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Mechanisms of inhibition by fluoride of urease activities of cell suspensions and biofilms of *Staphylococcus epidermidis, Streptococcus salivarius, Actinomyces naeslundii* and of dental plaque

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Background/aims: Fluoride is known to be a potent inhibitor of bacterial ureases and can also act in the form of hydrofluoric acid as a transmembrane proton conductor to acidify the cytoplasm of intact cells with possible indirect, acid inhibition of urease. Our research objectives were to assess the inhibitory potencies of fluoride for three urease-positive bacteria commonly found in the mouth and to determine the relative importance of direct and indirect inhibition of ureases for overall inhibition of intact cells or biofilms.
Methods: The experimental design involved intact bacteria in suspensions, mono-organism biofilms, cell extracts, and dental plaque. Standard enzymatic assays for ammonia production from urea were used.
Results: We found that ureolysis by cells in suspensions or mono-organism biofilms of *Staphylococcus epidermidis*, *Streptococcus salivarius* or *Actinomyces naeslundii* was inhibited by fluoride at plaque levels of 0.1–0.5 mM in a pH-dependent manner. The results of experiments with the organic weak acids indomethacin and capric acid, which do not directly inhibit urease enzyme, indicated that weak-acid effects leading to cytoplasmic acidification are also involved in fluoride inhibition. However, direct fluoride inhibition of urease appeared to be the major mechanism for reduction in ureolytic activity in acid environments. Results of experiments with freshly harvested supragingival dental plaque indicated responses to fluoride similar to those of *S. salivarius* with pH-dependent fluoride inhibition and both direct and indirect inhibition of urease.
Conclusion: Fluoride can act to diminish alkali production from urea by oral bacteria through direct and indirect mechanisms.

Key words: *Actinomyces naeslundii*; dental plaque; fluoride; *Staphylococcus epidermidis*; *Streptococcus salivarius*; urease

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The production of ammonia by urease-positive bacteria is thought to play an important part in the ecology of the human microbiota in health and disease. In the oral cavity, ammonia production from urea can have beneficial effects in neutralizing acids produced by bacteria in dental plaque and facilitating plaque pH rise, with resultant reductions in cariogenicity (5). However, ammonia production in the mouth is thought to have detrimental effects as well, enhancing the formation of tartar or calculus (27), inducing inflammation at the gingival margin and enhancing inflammation in periodontal pockets (4). Ureolysis has been found (14) to protect *Actinomyces naeslundii* against acid damage and allows the organism to use urea as a nitrogen source for growth. Shu and coworkers (19) recently showed that ammonia production from urea can be important for maintaining bacterial community diversity in multiorganism biofilms by allowing acid-sensitive noncariogenic and periodontal bacteria to survive while cohabiting with acid-tolerant organisms.

The major urease-positive, gram-positive bacteria in the mouth include the streptococci *Streptococcus salivarius* and *Streptococcus vestibularis*, both of which are associated with the microbiota of soft tissues rather than that of teeth. In biofilms associated with teeth, the major gram-positive urease producers are *A. naeslundii* and staphylococci, mainly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (20, 26). *S. epidermidis* was found (18) to be the most ureolytically active organism in plaque.

In other parts of the body, ureolysis can have a number of adverse effects; for example, urease is considered a virulence factor for *Helicobacter pylori,* allowing it to persist in the acid environment of the stomach (3). Ureolytic bacteria are also thought to play major roles in the formation of kidney stones (8). The urease-positive bacteria involved in kidney stone formation include gram-negative *Proteus mirabilis*, other enterics, and Ureaplasma organisms, and also gram-positive staphylococci and corynebacteria. Urease-positive staphylococci are important in urinary infections, and urease is considered a virulence factor (10) for the organisms.

The results of previous studies (5) have indicated that fluoride can inhibit alkali production by oral bacteria from arginine, catalyzed by the arginine-deiminase system, and from urea, catalyzed by urease. Fluoride is a direct inhibitor of bacterial ureases (16, 23) and readily crosses the cell membrane in response to ΔpH when the cytoplasmic pH is higher than that of the environment. Fluoride then would be expected to inhibit urease in the cytoplasm directly. However, fluoride also acts to dissipate ΔpH across the cell membrane and to acidify the cytoplasm (13). Since ureases are acid-sensitive enzymes, acidification of the cytoplasm could result in indirect inhibition by fluoride.

In this manuscript, we considered the two types of fluoride inhibition, direct and indirect, for organisms commonly found in the oral microbiota and exposed to fluoride on a daily basis. We also compared the effects of fluoride on ammonia production by suspension cells and biofilms, as most of the organisms in the mouth live in biofilms or large aggregates. Not only oral bacteria but also bacteria in the urinary track may be exposed regularly to fluoride, as fluoride is excreted mainly through the kidneys. Therefore, our results may be pertinent to the microbiota of many parts of the body.

Material and methods

Bacteria

A. naeslundii ATCC 12104, *S. epidermidis* (University of Rochester strain), and *S. salivarius* ATCC 13419 were grown routinely in our laboratory by weekly culture on tryptic soy agar plates (Difco Laboratories, Detroit, MI) and stored as stock suspensions in 50% (v/v) aqueous glycerol solution at − 70°C.

Intact cells and cell extracts

The organisms were grown in suspension cultures in TY medium containing 3% (w/v) tryptone (Difco) and 0.5% (w/v) yeast extract (Difco) supplemented with 1% (w/v) glucose. They were harvested during the late exponential phase of growth by means of centrifugation in the cold for 15 min at $6362 \times g$. The harvested cells were used for assays of ammonia production as intact cells or were used for preparation of cell extracts.

