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^{2}H-NMR studies on ether lipid-rich bacterial membranes: deuterium order profile of *Clostridium butyricum*

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Palmitic acid specifically deuterated at different carbon atoms, has been incorporated biosynthetically into the membrane lipids of *Clostridium butyricum*. The lipids of this organism are rich in plasmalogens and their glycerol acetals and exhibit an unusual fatty acyl and alkenyl chain distribution with saturated chains mainly at the *sn*-2 position and unsaturated chains at the *sn*-1 position. The ordering of the deuterated hydrocarbon chains in whole cells was measured with deuterium nuclear magnetic resonance and was compared to the order profiles of isolated cell membranes and membranes formed from the total phospholipid extract. The shape of the order profiles was similar for all three membranes, but the absolute values of the order profiles in whole cells and isolated membranes were lower than those of the liposomal lipids. The order profiles have the same characteristic shape as those found for the lamellar liquid-crystalline phases of synthetic diacylphospholipids.

The order profiles of biological membranes have been studied by a variety of physical methods including EPR experiments with spin-labelled fatty acids, proton, carbon-13, and deuterium relaxation times measured by nuclear magnetic resonance (NMR), by measurements of the quadrupole splitting observed with deuterium-labelled fatty acids (reviewed in Refs. 1 and 2) and by ^{19}F-NMR [3]. Because the theory is well understood and the deuterium atom represents a nonperturbing probe, the measurement of quadrupole splittings has proven to be an invaluable technique. A variety of chemically synthesized phospholipids and two biological membranes have been studied by this method [2]. All of the order profiles examined thus far have been obtained with glycerolipids containing diacyl structures. These have included the synthetic phospholipids: dipalmitoylphosphatidylcholine [4,5], dimyristoylphosphatidylcholine [6,7], 1-palmitoyl-2-oleoylphosphatidylcholine [8,9], and dipalmitoylphosphatidylserine [10]. Among the biological membranes studied have been *Escherichia coli*, which contains mainly diacylphosphoglycerides [11], and *Acholeplasma laidlawii*, which principally contains diacylphosphoglycerides and glycosyldiacylglycerols [12].

The membranes of a phylogenetically diverse group of organisms contain plasmalogens, *sn*-1-alk-1′-enyl-2-acyl-3-phosphoglycerides, usually

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mixed with diacyl lipids, in varying proportions. Among prokaryotes, obligatory anaerobic bacteria are often rich in these lipids [13]. Plasmalogens are also commonly found in unicellular protozoa, multicellular invertebrates, and in most tissues of higher animals, in which they may represent from less than one to more than forty percent of the total phospholipid. Tissues rich in plasmalogens are the brain, peripheral nerves, and heart [14]. The specific functions of these phospholipids are unknown, although physical studies have revealed significant differences in their surface potential, gel to liquid-crystalline phase transitions, and polymorphic phase behavior from those of diacylphosphoglycerides with the same polar head groups [15,16,17]. It was therefore of interest to compare the orientational order of the phospholipids in membranes that are rich in ether lipids with those previously observed in membranes containing mainly diacyl lipids. *Clostridium butyricum*, which is rich in both plasmalogens and the glycerol acetal of plasmenylethanolamine, is particularly suitable for such studies. When grown in the absence of biotin, its growth depends on the inclusion of fatty acids in the medium. Under these conditions all of the membrane lipids become highly enriched in the fed fatty acids [16,18]. We have examined the order profile of *Clostridium butyricum* grown on selectively deuterated palmitic acid.

Deuterated fatty acids were prepared as described previously [4,9]. *Clostridium butyricum* ATCC 19398 was maintained on a casamino acid-glucose medium containing biotin [19]. For incorporation of deuterated fatty acids, the cells were first grown for at least two subcultures on a vitamin-free medium containing a non-labelled fatty acid and the final culture, usually 5 liter, was grown with a specifically labelled fatty acid. When the labelled fatty acid is palmitic acid, unlabelled oleic acid is added to promote growth [18]. The final total concentration of fatty acids was 20 mg/l. The fatty acids, as 5% solutions in ethanol, were added after the medium was autoclaved.

