# Characterization of Membrane Lipids of a General Fatty Acid Auxotrophic Bacterium by Electron Spin Resonance Spectroscopy and Differential Scanning Calorimetry

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*Received July 27, 1984*

ABSTRACT: Lipids in the plasma membrane of the general fatty acid auxotroph *Butyrivibrio* S2 pack as a bilayer that is characterized by a high order and high motional anisotropy and a low membrane fluidity compared to mammalian plasma membranes. Lipid packing as determined by the electron spin resonance (ESR) order parameter and membrane fluidity as measured by ESR correlation times are, however, comparable to those of other bacterial membranes. Membranes of the organism grown with saturated fatty acids of well-defined hydrocarbon chain length undergo a broad reversible endothermic phase transition, the peak temperature of which is well below the growth temperature; the end-point temperature of this thermal transition approximately coincides with the minimum temperature supporting significant growth of the organism. The lipid phase transition is also reflected in the temperature dependence of various ESR parameters, whereby the transition temperature thus derived is higher than the peak temperature of the endothermic transition but still lower than the growth temperature. ESR and calorimetry evidence taken together suggest that the endothermic transition is a gel to liquid-crystal transition and that, at the growth temperature, the plasma membrane of *Butyrivibrio* S2 is in the liquid-crystalline state. Similar values were measured for the order parameter of cell membranes of *Butyrivibrio* S2 regardless of whether the organism was grown on myristic, palmitic, or stearic acid. *Butyrivibrio* S2 has a mechanism enabling it to maintain membrane packing and fluidity at a fairly constant level. The same appears to be true if, in the presence of a given fatty acid, the growth temperature of the organism is varied. Novel mechanisms by which this is accomplished are discussed that are different from other microorganisms in the sense that *Butyrivibrio* S2 can manipulate neither chain length nor unsaturation of membrane fatty acids.

The obligately anaerobic rumen bacterium *Butyrivibrio* S2 is a general fatty acid auxotroph whose growth is supported by the addition of any one of a series of straight-chain saturated ($C_{13}$–$C_{18}$) or monoenoic fatty acids to the growth medium (Hazlewood & Dawson, 1979). Growth-promoting fatty acid is incorporated into membrane lipids comprising phosphoglycolipids, phospholipids, and glycolipids either unchanged, as an ether-linked alkenyl grouping, or as a novel long-chain terminal dicarboxylic acid with midchain vicinal dimethyl groups (diabolic acid) (Klein et al., 1979). When growth is promoted by a saturated fatty acid, membrane lipids of *Butyrivibrio* S2 are completely devoid of acyl chain unsaturation but always contain significant quantities of esterified butyric acid, which is derived from hexose by fermentation (Hauser et al., 1979; Hazlewood et al., 1980a).

Previous work with *Butyrivibrio* S2 has been primarily concerned with the elucidation of the structure of complex lipids (Hazlewood et al., 1980a; Clarke et al., 1980) or novel hydrophobic moieties contained therein (Klein et al., 1979). This paper describes the results obtained in a study of the physical properties of both intact membranes and extracted lipids of the organism.

## Materials and Methods

*Culture Conditions and Preparation of Samples.* *Butyrivibrio* S2 was cultured in fatty acid free liquid medium containing 0.25–0.4% (w/v) galactose, essentially as described previously (medium 3; Hazlewood & Dawson, 1979). The different fatty acids (20–30 μg/mL) used to promote growth were dispersed in the medium by ultrasonication in the presence of sodium taurocholate (300–400 μg/mL) or by ultrasonication alone; in some experiments, fatty acids were added esterified in the structure of the nonionic detergent Tween 80 (1 mg/mL). Cultures were inoculated with 5% (by volume) inoculum, grown to maximum turbidity in the same medium, and were incubated at 39 °C (45 °C for stearic acid containing medium) for 16–18 h. Myristic, pentadecanoic, palmitic, heptadecanoic, stearic, and *trans*-vaccenic acids were obtained from Sigma Chemical Co. (Poole, U.K.). Bacterial cells were collected by centrifugation (20000*g*, 4 °C, 20 min). Plasma membranes having a lipid content of 55% on the basis of P analysis were prepared as described by Hazlewood et al. (1983) and were exhaustively dialyzed against distilled water before freeze-drying. Total lipids were extracted as described by Clarke et al. (1976). The main lipid (lipid 14) produced by *Butyrivibrio* S2 cultured with palmitic acid was extracted and purified as described previously (Clarke et al., 1980); it appeared as a single component when tested in a number of thin-layer chromatography (TLC) systems. Lipid phosphorus was determined by the method of Bartlett (1959).

*Electron Spin Resonance Spectroscopy.* Spin-labeled fatty acids 5-doxyl- and 16-doxylstearic acid with the 4,4-dimethyl-3-oxazolidinyl-1-oxy group attached to C-5 and C-16 of stearic acid, respectively, were obtained from Syva, Palo Alto, CA. Introducing the spin-labels into the membrane of *Butyrivibrio* S2 was performed by drying the spin probe down from chloroform/methanol (2:1 by volume) solution in a round-bottom flask. Washed cells or purified plasma mem-