Cell extracts were obtained by disrupting cells in a Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK) homogenizer. Each pellet was washed and resuspended in 50 mM KH_2PO_4 buffer (pH 7.0) in a tube containing half the pellet volume of 106 μm diameter glass beads (Sigma, St. Louis, MO). The cells were homogenized for 15 (*S. salivarius and S. epidermidis*) or 20 s (*A. naeslundii*) cycles and put in ice for 2 min during the intervals. Phase-contrast microscopy was used to check that disruption of cells was complete. The extracts (supernatants) were recovered by centrifugation for 10 min at $16{,}110 \times g$ in a microcentrifuge.

Biofilms

Biofilms were grown on standard glass microscope slides in fed-batch cultures as described previously (15, 17). The biofilms were formed in multislide culture boxes containing TY medium supplemented with 1% (w/v) sucrose and harvested after 6–8 days of growth. For *S. epidermidis* biofilms, the slides were initially coated with human saliva from two authors to facilitate bacterial adherence. Before use, saliva was centrifuged, filtered, and heat inactivated for 30 min at 65°C.

Some *S. salivarius* biofilms were dispersed in buffer to produce cell suspensions as described by Burne & Marquis (6). Mature, intact biofilms were washed by immersion in 50 mM KH_2PO_4 buffer (pH 7.0) and the biofilm mass then scraped from the slides with a sterile spatula into the same buffer solution. The biomass in suspension was homogenized using a Branson Sonifier Cell Disruptor 200 (Branson Sonic Power, Danbury, CT) for 20 s on ice. To check biofilm disruption with consequent separation of individual cells, the suspensions were observed with phase contrast light microscopy. The observations indicated no damage to individual cells that would have resulted in lowering of the cell refractive index or diminution of phase contrast.

Dental plaque

To assess the effects of fluoride on the urease activity of supragingival dental plaque, we collected plaque from the available surfaces of the teeth of the authors and a colleague, who did not brush their teeth after breakfast on the morning of collection. The plaque samples were pooled into a 50 mM KH_2PO_4 buffer solution (pH 7.0) to give sufficient material for multiple samples in a single experiment. The plaque suspensions were vortexed to break up large clumps.

Urease assays

Urease activity was assayed by determining ammonia production by cells in response to added urea as substrate. Ammonia was measured with the Roche/R-Biopharm Ammonia Kit (Darmstadt, Germany) based on reduction of 2-ketoglutarate to glutamate in the presence of ammonia with loss of reduced NADPH catalyzed by the glutamate dehydrogenase

enzyme. The oxidation of NADPH was assessed from changes in absorbance of light of 340 nm wavelength.

For assays with intact cells, the pellets were washed and resuspended in 50 mM KH_2PO_4 buffer (pH 7.0) to yield suspensions with 6.0 mg (*A. naeslundii*), 2.0 mg (*S. salivarius*), and 0.5 mg (*S. epidermidis*) cell dry weight/ml. Cell extracts were added to the same buffer solution, yielding 50% v/v (*A. naeslundii*), 25% v/v (*S. salivarius*), and 5% v/v (*S. epidermidis*) suspensions.

Each slide of intact biofilm was washed by immersion in 50 mM KH_2PO_4 buffer (pH 7.0) and then kept in fresh buffer to be used for ammonia assays. Dispersed biofilms of *S. salivarius* were assayed in buffered suspensions containing approximately 2.5 mg biofilm dry weight/ml. Dental plaque samples were also assayed in buffered suspensions with approximately 0.3 mg plaque dry weight/ml.

Control intact cells or cell extracts, control intact or dispersed biofilms or plaque were pH adjusted with HCl to values of 4.0, 5.0 or 6.0 determined with a glass electrode. They were then incubated for 5 min prior to the addition of excess urea (25 mM). Test suspensions exposed to different concentrations of sodium fluoride (NaF) were adjusted to the desired pH values and were incubated with fluoride for 5 min before urea addition. After the addition of urea, pH was kept nearly constant by intermittent additions of HCl, if necessary, during the whole assay, which lasted between 1 and 5 h. Ammonia production was measured using the samples taken during the assays.

To assess weak-acid effects of fluoride ($pK_a = 3.15$), two organic weak acids, indomethacin ($pK_a = 4.5$) and capric acid ($pK_a = 4.2$), were used separately at different concentrations in test suspensions to allow comparison of organic-acid effects with those of fluoride. Suspensions treated with organic weak acids were handled in the same way as those treated with fluoride. However, controls contained 0.5% ethanol, which did not affect ureolytic activity because the organic weak acids were dissolved in ethanol, in which they are more soluble than in water.

In another type of experiment, dental plaque samples were suspended in 50 mM KCl and 1 mM $MgCl_2$ solution. The pH of the suspension was adjusted to a desired value and kept constant for 5 min before urea addition. The pH was then allowed to rise. The pH values for both control and fluoride-treated suspensions were recorded during the assay, as well as ammonia production.

Cell and biofilm dry weights were assessed following procedures described by Belli & Marquis (2). Protein assays were performed as described by Lowry et al. (11).