The cells were harvested and washed as described in Ref. [16]. Cultures were examined by phase contrast or dark field microscopy for the presence of spores or contaminants. Methods for lipid extraction with chloroform-methanol solutions were basically those of Folch et al. [20]. Phospholipids were separated from neutral and glycolipids by column chromatography on silicic acid [21]. Membranes were prepared from freshly grown cells disrupted in a French pressure cell as described [22], with the omission of the $30\,000 \times g$ centrifugation step. The membrane pellet was washed with deuterium-depleted water. Cells to be directly examined by ^{2}H-NMR were also washed with deuterium-depleted water. Methods for studying the polar lipid, acyl, and alkenyl chain compositions have been described [21]. Phosphorus was measured by the method of Bartlett [23].

For ^{2}H-NMR measurements of lipids, 5 to 20 mg of the desired lipid was dissolved in methylene dichloride-methanol (2 : 1, v/v) and transferred to a sample tube. After the solvent was removed under a stream of nitrogen, the sample was placed under high vacuum until no further change in weight was observed. The dry lipid was resuspended in either pure deuterium-depleted water or 10 mM potassium phosphate buffer, (pH 7.0) containing 5 mM EDTA. The final water content was adjusted to 50% (w/w). To obtain liposomes, the sample was subjected to repeated vortex mixing under nitrogen with several freeze-thaw cycles. For the examination of membranes, approximately 0.5 to 1.0 ml of the $100\,000 \times g$ pellet was transferred directly into a NMR tube. An aliquot of 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA was added so that the final concentration of EDTA was 1 to 2 mM. For whole cell measurements, about 1 ml of freshly harvested and washed cells were transferred under nitrogen, directly into a NMR tube, which was stoppered. Harvesting and washing of these cells was carried out under nitrogen in closed screw cap centrifuge bottles and tubes.

All NMR measurements were carried out on a Bruker Spectrospin CXP-300 spectrometer operating at 46.1 MHz using a quadrupole echo sequence. The pulse width for a 90° pulse was 3.6 μs, the spectral width was 100 kHz and the recycle time was 0.25 s. Typical measuring times were 2 to 4 h for the membranes and whole cells and about 1 h for lipid suspensions.

When *Clostridium butyricum*, a biotin auxotroph, is grown in media devoid of biotin supple-

mented with saturated and unsaturated fatty acids, the saturated moieties are mostly incorporated into the *sn*-2 acyl chains of the diacylphospholipids, plasmalogens, and the glycerol acetal of plasmenylethanolamine. Unsaturated fatty acids are mainly incorporated into the *sn*-1 acyl chains of the diacyl phospholipids, the alk-1-enyl chains of the plasmalogens, and the alkyl chains of the glycerol acetal of the plasmalogen [18]. Thus, an unusual 1-unsaturated, 2-saturated asymmetry is seen in most, if not all of the major lipid classes. Data on the incorporation of deuterated fatty acids into cellular phospholipids is presented in Table I.

It can be seen that deuterium-labelled palmitic acid was predominantly incorporated into the phospholipid acyl chains. The aldehyde moieties, which include the alkenyl chains of the plasmalogens and the alkyl chains of the glycerol acetal of plasmenylethanolamine, were over 95% unsaturated or cyclopropane chains. Parallel studies in which the various lipid fractions were isolated from larger cultures, and the distribution of acyl chains in the diacyl phosphatidylethanolamine was determined, have shown that palmitic acid was largely incorporated into the *sn*-2 chains. An average of 25% of the phosphatidylethanolamine *sn*-1 acyl chains were palmitic acid. The plasmalogen content of the phosphatidylethanolamine fraction was from 50 to 70%. The mixed phosphatidylglycerol plus cardiolipin fraction had 25 to 30% plasmalogen [24].

