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# Unsaturated Fatty Acid Requirement in *Escherichia coli*: Mechanism of Palmitate-Induced Inhibition of Growth of Strain WN1

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**Summary.** The minimum requirement for unsaturated fatty acids was investigated in *E. coli* using a mutant impaired in the synthesis of vaccenic acid. Exogenously supplied palmitic acid was incorporated by this mutant which led to a reduction in the proportion of cellular unsaturated fatty acids. Growth was impaired as the level of saturated fatty acids approached 76% at 37 °C and 60% at 30 °C. The basis of this growth inhibition was investigated. Most transport systems and enzymes examined remained active in palmitate-grown cells although the specific activities of glutamate uptake and succinic dehydrogenase were depressed 50%. Fluorescent probes of membrane organization indicated that fluidity decreased with palmitate incorportation. Temperature scans with parinaric acid indicated that rigid lipid domains exist in palmitate-grown cells at their respective growth temperature. Freeze-fracture electron microscopy confirmed the presence of phase separations (particle-free areas) in palmitate-grown cells held at their growth temperature prior to quenching. The extent of this separation into particle-free and particle-enriched domains was equivalent to that induced by a shift to 0 °C in control cells. The incorporation of palmitate increased nucleotide leakage over threefold. The cytoplasmic enzyme β-galactosidase was released into the surrounding medium as the concentration of unsaturated fatty acid approached the minimum for a particular growth temperature. Lysis was observed as a decrease in turbidity when cells which had been grown with palmitate were shifted to a lower growth temperature. From these results we propose that leakage and partial lysis are the major factors contributing to the apparent decrease in growth rate caused by the excessive incorporation of palmitate. Further, we propose that membrane integrity may determine the minimum requirement for unsaturated fatty acids in *E. coli* rather than a specific effect on membrane transport and/or membrane-bound enzymes.

**Key words** lipids · membranes · *Escherichia coli* · temperature adaptation · fatty acids · phase separations

## Introduction

Both eukaryotic and prokaryotic cells are known to alter their lipid composition in response to growth temperature [5, 11, 12]. These changes are particularly well-documented in the bacterium *Escherichia coli* [3, 5, 19, 23]. In *E. coli* a mixture of saturated and unsaturated fatty acids is normally synthesized and assembled to form four major molecular species with respect to fatty acid arrangement (16:0+16:1, 16:0+18:1, 18:1+16:1, 18:1+18:1) which constitute over 95% of the total lipids [3, 29]. A balanced mixture of these molecular species is synthesized during growth. Excessive levels of molecular species containing two *cis*-18:1 acyl chains have been shown to inhibit growth, increase cellular permeability, and to facilitate the inactivation of several membrane-bound enzymes [2]. At the other extreme, starvation for unsaturated acyl chains also leads to growth inhibition and ultimately cellular death [7, 14, 18]. Under these starvation conditions, the minimum requirement for unsaturated fatty acids appears to be 15% [6]. However, the possible simultaneous inhibition of phospholipid synthesis by the limited availability of acyl chains is also inherent in this starvation approach [14].

In this study, we have examined the minimum requirement for unsaturated fatty acids using a *fad* F mutant [13] of *E. coli* which synthesizes both saturated and unsaturated fatty acids. With this strain, exogenously supplied palmitic acid was incorporated to high levels in membrane lipids leading to an impairment of growth and membrane function.

## Materials and Methods

### *Strains and Growth Conditions*

Two strains of *E. coli* K-12 were used in this study; strain WN1 (*fad* F), which is defective in the synthesis of *cis*-vaccenic acid [13, 25], and strain NL1, a *lac* $i^-$ transductant of strain WN1 which was prepared using bacteriophage Plvir as previously described [4]. Cells were grown in a reciprocating water bath at either 30 or 37 °C in modified Luria broth [21; tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter; Brij 58, 1 g/liter; no carbohydrate added]. Fatty acids (20 mg/liter were added where indicated. Growth was monitored by measuring optical density at 550 nm using a Bausch and Lomb Spectronic 70 spectrophotometer. Strain NL1 was used exclusively in experiments involving *lac* permease or β-galactosidase leakage.

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### *Enzyme Analysis*

Cells were grown following a 1:250 dilution of an overnight culture into fresh medium and were harvested during exponential growth (OD 0.6). These were washed in Tris-HCl (0.01 M, pH 7.8) and broken using an ultrasonic disruptor (Heat Systems, Inc., Plainview, New York). Total membranes were collected by centrifugation ($100{,}000 \times g$, 1 hr) and used for enzyme assays on the day of preparation. Colorimetric assays for NADH oxidase, succinic dehydrogenase and D-lactate oxidase were performed at 37 °C as described by Osborn et al. [26] using a water-jacketed cuvette and a Beckman model 53 spectrophotometer. NADH oxidase and succinic dehydrogenase assays were also performed by monitoring $O_2$ consumption using a Gilson microelectrode chamber and a Yellow Springs Instruments (Yellow Springs, Ohio) $O_2$-monitor equipped with a Clarke-type electrode as described by Baldassare et al. [2]. The electrode chamber was maintained at 37 °C using a Haake circulator. ATPase activities with $Ca^{++}$ (10 mM) and $Mg^{++}$ (10 mM) were determined as described by Evans [10] at 37 °C. Total β-galactosidase activity was measured as described by Sullivan et al. [32]. Protein was measured by the method of Lowry et al. [20]. Activities were reported as μM/mg protein per min.

