotropic transition in the outer membrane occurs at increasingly higher temperatures as the growth temperature is elevated. Since the temperature range over which the outer membrane can adapt to remain in a mixed (gel + liquid crystalline) lipid state is identical to the temperature range over which growth occurs (see Chapters II and III) it is proposed that outer membrane structure delimits the growth range of E. coli.

It is quite conceivable that the adaptability of outer membrane structure may be crucial for the cell's ability to grow over a broad range of temperatures. Outer membrane proteins associate in a specific manner to form pores or transmembrane channels (8, 9). Such pore structure may in turn require a specific lipid structure. In the mixed (gel + liquid crystalline) lipid state the resulting high lateral compressibility should enable the membrane to accomodate newly synthesized protein without large volume changes and should more readily allow for conformational changes (10) thus favoring pore formation. Indeed it has been suggested that the functioning of ion pores in neuronal membranes also require a mixed lipid state (11). Anesthesia is thought to result as a consequence of the melting of gel phase lipid (12). This suggests that anesthetics could also melt gel phase lipid in E. coli outer membranes and in turn inhibit growth.

In this chapter it is reported that low concentrations of two anesthetics, procaine and phenylethanol (PEA), decreases

the phase transition temperature of E. coli outer membranes and concomitantly decrease the maximum temperature of growth. The effects of these compounds on growth rate are greater at high temperatures where only a small amount of gel phase lipid exists and minimal or nonexistent at low temperatures. These results substantiate the hypothesis that outer membrane structure is critical in determining the temperature range of growth of E. coli. Further, it is demonstrated in this chapter that the temperature limits of growth of E. coli can be modulated by compounds that alter outer membrane structure.

Materials and Methods

The growth of E. coli strain W1485F$^-$, isolation and characterization of membranes, and electron spin resonance spectroscopy was as described in Chapter II except for the addition of either PEA or procaine (0.1% or 10mM final concentration respectively) to the growth media and to all buffers. The degree of purity of cytoplasmic and outer membranes equalled or exceeded that described in Chapter II. As judged by membrane protein concentration and by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (13), neither anesthetic affected the incorporation of major outer membrane proteins.

To assess the possibility that growth in PEA or procaine leads to compensatory changes in membrane architecture, outer membranes isolated from cells grown in the absence of

anesthetics were washed and resuspended in either procaine (10mM) or PEA (0.1%) prior to spin labelling. These membranes exhibited identical transitions compared to those grown and isolated in the presence of anesthetics.

Results and Discussion

Electron spin resonance (ESR) spectroscopy of spin labelled outer membranes isolated from cells grown in the presence of either PEA or procaine revealed differences in structural transitions compared to those of control outer membranes from cells grown at the same temperatures. As shown in Figure 1, the low temperature (gel → gel + liquid crystalline) transitions in outer membranes from cells grown in the presence of PEA at 20, 30 and 37°C were nearly identical, and were identical to those of control membranes (see Chapter II). In contrast, the high temperature (gel + liquid crystalline → liquid crystalline) transition changed with growth temperature (Figure 1) and occurred at lower temperatures than in control outer membranes from cells grown in the absence of anesthetic. As can be seen in Figures 1 and 2, the difference between the high temperature transition, T_U and the growth temperature, T_G, decreased linearly with increasing growth temperature. For outer membranes isolated from cells grown in the presence of anesthetic, this difference approached zero at a growth temperature of 41°C. Thus, at a growth temperature of 41°C, the membrane would be expected to exist essentially in a

Figure 1. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in E. coli outer membranes labelled with 5-doxyl stearate. The hyperfine splitting parameter is related to the rotational mobility of the spin label and therefore reports the local fluidity of the membrane lipids. High values of $2T_{\parallel}$ reflect low fluidity. Breaks in the temperature dependence of $2T_{\parallel}$ have been correlated with lipid phase separations or lipid phase transitions (see Chapter II). Membranes were isolated from cultures of E. coli W1485F$^-$ grown at a) 20° b) 30° and c) 37°C in M9-glucose minimal medium supplemented with 0.1% PEA. The membranes were isolated as previously described except that 0.1% PEA was present in all buffers used during the membrane isolation. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

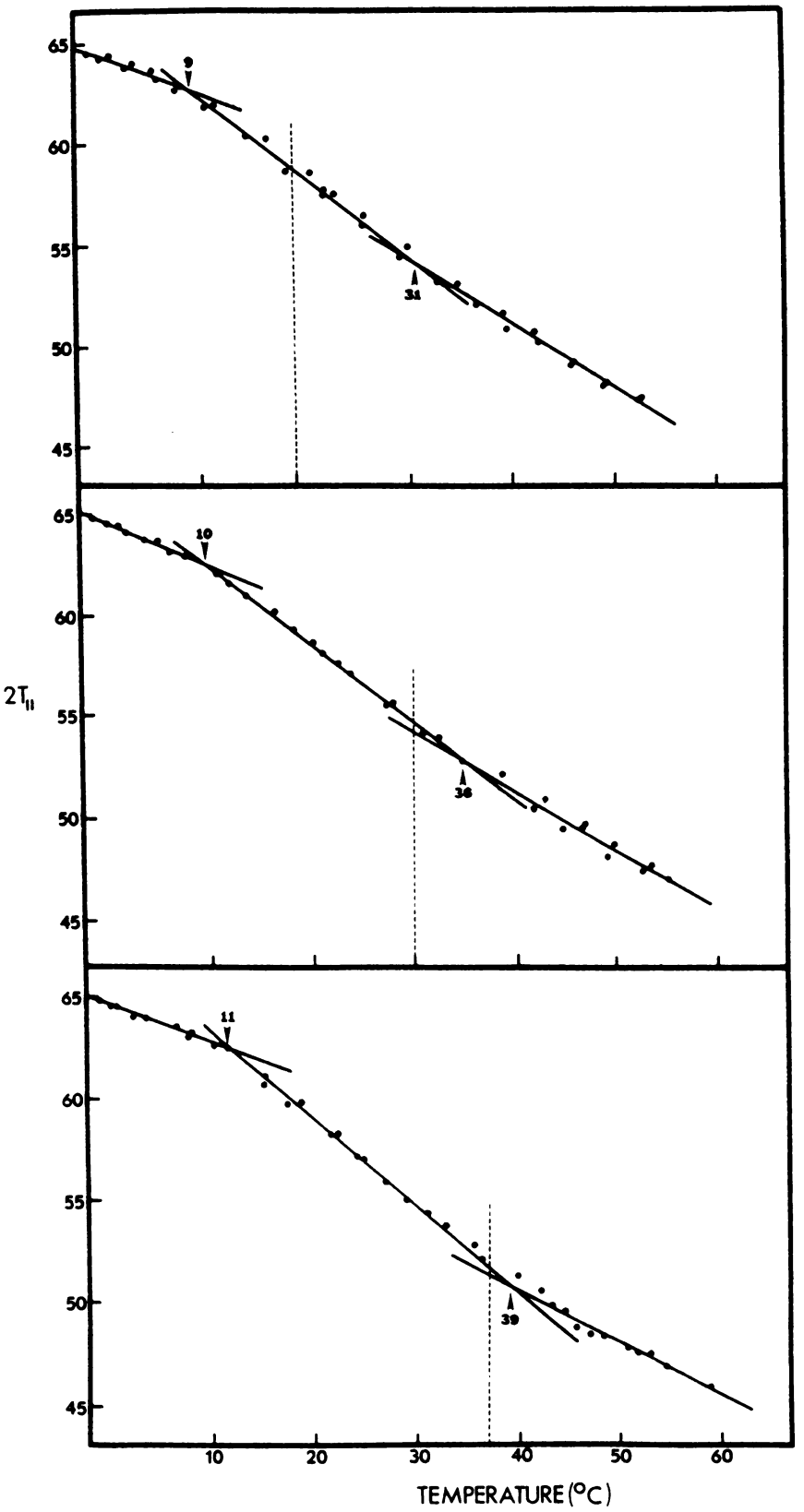
65
60
55
50
45
9
31
10
38
11
39
$2T_{\perp}$
10
20
30
40
50
60
TEMPERATURE (°C)