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Table I: Maximum Hyperfine Splittings ($2T_{\parallel}$) and Order Parameter ($S_{33}$) of 5-Doxylstearic Acid Spin-Label Incorporated into Cell Membranes and into Liposomes Made from the Total Lipids Extracted from *Butyrivibrio* S2

| membrane | fatty acid[a] | $2T_{\parallel}$ (G)[b] 37 °C | $2T_{\parallel}$ (G)[b] 47 °C | $S_{33}$[b] 37 °C | $S_{33}$[b] 47 °C |
|---|---|---|---|---|---|
| cell membranes | myristic acid | 57 | 55.5 | 0.68 | 0.63 |
| liposomes[c] | | | | | |
| with EDTA | | 45 | 43 | 0.43 | 0.40 |
| without EDTA | | 53 | 51 | 0.59 | 0.58 |
| cell membranes | palmitic acid | 55 | 54 | 0.67 | 0.63 |
| liposomes without EDTA | | 50–53[d] | 49–52[d] | 0.58 | 0.57 |
| cell membranes | stearic acid | 56 | 53 | 0.67 | 0.62[e] |
| liposomes with EDTA | | 45 | 42 | 0.44 | 0.38 |

[a] Fatty acid added to the growth medium. [b] The error of the measurement was ±1 G ($2T_{\parallel}$) and ±0.02 ($S_{33}$). [c] Liposomes were dispersed in $H_2O$ or 0.15 M NaCl with or without ~10 mM EDTA. [d] The range given represents the variation observed. [e] ESR spectra could not be obtained at temperatures >47 °C because of the spin-label becoming unstable.

branes in 0.1 M sodium phosphate buffer, pH 7.4, were then added to the flask, and equilibration of the probe with membrane lipids was facilitated by gentle shaking. Prior to labeling, whole cells were washed with 0.1 M sodium phosphate buffer, pH 7.4, at least 3 times to remove traces of cysteine, an essential component of the growth medium that was found to destroy the nitroxide radical of the spin-labeled fatty acids. The amount of spin-labeled fatty acid was calculated to give a molar ratio of membrane lipid:spin-label of 100–200:1 assuming an average $M_r$ of 1500 for the membrane lipids. In order to label total lipids with spin-labeled fatty acid, the extracted bacterial lipids were mixed with the label in chloroform/methanol (2:1 by volume) in a molar ratio of 100–200:1. The solvent was removed under vacuum at room temperature, and the dried lipid film was subsequently dispersed in distilled water or 0.15 M NaCl solution at 45–50 °C, sometimes with the addition of ethylenediaminetetraacetic acid (EDTA) (~0.4% w/v). ESR spectra were recorded at about 9.16 GHz with a Varian X-band spectrometer (Model E-104A) fitted with a variable-temperature device. The spectra were evaluated in terms of order parameters and orientational correlation time, $\tau$, as reported previously (Hauser et al., 1982).

*Differential Scanning Calorimetry.* Calorimetric studies were carried out with a Perkin-Elmer DSC-2 differential scanning calorimeter (Norwalk, CT). Lipids (3–5 mg) were weighed into the sample pan and dispersed by adding 50 μL of water or 0.15 M NaCl. Purified plasma membrane from *Butyrivibrio* S2 was dispersed in 0.1 M sodium phosphate buffer, pH 7.4, or 0.15 M NaCl solution sometimes containing 0.1% (w/v) EDTA and 0.02% (w/v) sodium azide, prior to transfer to the DSC sample pan.

## Results

*ESR Spin-Labeling.* At a given temperature qualitatively similar ESR spectra were obtained for 5-doxylstearic acid incorporated into the membranes of *Butyrivibrio* S2 (Figure 1A) and into liposomes made from their extracted lipids (Figure 1B). Examining a range of temperatures in this manner showed that above about 30 and 20 °C, respectively, the spectra from membranes and from the liposomes are typical for the spin-labeled molecule undergoing rapid but anisotropic motion as is the case when the spin-label is incorporated in smectic lamellar phases of lipid bilayers. Below these temperatures, the line shape of the spectra approached

