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THE INFLUENCE OF pH AND TEMPERATURE ON THE LIMITED ROTATIONAL FREEDOM OF THE STRUCTURED WATER AND LIPID HYDROCARBON CHAINS OF NATURAL MEMBRANES*

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SUMMARY

1. High resolution nuclear magnetic resonance spectroscopy was used to study the effects of pH and temperature on the mobility of the nonpolar part of the membrane lipids, and on the irrotationally bound water at their polar interphase in living cells of *Nocardia asteroides* and *Mycobacterium smegmatis*.

2. pH increments from 5.0 to 8.5 decreased the line width at half peak height of the lipid signal (increase of the lipid mobility) with a concomitant increment in water outflow and leakage of cell constituents.

3. Temperature increments from 2 to 42° increased the lipid mobility at the same time that hydrogen bond splitting occurred among the water molecules.

4. It is suggested that a reduction in the degree of ordered structure of the water molecules at the interphase (increment in water mobility) brings about a correlated increment in lipid mobility which seems to be related to an increased cell permeability (reduction in cell hydrophobicity).

5. The reduction in cell hydrophobicity produced a correlated decrement in the fluorescence of the fluorescent probe 1-anilinonaphthalene-8-sulfonate utilized for studying hydrophobic interactions at the cell membrane.

INTRODUCTION

Since nuclear magnetic resonance (NMR) spectra and relaxation times are influenced by the chemical and physical environment surrounding individual protons, the study of living cells with NMR can help to elucidate certain of the basic characteristics of the structured water at cell interphases and of the lipid phase of natural membranes. In previous communications, I have reported that live microorganisms gave high resolution NMR signals[1], and evidence was presented showing that the 1.3 ppm signal (relative to tetramethylsilane standard) found in *Nocardia asteroides*

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; NMR, nuclear magnetic resonance.

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is due to the cellular lipids and not to other constituents[2]. Recent work of other investigators utilizing spin-labels for the study of membranes also provides evidence for the mobility of long hydrophobic chains of phospholipids[3]. Broadening of the water and lipid signals of Nocardia cells was interpreted as due to hindrance of molecular motion of protons at the cell interphase, because during the salt-breaking effect on the cellular water, there was also an increment in lipid mobility[4]. It was also shown that in cells grown at pH 7.0 on solid media, or in cells exposed to Locke's solution (pH 7.0), the resonance signal belonging to the lipid hydrocarbon chain was very weak and often undetectable. The local magnetic field near a particular nucleus is determined by a number of factors including the polarization of remote parts of the sample, magnetic moments (nuclear and electronic) of neighboring molecules, and intramolecular effects due to other nuclei and electrons in the same molecule. Therefore, the effects of both pH and temperature were studied, and in the present communication I describe alterations in the mobility of the nonpolar part of the membrane lipids and the irrotationally bound water at their polar interphase when the external pH or the temperature are varied. Modifications of cell permeability apparently related to such lipid- and water-altered mobility are also presented and discussed.

MATERIALS AND METHODS

In general the same methodology as previously described[2] has been utilized.

Microorganisms

Because of its high lipid content and ease of handling, an actinomycete of the genus Nocardia was used. *N. asteroides* N-51, from the collection of the School of Medicine, University of Mexico, was used throughout the present study. The stock cultures were maintained and grown on Sabouraud dextrose slants at 28° for 10–12 days. Approx. 100 mg wet wt. of cells were obtained by scraping the contents off three slants (16 cm × 150 mm² tubes). Samples of 100 mg wet wt. of cells were placed in measured amounts (6 ml) of each of the different test solutions and exposed for 60 min at room temperature (about 23°). The cells were spun down and the supernatants separated and stored. The cellular pellet was packed into the microcell for NMR analysis. The cells remained viable after such a procedure as tested by subculturing them in Sabouraud plates. The supernatants were used to measure the cellular leakage by determining their absorption in a Hitachi–Perkin-Elmer double-beam spectrophotometer (124 Model) employing 3-ml cuvettes. The test solutions were: sodium phosphate, ionic strength 0.150, at various pH's from 5.0 to 8.5. To study the effect of temperature, cells directly collected from the surface of solid culture medium (Sabouraud dextrose slants adjusted to pH 7.5 before inoculation) were placed in the NMR microcells in order to detect changes mainly in the cellular water. The NMR spectrometer was a Varian A, 60-Mcycles/sec system. The band positions were taken relative to an external reference of 5 % tetramethylsilane in carbon tetrachloride as zero. All the results were obtained in NMR microcells (Varian Associates) with their pressure equilibration channel enlarged in diameter in order to expel easily the excess of interstitial water from the cellular pellets. The line widths reported are almost always averages for a total of at least eight scans up and

down the field. Temperature studies were done with a Varian Model V 60-40 temperature regulator. Actual probe temperatures were calculated from the chemical shifts of ethyleneglycol, before and after measurement of the sample. The time between preparation of samples and determination of spectra did not exceed 2 h except when the effect of time was also under study.

