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Differences in Interactions of Benzoic Acid and Benzoate with Interfaces

Benjamin J. Peters[†], Allison S. Groninger[‡], Fabio L. Fontes^{§,||}, Dean C. Crick^{§,||}, and Debbie C. Crans^{†,§}

[†]Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523, United States

[‡]Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523, United States

[§]Cell and Molecular Biology Program, Colorado State University, Fort Collins, Colorado 80523, United States

^{||}Mycobacteria Research Laboratories, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523, United States

Abstract

The interaction of benzoic acid and benzoate with model membrane systems was characterized to understand the molecular interactions of the two forms of a simple aromatic acid with the components of the membrane. The microemulsion system based on bis(2ethylhexyl)sulfosuccinate (AOT) allowed determination of the molecular positioning using 1D NMR and 2D NMR spectroscopic methods. Benzoic acid and benzoate were both found to penetrate the membrane/water interfaces; however, the benzoic acid was able to penetrate much deeper and thus is more readily able to traverse a membrane. The Langmuir monolayer model system, using dipalmitoylphosphatidylcholine, was used as a generic membrane lipid for a cell. Compression isotherms of monolayers demonstrated a pH dependent interaction with a lipid monolayer and confirming the pH dependent observations shown in the reverse micellar model system. These studies provide an explanation for the antimicrobial activity of benzoic acid while benzoate is inactive. Furthermore, these studies form the framework upon which we are investigating the mode of bacterial uptake of pyrazinoic acid, the active form of pyrazinamide, a front line drug used to combat tuberculosis.

Graphical Abstract

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.langmuir.6b02073](https://doi.org/10.1021/acs.langmuir.6b02073).
Calculated pK_a values; 1H NMR and 1H - 1H NOESY NMR spectra (PDF)

Notes

The authors declare no competing financial interest.

INTRODUCTION

As one of the most commonly used food preservatives, benzoic acid (HB) can prevent the spoilage of foods caused by contamination with bacteria^{1–4} and eukaryotes.^{1,5–7} HB (Figure 1a) is a small, planar, weak aromatic acid that is a powerful agent in preserving food in acidic environments, but ineffective at higher pH, when the proton is lost.¹ As long as the pH of the food is close to the pK_a of HB (4.2),⁸ or less, HB protects food against the growth of contaminating organisms.^{1,2,6,7} As a member of a class of compounds called uncouplers,^{9–11} when HB is protonated, it can alter the pH gradient across the membrane of cells³ and, ultimately, lead to an alteration of the cytoplasmic pH.¹² The ability of HB to acidify the interior of the cell (the cytoplasm) is not only dictated by the pH but also by the nature of the membrane, the ion potential and the cell type.¹ For example, Gram-positive bacteria are much more susceptible to HB [minimum inhibitory concentration (MIC) 100 mg/mL] compared to Gram-negative bacteria (MIC 1600 mg/mL).¹ In this Article, we show that the dependence of the antimicrobial properties of HB on protonation can be understood by considering the interaction of HB or benzoate (B^-) with membranes.

Antimicrobial activity that is dependent on pH is not limited to HB. Other small carboxylic acids have a similar pH dependence and effect on bacteria and yeast.^{2,7} An example, similar in structure to HB, is pyrazinoic acid (Figure 1b), the active form of the antitubercular drug pyrazinamide (Figure 1c).¹³ Pyrazinoic acid collapses the pH gradient across the *Mycobacterium tuberculosis* membrane at pHs 5.5 and 6.5 in a pH dependent manner.¹⁴ Near neutral pH, pyrazinoic acid has been reported to accumulate on the outside of a mycobacterial membrane or near the extracellular wall, but is able to enter the bacterium at a lower pH.¹⁵ HB has also been shown to have a pH dependent effect on *Mycobacterium*

tuberculosis with an MIC of 111 mg/L at pH 6.8 and 37 mg/L at pH 5.5.⁴ Thus, there are similarities between HB and pyrazinoic acid, in biological effects, which appear to be related to the physical similarities of these compounds (Figure 1).

While there is a large body of literature on HB's biological effects and chemical properties, limited information is available on the interaction of HB at a water-membrane interface. Phospholipid and surfactant interfaces have hydrophobic and hydrophilic components and, since, HB is able to dissolve in both organic nonpolar solvents as well as water,¹⁶ it should readily traverse the membrane.^{17,18} The interaction of HB with a surfactant interface was previously studied using both cationic and anionic micelles.¹⁹ Absorbance and fluorescence spectroscopies were used to demonstrate that association of HB and B⁻ resulted in changes in the orbital levels, as determined by the shifts in the absorbance and fluorescence signals.¹⁹ Here we explore these molecular interactions in detail using different model membrane systems and analytical techniques at varying pH values.