Figure 1.

Figure 2. The difference between the growth temperature T_G, and the upper transition, T_U, (T_U-T_G) in outer membrane isolates as a function of growth temperature. Δ——Δ outer membranes from cells grown in M9 glucose minimal medium, ---- outer membranes from cells grown in M9 glucose medium containing either 0.1% PEA (closed circles) or 10mM Procaine (open circles).

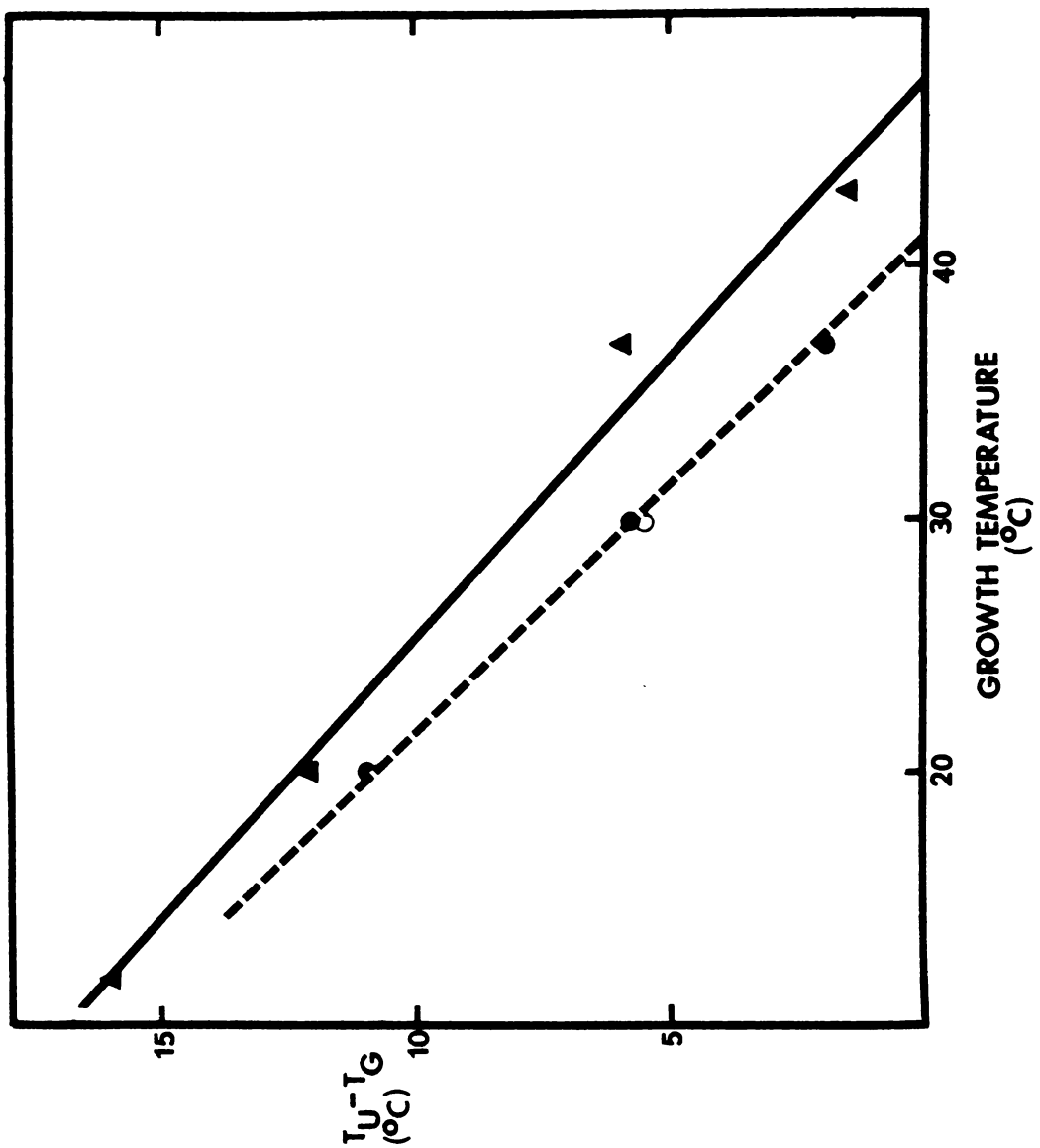

Figure 2.

liquid crystalline lipid state. In other words, under the conditions of the experiments reported here, outer membranes from cells grown in the presence of PEA or procaine are maintained within their transition, i.e., in a mixed lipid state, up to a growth temperature of approximately 41°C. This is in contrast to the hypothetical limit of 47°C for the mixed state of outer membranes from control cells grown in the absence of perturbant (Figure 2).