### *Transport Studies*

For transport studies, overnight cultures were diluted into fresh medium with detergent alone (control) or with detergent and palmitate. Cells were harvested at OD of 0.6 by centrifugation ($7{,}000 \times g$, 5 min), washed three times with buffer (50 mM $K_2HPO_4$, 0.5 mM $MgCl_2$, 300 mg/ml chloramphenicol H, 6.9) and resuspended to the original volume in this buffer containing 3% glycerol and 50 mM NaCl. Cells were preincubated for 10 min at 37 °C. Transport assays were initiated by the addition of radiochemicals (final concentration: L-[$U^{14}C$] glutamate, 20 μM, 2 μCi/μM; L-[$U^{14}C$] proline, 20 μM, 2 μCi/μM; or L-[$U^{3}H$]-leucine, 100 μM, 4 μCi/μM). Duplicate samples (0.5 ml each) were removed at 20-sec intervals, harvested by vacuum filtration on cellulose nitrate filters, and washed with 4 ml of 0.1 M $LiCl_2$. All filters were pretreated with buffer containing 50 mM amino acid. Filters with cells were dried and counted using a Beckman scintillation counter.

The *lac* permease and cryptic transport of β-galactosidase were measured using strain NL1. Cells were grown to an OD of 0.4, washed twice by centrifugation and resuspended in fresh medium. Cells were assayed *in vivo* under growth conditions by the addition of *o*-nitrophenol-β-D-galactopyranoside (2.0 mM) for total uptake. Cryptic transport was measured using 2.0 mM thiodigalactoside [11, 32]. Uptake was terminated by the addition of 2 volumes of 0.6 M sodium carbonate. Absorbance was measured at 420 nm and permease activity was computed as the difference between these two activities.

### *Lipid Analysis*

Batch cultures (50 ml) were harvested by centrifugation, washed once with fresh Luria broth containing detergent and twice with 0.01 M Tris-HCl buffer (pH 7.8). Cells were inactivated by resuspension in methanol (4 ml) followed by incubation at 45 °C for 15 min. After cooling, chloroform (8 ml) was added and the cells were extracted overnight. The lipid extract was washed and analyzed for total fatty acid composition and for the positional distribution of fatty acids as previously described [3, 15, 29]. For phospholipid analysis, cells were grown for 5 generations with 0.8 μCi/ml of added $^{32}PO_4^{2-}$. Phospholipid components were separated by thin-layer chromatography, scraped and counted [16].

### *Fluorescence Depolarization*

The fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a comparative measure of the fluidity of membranes [9, 17]. Membranes were prepared by the freeze/thaw method of DiRienzo and Inouye [8]. Membranes were suspended in Tris-HCl buffer (0.01 M, pH 7.8) at a final concentration of 50 μg/ml of protein containing DPH at a final concentration of $1 \times 10^{-6}$ M. Protein was measured by the method of Lowry et al. [20]. Polarization was measured using a SLM series 4,000 polarization fluorimeter (Urbana, Ill.). Temperature was controlled to within 0.05 °C using a Neslab refrigerated circulator and monitored within the sample cuvette [9]. Both *cis*-parinaric acid and *trans*-parinaric acid were employed exactly as described for DHP except that the excitation wavelength was 323 nm. Melting profiles of membranes containing parinaric acid were prepared using a Houston Instrument (Houston, Texas) X-Y recorder to continuously plot $I_{\parallel}/I_{\perp}$ as a function of cuvette temperature (2 to 43 °C, 1.5 °C per min). The output ratio was initially adjusted to 0.4 at 43 °C with membranes from detergent-grown cells. Membranes from cells grown with palmitic acid were examined without readjustment. Heating profiles appeared similar to cooling profiles. Only cooling profiles are presented.

### *Electron Microscopy*

Strain WN1 was grown with detergent alone and detergent plus palmitic acids. Cells were grown for 4 to 5 generations and harvested by centrifugation at room temperature. The pellet was mounted, held for 1 hr at the indicated temperature and rapidly frozen using a propane jet freezer like that described by Mueller et al. [24]. Specimen carriers and other tools used to handle cells were also maintained at the experimental temperature until the instant of freezing. This method eliminated the need for fixation and cryoprotection agents. Freeze-fracturing was accomplished using a Balzers freeze-etch device and were examined with a JEOL 100 C-X electron microscope.

### *Leakage*

For measurements of nucleotide leakage, cells were harvested at 0.6 OD, washed twice at ambient temperature in 0.1 M phosphate buffer (pH 7.2) and resuspended to their original volume in buffer. This suspension was incubated at 37 °C in a reciprocating water bath. Samples (2 ml) were taken at 15-min intervals and the cells removed by centrifugation ($7{,}000 \times g$, 3 min). Nucleotides were measured as absorbance at 262 nm, in the supernatant. Spectra of released material were very similar to those of adenine nucleotides. No obvious protein shoulder was present. Total nucleotides were released from cells by the addition of 0.2 ml of 70% perchloric acid followed by incubation at 37 °C for 1 hr. Leakage was expressed as the percentage of total cellular nucleotides.

β-galactosidase leakage into the growth medium was monitored by removing samples at various times during growth. Cells were removed by centrifugation ($10{,}000 \times g$, 5 min). Supernatants were stored in ice and assayed together for β-galactosidase activity. Results are expressed as an increase in $A_{420}$ per min per ml of culture media.