Results

Effects of acidification and fluoride on ammonia production by intact cells and mono-organism biofilms

The data presented in Table 1 indicate that suspension cells of *S. epidermidis* under the specific experimental conditions at pH 6.0 have some 70 times the ureolytic specific activity of cells of *S. salivarius* and some 400 times the activity of cells of *A. naeslundii*. The test periods for assessing urea degradation were extended for the latter two organisms because of their low specific activities. In addition, *S. epidermidis* ureolysis was less sensitive to acidification, most likely because higher ureolytic activity was protective against acid inhibition. It has been found that urease levels, for example for *S. salivarius*, vary depending on growth conditions (21), but the differences here are still considerable for cells grown under similar conditions. In these experiments, saturating levels of urea were used to obtain maximal ureolytic activities.

For all three organisms, ureolysis by biofilms was significantly less than that for cells in suspensions, but the activity was somewhat less sensitive to environmental pH. Ureolysis by biofilms of *S. epidermidis* was some 47 times that of *S. salivarius* and some 85 times that of *A. naeslundii*. The finding that dispersal of biofilms of *S. salivarius* did not greatly enhance ureolytic activity suggests that the diffusion of urea into the biofilms was not the main factor limiting activity.

Fluoride was found to be a potent inhibitor of ammonia production by both intact cells and biofilms in a pH-dependent manner. Inhibitory effects of fluoride on ammonia production by intact cells in suspensions of the test organisms during incubation with excess urea are shown in Fig. 1. In intact cells of *S. epidermidis* in suspensions (Fig. 1A) at a maintained pH value of 6.0, fluoride inhibited ureolysis, but high concentrations were required for significant effects – the 50% inhibitory concentration was greater than 1.0 mM. At pH 5.0, control urease activity was basically the same as that at pH 6.0. However, the sensitivity to fluoride was greatly increased, and the 50% inhibitory concentration was only about 0.1 mM. At pH 4.0, control urease activity was still some 70% that at pH 6.0, but fluoride sensitivity was increased so much that the fluoride concentration for 50% inhibition of pH 4 activity was only about 0.01 mM.

Ureolysis by *S. salivarius* was more sensitive to environmental acidification and to fluoride (Fig. 1B) than ureolysis by *S. epidermidis*. At a pH value of 5.0, the total ammonia output by *S. salivarius* was about half that at a pH value of 6.0, and ureolysis was almost completely inhibited by NaF at a level of only 0.1 mM. At a pH value of 6.0, this level of fluoride caused somewhat more than 50% inhibition. At a pH value of 4.0, ammonia production from urea by *S. salivarius* was greatly reduced, and fluoride further reduced ureolysis to even lower values.

The pattern of fluoride inhibition of ureolysis by intact cells of *A. naeslundii* was closer to that for *S. epidermidis* (Fig. 1A) than for *S. salivarius* (Fig. 1B). Thus, ammonia production from urea was relatively insensitive to fluoride at pH 6.0, was more than 50% inhibited by 0.2 mM NaF at pH 5.0, and was nearly completely inhibited by 0.2 mM NaF at pH 4.0. Ureolysis by intact cells of *A. naeslundii* was less acid sensitive than ureolysis by

Table 1. Ammonia production by suspension cells and biofilms of *S. epidermidis*, *S. salivarius* and *A. naeslundii* at constant pH values

Species	Ureolysis by	pH 6.0	pH 5.0	pH 4.0
S. epidermidis	Intact cells in suspensions (1 h)	174.0 ± 23.6*	172.8 ± 20.9	123.8 ± 30.7
	Intact biofilms (1 h)	22.1 ± 5.0§	33.9 ± 15.3	36.6 ± 4.9
S. salivarius	Intact cells in suspensions (2 h)	4.9 ± 1.4	2.5 ± 0.7	0.7 ± 0.3
	Intact biofilms (3 h)	1.4 ± 0.3	1.4 ± 0.6	1.0 ± 0.6
	Dispersed biofilms (3 h)	1.3 ± 0.3	0.7 ± 0.2	0.3 ± 0.1
A. naeslundii	Intact cells in suspensions (2 h)	0.9 ± 0.6	0.6 ± 0.3	0.3 ± 0.1
	Intact biofilms (5 h)	1.3 ± 0.2	1.0 ± 0.05	0.9 ± 0.06

*Ammonia production – μmol/mg cell dry weight.
§Ammonia production – μmol/mg biofilm dry weight.

