The Effects of Alkalinity and Hypertonicity on the Morphology and Motility of *Leptospira interrogans* (*biflexa*) strain B16

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SUMMARY

Effects of alkalinity and hypertonicity on the motile behaviour of *Leptospira interrogans* (*biflexa*) B16 were observed, quantified, and compared with effects previously shown by similar factors on the motility of eubacteria. *Leptospira interrogans* tolerated relatively high concentrations of hydroxide ions. Motility similar to that in controls was observed at pH values up to 9.8; but at pH 10.0 motility declined sharply with time of exposure, and there was structural alteration, visible as a blebbing of the cell envelope. Unlike the behaviour of eubacteria, immobilization of *L. interrogans* induced by hydroxide ions could not be reversed by lowering the pH. It is suggested that by restricting entry of hydroxide ions, the cell envelope protects its motility apparatus from adverse effects.

Leptospira interrogans was completely immobilized in 0.5 M and 1.0 M-sucrose solutions. Unlike the eubacteria, leptospire were incapable of spontaneous reversion to motile forms and resumption of motility was dependent on both concentration and time of exposure to sucrose. Deuterium oxide did not affect movement, suggesting that even though leptospire endoflagella and the exoflagella of eubacteria are analogous, the motile behaviour of *L. interrogans* is significantly different from that of eubacteria.

INTRODUCTION

Although the motion of leptospire has fascinated bacteriologists for decades, little is known about their motility and how it is affected by environmental factors. Furthermore, descriptions of leptospiral motility are generally subjective and qualitative rather than quantitative.

Kaiser & Doetsch (1975) showed that translational movement of *Leptospira interrogans* (*biflexa*) B16 was enhanced in viscous solutions. In SM4 medium supplemented with methyl cellulose, there was a marked increase both in the number of individual organisms exhibiting translational movement and in the velocity they attained, with maxima at viscosities exceeding 300 cP. The motile behaviour observed in such very viscous solutions contrasts markedly with that of flagellated bacteria, which show an increase in velocity as viscosity is elevated to 2 to 5 cP (depending on the organism and its flagellar arrangement), but then a rapid decrease in velocity at higher viscosities (Schneider & Doetsch, 1974). Thus environmental viscosity affects the motility of leptospire differently from that of flagellated bacteria.

Two other environmental factors known to influence the motility of eubacteria are pH and hypertonicity. Alkaline pH, in the range 9.4 to 10.0, causes immobilization of various Gram-negative bacteria, presumably by paralyzing their flagella-activating system (Schuetze & Doetsch, 1967). Reversal of paralysis may readily be accomplished by lowering the pH. Plasmolysis of eubacteria with either sucrose solutions (Okrend & Doetsch, 1969)

or $^3\text{H}_2\text{O}$ (Zipf & Doetsch, 1973) also reversibly inhibits bacterial motility, probably by causing a disruption between the cytoplasmic membrane and flagella. Resumption of motility occurs either spontaneously with time or on deplasmolysis (Okrend & Doetsch, 1969).

We describe attempts to quantify the effects of alkalinity and hypertonicity on leptospiral motility, and to compare them with those observed with eubacteria.

METHODS

Culture maintenance. *Leptospira interrogans* (*biflexa*) strain B16, used throughout this study, was graciously provided by Dr C. D. Cox, University of Massachusetts, Amherst, Massachusetts, U.S.A. Strain B16 is a freshwater isolate capable of excellent growth in synthetic media (Henneberry, Baseman & Cox, 1970) and was chosen for study because it is easy to maintain, avirulent, and does not require animal serum. For motility experiments, screw-capped culture tubes containing 10 ml SM4 medium (Henneberry *et al.*, 1970) were inoculated with 0.5 ml stock culture and incubated in a temperature-controlled oscillating shaker at 30 °C for 48 or 72 h. Leptospire had optimum motility during this time.

Preparation of alkaline test solutions. 3-(Cyclohexylamino) propane sulphonic acid (CAPS; Polysciences, Warrington, Pennsylvania, U.S.A.; pK_a 10.4 at 20 °C) was required at 7.5×10^{-3} M to maintain a stable alkaline pH throughout the experiment, without adversely affecting the motion or growth of *L. interrogans*. Immediately before use, 100 ml quantities of the buffer were adjusted to various pH values between 7.3 and 11.0 using 1 M-NaOH, NH_4OH or KOH. pH measurements were made with a Radiometer pH meter model 28 (Radiometer Corp., Copenhagen, Denmark).