To fully characterize the interaction of HB with model membrane systems, it is necessary to consider both HB and B⁻. The model membrane systems used in this study include microemulsions, and Langmuir monolayers, which mimic one leaflet of a cell membrane (Figure 2). The microemulsion system is a ternary system, which contains self-aggregates in the form of structures containing the surfactant surrounding the water pool called reverse micelles (Figure 2a). In this system, it is possible to obtain molecular information on the specific placement and interactions of molecules at an interface using the spectroscopic signature of the system.^{22–29} The reverse micelles allow for placement of the drug by spectroscopic characterization of the micellar interfaces by changing the ratio of water to surfactant (w_0).^{21,22,27,30–33} Although this system is powerful in providing the molecular detail, Langmuir monolayers of dipalmitoylphosphatidylcholine (DPPC) are frequently used models of eukaryotic cell membranes. Langmuir monolayers have been used previously for a variety of studies, detailing interactions of lipids with cholesterol,³⁴ phospholipids, fatty acids,³⁵ proteins,^{36,37} and drugs.^{38–41} By measuring the surface pressure of a phospholipid monolayer as a function of area per molecule, it is possible to compare a compound's ability to interact with the phospholipid monolayer and determine if it is affecting the rigidity of the monolayer or the packing of the phospholipids.⁴¹ With the combination of these two techniques, it is possible to obtain an understanding of the interactions of small molecules at a surfactant/phospholipid interface.⁴¹

Using the reverse micelle microemulsion model system, we demonstrate the molecular placement of both HB and B⁻ at the membrane interface, an observation that is supported using Langmuir monolayers. This study demonstrates the importance of the protonation state of HB and other weak acids, and thus pH, on their interactions with membrane interfaces.

EXPERIMENTAL SECTION

Materials and Methods.

Most reagents were used without further purification including HB (Sigma-Aldrich, 99.5%), 2,2,4-trimethylpentane (isooctane, Sigma-Aldrich, 99.0%), deuterium oxide

(D₂O, Cambridge Isotope Laboratories, 99.9%), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids Inc., >99%), *d*₆-dimethyl sulfoxide containing tetramethyl silane (*d*₆-DMSO, Cambridge Isotope Laboratories, 99.9% + 0.05% TMS), activated charcoal (Sigma-Aldrich, 8–20 mesh), methanol (Omnisolve, 99.9%), 3-(trimethylsilyl)propane-1-sulfonic acid (DSS, Wilmad.), hexane (Fisher Scientific, 99.9%), and isopropanol (EMD, 99.8%). Bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT, Aldrich, 99.8%) was purified using activated charcoal and methanol to remove acidic impurities as described previously.⁴² All pH measurements were conducted using a Thermo Orion 2 Star pH meter with a VWR semimicro pH probe. When conducting the NMR experiments, deuterium oxide was used in the presence of aqueous solutions and the pH was adjusted to consider the presence of deuterium (pD = 0.4 + pH).⁴² The pD is customarily referred to as pH and therefore we refer to pD as pH.⁴²

Preparation of Aqueous Solutions of HB for NMR Experiments.

A stock solution of HB was prepared by dissolving HB (0.031 g, 0.25 mmol) in D₂O (25.00 mL). This 10.0 mM solution was then pipetted into aliquots and the pH of each solution was adjusted using NaOD or DCl (1.0 and 0.1 M).

Preparation of AOT-Isooctane Stock Solution and Reverse Micelles.

The 750 mM AOT stock solution was prepared by dissolving AOT (8.34 g, 18.8 mmol) in 25.00 mL of isooctane and vortexed until clear. A range of w_0 values from 8 to 20 were prepared by adding specific volumes of the aqueous HB aliquots to a 750 mM AOT stock solution and vortexing the microemulsion until clear.

¹H NMR Studies on Reverse Micelle and D₂O Samples.

The ¹H NMR experiments were performed using a 400 MHz Varian NMR spectrometer using standard parameters at 25 °C. The aqueous samples were referenced to an external DSS sample. Reverse micelle samples were referenced to the isooctane methyl peak (0.904 ppm) as previously reported,⁴² and data was processed using MestreNova NMR processing software version 10.0.1 and OriginPro version 9.61. The p*K*_a of the HB/B[−] pair was determined from the titration curve prepared by plotting the chemical shifts at different pH values.

B[−] in Reverse Micelles and the ¹H–¹H 2D NOESY Experiments.