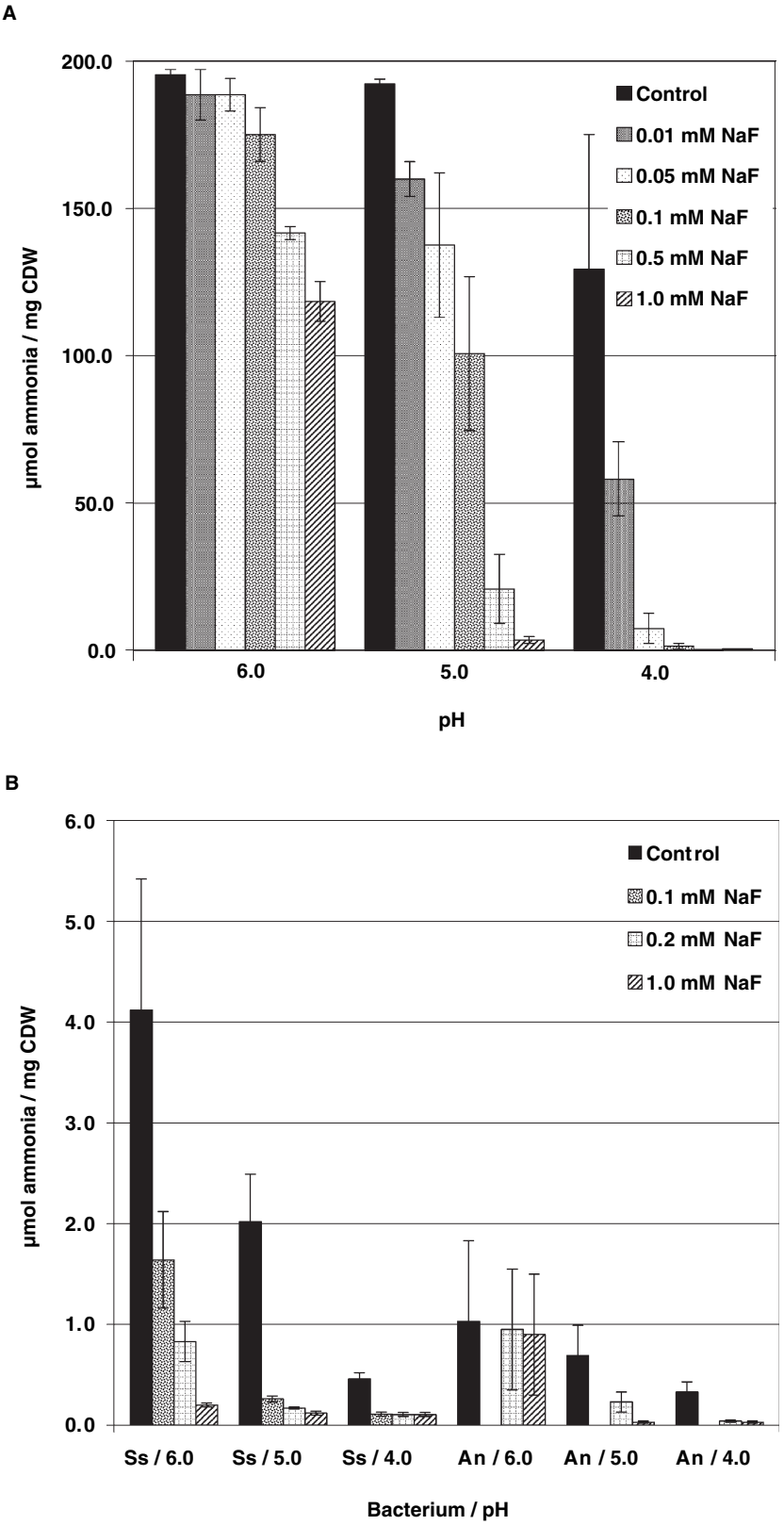
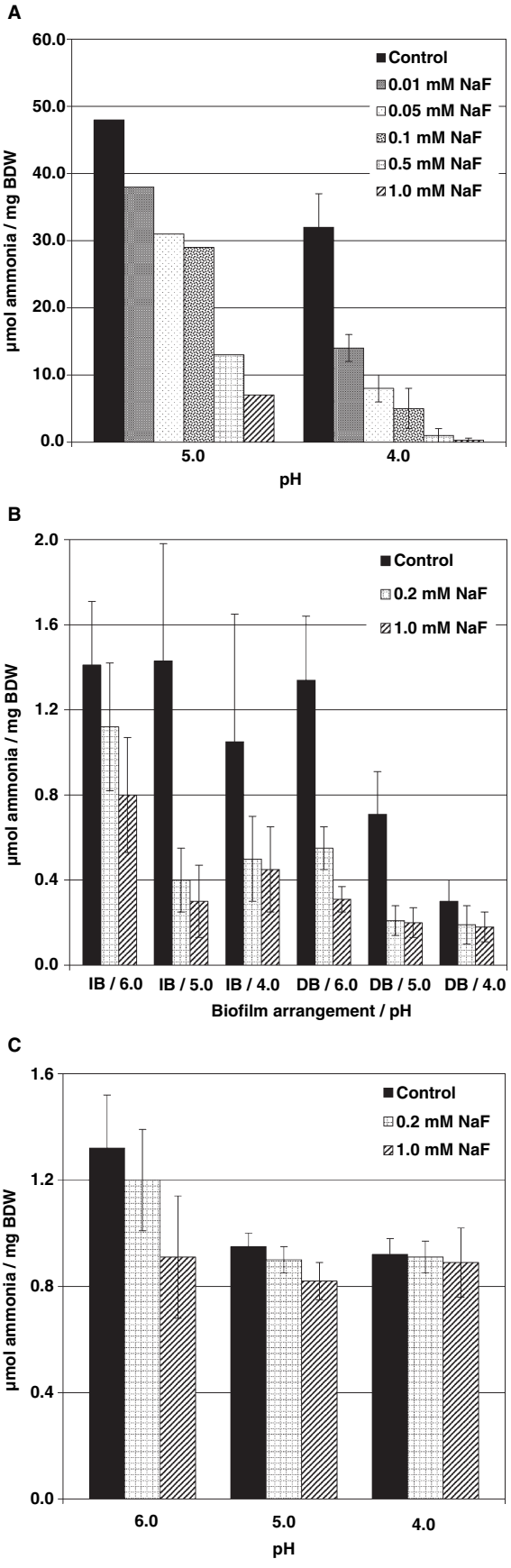


Fig. 1. A) Inhibition by fluoride of ammonia production by resting cell suspension of *S. epidermidis* at 1 h at the indicated pH values. B) Urease inhibition of *S. salivarius* (Ss) and *A. naeslundii* (An) at 2 h. Error bars show standard deviations with $n \geq 3$. CDW, cell dry weight. *A. naeslundii* was not assayed for effects of 0.1 mM fluoride.

