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Acknowledgments

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Abstract

Various aspects of the synthesis and function of fatty acids in the phospholipids of Escherichia coli K12 were investigated. The fatty acid compositions of the three major phospholipids; phosphatidylethanolamine, phosphatidylglycerol and cardiolipin; were determined during growth in media differing in NaCl concentration. Stationary phase cells grown in 0.3-0.6 M NaCl contained phospholipids with higher levels of cyclopropane and saturated fatty acids compared to cells grown in the absence of NaCl, but no appreciable differences were found in early exponential cultures. The differences, which were similar to those observed after growth at elevated temperature, were likely due to an increase in osmolality, since similar results were obtained after growth in high concentrations of NaCl, KCl, $MgCl_2$ and sucrose.

A protein synthesis requirement for optimal cyclopropane fatty acid synthesis in stationary phase was indicated, since exposure of pre-stationary cells to chloramphenicol resulted in reduced synthesis of cyclopropane fatty acids. Assay of S-adenosylmethionine synthetase activity (required for cyclopropane fatty acid synthesis) revealed reduced levels of this enzyme in cells grown in tryptone broth compared to cells grown in minimal medium. In vivo experiments failed to correlate the influence of high osmolality with growth conditions that have been reported to result in similar changes in lipid fatty acid composition, such as low pH, phosphate limitation and nitrogen limitation.

Artificial membranes composed of synthetic phosphatidylcholines containing the cyclopropane fatty acids, lactobacillic and

dihydrosterculic acids, melted at higher temperatures than those containing their unsaturated fatty acid analogues, cis-vaccenic and oleic acid. However, membrane melting temperature studies performed on E. coli lipids differing in cyclopropane and unsaturated fatty acid content, resulted in transition temperature profiles that were either extremely broad or resolved into two peaks which could not be readily interpreted in terms of fatty acid content.

Résumé

Divers aspects de la synthèse et de la fonction des acides gras des phospholipides ont été étudiés chez Escherichia coli K-12. La composition des acides gras des principaux phospholipides, le phosphatidyléthanolamine, le phosphatidylglycérol et le cardiolipide, a été établie au cours de la croissance dans des milieux différant par la concentration de NaCl. Les cellules en phase stationnaire cultivées dans 0.3-0.6 M NaCl avaient un taux plus élevé d'acides gras cyclopropaniques et insaturés dans les phospholipides que les cellules cultivées en l'absence de NaCl, mais aucune différence appréciable n'a été observée dans les cultures en début de la phase exponentielle de croissance. Les différences, qui sont semblables à celles qui ont été observées après croissance à température élevée, sont probablement dues à une augmentation de l'osmolalité puisque des résultats semblables ont été obtenus après croissance dans des concentrations élevées de NaCl, de KCl, de $MgCl_2$ et de sucrose.

Les résultats indiquent qu'une synthèse protéique est requise pour une synthèse optimale des acides gras cyclopropaniques puisque l'addition de chloramphénicol à des cultures avant la phase stationnaire réduit la synthèse des acides gras cyclopropaniques. L'essai de l'activité de la S-adénosylméthionine synthétase (requise pour la synthèse des acides gras cyclopropaniques) a montré un niveau plus faible de cet enzyme dans les cellules cultivées dans un bouillon à la tryptone que dans les cellules cultivées dans un milieu minimal. Les expériences in vivo n'ont pas montré de corrélation entre l'influence de l'osmolalité élevée et des conditions de croissance, telles qu'un pH peu élevé, une limitation en phosphate ou en azote, qui ont été rapportées comme conduisant à des changements semblables dans la composition des acides gras des

phospholipides.

Des membranes artificielles composées de phosphatidylcholines contenant des acides gras cyclopropaniques, les acides lactobacillique et dihydrosterculique, ont un point du fusion plus élevé que celles contenant les acides gras insaturés analogues, les acides vaccénique et oléique. Les études sur la température de fusion des membranes préparées avec les lipides de E. coli et contenant des proportions différentes d'acides gras cyclopropaniques et insaturés ont, cependant, donné des profils de températures de transition qui étaient extrêmement étendus ou bien présentaient deux maxima qui ne pouvaient pas être facilement interprétés en fonction de la composition des acides gras.

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The speculative mind of man moves forward in great revolutions, like a point on the rim of a turning wheel, and if now the point is forward, it cannot remain so for long because the wheel, and the cart which it carries, must move ahead, and as they do so the point on the rim moves backward. This oscillating movement whose temporary position we can rarely discern, is what we call the process of civilization.

James A. Michener

TABLE LEGENDS

TABLE LEGENDS (Cont'd)

TABLE LEGENDS (Cont'd)

FIGURE LEGENDS

FIGURE LEGENDS (Cont'd)

FIGURE LEGENDS (Cont'd)

1.0 Introduction

1.1 Overview

A surprising finding in a study of the lipids of several mutants of Escherichia coli K12 with putative membrane defects,(Bilsky and Armstrong, 1973), was the alteration in phospholipid fatty acid composition of the cells in response to growth in media of different salt concentration. Compared to cells grown in tryptone broth alone, cells grown to stationary phase in 0.3 - 0.6 M NaCl exhibited phospholipids with higher cyclopropane and saturated fatty acid contents (McGarrity and Armstrong,1975). Since examination of the phospholipids, phospholipid fatty acids and lipopolysaccharides of mutants and wild-type revealed little difference, our studies became directed toward investigation of the metabolism and function of cyclopropane fatty acids in E. coli.

The present study is concerned with an aspect of a very puzzling problem - the regulation of fatty acid composition in bacterial membranes as a function of environmental conditions. Relatively few studies have dealt with either the regulatory mechanisms responsible for producing large amounts of cyclopropane fatty acids under certain conditions but not under others, or the selective advantage conferred on organisms producing these energetically expensive components (3 moles of adenosine triphosphate/mole of cyclopropane fatty acid). This thesis has been addressed to these problems, as well as the effect of salt on phospholipid fatty acid composition in E. coli.

1.2 Occurrence of Cyclopropane Fatty Acids

Cyclopropane fatty acids are synthesized by the enzymatic

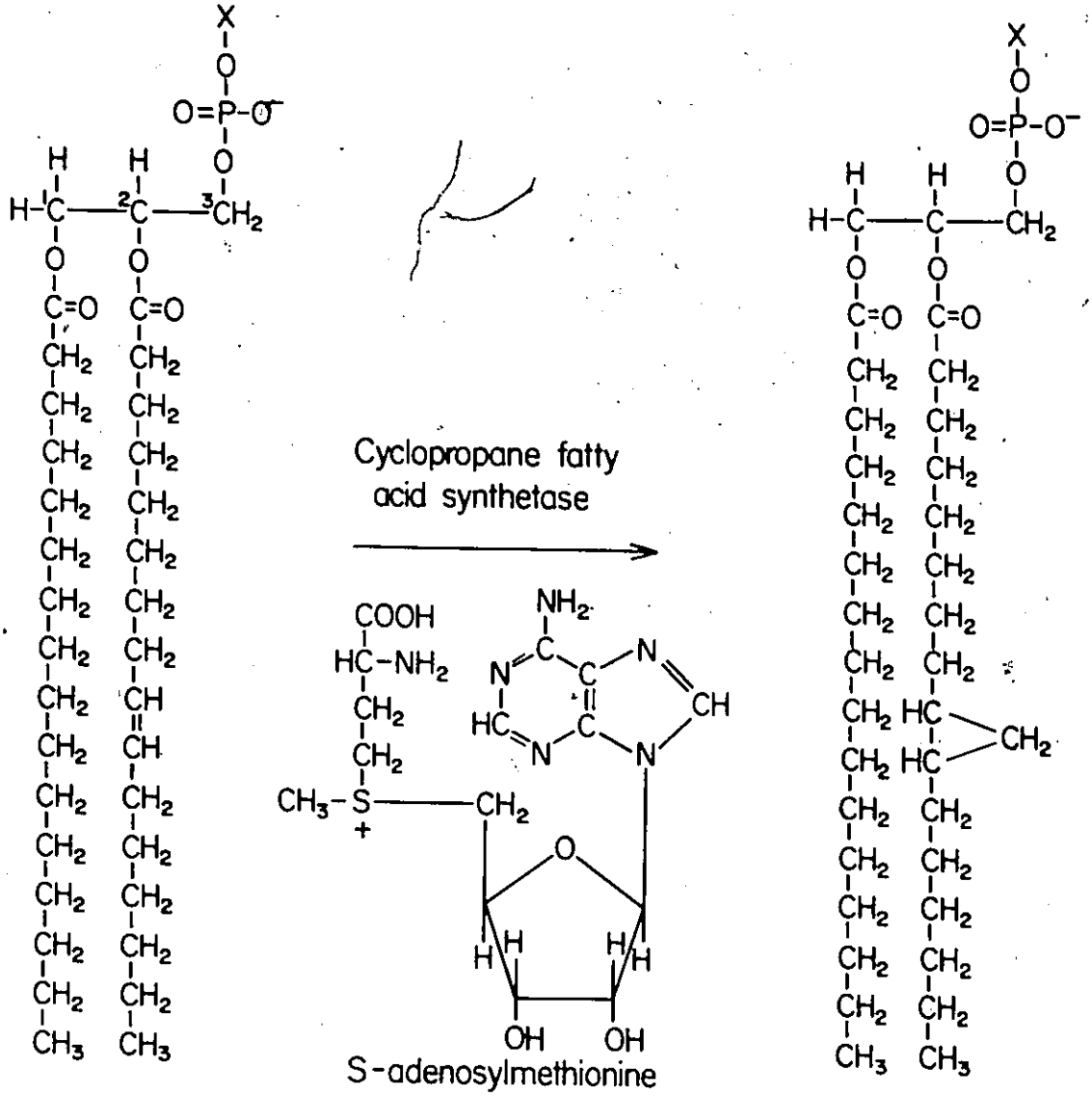
introduction of methylene bridges across the unsaturated sites of fatty acids acylated in phospholipids (Fig. 1). In E. coli, lactobacillic acid (cis-11,12-methylene octadecanoic acid) and cis-9,10-methylene hexadecanoic acid are derived from their unsaturated fatty acid analogues, cis-vaccenic and palmitoleic acid.

Cyclopropane fatty acids are found in a wide variety of organisms, but are particularly prevalent in bacteria. Some of these organisms include protozoa (Christie,1970); seed oils and plant leaves (Christie, 1970; Kuiper and Stuiver, 1972); females of spirostreptid millipedes (van der Horst and Oudejans,1973); sheep rumen tissues (Body, 1972); E. coli (Dauchy and Asselineau,1960; Law, 1961; Kaneshiro and Marr, 1961); Agrobacterium tumefaciens (Hofmann and Tausig,1955); Serratia marcescens (Zalkin and Law,1962; Kates and Adams,1962; Bishop and Still, 1963 a,b); Clostridium butyricum (Goldfine and Bloch,1961); Pleuropneumonia-like organisms (O'Leary,1962); Streptococcus lactis (MacLeod et al,1962); Streptococcus faecalis (Jungkind and Wood, 1974); Lactobacillus sp. (Hofmann et al, 1952; Hofmann and Sax, 1953; Hofmann et al, 1955); Pasturella pestis (Asselineau, 1961); Bacillus subtilis (Asselineau,1961); Pseudomonas fluorescens (Crowfoot and Hunt, 1970); Lactobacillus casei (Chalk and Kodicek,1961) Salmonella typhimurium (Gray,1962); Aerobacter aerogenes (O'Leary,1962). Vibrio cholerae (Brian and Gardner, 1968) and Proteus sp. (Nesbitt and Lennarz, 1965).

1.3 Discovery and Identification of Cyclopropane Fatty Acids

The trivial name, lactobacillic acid, was assigned by Hofmann et al (1950, 1952, 1957) to a major fatty acid component in the phospholipids of Lactobacillus arabinosus and other lactic acid bacteria. Subsequent

Figure 1. Synthesis of cyclopropane fatty acids. See text for details.


Cyclopropane fatty
acid synthetase
S-adenosylmethionine

isolation and characterization based on chemical and physical properties led to identification as cis-11, 12-methylene octadecanoic acid (Hofmann et al, 1954, 1957; Marco and Hofmann, 1956). O'Leary (1959), in a radioactive labelling experiment, found that cis-vaccenic acid (cis-11, 12-octadecanoic acid), the predominant unsaturated 18-carbon fatty acid of the lactic acid bacteria, is the metabolic precursor of lactobacillic acid.

Kaneshiro and Marr (1961) and Chalk and Kodicek (1961) isolated and characterized a second cyclopropane fatty acid, cis-9, 10-methylene hexadecanoic acid, from E. coli. It was the same seventeen-carbon fatty acid observed by Dauchy and Asselineau (1960) and Law (1961) in E. coli and by Asselineau (1961) in Pasturella pestis and Bacillus subtilis. Identification of both lactobacillic and cis-9,10-methylene hexadecanoic acids in E. coli lipid extracts (Chalk and Kodicek, 1961) was based on paper and gas liquid chromatography, susceptibility to acid hydrolysis and radioactive uptake of ^{14}C derived from (Me-^{14}C)-methionine. The work was extended by Kanshiro and Marr (1961), who isolated cis-9, 10-methylene hexadecanoic acid by gas liquid chromatography and characterized it by comparison to a synthetic product according to melting point, infrared absorption, chromatographic mobility in gas liquid chromatography, and by analysis of products after mild oxidation and reduction.

1.4 Origin of the Methyl Group

The identification of S-adenosylmethionine (SAM) as the methyl donor in cyclopropane fatty acid synthesis (Fig. 1) is based on considerable evidence. An early report by Liu and Hofmann (1962)

indicated that the methylene bridge of lactobacillic acid could be suppled by both methionine and formate. Later, in in vitro experiments, Law et al (1963) found that the addition of a number of unlabelled one-carbon donors, including sodium propionate, L-serine, sodium formate, formaldehyde, methanol and glycine, did not result in a decrease in incorporation of ^{14}C from labelled methionine into the cyclopropane fatty acids of lactic acid bacteria, suggesting methionine as the sole carbon donor. In addition, O'Leary (1959) and Law et al (1963) found that mutants of E. coli unable to convert one-carbon compounds to methionine methyl groups demonstrated very little ability to utilize methyl groups, other than that of methionine, for the synthesis of cyclopropane fatty acids. Though Henderson et al (1965) found that low cellular levels of folic acid in lactobacilli resulted in decreased cyclopropane fatty acid levels, Jungkind and Wood (1974a) found that folic acid did not dilute the incorporation of (^{14}C)-methyl from labelled methionine into cyclopropane fatty acids during growth of Streptococcus faecalis.

By following the fate of deuterium labelled methionine methyl groups, Pohl et al (1963) demonstrated in E. coli, that the methyl carbon of methionine and two hydrogen atoms are transferred to the fatty acid unsaturated sites. In assays of cyclopropane fatty acid synthetase involving crude soluble extracts of Serratia marcescens, Zalkin et al (1963) established that serine, formate and formaldehyde were very poor substrates, and that S-adenosylmethionine was a much better substrate than methionine.

1.5 Cyclopropane Fatty Acid Synthetase Substrate Specificity

The fatty acid substrate of cyclopropane fatty acid synthetase

appears to be an intact phospholipid. Zalkin et al (1963) tested the activity of cyclopropane fatty acid synthetase, from a cell free homogenate of Clostridium butyricum, with several fatty acid containing substrates. Little to no enzyme activity was observed with addition of the substrates palmitoleoyl-coenzyme A, palmitoleic acid or synthetic dipalmitoyl phosphatidylethanolamine. Highest activities were recorded in assays containing exponential phase phospholipids rich in unsaturated fatty acids. A stationary phase phospholipid preparation, in which a substantial proportion of the unsaturated sites had been methylated, acted as a poor substrate. Later, Thomas and Law (1966) demonstrated that a diether analogue of phosphatidylethanolamine, 1,2-di-(octadecenoyl)-3-(2-aminoethyl) phosphorylpropane, acted as a good substrate in an in vitro assay of C. butyricum extracts.

Since the enzyme methylates only phospholipids derived from sn-glycero-3-phosphate, it shows a high degree of specificity similar to that of phospholipase A (van Deenen and de Haas 1963). The enzyme also has some positional specificity. Unsaturated fatty acids in the oc- or 1-position of the phospholipid are more readily methylated by the C. butyricum enzyme (Thomas and Law, 1966; Hildebrand and Law, 1964). However, in vitro assays of the enzyme demonstrated that unsaturated acyl residues in both positions can be methylated, though the acids in unfavoured positions are methylated at slower rates (Hildebrand and Law, 1964).

Current evidence indicates that there is an absolute specificity for the cis isomer. Unsaturated fatty acid auxotrophs do not synthesize cyclopropane fatty acids when grown on trans-unsaturated fatty acids while they do so when grown on the cis-isomer (Silbert et al, 1968). Phospholipids such as phosphatidylethanolamine and phosphatidyl-glycerol containing the major unsaturated fatty acids of E. coli,

cis-vaccenic and palmitoleic acid, as well as oleic acid (Thomas and Law 1966) are good substrates.

1.6 Cyclopropane Fatty Acid Synthetase Activity

Investigation of the kinetics and substrate specificity of cyclopropane fatty acid synthetase has been hampered by the absence of reproducible assay systems. One of the serious problems encountered in developing a suitable assay has been dispersal of the phospholipid since phospholipids have limited solubility in aqueous systems. In the method developed by Goldfine in 1966, the phospholipid was precipitated on filter paper in order to maximize surface exposure by spreading the substrate over the large area of the filter paper fibers. In other methods, the phospholipid was dispersed as liposome preparations (Fleischer and Klowawen, 1961) or micellar dispersions of sonicated lipids (Thomas and Law, 1966; Cronan, 1968). The artificial membrane technique was found to be most reproducible and yielded highest activities when microbial phospholipids were used as substrate. However, the filter technique yielded better results with phospholipid substrates which are particularly difficult to disperse and assays of enzyme activity were unaffected by addition of anionic detergents and divalent cations (Thomas and Law, 1966).

The activity of the enzyme may be dependent upon the surface area as well as the charge of the dispersed phospholipid. Chung and Law (1964) found a suitably dispersed phosphatidylethanolamine contaminated with an anionic surface active agent the most effective substrate for the enzyme in C. butyricum extracts. The authors suggested that the anionic charge is likely important in matching the surface charge of the phospholipid to that of the enzyme, since cationic detergents, which are equally effective in dispersing lipid, inhibit enzyme activity

(Chung and Law,1964). Moreover, phosphatidylglycerol, which is negatively charged, can also increase enzyme activity (Thomas and Law, 1966). However, these authors found that anionic detergents did not effect enzyme assays on filter discs. The enzyme activity actually decreased with increasing concentration of phosphatidylserine, which is more negatively charged than phosphatidylethanolamine. When phosphatidylglycerol was used as a substrate, addition of divalent cations (calcium, magnesium etc.), resulted in a two fold increase in rate. Thus, conditions favouring maximum rates of enzyme activity can vary a great deal depending on both the mode of dispersal and type of phospholipid.

Zalkin et al (1963) observed maximal activity of cyclopropane fatty acid synthetase in C. butyricum and S. marcescens extracts in pH 7.0 buffer. Due to the high endogenous lipid content in crude cell free homogenates of E. coli and S. marcescens, optimal lipid concentrations could not be determined for the enzyme of these organisms. Cronan (1968) was able to demonstrate only a slight increase in activity due to addition of lipid in fresh stationary phase extracts. In active preparations, the maximum enzyme activity in E. coli extracts was found to be similar to that of S. marcescens, 0.5-1.0 m μmole/mg of protein/hour, which is about ten fold lower than that observed in C. butyricum extracts. Highest enzyme activities were observed in C. butyricum extracts incubated in 6 mM phospholipid and 0.5 mM S-adenosylmethionine (Zalkin et al, 1963).

Zalkin et al (1963) examined the effect on cyclopropane fatty acid synthetase activity of several inhibitors which have been reported to decrease the activity, in liver extracts, of enzymes involved in

methylation of phosphatidylethanolamine in the synthesis of phosphatidylcholine (Bremer and Greenberg, 1961 a,b). Inhibition of activity by p-hydroxymercuribenzatol in S. marcescens and C. butyricum preparations was reversed by addition of cysteine. Iodoacetate increased activity in S. marcescens extracts but caused a decrease in C. butyricum extracts. Enzyme activity was unaffected by addition of mercaptoethanol or S-adenosylhomocysteine.

1.7 Metabolic Control of Cyclopropane Fatty Acid Synthesis

Since unsaturated fatty acids are abundant at most stages in growth, cyclopropane fatty acid synthesis is likely dependent upon intracellular cyclopropane fatty acid synthetase activity and S-adenosylmethionine levels. Early work (Zalkin et al, 1963) failed to reveal low molecular weight or loosely bound activators or inhibitors of cyclopropane fatty acid synthetase; dialysis of crude enzyme extracts did not result in altered cyclopropane fatty acid synthetase activity. However, these studies did not rule out the possibility of tightly bound, short lived or high molecular weight regulators.

Decreasing oxygen tension during growth of Pseudomonas fluorescens resulted in a marked increase in cyclopropane fatty acid synthetase activity (Crowfoot and Hunt, 1970). Since removal of oxygen with thioglycolate in in vitro assays had no effect on enzyme activity, and normal enzyme activity was observed in cultures subjected to low oxygen tension, but treated with the protein synthesis inhibitor, chloramphenicol, the authors speculated that the enzyme was induced, although they failed to identify a likely inducer. On the other hand, Cronan (1968) did not observe differences in enzyme activity in E. coli cell extracts grown to late exponential and stationary phase, although a several fold increase in cyclopropane fatty acid levels

was observed during this stage in growth. At this point, the evidence is too scanty to indicate that cyclopropane fatty acid synthetase is regulated by induction, since alternative possibilities, such as a difference in protein turnover, exist for even the P. fluorescens study.

Several authors have explored the possibility that cyclopropane fatty acid synthesis is dependent upon the intracellular concentration of S-adenosylmethionine. Studies of auxotrophs with impaired ability to synthesize adenosine triphosphate, methionine or S-adenosylmethionine have not resolved this question. Several E. coli mutants with impaired S-adenosylmethionine synthetase produced cyclopropane fatty acid levels equivalent to the levels in the parent strain (Cronan et al, 1974). However, in Agrobacterium tumefaciens, limiting methionine levels caused a decrease in the accumulation of cyclopropane fatty acids (Kanshiro, 1968), possibly due to preferential utilization of the limited methionine for N-methylated lipids and protein synthesis.