Moreover, the decrease in T_U of the outer membrane is mirrored by a decrease in the maximum temperature of growth (Figure 3). The growth rates of cells incubated at low temperatures (<30°C) in the presence of anesthetic were essentially identical to those of cultures without perturbant. This is consistent with the model presented in Chapters II and III since at these lower growth temperatures the outer membrane's high temperature transition (T_U) lies well above the incubation temperature and the membrane should exist well within its transition. However, as the cells are grown above 30°C in the presence of anesthetic, growth is slower and is completely inhibited at 42°C. This temperature is essentially identical to the upper limit that the cells can maintain the outer membrane within its transition when anesthetic is present. The concurrent decreases in the upper temperature limit of growth and in the high temperature limit of the transition of the outer membrane upon addition of anesthetic renders further evidence that the temperature range over which the outer membrane

Figure 3. Growth rates of cultures of E. coli W1485 incubated at various temperatures were determined by monitoring the optical density of the cultures of 560nm. ——Cultures growing in M9 minimal medium containing 0.4% glucose. ---- Cultures growing in M9 minimal-glucose medium supplemented either with 0.1% PEA (closed circles) or 10mM procaine (open circles)

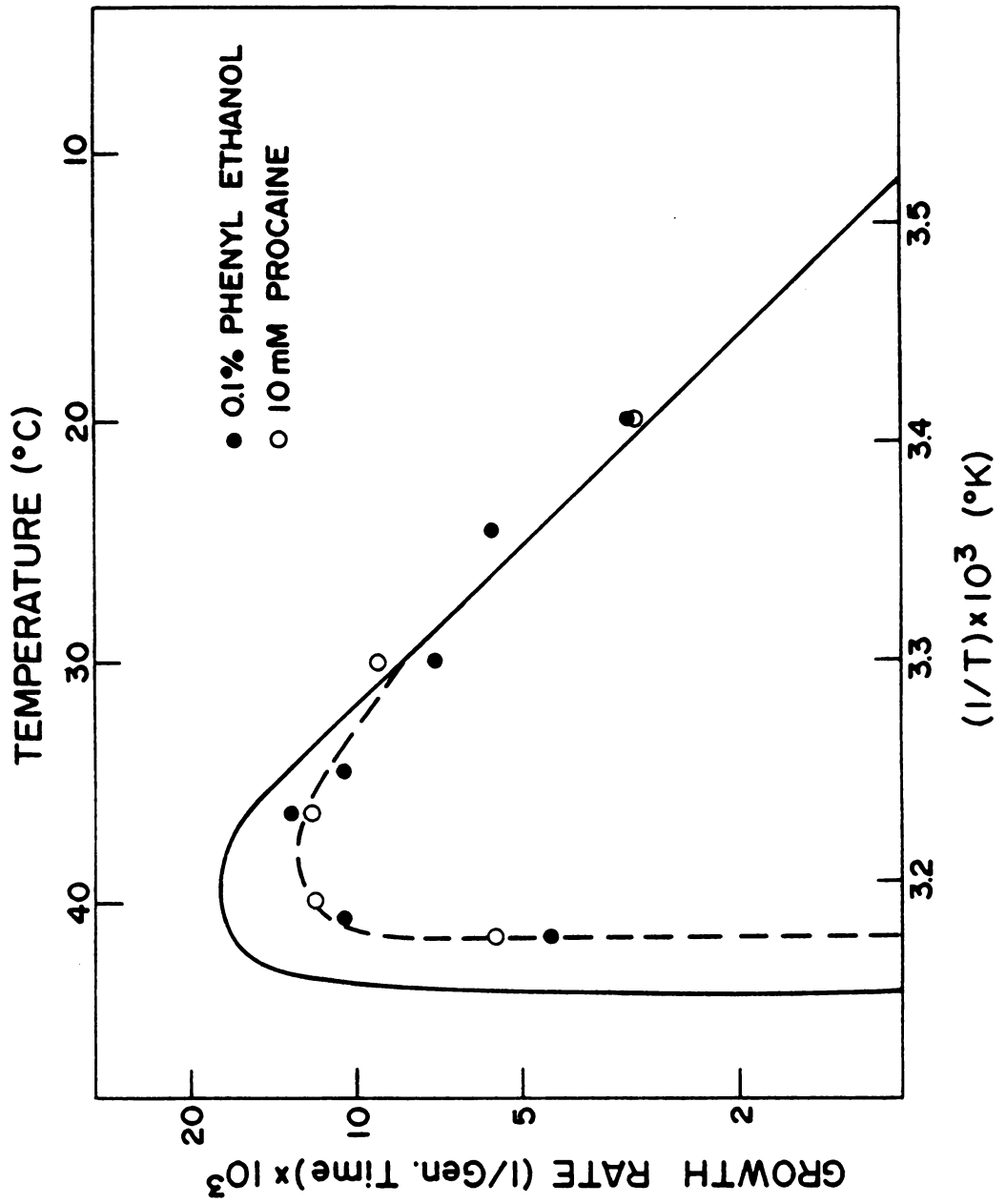
TEMPERATURE (°C)
10
20
30
40
● 0.1% PHENYL ETHANOL
○ 10 mM PROCAINE
GROWTH RATE (1/Gen. Time) x 10³
20
10
5
2
(1/T) x 10³ (°K)
3.5
3.4
3.3
3.2

Figure 3.

can exist within its transition determines the temperature range over which growth can occur.

Structural transitions of spin labelled cytoplasmic membranes were practically identical when membranes were isolated from cells grown either in the presence or absence of perturbants. These findings suggest that the outer membrane is more sensitive to low concentrations of anesthetics than is the cytoplasmic membrane. At concentrations of PEA higher than those used here, the synthesis of outer membrane proteins is inhibited (14) presumably through the involvement of cytoplasmic membrane function.