S. salivarius cells but more acid sensitive than *S. epidermidis* cells (Table 1).

Ureolysis per unit of dry weight by *S. epidermidis* in biofilms at constant pH values of either 5.0 or 4.0 was slower (Fig. 2A) than that by cells in suspensions. For biofilms not exposed to fluoride at pH 5.0, ammonia production from urea was approximately 34 µmol/mg biofilm dry weight per hour, compared with a value of approximately 174.0 µmol/mg cell dry weight per hour for cells in suspensions (Table 1). Part of the difference may be due to the biofilms being richer in carbohydrate than are cells from suspension cultures, which were found to be richer in protein, as assessed by the Lowry (11) method per biomass unit. Part of the difference may also be due to reduced diffusion of urea into biofilms (22), but this effect seems to be minor for biofilm ureolysis. The data in Table 1 show that ureolysis by intact biofilms of *S. epidermidis* was not inhibited by acidification, even at pH 4.0. The fluoride effect in biofilms was clearly pH-dependent, as shown in Fig. 2. Ureolysis by biofilms of *S. epidermidis* at a pH value of 6.0 was relatively insensitive to fluoride (data not shown), but at a pH value of 5.0 (Fig. 2A), the 50% inhibitory concentration was between 0.1 and 0.5 mM. At a pH value of 4.0, 50% inhibition was caused by only about 0.01 mM fluoride. Thus, plaque levels of 0.1–0.5 mM fluoride (7, 24) appear to be highly inhibitory for urease at the pH values (around 4.0) of acidified plaque, particularly those of cariogenic plaque.

Ureolysis by mono-organism biofilms of *S. salivarius* was found to be only somewhat less sensitive to fluoride inhibition than ureolysis by cells in suspensions (Fig. 2B). At pH 6, the 50% inhibitory concentration was somewhat greater than 1.0 mM, but at pH values of 5.0 and 4.0, 0.2 mM fluoride was sufficient to cause more than 50% inhibition. Again, although ureolysis by organisms in biofilms was somewhat more resistant to fluoride, major inhibition still occurred at what are considered plaque levels of fluoride. Also, when intact biofilms of *S. salivarius* were dispersed to release biofilm cells into suspensions, ureolysis by these cells in the presence of fluoride was similar to that of *S. salivarius* cells from suspension cultures (Fig. 2B) in terms of sensitivity both to acidification and to fluoride in a pH-dependent manner. The amount of ammonia produced at pH 5.0 was about half of the ammonia produced at pH 6.0 and 0.2 mM fluoride caused more than 50% inhibition of ureolysis.



Intact biofilms of *A. naeslundii* proved to be less sensitive to fluoride and acidification than were intact cells in suspensions (Fig. 2C). At pH values of 5.0 and 4.0, ureolysis by biofilms was not very sensitive to acidification or to fluoride, even at a high level of 1.0 mM. However, the rates of ammonia production by biofilms under these conditions was so low that the incubation period had to be increased to 5 h for accurate ammonia assays.

Effects of acidification, fluoride and organic weak acids on urease activity in cell extracts

Fluoride can bind directly to urease, at least that of *Klebsiella aerogenes*, and inhibit its activity in a pH-dependent manner (23). The data presented in Fig. 3A show that urease activity in cell extracts of *S. salivarius* and *A. naeslundii* was higher at pH 6.0 than at pH 5.0; there was essentially no activity at pH 4.0 (data for pH 4 not shown). The enzyme in extracts was highly sensitive to fluoride, even at pH 6. At pH 5, activity was sufficiently reduced by acid inhibition to make it difficult to show large fluoride effects. The organic weak acids indomethacin and capric acid, in contrast to fluoride, were not direct inhibitors for urease in cell extracts (Fig. 3B).

Effects of organic weak acids on ammonia production by intact cells and by biofilms

We assessed inhibitory effects of indomethacin and capric acid, which are not direct inhibitors for urease in cell extracts, to determine whether ureolysis by intact bacteria can be affected by cytoplasmic acidification caused by the organic acids through discharge of ΔpH across the cell membrane (13). As shown by the data presented in Fig. 4A and B, the patterns of inhibition for the two organic weak acids were similar to that for fluoride for *S. epidermidis* intact cells in suspensions. Indomethacin was approximately as potent as fluoride for inhibition of ammonia

Fig. 2. Inhibition by fluoride of ammonia production from urea at constant environmental pH values: A) by *S. epidermidis* intact biofilms for 1 h, B) by intact *S. salivarius* biofilms for 3 h (IB), or by dispersed (DB) biofilms of *S. salivarius* for 3 h, C) by intact biofilms of *A. naeslundii* for 5 h . Error bars show standard deviations with $n \geq 3$. BDW, biofilm dry weight. Mean values of two separate assays are presented for *S. epidermidis* (A) at pH 5.0.

