DOE/ER/14003-7

Energetics and kinetics of anaerobic aromatic and fatty acid degradation

Progress Report

March, 1992 to June, 1995

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June 23, 1995

Prepared for the U. S. Department of Energy under contract number DE-FG05-89-ER14003

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I. Summary.

Factors affecting the rate and extent of benzoate degradation by anaerobic syntrophic consortia were studied. Cocultures of a syntrophic benzoate degrader, strain SB, with a hydrogen/formate-using sulfate reducer degraded benzoate to a threshold that depended on the amount of substrate and acetate present. The benzoate threshold was not a function of the inhibition of benzoate degradation capacity by acetate or the toxicity of the undissociated form of acetate. Rather, a critical or minimal Gibb's free energy value may exist where thermodynamic constraints preclude further benzoate degradation. A sensitive assay to detect low formate concentrations was developed to measure the formate levels when the benzoate threshold was reached. We showed that increased acetate concentrations, even when hydrogen and formate levels are low, affects the extent of benzoate degradation, implicating the importance of interspecies acetate transfer.

In addition to benzoate, various saturated and unsaturated fatty acids, 2-methylbutyrate, and methyl esters of fatty acids supported growth in coculture with a hydrogen-using partner. SB is the only syntrophic bacterium known to use both benzoate and fatty acids. Phylogenetic analysis showed that SB clustered with sulfate reducers in the delta subclass of the *Proteobacteria*. SB grew well in coculture with *Desulfoarculus baarsii*, a sulfate reducer that uses formate but not hydrogen. This unequivocally shows that SB can grow by interspecies formate transfer.

Biochemical studies on the fatty acid-degrading, syntrophic bacterium, *Syntrophomonas wolfei*, showed that the D-isomer of 3-hydroxybutyryl-CoA needed for poly-3-hydroxyalkanoate (PHA) synthesis is made by an acetoacetyl-CoA reductase rather than a stereospecific enoyl-CoA hydratase. The genes for PHA synthesis in *S. wolfei* have been cloned into *Escherichia coli*. *S. wolfei* contains a *c*-type cytochrome that may be involved in electron transport and energy conservation during growth with crotonate. *S. wolfei* also contains low levels formate dehydrogenase activity. Butyrate-grown cells are enzymatically equipped to produce hydrogen rather than formate.

In addition, we have shown that some metal-reducing bacteria can grow by dissimilatory cobalt reduction, and have isolated a phylogenetically distinct dissimilatory iron-reducing bacterium. The physiology and phylogeny of several new halophilic anaerobes was studied. Finally, experiments using glass beads of different sizes showed that pore size restricts that penetration and growth of bacteria, suggesting that pore size is an important factor governing the dispersal and activity of microorganisms in subsurface environments.

II. Work Accomplished.

A. Kinetics and energetics of benzoate degradation.

Many times, initial transformation reactions result in the conversion of diverse aromatic compounds to benzoic acid. Thus, the factors that affect the rate and extent of benzoate degradation may also influence the anaerobic degradation of other aromatic compounds. In methanogenic environments, benzoate is degraded to acetate, H_2, and formate by syntrophic bacteria (7).

The degradation of benzoate is thermodynamically unfavorable unless the reaction is coupled to the use of hydrogen and/or formate by organisms such as methanogens. We have isolated a new syntrophic bacterium, strain SB, that degrades benzoate only in coculture with a H_2/formate-using bacterium such as *Desulfovibrio* strain G-11 according to equation a.

$$benzoate^- + 6\ H_2O \text{ -----> } 3\ acetate^- + HCOO^- + 2\ H_2 + 3\ H^+ \qquad (a)$$
$$\Delta G^{o'} = +\ 69.2\ kJ/mol$$

We have found that cocultures of SB with G-11 degrade benzoate to a threshold concentration below which no further benzoate degradation occurs, even after extended incubations (6, 12).