The differential sensitivity of the outer membrane to PEA and procaine may reflect a complex supramolecular structure. Recently a model has been put forth which describes the outer membrane in terms of polymer domains (15). Microdomains which exist within this network apparently cannot be detected by x-ray diffraction (16) but participate in the membrane phase transition (6, 7, 17). Other evidence suggests that membrane lipid domains are created by long range lipid-protein interactions (18). Such a "polymer-network" model of the outer membrane could explain increased sensitivity to anesthetics in terms of the disruption of lipid-protein interactions as has been described by Mazzanti et. al. (19). It is possible that the disruption of outer membrane domains by local anesthetics could result in the inhibition of the initiation of DNA synthesis. Nagai (20) has described an association of replicative

origin DNA with outer membrane proteins. And procaine has been shown to enhance hyperthermic killing of E. coli (21) possibly through a mechanism that involves DNA.

Although the structural transition of the outer membrane changes as function of growth temperature (Chapter II), these changes have been shown to require components other than the phospholipid (Chapter III). Lipopolysaccharides derived from cells grown at increasingly higher temperatures exhibit elevated transition temperatures (22). That spin labels which partition only into domains of the outer membrane devoid of lipopolysaccharide (23-25) may be detecting these changes (Chapter II) gives further evidence to the complexity of outer membrane structure.

In summary, it appears that low concentrations of local anesthetics inhibit growth of E. coli by disrupting the mixed (gel + liquid crystalline) lipid state in the outer membrane. This effect is reversed at lower growth temperatures consistent with a mechanism involving the restoration of gel phase lipid. A similar reversal of anesthetic effect in the squid axon brought about by a lowering of temperature has been described (26). More generally, our results indicate that the growth limits of Gram negative bacteria can be altered and restricted by compounds which preferentially alter the structure of the outer membrane. Consequently, the possibility exists of developing more efficacious outer membrane perturbants which might be pharmacologically important in modifying and controlling Gram negative infective

agents.

Summary

The local anesthetics procaine and 2-phenylethanol lowered the phase transition temperature of purified outer membranes of Escherichia coli. Concomitantly, these anesthetics lowered the maximum temperature of growth without affecting growth at low temperatures. The phase transition of the cytoplasmic membrane was not affected. These data substantiate the hypothesis that the temperature range over which the cell can maintain the outer membrane in a mixed (gel + liquid crystalline) lipid state determines the temperature range over which growth can occur.

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APPENDICES

APPENDIX A

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

Osmotic fragility and mechanical deformability of human erythrocytes are altered by low concentrations of a variety of membrane stabilizing drugs (1). Such drugs have the potential for protecting erythrocytes from hemolysis and for increasing 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 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 which is closely associated with proteins and devoid of cholesterol in the human erythrocyte membrane (2, 3), electron spin resonance spectroscopy of erythrocytes labelled with 5DS should reveal structural information specifically related to protein-lipid interactions.

It is reported here that 5DS and 5-doxyl stearate methyl ester (5DS-ME) labelled human erythrocytes exhibit discontinuities in the temperature dependence of electron spin resonance (ESR) spectral parameters. These temperature induced changes in membrane structure correlate with other reported temperature dependent phenomena of erythrocytes which are presumed to reflect lipid state changes (4-8). A variety of membrane stabilizing agents are shown to affect the temperature dependence of these measured spectral parameters. Thus membrane stabilization induces a structural rearrangement in the erythrocyte membrane predominantly involving a change in protein-lipid interactions.

Material 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 300m0smole NaCl. In

order to remove serum albumin and other potential binding substances of the spin probe, the reserved 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, CA) were dissolved as a 30 mM solution in absolute ethanol. The labelling procedure used was that described in Chapter II except that the ethanol was evaporated prior to addition of erythrocytes. Drugs were added following labelling. In order to standardize drug concentrations with respect to the stabilizing effect, concentrations were selected which caused approximately equivalent degrees of submaximal protection against hypotonic hemolysis (9,10). The final probe concentration was approximately 0.4 μmoles/mg membrane protein. At this concentration 5DS has been reported to minimally perturb erythrocytes (11) and to localize in lipid domains in close association with proteins (2, 3).

All ESR studies were performed by standard methods as described in Chapter II. 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. When spin labelled preparations are placed in the resonant cavity of an ESR spectrometer they are exposed to microwaves of a constant frequency while held in a linearly varying magnetic field. Microwaves are thus absorbed at three points in the magnetic field corresponding to the resonant characteristics of the unpaired electrons. These characteristics are dependent on intermolecular influences. A typical spectrum is displayed as the first derivative of microwave absorption plotted against an increasing magnetic field in gauss (See Figure 1). The distance between the low field and high field absorption peaks, $2T_{\parallel}$ (or 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 electrons tend to average resulting in lower values, of $2T_{\parallel}$ which reflect a more fluid environment. The hyperfine splittine 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 fluid environments (12).

In studies reported here $2T_{\perp}$ could be determined in 5DS labelled preparations above about 12°C. This parameter is used along with $2T_{\parallel}$ to calculate the order parameter S. 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

Figure 1. Electron spin resonance spectra of human erythrocytes labelled with 5-doxyl stearate. The spectra were taken at the temperatures indicated. Scan range was 100 Gauss. Absence of free probe is indicated by 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.