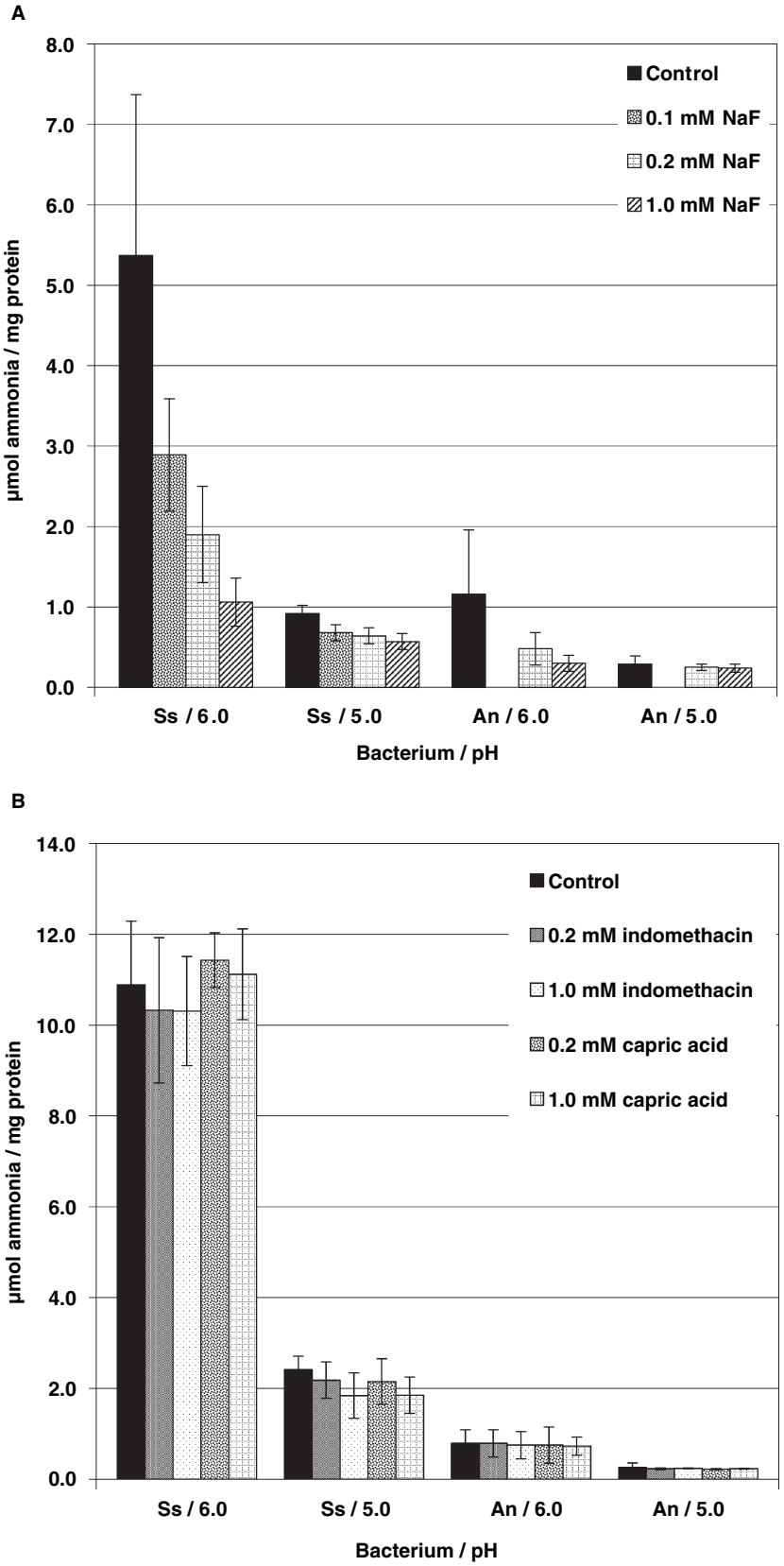
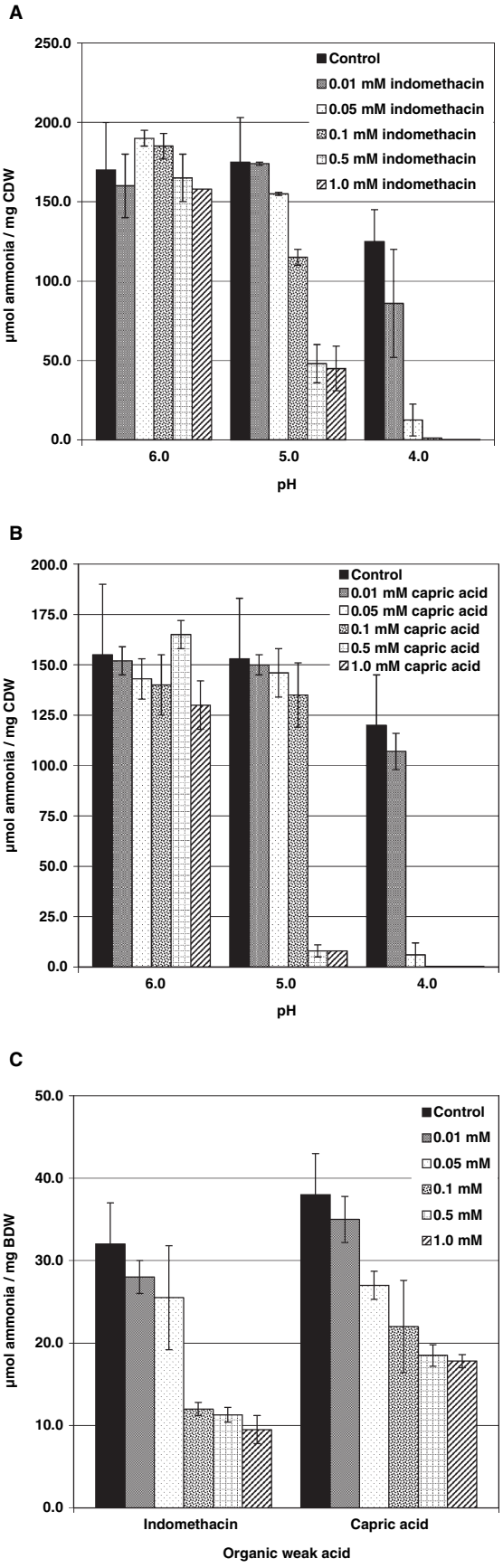