### *Chemicals*

Brij 58, Tris buffer, biochemicals and all fatty acids were obtained from the Sigma Chemical Company (St. Louis, Mo.). DPH was obtained from the Aldrich Chemical Company (Milwaukee, Wisc.). Parinaric acid was obtained from Molecular Probes, Inc. (Plano, Texas). Radiochemicals were obtained from the Amersham Corporation (Arlington Heights, Ill.).

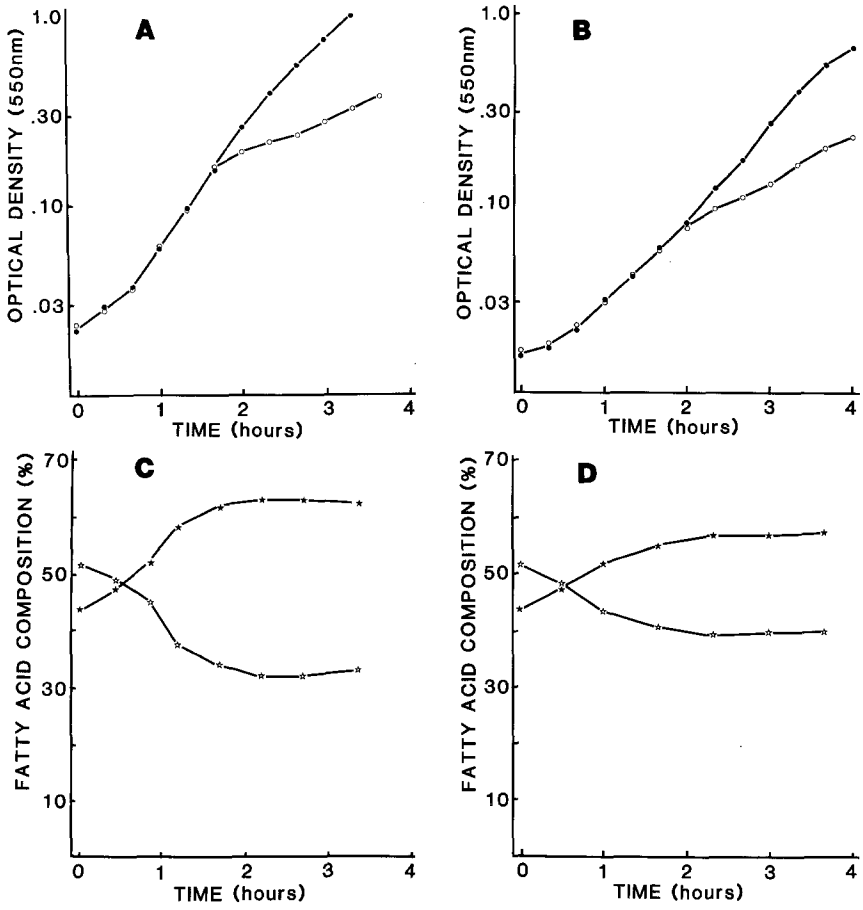


**Fig. 1.** Effects of palmitic acid on growth and lipid composition. An overnight culture at each temperature was diluted into fresh medium and split into 2 parts. Palmitate plus detergent was added to one and detergent alone to the other (control). Optical density was monitored and samples were removed for fatty acid analysis. The fatty acid compositions of the cells containing detergent alone remained relatively constant and are not shown. (*A*) Growth at 37 °C; (*B*) growth at 30 °C; (*C*) fatty acid composition of cells grown at 37 °C with palmitate; (*D*) fatty acid composition of cells grown at 30 °C with palmitate. Symbols: ● detergent alone; ○ detergent plus palmitate; ✶ saturated fatty acids (predominantly palmitate); ☆ unsaturated fatty acids

## Results

### *Effects of Exogenous Palmitic Acid on Growth and Lipid Composition*

The addition of palmitic acid, a natural constituent of *E. coli* phospholipids, to cultures of strain WN1 at either 30 or 37 °C resulted in a partial inhibition of growth which began after approximately three mass doublings (Fig. 1, *A* and *B*). During this initial growth period, the level of saturated fatty acids present in cellular membranes increased reaching a maximum near the time of onset of inhibition (Fig. 1, *C* and *D*; Table 1).

Cells which were grown at 37 °C with palmitate achieved higher levels of palmitic acid incorporation (72%) than did cells grown at 30 °C (56%). When cells which had been grown at 37 °C for three generations with exogenous palmitic acid were shifted to 30 °C, the inhibition of growth was exaggerated and was accompanied by partial lysis (Fig. 2). Similar results were obtained when cells grown at 30 °C with palmitate were shifted to 23 °C (*not shown*). In both cases, growth resumed when cells were returned to the original temperature prior to this lysis. Thus, the levels of palmitic acid incorporated at each respective temperature approach the maximum levels tolerated at that temperature. The effects of other fatty acids which are natural constituents of *E. coli* lipids (myristic, palmitoleic, vaccenic) were also examined. Although readily incorporated (Table 1), none of these fatty acids were found to inhibit growth at 37 °C. The inclusion of vaccenic acid in combination with palmitic acid prevented growth inhibition.