Thresholds for the biodegradation of synthetic and natural organic compounds, inorganic nutrients, and anaerobic hydrogen utilization have been reported. The existence of thresholds may explain the persistence of trace levels of many organic compounds in natural waters and are important for risk assessment concerning the fate of anthropogenic compounds in the environment. Although the importance of substrate thresholds in microbial ecology is clear, little is known about the cause of thresholds. We have used our discovery of a benzoate threshold to study the causes of substrate thresholds. Since the benzoate threshold is probably the result of a thermodynamic limitation, this may be a useful experimental system to test whether a minimal or critical Gibb's free energy value exits for ATP synthesis as hypothesized by Thauer and Morris (11).

Benzoate degradation by the SB/G-11 coculture reached a threshold value that depended on the amount of substrate (6) and acetate added (12). The buildup of inhibitory levels of formate or hydrogen as a cause of the threshold was excluded (Table 1). The free energy change for benzoate degradation when the threshold was reached was favorable (Table 1). It may be that there is a "critical" of minimal Gibb's free energy value, above which no further benzoate degradation can occur. A value of about 17 to 23 kJ/mol has been proposed as the minimum energy quantum needed to translocate a proton across an energized membrane, equivalent to about 1/4 to 1/3 of an ATP (10). The $\Delta G'$ for benzoate when the threshold is reached is close to this minimum energy value.

Consistent with a thermodynamic explanation for the threshold, the addition of an acetate-using bacterium to cell suspensions with a benzoate threshold resulted in further acetate and benzoate degradation (12). Secondly, no threshold was observed when a thermodynamically more favorable electron acceptor was used (nitrate versus sulfate) even though the overall kinetics of benzoate degradation were much slower with nitrate than with sulfate as the electron acceptor (12). This experiment also excluded toxicity of the undissociated form of acetate as the cause of the threshold. A threshold was not observed when nitrate was substituted for sulfate as the electron acceptor, even though both suspensions had the same initial concentration of acetate, and consequently, the same initial concentration of the undissociated form of acetate.

Increasing acetate concentrations inhibited benzoate degradation (12). The apparent V_{max} and K_m for benzoate degradation decreased with increasing

acetate concentration, but the benzoate degradation capacity (V_{max}/k_m) remained comparable. Simulation studies showed that an uncompetitive inhibition model modified to include a term for the threshold best explained the observed inhibition pattern and that the apparent K_i for acetate inhibition of benzoate degradation was 10.0±0.6 mM (12). Simulation studies based on a variety of inhibition models did not predict a benzoate threshold, even when the initial acetate concentration and the values for V_{max}, k_m, and k_i were varied within reasonable limits. Thus, the threshold was not the result of acetate inhibition of the benzoate degradation capacity. Figure 1 shows an example of this approach where the rate of benzoate degradation with 65 mM acetate is shown. The simulated benzoate degradation curve was generated using an uncompetitive inhibition model (equation b) and the values for V_{max}, k_m, k_i, and S_o obtained from experiments with 65 mM acetate.

$$V_{max}/(1+I/k_i)\, t = S_o - S + K_m/(1 + I/k_i)\ln (S_o/S) \qquad (b)$$

V_{max} is the maximal velocity, K_m is the half-saturation Michealis-Menten constant, K_i is the inhibition constant, I is the inhibitor concentration, S is the substrate concentration, S_o is the initial substrate concentration, and t is time. The above model was modified to include a substrate threshold (12).

An important implication of this work is that the extent of benzoate degradation can be estimated from thermodynamic considerations. If a critical $\Delta G'$ for benzoate degradation exists, then the extent of benzoate degradation can be estimated from the concentrations of acetate, hydrogen and formate at the end of the experiment (12). Fairly good agreement exists between the experimentally obtained benzoate threshold values and those predicted using $\Delta G'_{crit}$ (12). Our work shows that both the rate and extent of substrate degradation by syntrophic consortia are controlled by the efficient removal of end products. In particular, we highlight the need for interspecies acetate transfer.