Fig. 3. A) Inhibitory actions of fluoride on ammonia production for 2 h at constant pH values from urea by cell extracts of *S. salivarius* (Ss) or *A. naeslundii* (An). B) Inhibitory actions of organic weak acids for 2 h at constant pH values. Error bars show standard deviations with $n = 3$. *A. naeslundii* (A) was not assayed for effects of 0.1 mM fluoride.

production at pH 4.0, as found previously for inhibition of acid production by mutans streptococci (1, 12); capric acid was slightly less potent. Ammonia production at pH 4.0 by biofilms of *S. epidermidis* (Fig. 4C) was inhibited by either of the acids, but biofilms were more resistant to the acids than suspension cells, as might be expected from the greater resistance of biofilms to environmental acidification. However, it did appear that fluoride inhibition of urease activity of intact cells of *S. epidermidis* can involve indirect effects caused by discharge of ΔpH, as well as direct inhibition of urease enzyme.

Ammonia production by intact cells of *S. salivarius* was affected differently by the organic weak acids (Fig. 5A) than by fluoride. At constant pH 6.0, there was almost no inhibition of ammonia production at any concentration of the organic acids tested, in contrast to the inhibition found for fluoride at the same environmental pH. Only at pH 4.0 was 50% inhibition induced by 0.2 mM indomethacin or capric acid. Therefore, fluoride inhibition of ureolysis in this organism appeared to be due more to binding to urease and less to cytoplasmic acidification.

The effects of weak acids on ureolysis by cells of *A. naeslundii* (Fig. 5B) in suspensions differed from those of *S. salivarius* and *S. epidermidis* cells. Apart from an unexplained but repeatable finding that 0.2 mM capric acid actually stimulated urease activity for *A. naeslundii* at pH 6, the organic acids were inhibitory at each pH value tested. As described above, inhibition of ureolysis by intact cells of *A. naeslundii* in suspensions due to indomethacin showed an opposite picture from that in the presence of fluoride at pH 6.0 (Fig. 1B and 5B). At this pH value, ammonia production was more than 50% inhibited by only 0.2 mM indomethacin, although 1.0 mM was only somewhat more inihibitory for ureolysis. Also at pH 6.0, 1.0 mM capric acid was 50% inhibitory for ureolysis, whereas 0.2 mM did not cause any inhibition but did cause an unexplained increase in ammonia production. At pH 5.0 and 4.0, indomethacin and capric acid were highly inhibitory for ammonia production, but the latter seemed to be less potent, while fluoride was much more effective than either organic weak acid (Fig. 1B). Thus, the overall conclusion is that fluoride inhibits ammonia production in *A. naeslundii* both by the indirect mode through cytoplasmic acidification and by direct binding to the active site of urease.



Effects of acidification, fluoride and organic weak acids on ammonia production by human dental plaque

Ammonia production from freshly harvested, supragingival dental plaque with urea added as substrate was assayed over a 2-h period. In pH-rise assays, ammonia production was sufficient in control suspensions to lead to rapid pH rise from the initial pH value of 4.0 to a final pH of 7.8 with an increase in ammonia concentration from 3.8 to 26.0 µmol/mg plaque dry weight. Both pH rise and ammonia production were essentially stopped by 1.0 mM fluoride, except for a minor initial burst of production. The plaque may have contained food debris from the host diet, but the pH rise without added urea was minimal, as was ammonia production with the type of plaque used, obtained hours after the last meal and resuspended to phosphate buffer.

In constant-pH assays, ammonia production was inhibited in a pH-dependent manner by fluoride, 0.2 mM NaF causing significant inhibition and 1.0 mM NaF major inhibition (Fig. 6). At pH 6.0, 0.2 mM fluoride was 25% inhibitory for ammonia production, whereas at pH 5.0, inhibition increased to 40% and at pH 4.0 to some 50% (Fig. 6A). The level of ammonia production per mg plaque dry weight was similar to that for *S. salivarius* cells in suspensions and well below the level for *S. epidermidis*. Of course, the plaque microbiota is heterogeneous, but with the plaque samples used, significant ammonia production from urea occurred. Indomethacin and caprate also inhibited ammonia production by dental plaque but were less effective than fluoride (Fig. 6B). The data suggest that fluoride inhibition of ammonia production by human dental plaque is due more to direct inhibition involving binding to the active site of ureases than to indirect action associated with acidification of cell cytoplasm.

Discussion

In the mouth, the effects of fluoride and organic weak acids on bacteria in saliva would be expected to be muted because of

Fig. 4. A) Inhibition by indomethacin of ammonia production from urea in 1 h by intact cells of *S. epidermidis* in suspensions. B) Inhibition by capric acid for cell suspensions in 1 h. C) Inhibition by both organic weak acids for production by intact biofilms at a constant pH value of 4.0 for 1 h. Error bars show standard deviations with n ≥ 3.