To prepare the 200 mM B[−] aqueous stock solution (43 mM in overall sample), HB (0.0050 g, 0.040 mmol) was dissolved into 2 mL of doubly deionized H₂O (ddiH₂O) as the pH was adjusted to 7.8 using 1.0 and 0.1 M of HCl and NaOH. The 750 mM AOT stock solution was prepared by dissolving the sodium salt of AOT (0.333 g, 0.750 mmol) into 1 mL of 95% isooctane/5% cyclohexane (v/v). The reverse micelles were prepared by mixing 215 μL of the B[−] aqueous stock solution with 785 μL of AOT stock solution and vortexed until clear.

The ¹H–¹H NOESY NMR experiment was conducted using a 500 MHz Varian NMR at 25 °C with 32 scans per transient and 200 transient pairs in the f1 dimension with a standard pulse sequence with a mixing time of 200 ms locking onto *d*₁₂-cyclohexane similar to that reported previously.²⁶ The spectrum was referenced to the upfield isooctane methyl peak at

0.904 ppm as previously reported⁴² and the data was processed using MestreNova NMR processing software version 10.0.1.

HB in Reverse Micelles and the ^1H – ^1H 2D NOESY Experiments.

To prepare the aqueous 200 mM HB sample, HB (0.0053 g, 0.043 mmol) was weighed and 215 μL of D_2O was added to the solid. To that mixture, 785 μL of 750 mM AOT in isooctane was added and the suspension was vortexed until clear to form w_0 16 reverse micelles. The concentration of the HB in the water pool of the reverse micelles was 200 mM but when considering the overall solution (isooctane and H_2O) the concentration was 43 mM. The pH of this aqueous solution was determined by comparing the chemical shifts of an aqueous saturated HB solution pH ^1H NMR spectrum. We and others have previously found effects on $\text{p}K_a$ values near interfaces^{43,44} so the pH values reported here are for the aqueous solution prior to the addition to the reverse micelle.

The sample was analyzed using a ^1H – ^1H NOESY NMR experiment using a 500 MHz Varian NMR at 25 °C as described above for B^- .

Reverse Micelle Sample Preparation for Dynamic Light Scattering (DLS) Measurements.

HB (0.012 g, 0.10 mmol) was dissolved into 10 mL of ddiH_2O to prepare a 10.0 mM stock solution. The pH of aliquots (5 mL) of the HB stock solution were adjusted with 1.0 and 0.1 M of HCl and NaOH, respectively. The 100 mM AOT stock solution was prepared by dissolving purified AOT (8.89 g, 20.0 mM) into 200 mL of isooctane and vortexed until clear. The reverse micelles were then prepared by adding specific volumes of HB solution at different pH's or ddiH_2O to specific volumes of AOT stock solution and vortexed until clear to prepare reverse micelle sizes of w_0 20, 16, 12, and 8.

To prepare the 200 mM (5.72 mM overall) samples of HB, 0.0035 g (0.029 mmol) HB was dissolved into 5 mL of w_0 16 reverse micelle solution by vortexing until the solution was clear. Similarly, to prepare the 200 mM B^- solution, 0.049 g (0.40 mmol) of HB was dissolved in 2 mL of ddiH_2O while the pH was adjusted to 9.0 using HCl and NaOH (1.0 and 0.1 M). Then 5 mL of w_0 16 reverse micelle solution was prepared by mixing the 200 mM B^- solution with 100 mM AOT solution.

The 1 cm $\times$ 1 cm glass cuvettes with Teflon cap used for the DLS measurement were cleaned by first rinsing 3 times with isooctane followed by rinsing three times with the sample solution to be measured. The cuvettes were then filled with 1 mL of a reverse micelle sample and capped. Identical results were obtained whether samples were filtered or not. Each sample was made in triplicate and measured according to the following section.

DLS Measurement of Reverse Micelle Solutions.

DLS measurements were conducted using a Zetasizer nano-ZS at 25 °C. Each measurement consisted of a 700 s equilibration time followed by 10 acquisitions consisting of 15 scans for each acquisition. The data was analyzed using Zetasizer software and the values reported are the average of triplicate samples measurements.

Langmuir Monolayer Film Preparation.