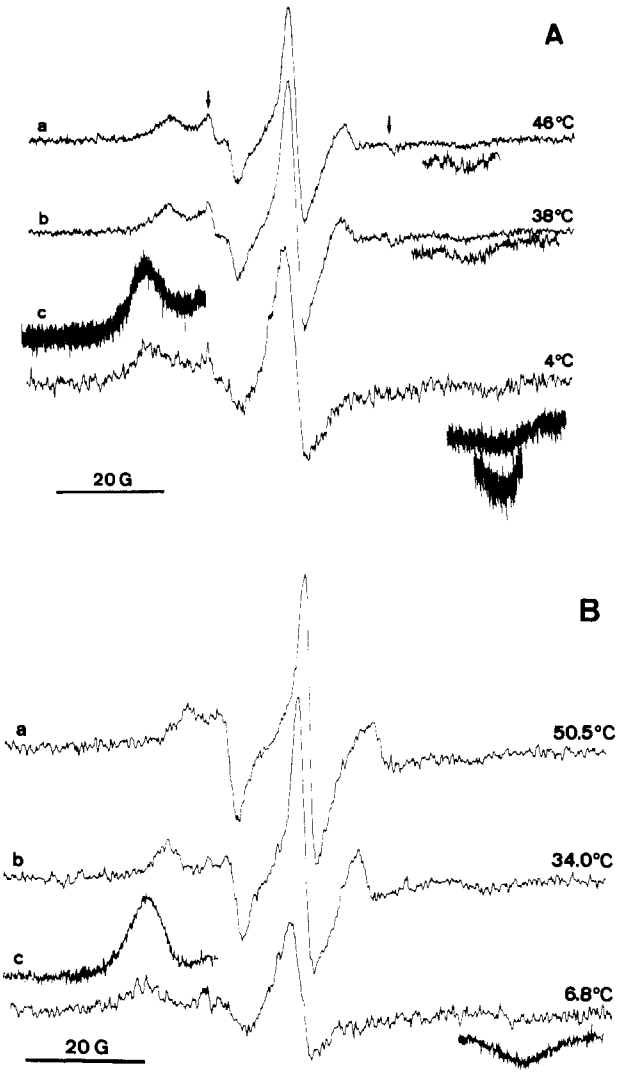


FIGURE 1: (A) ESR spectra of 5-doxylstearic acid incorporated into the plasma membrane of *Butyrivibrio* S2 dispersed in 0.1 M sodium phosphate buffer, pH 7.4. Cells (112 mg dry weight) grown in the presence of palmitic acid were washed repeatedly in phosphate buffer and finally dispersed in 0.4 mL of the same buffer to yield a lipid concentration of ~18 mM. 5-Doxylstearic acid was incorporated to give a lipid:spin-label ratio of ~100:1. ESR spectra a–c were recorded at 46, 38, and 4 °C, respectively. Vertical expansions of the high- and low-field part of the spectra (for instance, in spectrum c) were used to derive the maximum hyperfine splitting $2T_{\parallel}$. The arrows mark signals arising from free spin-label. (B) ESR spectra of 5-doxylstearic acid incorporated into the total lipids extracted from cells grown in the presence of palmitic acid. The lipids containing the spin probe were dispersed in water or 0.15 M NaCl solution. The lipid concentration was 10 mg/mL (6.7 mM); the lipid:spin-label molar ratio was 100:1. Vertical expansions of the high- and low-field part of the spectra were used to derive $2T_{\parallel}$. Spectra a–c were recorded at 50.5, 34, and 6.8 °C, respectively.

that of a powder spectrum with values of $T_{\parallel} \rightarrow T_{zz}$ (cf. Figures 1Ac,Bc and Figure 2A). The spectra shown in Figure 1A are for cells grown in the presence of palmitic acid at 39 °C. Qualitatively and quantitatively similar spectra were obtained when 5-doxylstearic acid was incorporated into cells grown with myristic or stearic acid. Values for the maximum hyperfine splittings $2T_{\parallel}$ and the order parameter $S_{33}$ derived from the ESR spectra are presented in Table I. These values, which are a measure of the anisotropy of motion, are significantly higher in cell membranes than in liposomes made from their extracted lipids. Furthermore, the values appear to be independent of the exogenously supplied fatty acid. This is not only true for temperatures at or near the growth temperature,

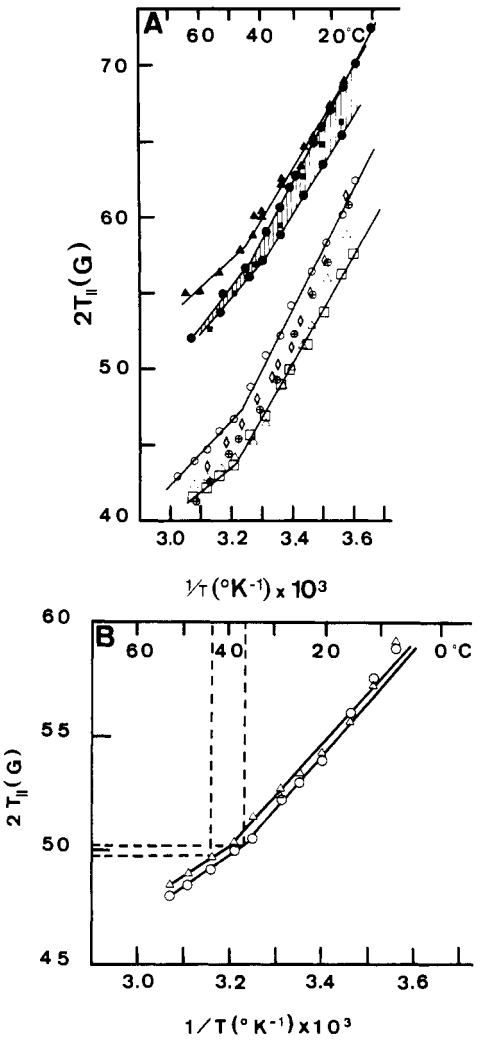


FIGURE 2: (A) Maximum hyperfine splittings $2T_{\parallel}$ as a function of $1/T$ ($K^{-1}$) for cells grown with different fatty acids (solid symbols) and liposomes made from their extracted lipids (open symbols). Cells and liposomes were labeled with 5-doxylstearic acid as described under Materials and Methods. Experimental conditions are given in the legend to Figure 1. Cells were grown at 39 °C with myristic acid (▲), with palmitic acid (●), and with palmitic acid in the presence of taurocholate in the growth medium (■). Apparently, the presence of taurocholate had no effect on the $2T_{\parallel}$ values. Different cell preparations grown with palmitic acid were investigated over a period of about 1 year; the observed spread in $2T_{\parallel}$ values is represented by the hatched area. The $2T_{\parallel}$ values for cells grown on stearic acid at 45 °C would lie within the hatched area; they have been omitted in order to avoid crowding. The open symbols represent $2T_{\parallel}$ values measured in dispersions of lipids extracted from cells grown with myristic acid (△), pentadecanoic acid (◇), palmitic acid (○), heptadecanoic (margaric acid (⊗), and *trans*-11-octadecenoic (vaccenic) acid (□). The data obtained with lipids extracted from cells grown with stearic acid are not included in the plot for the sake of clarity but would be close to those obtained with vaccenic acid. Lipids were dispersed in $H_2O$ or 0.15 M NaCl containing 0.01 M EDTA. Statistical analysis of the data shows that each set of data points is best fitted by two straight lines, and the solid lines represent least-squares fit to the experimental data. The temperature at which the two straight lines intersect represents the lipid transition temperature, and values thus derived are summarized in Table II. In order to avoid crowding, solid lines are only plotted for lipids extracted from cells grown with palmitic acid (○) and vaccenic acid (□). (B) Maximum hyperfine splittings $2T_{\parallel}$ as a function of $1/T$ for dispersions of lipids extracted from cells grown with palmitic acid at 36 (○) and 44 °C (△). In contrast to (A), the lipids were dispersed in $H_2O$ or 0.15 M NaCl in the absence of EDTA. The data points at temperatures <ts 20 °C appear to deviate from the straight line fitted to the data points above this temperature; the origin of this deviation is unknown. The horizontal dashed lines give the $2T_{\parallel}$ values measured at the growth temperature of the two lipid dispersions, at 36 and 44 °C, respectively.