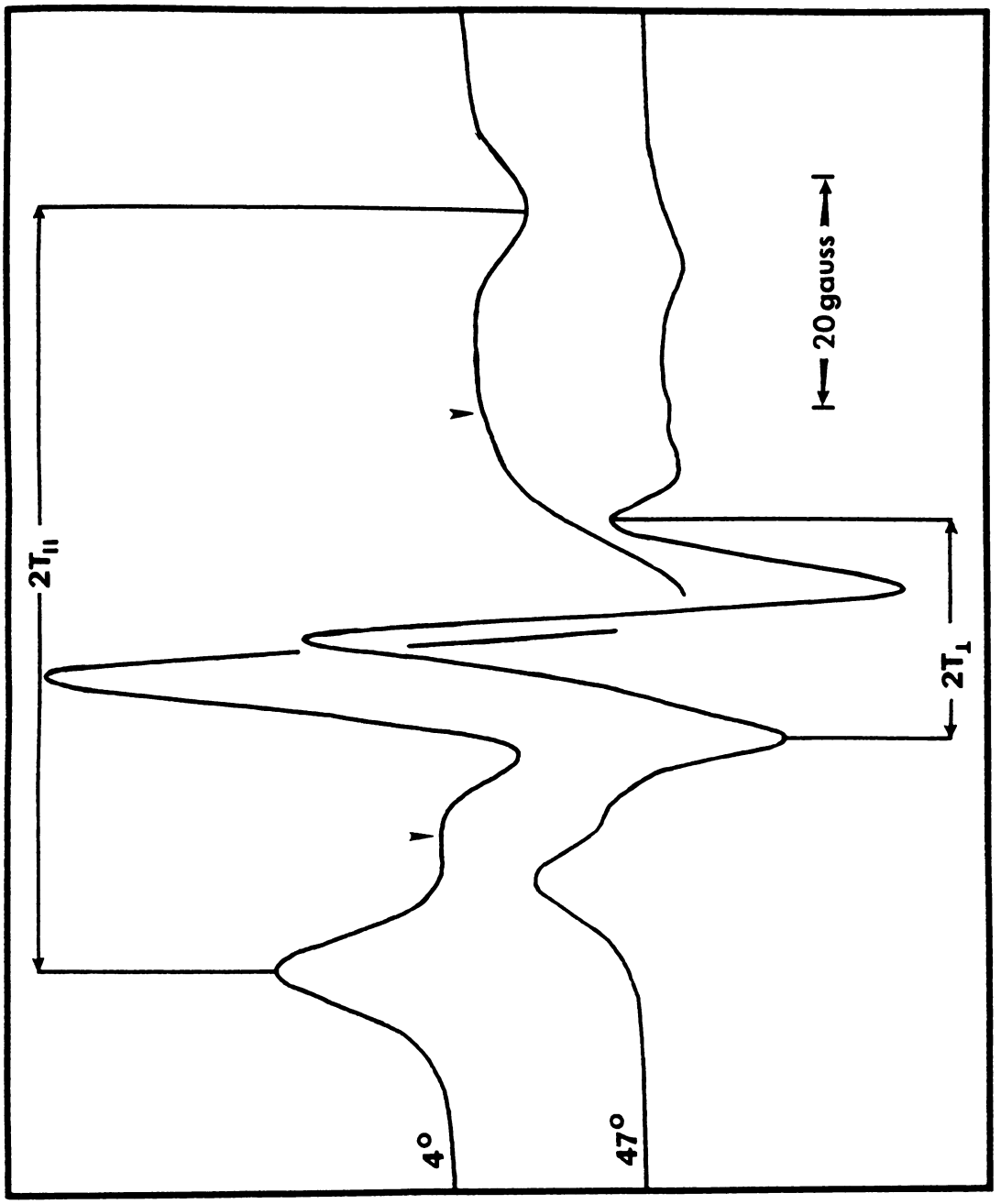

Figure 1.

random sample S=0 (13).

The hyperfine coupling constant also calculated from the directly measured $2T_{\parallel}$ and $2T_{\perp}$ parameters is considered to reflect local polarity (14) 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., and McGroarty, E., submitted). Briefly, a B-spline (a piece-wise set of polynomials which 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 (See Appendix B). 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 individuals.

Results

Control Erythrocytes

ESR spectra of 5DS labelled 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 2). 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 probe which is not charged causes less pertubation of the cells. Spectra of 5DS-ME labelled cells allowed determination of the hyperfine coupling constant which indicated that the probe was in an environment similar to that of 5DS labelled cells at temperatures up to approximately 28°. Above that temperature the spectra changed in such a manner that the spectral parameters were difficult to measure.

In spectra of 5DS-ME labelled cells recorded between 0 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

Figure 2. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in erythrocytes labelled with 5-doxyl stearate. The arrow indicates the transition temperature.

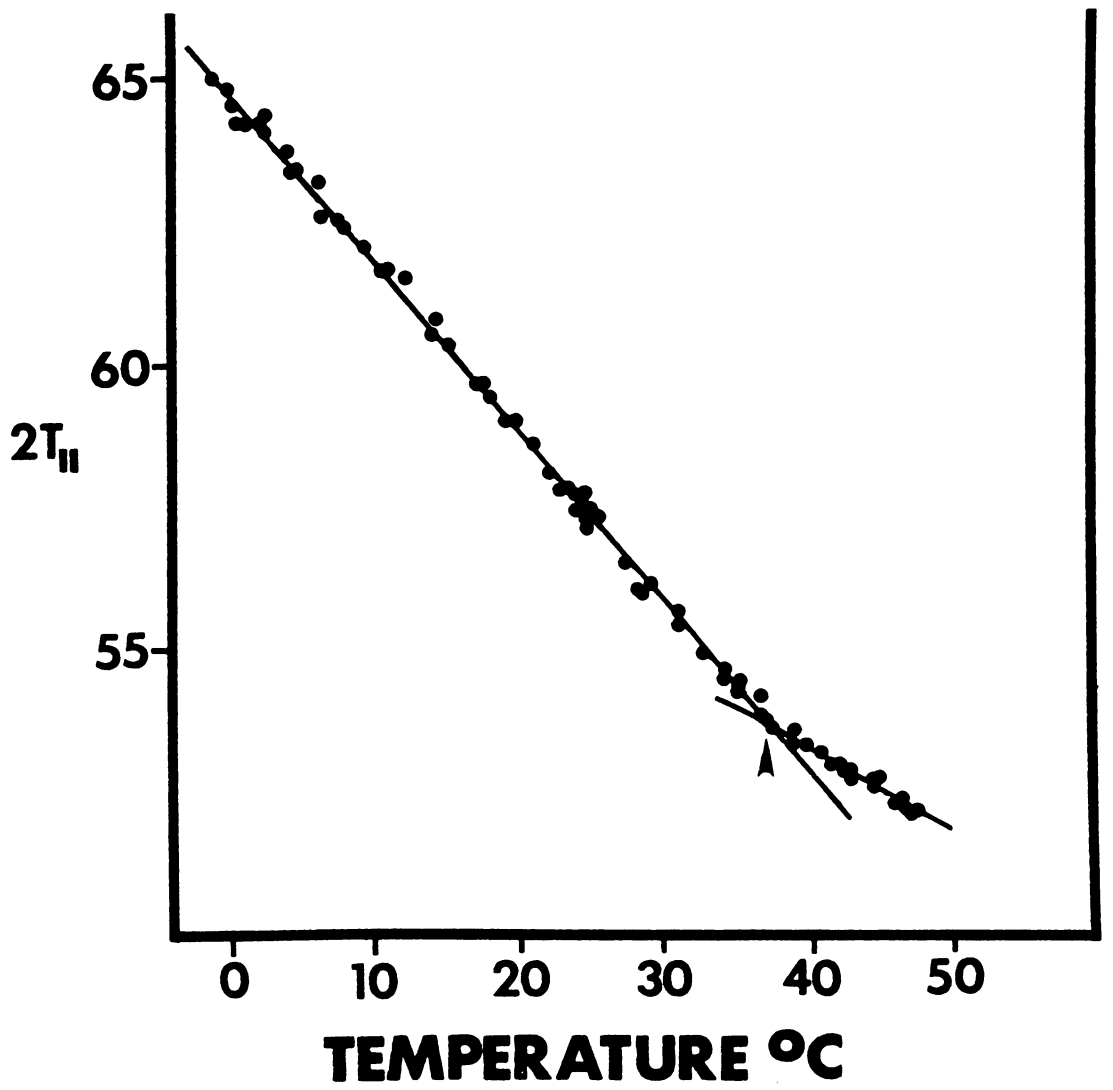

Figure 2.