Langmuir monolayer films were prepared using a Langmuir Kibron μ troughXS (59 mm wide and 232 mm long). The subphase consisted of ddiH₂O (25 mL) or 1.0 mM HB (0.0031 g, 25 μ mol) at pH 7.0 or 3.0. The pH was adjusted using 1.0 or 0.1 M HCl and NaOH. The lipid stock solution consisted of 2.0 mg (2.7 μ mol) of DPPC in 1 mL of *n*-hexane/isopropanol (3:2, v/v). In a dropwise manner, lipid stock solution (19 nmol) was applied to the surface of the subphase and left to equilibrate for 10 min to allow the solvent to evaporate. The trough barriers were then compressed at a constant rate of 35 mm min⁻¹. Surface pressure of the compression isotherms was measured at 25 °C by a modified Wilhelmy plate method, where a wire probe was used in place of a plate. The results of each measurement are reported as an average of three trials. The compression modulus of each sample was calculated as reported previously.⁴¹

RESULTS AND DISCUSSION

NMR Spectra of Aqueous HB/B⁻.

The spectra of HB in aqueous solution was recorded at pH values ranging from 1.2– 9.3 to yield the titration shown in Figure S1. Between the pH values of 3.6 and 6.0, the three HB aromatic proton peaks shift upfield (–0.17 ppm (H_a), –0.07 ppm (H_b), and –0.14 ppm (H_c)), whereas below pH 3.6 and above pH 6.0 the aromatic HB/B⁻ proton peaks do not shift. These results are interpreted as HB deprotonating as the pH increases above 3.6 forming B⁻. Once the pH reaches 6.0, all of the HB has been converted to B⁻. Using the chemical shifting of H_a, the pK_a was found to be 4.7, which is in agreement with the reported value of 4.65 (D₂O, see Table S1).⁸

NMR Spectra of HB/B⁻ in Reverse Micelles.

Figure 3 shows the ¹H NMR spectra of HB and B⁻ aromatic protons in D₂O and reverse micelles of varying sizes (*w*₀ 20, 16, 12, and 8). The aromatic proton peaks in HB shift from D₂O (Figure 3a): where the H_a proton peak shifts downfield (+0.6 ppm) relative to the peak in reverse micellar solution, H_c and H_b shift upfield (–0.26 and –0.15 ppm, respectively).

The upfield shifting of HB protons (Figure 3a), H_c and H_b, is consistent with reduction in solvent polarity. This would place HB within the AOT aliphatic chains or the isooctane. The slight downfield shift for the H_a proton of HB is most likely caused by the difference in hydrogen bonding with D₂O vs the hydrogen bonding of the carboxylic acid of HB with AOT. When considering that the peaks of HB become sharper with a decrease in size of reverse micelles, HB would be associated with the AOT aliphatic chains; otherwise, there would be no change in the peak broadness with a change in the reverse micellar size. Hydrogen bonding of carboxylic acids in dimeric structures has been reported under aprotic conditions, such as in organic solvents.^{17,18} Although the reverse micelle microemulsion is a heterogeneous environment, and it is conceivable that HB forms dimers under these conditions, the samples contain few HB molecules requiring a high affinity to form when less than one molecule is present in each reverse micelle (see Table 1). When comparing the NMR spectrum of HB in isooctane and isooctane/d₁₂-cyclohexane (95%/5%), where it is predicted to form dimers, to the spectrum of HB in reverse micelles (Figure S2), the spectra

are not identical. The difference in chemical shifts of H_c and H_b in Figure S2 of the dimer in organic solvent and the spectrum observed in the reverse micellar microemulsion suggests that HB is in its monomeric form in the microemulsion.

The shifting of the proton signals for B^- (Figure 3b) exhibits a more gradual downfield shifting pattern for H_a from D_2O to smaller reverse micelles (+0.11 ppm). H_c and H_b protons both have a gradual upfield shifting (−0.12 ppm and −0.05 ppm respectively), coalescing in the w_0 8 spectrum (Figure 3b). All of the peaks in w_0 20 reverse micelles align closely with the peaks in D_2O , while the peaks begin to differ from the D_2O spectrum as the reverse micelles get smaller. Thus, we conclude that B^- is most likely located in the bulk water pool in large reverse micelles and the shifting at lower reverse micelles indicates a size related penetration of B^- into the water/surfactant interface (Stern layer).

NMR Titration of HB in Reverse Micelles.

As can be seen in Figure 4, a titration of HB in a reverse micelle of w_0 16 (hydrodynamic radius of ~4.2 nm) was conducted and spectra in D_2O at low and high pH values were added for comparison. In Figure 4, the H_a peak shifts upfield (−0.19 ppm) as the pH is raised; however, the magnitude of the shifting is very different, suggesting that it is not simply residing in D_2O (see Figure 3). This is consistent with the observation that both H_c and H_b signals in HB and B^- shift downfield in the reverse micelles as pH is raised, in contrast to the D_2O peaks.