1.8 Influence of Growth Conditions on Cyclopropane Fatty Acid Production

The ratio of cyclopropane fatty acids to unsaturated fatty acids in bacterial lipids can be influenced by the stage and conditions of growth. Highest ratios are observed at advanced stages, late exponential and stationary phase in E. coli (Marr and Ingraham, 1962; Law, et al, 1963; Knivett and Cullen, 1965), Agrobacterium tumefaciens (Law et al, 1963), Lactobacillus arabinosus (Groom and McNeil, 1961), Serratia marcescens (Law et al, 1963; Kates et al, 1964) and Streptococcus faecalis (Jungkind and Wood, 1974a).

Low oxygen pressure increases cyclopropane fatty acid levels in

E. coli (Knivett and Cullen, 1965) and Pseudomonas fluorescens (Crowfoot and Hunt, 1970). Other conditions resulting in elevated cyclopropane fatty acid levels include low pH in E. coli (Knivett and Cullen, 1965) and Streptococcus faecalis (Jungkind and Wood, 1974), and high NaCl concentration in E. coli (McGarrity and Armstrong, 1975).

Cyclopropane fatty acid production in stationary bacterial cultures can also be influenced by the growth-limiting medium component. In E. coli, higher amounts of cyclopropane fatty acids are produced if nitrogen (Marr and Ingraham, 1962; Knivett and Cullen, 1967) or phosphate (Knivett and Cullen, 1967) is growth-limiting. Reduced amounts are observed if magnesium (Knivett and Cullen, 1965, 1967), sulfate (Knivett and Cullen, 1967) or the carbon source (Marr and Ingraham, 1962; Shaw and Ingraham, 1965, Knivett and Cullen, 1967) is growth limiting. Growth in the presence of high citrate concentrations resulted in less cyclopropane fatty acids, but this is likely due to complexing of magnesium (Knivett and Cullen, 1965).

1.9 Role of Cyclopropane Fatty Acids

An absence or low level of cyclopropane fatty acids has often been associated with defects in cell wall synthesis or decreased viability under adverse conditions. For example the L-forms of Proteus P18 and filamentous forms of E. coli B contain less cyclopropane fatty acid (Nesbitt and Lennarz, 1965; Weinbaum and Panos, 1966). In contrast, a typical corrugated Vibrio cholerae (termed rugose) which accumulates in aging cultures exhibits unusual resistance to adverse environmental conditions and has elevated levels of cyclopropane fatty acids (Brian

and Gardner, 1968; Brian et al,1966). Jungkind and Wood (1974b) suggested that group D streptococci are more resistant to high temperatures and NaCl concentration than group A (Deibel, 1964) due to the presence of cyclopropane fatty acids in group D.

The idea that cyclopropane fatty acids confer an advantage on organisms living in adverse conditions has also been proposed to explain their presence in higher organisms. Oudejans et al (1976) suggested that the function of cyclopropane fatty acids in the millipede Graphidostreptus tumuliporous may be to protect the lipids from oxidation during vulnerable early larval stages exposed to a desicating arid environment. Kuiper and Struvier (1972) speculated that the presence of cyclopropane fatty acids is a physiological adaptation of early spring and drought-tolerant plants. For example, increasing membrane fluidity resulting from substitution of cyclopropane fatty acids for equivalent chain lengthened saturated fatty acid analogues could increase membrane flexibility which they thought to be important in frost hardiness.

Since cyclopropane fatty acids are more abundant in stationary phase bacteria than actively metabolizing exponential cells, most authors have speculated that the selective advantage of these membrane components is related to a preservation or protective function. Law et al (1963) observed that the cyclopropane fatty acids turned over very slowly in stationary phase E. coli and Serratia marcescens. Cronan (1968) suggested that such reduced turnover could lead to decreased catabolic degradation, hence retention of an intact membrane. However, in a later report, he found essentially identical

turnover rates for unsaturated fatty acids and their cyclopropane analogues (Cronan et al, 1974).

Law et al (1963, 1971) proposed that replacement of unsaturated sites with cyclopropane rings may confer protection against oxidation or free radical formation. Although this proposal has not been disproven by experimental evidence, it seems unlikely since, as mentioned above, low oxygen pressure leads to increased cyclopropane fatty acid production (Section 1.7).

Other roles suggested for cyclopropane fatty acid production include a system for elimination of active methyl groups (Cronan et al, 1974) or a system altering membrane barrier function leading to increased resistance to lactic acid, an acidic product of glycolysis (Jungkind and Wood, 1974b). The first proposal seems unlikely since it is metabolically wasteful. The second proposal is appealing since low pH has been shown to favour cyclopropane fatty acid production, and a parental strain of Streptococcus faecalis with the ability to produce cyclopropane fatty acids has been shown to be more resistant to lactic acid than a mutant without this ability (Jungkind and Wood 1974b). However, Taylor and Cronan (1976) found equal susceptibility to lactic acid in both wild-type E. coli and a mutant strain unable to synthesize cyclopropane fatty acids.

The possibility that cyclopropane fatty acids may influence the physical properties of the membrane (Kito et al, 1973) has not been fully explored. The results of van Deenen (1965), Overath et al (1970) and Cullen et al (1971) have shown that the molecular packing of phospholipids with unsaturated fatty acids or their cyclopropane analogues

is similar. However, the melting temperatures of free cyclopropane fatty acids are higher than that of their unsaturated analogues. Substitution of cyclopropane fatty acids for unsaturated fatty acids in membrane phospholipid may result in a decrease in the fluidity of the membrane phospholipid. The fluidity of the membrane has been shown to influence the activity of a wide variety of membrane processes (Sandermann, 1978). Hence, the methylation of unsaturated fatty acids may have a regulatory role in bacterial membranes attenuating the fluidity of the membrane and therefore the activity of membrane functions in stationary phase.

1.10 Cyclopropane Fatty Acid Synthetase Mutants

The isolation of bacterial mutants devoid of cyclopropane fatty acid synthetase activity permitted comparisons to evaluate the role of cyclopropane fatty acids. Jungkind and Wood (1974a,b) found that several Streptococcus faecalis mutants resistant to the anti-folic acid drugs, amethopterin and pyrimethamine, were unable to synthesize cyclopropane fatty acids. Taylor and Cronan (1976) isolated two Escherichia coli K_{12} mutants by a more direct procedure involving (^{3}H-methyl)-L-methionine suicide.

Jungkind and Wood (1974b) found that growth of the S. faecalis mutants was curtailed, compared to the parental strain, at 47°C, with 3.5% NaCl at 37°C, with 3.5% NaCl at 47°C or with sodium deoxycholate (200 ug/ml). Survival of stationary phase mutant cells decreased when they were exposed to 3.5% NaCl at 47°C, sodium deoxycholate (400 ug/ml) and pH 4.0 sodium lactate (Jungkind and Wood 1974b). However, Taylor and Cronan (1976) found no difference from the wild-type in sensitivity of their E. coli mutants to 0.1 M lactate (pH 4.0), high NaCl concentration, various detergents, amethopterin, rapid

freezing and drying and lysis of spheroplasts by Brij 58.

1.11 Objectives

The main objectives of this thesis were to investigate the control of membrane phospholipid fatty acid composition in E. coli in response to growth in media of high osmolality and to determine the effect of cyclopropane fatty acids on the membrane physical properties. Studies on the regulatory mechanisms controlling cyclopropane fatty acid levels as well as the role of these fatty acids in E. coli membrane physiology were emphasized. The principal investigations have dealt with:

1. The influence of osmolality and temperature on the fatty acid composition of the phospholipids of E. coli during growth.
2. Elucidation of the growth limiting factor responsible for termination of growth of E. coli in tryptone broth and its possible role in the control of membrane phospholipid fatty acid composition.
3. The effect of the protein synthesis inhibitor, chloramphenicol, on late stationary phase fatty acid composition.
4. The role of S-adenosylmethionine synthetase and cyclopropane fatty acid synthetase activities in mediating the observed differences in cyclopropane fatty acid composition due to growth in media of high osmolality.
5. The transition temperatures of E. coli membranes varying in cyclopropane and saturated fatty acid levels.
6. The melting temperatures of synthetic lecithins acylated with unsaturated fatty acids and their fatty acid analogues.

Unfortunately, those objectives have been met with varying success. However, both conclusive and inconclusive results have been included with the hope that they will provide in analysis framework for the design of further studies.

2.0 Materials and Methods

2.1 Bacteria

The lipids of a multiple auxotroph of Escherichia coli K12, AW405, (Armstrong et al 1967) were investigated. AW405 requires histidine, leucine, threonine and vitamin B_1 for growth. Other distinguishing characteristics include inability to metabolize the sugars lactose, galactose, arabinose and xylose; and resistance to streptomycin and to the bacteriophages T1, T5 and T6.

2.2 Media

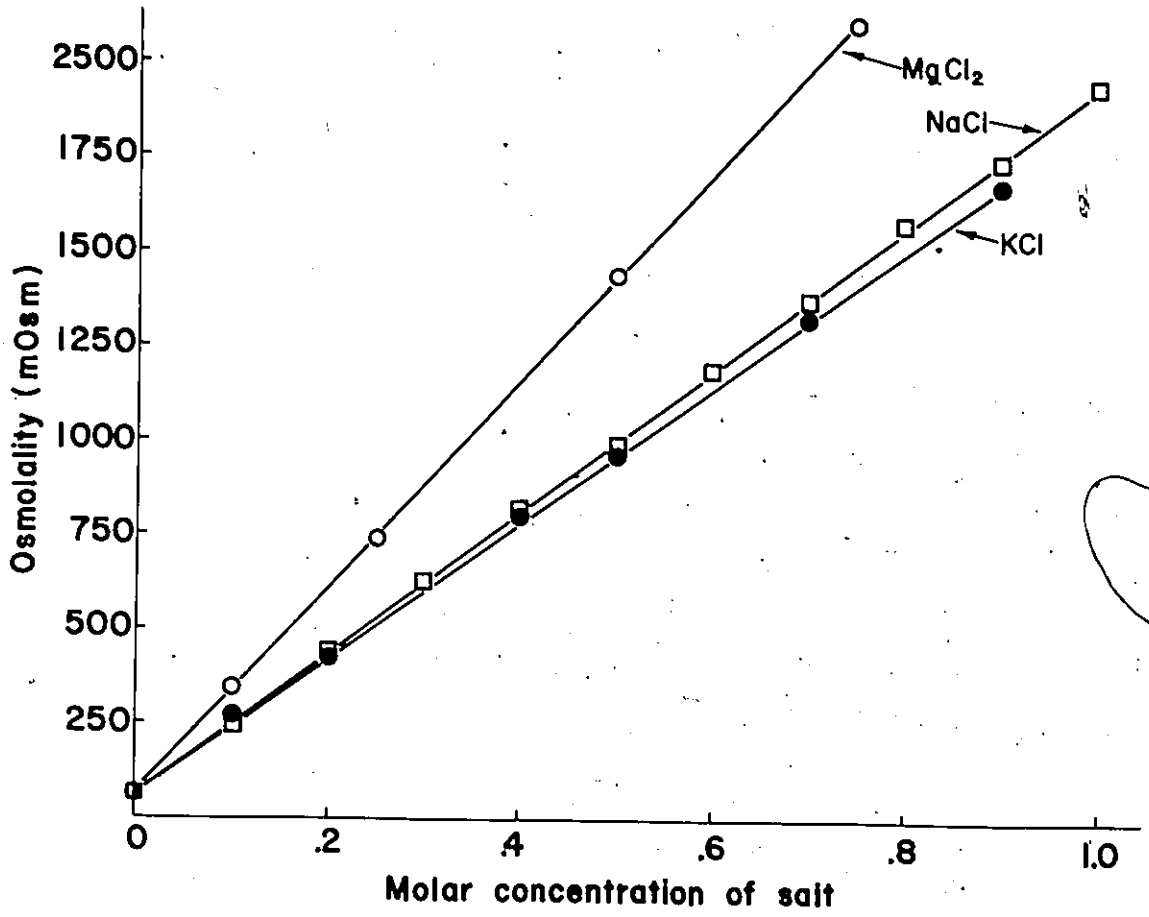
AW405 was grown in either minimal medium or a 1% solution of tryptone (Difco) broth. Minimal medium contained 11.2 gm K_2HPO_4, KH_2PO_4 2.0 gm $(NH_4)_2SO_4$, 1.0 ml M $MgSO_4$ and 1.0 ml of 50 mg/100 ml $Fe_2(SO_4)_3$ in 870 ml; 100 ml of 10% glucose; 10 ml each of filter sterilized 1% threonine, 1% leucine and 1% histidine; and 1.0 ml of 1 mg/ml vitamin B_1. Agar plates contained 1.5% agar and 1% tryptone or minimal medium. E. coli B and wild type K12 were grown in the same media but without separate addition of histidine, leucine or thronine. The concentrations of other additives, such as KCl and NaCl, for particular experiments, are indicated in the results. H_1 buffer contained only the salts of minimal medium.

Osmolality was measured by A. Bilsky (1972) in an Advanced Osmometer (Advanced Instruments, Inc., Newton Highlands, Massachusetts). The standard curves obtained for NaCl, KCl and $MgCl_2$ in tryptone broth (60 mOsm) can be found in Figure 2.

2.3 Growth

In most experiments, several 2,800 ml Fernbach flasks, each

Figure 2. Osmolality of $MgCl_2$, NaCl and KCl solutions.


2500
1750
1500
1250
1000
750
500
250
Osmolality (mOsm)
0
.2
.4
.6
.8
1.0
Molar concentration of salt
MgCl2
NaCl
KCl

containing 500 ml of medium, were inoculated with fresh stationary phase culture and incubated with shaking in a New Brunswick Psychotherm. For experiments requiring batch cell preparations, up to 22 liters of medium were inoculated with 250 ml of fresh stationary phase culture along with 5 ml of octanol (added as antifoaming agent) and incubated with aeration in a 25 liter capacity New Brunswick fermentor. 50-250 ml Erlenmeyer flasks containing 10-50 ml of medium were used to maintain cultures and in experiments indicated in the results. Growth was monitored by following absorbance at 650 nm in 18 x 150 mm tubes with a Coleman Junior II spectrophotometer.

2.4 Chemicals

Methyl esters of capric, lauric, myristic, palmitic, palmitoleic, stearic and cis-vaccenic acid were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Phosphatidylethanolamine and cardiolipin were obtained from Calbiochem (San Diego, Calif.) Phosphatidylglycerol was a gift of M. Kates. An equimolar specific activity mixture of ^{14}C-labelled amino acids was obtained from Amersham/Searle (Toronto, Canada).

2.5 Thin Layer Chromatography

Using a Desaga adjustable applicator (Heidleberg, West Germany), 0.25-0.5 mm layers of silica gel H (Brinkman, Canada) slurried, in water (45:120, w/v), were spread on 20x20 or 20x5 cm glass plates. After drying for several hours at room temperature, the plates were activated at 110-120°C for one hour, cooled and stored in a vacuum desicator over $CaSO_4$ (Drierite). Lipid mixtures were spotted on plates about 3 cm from the bottom (origin) and then developed in solvent equilibrated tanks (lined on three sides with filter paper) containing up to 100 ml of

solvent. The compositions of the most commonly used solvent systems are listed in Table 1. Solvent systems A, C and D were derived from systems used by Skipski and Barclay (1969) whereas system D was first described by Rouser et al (1969). Plates were developed until the solvent front was about 2 cm from the top of the plate.

The neutral lipids, (isoprenoids, respiratory pigments, fatty acid glycerides, etc.) were separated from the phospholipids by thin layer chromatography in solvent system A (Fig. 3). Phosphatidylethanolamine was separated from the remaining phospholipids in solvent B, and cardiolipin was separated from phosphatidylglycerol in system C (Fig. 3). Solvent system D was used to examine the purity of synthetic lecithin preparations.

The lipid spots separated on thin layer chromatograms were visualized using the following staining procedures:

a) 50% sulfuric acid. Plates were sprayed and then heated at 180°C. Organic compounds were charred brown to black.

b) Dittmer-Lester molybdenum blue reagent. Plates were sprayed lightly. Phosphate containing lipids appeared blue (Dittmer and Lester 1964).

c) 0.2% ninhydrin in butanol saturated with water. Plates were sprayed and then heated. Lipids with free amino groups appeared red to violet (Skipski et al, 1962).

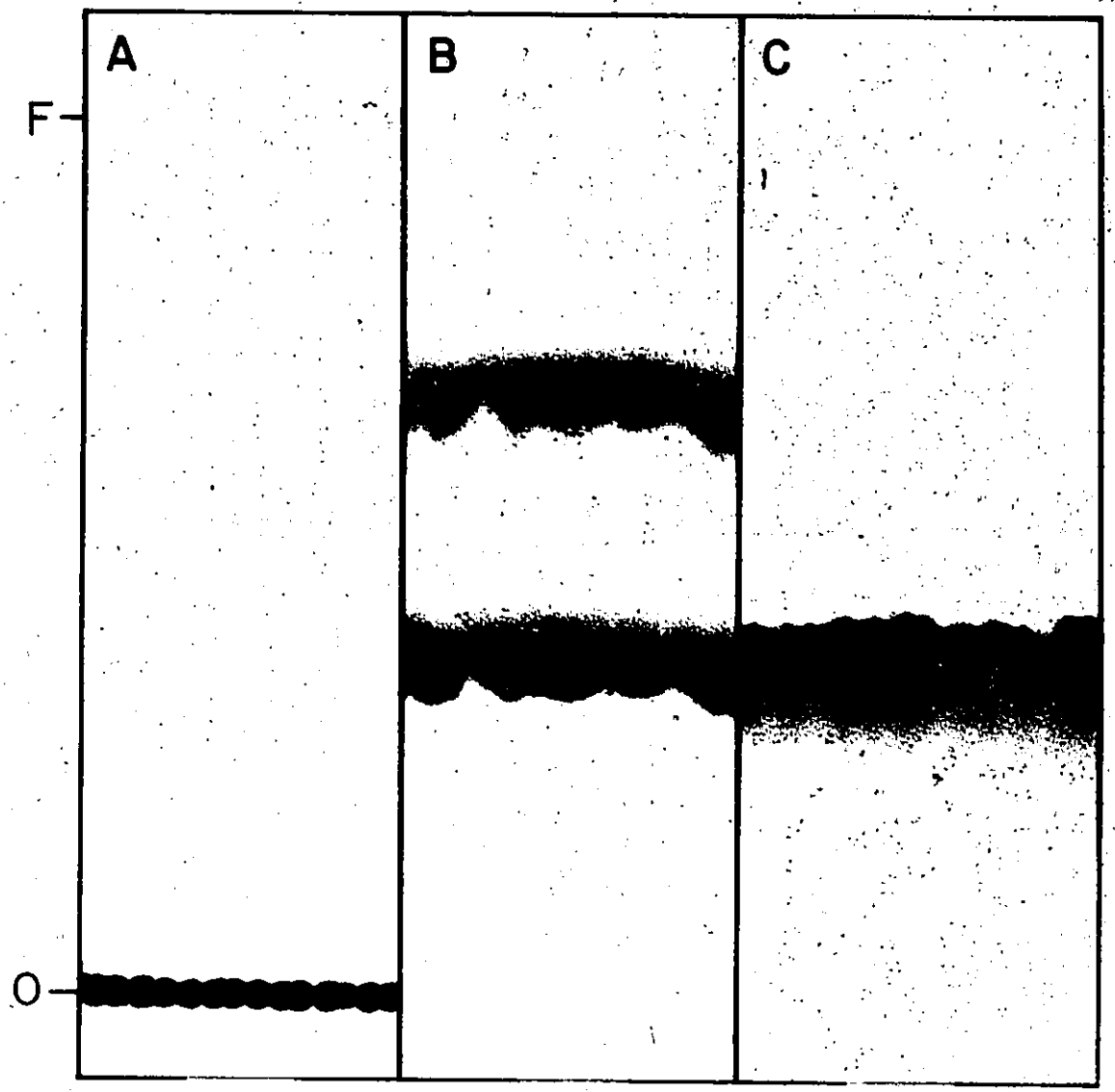
d) Periodate-Schiff reagent. Plates were sprayed with 0.2% aqueous sodium periodate, left for 15 minutes, exposed to SO_2, sprayed with Schiff reagent and exposed to SO_2. Lipids with vicinal hydroxyls, eg. phosphatidylglycerol, appeared red (Schneider, 1966).

Table 1

Thin layer chromatography solvents

Solvent	Ratio (v/v)			
	A	B	C	D
Chloroform	-	65	80	25
Petroleum Ether	60	-	-	-
Methanol	-	50	26	15
Acetone	20	-	-	-
Acetic Acid	-	-	4	4
Ammonia (30%)	-	2.5	-	-
Water	-	-	0.3	2

Figure 3 Autoradiograms of thin-layer chromatograms of ^{14}C-labeled phospholipids from E. coli AW405. Solvent systems are described in Table 1. In A, the phospholipids, which remained at the origin (O), were separated from neutral lipids. In B, phosphatidylethanolamine (lower band) was separated from phosphatidylglycerol and Cardiolipin (upper band). In C, phosphatidylglycerol (lower band) was separated from cardiolipin (upper band). F, solvent front.


A
B
C
F
O

e) Iodine vapour. Plates were exposed to iodine vapour. Most lipids (particularly those containing unsaturated sites) appeared yellow to brown (Mangold and Malins, 1960).

f) Rhodamine 6G. Plates were sprayed with a 0.05% Rhodamine 6G in 95% ethanol and then examined under ultraviolet light. Lipids fluoresced. (Wagner et al, 1961).

Lipids detected on thin layer chromatograms with iodine vapour or Rhodamine 6G could be quantitatively recovered. 1 to 2 gm of silica gel containing lipids were scraped from thin layer chromatograms into the stem of a glass funnel blocked with glass wool. Lipids were then eluted with 50 ml methanol-chloroform, 2:1 (v/v).