In experiments with EDTA, 5×10^{-3} M-disodium EDTA was prepared in 100 ml 7.5×10^{-3} M-CAPS buffer and the final pH was adjusted to 7.3 or 9.6 with 1 M-NaOH.

Preparation of alkaline suspensions. Millipore Swinnex-25 filter holders (SX HA 02505) were modified so that 10 ml syringes could be coupled at each end, and used to force fluids in either direction through the holder without rupturing the filter. Using these, the organism could be separated from its growth medium, washed, and resuspended in test solution without centrifuging (which might have caused structural damage to the leptospiral cell envelope and thereby a change in motility). Repeated filtering, washing and resuspension did not affect leptospiral morphology or motion.

Samples (2 ml) of a 48 h culture of *L. interrogans* were pipetted into the barrel of a 10 ml syringe coupled to a modified Swinnex-25 filter holder, containing a 25 mm diam. Millipore filter, pore size $0.45 \mu\text{m}$. The growth medium was gently forced through the filter so that the leptospire collected on the filter surface. The filter holder was then inverted, another syringe barrel was inserted (on the opposite side to the first one), and 4 ml test solution was forced through the filter to resuspend the organisms which were collected in a clean culture tube. This served as a wash step. The washed suspension was passed in the same manner through a second filter, and the leptospire were again resuspended in the test solution.

Motility studies. Leptospiral test suspension ($10 \mu\text{l}$) was placed on a 75×25 mm glass slide, and covered with a 22×22 mm coverslip (0.19 to 0.25 mm thick), sealed at its edges with petroleum jelly to prevent currents and 'drifting'. The slide was examined immediately under dark-field illumination using a Zeiss Photomicroscope I equipped with a $40 \times$ oil-immersion apochromatic objective. Approximately 200 organisms were counted at random locations and the ratio of motile organisms, i.e. those having either translational or non-translational motion, to total organisms was determined. The first determination was made

immediately after the organism had been suspended in the test solution and subsequent determinations were carried out after 5, 10, 15, 30 and 60 min. Tests were performed in triplicate.

Hypertonicity studies. Sucrose was dissolved in 100 ml 10^{-3} M-3-(*N*-morpholino) propane sulphonic acid (MOPS; Polysciences) to give a final concentration of 0.5 M or 1.0 M, and the final pH was adjusted to 7.3 with 1 M-NaOH. Bacteria from 2 ml of culture were washed and resuspended in these sucrose solutions and 10 μ l portions were examined at intervals to determine the percentage of motile organisms.

For recovery experiments, the leptospire were exposed to 0.5 M or 1.0 M-sucrose for 5, 15, 30 or 60 min, after which, the sucrose was removed and the organisms were washed and resuspended in 4.0 ml of 10^{-3} M-MOPS buffer or SM4 medium. Samples were again examined at intervals to determine the percentage of organisms recovering. Neither the motility nor the morphology of the organism was affected by washing twice with 10^{-3} M-MOPS buffer.

For studies of the effect of suspension in deuterium oxide, bacteria from 0.5 ml of culture were washed and resuspended in 2.0 ml 99.8 % (v/v) $^2\text{H}_2\text{O}$ (pH 6.5). Samples were examined microscopically at intervals for up to 2 h and the percentage of organisms retaining motility was determined.

Dark-field photography. Photographs were taken using a Zeiss Photomicroscope II equipped with a Zeiss microflash device and a Ukatron UN-60 microflash generator. A 60 Ws discharge was used, except for time exposures, which used a 6 V focusing light and four separate 30 Ws discharges fired manually at 5 s intervals. Dark-field illumination was used with a 40 $\times$ or 100 $\times$ oil-immersion apochromatic objective, a 3.2 $\times$ projective, and a 1.25, 1.6 or 2.0 $\times$ optovar. Kodak Tri-X Pan film, ASA 400, was used.

RESULTS

Effect of alkalinity on leptospiral motion

When leptospire were washed and resuspended in 7.5×10^{-3} M-CAPS buffer, adjusted to various pH values with 1 M-NaOH, their motion was affected as shown in Table 1. Even with exposures of up to 1 h at pH 9.8, over 90 % of the organisms showed some form of translational or non-translational motion. When the pH was raised to 10.0, however, the percentage of leptospire showing movement declined drastically with time, ranging from 90 % motility after 5 min to only 7 % motility after 1 h. At pH 10.6, all motion stopped within the first 5 min of exposure.