The shifting pattern of H_a in reverse micelles of B^- is similar to the shifting pattern of H_a in D_2O . H_c and H_b peaks at low pH are significantly further upfield from the peaks in the D_2O spectrum, suggesting a placement different from bulk D_2O . As the pH is increased, the peaks shift toward the peaks in the D_2O spectrum consistent with deprotonation of the HB to form B^- , consistent with a placement near the Stern layer for the deprotonated species.

The pK_a value for HB was calculated for the different reverse micellar systems investigated and compared to aqueous HB in D_2O (Table S1). In Figure 5, the titration points are shown and the pK_a values for different sizes of reverse micelles were found to be 4.0 (w_0 20), 4.1 (w_0 16, 12), and 3.7 (w_0 8). The pK_a values were determined using H_a chemical shifting because H_a shifted the most in all the spectra. The pK_a values from the other protons were calculated and show a similar trend as H_a , except when signal overlap at the w_0 8 (see Table S1). The difference in pK_a from D_2O (−0.6 to −0.9) is most likely caused by the interactions with the interface of the reverse micelle. Within all the sizes of reverse micelle tested, a similar shifting pattern was observed for the aromatic protons of HB/ B^- as pH was altered, with less shifting occurring as the reverse micelles became smaller.

1H – 1H NOESY Spectra of HB/ B^- in w_0 16 Reverse Micelles.

Since 1D chemical shifting is limited, additional information on the placement of HB and B^- was sought using 2D NMR experiments.^{26,42} For each of these spectra, the concentration of HB and B^- was increased to 200 mM to compensate for a lower sensitivity of the experiments as compared to a 1D 1H NMR experiment. Partial 1H – 1H NOESY spectra of HB/ B^- are shown in Figure 6 to focus on chemical shift regions of interest (see full spectrum in Figure S3). The protons of HB only have cross peaks corresponding to an interaction with

either an AOT methyl or isooctane methyl protons (Figure 6a). In contrast, B^- shows off diagonal cross peaks between protons and the large water peak at 4.6 ppm (Figure 6b).

In Figure 6, there are off-diagonal cross peaks between H_a and H_b/H_c which show that these protons are near each other and serves as an internal control. For a compound buried up in the interface layer it is to be expected that there are much smaller off diagonal cross peaks between the aromatic HB or B^- protons and the water peak. These peaks relate to a direct interaction between the HB or B^- anion and the water pool of the reverse micelle. Figure 6a of HB shows large off-diagonal cross peaks of the H_a and H_b/H_c protons, as expected for the internal check, but much smaller off-diagonal cross peaks relating to the methyl peak at about 1 ppm in the f2 dimension. The cross peaks at about 1 ppm indicate an interaction of HB with methyl protons that may be associated with the isooctane or methyls on the AOT. The intensity of this signal and others in this spectrum is weak, presumably indicative of a low theta angle and thus low NOE intensity of all associated signals or that the interaction between the components is weak.

Similar considerations should be made in Figure 6b. Offdiagonal cross peaks are observed at 1.2, 1.5, and 4.5 ppm in the f2 dimension with the aromatic H_a B^- protons. These signals are consistent with interaction with the water peak as well as the methyl groups at the end of the AOT main chain. Similar signals suggesting proximity with the H_b and H_c protons are not observed; only an off-diagonal cross peak with the water is observed for these protons. Combined, these data suggest directionality in the positioning of the B^- anion. It is somewhat unexpected that the B^- would have the H_a interacting more with the end of the AOT groups than the H_b and H_c protons and may be related to differential intensities of these signals. This observation is compounded by the potential observation of a second population of B^- protons (7.8 ppm), which are not present in a high concentration, but the lifetime of the species is significant because a signal is observed on the diagonal. These off-diagonal cross peaks are in a different phase than the diagonal peaks and as such are reporting on an interaction different than that observed between protons on the aromatic ring with one another. Indeed this is evidence for a temporary existence of this population of B^- associating with the H_2O molecules.

Combined, this data shows a direct interaction of HB and B^- with the interface of the reverse micelle and the water pool, respectively. The HB species penetrating into the reverse micelle interface because the aromatic HB protons are associating with the AOT methyl groups and/or isooctane. The B^- species is interacting with parts of the AOT that are closer to the headgroup and shows direct interaction with the H_2O . The experimental data in Figure 6 shows that HB and B^- are located at different positions in the reverse micelle and that there is a clear protonation state dependence for the penetration of HB/ B^- .

DLS of HB/ B^- in Reverse Micelles.