2.6 Autoradiograms

Labeled phospholipids were obtained by growing a culture of AW405 in tryptone, to which a mixture of ^{14}C-labelled amino acids had been added. Autoradiograms from chromatograms of the ^{14}C-labeled lipids were obtained by developing Kodak X-ray film which had been exposed from three days to several weeks, depending on the amount of lipid present.

2.7 Lipid Extraction

Lipid extraction was carried out according to Kates (1976). The method is a modified version of a technique used for extraction of fish lipids by Bligh and Dyer (1959). Cell cultures, cooled to 4°C on ice, were harvested by centrifugation at 3,300 x g for 20 minutes. Resultant pellets (up to 5 gm) were suspended (Vortex mixer) in about 60 ml of cold 0.15 M NaCl. The cells were then harvested at 2,000 G for 20 minutes in a 100 ml tared glass centrifuge tube. Assuming 0.8 ml

of water per gm wet cells, enough distilled water was mixed with the pellet to bring the total water content to 8 ml.

The cell suspension was then vigorously mixed with 30 ml methanol-chloroform (2:1 v/v) and centrifuged at 2,000 RPM for 20 minutes, which resulted in a pellet and a clear yellow supernatant containing most of the lipid. The pellet was washed twice with 38 ml aliquots of methanol-chloroform-water, 2:1:0.8 (v/v). The three supernatants were combined and then divided into three centrifuge tubes. To each was added, with stirring, 10 ml chloroform followed by 10 ml water. Upon centrifugation at 2,000 x g for 20 minutes, the mixture formed a biphasic system with a small amount of material at the interphase. The lower phase, containing most of the lipid, was carefully removed with a Pasteur pipet. After washing with two 20 ml aliquots of chloroform, the methanol-water upper phase along with the interphase material was discarded[a]. Solvent was removed from the combined extracts by flask evaporation in a Büchi Rotavapor-R (Switzerland) at 10 mm Hg pressure. After drying by flash evaporation with benzene, lipids, dissolved in chloroform, were stored below 0° in 15 ml teflon capped tubes. Less than 1% of the total lipid was contained in a fourth extraction of the pellet of a ^{14}C-labeled E. coli lipid preparation.

2.8 Paper Chromatography

After subjecting the phospholipid to mild alkaline deacylation according to Kates (1972), the polar products were spotted on 1 x 23

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[a] Alternatively the combined supernatants were poured into a 250 ml separatory funnel (glass lid and teflon stopcock). After addition with shaking of 30 ml chloroform and 30 ml H_2O, the mixture was allowed to separate into two phases at 4°C. After removal of the lower phase, the methanol and water upper phase was washed twice with two 60 ml volumes of chloroform.

inch paper strips (Whatman No. 1) and chromatographed descending in phenol-water, 100:38 (w/v) (Ferrari and Benson, 1961) or phenol saturated with water-ethanol-acetic acid, 50:5:6 (v/v) (Dawson, 1967). The chromatograms were air dried for two days and stained according to White and Frerman (1967) for vicinal hydroxyl groups, according to Burrows et al (1952) for amino groups and according to Clark (1964) for phosphate groups.

2.9 Fatty Acid Analysis

Fatty acid methyl esters were prepared according to Kates (1964). Samples were analyzed in a Hewlett Packard model 402 gas liquid chromatograph equipped with a flame ionization detector. Dual columns, 183 cm in length, were packed with 10% butanediol succinate on chromasorb W (Chromatographic Specialties, Brockville, Ontario). Nitrogen was used as a carrier gas. Weight percentages were computed according to the peak-height times retention-time method (Carroll, 1961).

2.10 Enzyme Assays

Cyclopropane fatty acid synthetase was assayed with a modified version of a technique originally developed by Cronan (1968). Cells, harvested by centrifugation, were suspended in 10 mM mercaptoethanol-0.1 M Tris buffer, pH 8.0 (30% cells in buffer, w/v), sonicated in several one minute bursts at 4°C, and centrifuged at 10,000 x G for 15 minutes to remove whole cells. Aliquots of the supernatant (about 15 mg protein/ml, as determined according to Lowry et al (1951), were assayed at 37°C in a solution containing 10 µmoles adenosine triphosphate, 0.3 mg of sonicated E. coli lipid[a], 100 µmoles $MgCl_2$ 100 µmoles 2-mercaptoethanol, 100 µmoles Tris (pH 7.0), 1.0 µmole

[a] Phospholipid rich in cis-vaccenic and palmitoleic acid was extracted from an AW405 20°C exponential culture.

methionine and 0.8 μC (^{14}C-methyl)-L-methionine (New England Nuclear). The final volume was 1.5 ml. The assay was initiated by adding the enzyme extract to the assay medium. After removal at fixed time intervals, 0.1 ml samples were heated at 100°C in 20% KOH: methanol (1:1) for fifteen minutes to saponify the lipids. A drop of phenolthalein was added and the reaction mixture was acidified with 6NHCl to pH 1.0 and extracted with three volumes of petroleum ether. After washing with three one ml portions of water, the samples were dried with a stream of N_2 and counted in Biofluor.

S-adenosylmethionine synthetase activity was assayed according to Greene et al (1970). Cells were harvested by centrifugation, washed with 0.02 M Tris·pH 7.6 (4°C) and resuspended in a sufficient volume of the same buffer to yield a concentration of 15 mg/ml protein. Cells were then toluenized by incubation at 37°C for 10 min in the presence of 0.05 volume of toluene. One ml assay mixtures containing 150 μmoles Tris·HCl pH 8.5, 100 μmoles $MgCl_2$, 8 μmoles glutathione, 20 μmoles L-methionine, 10 μmoles adenosine triphosphate, 0.8 μC(^{14}C-methyl)-L-methionine and 0.4 ml toluenized cells were incubated at 37°C. 10 μ litre samples at 0 and 60 minutes were spotted on 1 x 23 inch paper strips and run descending in n-butanol-acetic acid-water, 120:30:50 (v/v). After development, the chromatograms were cut into 2 cm strips which were counted in Econofluor in a Beckman LS233 liquid scintillation counter.

2.11 Phase Transition Studies

Membrane phase transitions were measured in whole cell and artificial membrane preparations with the fluorescent probe N-phenyl-

1-napthylamine (NPN) essentially according to Trauble and Overath (1973). Sonicated lipid vesicle dispersions contained 1 to 5 x 10^{-4} M lipid, 10^{-5}M NPN and 3% methanol in 17 mM sodium phosphate buffer (pH 7.0). 7-50 mg of dipalmitoyl lecithin (DPL) or E. coli lipid in chloroform was dried on the bottom of a 25 ml beaker under a stream of nitrogen. Residual chloroform was removed by placing samples under reduced pressure (20 Torr) for several hours. After addition of 5 ml distilled water, the samples were sonicated at 42°C and then diluted to 7 ml. Then, 0.09 ml of NPN in methanol (7.3 mg NPN/100 ml methanol) was added to 2.4 ml 20 mM sodium phosphate buffer, pH 7.0, and 0.5 ml of the lipid preparation. After vortex mixing and incubation at 42°C for 15 minutes, relative fluorescent intensity (Emmission, 420 nm; Excitation, 350 nm)[a] was measured with changing temperature on a Turner Fluorometer and recorded on an x - y recorder. When relative fluorescence I, is plotted versus temperature T, in an Arrhenius plot (log relative fluorescence intensity, I, versus $1/T^{\circ}K$); straight lines (with different slopes) corresponding to the two states below (1) and above (2) the phase transition are obtained (Figure 3). A line parallel to the y-axis (ΔI) joining line 1 to line 2 such that numerus a = numerus b passes through the membrane melting temperature (Tauble and Overath, 1973).

For whole cell preparations, 2.91 ml suspensions of E. coli in 20 mM sodium phosphate buffer, pH 7.0, containing from 0.5-1.0 mg of lipid, were mixed with 0.09 ml of NPN in methanol (24.1 mg NPN/100 ml methanol), sonicated at 0°C for 10 seconds and then incubated at 37°C

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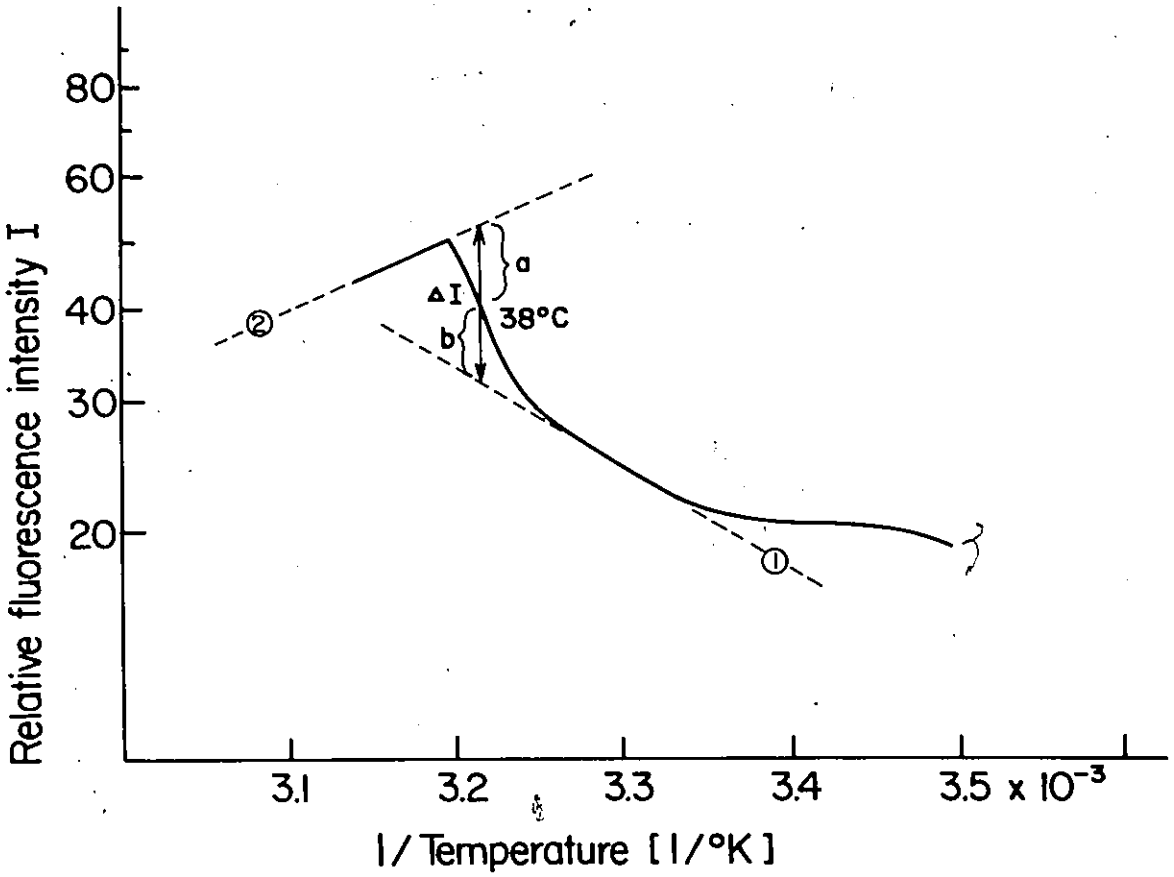
[a] In most experiments a 360 nm narrow pass filter was used for excitation along with a 415 nm sharp cut and 2% neutral density filter for emmission.

Figure 4 Determination of the membrane melting temperature of sonicated dipalmitoyl lecithin vesicles using NPN fluorescence.


Relative fluorescence intensity I
80
60
40
30
20
a
ΔI
38°C
b
②
①
3.1
3.2
3.3
3.4
3.5 x 10⁻³
1/ Temperature [1/°K]

for 10 minutes. Phase transition temperatures were determined from relative fluorescence versus temperature data as above.

Membrane phase transitions of artificial liposome preparations were measured using differential scanning calorimetry (Ladbrooke and Chapman, 1969). Up to 10 mg lipid was stirred with distilled water (less than 1:1, w/v) or ethylene glycol-water (less than 2:1, w/v) in aluminum pans at 37°C. After incubation at 37°C for 30 minutes, the pans were sealed with alluminum lids by dry welding. Differential heat flow versus temperature was measured using a Perkin Elmer DSC-2, Differential Scanning Calorimeter, (Norwalk, Conn).

2.12 Synthesis of Cyclopropane Fatty Acids

The cyclopropane fatty acids, cis-9,10-methylenehexadecanoic, lactobacillic and dihydrosterculic acids; were synthesized from their unsaturated fatty acid analogues; palmitoleic, cis-vaccenic and oleic acids; according to Simmons and Smith (1959). A mixture of 2.2 gm of zinc copper couple (0.03 mole of zinc), 500 mg of iodine, (0.002 mole), 5.36 gm of methylene iodide (0.02 mole), 0.01 mole of methyl fatty acid (99% pure) and 10 ml of anhydrous ether was stirred under reflux for 48 hours. The cool reaction mixture was filtered and the filtrate was washed successively with 5% hydrochloric acid, water and 5% sodium sulfite solution. After removal of the ethyl ether by flash evaporation, residual water was removed from the sample by flash evaporation with benzene. Gas liquid chromatography of the products revealed 77 to 94% of the unsaturated fatty acids were converted to their corresponding

cyclopropane analogues. Other components in the product mixtures included the unsaturated fatty acid and a component[a] with mobility on butanediol succinate intermediate between the unsaturated and cyclopropane fatty acid.

Cyclopropane and unsaturated fatty acid methyl esters were separated by argentation silica gel column chromatography according to Pohl et al (1963). 5 gm of silver nitrate in 100 ml of distilled water was slurried with 95 gm of 60 - 200 mesh silica gel (Baker)[b]. The mixture was then dried overnight at 120°C and stored in a tightly sealed brown bottle. 20 gm of the gel was slurried in 3% ethyl ether in hexane (v/v) and poured into a 1 X 15" column stoppered with glass wool and fitted with a teflon stopcock. Up to 1 gm of sample (cyclopropane and unsaturated fatty acid methyl esters) was eluted with the same solvent in 5 ml fractions. Cyclopropane and saturated fatty acid methyl esters eluted with 25-100 ml of solvent whereas unsaturated components eluted with 75-350 ml of solvent.

The fractions were analyzed by either argentation thin layer chromatography or by GLC on butanediol succinate. Silver nitrate impreganted thin layer chromatography plates were prepared immediately before use by spraying the plates (Silica gel H) with 5% $AgNO_3$ in distilled water (w/v) or developing the plates in 5% $AgNO_3$ in acetonitrile (w/v) and activating the plates at 120°C for 60 minutes. Good separations between saturated and unsaturated fatty acids were obtained using plates prepared in either way when samples were developed in 5% ether in hexane, Table 2.

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[a] Likely the corresponding trans-cyclopropane fatty acid (Christie, 1970)

[b] The original technique employed 15% silver nitrate in silica gel columns but 5% columns were found equally effective and more economical.

Table 2. R_f values of saturated, unsaturated, and cyclopropane fatty acid methyl esters after development on silver nitrate impregnated silica gel H plates in 5% ethyl ether in hexane (v/v).[a]

Methyl Ester	R_f Value	
	Sprayed with 5% $AgNO_3$ in distilled Water.	Developed in 5% $AgNO_3$ in Acetonitrile.
Oleate	0.15	0.06
Stearate	0.43	0.24
Dihydrosteculate	0.44	0.23

[a] Solvent system adapted from Morris 1964.

The cyclopropane fatty acid methyl esters were found to be 93-96% pure. The methyl esters were converted to free fatty acids using the saponification procedure described by Kates (1972). Crystallization at -10°C from petroleum ether did not result in further purification. The overall yield, based upon the amount of unsaturated fatty acid subjected to the Simmons and Smith reaction, was about 25-30%. The free fatty acids were stored at -20°C dissolved in chloroform.

2.13 Synthesis of Phosphatidylcholines

Phosphatidylcholines, containing unsaturated or cyclopropane fatty acyl esters, were synthesized from the cadmium chloride adduct of sn-glycero-3-phosphorylcholine ($GPC \cdot CdCl_2$) and free fatty acids according to Warner and Benson (1977). $GPC \cdot CdCl_2$ was prepared from commercial egg yolk lecithin according to Chadha (1970). The synthetic phosphatidylcholines were purified according to Pugh and Kates (1975). Purified samples ran as single components on thin layer chromatograms that cochromatographed with purified egg yolk lecithin[a] when developed in chloroform-methanol-acetic-water, 25:15:4:2 (v/v). After deacylation according to Kates (1972), the water soluble product (GPC) ran as a single component that cochromatographed with the deacylated product of purified egg yolk phosphatidylcholine on paper chromatograms developed in the solvent systems already noted (Section 2.8). The fatty acid compositions of the synthetic phosphatidylcholines prepared as above are contained in Table 3.

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[a] The egg yolk lecithin was purified by the procedure of Rhodes and Lee (1957).

Table 3. Fatty acid compositions of synthetic phosphatidylcholines aylated with cis-vaccenic, dihydrosterculic, lactobacillic and cis-9,10-methylene hexadecanoic acids.

Fatty Acid	Main Fatty Ester			
	cis-vaccenate	dihydro-sterculate	lacto-bacillate	cis-9,10-methylene hexadecanoate
Palmitoleate		0.3		1.2
Unknown a*				4.8
cis-9,10-methylene hexadecanoate				94.0
Stearate	1.0			
cis-Vaccenate	99.0		2.7	
Unknown b*			0.7	
Lactobacillate			95.8	
Oleate		1.1		
Unknown c*		4.2		
Dihydrosterculate		92.8		
Unknown d		1.6		

* Likely trans-cyclopropane fatty acids, Christie (1970).

3.0 Results

3.1 Growth

Within the temperature and osmolality range of interest in this study (20-42°C and 80-1500 mOsm), the growth of E. coli AW405 in tryptone broth was found to be indistinguishable from a motile isolate of AW405, A014, described by Bilsky (1972). At 30°C, the cells grew exponentially at low cell densities with a doubling period of 1.04 hour (Fig. 5). Increasing NaCl concentrations beyond 0.6 M resulted in departure from exponential growth at lower cell densities, as well as decreased stationary cell yields. The influence of NaCl and KCl on growth was identical. Little to no growth was observed in cultures grown in 1 M NaCl, 1.2 M sucrose or 0.4 M $MgCl_2$. The absence of growth in 0.4 M $MgCl_2$ (~1200 mOsm) cannot be explained on the basis of the osmolality alone, since growth was observed at higher osmotic pressures in cultures with NaCl as the osmotic agent.

3.2 Lipid Composition

Exhaustive lipid extraction revealed that the total lipid content was 1.5 to 2.1% of the wet weight, or 7.8-10.8% of the dry weight, of AW405 pellets. Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, the major phospholipid components, were isolated in the ratio 76.4 ± 6.7 : 22.2 ± 6.7 : 1.4 ± 0.6 (w/w) as determined by the gas chromatography internal standard method (Kates 1972). The relative amounts did not differ significantly between the different media or between exponential and stationary phase. Identifications were based on the mobilities of the phospholipids on thin layer chromatograms (Table 4) and of their deacylated products on paper chromatograms (Table 5). Good agreement was obtained between the R_f values of the

Figure 5 Growth curves for AW405 in 1% tryptone broth with different NaCl concentrations.

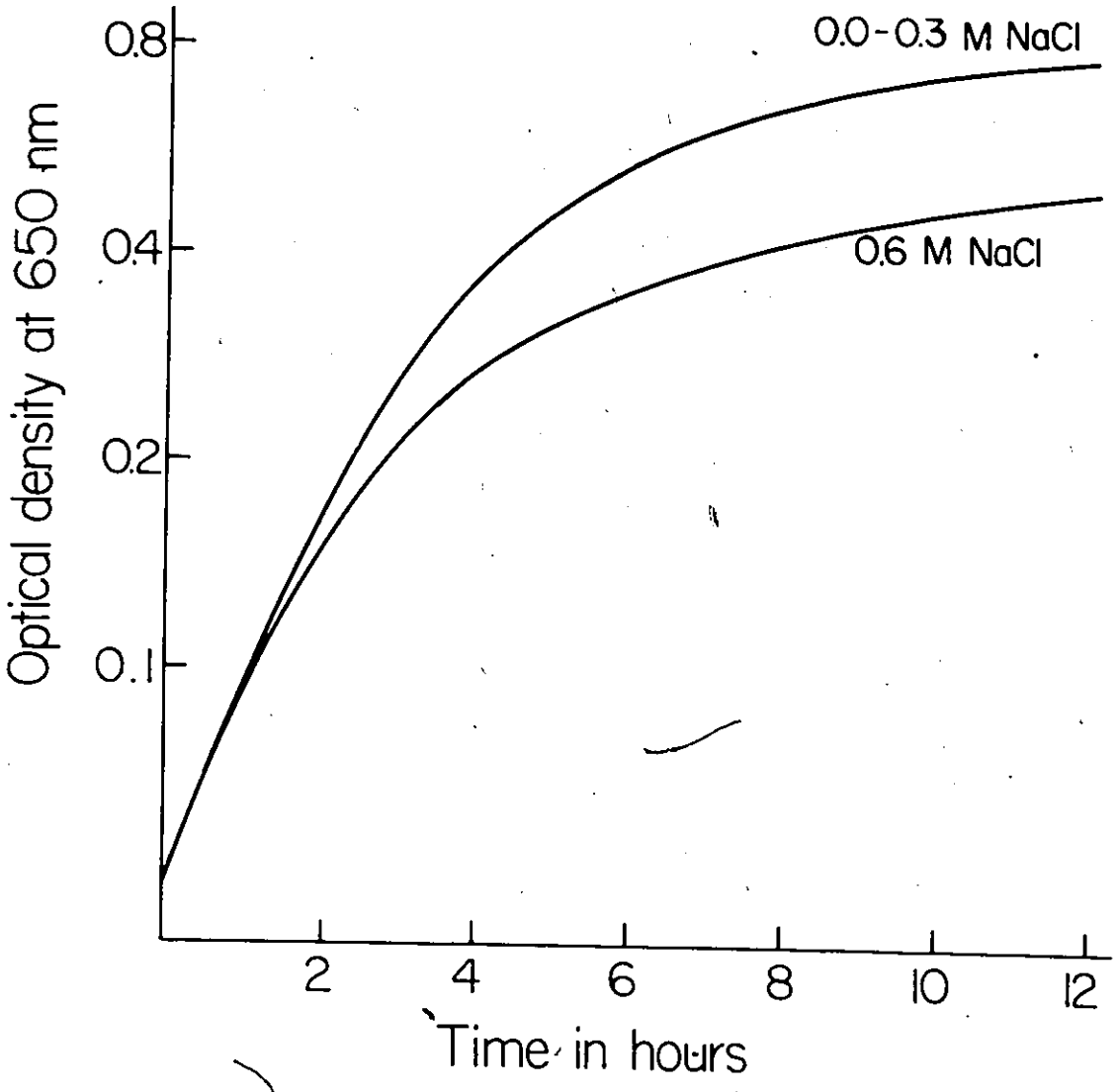

0.8
0.4
0.2
0.1
Optical density at 650 nm
0.0-0.3 M NaCl
0.6 M NaCl
2
4
6
8
10
12
Time in hours

TABLE 4

R_f values of the major phospholipids of E. coli AW405 in various solvent systems

Phospholipid	R_f value in solvent					
	A[a]		B[b]		C[c]	
	AW405	Standard	AW405	Standard	AW405	Standard
Phosphatidylethanolamine	0.0	0.0	0.38	0.37	0.33	0.30
Phosphatidylglycerol	0.0	0.0	0.66	0.64	0.47	0.41
Cardiolipin	0.0	0.0	0.90	0.92	0.78	0.87

[a] Petroleum ether-acetone, 3:1 (v/v)

[b] Chloroform-methanol-30% ammonia, 65:50:25 (v/v)

[c] Chloroform-methanol-acetic acid-water, 80:26:4:0.3 (v/v)

TABLE 5

PAPER CHROMATOGRAPHIC MOBILITIES OF WATER-SOLUBLE PRODUCTS OF DEACYLATION OF PG, PE AND CL ISOLATED FROM AW405.