i.e., above the phase transition temperature (cf. Tables I and II), but appears to hold for the total temperature range investigated, at least within the experimental error (see Figure 2 and Discussion). This result indicates that, irrespective of the fatty acyl chain length of the membrane lipids, the membrane packing and fluidity (as monitored by $2T_{\parallel}$ or $S_{33}$) are maintained at a fairly constant level. Liposomes dispersed in $H_2O$ or 0.15 M NaCl containing EDTA usually gave lower $2T_{\parallel}$ and $S_{33}$ values than aqueous dispersions without EDTA (Table I). This may be due to the extracted lipids containing residual metal ions bound to their polar group.

The temperature dependence of the hyperfine splittings $2T_{\parallel}$ derived from the ESR spectra (e.g., Figure 1) is shown in Figure 2. Over the whole temperature range (0–60 °C) investigated, significantly larger $2T_{\parallel}$ values were measured for cell membranes (closed symbols, Figure 2A) as compared to the lipid dispersions (open symbols, Figure 2A). Furthermore, the $2T_{\parallel}$ values for cells grown with myristic, palmitic, or stearic acid agreed within 2–3G, and this was found to hold for the total temperature range (Figure 2A; Hazlewood et al., 1980b). The same was true for the $2T_{\parallel}$ values measured with liposomes made from the lipid extract of cells grown with different fatty acids (Figure 2A). Liposomes made from the lipid extract of cells grown with palmitic acid at 36 and 44 °C gave very similar $2T_{\parallel}$ values below ~20 °C. However, above ~30 °C the $2T_{\parallel}$ values of the lipids from cells grown at 44 °C are slightly, but significantly higher (Figure 2B). The $2T_{\parallel}$ values determined at the individual growth temperatures of 44 and 36 °C are then 49.7 and 50.3 G, respectively, and are in good agreement within the error of the measurement. Figure 2B also shows that at temperatures above 20 °C the $2T_{\parallel}$ values are significantly higher than the corresponding values of the lipid dispersions shown in Figure 2A. As mentioned before, this was usually observed for aqueous lipid dispersions in the absence of EDTA.

Statistical analysis of the data presented in Figure 2A shows that each set of data points is best fitted by two straight lines. The data for liposomes dispersed in $H_2O$ (Figure 2B) exhibit a more complex relationship with probably two discontinuities. The discontinuity in the plots of $2T_{\parallel}$ against reciprocal temperature was previously proposed (Hauser et al., 1979) to reflect a lipid transition, probably some kind of gel to liquid-crystal transition. The temperature at which the discontinuity (proposed transition) occurred was different for each of the fatty acids used to promote growth of *Butyrivibrio* S2 (Table II). Inspection of this table shows that the transition temperature is related to the growth temperature occurring a few degrees below this temperature except for pentadecanoic acid, where the lipid transition was ~12 °C below the growth temperature. There was, however, good agreement (within 1 or 2 °C) between the transition temperature determined in cells and that measured in liposomes made from their extracted lipids. Considerably lower transition temperatures were observed when the lipid dispersion contained EDTA (Table II). Residual metal ions bound to the lipid polar group could be responsible for this difference. Metal ion complexes are known to raise the temperature of the lipid phase transition (Papahadjopoulos et al., 1977; Hauser & Shipley, 1984).

ESR spectra of 16-doxylstearic acid incorporated into the lipids extracted from cells grown with palmitic acid are shown in Figure 3. Similar spectra were obtained when this label was incorporated directly into cells grown with palmitic acid (data not shown). At temperatures >37 °C, the spectra from both cells and lipid extracts were interpreted in terms of rapid and to a first approximation isotropic motion. In this tem-

Table II: Transition Temperatures Derived from ESR Measurements

| fatty acid[a] | transition temp (°C) | | growth temp (°C) | min temp supporting significant growth of organism (°C)[b] |
|---|---|---|---|---|
| | lipid extract | cells | | |
| myristic acid (14:0) | 35 (31)[c] | 35 | 39 | 35 |
| pentadecanoic acid (15:0) | 27 (22) | | 39 | 33 |
| palmitic acid (16:0) | 34 | | 36 | |
| palmitic acid (16:0) | 36 | 34 (30–36)[d] | 39 | 33 |
| palmitic acid (16:0) | 39 | | 44 | |
| heptadecanoic (margaric) acid (17:0) | 36 (31) | | 39 | 31 |
| stearic acid (18:0) | 42 | 43 | 45 | 39 |
| *trans*-11-octadecenoic (vaccenic) acid (18:1) | 38 | | 39 | |