Finally, we have characterized the kinetics of benzoate degradation by pure cultures of sulfate-reducing bacteria. The V_{max} and K_m for benzoate degradation by *Desulfotomaculum sapomandans* were 8.4± 0.7 nmol/min/mg and 8.6±0.1 μM, respectively, and that for *Desulfovibrio multivorans* were 38.9±4.6 nmol/min/mg and 6.4±1.0 μM, respectively. The V_{max} and k_m for benzoate degradation by SB in coculture with *Desulfovibrio* strain G-11 were 110.9±5.3 nmol/min/mg and 41.7±3.9 μM, respectively (12). The capacities for benzoate degradation (V_{max}/k_m) by the sulfate reducers and SB were comparable, 2.6 per min for SB cocultures and 1.0 to 6.0 per min for the sulfate reducers. Thus, syntrophic associations should be able to compete for benzoate with sulfate-reducing bacteria when sulfate concentrations are high. However, it appears that the degradation of fatty and aromatic acids in sulfate-reducing sediments does not involved syntrophic interactions (2). Why syntrophic associations are not involved in the degradation of these compounds under sulfate-reducing conditions needs further study.

The following papers on this work have been published or have been submitted:

B. T. Hopkins, M. J. McInerney, and V. Warikoo. 1995. Evidence for an anaerobic syntrophic benzoate degradation threshold and isolation of the syntrophic benzoate degrader. Appl. Environ. Microbiol. 61: 526-530. (reprint enclosed)

V. Warikoo, M. J. McInerney, J. A. Robinson, and J. M. Suflita. 1995. Interspecies acetate transfer influences the extent of anaerobic benzoate degradation by syntrophic consortia. Appl. Environ. Microbiol. submitted (manuscript enclosed).

B. Characterization of Strain SB.

Phylogenetic characterization of strain SB suggests that SB clusters in the delta subclass (V. K. Bhupathiraju, M. J. McInerney, R. S. Tanner, and C. R. Woese, unpublished data) as does *Syntrophobacter wolinii* (5) and the two described species in the genus *Syntrophus* (13). The fatty acid-degrading, syntrophic bacteria form a distinct line of decent in the gram-positive phylum (14, 15). Thus, several phylogenetic groups of syntrophic bacteria exist. Another unusual property of SB is that fatty acids, unsaturated fatty acids, 2-methylbutyrate, and methyl esters of fatty acids support growth in coculture with strain G-11 (3). This is the only syntrophic bacterium known to use both fatty acids and benzoate.

The pathway for benzoate metabolism by strain SB was studied by demonstrating the presence of key enzymatic activities (3). Benzoyl-CoA synthetase, glutaryl-CoA dehydrogenase, several ß-oxidation enzymes, and the enzymes needed for substrate-level phosphorylation were detected. These studies suggest that the metabolism of benzoate by SB is similar to that of other benzoate-degrading anaerobes. A manuscript summarizing these studies will be ready for submission in the fall of this year.

C. Role of formate in benzoate metabolism.

Whether hydrogen, formate or both serves as the interspecies electron carrier in syntrophic associations has been a matter of considerable interest in the last several years. We have found that with butyrate-degrading syntrophic cocultures of *Syntrophomonas wolfei* and *Methanospirillum hungatei*, both organisms are enzymatically equipped for hydrogen rather than formate use (N. Q. Wofford and M. J. McInerney, unpublished data). Cells of each organism were separated from each other by Percoll gradients and the activities of hydrogenase and formate dehydrogenase were measured. In *S. wolfei*, the levels of hydrogenase were about 900 times higher than those of formate dehydrogenase. Hydrogenase activity was about 400 times greater than formate dehydrogenase activity in *M. hungatei*. Formate dehydrogenase activity measured by substrate disappearance, radioactive exchange, or spectrophotometrically gave similar results. We have also shown that *S. wolfei*

grows as well in association with a methanogen that uses only hydrogen, *Methanobacterium bryantii* (N. Q. Wofford and M. J. McInerney, unpublished data). Thus, formate transfer does not appear to be required for syntrophic butyrate metabolism.

We have developed a sensitive assay to detect formate (12). Using this method, we found that the formate concentration is about 7 to 8 μM when the benzoate threshold is reached. We have also found that strain SB grows rapidly in coculture with *Desulfoarculus baarsii*, a sulfate reducer that uses formate and acetate, but not hydrogen (V. Warikoo and M. J. McInerney, unpublished data). Since interspecies hydrogen transfer cannot occur in this coculture, this unequivocally shows that SB can grow by interspecies formate transfer.