Fig. 1 shows the order profiles of the incorporated fatty acids in whole cells, isolated membranes, and liposomes reconstituted from the extracted polar lipid fraction. Data were obtained at 37°C which is above T_m for phosphatidylethanolamine ($T_m = 14°C$), the glycerol acetal of plasmenylethanolamine ($T_m = 31°C$), and that of the phosphatidylglycerol/cardiolipin fraction from cells grown on palmitate/oleate (60:40 w/w) (Ref. 24, and unpublished data). All three profiles exhibit the same shape previously seen with liposomes containing synthetic diacylphosphoglycerides, and in biological membranes rich in acylglycerolipids [2,3]. There is a region of almost constant [S_{mol}] extending from the C-4 segment to the C-9 segment, decreasing towards the C-13 segment. Both qualitatively and quantitatively these order profiles compare well with those observed for 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) liposomes [8,9]. With POPC the maximum [S_{mol}] value was about 0.4 around the C-6/C-7 segment, which agrees well with our value of 0.47 for the C-7 segment of the liposomes. This coincidence is quite unexpected since POPC is a synthetic lipid with a well-defined fatty acid com-

TABLE I

ALIPHATIC CHAIN COMPOSITION OF *CLOSTRIDIUM BUTYRICUM* GROWN ON DEUTERIUM-LABELLED FATTY ACIDS

Cells grown on palmitic acid/oleic acid (60:40, w/w)

Position of label palmitic acid	Acyl chains (wt% 16:0)	Aldehydes [a] (wt% 16:0)
4,4	65.1 [b]	4.6 [b]
7,7	67.3	3.5
9,9	64.9	2.0
12,12	51.5	1.9
13,13	57.0	1.2

[a] Aldehydes are derived from the alk-1-enyl chains of the plasmalogens and the alkyl chains of the glycerol acetal of plasmenylethanolamine.

[b] Most of the remainder of the acyl and aldehyde chains was 18:1 plus C_{19} cyclopropane, presumably *cis*-9,10-methylene octadecanoic acid.

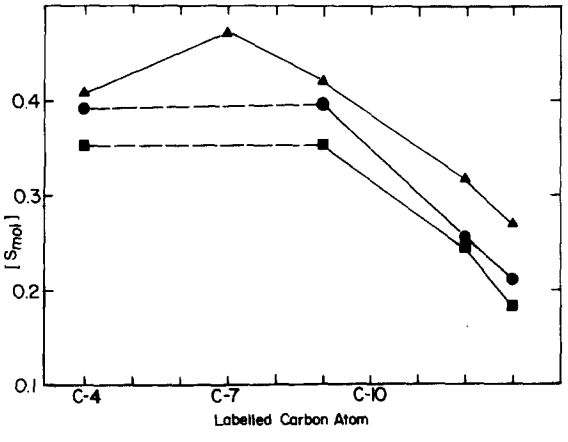

Fig. 1. Variation of the deuterium order parameter [S_{mol}] with segment position for *C. butyricum*. ▲, Lipid dispersion; ●, intact cells; ■, membrane preparation. Measuring temperature 37°C. Samples were prepared as detailed in the text. [S_{mol}] was calculated from the quadrupolar splittings as described previously [11], with $[S_{mol}] = -2S_{C\text{-}^2H}$.