[a] Fatty acid present in the growth medium. [b] Significant growth was defined as an increase in bacterial dry weight of >0.05 mg/mL during 20-h incubation. [c] Cell membrane (30 mg/mL ≃ 18 mM lipids) were dispersed in 0.1 M sodium phosphate buffer, pH 7.4; all lipids were dispersed in $H_2O$ at 10 mg/mL (≃7 mM). Values in parentheses refer to liposomes dispersed in $H_2O$ containing 10 mM EDTA. [d] Mean value of four different cell preparations; the range in parentheses gives the extreme values. All values were independent of the presence of taurocholate added to the growth medium.

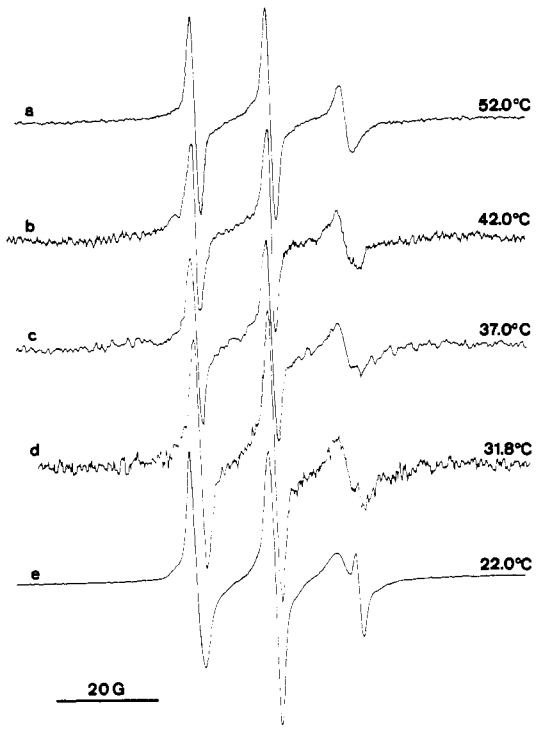


FIGURE 3: Temperature dependence of the ESR spectra of 16-doxylstearic acid incorporated in the total lipids extracted from *Butyrivibrio* S2 cells grown with palmitic acid. The lipids (6–10 mg) were dispersed in 1 mL of water or 0.15 M NaCl, 0.1% (~3 mM) EDTA, and 0.02% $NaN_3$ as described under Materials and Methods. The lipid:spin-label molar ratio was about 250:1. The numbers on the right give temperatures at which the spectra were recorded.

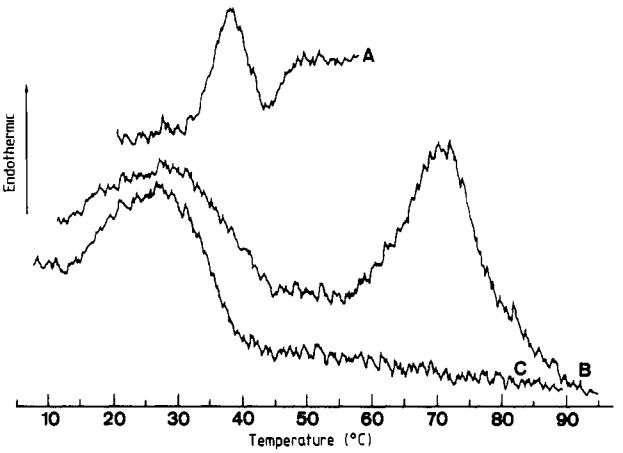


FIGURE 4: Differential scanning calorimetry (DSC) curves for the purified plasma membrane of *Butyrivibrio* S2 dispersed either in 0.1 M sodium phosphate buffer, pH 7.4, or in 0.15 M NaCl, pH 8.6, containing 0.1% (~3 mM) EDTA and 0.02% $NaN_3$. A slurry of about 20 mg of plasma membrane from cells grown in the presence of palmitic acid in 0.1 mL of buffer was prepared (see Materials and Methods). The slurry was immediately transferred to the DSC pan and the pan hermetically sealed. Heating curves were recorded at 5 °C/min; (A) first heating run, which was interrupted at about 55 °C; (B) subsequent heating run after the sample was cooled to 0 °C; (C) repeat of (B) after the sample was cooled to 0 °C.

perature range orientational correlation times $\tau$ were calculated according to the theory of Kivelson [1960; cf. Stone et al. (1965)]. The $\tau$ values were similar for 16-doxylstearic acid in cell membranes and in liposomes made from their lipid extract. At 37 °C, a value of $\tau = (1.5 \pm 0.2) \times 10^{-9}$ s was obtained, which is in good agreement with values measured in other bacterial membranes [see, for instance, Esser & Lanyi (1973)]. At temperatures <37 °C, the upfield peak appears to broaden, and at room temperature it is clearly resolved into two components. The hyperfine splitting constant $a_N$ derived from spectral simulations was 14.3 and 15.8 G for the broad and narrow components, respectively. The former value is representative of an apolar environment while the latter is characteristic of water, indicating that at temperatures below ~37 °C part of the spin-label is squeezed out into the water.