A manuscript on the role of formate and hydrogen in butyrate metabolism by cocultures of *S. wolfei* with *M. hungatei* will be ready for submission in the fall of this year. Further experiments on the time course of benzoate degradation and product formation are needed before the manuscript on the SB-*D. baarsii* coculture work can be prepared.

D. Metabolism of fatty acids by *S. wolfei*.

During the first two years of the project, work on the enzymology of fatty acid degradation and poly-3-hydroxyalkanoate (PHA) synthesis was completed (1, 8, 9). We studied the enzymes involved in crotonate metabolism by pure cultures of *S. wolfei* and showed that crotonate-grown cells had a crotonyl-CoA: acetate CoA transferase activity that was not detected in butyrate-grown cocultures (9). This suggested that the long lags observed before *S. wolfei* grew with crotonate were initially due to its inability to activate crotonate. Formate dehydrogenase activity was detected in pure cultures of *S. wolfei*, but the activity was low, suggesting a biosynthetic rather than a catabolic role for this enzyme. We also found that *S. wolfei* contains a *c*-type cytochrome that may be involved in electron transfer system since the molar growth yields of *S. wolfei* with crotonate are much higher than those of other crotonate-using anaerobes.

S. wolfei synthesizes poly-3-hydroxyalkanoates using an acetoacetyl-CoA synthetase reaction (1). This enzyme was partially purified and the most pure fractions used either NADH or NADPH as the electron donor. These studies showed that *S. wolfei* makes D-(-)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase activity rather than by a stereospecific enoyl-CoA hydratase and that the reducing equivalents required for PHA synthesis from acetoacetyl-CoA can be supplied from NADH made in ß-oxidation.

S. wolfei uses two pathways for the synthesis of PHA (8). Early in growth, PHA is made directly from a ß-oxidation intermediate without cleavage of a carbon-carbon bond. During the latter stages of growth, PHA is made from the condensation and subsequent reduction of two acetyl-CoA molecules. ß-Ketothiolase is the key enzyme in that controls the synthesis of PHA from acetyl-CoA . This enzyme was purified to near homogeneity from *S. wolfei* (K. Kealy, M. S. Thesis, University of Oklahoma, 1992). The enzyme has a homotetrameric subunit composition. It uses acetoacetyl-CoA but not 3-ketooctanoyl-CoA, indicating that it is specific for short-chain, 3-ketoacyl-CoA substrates. The condensation reaction is strongly inhibited by CoA (apparent k_i

of 3.6 μM) while the thiolysis reaction is competitively inhibited by acetyl-CoA (apparent k_i of 500 μM). This indicates that the synthesis of PHA from acetyl-CoA is favored when the concentration of free CoA is low, and the acetyl-CoA concentration is high. These conditions presumably occur during the latter stages of growth.

Finally, I was able to clone the genes for PHA synthesis from *S. wolfei* into *Escherichia coli* (8).

The following manuscripts have been published or are in preparation.

M. J. McInerney, D. A. Amos, K. S. Kealy, and J. D. Palmer. 1992. Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. FEMS Microbiol. Rev. 103: 195-206. (reprint attached)

M. J. McInerney, and N. Q. Wofford. 1992. Enzymes involved in crotonate metabolism in *Syntrophomonas wolfei.* Arch. Microbiol. 158: 344-349. (reprint attached)

D. A. Amos, and M. J. McInerney. 1993. Formation of D-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase in *Syntrophomonas wolfei* subsp. *wolfei.* Arch. Microbiol. 159: 16-20. (reprint attached)

K. S. Kealy, and M. J. McInerney. 1995. Purification and properties of a ß-ketothiolase from *Syntrophomonas wolfei.* in preparation for Arch. Microbiol.

E. Other projects.

Students that enter my laboratory are given a project involving the isolation an interesting type of microorganism or a project concerning subsurface microbiology to gain experience in anaerobic cultivation methods and expertise in microbial diversity and physiology. Several of these projects have lead to publications that were partially supported by this grant.