position whereas the order profiles in Fig. 1 represent the combined effects of three different lipid classes, a more heterogeneous hydrocarbon chain composition, and also some variability in the point of attachment (*sn*-1 and *sn*-2) of the incorporated fatty acids. The good agreement between the order profile of the synthetic POPC and the membranes and lipids of *C. butyricum* is indicative of a general average lipid conformation which is largely independent of details in their chemical structures. For reasons not yet understood, no useful signal was obtained in deuterium NMR studies on the membranes and whole cells grown with [7,7-$^2H_2$]palmitic acid. Fig. 2 shows representative ^{2}H-NMR spectra of the cells grown on [9,9-$^2H_2$]palmitic acid/oleic acid (60:40, w/w), and their membranes and lipids. Inspection of the ^{2}H-NMR spectra of the cells and membranes and of the order profiles shows that only one doublet is obtained for all segments beyond C-2. Since the deuterium label is predominantly in the *sn*-2 chains of the diacyl and ether lipids, we may conclude that in mixed ether/diacyl lipid membranes, the ordering pattern of these chains of the two types of lipids is virtually the same as in pure POPC bilayers. A further significant finding of these studies is that the [S_{mol}] values of the fatty acids in whole cells and isolated membranes are generally somewhat lower than those of the liposomal phospholipids.

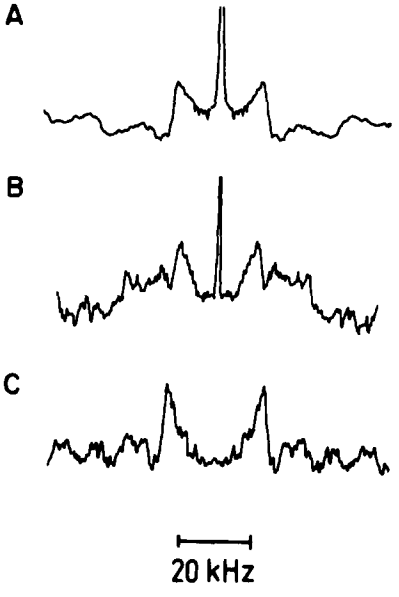

Fig. 2. ^{2}H-NMR spectra (46.1 MHz) of (A) whole cells, (B) membranes, and (C) dispersed phospholipids of *C. butyricum* grown on [9,9-$^2H_2$]palmitic acid/oleic acid (60:40, w/w). The measuring temperature was 37°C. Samples were prepared as detailed in the text. 50000 scans, 100 kHz spectral width, 350 Hz line broadening. The spectra were symmetrized.

Although their lipid compositions differ greatly, *E. coli, C. butyricum,* and *A. laidlawii* deuterium order profiles are remarkably similar. The presence of approximately 40% plasmalogens and 24 to 31% glycerol acetal of plasmalogen in the membranes of *C. butyricum* appears to affect the ordering pattern very little. These data were obtained with lipids and membranes containing labelled palmitic acid, which was mainly present in the *sn*-2 acyl chains of the various lipid classes. Thus conclusions concerning the *sn*-1 acyl, alkenyl and alkyl chains will have to await studies in which these chains have been specifically labelled. As we have shown [24], this can be accomplished by growing these cells with deuterium-labelled unsaturated fatty acids combined with unlabelled saturated fatty acids (unpublished results).

In addition to considerable differences in lipid composition, there are major structural dissimilarities between the organisms in which the deuterium order profile has been determined. *A. laidlawii* has a single plasma membrane, but no cell wall. *E. coli,* a Gram-negative organism, has both a cytoplasmic membrane and an outer membrane, which is outside the rigid peptidoglycan layer. Thus far the order profile has only been obtained with whole *E. coli* cells, but the difference in the order parameters of inner and outer membranes labelled either near the middle of the acyl chains [25], or with perdeuterated fatty acids [26], are relatively small. *C. butyricum,* like *A. laidlawii,* has only a single cytoplasmic membrane, however, it is surrounded by a thick, rigid cell wall. The order profile of the membrane lipids appears to be little affected by the supermolecular organization of the cell envelope. Importantly, in all of the systems studied, the presence of the non-lipid constituents of the cell membrane appears to have a disordering effect on the membrane lipids. This effect has been discussed extensively [2]. The membrane proteins have uneven surfaces at the atomic level, and the fit between lipid hydrocarbon chains and these

surfaces cannot be very close along the length of the chains. On the NMR time-scale this appears to lead to spatial disorder. There may also be density fluctuations along the protein surface itself.

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