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

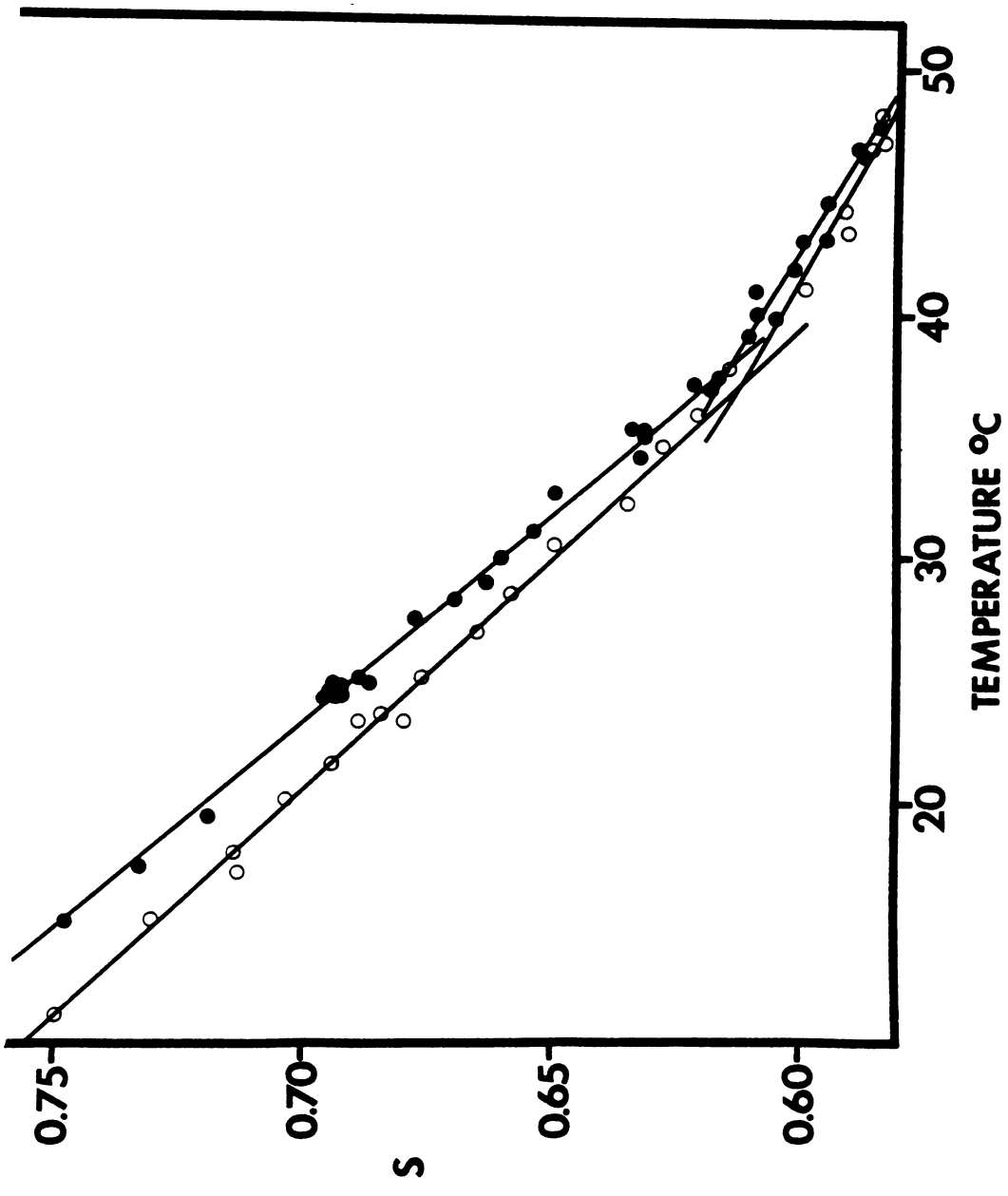

Figure 3.

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° and a second at about 37° (See Table 1).

Effects of Membrane Stabilizing Agents

In order to further characterize erythrocyte membrane mediated phenomena, we analyzed the effect on membrane structure of compounds which are known to protect human erythrocytes from osmotic hemolysis: propranolol, diazepam, chlorpromazine (9) and pluronic polyols (polyoxypropylene-poly-oxyethylene condensates) (10). 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 labelled erythrocytes caused a slight decrease in the upper transition temperature as measured using $2T_{||}$ and S (See Table 1). In addition a low temperature transition could be detected with 5DS when propranolol was added (Figure 4). 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 labelled cells in the presence of diazepam, chlopromazine or Pluronic F68. All of these agents reduced the upper transition temperature but did not appreciably alter the lower transition

Table 1. Transition temperatures (°C) of erythrocyte membranes using ESR spectroscopy.

SAMPLE		T_L[b]	T_U[c]
Control		14.5	36.5
Propranolol[a]	($5x10^{-4}$M)	14.6	35.1
Diazepam	($3x10^{-4}$M)	14.7	32.9
Chlorpromazine	($5x10^{-5}$M)	13.2	34.2
Pluronic F68	($7x10^{-5}$M)	14.0	33.6

a. Perturbants were added to give a final concentration as indicated.
b. lower transition temperature determined with 5-doxyl stearate methyl ester (5DS-ME) by measuring $2T_{\parallel}$ as a function of temperature.
c. upper transition temperature determined with 5DS by measuring both $2T_{\parallel}$ and S (order parameter) as a function of temperature.