V. K. Bhupathiraju, A. Oren, P. K. Sharma, R. S. Tanner, C. R. Woese, and M. J. McInerney. 1994. *Haloanaerobium salsugo* sp. nov., a moderately halophilic anaerobic bacterium from subterranean brine. Int. J. System. Bacteriol. 44: 565-572. (reprint enclosed)

Although the glucose fermentation products of *H. salsugo* suggested that it was a *Halobacteroides* species, 16S rRNA sequencing showed that it was phylogenetically most closely related to *Haloanaerobium* species. We have recently found that the type strain of *Halobacteroides acetoethylicus* should be placed within the genus *Haloanaerobium*. We are also completing physiological and phylogenetic studies of three other isolates. A draft of a manuscript on this latter work will be submitted for publication in the fall of this year.

F. Caccavo, Jr., D. L. Lonergan, D. R. Lovley, M. Davis, J. F. Stolz, and M. J. McInerney. 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. 60: 3752-3759.

A new metal-reducing bacterium that uses both acetate and hydrogen as electron donors, and that can grow by dissimilatory cobalt reduction is described. *G. sulfurreducens* is the first metal reducer described that can use both hydrogen and acetate. The ability of *G. sulfurreducens* to use elemental sulfur as an electron acceptor provides further evidence of the interrelationship between the iron and sulfur cycles.

F. Caccavo, Jr., R. A. Rossello-Mora, W. Ludwig, K. H. Schleifer, and M. J. McInerney. 1995. *Geovibrio ferrireducens*, a novel dissimilatory Fe(III)-reducing bacterium. in preparation for Arch. Microbiol.

A phylogenetically distinct metal-reducing bacterium is described. Along with *Flexistipes sinusarabici*, it forms a separate line of descent within the eubacteria. Recent work in collaboration with J. D. Coates shows that *G. ferrireducens* uses proline and propionate as electron donors for dissimilatory iron reduction. This is the first report of a metal reducer that uses fatty acids other than formate or acetate.

G. Trebbau de Acevedo and M. J. McInerney. 1995. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. J. Indust. Microbiol. in press.

A Venezuelan student had a hypothesis that the unusual lipids found in the *Archea* could be effective as emulsifiers or biosurfactants in enhanced oil recovery. She set up several enrichments and isolated thermophilic eubacteria and archaebacteria that emulsified oil. Further characterization of a methanogenic strain showed that the emulsifier was a cell-wall associated protein. This work provides an explanation why the stimulation of microbial growth in the reservoir results in increased oil production.

P. K. Sharma, and M. J. McInerney. 1994. Effect of grain size on bacterial penetration, reproduction, and metabolic activity in porous glass bead chambers. Appl. Environ. Microbiol. 60: 1481-1486.

The paper shows that growth and metabolism of bacteria in small pores is restricted. Thus, pore size may be an important factor governing the penetration and dispersal of microorganisms in subsurface reservoirs and, consequently, the biodegradation of organic compounds in these reservoirs.

III. References

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3. Bhupathiraju, V. K., and M. J. McIneney. 1995. Characterization of a syntrophic benzoate-degrading bacterium: enzymes involved in benzoate degradation. Abst. Ann. Meet. Amer. Soc. Microbiol. Q-217, p. 438. Amercian Society for Microbiology, Washington, D. C.

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5. Harmsen, H., B. Wullings, A. D. L. Akkermans, W. Ludwig, and A. J. M. Stams. 1993. Phylogenetic analysis of *Syntrophobacter wolinii* reveals a relationship with the sulfate-reducing bacteria. Arch. Microbiol. 160: 238-240.

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10. Schink, B. 1991. Syntrophism among prokaryotes, pp. 276-299. In: A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (eds.), The prokaryotes, 2nd Ed. A handbook on the biology of bacteria: ecophysiology, isolation, identification, and applications. Springer-Verlag, New York.

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12. Warikoo, V. M. J. McInerney, J. A. Robinson, and J. M. Suflita. 1995. Interspecies acetate transfer influences the extent of anaerobic benzoate degradation by syntrophic consortia. Appl. Environ. Microbiol. submitted.

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14. Zhao, H., D. Yeng, C. R. Woese, and M. P. Bryant. 1990. Assignment of *Clostridium bryantii* to *Syntrophospora bryantii* gen. nov. comb., based on 16s rRNA sequence analysis of its crotonate-grown pure culture. Int. J. Syst. Bacteriol. 40: 40-44.

15. Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant. 1993. Assignment of the fatty acid-ß-oxidizing syntrophic bacteria to *Syntropphomonadaceae* fam. nov. on the basis of 16S rRNA sequence analysis. Int. J. System. Bacteriol. 43: 278-286.

Figure 1. Simulation study to determine whether acetate inhibition results in a threshold for benzoate degradation. Symbols: (•) simulation results; (o) *experimental data.*

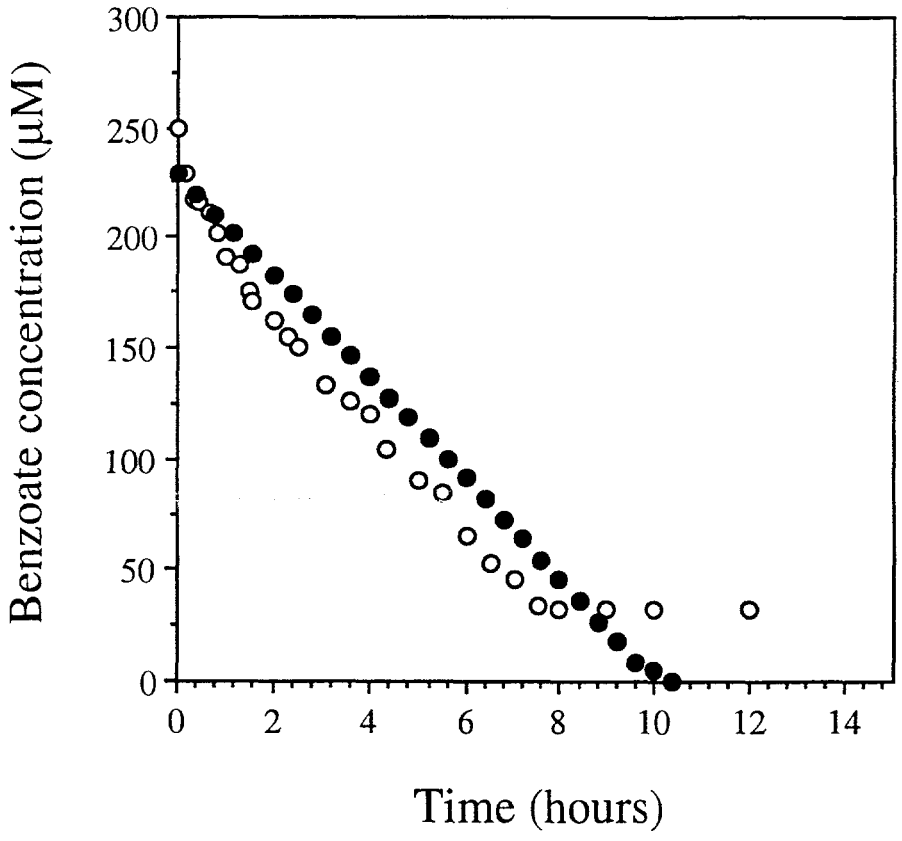

Table 1. The effect of acetate on the benzoate threshold value and the energetics of benzoate degradation by cell suspensions of strain SB with *Desulfovibrio* strain G-11[a].

Additions	Final concentrations				$\Delta G'$ [b]
	Benzoate	Acetate	Hydrogen	Formate	
(mM)	(μM)	(mM)	(Pa)	(μM)	(kJ mol^{-1})
Acetate					
0.0	<0.2	3.0 (0.6)	0.21 (0.06)	8.95 (0.0)	-42.2 (2.8)
10.0	<0.2	13.2 (1.5)	0.08 (0.09)	7.13 (1.6)	-36.4 (1.9)
20.0	2.3 (0.5)	22.0 (2.5)	0.40 (0.03)	8.07 (0.5)	-30.1 (2.0)
65.0	29.8 (0.5)	65.7 (0.2)	0.30 (0.10)	7.22 (1.5)	-30.5 (0.8)
Sodium chloride					
65.0	<0.2	3.1 (0.0)	0.22 (0.0)	9.1 (0.4)	-41.5 (0)

[a] Values are means with the ranges given in parentheses.

[b] A value of 0.1 μM of benzoate was assumed in the calculations where final benzoate concentration was below detection limit.