PARENT LIPID	DEGRADATIVE PRODUCT	Rf VALUE x 100			
		PEAW+	PEAW+ (Lit)++	PW*	PW* (Lit)++
PE(St)	GPE	63	66	62	62
PE(Is)	GPE	64		61	
PG(St)	GPG	51	51	49	46
PG(Is)	GPG	51		41	
CL(St)	GPGPG	36	28	19	18
CL(Is)	GPGPG	33		18	

PW* saturated phenol-water (100:38 W/V)

PEAW+ Phenol saturated with water-ethanol-acetic acid (50:5:6:)

PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; (St), standard; (Is), isolated; GPE, glycerylphosphorylethanolamine; GPG, glycerylphosphorylglycerol; GPGPG, glycerylphosphorylglycerylphosphoryl-glycerol. (Lit)++ - Kates (1972)

isolated phospholipids, standards and reported values (Kates 1972). The samll discrepancies may be attributed to differences in methodology, or to differences in fatty acid composition. For example, bovine cardiolipin is less polar than E. coli cardiolipin due to its longer chain length fatty acid composition. Colour development of the phospholipids on thin layer chromatograms with specific staining reagents was consistent with the proposed identities (Table 6).

The fatty acid content of E. coli phospholipid consisted primarily of palmitic, palmitoleic, cis-vaccenic, cis-9,10-methylenehexadecanoic and lactobacillic acids, along with lesser amounts of myristic, stearic, lauric and unidentified fatty acids. The fatty acids were identified by comparison of retention times relative to palmitate with those obtained for standards as well as published values (Table 7). Mild oxidation of the fatty acid extract (Kates 1972) resulted in a decrease in the relative proportion of palmitoleic and cis-vaccenic acids consistent with their unsaturated structures (Table 8). Acetylation of the fatty acids (Kates 1972) did not affect the fatty acid composition indicating an absence of hydroxy-fatty acids (Table 8).

3.3 Phospholipid Fatty Acid Composition During Growth

The fatty acid compositions of the phospholipids of AW405, during growth at 30°C in tryptone broth, are listed in Tables 9-11. Cardiolipin (Table 11) contained the highest proportion of unusual fatty acids (up to 16.9%) and the least amount of cis-vaccenic acid (as low as 11.7%) whereas phosphatidylglycerol (Table 10) contained the highest proportion of cis-vaccenic acid (up to 33.5%).

In general, the decrease in growth rate at the end of the exponential

TABLE 6

Staining characteristics of the phospholipids of E. coli

Phospholipid	Lipid Isolate Thin Layer Chromatography			Deacylated Product Paper Chromatography		
	Ninhydrin Stain (1)	Phosphate Stain (2)	Periodate Schiff Stain (3)	Ninhydrin Stain (4)	Phosphate Stain (5)	Vicglycol Stain (6)
Phosphatidylethanolamine	+	+	-	+	+	+
Phosphalidylglycerol	-	+	+	-	+	+
Cardiolipin	-	+	-	-	+	+

+ indicates a positive reaction; - indicates a negative reaction

Staining techniques: 1, Skipski et al (1962); 2, Dittmer and Lester, (1964); 3, Schneider (1966); 4. Burrows et al, (1952); 5, Clark, (1964) and 6, White and Frerman, (1964).

TABLE 7

GLC retention times relative to methyl palmitate of E. coli AW405 phospholipid fatty acid methyl esters on 10% butanediol succinate.

Fatty acid methyl ester		Retention relative to methyl palmitate (tn/t_{16})		
Name	Abbreviation	AW405[b]	Standards	Literature[d]
Laurate	C12:0	0.30	0.30	-
Myristate	C14:0	0.54	0.55	0.51
Palmitate	C16:0	1.00	1.00	1.00
Palmitoleate	C16:1	1.13	1.14	1.14
cis-9,10-Methylene hexadecanoate	C17:Δ^a	1.54	1.58	1.63
Stearate	C18:0	1.85	1.90	1.97
cis-Vaccenate	C18:1	2.09	2.15	-
Lactobacillate	C19:Δ^a	3.16	3.38[c]	3.18

aΔ; cyclopropane

b Unidentified fatty acids with relative retention times 0.73, 1.36, 2.34 and 2.90 are also found in usually trace amounts.

c Synthetic

d Kates (1972)

TABLE 8

Susceptibility of E. coli fatty acids[a] to oxidation and acetylation.

Fatty Acid	Treatment		
	Control	Oxidation	Acetylation
Laurate	0.1	0.1	0.1
Myristate	2.8	3.3	2.8
Palmitate	46.4	51.0	47.0
Palmitoleate	10.0	5.2	10.3
cis-9,10-Methylene hexadecanoate	27.9	30.7	27.6
Stearate	1.2	1.8	1.1
cis-Vaccenate	10.3	5.3	10.1
Lactobacillate	1.2	2.7	1.2

[a] Extracted from a stationary E. coli culture

[b] According to Kates (1972)

The retention times relative to palmitate of the unidentified fatty acids were 0.73, 1.35, 1.64, 2.32 and 2.97.

Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 9

Distribution of fatty acids in phosphatidylethanolamine during growth of AW405 in 1% tryptone.

Fatty Acid	Optical Density				
	0.2	0.4	0.6	0.8	Stationary
Laurate	0.3	0.4	0.2	0.1	0.2
Myristate	5.5	3.9	3.0	3.4	3.2
Palmitate	36.5	34.2	37.0	35.1	38.5
Palmitoleate	33.5	33.9	32.5	30.6	21.7
cis-9,10-methylene hexadecanoate	4.4	3.0	4.7	10.3	15.3
Stearate	0.8	0.3	0.4	-	0.4
cis-Vaccenate	18.2	24.1	21.1	18.7	18.1
Lactobacillate	0.4	-	-	-	1.1
Unidentified	0.4	0.2	1.1	1.8	1.5

The retention times relative to Palmitate of the unidentified fatty acids were 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 10

Distribution of fatty acids in phosphatidylglycerol during growth of AW405 in 1% triptone.

Fatty Acid	Optical Density				
	0.2	0.4	0.6	0.8	Stationary
Laurate	0.6	0.4	0.3	-	0.1
Myristate	4.3	2.2	1.9	2.2	2.2
Palmitate	31.7	33.2	32.6	36.7	35.5
Palmitoleate	29.5	25.4	25.7	28.0	21.0
cis-9,10-methylene hexadecanoate	1.8	1.6	2.2	2.4	8.2
Stearate	1.5	1.3	0.8	-	0.6
cis-Vaccenate	28.1	34.1	33.5	29.3	30.0
Lactobacillate	-	-	0.4	-	1.8
Unidentified	2.5	1.8	2.6	1.4	0.6

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 11

Distribution of fatty acids in cardiolipin during growth of AW405 in 1% tryptone.

Fatty Acid	Optical Density				
	0.2	0.4	0.6	0.8	Stationary
Laurate	2.5	2.9	1.5	0.2	-
Myristate	7.1	5.9	5.3	1.4	5.0
Palmitate	33.9	31.7	33.6	33.6	39.3
Palmitoleate	17.3	19.4	22.4	25.8	20.5
cis-9,10-methylene hexadecanoate	3.3	2.4	2.6	5.0	9.5
Stearate	5.9	5.2	4.5	0.8	3.0
cis-Vaccenate	11.7	15.6	17.9	22.6	17.6
Lactobacillate	1.4	-	-	-	0.8
Unidentifed	16.9	16.9	12.2	10.6	4.3

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

phase, (Fig. 5) coincided with a decline in the proportion of the unsaturated fatty acids, as palmitoleic and cis-vaccenic acids were converted to cis-9,10-methylenehexadecanoic and lactobacillic acids. Relatively small amounts of lactobacillic acid were synthesized, but the rise in cis-9,10-methylenehexadecanoic acid and concomitant decrease in palmitoleic acid was quite apparent (Tables 9-11). During this stage in growth, there was also a small but significant increase in the proportion of palmitic acid at the expense of cis-vaccenic and lactobacillic acid, as well as a decrease in the proportion of unusual fatty acids in cardiolipin.

3.4 Influence of Salt on Lipid Composition

The phospholipids of AW405 were examined during growth at 30°C in tryptone broth supplemented with 0-0.6 M NaCl. The ratio of phospholipids; phosphatidylethanolamine, phosphatidylglycerol and cardiolipin; throughout growth, and the fatty acid compositions of the early exponential phase phospholipids, were quite similar (Tables 12-15). However, the phospholipid fatty acid compositions at advanced stages in growth were substantially different. The same trends observed during growth in 1% tryptone were observed: an increase in the cyclopropane fatty acids, a decrease in palmitoleic and cis-vaccenic acids, an increase in palmitic acid, and a decrease in unidentified fatty acids in cardiolipin. However, in the presence of salt, the differences between early exponential and stationary phase were much more pronounced. For example a 2-3 fold increase in the proportion of cis-9,10-methylene hexadecanoic acid was observed in cells grown in 0.6 M NaCl compared to cells grown in the absence of added salt. The changes in

TABLE 12

Effect of growth medium NaCl concentration on the fatty acid composition of phosphatidylethanolamine.

Fatty Acid	Exponential			Stationary		
	None	0.3M	0.6M	None	0.3M	0.6M
Laurate	0.3	0.7	0.4	0.2	0.1	0.3
Myristate	5.5	4.3	4.8	3.2	2.9	4.9
Palmitate	36.5	34.3	34.8	38.5	41.6	43.5
Palmitoleate	33.5	35.3	34.4	21.7	3.9	2.2
cis-9,10-Methylene hexadecanoate	4.4	3.1	6.2	15.3	32.3	33.2
Stearate	0.8	0.7	0.6	0.4	0.9	0.9
cis-Vaccenate	18.2	21.1	18.1	18.1	11.1	7.2
Lactobacillate	0.4	T	T	1.1	5.0	5.0
Unidentified	0.4	0.5	0.7	1.5	2.2	2.8

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 13

Effect of growth medium NaCl concentration on the fatty acid composition of phosphatidylglycerol.

Fatty Acid	Exponential			Stationary		
	None	0.3M	0.6M	None	0.3M	0.6M
Laurate	0.6	0.7	0.2	0.1	0.1	0.1
Myristate	4.3	2.8	3.0	2.2	2.6	2.8
Palmitate	31.7	31.9	36.3	35.5	40.1	41.9
Palmitoleate	29.5	28.6	21.1	21.0	8.4	4.5
cis-9,10 Methylene hexadecanoate	1.8	2.2	3.2	8.2	21.2	24.2
Stearate	1.5	1.7	1.1	0.6	1.0	1.1
cis-Vaccenate	28.1	29.4	26.9	30.0	19.4	14.7
Lactobacillate	0.3	0.3	0.6	1.8	4.6	7.2
Unidentified	2.2	2.4	7.6	0.6	2.6	3.5

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.

Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 14

Effect of growth medium NaCl concentration on the fatty acid composition of cardiolipin.

Fatty Acid	Exponential			Stationary		
	None	0.3M	0.6M	None	0.3M	0.6M
Laurate	2.5	4.0	1.5	T	0.7	1.0
Myristate	7.1	7.5	7.1	5.0	4.4	6.7
Palmitate	33.9	30.5	33.8	39.0	38.8	39.6
Palmitoleate	17.3	19.7	19.2	20.5	6.8	8.5
cis-9,10-Methylene hexadecanoate	3.3	3.4	3.2	9.5	18.2	18.9
Stearate	5.9	6.1	7.6	3.0	9.4	5.6
cis-Vaccenate	11.7	11.8	14.7	17.6	12.1	9.1
Lactobacillate	1.4	0.8	T	0.8	2.3	2.8
Unidentified	16.9	16.2	12.9	4.6	7.3	7.8

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% for separate runs of the same sample.

TABLE 15

Influence of NaCl on the Fatty Acid Composition of *E. coli* Phospholipids

Fatty Acid	Exponential			Stationary		
	None	0.3M	0.6M	None	0.3M	0.6M
Laurate	0.5	0.5	T	T	T	T
Mysistate	4.3	3.6	3.8	3.8	3.1	4.1
Palmitate	29.0	31.0	34.7	43.8	42.5	44.9
Palmitoleate	36.2	34.2	32.5	16.0	2.3	3.0
cis-9,10-Methylene hexadecanoate	1.3	2.0	3.5	10.9	27.3	26.1
Stearate	0.6	0.9	1.3	T	T	
cis-Vaccenate	26.1	26.5	22.5	19.6	10.2	9.1
Lactobacillate	T	T	T	2.5	7.4	5.6
Other	2.0	1.3	1.7	3.4	7.2	7.2

The retention times relative to palmitate of the unidentified fatty acids are 0.73, 1.35, 1.64, 2.32 and 2.97.
Values are expressed as weight percentage of total fatty acids and are reproducible within ± 1% ofr separate runs of the same sample.

fatty acid composition also occurred at lower cell densities and were much more abrupt (Fig. 6). Growth to stationary phase, in the presence of 0.3 - 0.6 M NaCl, also resulted in decreased long chain fatty acids (fatty acids 18 carbons in length or longer) and decreased cyclopropane and unsaturated fatty acids when combined (Table 16).

3.5 Effect of osmolality on fatty acid composition

To determine if the alterations in fatty acid composition observed in cultures grown in the presence of NaCl were due to differences in osmolality, the influence of other osmotic agents, such as KCl, $MgCl_2$ and sucrose, was examined. Phospholipid fatty acid compositions were examined in early exponential phase and late stationary phase (Tables 17-18). For the same stage of growth and osmolality, the fatty acid compositions of cells, grown in tryptone and NaCl, KCl or $MgCl_2$ were almost identical (Tables 17-18). However, even in exponential phase, higher levels of cyclopropane fatty acids were observed in cultures grown in 0.6 M sucrose (about 6 fold higher in exponential phase). In late stationary phase almost all of the unsaturated fatty acids had been converted to cyclopropane fatty acids (Table 18). Qualitatively, the results reveal that osmolality may be a key factor, but that other factors, such as permeability and charge, may also play some part since growth in sucrose did not result in the same phospholipid fatty acid composition as growth in other media of the same osmolality.

3.6 Effect of Temperature on Fatty Acid Composition

Several authors have reported that increasing the incubation temperature of E. coli cultures results in membrane phospholipid fatty acid compositions reduced in cis-vaccenic acid but enriched in palmitic and cyclopropane fatty acids. This trend was observed in E. coli (Haest et al 1969; Shaw and Ingraham 1965; Sinensky 1971; Kito et al 1972,

Figure 6. Change in palmitoleic and cis-9,10-methylene hexadecanoic acid levels in phosphatidylethanolamine during growth in 0 and 0.6 M NaCl. Palmitoleic acid during growth in 0.0 M (●---●) and 0.6 M (⊙—⊙) NaCl. Cis-9,10-methylene hexadecanoic acid during growth in 0.0 (x---x) and 0.6 M (⊗—⊗) NaCl.

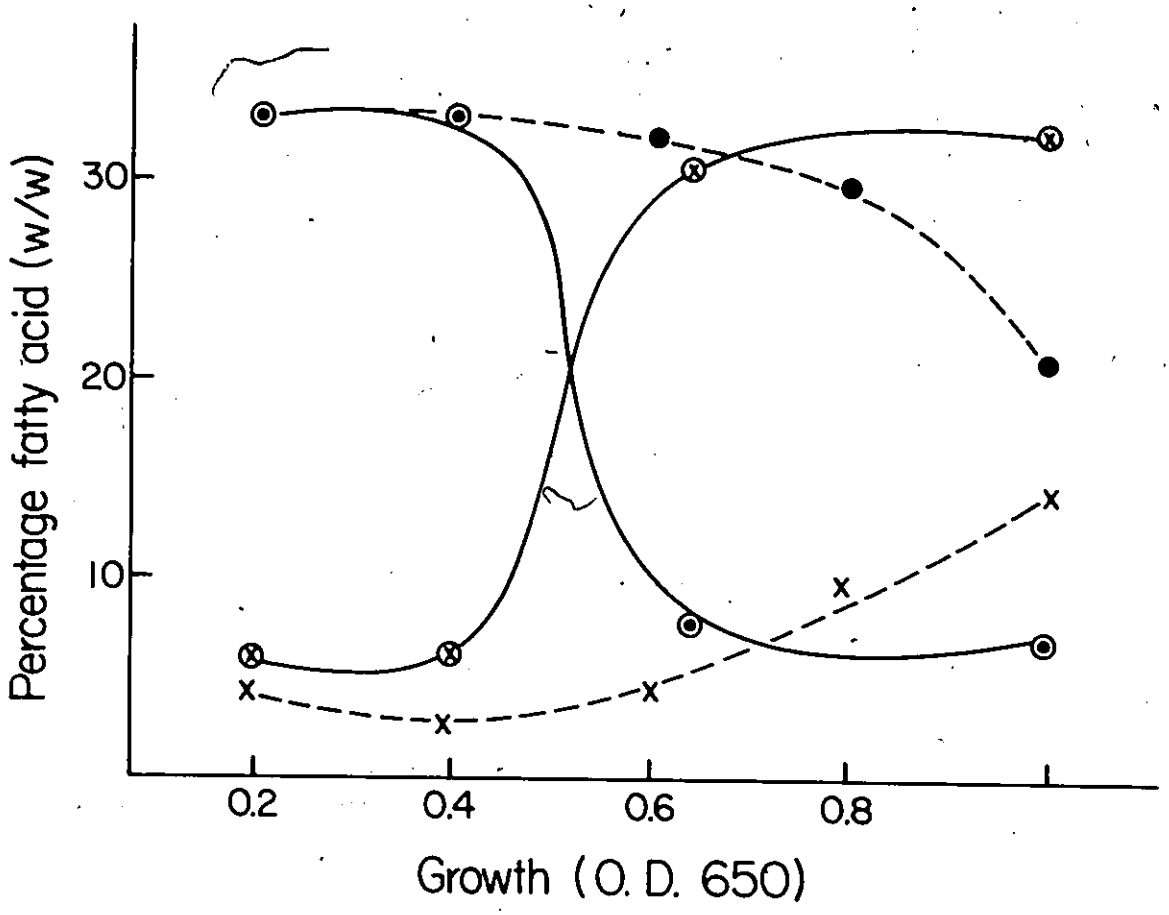

Percentage fatty acid (w/w)
30
20
10
0.2
0.4
0.6
0.8
Growth (O. D. 650)

TABLE 16

Effect of salt and stage in growth on cyclopropane, unsaturated and long chain fatty acid levels of E. coli lipids.

	0.0MNaCl			0.3MNaCl			0.6MNaCl	
	Optical Density$_{650}$							
	0.4	0.6	stat.	0.4	0.6	stat.	0.4	stat.
Phosphatidylethanolamine								
% Unsaturated and Cyclopropane[a]	61.0	58.3	56.2	58.2	59.4	52.3	51.0	47.7
% Cyclopropane[b]	3.0	4.7	16.4	7.3	10.0	37.3	16.8	38.2
% Long Chain	24.8	22.3	20.3	24.2	25.1	18.6	24.4	14.5
Phosphatidylglycerol								
% Unsaturated and Cyclopropane[a]	61.1	61.8	61.0	59.5	58.3	53.5	51.1	51.6
% Cyclopropane[b]	1.6	2.6	10.0	4.6	3.8	25.8	11.3	32.4
% Long Chain	36.4	36.3	31.4	36.6	35.2	26.2	28.9	24.3
Cardiolipin								
% Unsaturated and Cyclopropane[a]	38.9	43.2	48.4	34.3	49.7	39.5	40.3	39.3
% Cyclopropane	3.9	2.6	10.3	5.1	5.3	20.5	7.7	21.7
% Long Chain	31.5	29.8	22.7	29.2	33.7	26.1	22.3	20.4

[a] Sum of palmitoleic, cis-9,10-methylenehexadecanoic, lactobacillic and cis-vaccenic acids.

[b] Sum of cis-9,10-methylenehexadecanoic and lactobacillic acids

[c] Sum of fatty acids of length 18 carbons or more.

TABLE 17

Effect of NaCl, KCl, $MgCl_2$ and sucrose on the fatty acid composition of early exponential E. coli phospholipids.