*Differential Scanning Calorimetry.* The thermal behavior of the plasma membrane of *Butyrivibrio* S2 grown with palmitic acid was reproducible and is shown in Figure 4. The membranes were first cooled to about 0 °C, and the initial heating run gave a broad endothermic transition (onset temperature ~33 °C, peak temperature ~38 °C; curve A of Figure 4). Upon cooling, a very broad exothermic transition was observed spread out between 27 and 5 °C (data not shown). Subsequent heating runs exhibited a reproducible broad endothermic transition at a lower temperature than that observed on initial heating. The endothermic transition occurred between about 15 and 43 °C with a peak temperature of about 28 °C. Further heating produced another broad endothermic transition with a peak at about 70 °C (curve B of Figure 4). The latter transition was irreversible, and repeated cooling and heating cycles showed only the low-temperature transition at ~28 °C (cf. curves B and C of Figure 4). The high-temperature transition was absent in the heating curves of the lipid extract of *Butyrivibrio* S2 (see Figure 5). On the basis of these observations, the high-temperature transition was assigned to the denaturation of membrane proteins.

The thermal behavior of the total lipids extracted from *Butyrivibrio* S2 grown with palmitic acid is shown in Figure 5. The heating curve (Figure 5A) of the fully hydrated lipid

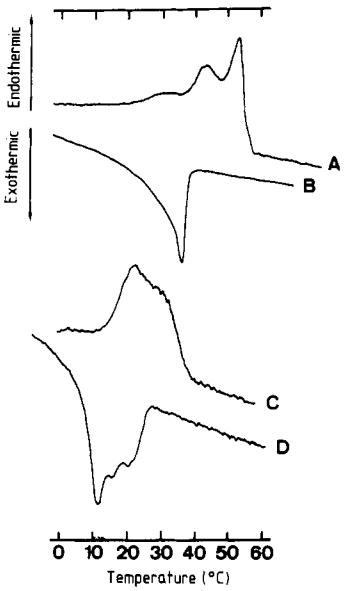


FIGURE 5: Differential scanning calorimetry heating and cooling curves for the total lipids and lipid 14 of *Butyrivibrio* S2 grown with palmitic acid. Lipids were extracted as described under Materials and Methods. A total of 3.5 mg of total lipid was weighed into the sample pan and dispersed in 0.05 mL of 0.15 M NaCl, pH 8.6, containing 0.1% (3 mM) EDTA and 0.02% $NaN_3$. The pan was sealed immediately and transferred to the DSC instrument and heating (A) and cooling (B) curves recorded. A $CHCl_3/CH_3OH$ (9:1 by volume) solution containing 2 mg of pure lipid was pipetted into a DSC pan and dried under vacuum. To the dried waxy lipid, 0.05 mL of aqueous medium was added and the pan hermetically sealed. Heating and cooling curves are shown in (C) and (D), respectively.

dispersion consists of at least three endothermic transitions in the temperature range 10–60 °C. This is true for dispersions in $H_2O$ or in 0.15 M NaCl solution containing about 3 mM EDTA. The approximate temperature of the first peak, which is the broadest and not completely resolved, is 30–35 °C; the temperature of the other two well-resolved peaks is 45 and 54 °C, respectively. On cooling, a broad exothermic transition was observed with a peak temperature of about 36 °C (Figure 5B). Repeated heating and cooling cycles showed essentially the same behavior as that depicted in Figure 5A,B indicating that the thermal transitions are reversible.

The differential scanning calorimety curves for the predominant lipid (lipid 14; Clarke et al., 1980) purified from *Butyrivbrio* S2 cells grown with palmitic acid are shown in Figure 5C,D. Heating the fully hydrated lipid from 0 °C gave a broad endothermic transition between about 15 and 40 °C with peaks at about 20 and 30 °C. It is noteworthy that for aqueous dispersions of the pure lipid the heating (Figure 5C) and the cooling curves (Figure 5D) are both composite, consisting of at least two and three components, respectively. The onset of the exothermic transition is at ~27 °C, the peak is at ~12 °C, and the transition is completed at ~4 °C. The thermal transitions observed with aqueous dispersions of pure lipid 14 are reversible, and repeated heating and cooling cycles gave essentially the same pattern as that shown in Figure 5C,D. On the basis of a comparison of the heating curves of the total lipid extract (Figure 5A) and of lipid 14 (Figure 5C), the broad, low-temperature transition observed with the total lipid extract (Figure 5A) may be attributed to lipid 14.

The plasma membrane of *Butyrivibrio* S2 cultured in a medium containing Tween 80 as the source of a number of different esterified fatty acids showed a different thermal behavior. There was no apparent endothermic transition between 7 and 50 °C; the only endothermic transition observed with these membranes was the broad, high-temperature transition at about 60–70 °C attributable to membrane proteins.

## Discussion

*Butyrivibrio* S2 isolated from the ovine rumen is unable to synthesize long-chain fatty acids. Saturated fatty acids added to the growth medium are incorporated, without desaturation, into plasmalogen-type lipids, either unchanged, as the corresponding fatty aldehyde, or as a new long-chain dicarboxylic acid (diabolic acid) (Hazlewood & Dawson, 1979; Hauser et al., 1979) (cf. Figure 6). This gave us an opportunity to study the physical state of a membrane containing hydrophobic moieties derived from a fatty acid precursor with a single well-defined chain length. The chemical formula of the predominant complex lipid 14 is give in Figure 6. The lipid can be viewed as consisting of two parts: (1) *sn*-1-alkenyl-glycero-3-phospho-1′-*sn*-glycerol and (2) the butyroyl ester of *sn*-1-alkenyl-3-galactosylglycerol. These two parts are linked together through a single diabolic acid residue of 32 C atoms, which is esterified to the *sn*-2 positions of the two glycerol groups. The butyroyl group esterified to the 3′-hydroxy-*sn*-glycerol can be replaced by a palmitoyl group [lipid 11; for details of the chemical structure of diabolic acid containing phospholipids occurring in *Butyrivibrio* 52, see Clarke et al. (1980)].