RESULTS AND DISCUSSION

The effect of pH on the limited mobility of the structured water and lipid hydrocarbon chains in N. asteroides

The effect of pH on the cell lipid chains as the pH was increased from 5 to 8.5 in phosphate buffer solutions at 23° is shown in Fig. 1. The line widths at half peak height, in cycles/sec of the lipid signals, are plotted against the pH. The line width remained almost constant between pH 5 and 6. The initial slight fall at pH 7.0 was followed by an abrupt fall at pH 8.5, indicating an increment in lipid mobility. At alkaline pH the net negative charge at the interphase was increased and the repulsion of the vicinal negative groups must also have increased the water mobility.

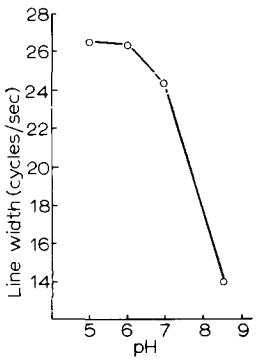


Fig. 1. Line widths at half peak height of the lipid signal arising from living *N. asteroides* cells *vs.* pH. Concn., 100 mg wet wt. of cells cultured on Sabouraud medium for 9 days.

In support of this assumption were the narrowing and heightening of the cell-water peak which rose from 36 mm at pH 5–6 to 42 mm at pH 7.0 and finally to 119 mm at pH 8.5. There was no appreciable change in the water-line position which remained at about 4.8 ppm (Fig. 2, Peak 1).

Care must be taken with cells exposed to alkaline solutions because water is released from the cells as a function of time and this can cause disruption of the cell pellet in the NMR microcell and thus can give rise to peak distortions. Repacking of the pellet shows a smaller area under the cell-water peak indicating that water has effectively been removed from the cell. Because the ionic strength is similar for all tested solutions, the water outflow must reflect a change in water permeability which is facilitated when the net negative charge at the interphase is increased at alkaline pH. These results are in agreement with the suggestion that the entry of water into the membrane requires the presence of a polar group which is ionized at neutral and alkaline pH's but is undissociated at pH 5.0; this assumption is supported by the evidence that the permeability coefficients for water transport across frog skin are reduced at pH 5.0 (refs. 5 and 6).

After the cell pellet hat been repacked, there was a reduction in the microcell volume resulting in the collapse of the lipid signal but to a lesser degree than that of the water signal. In any case the lipid mobility, as judged by the line width decrement, remained higher at pH 8.5 than at pH 7.0, 6.0 or 5.0. After 4–6 h, the signal belonging to the lipid chains became a triplet in the cells exposed at pH 8.5 but not in the others (Fig. 2, Peak 2). After prolonged intervals (12–24 h) the lipid signal broadened. That the bulk of the Nocardia cells remained alive after all the above-mentioned manipulations was demonstrated by subculturing them from the NMR microcell pellets. The leakage of cell constituents as detected by the absorbance of the supernatants at 260 nm also indicates that the cell permeability is higher at alkaline pH. The cellular leakage measured as absorbance at 260 nm and expressed in absorbance units was 0.070 for the supernatant of the cells exposed to pH 5–6; 0.193 at pH 7.0; 0.221 at pH 7.4; and 0.236 for those exposed at pH 8.0.