Fatty Acid	None	0.3M NaCl	0.3M KCl	0.6M NaCl	0.6M KCl	0.2M $MgCl_2$	0.6M Sucrose
Laurate	0.5	0.5	0.5	T	0.7	0.6	0.6
Myristate	4.3	3.6	3.4	3.8	3.9	2.6	3.2
Palmitate	29.0	31.0	31.9	34.7	33.9	32.3	37.9
Palmitoleate	36.2	34.2	33.4	32.5	33.1	30.5	8.4
cis-9,10-Methylene hexadecanoate	1.3	2.0	2.2	3.5	3.4	4.7	24.2
Stearate	0.6	0.9	0.9	1.3	1.1	0.9	0.9
cis-Vaccenate	26.1	26.5	26.6	22.5	22.2	27.3	17.4
Lactobacilliate	T	T	T	T	T	T	5.4
Unidentified	2.0	1.3	1.1	1.7	1.7	1.1	2.0

TABLE 18

Effect of NaCl, KCl, $MgCl_2$ and sucrose on the fatty acid composition of late stationary *E. coli* phospholipids.

Fatty Acid	None	0.3M NaCl	0.3M KCl	0.6M NaCl	0.6M KCl	0.2M $MgCl_2$	0.6M Sucrose
Laurate	T	T	T	T	T	T	T
Myristate	3.8	3.1	2.9	4.1	3.9	2.5	6.0
Palmitate	43.8	42.5	40.6	44.9	41.9	40.3	45.1
Palmitoleate	16.0	2.3	1.8	3.0	3.4	2.1	1.0
cis-9,10-Methylene hexadecanoate	10.9	27.3	30.6	26.1	30.2	33.0	28.0
Stearate	T	T	T	0.7	T	0.6	0.9
cis-Vaccenate	19.6	10.2	9.8	9.1	9.6	7.5	1.9
Lactobacillate	2.5	7.4	9.9	5.6	6.8	11.7	14.9
Unidentified	3.4	7.2	4.4	6.5	4.2	2.3	2.2

Marr and Ingraham 1962; Cronan 1975) in Pseudomonas fluorescens (Cullin et al 1971) in Serratia marcescens (Kates and Hagen 1964) and in Streptococcus faecalis (Jungkind and Wood 1974a). Since growth in media differing in salt concentration resulted in similar differences in stationary phase lipids, the phospholipid fatty acid compositions of cultures of AW405 grown from 20-42°C were examined in early exponential and stationary phase to compare the two effects (Table 19). Cultures were inoculated with a sufficiently small number of cells to permit at least eight doubling periods in early exponential phase before harvesting, in order to allow sufficient time for the cultures to adjust to the new growing conditions.

Qualitatively, growth of AW405 over the 20-42°C temperature range resulted in the same differences in fatty acid composition reported earlier for cell growth in 0-0.6 M NaCl (Table 15). Higher growth temperatures resulted in less cis-vaccenic and more palmitic and cyclopropane fatty acids. However, differences were small in exponential lipids, as they were for cells grown in media with different osmolalities. Stationary phase phospholipids of cells grown at 20°C contained less vaccenic acid and more palmitic and cyclopropane fatty acids than the early exponential culture grown at 42°C, a trend also observed in the osmolality study.

3.7 Growth Conditions and Possible Growth Limiting Factors

Differences in lipid cyclopropane fatty acid levels in bacteria have been related to growth conditions as well as the growth limiting components in minimal media (See Section 1.8). We became curious as to whether the effect of osmolality was somehow related to the other growth conditions which have been shown to influence cyclopropane fatty acid synthesis in E. coli. As a first step, we tried to identify the

Table 19

Effect of temperature and stage in growth on the fatty acid composition of *E. coli* lipids[d].

Fatty Acid	Temperature					
	20°C		30°C		42°C	
	Optical Density$_{650}$					
	0.12[a]	0.80[b]	0.17[a]	0.80[b]	0.05[a]	0.82[b]
Laurate	0.7	T[c]	0.6	T[c]	0.5	0.1
Myristate	3.2	3.6	3.8	2.7	3.7	2.3
Palmitate	28.1	37.9	30.8	37.6	32.9	43.0
Palmitoleate	36.5	25.7	35.1	13.9	35.9	8.7
cis-9,10-Methylene hexadecanoate	0.5	8.7	1.4	17.1	2.7	29.1
Stearate	1.5	0.9	0.9	3.3	1.0	0.9
cis-Vaccenate	28.5	21.2	26.8	18.2	22.3	9.2
Lactobacillate	T[c]	2.1	T[c]	2.1	T[c]	2.5
Unidentified	1.0	T	0.6	5.1	1.0	4.2

[a] Early exponential cultures

[b] Early stationary phase cultures

[c] T indicates trace amount

[d] All cultures grown in 1% tryptone and 0.3 M NaCl

changes in growth conditions, during growth in tryptone broth and 0.3 M NaCl, resulting in cessation of AW405 growth (the growth limiting factors).

Elevated levels of cyclopropane fatty acids in stationary phase E. coli cultures have been observed when nitrogen or phosphate is growth limiting, a pattern similar to that observed for growth in tryptone broth at high NaCl concentrations. However, addition of either phosphate (as 0.01 mM sodium phosphate buffer, pH 7.0) or nitrogen 0.2% (as ammonium sulfate) to tryptone broth with 0.3 M NaCl did not result in further growth, indicating that these components were not growth limiting.

Reduced levels of stationary phase cyclopropane fatty acids, due to a cessation of cyclopropane fatty acid production at the onset of stationary phase, were reported in E. coli cultures with limiting magnesium, sulfate or carbon source (Knivett and Cullen 1967). Since cyclopropane fatty acids increased with high NaCl concentration, the above seemed unlikely candidates as growth limiting factors under these growth conditions. Nevertheless, growth was not increased in tryptone and 0.3 M NaCl cultures supplemented with 0.2% ammonium sulfate or 0.1 µM $Fe_2(SO_4)_3$. Carbon source was not limiting since addition of amino acids to 3 mg/ml did not increase growth and filter sterilized spent media permitted growth of E. coli B to O.D. 0.4 but no growth of AW405.

Other possible growth limiting factors that were considered included starvation for a required amino acid or accumulation of a toxic product. Addition of the required amino acids, histidine threonine and leucine (to 1 mg/ml), either alone or in combination,

did not increase growth. Dilution of a late stationary phase culture with an equal volume of fresh media resulted in a new round of growth terminating in a final cell density only 10% less than that achieved by the parent culture. Therefore, cessation of growth due to accumulation of a toxic product is unlikely.

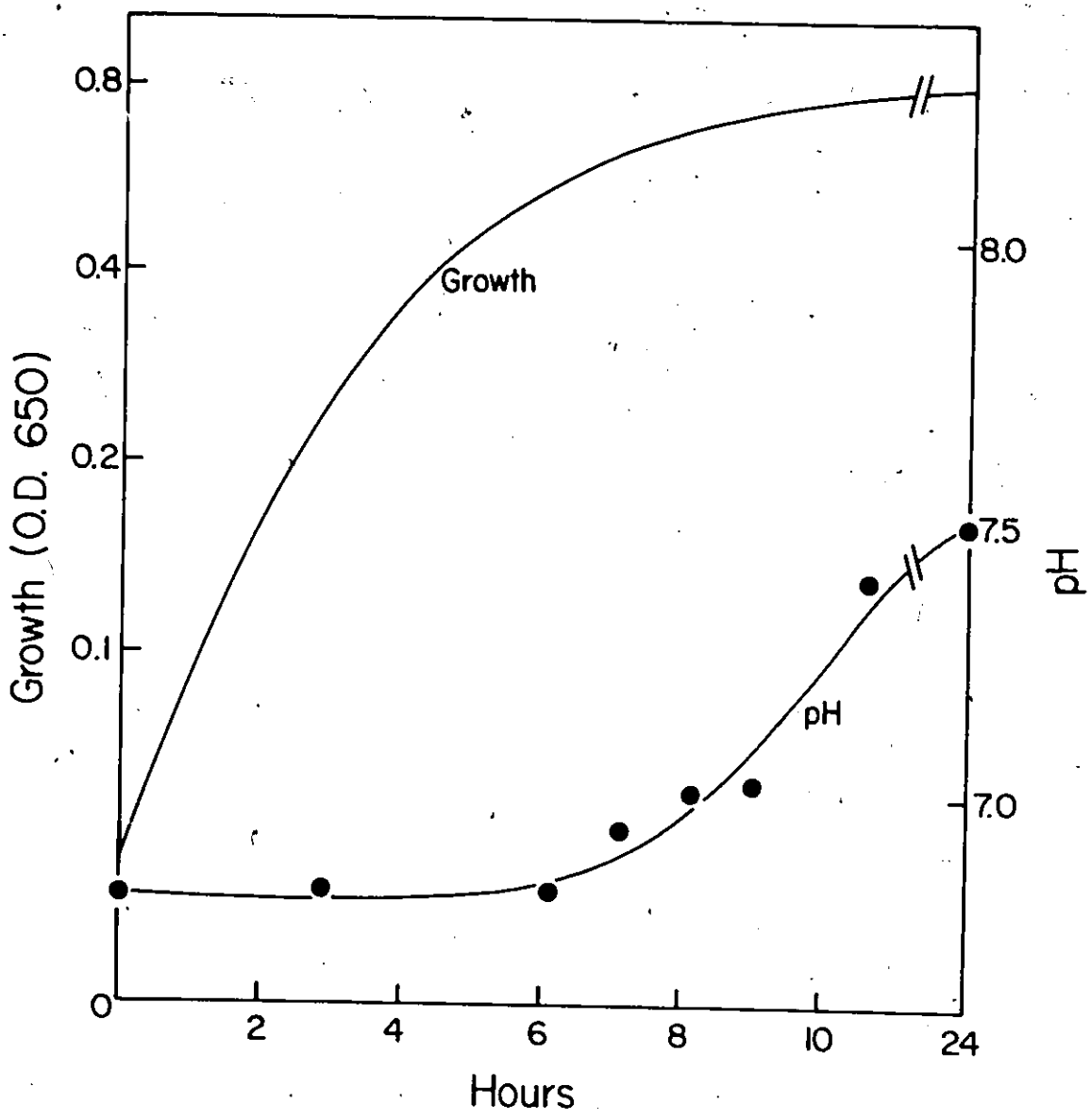
Stationary phase cyclopropane fatty acid production can also be enhanced by growth at elevated temperature, low oxygen pressure or low pH (see Section 1.8). As reported earlier, the influence of osmolality on fatty acid composition mimics the influence of temperature. Undoubtedly oxygen pressure is somewhat reduced in medium of high osmolality but this was judged insufficient by Knivett and Cullen (1967) to affect cyclopropane fatty acid production. To maximize oxygen pressure AW405 was grown in large Erlenmeyer or Fernbach flasks with a medium to flask volume ratio of less than 1:5 with rapid rotary mixing. A gradual increase in pH from pH 6.8 to pH 7.5 was observed at late stages in growth (Figure 7) negating the possibility that the increase in cyclopropane fatty acids in stationary phase is related to a drop in pH.

There appears to be no obvious relationship between the influence of osmolality and other growth conditions that have been observed to influence lipid fatty acid composition in bacteria. Since we were unable to identify the growth limiting conditions in cultures grown in tryptone, the identities of these remains an interesting question.

3.8 Regulation of cyclopropane fatty acid synthesis

The extent to which unsaturated fatty acids are converted to cyclopropane fatty acids in stationary phase is likely dependent upon the availability of S-adenosylmethionine and cyclopropane fatty acid

Figure 7 Change in the pH of the medium during growth of AW405 in 1% tryptone broth and 0.3 M NaCl.


0.8
0.4
0.2
0.1
0
Growth (O.D. 650)
Growth
pH
8.0
7.5
7.0
pH
2
4
6
8
10
24
Hours

synthetase activity. To test if post-exponential protein synthesis of one or more of the enzymes involved in S-adenosylmethionine synthesis, or cyclopropane fatty acid synthetase, is required for normal rates of cyclopropane fatty acid synthesis, late- and post-exponential cultures of AW405 were treated with the protein synthesis inhibitor, chloramphenicol. Cultures grown in tryptone broth with 0.6 M NaCl were treated with chloramphenicol at the end of the exponential stage and in the post-exponential stage of growth (Figure 8). These are the stages immediately before and during the period of highest cyclopropane fatty acid accumulation.

In the late-exponential and post-exponential cultures a three fold decrease and little change, respectively, in the ratio of cyclopropane to unsaturated fatty acids after two hours incubation with chloramphenicol were observed (Table 20). In contrast, substantial increases in the ratio (0.22 to 0.28 and 0.28 to 0.48) were observed in untreated controls (Table 20). Since there was little increase in weight of fatty acids in either treated sample during the incubation period, the decrease in cyclopropane fatty acids in the late exponential treated sample could be due to a rapid rate of fatty acid turnover which is curtailed in the post-exponential sample. Hence the results indicate a requirement for continued protein synthesis if high levels of cyclopropane fatty acids are to be produced during late stages in growth.

Assay of cyclopropane fatty acid synthetase revealed activity in cells grown in minimal medium, 0.12 mμMoles cyclopropane fatty acid/mg protein/hour, but no activity in cells grown in tryptone broth (Figure 9). However, the crude assay solutions of tryptone grown cells contained little to no S-adenosylmethionine as shown by paper

Figure 8. Influence of chloramphenicol (cm) on the growth of AW405 in 1% tryptone and 0.6 M NaCl broth.

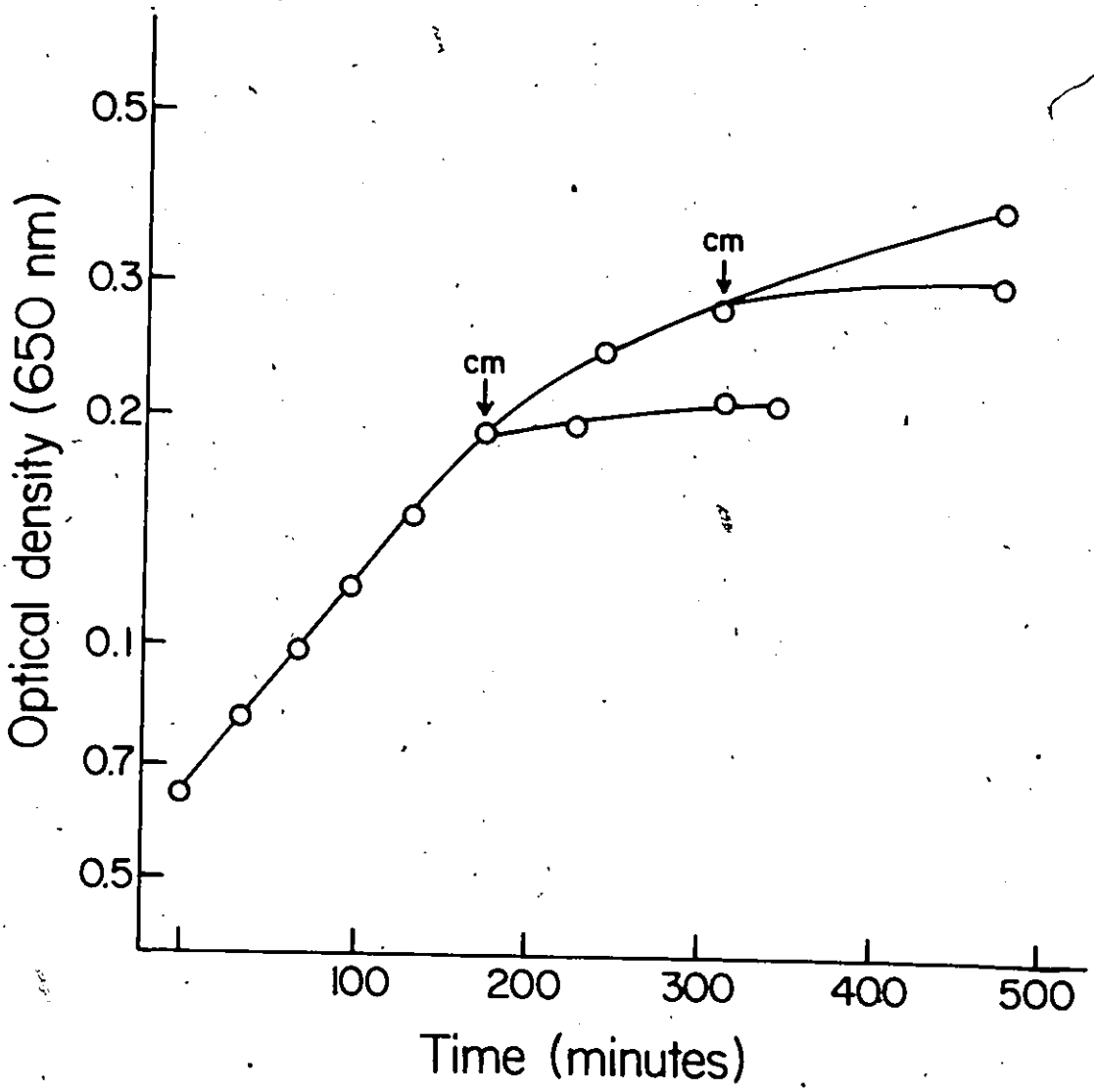

Optical density (650 nm)
0.5
0.3
0.2
0.1
0.7
0.5
cm
cm
100
200
300
400
500
Time (minutes)

Table 20

Influence of chloramphenicol on cyclopropane fatty acid synthesis.

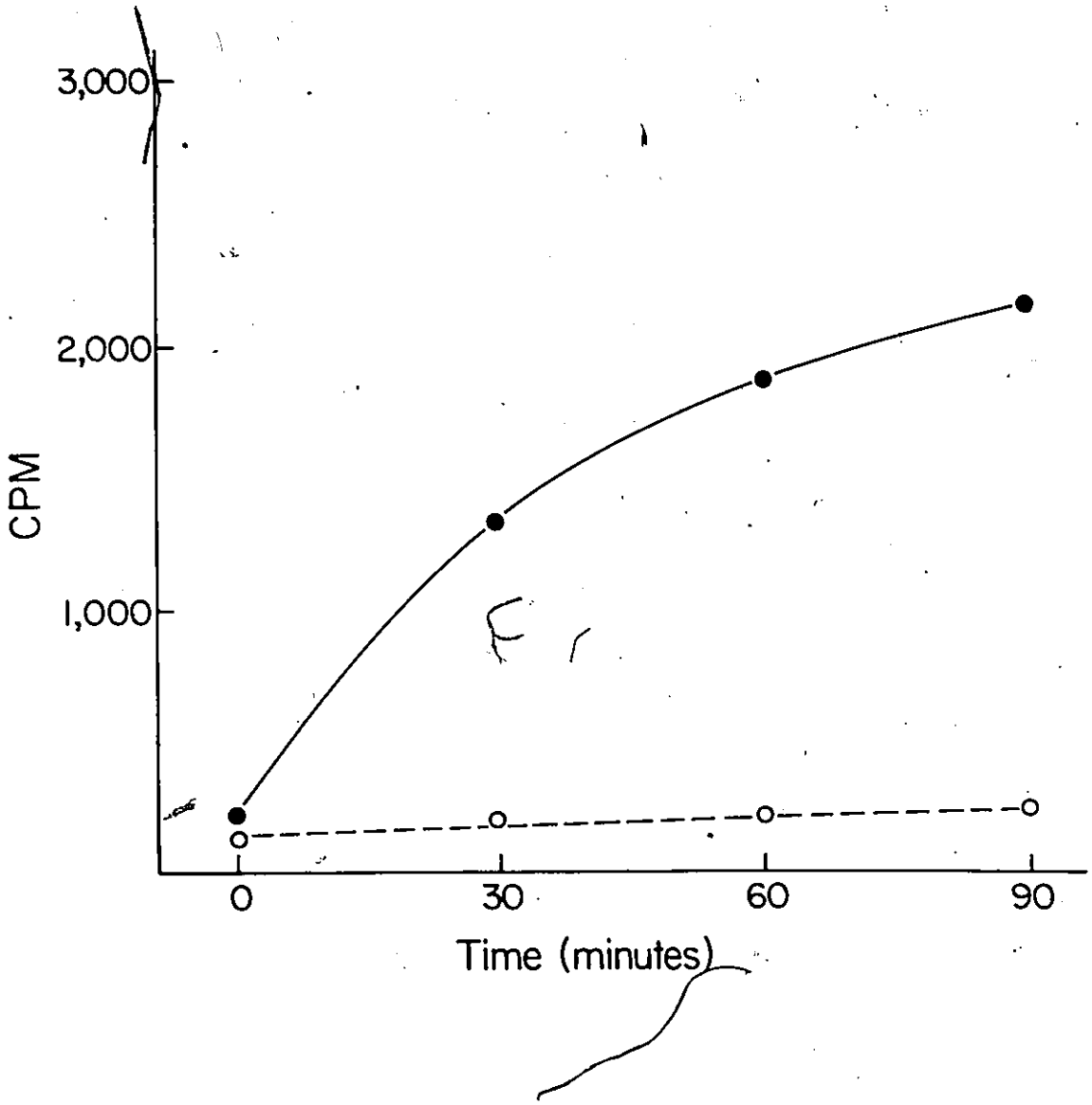
Fatty Acid	Optical Density$_{650}$ 0.2			Optical Density$_{650}$ 0.3		
	Initial 0 min	Treated[c] 120 min	Untreated 120 min	Initial 0 min	Treated[c] 120 min	Untreated 120 min
% Cyclopropane[a]	8.2	2.8	9.9	9.9	9.8	13.0
% Unsaturated[b]	37.7	38.1	35.4	35.4	31.9	27.1
Cyclopropane/Unsaturated	0.22	0.07	0.28	0.28	0.31	0.48
Total Weight (µg/ml)	56.5	60.1	70.7	70.7	72.6	93.9

[a] Sum of cis-9,10-methylenehexadecanoic and lactobacillic acids

[b] Sum of palmitoleic and cis-vaccenic acid.

[c] 100 µg/ml of chloramphenicol added.

Figure 9. Cyclopropane fatty acid synthetase activity in cell extracts of E. coli AW405 after growth in minimal medium (● — ●) and tryptone broth (o---o). Enzyme activity was measured by monitoring the incorporation of radioactive label from ($^{14}CH_3$)-methionine into E. coli phospholipids.