The positional distribution of acyl chains was compared in lipids from strain WN1 grown with and without palmitic acid (Table 2). Following growth in the absence of supplemental palmitic acid, 16:0 and 16:1 were present primarily at the 1 and 2 positions, respectively. However, following growth in the presence of palmitic acid, 16:0 was found in both the 1 and 2 positions. The results indicated that during growth in the absence of supplemental palmitic acid, the bulk of the phospholipid molecules in strain WN1 were identical in their acyl chain arrangement and composition (1-palmitoyl-2-palmitoleoyl diglycerides). However, following growth with supplemental palmitic acid, a second major phosphoglyceride species was also particularly abundant in cells grown at 37 °C, 1,2-dipalmitoyl diglyceride. Despite the changes

**Table 1.** Fatty acid composition of strain WN1 grown with various acyl supplements

| Growth conditions | | Composition (%)[a, b] | | | |
|---|---|---|---|---|---|
| Supplement | Temperature (°C) | 14:0 | 16:0 | 16:1 + Δ17 | 18:1 |
| Detergent control | 37 | 9 | 44 | 46 | 0 |
| Myristic | 37 | 25 | 33 | 39 | 0 |
| Palmitic | 37 | 5 | 72 | 24 | 0 |
| Palmitoleic | 37 | 12 | 36 | 52 | 0 |
| Vaccenic | 37 | 9 | 24 | 27 | 40 |
| Detergent control | 30 | 6 | 41 | 53 | 0 |
| Palmitic | 30 | 3 | 57 | 40 | 0 |
| Palmitoleic | 30 | 12 | 30 | 58 | 0 |
| Vaccenic | 30 | 7 | 33 | 28 | 41 |

[a] Cells were grown for 4 to 5 generations with the indicated supplements. Results represent an average of duplicate determinations.

[b] Abbreviations: 14:0, myristic acid; 16:0, palmitic acid; 16:1 + Δ17, palmitoleic acid plus *cis*-9,10-methylene hexadecenoic acid; 18:1, vaccenic acid.

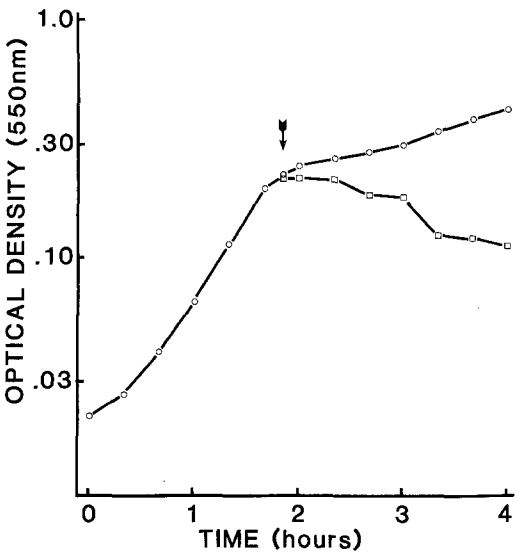


**Fig. 2.** Effect of a temperature shift-down on palmitate-grown cells. An overnight culture was diluted into fresh medium containing detergent plus palmitate and incubated at 37 °C. This culture was split into 2 parts after 2 hr (arrow) and half was incubated at 30 °C while the other half served as a control at 37 °C. Symbols: ○ cells growing at 37 °C; □ cells shifted at arrow from 37 to 30 °C

in acyl chain composition which occurred during growth with palmitic acid, phospholipid composition was unaffected (phosphatidylethanolamine 84%; phosphatidylglycerol) 8%; and cardiolipin 8%).

*Bulk Membrane Fluidity*

The relative fluidity of control (detergent-grown) and palmitate-grown cells was compared using DPH

**Table 2.** Positional distribution of acyl chains

| Growth conditions | | Position | Composition (%)[a, b] | | |
|---|---|---|---|---|---|
| Supplement | Temperature (°C) | | 14:0 | 16:0 | 16:1 + Δ17 |
| Detergent control | 30 | 1 | 10 | 85 | 5 |
| | | 2 | 3 | 1 | 96 |
| Detergent control | 37 | 1 | 9 | 89 | 3 |
| | | 2 | 7 | 9 | 85 |
| Palmitate | 30 | 1 | 3 | 97 | 0 |
| | | 2 | 4 | 15 | 81 |
| Palmitate | 37 | 1 | 2 | 98 | 0 |
| | | 2 | 6 | 38 | 56 |

[a] Results represent an average of duplicate determinations.

[b] Abbreviations of fatty acids are as in Table 1.

**Table 3.** Comparison of relative membrane fluidity (polarization)

| Growth conditions | | Polarization[a, b, c] | |
|---|---|---|---|
| Supplements | Temperature (°C) | 37 °C | 30 °C |
| Detergent alone | 30 | 0.279 ± 0.002 | 0.309 ± 0.002 |
| Detergent + palmitic acid | 30 | 0.318 ± 0.001 | 0.347 ± 0.001 |
| Detergent alone | 37 | 0.290 ± 0.001 | N.D. |
| Detergent + palmitic acid | 37 | 0.337 ± 0.001 | N.D. |

[a] Assay temperatures, 37 and 30 °C.

[b] Values represent average ± standard deviation of a minimum of three separate determinations. Each determination represented an average of 10 measurements.

[c] $\text{Polarization} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$.

(Table 3). The polarization of isolated membranes was higher (more rigid) when assayed at 30 °C than when assayed at 37 °C. Membranes from control cells grown at 37 °C were more rigid (higher polarization) than membranes from cells grown at 30 °C, consistent with the increased abundance of saturated fatty acids in cells grown at 37 °C (Table 1). Membranes from palmitate-grown cells were more rigid than those from control cells. Cells grown at 37 °C with palmitate contained more rigid membranes than those grown with palmitate at 30 °C, again reflecting the differences in acyl chain composition (Table 1). The increase in polarization caused by growth with exogenous palmitate was approximately equivalent to the increase in polarization caused by a 9 °C descrease in assay temperature for cells grown at 30 °C and 11 °C for cells grown at 37 °C.