For further characterization of the interactions of HB/ B^- with reverse micelles, dynamic light scattering was used to document formation of reverse micelles and to determine if HB or B^- affected the reverse micelle size. Because such effects are sensitive to the concentration of the HB and B^- , studies were done both at low concentration (less than one molecule for each reverse micelle), and at high concentration (12.4 molecules for each

reverse micelle). At the low concentrations, it is most likely that HB is not in its dimeric form since the amount of HB per reverse micelle is less than 1 molecule.^{26,45} Table 1 lists the measured hydrodynamic radius, the radius for the water pool, the polydispersity, and the aggregation number that allows for calculation of the numbers of HB or B⁻ per reverse micelle. As shown in Table 1, HB and B⁻ does not affect the size of the reverse micelle at 10 mM but may have a slight effect at 200 mM HB or B⁻. Since the size of the reverse micelles even at 12.4 molecules per reverse micelle does not change, HB or B⁻ interacts with the interface without changing the interface of AOT reverse micelles or the 2–3 layers of water molecules that are adjacent to the Stern layer.²⁰ We conclude that the changes in the structural organization are small enough that the overall volume of the layer and the associated inner water pool do not change. The ability of HB and B⁻ to become part of the interface is consistent with observations in other systems such as dipicolinic acid,^{24,26} and a series of vanadium complexes,^{41,46} but different from a charged compound such as metformin that is exclusively at the interface.⁴⁷

Interactions of HB/B⁻ with Langmuir Monolayers.

HB and B⁻ interactions with lipid DPPC monolayers were investigated for comparison with the studies of the more anionic model membranes generated in AOT microemulsions. The interactions with the DPPC monolayer were studied by comparing the effect of having either HB or B⁻ in the subphase with a system containing only ddiH₂O at varying pH values. As seen in Figure 7a, at higher areas per molecule, HB increases the pressure of the film and B⁻ decreases the pressure between 30 and 80 Å²/molecule, which corresponds to the liquid expanded/condensed state of the lipid monolayer. The compression modulus was calculated from these isotherms (Figure 7b) and, at pH 2.9, the maximum compression modulus of the DPPC monolayer was reached at the same surface pressure for both HB and deionized and ddiH₂O subphases. The maximum compression modulus at pH 7.0 for DPPC monolayers with a subphase of 1 mM B⁻ and ddiH₂O and deionized reached a maximum at the same pressure, but the presence of B⁻ increased the maximum compression modulus.

In the presence of HB, the pressure increases on the monolayer when the monolayer is in the liquid expanded/condensed state. This is consistent with HB penetrating into the monolayer but does not affect the maximum compression modulus and is showing no effect on compressibility of the monolayer. On the other hand, B⁻ decreases the pressure in this same state, by reducing the overall compressibility (increases C_s^{-1}) of the DPPC monolayer. The decrease in pressure shows an interaction with the B⁻ that initially brings molecules in the DPPC monolayer together but, as the area decreases, it prevents the molecular packing as shown by the increase in C_s^{-1} . The decrease in compressibility is consistent with charge repulsion between the B⁻ and phosphate of the DPPC headgroup, while the phospholipids packing at higher areas per molecule of DPPC could be contributed to an ionic interaction between the choline of the DPPC and carboxylate of B⁻. When comparing pressures as a function of area to the control DPPC monolayers, the difference is small overall, but it does show a weak pH dependent interaction with the monolayer for HB and a stronger interaction of B⁻ with DPPC.

Evaluation of the Interactions of HB or B⁻ with Interfaces in Microemulsions and in Langmuir Monolayers.

The interactions of HB and B⁻ at a series of pH values were characterized using different methods to evaluate the effect that protonation state has on the interactions of HB and B⁻ with an interface. The ¹H and ¹H-¹H NOESY NMR reverse micelle experiments show a pH dependent penetration and that HB and B⁻ interact differently with the interface; HB was found to penetrate much deeper in the interface than B⁻. The DLS experiments showed that HB and B⁻ both had a minimal effect on the reverse micelle size, suggesting little effect on the structure of the reverse micelles interface. When looking at interactions of HB and B⁻ with DPPC monolayers, a pH dependent interaction was demonstrated, further confirming the pH dependence for HB and B⁻ for interaction with interfaces.