At the growth temperature of 39 °C the ESR spectra of both membranes of *Butyrivibrio* S2 and liposomes made from their lipid extract are characteristic of fast, but anisotropic motion. Assuming the lipids of *Butyrivibrio* S2 pack as a bilayer, the rapid motion represents rotation of the probe molecule about its long axis. The value of the order parameter $S_{33}$, which is a measure of the anisotropy of motion, is among the highest measured for biological membranes. At 39 °C the order in the membrane of *Butyrivibrio* S2 is comparable to

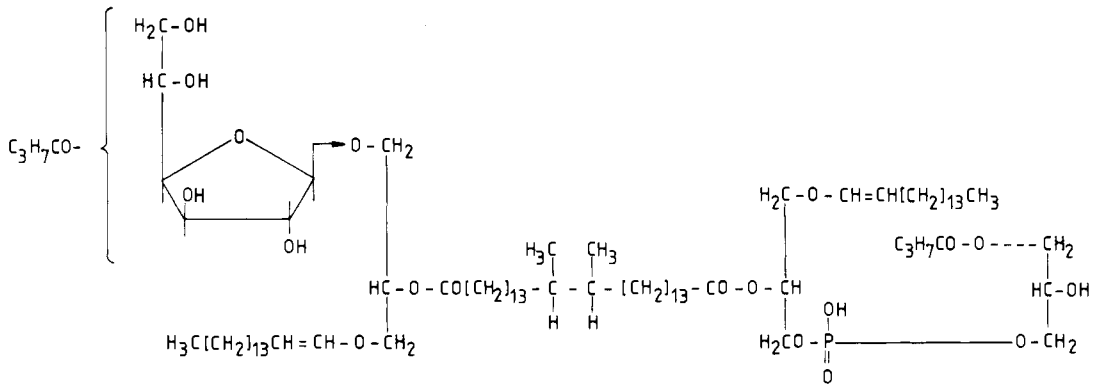


FIGURE 6: Structural formula of lipid 14. In a derivative of lipid 14, the butyroyl group esterified to the 3′-hydroxy-*sn*-glycerol is replaced by a palmitoyl group (lipid 11).

that of a dipalmitoylphosphatidylcholine bilayer in the gel state at about 30 °C (Hauser et al., 1979). The $S_{33}$ value is also comparable to those measured in other bacterial membranes (Esser & Lanyi, 1973; Plachy et al., 1974; Chignell & Chignell, 1975; Hauser et al., 1982). We can conclude that the packing of the lipids in this membrane is characterized by a high order or high motional anisotropy.

It is clear from Table I and Figure 2 that within the experimental spread (2–4 G) the $2T_{\parallel}$ values measured in membranes and liposomes made from their extracted lipids are independent of the fatty acid used to promote growth. The $2T_{\parallel}$ values appear to depend on the growth temperature. Unfortunately, only a limited range of growth temperatures could be tested since *Butyrivibrio* S2 has an upper limit to growth, which is between 44 and 46 °C, and a lower limit, which varies from 31 to 39 °C (Table II), depending on the nature of the fatty acid used to promote growth. We can conclude that *Butyrivibrio* S2 has a mechanism enabling it to maintain membrane packing and fluidity at a constant level when the hydrocarbon chain length of the added *n*-saturated fatty acid is varied from 14 to 18 carbons. Whatever the mechanism, it allows the bacterium to adjust the membrane packing (fluidity) to a level that, though high (low) compared to other membranes, is typical for bacterial membranes in the functional state, with motional characteristics reminiscent of the liquid-crystalline state. This is remarkable considering that the hydrocarbon chains of the lipids of this organism may consist of a simple, *n*-saturated long-chain fatty acid such as stearic acid. For instance, distearoyl phospholipids would be certainly in the crystalline or gel state at the growth temperature of *Butyrivibrio* S2. Possible mechanisms *Butyrivibrio* S2 uses to maintain the membrane fluidity at the required level have been discussed in some detail previously (Hauser et al., 1979; Hazlewood et al., 1980b). Essentially, the introduction of methyl branching in novel dicarboxylic acids (diabolic acids) and the esterification of hydroxyl groups of glycolipids with butyric acid are thought to be responsible for maintaining the required membrane fluidity. The present work has further revealed that, when growth is promoted by the addition of an unsaturated fatty acid (vaccenic acid), the transition temperature measured with extracted lipids falls within the range of values obtained for cells grown with *n*-saturated acids (Figure 2 and Table II). However, it is significant that in this case the organism is almost devoid of lipids containing esterified diabolic acid, which are predominant in cells grown with saturated fatty acids. Their place is taken by diglyceride galactosylphosphoethanolamine as a major component, together with an abundance of acetal plasmalogen containing phospholipids and galactolipids—complex lipids that are scarcely detectable in the saturated fatty acid grown organisms (N. G. Clarke, G. P. Hazlewood, and R. M. C. Dawson, unpublished data). Such an ability to qualitatively alter the lipid composition clearly represents a second highly novel adaptive mechanism enabling the auxotroph *Butyrivibrio* S2 to maintain membrane fluidity despite it being unable to manipulate the chain length and degree of unsaturation of membrane fatty acids in the manner of most microorganisms. The narrow temperature range over which growth of *Butyrivibrio* S2 occurred (e.g., 36–44 °C with palmitic acid) is consistent with the observed absence of a fatty acid desaturase and indicates that the available mechanisms for maintaining membrane fluidity only have scope for fairly limited adaptation; large deviations from the normal growth temperature (39 °C) are apparently not tolerated because the transition temperature for membrane lipids cannot be reduced below a certain minimum value for each growth-promoting fatty acids. In view of the fact that the temperature range within which *Butyrivibrio* S2 can grow is indeed very limited, we have avoided the term homeoviscous adaptation in our discussion.