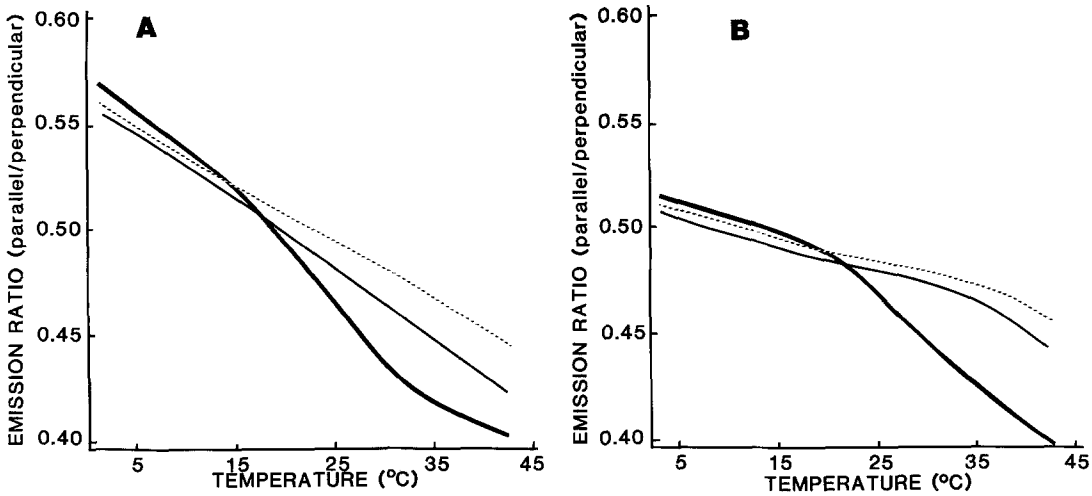


**Fig. 3.** Fluorescence depolarization of membranes from control and palmitate-grown cells. Relative changes in fluorescence depolarization are plotted as the emission ratio (parallel/perpendicular). Cells were harvested after 4–5 generations of growth with either detergent (control) or detergent plus palmitic acid. Temperature scans of fluorescence depolarization were determined using *cis*- and *trans*-parinaric acids as probes. (*A*) *cis*-parinaric; (*B*) *trans*-parinaric acid. Symbols: heavy line, control cells grown at 37 °C; thin solid line, cells grown at 30 °C with palmitic acid; broken line, cells grown at 37 °C with palmitic acid

Phase transitions in membranes from control and palmitate-grown cells were examined using either *cis*- or *trans*-parinaric acid (Fig. 3). These probes differ in that *cis*-parinaric acid partitions into both solid and fluid domains [31] and reflects bulk membrane properties, as does DPH. *Trans*-parinaric acid preferentially segregates into rigid membrane domains [28, 31]. The ratio $I_{\parallel}/I_{\perp}$ was used to monitor changes in relative probe mobility. This value is not equal to polarization but reflects the same trend [28]. The trends observed with membranes from cells grown with detergent at both 30 and 37 °C were similar (Fig. 3). With *cis*-parinaric acid two discontinuities were observed. These discontinuities occurred at approximately 36 and 11 °C and corresponded to the onset and completion of an organizational transition within the membrane. With *trans*-parinaric acid, less defined changes were observed with an onset near 40 °C and completion near 20 °C. Membranes from cells grown with palmitic acid were quite different. The onset of the phase transitions with both *cis*- and *trans*-parinaric acid occurred above 43 °C. Based upon a comparison of the $I_{\parallel}/I_{\perp}$ ratios, the transitions reflected by the *cis* probe appeared to be 15% and 30% completed at 43 °C in membranes from cells grown with palmitate at 30 and 37 °C, respectively. Similarly, the transition reflected by the *trans* probe appeared to be over 60 and 75% completed at 43 °C in membranes from cells grown with palmitate at 30 and 37 °C, respectively. These results suggest that substantial phase separations may exist in the membranes of palmitate-grown cells at their respective growth temperatures.

*Membrane Organization*

The gross organization of the plasma membranes from control (detergent-grown) cells and palmitate-grown cells was examined by freeze-fracture electron microscopy (Fig. 4). In control cells grown at 37 °C and held at 37 °C prior to freezing, intramembranous particles appeared as a reticulate pattern which uniformly covered the surface (Fig. 4*A*). Although not shown, control cells grown at 30 °C and frozen from this temperature appeared identical. When control cells were held at 0 °C for 1 hr prior to rapid freezing, large particle-free patches were observed, indicative of a phase separation (Fig. 4*B*). In contrast, membranes from cells grown with palmitic acid at 30 and at 37 °C exhibited large particle-free patches even when frozen from their respective growth temperature (Fig. 4, *C* and *D*). The size of these patches was further increased by holding at 0 °C prior to rapid freezing (*not shown*). Thus, phase separations appear to be present in the membranes of strain WN1 during growth in the presence of palmitic acid, consistent with our fluorescence results with *cis*- and *trans*-parinaric acid.