It is known that weak aromatic carboxylic acids interact at surfactant interfaces in both their anionic and neutral forms.¹⁹ Even the doubly deprotonated dipicolinic acid was found to penetrate deeply into the AOT reverse micelle interface.²⁶ Here, we show that B⁻ did not penetrate as deeply into the interface as dipicolinic acid²⁶ or dipicolinate vanadium complexes.⁴⁶ The location of B⁻ is more similar to that of ascorbate and the vanadium maltolato complex,⁴⁸ which may be caused by the different interactions with the AOT sulfonate. The penetration of HB is similar to the penetration of benzyl alcohol,²⁸ as well as the dianionic dipicolinate²⁶ and some vanadium complexes.⁴⁶ Studies were reported predicting the pK_a values of simple acids and bases near an interphase.⁴⁹ Previously, HB and B⁻ had been shown to interact at the interface of both anionic and cationic micelles but the specific molecular details and the difference in penetration and interactions into the surfactant interface were not explored.¹⁹ Phenols have been shown to exhibit a time dependent protonation caused by hydrogen bonding with the AOT.²⁷ Similar observations were not observed with HB/B⁻. We hypothesize this difference originates in the structural difference of phenol and benzoic acid molecules. A possible result of this difference could be that phenolates are penetrating deeper into the AOT interface than benzoate. Deep penetration into the interface would result in an increase in the pK_a value of the phenol because the protonated form would be the more stable form. The pK_a for benzoic acid changes only a modest amount and not as dramatic as the change in the pK_a value for phenol. The fact these compounds are impacted differently by the reverse micelle is consistent with a fundamentally different placement and interaction with the interface. We show here that HB is able to penetrate into a surfactant interface much deeper than the corresponding anion B⁻ but that both compounds penetrate the surfactant/lipid interface.

Structure and Biological Implications.

As a common preservative, it is important to investigate the uptake and interactions of HB with membrane interfaces. Importantly, HB is limited as a food preservative to acidic food and it follows that HB, rather than B⁻, is the active form.¹ We show here that HB will penetrate deeper into an interface than the deprotonated B⁻ and thus that HB is more readily able to penetrate a membrane. We illustrate the process in Figure 8.

Because cell membranes generally consist of bilayers and there is a difference in pH across the membrane,⁵⁰ the pH of both sides of the bilayer is important. In an acidic environment,

most cells will maintain a neutral pH in the cytosol to keep cellular function operating.^{50,51} When HB is approaching the membrane from the outside of the cell in an acidic environment, it can readily penetrate the membrane and reach the neutral cytoplasm. Deprotonation of HB in the higher pH environment will provide a proton which will reduce the cytoplasmic pH as is commonly observed for uncouplers.⁵⁰ These considerations may explain the relative intrinsic resistance of Gram-negative bacteria to HB. The Gram-positive bacteria have one membrane (Figure 8a), whereas the Gramnegative bacteria have two membranes (Figure 8b). Since HB deprotonates after passing through the first membrane, it will not be able to penetrate the second membrane (Figure 8b). If the periplasmic space acidifies, then HB could diffuse through both membranes and reach the cytosol in the Gram-negative bacterium as illustrated in Figure 8c, illustrating how the Gramnegative bacteria ultimately succumb to such compounds at higher concentration than Gram-positive bacteria.⁵⁰

The observed pH dependence of HB placement and biological properties is likely to extend to other aromatic carboxylic acids as well, and possibly even aliphatic acids.^{2,7,12} When considering compounds that have other functional groups, it is important to evaluate how those functional groups affect the overall physical properties of the compound and the environment.^{52,53} For instance, pyrazinoic acid is structurally very similar to HB, but the presence of the nitrogen atoms on the pyrazine ring dramatically changes the polarity and physical properties of the compound. This seemingly small difference alters the electronics and solubility of the molecule and, consequently, will alter interactions of the compound with a membrane interface. Detailed analyses are necessary to fully understand these seemingly simple but yet very complex systems in order to understand the mode of action of these simple aromatic compounds.

CONCLUSIONS

We find that HB and B⁻ have very different placements at the water membrane-like interface in two different model membrane systems. HB penetrates deeply while B⁻ will reside at the Stern layer in an AOT microemulsion system. This placement is supported by studies pH dependence is consistent in Langmuir monolayers, documenting that these results can be extended to interfaces prepared by membrane-lipids. These interactions are primarily attributed to the differences in charge (i.e., protonation state) and result in distinct interactions with the surfactant itself. In summary, we show here that the protonation state of a weak aromatic carboxylic acid can alter the interactions of a surfactant/lipid interface and that these observations can explain the properties of a very common food stabilizing agent, benzoic acid (HB).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of (a) benzoic acid (HB) with labeled aromatic protons, (b) pyrazinoic acid, and
(c) pyrazinamide.

a: Reverse Micelle

b: Langmuir Monolayer

Figure 2.