Table III: Comparison of Thermal Behavior of the Plasma Membrane of *Butyrivibrio* S2 Grown with Palmitic Acid, As Determined by ESR Spin-Labeling and Differential Scanning Calorimetry

| transition temp (°C) | | |
|---|---|---|
| cell membrane | lipid extract | method |
| 34 | 36 | ESR |
| 28 (range 15–43) | 30–35 | DSC |
| | 45 | |
| | 54 | |
| | 20–30 (broad transition)[a] | DSC |
| | 15–40 (transition range)[a] | |

[a] Thermal behavior of the lipid dispersion made from pure lipid 14, which is the predominant lipid of *Butyrivibrio* S2.

Below about 30 °C the line shape of the ESR spectra from spin-label incorporated into *Butyrivibrio* S2 reflects increasing immobilization. Below about 20 °C the line shape approaches that of a typical powder spectrum with $T_{\parallel}$ (>30 G) approaching the tensor component $T_{zz}$.

Discontinuities occur in the temperature dependence of $2T_{\parallel}$ (Figure 2), which depend on the fatty acid in the growth medium (Table II). In a previous paper (Hauser et al., 1979), these discontinuities were tentatively attributed to lipid phase transitions. Results shown in Figure 2 and 4 are summarized in Table III and provide evidence that the interpretation given before is correct. The transition is probably a gel to liquid crystal transition. The peak temperature of the endothermic transition in the cell membrane of *Butyrivibio* S2, which is measured reproducibly in the DSC, is lower than the discontinuity in the $2T_{\parallel}$ vs. $1/T$ plot (Table III). The latter is close to the end point of the thermal transition, which seems to coincide with the minimum temperature supporting growth of the organism (cf. Table II and Figure 4C).

Considering the complex structure of the predominant lipid (lipid 14, cf. Figure 6), the composite transition curve obtained with aqueous dispersions of this lipid is not surprising. The temperature range of the endothermic transition of lipid 14 is similar to that of the endothermic transition observed with the plasma membrane of *Butyrivibrio* S2 (cf. Figures 4 and 5 and Table III). The observation that the high-temperature transitions at about 45 and 54 °C observed with the total lipid extract (Figure 5) exceed the end point of the endothermic transition in *Butyrivibrio* S2 membranes is puzzling. These lipid transitions are also higher than the temperatures at which discontinuities are measured by ESR in membranes or their lipid extracts. We have no explanation for this phenomenon. Lipid tightly associated with integral membrane proteins could, however, be envisaged not to contribute to the lipid phase transition. On the other hand, the fact that there is good agreement between the transition temperatures measured by ESR spectroscopy in cells and in liposomes made from their extracted lipids indicates that the transition temperature is primarily a function of the lipid composition of the membrane with lipid–protein interactions playing a minor role. This conclusion is further corroborated by the finding that the correlation time $\tau$ for the motion of 16-doxylstearic acid in cell membranes and in liposomes made from their extracted lipids is similar. This indicates that the presence of integral membrane protein has little effect on the fast motion of the label located close to the center plane of the membrane. The membrane fluidity (microviscosity) in this region of the

membrane is, therefore, primarily determined by the lipid composition.

Cooling the membrane below the transition temperature may induce the formation of crystalline lipid clusters and concomitantly aggregation of membrane proteins. Upon subsequent heating, the lipid clusters will melt in a cooperative way with little interference from the presence of membrane proteins. This interpretation is consistent with the observation that plasma membranes from *Butyrivibrio* S2 cultured with Tween 80 as the source of fatty acid do not undergo an endothermic transition up to 50 °C. The heterogeneity of the fatty acid composition of Tween 80 may prevent crystallization and hence lipid clustering. An alternative explanation would be that, due to the fatty acid heterogeneity of Tween 80, the cooperativity of the thermal transition is drastically reduced and as a result the transition would be broadened beyond detection (Silvius & McElhaney, 1978).

The ESR results, together with the calorimetry data, provide evidence suggesting that the membrane of *Butyrivibrio* S2 at the growth temperature is in the liquid-crystalline state in spite of tightly packed, highly ordered lipid hydrocarbon chains. Consistent with this conclusion are the following observations: below about 20 °C, the onset temperature of the lipid phase transitions in *Butyrivibrio* S2 (Figure 4, Table III), 5-doxylstearic acid gives immobilized spectra, indicating that molecular motion has ceased. Coincidentally, a significant proportion of the 16-doxylstearic acid spin-label is squeezed out of the membrane; this is also true for the Tempo label (2,2 6,6-tetramethylpiperidinyl-1-oxy), which only exhibited a measurable distribution between the membrane and the aqueous phase above the phase transition temperature (Hazlewood et al., 1980b).

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