*Effects of Palmitate Incorporation on Membrane-Bound Enzymes and Active Transport*

Both active transport and the activities of a variety of membrane-bound enzymes were examined in control and palmitate-grown cells (Tables 4 and 5). Palmitate-grown cells were as efficient in concentrating leucine and proline as the control cells. NADH oxidase activities (colorimetric and polarigraphic), suc-

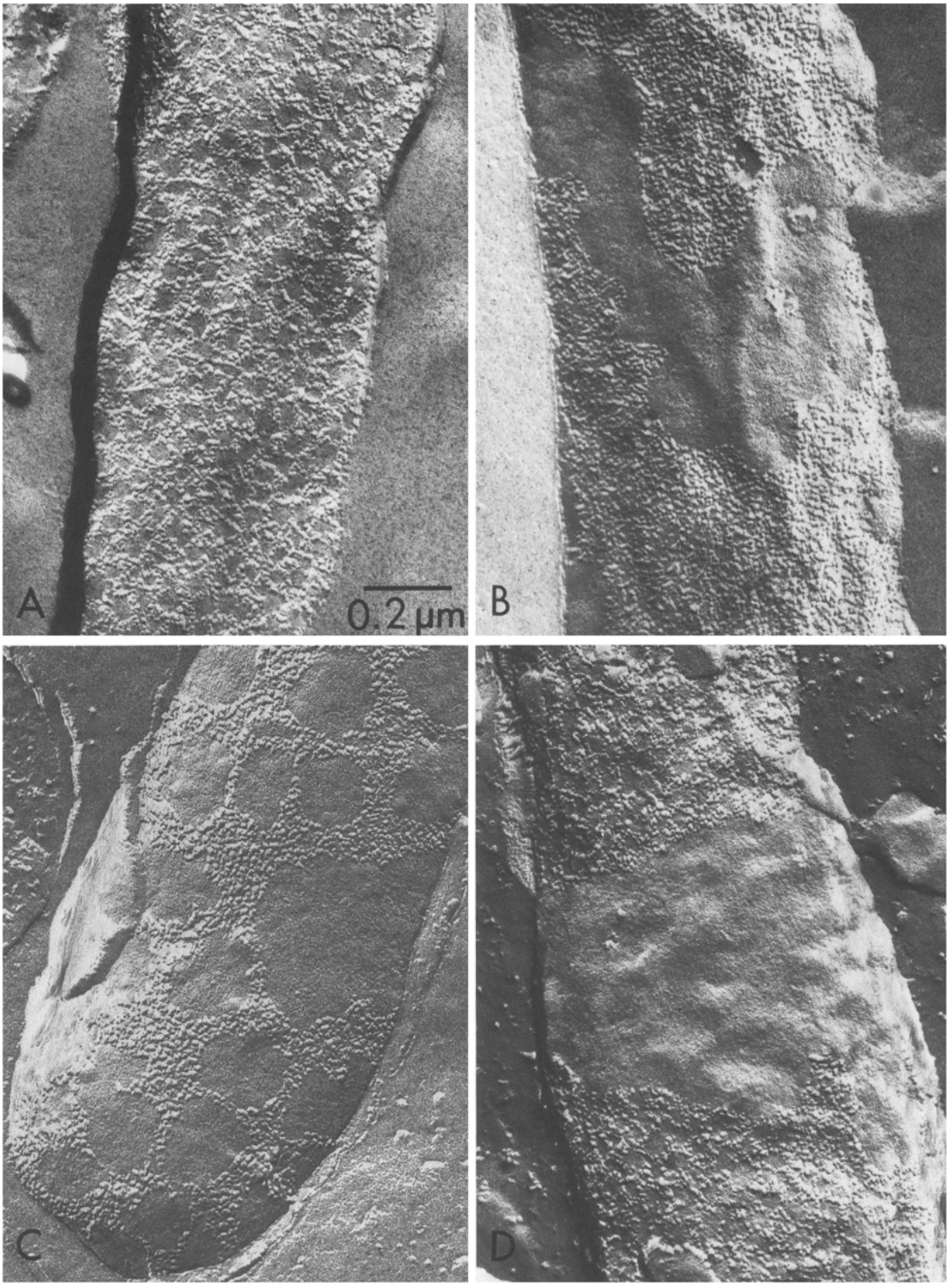


**Fig. 4.** Freeze-fracture electron micrographs of plasma membranes. (*A*) control cells grown at 37 °C and held at 37 °C prior to quenching; (*B*) control cells grown at 37 °C and held at 0 °C prior to quenching; (*C*) cells grown at 30 °C with palmitate and held at 30 °C prior to quenching; (*D*) cells grown with palmitate at 37 °C and held at 37 °C prior to quenching. Bar represents 0.2 μm

**Table 4.** Comparison of transport activity in membranes from control and palmitate-grown cells[a]

| Transport system | Uptake (nM/mg cell protein per min) | |
|---|---|---|
| | Detergent [Average ±SD (replicates)] | Detergent +palmitate [Average ±SD (replicates)] |
| Glutamate | 5.2 ±1.1 (4) | 2.7 ±1.2 (8) |
| Leucine | 1.7 ±0.2 (4) | 1.9 ±0.3 (4) |
| Proline | 3.5 ±0.3 (3) | 3.7 ±0.2 (3) |
| *Lac* Permease[b,c] | | |
| Cryptic | 0.013±0.003 (5) | 0.053±0.011 (4) |
| Active | 0.015±0.002 (4) | 0.055±0.008 (4) |

[a] Cells grown with detergent or with detergent and palmitic acid.
[b] Strain NL1, lac i⁻ transductant of strain WN1.
[c] Units: increase in absorbance (420 nm) per min per OD 550 of cells.