3,000
2,000
1,000
CPM
0
30
60
90
Time (minutes)

chromatography. Since the assay system (Cronan 1968) required endogenous S-adenosylmethionine synthesis the negative result could have been due to a lack of S-adenosylmethionine synthetase. Unfortunately, we were unable to synthesize ($^{14}CH_3$)-S-adenosylmethionine of sufficiently high specific activity for a successful assay of cyclopropane fatty acid synthetase by techniques not requiring endogenous S-adenosylmethionine synthetase.

Assay of the enzyme according to Greene et al (1970) confirmed the difference in S-adenosylmethionine synthetase activity between cells grown in minimal medium and tryptone broth. Although assays using toluenized cells are not precise, they are sufficiently accurate to reveal large differences in activity (Greene et al,1970). The results indicated that the S-adenosylmethionine activity in cells grown in minimal media was at least seven fold higher than that of cells grown in tryptone broth.

3.9 Membrane transition temperatures

Artificial and natural membranes have been reported to have melting characteristics dependent upon their fatty acid compositions (Cronan and Gelmann, 1975). Membranes rich in high melting point fatty acids (saturated, long chain,etc.) melt at higher temperatures than those rich in low melting point fatty acids (unsaturated, short chain,etc.). However, the role of cyclopropane fatty acids within this scheme has not been investigated. Upon comparison of the free fatty acid melting temperatures, it seemed reasonable to predict that phospholipids acylated with cyclopropane fatty acids would have melting temperatures intermediate between their unsaturated and saturated fatty acid analogues (Table 21).

To investigate this possibility, the transition temperatures of artificial lecithins composed of either cyclopropane or unsaturated fatty

Table 21

Melting temperatures of free fatty acids and their lecithin derivatives

Fatty Acid	Chain Length	Position of Double Bend or Cyclopropane Ring	Melting Temperature (°C)	
			Free Fatty Acid	Lecithin
Saturated				
Lauric	12	-	44.2	0[a]
Myristic	14	-	53.9	23[a]
Palmitic	16	-	63.1	41[a]
Stearic	18	-	69.6	58[a]
Unsaturated				
Palmitoleic	16	9	-0.5 to 0.5[d]	<-40
cis-Vaccenic	18	11	13.4	-23
Oleic	18	9	13.4	-22[b]
Cyclopropane				
cis-9,10-methylene hexadecanoic	16	9	9[d]	<-40
Lactobacillic	18	11	28-29	-7
Dihydrosterculic	18	9	38-41[c]	-7

[a] Values reported Labrooke and Chapman [1], DeKruyff et al. [20] and Phillips et al. [21].

b Value reported by Phillips et al. [21, 22].

c Value reported by Simmons and Smith [11].

d Kaneshiro and Marr [23].

acids were compared. Analysis by differential scanning calorimetry showed that lecithins acylated with lactobacillic (Figure 10) or dihydrosterculic (Figure 11) acids melted at $-7^{\circ}C$ in these liposome preparations, whereas lecithin acylated with cis-vaccenic acid melted at $-23^{\circ}C$ (Figure 12). Liposomes composed of lecithins acylated with cis-9,10-methylene hexadecanoic and palmitoleic acids did not melt above $-40^{\circ}C$, the lower limit of the temperature control of the calorimeter. These results indicated that substitution of cyclopropane fatty acids for their unsaturated fatty acid analogues in membranes should increase their melting temperatures and hence decrease their fluidity.

Since growth in high osmotic media to stationary phase resulted in partial replacement of the unsaturated fatty acids with higher melting point cyclopropane fatty acids and palmitic acid, it was suspected that these membranes would melt at higher temperatures. To test this possibility, the transition temperatures of AW405 membranes (whole cell and phospholipid bilayer preparations) were measured using the fluorescent probe, N-phenylnapthylamine (NPN), and differential scanning calorimetry. However, the extreme variability encountered from one measurement to another (even for the same membrane preparation) did not permit unequivocal interpretation of the transition data in terms of changes in phospholipid fatty acid composition due either to stage in growth or to growth in media of different osmolality.

In most cases, the differences in NPN fluorescence, indicative of phase transitions (Section 2.11), were small and in some cases not observed. Unfortunately, with the technique employed, we were unable to distinguish whether the weak response was due to a high noise to signal ratio or low cooperativity, ie. a small fraction of the lipid actually

Figure 10. Differential calorimeter scan of lecithin acylated with lactobacillic acid.

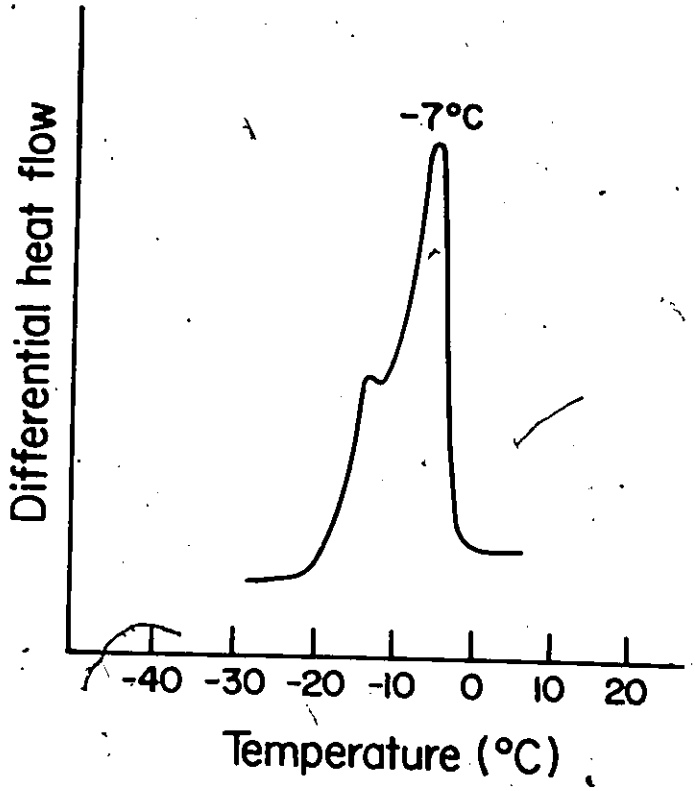

-7°C
Differential heat flow
-40
-30
-20
-10
0
10
20
Temperature (°C)

Figure 11. Differential calorimeter scan of lecithin acylated with dihydrosterculic acid.

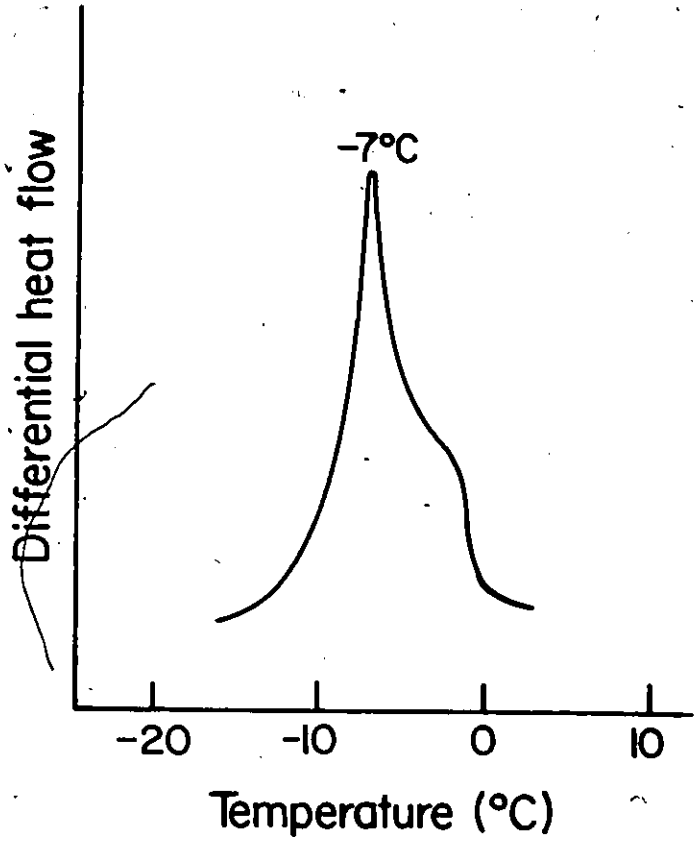
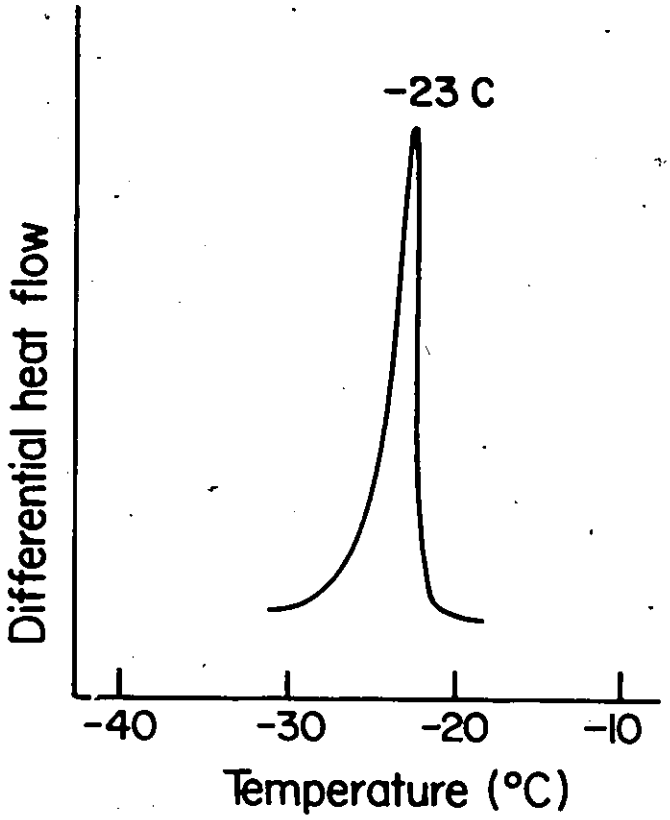

Differential heat flow
-7°C
-20
-10
0
10
Temperature (°C)

Figure 12. Differential calorimeter scan of lecithin acylated with cis-vaccenic acid.


-23 C
Differential heat flow
-40
-30
-20
-10
Temperature (°C)

participating in the phase transitions. The fluorescent response was not improved by running samples in distilled water, 20 mM phosphate buffer (pH 7.0) or 1 M NaCl, or by 10 fold variations in membrane phospholipid and probe concentration.

In those samples where transition profiles were detected, three different log relative fluorescence versus reciprocal temperature profiles were observed:

a) broad transitions with transition temperatures at $25.6 \pm 3.7^{\circ}$ (Figure 13).

b) double transitions at temperatures, 17.0 ± 3.9 and $28.0 \pm 4.4^{\circ}C$ (Figure 14).

c) single narrow transitions at temperatures $32.3 \pm 4.6^{\circ}C$ (Figure 15)

Profile (a) transitions were observed most frequently when examining freshly prepared exponential cells, whereas profile (b) and (c) were observed predominantly with stationary cell preparations after repeated heating ($40^{\circ}C$) and cooling ($4^{\circ}C$) cycles (Table 22). The possibility that the double transition profiles (b), reflected differences in transition temperature between inner and outer membranes appeared unlikely since all three profiles; (a), (b), and (c); were observed in artificial phospholipid membrane preparations which presumably have homogeneous lipid compositions (Figure 16).

Repeated attempts to determine membrane melting temperatures in whole cell preparations using differential calorimetry failed to reveal differential heat flow versus temperature profiles that could be unambiguously assigned in terms of phase transition behaviour. Phase

Figure 13. Log relative fluorescence versus temperature plots of whole cell membrane preparations with single transitions as detected using NPN fluorescence.

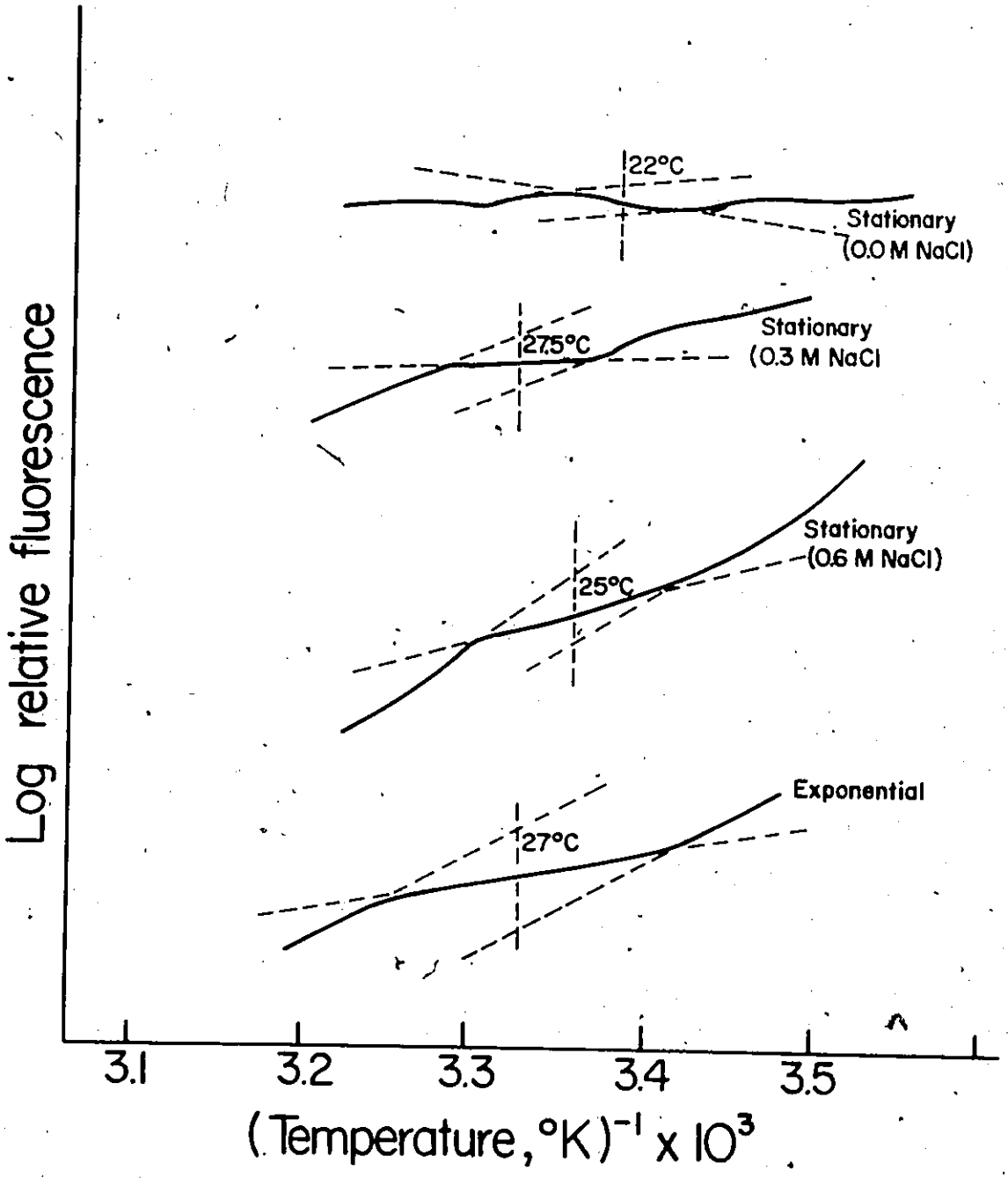

Log relative fluorescence
22°C
Stationary
(0.0 M NaCl)
27.5°C
Stationary
(0.3 M NaCl
25°C
Stationary
(0.6 M NaCl)
27°C
Exponential
3.1
3.2
3.3
3.4
3.5
(Temperature, °K)⁻¹ x 10³

Figure 14. Log relative fluorescence versus temperature plots of whole cell membrane preparations with double transitions as detected using NPN fluorescence.

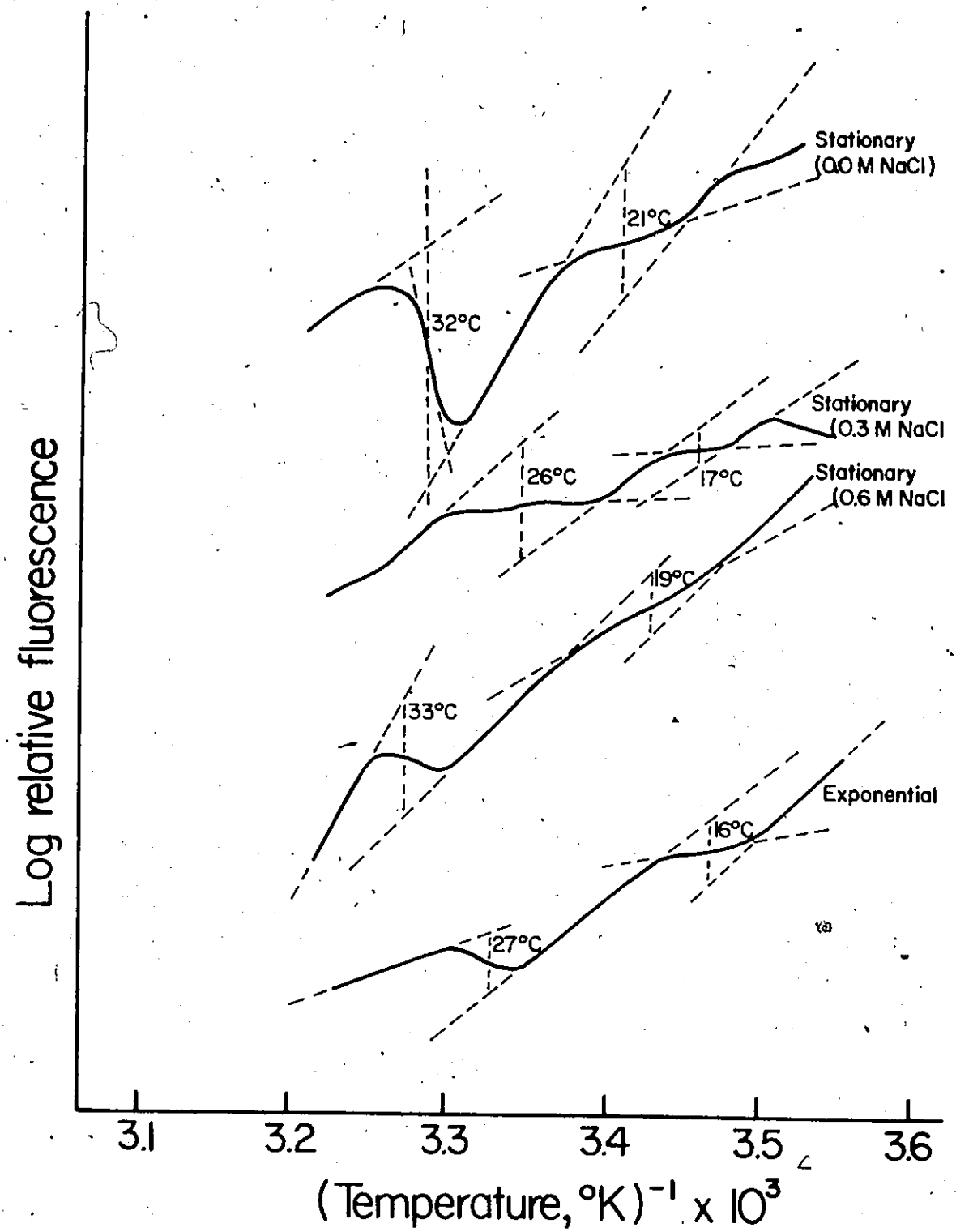

Log relative fluorescence
Stationary (0.0 M NaCl)
21°C
32°C
Stationary (0.3 M NaCl
26°C
17°C
Stationary (0.6 M NaCl
19°C
33°C
Exponential
16°C
27°C
3.1
3.2
3.3
3.4
3.5
3.6
(Temperature, °K)⁻¹ x 10³

Figure 15. Log relative fluorescence versus temperature plots of whole cell membrane preparations with single high temperature narrow transitions as detected using NPN fluorescence.

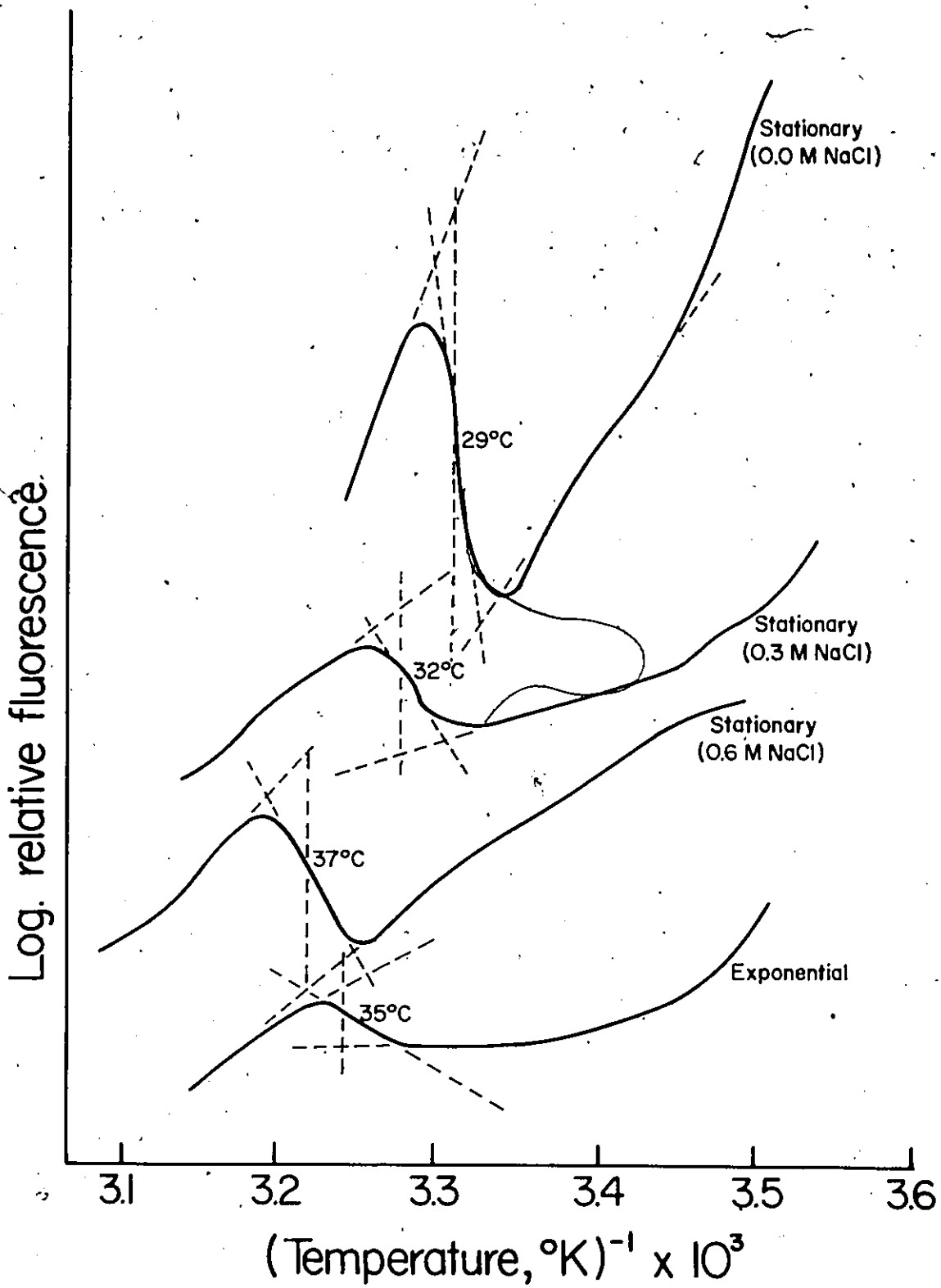

Stationary
(0.0 M NaCl)
29°C
Stationary
(0.3 M NaCl)
32°C
Stationary
(0.6 M NaCl)
37°C
Exponential
35°C
Log. relative fluorescence
3.1
3.2
3.3
3.4
3.5
3.6
(Temperature, °K)⁻¹ x 10³

Table 22

Transition temperatures[1] measured using the fluorescent probe, NPN.