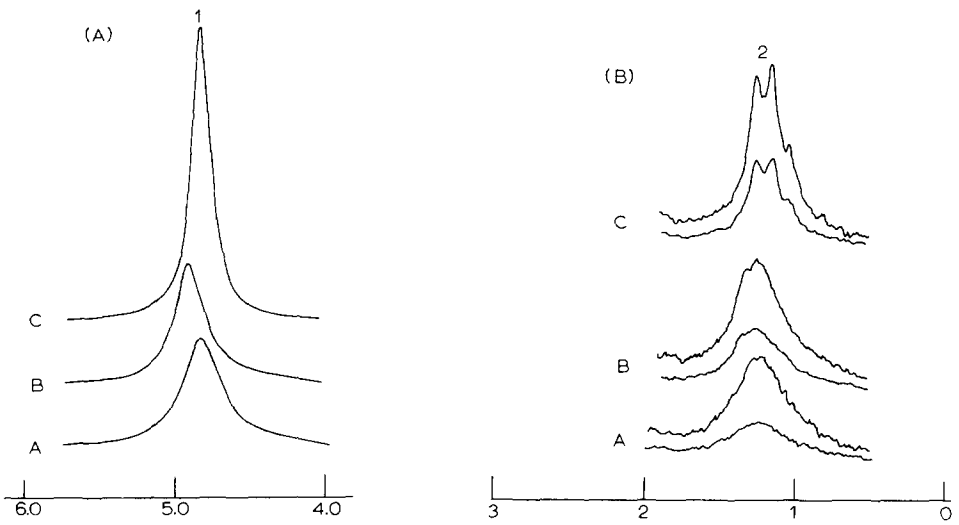
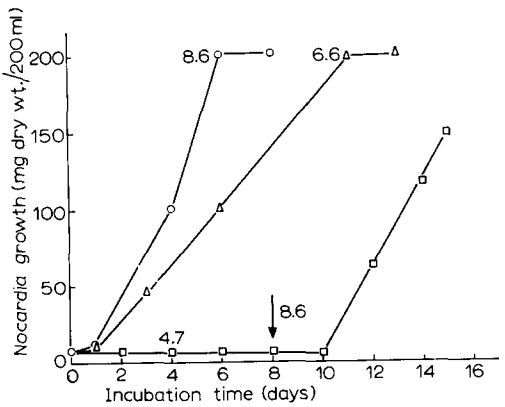


Fig. 2. Spectra of *N. asteroides* cells after exposure to phosphate buffer solutions pH 5–6 (A), 7.0 (B) and 8.5 (C). Peak 1 at about 4.8 ppm is due to water protons and Peak 2 at about 1.3 ppm is due to $(CH_2)_n$ protons. The spectra were recorded on the 60-Mcycles/sec instrument (single scan) at 31°. Spectrum at magnification × 1 for the water signal(s); × 10– × 20 for the lipid signal(s).

On the assumption that at alkaline pH the water permeability of the hydrophobic Nocardia increases, the effect of pH on their capacity for growing in liquid culture medium (nutrient broth, 2% glucose, 0.5% yeast extract and 0.5 mM phosphate) was tested. Fig. 3 shows that at pH 8.5 the cells grew faster than at pH 6.6 and dit not grow at all at pH 4.7. If after 8 days the pH of the culture medium was increased from 4.7 to 8.6 (by the addition of 1.0 M NaOH), the cells were able to resume normal growth. Since the Nocardia cells require the presence of phosphate for glucose uptake[7], and since glucose is the main carbon source in the medium, the results suggest that HPO_4^{2-} could be the polar group responsible for glucose uptake perhaps through reducing the cell hydrophobicity (increasing water permeability), thereby allowing adequate interaction of glucose with its transport system.

The effect of temperature on the limited mobility of the structured water and lipid hydrocarbon chains in N. asteroides

Fig. 4 shows the effect of temperature on the cell lipid chains as the temperature was increased from 2 to 43° at pH 7.5. The line widths at half peak height seemed to fall from a value of about 18.5 cycles/sec at 2° to values of about 12 cycles/sec at 43°. These observations are consistent with the idea that as the temperature increases the lipid chains become more mobile and at the same time the structured water at the cell surface becomes less stable. This can be substantiated from the observed shift of the cell-water peak displaced from 4.92 ppm at 2° to 4.58 ppm at 43°. This shift of the water signal can be interpreted as hydrogen bond splitting among the water molecules. Variations of line width with temperature, similar to those reported here for lipids of living cells, have been observed with NMR for the hydrophobic part of amphipatic molecules[8]. All these results, along with that previously reported[1,2,4], can be interpreted as follows: a higher degree of mobility of the lipid molecules when the structured water at their charged surface is removed leads to increased water permeability and leakage of cellular constituents.



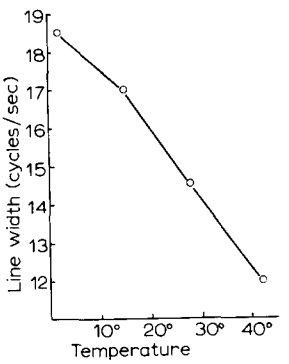


Fig. 3. Effect of pH on the capacity of *N. asteroides* cells to grow in liquid medium (nutrient broth, 2% glucose, 0.5% yeast extract and 0.5 mM phosphate). The arrow indicates the time at which the pH was adjusted from 4.7 to 8.6.

Fig. 4. Line widths at half peak height of the lipid signal arising from living *N. asteroides* cells *vs.* temperature. Concn. 100 mg wet wt. of cells cultured on Sabouraud medium for 9 days.