At pH 9.6 the leptospire appeared morphologically normal (Fig. 1*a*); but at pH 10.0 structural alterations were seen in conjunction with the cessation of motility. After 10 to 15 min in a pH 10.0 solution, small blebs developed at one end of the organism (Fig. 1*b*). After a longer exposure time, more organisms were observed to have blebs, many of which were larger in diameter (Fig. 1*c*). In organisms possessing large blebs, the intact 'non-blebbed' portion was significantly shorter than in organisms with only small blebs. After 1 h a number of spherical membrane-like particles or spherules were evident (Fig. 1*d*). Organisms with visible structural changes were seldom motile, but non-motile cells with no visible alteration in appearance also were observed.

At pH 10.6, all organisms ceased to move within the first 5 min of exposure and almost every one either possessed a bleb or was in a spherule form. Once most of the organisms either began forming blebs or stopped moving, the process could not be reversed by lowering the pH. Motility inhibition and structural damage as a result of alkalinity thus appear to

Table 1. *Effect of alkalinity on leptospiral motion*

Leptospire were suspended in 7.5×10^{-3} M-CAPS buffer, adjusted to the final pH given by addition of 1 M-NaOH. Motility is expressed as the percentage of leptospire showing either translational or non-translational motion of any kind. Each test was performed in triplicate, and the range of results is given in parenthesis.

Final pH	Exposure time (min)	Average motility (%)
7.3	5	95 (93-97)
	10	96 (95-97)
	15	95 (92-97)
	30	93 (92-94)
	60	94 (93-96)
9.6	5	95 (94-96)
	10	93 (92-95)
	15	93 (92-94)
	30	93 (92-94)
	60	94 (93-95)
9.8	5	97 (96-97)
	10	96 (95-97)
	15	96 (95-97)
	30	96 (95-97)
	60	97 (96-98)
10.0	5	90 (84-92)
	10	74 (65-86)
	15	50 (45-55)
	30	25 (19-29)
	60	7 (5-8)
10.6	5	0
	10	0
	15	0
	30	0
	60	0

be permanent. If the pH was increased to 11.0, all cells immediately lysed and assumed a 'moth-eaten' appearance (Fig. 1e).

It seems likely that the small blebs begin as a pulling away of the envelope from the protoplasmic cylinder at one end of the organism. This process continues down the entire length (hence the shortened appearance of cells with large blebs) until the organism assumes a spherical form, presumably with the protoplasmic cylinder folded up on itself within the bounding membrane. Similar changes have been reported in leptospire exposed to other adverse conditions (Auran, Johnson & Ritzi, 1972; Kiktenko, Bakulina & Levina, 1974).

To determine whether Na^+ ions specifically affected leptospiral motion, the same experimental procedures were carried out using KOH or NH_4OH instead of NaOH. The results are summarized in Fig. 2.

It seemed possible that the leptospiral membrane might play a role in the resistance to hydroxide ions. EDTA chelates Mg^{2+} from biological membranes and causes either a breakdown of the membrane or an alteration in its permeability (Indge, 1968). The experiments described above were therefore repeated in the presence of 5×10^{-3} M-disodium EDTA (Fig. 3). At pH 7.3, EDTA did not affect leptospiral motion. The results observed at pH 9.6 in the presence of EDTA, however, are strikingly different from those obtained in the absence of EDTA. In addition, blebs and spherules, such as those normally observed at pH 10.0 to 10.6, were frequently seen at pH 9.6 when EDTA was present in the suspending medium.

Fig. 1. *Leptospira interrogans*. (a) 'Normal' leptospiral appearance in CAPS buffer, pH 9.6. (b) Small bleb at one end of the cell after 15 min in CAPS buffer, pH 10.0. (c) Large bleb at one end of the cell after 30 min at pH 10.0: a spherule form is adjacent to the bleb form. (d) Spherule form, after 60 min exposure at pH 10.0. (e) Appearance in CAPS buffer, pH 11.0. (f) Appearance after 5 min in 0.5 M-sucrose and subsequent resuspension in SM4 medium. Bar marker represents 10 μ m.

Fig. 2

Fig. 3

Fig. 2. Leptospiral motility at pH 9.6 (●, ■, ▲) and pH 10.0 (○, □, △). The percentage of organisms counted showing motion of any kind is plotted against exposure time in the alkaline test solution. pH values were adjusted using 1 M solutions of NaOH (○, ●), KOH (□, ■) or NH₄OH (△, ▲).

Fig. 3. The effect of 5×10^{-3} M-EDTA on leptospiral motion at pH 7.3 and pH 9.6. The percentage of organisms counted showing motion of any kind is plotted against exposure time in the alkaline test solution. pH values were adjusted using 1 M-NaOH. ■, pH 7.3, no EDTA; ●, pH 9.6, no EDTA; ▲, pH 7.3, EDTA present; ○, pH 9.6, EDTA present.