Schematic diagram of an aerosol-OT reverse micelle present in a microemulsion (a). The bulk water pool, interface of water and surfactant (Stern layer), surfactant aliphatic chains, and organic solvent (isooctane) are labeled (A)–(D), respectively.^{20,21} Schematic diagram of a Langmuir monolayer (b) depicting a layer of phospholipids (pink) on an aqueous subphase (blue), and barriers for monolayer compression (black).

Figure 3.

1D ^1H NMR spectra of 10 mM HB (pH 1.2, a) and 10 mM B^- (pH 9.3, b) aromatic protons inside reverse micelles of varying sizes and D_2O . Peaks are labeled with corresponding protons on HB/ B^- (see Figure 1).

Figure 4. Titration from pH 1.2 to 9.3 of 10 mM HB/B $^-$ in w_0 16 reverse micelles with 10 mM HB/B $^-$ spectra at pH 1.2 and 9.3 for comparison. The protons corresponding to the aromatic protons of HB/B $^-$ are labeled according to the labeling in Figure 1.

Figure 5.

Chemical shifting as a function of pH for H_a (a), H_b (b), and H_c (c) in different sizes of reverse micelles with w_0 8, 12, 18 and 20 and D_2O .

Figure 6. ^1H - ^1H NOESY NMR spectra of 200 mM HB (a) and B^- (b) in w_0 16 reverse micelles. Both spectra were run using the typical ^1H - ^1H NOESY NMR pulse sequence and spectra were processed using a sine weighting function where the cross peaks and diagonal are shown using lines on the spectra. Important peaks are labeled and refer to Figure 1 in the Introduction for HB structure.

Figure 7.

Compression isotherm surface pressure measurements of DPPC monolayer as a function of area per molecule (a) with the subphase at either pH 7.0 or 2.9 and consisting of either 1 mM HB/B⁻ or ddiH₂O. The compression modulus (b) was calculated from the compression isotherm data as reported previously⁴¹ and was graphed as a function of surface pressure with the maximum C_s^{-1} values indicated by the arrows.

Figure 8. Illustration of HB diffusing across generalized Gram-positive (a) and Gram-negative (b, c) membranes. The Gram-negative membranes show a dependence on protonation state for HB to be able to cross the second membrane when the periplasm is at a higher pH (b) and at a lower pH (c).

Table 1.Comparing sizes of Reverse Micelles of Different w_0 with and without HB or B^{-a}

w_0	RM content	R_h (nm)	R_w (nm)	PDI	R_h reported (nm) ^b	η_{agg}	HB/B ⁻ per RM
20	water	4.7 ± 0.2	3.6 ± 0.2	0.3 ± 0.1	4.4	302	0
	10 mM B ⁻	4.4 ± 0.3	3.3 ± 0.3	0.6 ± 0.1	4.4	302	1.1
	10 mM HB	4.5 ± 0.4	3.4 ± 0.4	0.4 ± 0.1	4.4	302	1.1
16	water	4.1 ± 0.1	3.0 ± 0.1	0.3 ± 0.1	4.2	215	0
	10 mM B ⁻	4.4 ± 0.8	3.3 ± 0.8	0.4 ± 0.1	4.2	215	0.6
	10 mM HB	4.0 ± 0.3	2.9 ± 0.3	0.4 ± 0.1	4.2	215	0.6
	200 mM B ⁻	3.8 ± 0.1	2.7 ± 0.1	0.3 ± 0.1	4.2	215	12.4
	200 mM HB	4.4 ± 0.7	3.3 ± 0.7	0.4 ± 0.1	4.2	215	12.4
12	water	3.9 ± 0.4	2.8 ± 0.4	0.3 ± 0.1	3.7	129	0
	10 mM B ⁻	3.7 ± 0.2	2.6 ± 0.2	0.3 ± 0.1	3.7	129	0.3
	10 mM HB	3.5 ± 0.3	2.4 ± 0.3	0.4 ± 0.2	3.7	129	0.3
8	water	3.7 ± 0.9	2.6 ± 0.9	0.9 ± 0.1	3.2	72	0
	10 mM B ⁻	3.6 ± 0.8	2.5 ± 0.8	0.2 ± 0.1	3.2	72	0.1
	10 mM HB	3.6 ± 1.0	2.5 ± 1.0	0.2 ± 0.1	3.2	72	0.1

^aThe table outlines the contents of the reverse micelles (RM), their relative observed hydrodynamic radii (R_h), water pool radii (R_w), polydispersity index (PDI), aggregation number (η_{agg}), and molecules per reverse micelle as calculated using the aggregation number (# AOT/RMs);

^bLiterature value is from ref 33.