Figure 4. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in erythrocytes labelled with 5-doxyl stearate and subjected to $5x10^{-4}$M propranolol. The arrow indicates the transition temperature.

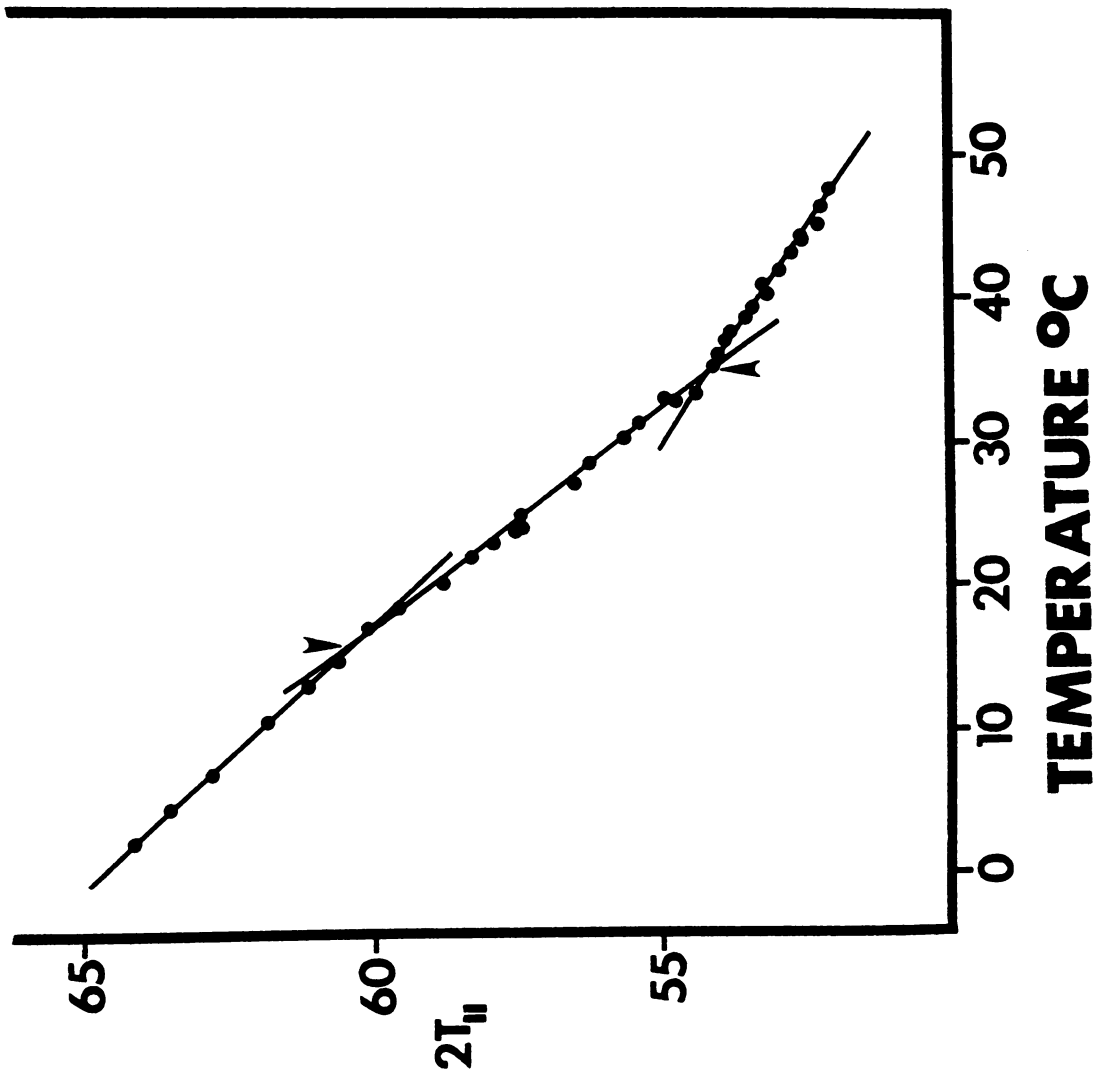

Figure 4.

temperature (Table 1). Furthermore, the addition of diazepam or chlorpromazine (but not Pluronic F68) allowed for the detection of the low temperature transition in 5DS labelled cells. In addition, the presence of diazepam was shown to significantly alter the order parameter as indicated in Figure 3. None of the other perturbants showed this effect. 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 (15, 16).

Discussion

Data presented here indicate that there are two thermally induced structural transitions in human erythrocyte membranes: one of these is found at 15°, the other at 37°C. These transitions are presumptively 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 (17), 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.

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 tryptophas fluorescence by spin labels showed discontinuities at 15 and 35°C (3). Laser raman spectroscopy (4) and viscosimetry (5) revealed a discontinuity at approximately 18°C. A discontinuity in the susceptibility of human erythrocytes to benzyl-lysolecithin has been reported to occur at about 15°C (6). Transfer of phospholipid from hemagglutinating virus of Japan to erythrocyte membranes was reported to begin at about 19°C and saturate at 37°C (7). Finally, the preservation of membrane lipid asymmetry by Mg^{2+} upon lysis has been shown to diminish about 18° and disappear at about 40°C (8). These data support the contention that temperature induced structural transitions occur in erythrocyte membranes and can be detected using spin labelling techniques. Apparently, these transitions occur in local areas not influenced by cholesterol.

In all cases the addition of the membrane stabilizing drugs to the spin labelled 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 (18), it appears likely 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 (19).

Addition of propranolol, chlorpromazine and diazepam (but not Pluronic F68) to erythrocytes allowed for the determination of a low temperature transition using 5DS, and caused hemolysis at high concentrations in isotonic saline (data not shown). Since Pluronic did not cause hemolysis at high concentrations, it is probably that it interacts with membrane in a unique fashion. 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 (15) can be correlated with a protein conformational change (20).

Propranolol, diazepam, chlorpromazine, and Pluronic F68 have been shown to protect erythrocytes against hypotonic hemolysis. Thus it is probable that the structure of specific membrane domains modulate the osmotic fragility of the intact cell. 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 (15, 21, 22), 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.