Effect of hypertonicity on leptospiral motion

Suspension in either 0.5 M or 1.0 M-sucrose completely inhibited leptospiral motion. Spontaneous recovery of movement was not observed even after 4 h. Furthermore, when inoculated into SM4 medium supplemented with either 0.5 M or 1.0 M-sucrose, *L. interrogans* did not grow. After 72 h, microscopic examination showed only cell debris.

When the organism was suspended in 0.5 M-sucrose for various periods of time and subsequently washed and resuspended in SM4 medium, some recovery of movement was observed, although it was never complete (Table 2). The average percentage of leptospires recovering dropped significantly as the time of exposure to sucrose increased. Following short exposure periods (5 or 15 min), recovery increased with time after resuspension in SM4 medium; but after longer periods of exposure to sucrose (30 or 60 min), the length of time spent in the recovery medium had no influence. Fewer leptospires were able to regain motility if 1.0 M instead of 0.5 M-sucrose was used as the plasmolysing agent. Similar results were observed if SM4 medium was substituted for MOPS buffer or if MOPS buffer was used as a recovery medium in place of SM4. This indicates that metabolites do not cause the motility inhibition or the subsequent reversals.

When suspended in 0.5 M-sucrose, most organisms showed no structural alteration, although a few were seen to have small blebs at one end. On removal of the sucrose, and subsequent resuspension in SM4 medium, however, many of the organisms which regained their motility were observed to have small blebs at one end of the cell, giving them a 'sperm-like' appearance (Fig. 1*f*). Even after 4 h such cells were still motile and occasionally exhibited translational movement. This indicates that leptospires were capable of motion even with some structural damage to the cell envelope.

Although it has been reported that $^2\text{H}_2\text{O}$ acts as a mild plasmolytic agent for many flagellated bacteria (Zipf & Doetsch, 1973), when *L. interrogans* was removed from its

Table 2. *Reversal of plasmolysis-induced inhibition of leptospiral motility*

Leptospires were exposed to 0.5 M-sucrose for different times, and then the sucrose was removed, and the organisms were washed and resuspended in SM₄ medium. Recovery is expressed as the percentage of total cells counted showing resumption of motion. Each test was performed in triplicate and the range of results is given in parenthesis.

Exposure to sucrose (min)	Motility (%)	Time in SM ₄ medium (min)	Average recovery (%)
5	0	5	55 (44-64)
		10	55 (45-63)
		15	58 (54-62)
		30	67 (59-77)
		60	67 (54-79)
		90	67 (56-75)
		120	70 (60-75)
15	0	5	36 (27-45)
		10	32 (29-36)
		15	40 (39-41)
		30	50 (45-53)
		60	47 (46-50)
		90	51 (49-52)
		120	50 (49-51)
30	0	5	21 (20-23)
		10	21 (17-24)
		15	24 (18-28)
		30	24 (20-28)
		60	22 (16-26)
		90	21 (15-24)
		120	20 (16-23)
60	0	5	3 (1-6)
		10	4 (2-10)
		15	4 (1-8)
		30	4 (0-8)
		60	3 (0-7)
		90	3 (0-6)
		120	3 (0-7)

growth medium, washed, and resuspended in 99.8 % ²H₂O, over 95 % of the leptospires remained motile.

DISCUSSION

Schuetze & Doetsch (1967) reported that various flagellated Gram-negative bacteria were immobilized at pH values of 9.4 to 10.0; for example, *Escherichia coli* was immobilized at pH 9.4, while *Pseudomonas fluorescens* was extremely active at pH 9.4, but non-motile at pH 10.0. On the other hand, *Serratia marcescens* was actively motile at pH 9.8. 'Paralysis' could readily be reversed by lowering the pH with acid; the movement of *E. coli* was stopped and started five times in 30 min simply by raising and lowering the pH. It was suggested that the pH-induced paralysis of eubacteria is an effect on the 'activating system' of their flagella rather than on the flagella themselves.

Leptospira interrogans tolerated relatively high concentrations of hydroxide ions. Motility similar to that in controls was consistently observed at pH 9.6 and continued at pH levels as high as 9.8. At pH 10.0, however, motility declined sharply as the time of exposure to the hydroxide ions increased. Structural alteration was observed concurrently with cessation of motility. Since similar effects were observed when either NaOH, KOH or

NH_4OH was used to adjust the pH, results can be attributed to the hydroxide ions and not the Na^+ , K^+ or NH_4^+ ions. Once most of the leptospire either stopped moving or showed a blebbing of the envelope, these processes could not be reversed by lowering the pH. Thus, hydroxide ion-induced inhibition of leptospiral motion appears to be permanent, in contrast to the reversible immobilization seen in flagellated eubacteria.