**Table 5.** Comparison of enzyme activity in membranes from control and palmitate-grown cells of strain WN1[a]

| Enzyme | Activity (μM/mg protein per min)[a,b] | |
|---|---|---|
| | Detergent | Detergent +palmitate |
| NADH oxidase (spectrophotometric) | 1.77 | 1.83 |
| NADH oxidase ($O_2$-electrode)[c] | 1.68 | 1.36 |
| Succinic dehydrogenase (spectrophotometric) | 0.54 | 0.58 |
| Succinic dehydrogenase ($O_2$-electrode)[c] | 0.69 | 0.28 |
| D-lactate oxidase | 0.19 | 0.17 |
| ATPase ($Ca^{++}$) | 0.12 | 0.11 |
| ATPase ($Mg^{++}$) | 0.13 | 0.10 |

[a] Cells grown with detergent or with detergent and palmitic acid.
[b] Average from 2 separate membrane preparations.
[c] μatoms $O_2$/mg protein per min.

cinic dehydrogenase and D-lactate oxidase activities (colorimetric), and ATPase activities were also equivalent to that of control cells. However, glutamate uptake and succinic dehydrogenase activity (using $O_2$ as the terminal electron acceptor) were approximately half as active in palmitate-grown cells as in control cells. Previous studies by Baldassare et al. [2] have shown that the succinic dehydrogenase activity using $O_2$ as an electron acceptor is particularly sensitive to changes in lipid composition. The *lac* permease activity and the rate of cryptic β-galactoside uptake appeared abnormally high in palmitate-grown cells, being threefold that of control cells. However, this apparent increase may be artifactual due to the leakage of β-galactosidase into the medium during incubation.

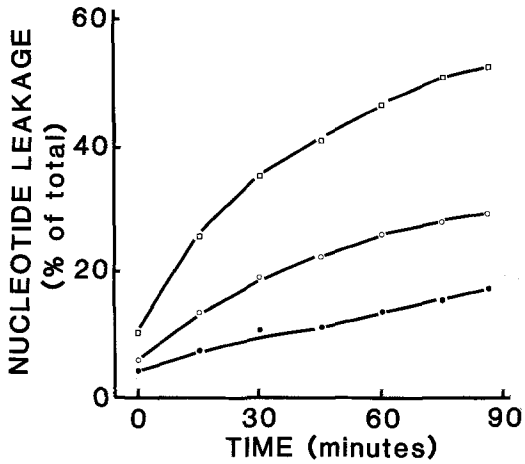


**Fig. 5.** Effects of palmitate incorporation on nucleotide leakage. Symbols: ● cells grown with detergent at 37 °C; ○ cells grown with detergent plus palmitate at 37 °C for 60 min prior to harvesting; □ cells grown with detergent plus palmitate at 37 °C for 200 min prior to harvesting

*Effects of Palmitate Incorporation on Cell Leakage*

The leakage of nucleotides into buffer was examined in palmitate-grown and control cells (Fig. 5). Cells which had been grown for 200 min with palmitate released 45% of their total nucleotides into the surrounding buffer within 1 hr as compared to 15% for control cells. Cells grown with palmitate for only 60 min contained less saturated fatty acid (Fig. 1) and exhibited an intermediate rate of nucleotide leakage. Cells were also grown with vaccenic acid, palmitoleic acid, and oleic acid. The rates of nucleotide leakage from these cells were similar to those shown for control cells.

Strain NL1, a *lac* i⁻ derivative of strain WN1, was used to investigate protein release during growth with and without palmitic acid (Fig. 6). Very little β-galactosidase activity was found in the medium of cells grown with detergent alone, at either 30 or 37 °C (2 to 4% of total activity). This soluble activity did not increase during the growth of control cells and probably represented protein which had been released by cell lysis in the overnight culture used as inoculum. In contrast, up to 1/3 of the total cellular β-galactosidase activity was present in the medium following growth with palmitic acid for four generations. The release of β-galactosidase activity into the surrounding growth medium began after approximately two mass doublings, preceeding the abrupt decline in the rate of growth and before the maximal level of palmitate had been reached in cellular membranes (Fig. 1). This release of β-galactosidase activity probably results from partial lysis of palmitate-grown cells.