Summary

Membrane structure in intact human erythrocytes was analyzed by electron spin resonance spectroscopy (ESR). The spin probes 5-doxyl stearate and 5-doxyl stearate methyl ester revealed thermally induced structural transitions in the membrane at 37 and 15°C. The addition of propranolol, diazepam, chlorpromazine, or Pluronic F68 all caused a decrease in the 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 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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APPENDIX B

USE OF ELECTRON SPIN RESONANCE TO STUDY BACILLUS MEGATERIUM SPORE MEMBRANES

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 stereospecific compound like L-proline (1). One model to explain these processes suggests the spore membrane(s) may be involved (3). To study this possibility electron spin resonance (ESR) spectroscopy has been used. What appears to be the first demonstration of biophysical changes in spore membranes that result from heat-activation and L-proline is reported here.

Materials and Methods

Isolation of Spore Membranes

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 are 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.

Spin Labelling

All ESR studies were done with freshly prepared membranes (0.4 ml) to which 4-5 µl of 30 mM 5-DS was added. The labelling techniques that were used have been previously described (see Chapter II). ESR spectra of such spin labelled preparations allow the determination of the hyperfine splitting parameter, $2T_{\parallel}$, which reports the local fluidity of the membrane lipids (7). 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., Stanford, CT) was used to monitor the temperature of the sample.

Data Analysis

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., submitted). Briefly, a B-spline (8) was used to provide a smooth fit for the ESR data and points of inflection were used to group data. Regression 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 (Chapter II). 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.

Results and 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 (9). The membranes

contained no peptidogylcan, 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 2) 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 exhibited a greater slope (-0.29 gauss/°C) than did 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. 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, the effect of adding in vitro a known trigger reagent, L-proline, to membranes from heat-activated spores was tested. The results (Figure 3) show a dramatic change in the upper transition temperature from 23° before to 31° after the addition of L-proline. When the same experiment

Figure 1. Temperature dependence of $2T_{\parallel}$ 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.

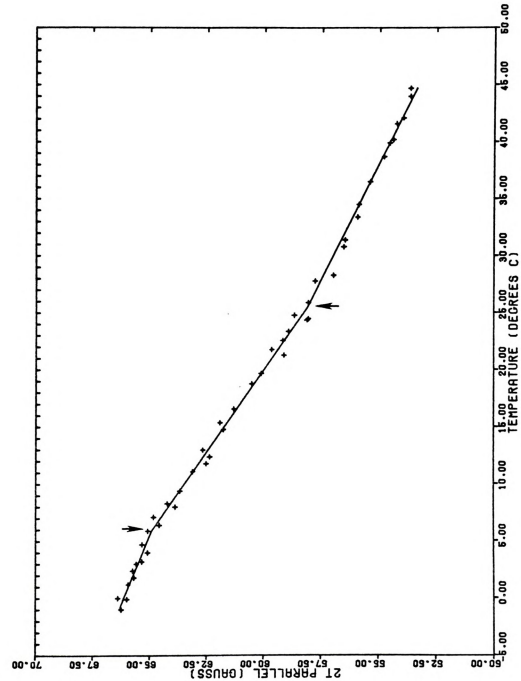
2T PARALLEL (GAUSS)
50.00
52.60
55.00
57.60
60.00
62.60
65.00
67.60
70.00
TEMPERATURE (DEGREES C)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00

Figure 1.

Figure 2. Membranes from heat-activated spores were isolated and analyzed as described in the legend of Figure 2.

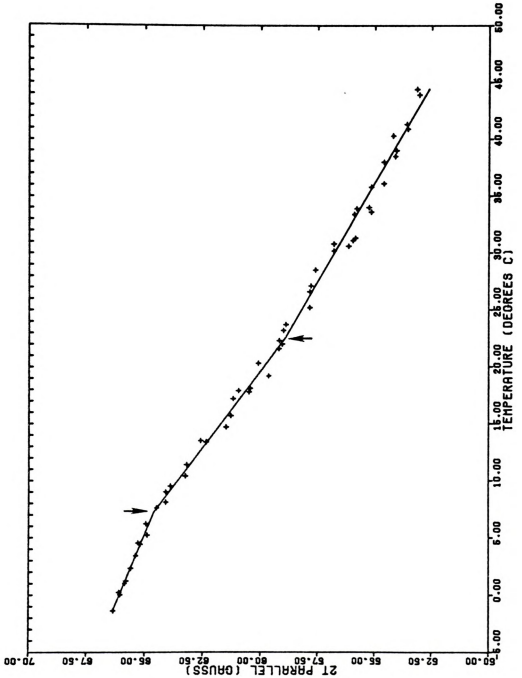
2T PARALLEL (GAUSS)
60.00
62.50
65.00
67.50
70.00
85.00
82.50
80.00
77.50
75.00
TEMPERATURE (DEGREES C)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00

Figure 2.

Figure 3. Effect of L-proline. Membranes from heat-activated spores were isolated and anaylzed as described in the legend of Figure 1 except for the presence of 30mM L-proline.

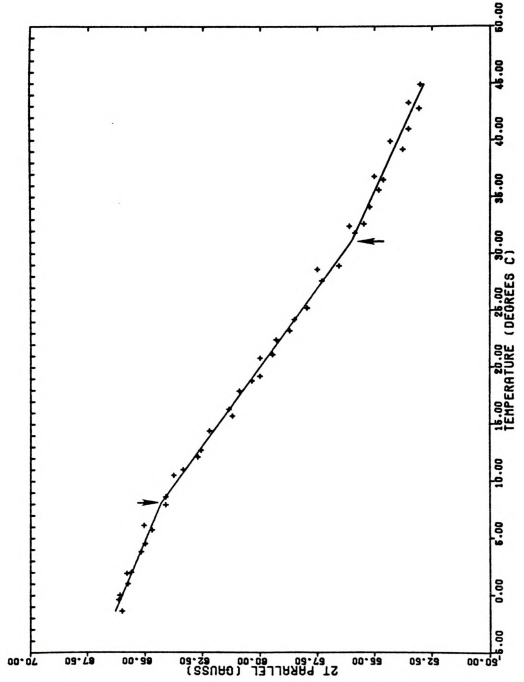
2T PARALLEL (GAUSS)
70.00
67.60
65.00
62.60
60.00
60.00
55.00
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
TEMPERATURE (DEGREES C)

Figure 3.

was done with D-proline (30mM) 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. While there is 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.

Although these data do not explain the mechanism of triggering germination, techniques described provide 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, it will be possible to study the interactions of L-proline with the trigger site both in vitro and in vivo.

Summary

Membranes from dormant and heat-activated spores were labelled 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.

References

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