This contrast in behaviour is probably due to different mechanisms of propulsion. The eubacteria propel themselves by exoflagella, and alkali-induced paralysis appears to arise from some reversible effect of the hydroxide ions on the flagella-activating system. Leptospire, on the other hand, possess endoflagella which probably serve as their organelles of locomotion (Birch-Andersen, Hovind Hougen & Borg-Petersen, 1973; Nauman, Holt & Cox, 1969). Hydroxide ion-induced immobilization is permanent and appears to be due, at least in part, to structural alterations in the cell envelope.

Evidence from the EDTA experiments implies that by restricting the entry of hydroxide ions, the cell envelope of *L. interrogans* protects its motility apparatus from any adverse effects. However, once the chemical structure or permeability of the cell envelope is altered, as with EDTA, pH levels which normally have no effect on motility can cause immobilization. In addition, structural alterations (such as bleb and spherule formation), normally seen only at high levels of pH, occur at lower pH values. We suggest, therefore, that the leptospiral cell envelope acts as a semipermeable membrane that prevents the entry of hydroxide ions; but that at a certain concentration (pH 10.0), the hydroxide ions themselves begin to cause either an alteration in the permeability of the cell envelope or a modification of its relationship with the protoplasmic cylinder.

Auran *et al.* (1972) reported that, following exposure to 1.0 M-NaCl, the envelope of *Leptospira* serotype *canicola* formed small blebs at one end of the organism. This process then continued along the length of the organism until it became spherical. The protoplasmic cylinder was observed folded back upon itself within the bounding membrane. Since identical forms (small blebs, large blebs and spherules) were observed with *L. interrogans* at pH values of 10.0 to 10.6, it is likely that similar events to those reported by Auran and co-workers occurred here. A plausible explanation for alkali-induced spherule formation and concomitant loss of motion assumes that there is, under normal conditions, an association between the wall of the protoplasmic cylinder and the cell envelope. High concentrations of hydroxide ions modify this association either by altering cell envelope permeability, thus allowing an influx of hydroxide ions, or by causing dissociation of the envelope from the protoplasmic cylinder. As a result, the loosened membrane assumes a spherical form of lowest free energy and forms what might be termed a 'pseudoprotoplast'. During spherulation, motion ceases, either as a direct result of the structural displacement of the envelope or as a consequence of ion-induced inhibition of metabolic reactions associated with motility. Since the endoflagella lie wrapped around the protoplasmic cylinder in the area between the wall and the cell envelope, any displacement of the envelope from the protoplasmic cylinder might prevent their normal functioning. It is equally possible that the hydroxide ions, at high enough concentrations, alter the permeability of the envelope and inhibit motility-associated metabolic reactions independently of the spherulation process.

A second environmental factor shown to affect the motility of leptospire is hypertonicity. *Leptospira interrogans* was completely immobilized in 0.5 M and 1.0 M solutions of sucrose. Unlike eubacteria, which, on plasmolysis with sucrose, revert to motile forms both spontaneously and when deplasmolysed (Okrend & Doetsch, 1969), leptospire showed no spontaneous reversal of hypertonicity-induced motility inhibition. In fact, the degree of

reversal following exposure varied with both the concentration and the time of exposure to the sucrose.

Okrend & Doetsch (1969) suggested that during plasmolysis displacement of the cytoplasmic membrane disrupted flagellar function in eubacteria. This could not be confirmed microscopically, however, with *L. interrogans* because of their narrowness ($0.1\ \mu\text{m}$) and their resistance to staining. Electron microscopy was not attempted because of difficulties normally encountered in resolving the cytoplasmic membrane and cell wall of leptospires and because of the uncertainty of proving that membrane displacement, if observed, occurred as a direct result of the sucrose and not the fixation and dehydration procedures. Evidence indicated that inhibition of motility may have had some biochemical basis (possibly from dehydration effects) since there was no multiplication and the organisms lysed upon incubation in SM4 medium supplemented with $0.5\ \text{M}$ or $1.0\ \text{M}$ sucrose. The manner in which hypertonicity causes the detrimental effects is at present unknown.

Although leptospires possess endoflagella which are morphologically and chemically analogous to the exoflagella of eubacteria, their behaviour in response to various environmental factors differs. These behavioural differences are due, most likely, to the unique morphology of leptospires.

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