An identical series of experiments was conducted using *Mycobacterium smegmatis* ATCC 101, and similar results were obtained. The NMR water and lipid signals from this microorganism increased significantly at alkaline pH or when the temperature was raised and *vice versa*; pH or temperature decrements led to a reduced water and lipid mobility. The absorbance of the supernatants at alkaline pH also indicates an increment in the leakage of cell constituents. The fact that Ca^{2+} extrudes water from the cells without increasing the leakage of cellular constituents[2] suggests that Ca^{2+} mainly dehydrates the cell membrane rendering it more hydrophobic.

In order to obtain further evidence on these last points, the interaction of the fluorescent probe 1-anilinonaphthalene-8-sulfonate (ANS) with living microorganisms was studied. ANS has been described as a probe for hydrophobic–hydrophilic

interactions in erythrocyte membranes[9]. As suggested by the NMR results, a reduction in the degree of ordered structure of the water molecules at the interphase (increment in water mobility) caused by increments in pH or temperature produced a correlated increment in lipid mobility and increased the cell permeability (enhanced leakage and water outflow) through reducing the cell hydrophobicity. This should lead to a decrease in the fluorescence of ANS when the pH or temperature are raised and *vice versa* owing to the variations in the hydrophobic characteristics of Nocardia. Ca^{2+}, which seems to increase the cell hydrophobicity and reduce the cell permeability (leakage)[2], should also give rise to increments in the fluorescence of ANS.

A suspension of water-washed living Nocardia containing 9.0 mg dry wt. of cells per ml was prepared and exposed to 20 μM ANS under various experimental conditions: pH from 5 to 8; temperature from 1 to 26°; as well as 30–60 mosM $CaCl_2$ or NaCl. The binding of ANS to the microorganisms was measured by determining the fluorescence of the cell suspensions in a Turner spectrofluorimeter Model 111-50 R. Since ANS does not fluoresce in aqueous solutions, the appearance of fluorescence indicates the interaction of the dye molecules with the microorganisms.

Fluorescence of the microorganisms is shown to be highly dependent on pH and temperature (Table I). The fluorescence of ANS bound to the microorganisms

TABLE I

EFFECT OF ADDED SALTS, pH AND TEMPERATURE ON THE RELATIVE FLUORESCENCE INTENSITIES AND BINDING OF ANS TO *N. asteroides*.

The ANS concn. was 20 μM. The *N. asteroides* cells were water-washed and the cell concentration varied from 8.4 to 9.3 mg dry wt. For the pH experiments, 0.15 M "Trizma·HCl" was adjusted at the desired pH with 0.15 M "Trizma base".

System	*Relative fluorescence*	*ANS bound (μmoles/g dry wt. of cells)*
Water-washed cells	0	0
+ 30 mosM NaCl	15.0	0.66
+ 60 mosM NaCl	27.0	1.53
+ 30 mosM $CaCl_2$	54.0	2.44
+ 60 mosM $CaCl_2$	63.0	2.86
Water-washed cells		
at pH 8.0	15.0	
at pH 7.4	18.0	
at pH 7.0	21.0	
at pH 5.0	28.5	
Water-washed cells		
at 26°	3.0	
at 16°	9.0	
at 6°	12.0	
at 1°	15.0	
Water-washed cells + 30 mosM $CaCl_2$		
at 26°	54.0	
at 16°	71.0	
at 6°	91.5	
at 1°	97.5	

increased about 5-fold as the temperature was lowered from 26 to 1°; and increased about 2-fold as the pH was lowered from 8.0 to 5.0. A marked enhanced fluorescence was also observed in the presence of 30–60 mosM $CaCl_2$ as compared with that obtained with the same concentration of NaCl. This increase in fluorescence seems to be due mainly to an increase in the number of molecules bound to the cells rather than to an increment in quantum yield. The amount of ANS bound to the cells was calculated after determination of the free ANS in the supernatant obtained after centrifugation. The free ANS was measured by the fluorescence of an aliquot of the supernatant in dioxane (final concentration 50% dioxane in water) and compared against a standard curve of fluorescence of ANS in 50% dioxane in water. The details of this procedure will be published[10].

The decrease in the amount of structured water at the interphase *plus* the increment in the lipid mobility as well as charge neutralization must facilitate the entrance of ANS molecules into the lipid barrier. Moreover, at alkaline pH, when the hydrophobicity becomes reduced (as indicated by the NMR results obtained in the present study), the fluorescence of ANS in erythrocyte membranes also decreases (C. GITLER, personal communication).

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