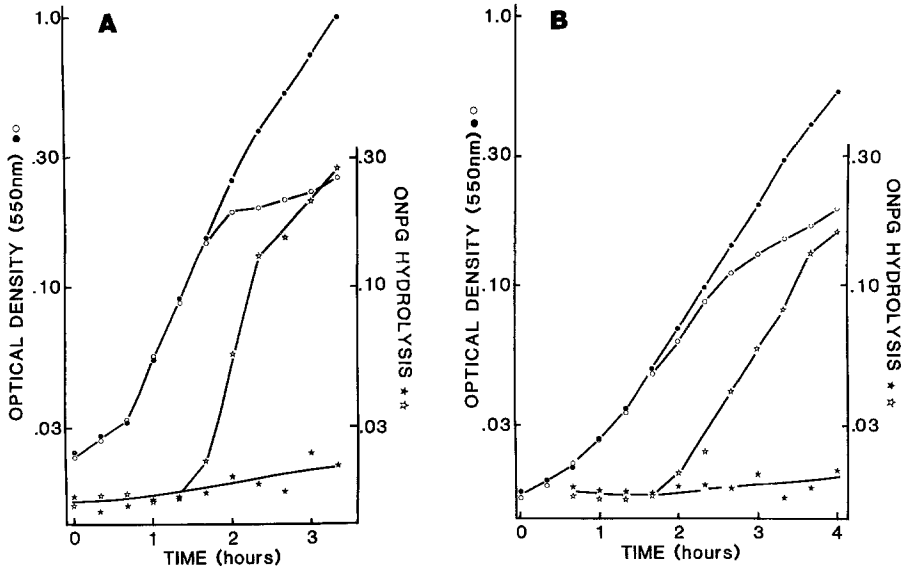


**Fig. 6.** Effect of palmitate on the growth of strain NL1 and the release of β-galactosidase into the medium. Cells were diluted into fresh medium and incubated with detergent and detergent plus palmitate. Samples were removed for the measurement of optical density and released β-galactosidase activity (ONPG-hydrolysis in the cell-free supernatant). (*A*) 37 °C; (*B*) 30 °C. Symbols: •, ★ control cells with detergent; ○, ☆ cells containing detergent plus palmitate

## Discussion

Previous studies have proposed that a minimum of 15 to 20% unsaturated fatty acid is required for the growth of *E. coli* based upon the starvation of unsaturated fatty acid auxotrophs [5, 6, 18]. Our results, using a different approach, are in general agreement. During the growth of strain WN1 with exogenous palmitic acid, lipids containing low levels of unsaturated fatty acids were synthesized. At 37 °C, growth was impaired as the proportion of unsaturated fatty acids in the cellular lipids declined to 24%. At 30 °C, higher levels of unsaturated fatty acids were required and growth was impaired as the proportion of unsaturated fatty acids declined to 40%. These minimum requirements for unsaturated fatty acids are well below the levels of unsaturated fatty acids synthesized by wild type *E. coli* at either 30 or 37 °C [3, 23].

Membrane organization and membrane function were examined to determine the basis of growth impairment by palmitic acid. Bulk membrane viscosity (polarization of DPH) increased during growth with palmitic acid and reached similar values at 30 and at 37 °C with polarizations of 0.347 and 0.337, respectively. This may reflect a physical limitation on the incorporation of palmitic acid. Extensive phase separations were observed in freeze-fractured preparations of cells grown with palmitic acid and held at their respective growth temperature prior to quenching. These particle-free areas probably represent domains enriched with the high melting lipid species containing two saturated acyl chains. Temperature scans of *cis*- and *trans*-parinaric acid-containing membranes provided further evidence that rigid domains exist in membranes from palmitate-grown cells at their respective growth temperature. Many of the membrane-bound enzymes and transport systems examined were as active in palmitate-grown cells as in the control cells despite the presence of phase separations. Only two of these functions were less active in cells containing high levels of saturated fatty acid, succinic dehydrogenase activity (with $O_2$ as the electron acceptor) and glutamate uptake. In contrast, membrane permeability and integrity were adversely affected by palmitate incorporation. The nucleotides were released from palmitate-grown cells into surrounding buffer much more rapidly than were nucleotides from normal cells. In addition, palmitate incorporation facilitated the loss of the cytoplasmic enzyme, β-galactosidase. It is likely that this leakage and failure of the essential plasma membrane barrier are the principal causes for the decrease in growth in the presence of palmitic acid. Phase separations have been shown to increase membrane leakage [22, 27] and probably contribute to membrane instability. This physical limitation of membrane integrity is consistent with the requirement for higher levels of unsaturated fatty acids at 30 °C. Phase separations are promoted by lower temperatures; consequently, the lysis of cells grown with palmitic acid when shifted to lower temperatures may reflect a limitation on the extent of phase separation which can be tolerated while still maintaining cellular integrity. Recent studies by Akutso et al. [1] have shown that the requirement for fatty acid unsaturation in unsaturated fatty acid auxotrophs of *E. coli* may be further decreased by the addition of osmotic stabilizers to the growth medium. Thus, the requirement for the physical stability

of the membrane may serve to determine the minimum requirement of *E. coli* for unsaturated fatty acids under a particular set of growth conditions, rather than the impairment of specific enzymatic functions associated with the membranes.

The recent results of Silvius et al. [30] using *Acholeplasma laidlawii* lead us to question the need for acyl chain diversity in bacteria. This strain was grown to produce fatty acid homogeneous membranes, containing a single type of phospholipid molecular species with respect to acyl chains. Strain WN1 and probably all *fad* $F^-$ strains of *E. coli* are unique among *E. coli* strains in that over 90% of their phospholipids contain a saturated acyl chain at the 1 position and palmitoleic acid at the 2 position. Thus, the bulk of the lipids in this organism is also composed of a single molecular type with respect to acyl chains. This homogeneity of molecular species in *E. coli* did not cause a major impairment of growth.

Relatively well-defined phase transitions were observed in the membranes of strain WN1 using *cis*-parinaric acid, comparable to those previously observed for unsaturated fatty acid auxotrophs [33, 34]. Extensive phase separations into particle-free and particle-rich regions were observed by freeze-fracture electron microscopy when cells were held at 0 °C prior to quenching. Considering the lipid homogeneity, these phase separations could not have involved a segregation of lipid species based on acyl chain type. These separations may reflect, in part, a segregation of phospholipid types. In addition, these may represent an exclusion of the larger protein species during the formation of solid domains, analogous to the concentration of a solute during the freezing of an aqueous solution.

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