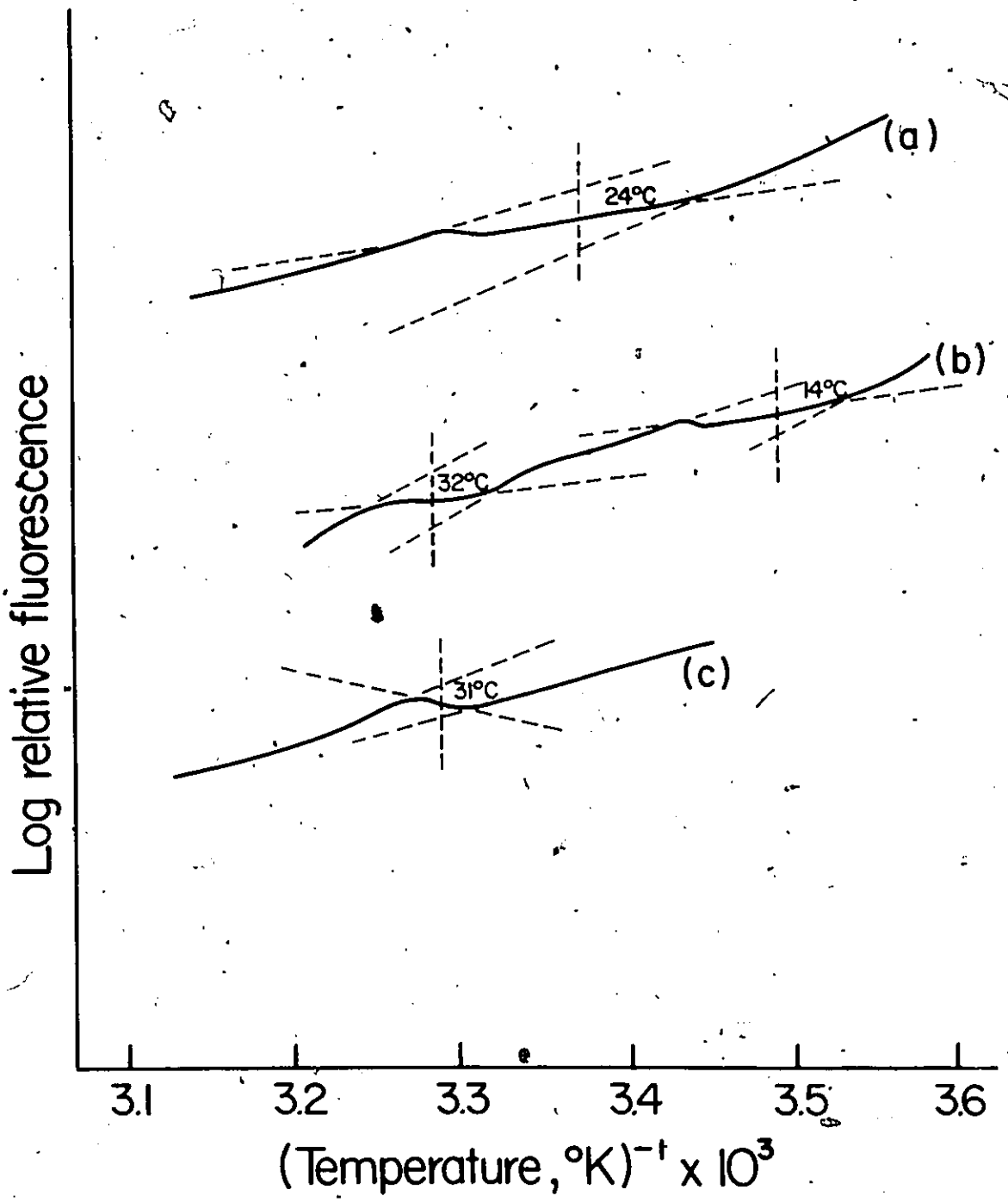
Description	Profile[2]	Mean	Standard Deviation	No. of Samples[3]	
				Exponential Phase	Stationary Phase
Double Lower	(b)	17.0	3.9	4	17
Single Broad	(a)	25.6	3.6	8	8
Double Upper	(b)	28.0	4.4	4	17
Single Narrow	(c)	32.3	4.6	2	3

[1] Measured in ^{o}C.

[2] See text

[3] Cells grown in 1% tryptone broth and 0-0.6 M NaCl

Figure 16. Log relative fluorescence versus temperature plots of sonicated phospholipid preparations. Stationary phase cultures grown in 0.3 M NaCl, a; 0.0 M NaCl, b and 0.3 M NaCl, c.


Log relative fluorescence
(a)
24°C
(b)
14°C
32°C
(c)
31°C
3.1
3.2
3.3
3.4
3.5
3.6
(Temperature, °K)$^{-1}$ x 10^3

transition patterns (if present) were obscurred by shifting base lines and electronic spiking patterns at the high sensitivity settings required to observe transition temperatures in the membranes which contained less than 1 mg of total phospholipid.

On the other hand, phase transition profiles in artificial liposome preparations made from E. coli phospholipids were detected by differential calorimetry (Figure 17 to 21). The extreme broadness of transitions and resultant difficulties in establishing base lines negated the possibility of establishing the degree of phospholipid cooperativity but patterns resembling profiles (b) and (c) in the fluorescent study were obtained in both heating and cooling patterns. Transitions were especially apparent in cooling curves, likely due to supercooling resulting in the rapid release of heat. The slow absorption of heat encountered in heating curves resulted in transition profiles not easily discernible from the base line.

Cooling scans revealed lower transitions centering at 13-15°C and upper transitions at 23-26°C in exponential (Figure 17) and stationary (Figure 18) AW405 phospholipid liposomes from cells grown with or without added salt. The upper transition was much more pronounced in stationary phase than exponential phase preparations. Heating curves revealed double transitions but at higher temperatures, ie. 19-23°C and 30-32°C (Figure 19). In contrast to the above, an upper temperature transition at 29°C in cooling curves, (Figure 20), and 31°C in heating curves (Figure 21) were obtained in scans of liposomes prepared from AW405 phospholipids extracted from cells grown in the presence of 0.6 M sucrose.

Figure 17. Differential calorimeter scans of liposomes prepared from exponential phospholipids (cooling runs).

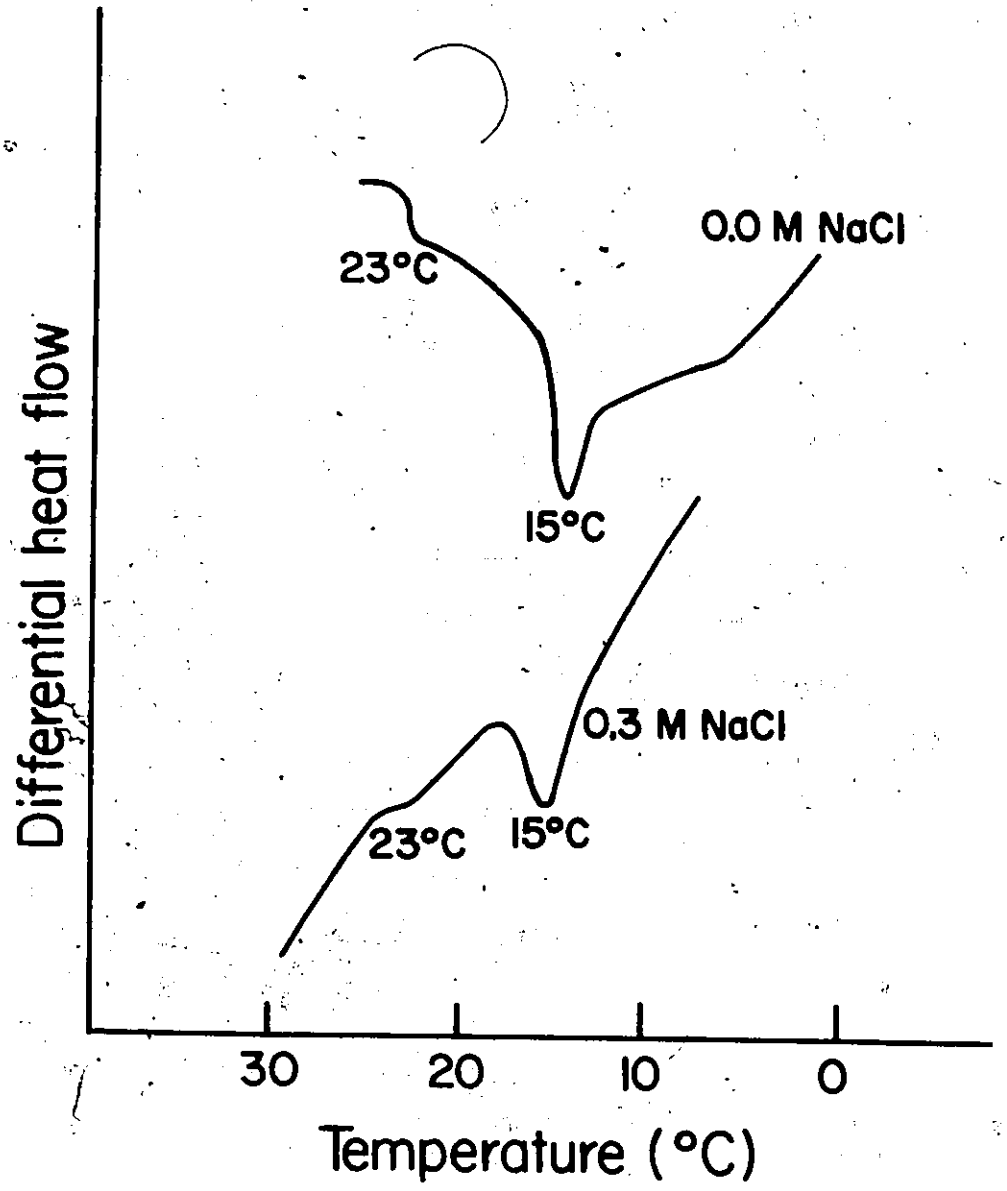

Differential heat flow
23°C
0.0 M NaCl
15°C
0.3 M NaCl
23°C
15°C
30
20
10
0
Temperature (°C)

Figure 18. Differential calorimeter scans of liposomes prepared from stationary phase phospholipids (cooling runs).

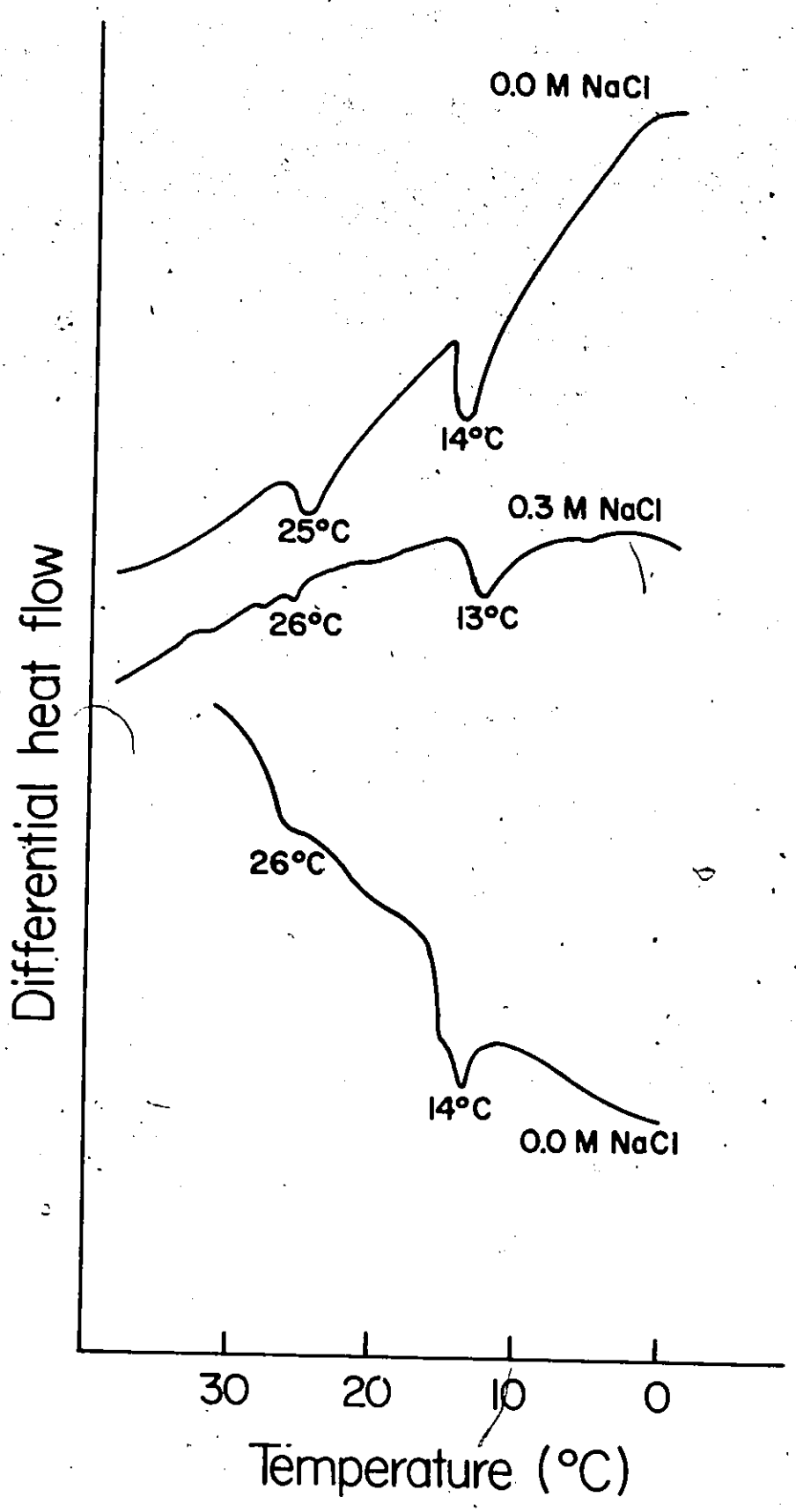

0.0 M NaCl
14°C
25°C
0.3 M NaCl
26°C
13°C
26°C
14°C
0.0 M NaCl
Differential heat flow
30
20
10
0
Temperature (°C)

Figure 19. Differential calorimeter scans of liposomes prepared from AW405 phospholipids (Heating runs).

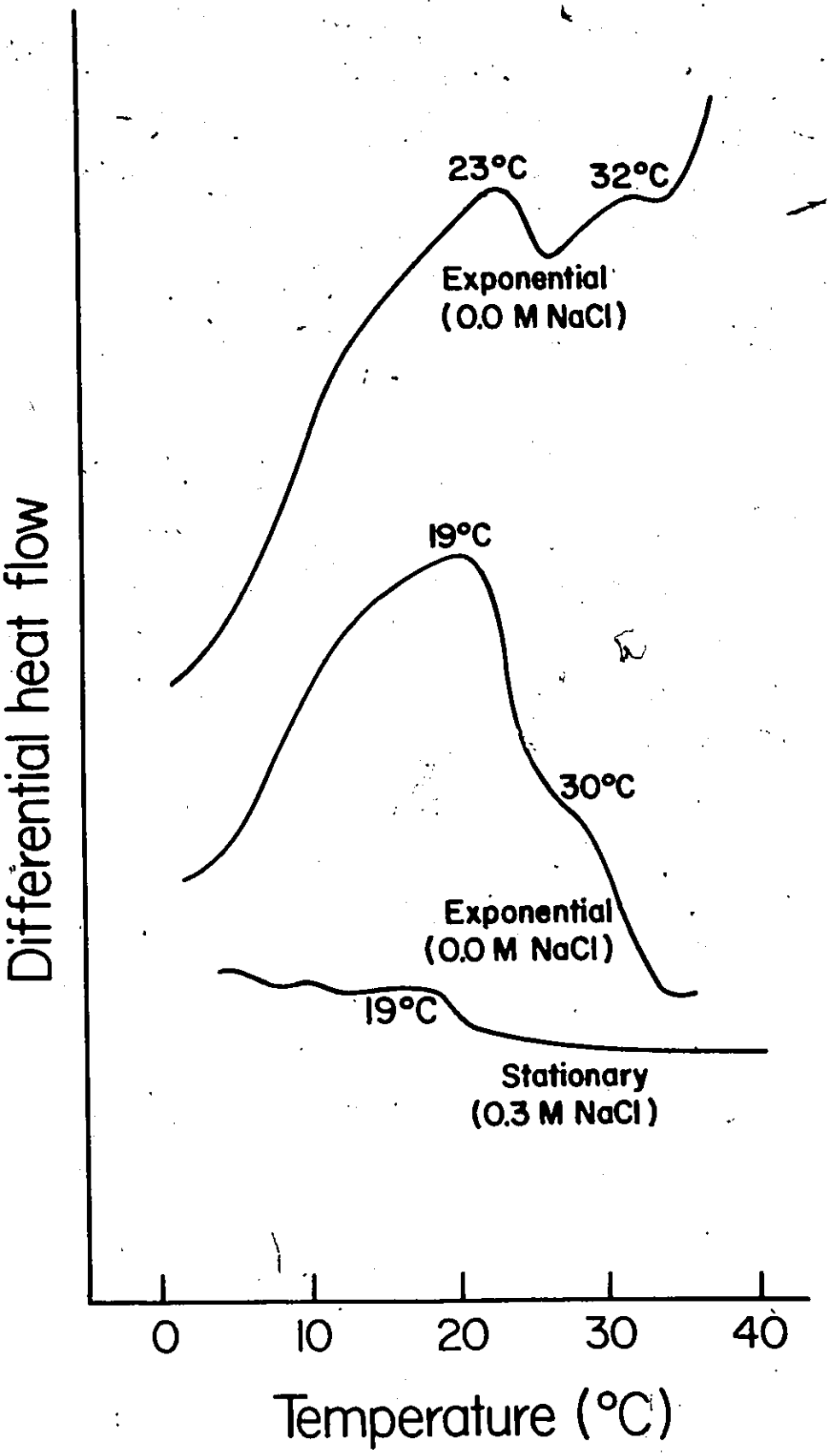

23°C
32°C
Exponential
(0.0 M NaCl)
19°C
30°C
Exponential
(0.0 M NaCl)
19°C
Stationary
(0.3 M NaCl)
Differential heat flow
0
10
20
30
40
Temperature (°C)

Figure 20. Differential calorimeter scan of a stationary phase phospholipid preparation from cells grown in the presence of 0.6 M sucrose (Cooling curve).

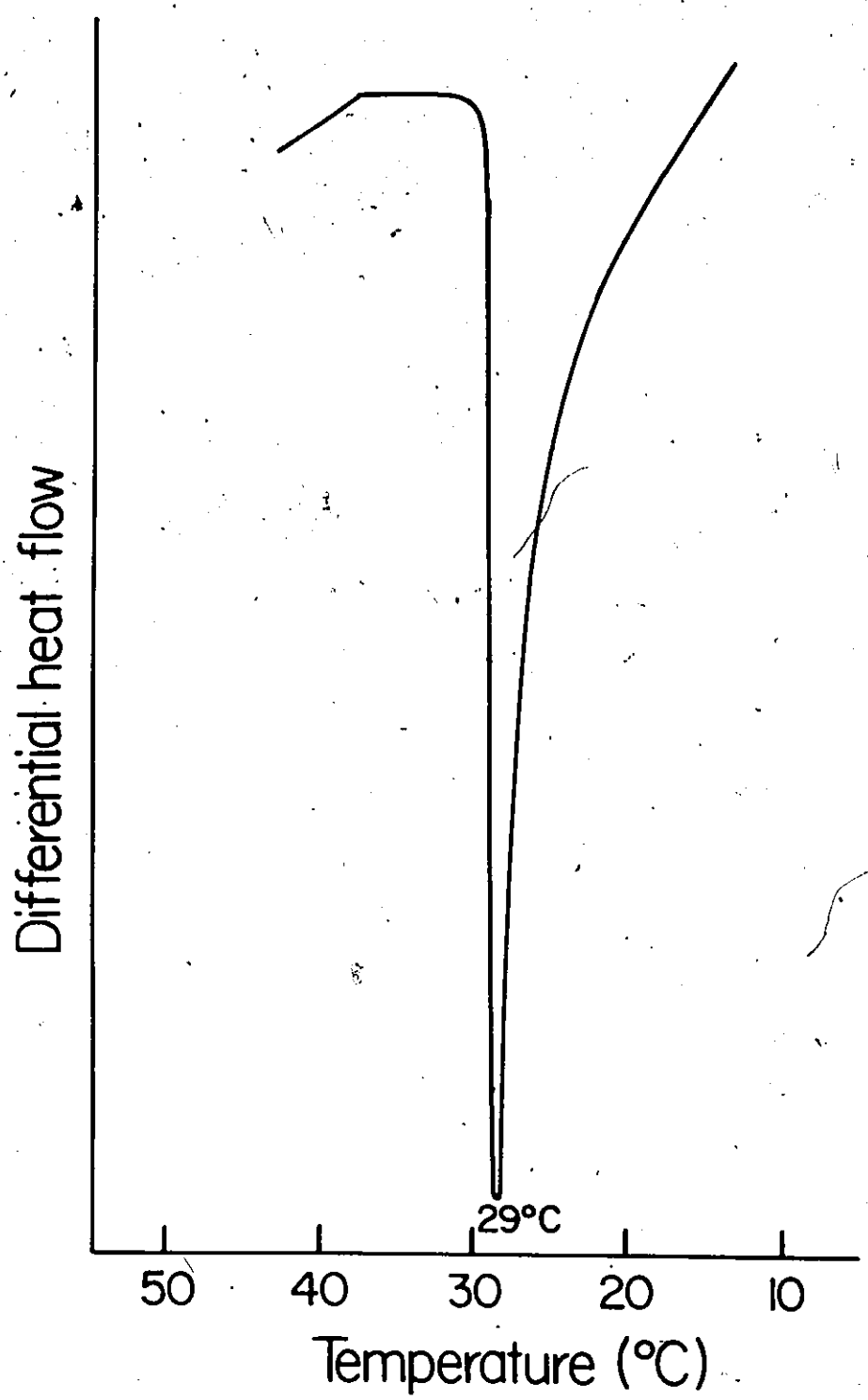
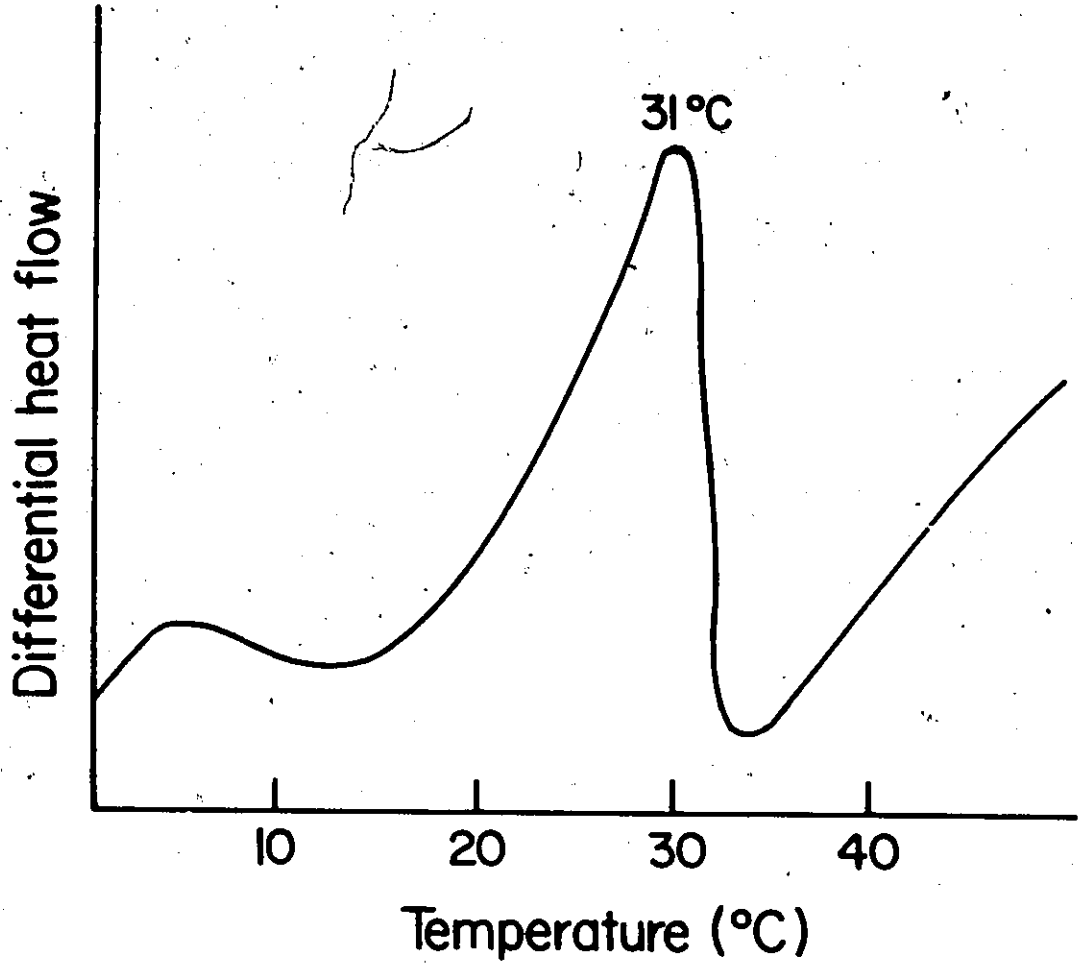

Differential heat flow
29°C
50
40
30
20
10
Temperature (°C)

Figure 21. Differential calorimeter scan of a stationary phase phospholipid preparation from cells grown in the presence of 0.6 M sucrose (Heating curve).


31°C
Differential heat flow
10
20
30
40
Temperature (°C)

4.00 Discussion

In our results, we have demonstrated that the changes in phospholipid fatty acid composition during growth of E. coli at high osmolality are qualitatively similar to those observed during growth at high temperature. Under both conditions there was a marked increase in the relative amounts of cyclopropane fatty acids and a decrease in saturated fatty acids. Lecithins acylated with cyclopropane fatty acids melted at higher temperatures than those containing their unsaturated fatty acid analogues. However, we were unable to demonstrate any difference in the physical properties of E. coli membranes due to substitution of cyclopropane fatty acids for unsaturated fatty acids in E. coli phospholipids.

4.1 Fatty Acid Regulation in Response to Osmolality and Temperature

The results have indicated that a rise in cyclopropane fatty acids in the phospholipids of AW405 is observed, with a decline in the combined percentage of lactobacillate and cis-vaccenate in stationary phase phospholipids, when cells were grown in media of high salt concentration (Section 3.4 to 3.5). The response is similar to that observed at elevated temperatures (Section 3.6). This implies a relationship between the cellular mechanisms regulating the unsaturated to saturated fatty acid levels in response to temperature and salt concentration. In both cases, the exponential phase phospholipid fatty acid compositions were similar (Section 3.5 to 3.6) but stationary phase phospholipids showed pronounced differences. Increased salt concentrations resulted in an increase in cyclopropane fatty acids and a reduction in unsaturated fatty acids. There was also a slight decrease in the combined percentage of unsaturated and cyclopropane fatty acids and a decrease in average chain length (Section 3.4 and 3.6).

Qualitatively similar results were obtained when cells were grown in the presence of NaCl, $MgCl_2$, KCl and sucrose so that the altered fatty acid compositions observed are likely a response to increasing osmolality (Section 3.5). The ratio of cyclopropane fatty acids to unsaturated fatty acids acylated to the phospholipids of bacterial membranes in stationary phase has been reported to increase under a number of growth limiting conditions (Section 1.8) but we were unable to find a direct correlation between the effect of osmolality and these growth-limiting factors (Section 3.7).

Several different metabolic sites have been suggested to control the proportions of the fatty acids acylated to E. coli phospholipids. For example, the unsaturated to saturated fatty acid ratio could be controlled in response to temperature at the level of fatty acid synthesis. In support of this proposal, the ratio of saturated to unsaturated fatty acids, synthesized in vitro or accumulated in vivo in cells blocked in phospholipid synthesis, was reported to increase with incubation temperature (Cronan, 1975 and Okuyama et al, 1977). Alternatively, the ratio could be controlled at the level of phospholipid synthesis. Cells, starved for glycerol phosphate, synthesized phospholipids with higher proportions of unsaturated fatty acids than their precursor free-fatty-acid pools (Cronan, 1975) and in vitro assays of the acyltransferase system demonstrated preferential incorporation of saturated fatty acids at higher temperatures (Sinensky, 1971).

Implied in the above control mechanisms is a simple relationship involving one or more thermolabile enzymes resulting in altered substrate specificity or enzyme activity. If such were the case, then the phospholipid fatty acid compositions of membranes in cells grown at different temperatures would consistently reflect the

temperature correlation. However, we found a less than 6% decrease in cis-vaccenate levels in exponential cells grown at 42°C compared to cells grown at 20°C, and very little difference between 20°C and 30°C exponential phospholipid fatty acid compositions (Section 3.6). In fact, the stationary cells grown at 20°C contained a smaller proportion of combined unsaturated and cyclopropane fatty acids, and more palmitic acid, than the exponential phase culture grown at 42°C. Kito et al (1972) reported similar results for a strain of E. coli although the relative amount of cis-vaccenate ranged from 9-13% over a 20-40°C temperature range among the three phospholipids. However, in both studies, large differences in saturated to combined unsaturated and cyclopropane fatty acid ratios were observed in stationary phase cell phospholipids over these temperature ranges (Section 3.6; Kito et al 1972).

Recently Stein and Block (1976) have demonstrated that B-hydroxy-decanoylthioester dehydrase, the enzyme responsible for introducing the double bond into unsaturated fatty acids (Cronan and Vagelos, 1972). is inhibited by the novel nucleotide, guanosine 5'-diphosphate-3' diphosphate (ppGpp). It is conceivable that inhibition of unsaturated fatty acid synthesis by elevated concentrations of this nucleotide could account for the lower unsaturated to saturated fatty acid proportion observed in E. coli phospholipids in late stages of growth. Increased concentrations of this nucleotide at late stages in growth of E. coli have actually been observed, and are thought to inhibit RNA synthesis as well as phospholipid synthesis (Cronan, 1978). Our results are compatable with this proposal, since decreased ratios of unsaturated to saturated fatty acids in E. coli phospholipids were observed when ppGpp concentrations were expected to be increased.

Such a control mechanism, dependent upon ppGpp inhibition of unsaturated fatty acid synthesis, seems more attractive to us than control by either of the thermo-regulatory modes suggested above, which are inconsistent with our data. This possibility should certainly be examined more closely.

4.2 Control of Cyclopropane Fatty Acid Levels

The final proportions of cyclopropane fatty acyl esters observed in the phospholipids of E. coli could be dependent upon cyclopropane fatty acid synthesis activity or S-adenosylmethionine synthesis. Cronan (1968) found consistently high cyclopropane fatty acid synthetase activity throughout growth in E. coli, but his study and others (Section 1.7) did not rule out the possibility that the enzyme could be activated by a low molecular weight regulator such as ppGpp. This latter possibility should be considered more closely since our results have shown that high levels of cyclopropane fatty acids in phospholipids are almost invariably found with high levels of saturated fatty acids (Sections 3.4-6), and the phospholipid unsaturated to saturated fatty acid ratio may be influenced by the nucleotide.

Intracellular concentrations of S-adenosylmethionine, the methyl donor of cyclopropane fatty acid synthetase, could be reduced by low S-adenosylmethionine synthetase activity or by low levels of its metabolic precursors, adenosine triphosphate and methionine. Holloway et al (1970) found that growth of E. coli in media concentrations of methionine as low as 10^{-5} M resulted in repression of S-adenosyl-methionine synthetase activity. A 10-12 fold decrease in enzyme activity was also observed by these authors when cells were grown in a complete medium (containing tryptone, yeast extract and salt) presumably due to exogenous methionine. Addition of chloramphenicol (40 µg/ml) stopped growth and prevented an increase of enzyme activity in repressed cells after removal of methionine. On this basis, the

authors speculated that protein synthesis is required for the derepression process.

Maximal cyclopropane fatty acid synthetase rates in *in vitro* assays were obtained at concentrations of S-adenosylmethionine in excess of 0.5 mM (Zalkin *et al* 1963), so that decreased enzyme activity would be expected at intracellular concentrations less than this level. Decreased S-adenosylmethionine concentrations, leading to lower rates of cyclopropane fatty acid synthesis, could result if S-adenosylmethionine synthetase activity was curtailed by methionine repression. Moreover, the growth-condition variations in phospholipid cyclopropane fatty acid concentrations could reflect the extent to which the enzyme is repressed.

In agreement with Holloway *et al* (1970), our results have shown that the S-adenosylmethionine synthetase activity of *E. coli* cells grown in tryptone broth is lower than that of cells grown in minimal medium (Section 3.8). Exposure of an early post exponential culture to chloramphenicol resulted in a lower cyclopropane to unsaturated fatty acid ratio in the phospholipids, which could indicate that derepression of S-adenosylmethionine synthetase is required for normal rates of cyclopropane fatty acid synthesis. However, measurement of the actual intracellular S-adenosylmethionine concentrations in cells grown under those conditions in which cyclopropane fatty acid synthesis rates have been reported to vary is required before any credence should be given to these proposals. Moreover, Cronan *et al* (1974) found little to no difference in the rates of cyclopropane fatty acid synthesis in several strains of *E. coli* with a 20 fold variation in S-adenosylmethionine synthetase activity.

S-adenosylmethionine levels, and hence rates of cyclopropane fatty acid synthesis, could also be dependent upon the intracellular

concentrations of the metabolic precursors, adenosine triphosphate and methionine. Preiss and co-workers (1966) have proposed that glycogen accumulation in stationary phase bacteria requires elevated levels of adenosine triphosphate. Consistent with this proposal, Dietzler et al (1974) found a 4.17 fold increase in glycogen synthesis was accompanied by a 50% increase in adenosine triphosphate levels at the cessation of growth in an E. coli culture starved for nitrogen. If glycogen accumulation can be used as an indicator, high adenosine triphosphate levels would be expected if growth is limited by phosphate, sulfate or nitrogen starvation, or low pH; but low concentrations would be expected if the carbon source is limiting (Dietzler 1974). With the exception of sulfate starvation, glycogen and cyclopropane fatty acid accumulation are observed in cells grown under the same growth limiting conditions (Section 1.8). If sulfate limitation results in methionine depletion in post exponential cultures, one could argue that cyclopropane fatty acid accumulation is dependent upon methionine and adenosine triphosphate synthesis, although considerably more evidence would be required to prove it. It would be interesting to examine what effect other growth conditions known to affect cyclopropane fatty acid synthesis (such as magnesium limitation, high citrate concentration, temperature and osmolality) would have on glycogen accumulation.

4.3 The Role of Cyclopropane Fatty Acids in E. coli

The physico chemical properties of E. coli membranes are thought to be attenuated through changes in the relative amounts of unsaturated and saturated fatty acids in the membrane phospholipids (Cronan and Gelmann, 1975) but the role of cyclopropane fatty acids remained obscure. Law et al (1963) suggested that they protect unsaturated sites from oxidation, yet cyclopropane fatty acid production is enhanced by anaerobiosis (Knivett and Cullen, 1965). Cronan (1968) first suggested

that cyclopropane fatty acids might be protected from degradation due to a reduced rate of turnover but later, Cronan (1974) showed that this is not the case. To date, it has not been established whether the contribution of cyclopropane fatty acids to membrane fluidity more nearly resemble that of unsaturated or saturated fatty acids. Several workers (Marr and Ingraham, 1962; Kito et al 1972; Broeckman and Steenbakers, 1974) have grouped unsaturated and cyclopropane fatty acids together in their considerations.

Since the changes in fatty acid composition, in response to growth at elevated temperatures, are thought to increase membrane melting temperatures, it was suggested that substitution of cyclopropane fatty acids for unsaturated fatty acids could have a similar effect (Section 1.9). Measurement of the melting temperatures of synthetic lecithins acylated with the cyclopropane fatty acids, lactobacillate and dihydrosterculate revealed that these phospholipids melted at temperatures 16°C higher than lecithins acylated with their unsaturated fatty acid analogues, oleate and cis-vaccenate (Section 3.10). Therefore, substitution of cyclopropane fatty acids for unsaturated fatty acids in E. coli membrane phospholipids should tend to increase their melting temperatures and hence their fluidity. Since the fluidity of the membrane has been shown to influence the activity of a wide variety of membrane processes (Sandermann, 1978), the methylation of unsaturated fatty acids may have a regulatory role in bacterial membranes attenuating the fluidity and therefore the activity of membrane functions in stationary phase.

4.4 Transition Profiles of E. coli Membranes During Growth

The physico chemical properties of bacterial membranes are thought

to be maintained such that the lipid bilayers are sufficiently fluid to permit the movement of integral proteins and yet "solid" enough to maintain a structural role as an osmotic barrier (Esfahani et al, 1971). This theory which is based on the "fluid mosaic" model of Singer and Nicolson (1972), has been used to explain the preferential incorporation of higher melting point fatty acids in cells growing at elevated temperatures and in unsaturated fatty acid auxotrophs growing on low melting point fatty acids (Cronan and Vagelos, 1972; Esfahani et al, 1969, 1971). Due to the similarity of the changes in fatty acid composition in response to growth at high temperatures and osmolalities, the melting characteristics of E. coli phospholipids from cells grown in media varying in osmolality were examined (Section 3.9).

Unfortunately, an analysis of the membrane phase transition data, reported in Section 3.9, in terms of fatty acid composition, is difficult. The problems arise from the variability of the results and the lack of a supportive data base on the physical properties of artificial and natural membranes with highly heterogeneous fatty acid compositions. Most work on the melting characteristics of artificial membranes has focused on systems composed of as few as one or two lecithin species (Ladbrooke and Chapman 1969; Melchior and Steim 1976). Similarly, most work on E. coli membranes has involved unsaturated fatty acid auxotrophs with simplified fatty acid compositions in which one to two fatty acids predominated (Overath et al 1970; Nishihara et al 1975; Overath and Träuble 1973; Träuble and Overath 1973). Hence, extrapolation of these results to the present system, with three or more major fatty acid components distributed among three phospholipids, is tenuous.

Ladbrooke and Chapman (1969) formulated a number of empirical rules based upon lipid phase transition data. Lipids acylated with unsubstituted saturated fatty acids have higher melting points than those with unsaturated, cyclopropane or branched chain fatty acids of the same chain length. Melting points are further decreased by increased numbers of branch points, unsaturated sites or cyclopropane rings along the length of the fatty acid chains. Acylation with longer chained length but otherwise homologous fatty acids results in lipids with higher transition temperatures. Lipids acylated with trans-unsaturated fatty acids have melting points intermediate between homologous saturated and cis-unsaturated fatty acids. Heterogeneity of fatty acid composition results in lipids with broad transitions and, in extreme cases, phase separation resulting in more than one transition temperature.

The occurrence of phase separation in heterogeneous phospholipid mixtures is also dependent upon the distribution of the fatty acids within the phospholipid molecules as well as the melting temperatures of the fatty acids. For example, Phillips et al (1972) reported that dioleoyl, 1-stearoyl-2-oleoyl and distearoyl lecithins melted with single sharp transitions at -22, 3 and 58°C respectively. However, two transitions, indicating lateral phase separation, were observed at -22°C and from 30-53°C for an equimolar mixture of dioleoyl and distearoyl lecithins.

Some indication of the fatty acyl arrangement of the major phospholipid species in E. coli can be obtained on the basis of the phospholipid fatty acid composition and the results of studies on the positional specificity of E. coli acyltransferases. According

to these studies (Van Deenan 1965; Cronan and Gelmann 1975; Sinensky 1971; Nishihara et al 1975; Silbert 1970), palmitic and palmitoleic acids are found almost exclusively in the one and two positions (Figure 1) respectively, whereas myristic and cis-vaccenic acids have broad positional specificity (found to both positions). Therefore, both monounsaturated and di-unsaturated phospholipid species would be expected in early exponential phase cultures (Table 17). Accordingly, the mono-unsaturated species would make up about 60% of the phospholipids and these would have palmitate at position one and either cis-vaccenate or palmitoleate at position two. The di-unsaturated species, making up as much as 30% of the total would consist of cis-vaccenate at position one and either cis-vaccenate or palmitoleate at position two. On the other hand, late stationary phase cultures, grown in 0.6 M sucrose, would contain very little di-unsaturated phospholipid (Table 18). About 90% of the phospholipid species would consist of palmitate at position one and cis-9,10-methylene hexadecanoate or lactobacillate at position 2.

The phase transition behaviour, reported in Section 3.9, for E. coli phospholipids can then be rationalized according to the above analysis framework. The single broad transition of 25.6 ± 3.6°C observed for exponential and early stationary phase cell phospholipids could be due to melting of the membrane as a single unit. The broadness of the response and the incremental difference in fluorescence observed are consistent with artificial membrane studies on heterogeneous systems (Ladbrooke and Chapman 1969). Both NPN fluorescence and differential calorimetry revealed double transition profiles for the phospholipids of cells grown from exponential to

stationary phase. The two melting temperatures may indicate partial phase separation of phospholipid patches in the membranes which are rich and poor in di-unsaturated fatty acyl phospholipids. The single upper temperature transitions observed in differential calorimetry and NPN fluorescence studies could correspond to a more extensive phase separation in which the lower transition would appear at temperatures lower than the measured range of these studies. Alternatively, in the case of phospholipids from cultures grown in 0.6 M sucrose, the lower transition may have disappeared due to the absence of di-unsaturated phospholipid species.

If the above analysis is essentially correct, then the physico-chemical properties of E. coli membranes likely change substantially during growth. Late stationary phase E. coli membranes would have much more crystalline character than early exponential membranes due to the loss of di-unsaturated phospholipids. Methylation of the remaining unsaturated fatty acids resulting in phospholipids rich in higher melting cyclopropane fatty acids would likely further increase their crystalline character.

Numerous studies have reported the effects of membrane fluidity on membrane functions in E. coli. Many of these have been extensively reviewed (Cronan and Gelmann 1975; Cronan 1978; Sandermann 1978). In general, a more crystalline (less fluid) membrane is expected to result in decreased permeability to salts; decreased transport of lactose, proline and other nutrients; decreased rate of growth and increased resistance to lysis in E. coli. These characteristics could be advantageous in stationary phase where the preservation of cell integrity, energy and metabolic pools could confer a selective advantage.

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