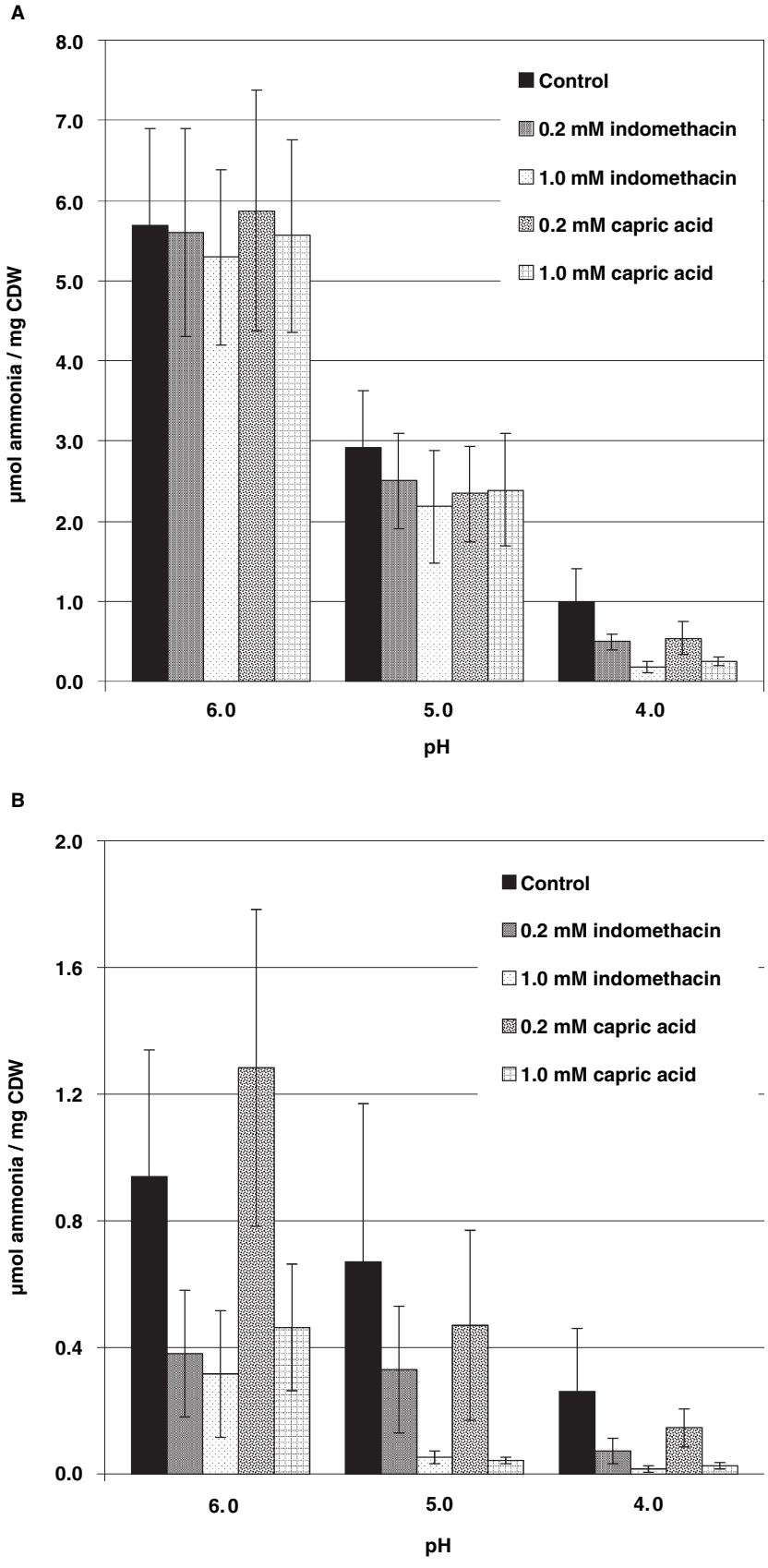


Fig. 5. Inhibition of ammonia production by intact cells of A) *S. salivarius* or B) *A. naeslundii* suspension caused by organic weak acids at constant pH values during 2 h. Error bars show standard deviations with $n = 3$.

pH values near neutrality and low fluoride or organic weak acid levels. There is also an unimpeded interaction of urea in saliva and crevicular fluid with ureolytic bacteria such as *S. salivarius*, and the urea is thus rapidly degraded. However, in plaque, ureolysis would be much more affected because of acidification to pH values as low as 4 and concentration of weak acids by plaque to some 100 times salivary levels, at least for fluoride. Salako & Kleinberg (18) have associated the direct supply of secreted urea to plaque with proximity to salivary gland ducts. These same plaque locations are also prone to calculus formation and selection of ureolytic bacteria, including gram-positive staphylococci and *A. naeslundii*, as well as gram-negative bacteria, including *Haemophilus parainfluenzae*. The situation regarding ureolysis and the tongue microbiota is less well defined, but because of the diversity of bacteria on the dorsum of the tongue, acidification and weak acids would be expected to have significant effects associated with urease-positive bacteria in the microbiota, including *S. salivarius*.

Normal levels of fluoride in dental plaque have been found to be between 0.1 and 0.5 µmol/g plaque wet weight, or about 100 times the levels in saliva (7, 24). These levels correspond to about 0.1–0.5 mM, as plaque is made up mainly of water, both within bacteria and in the interstitial polymer matrix. Moreover, the fluoride level in plaque can become elevated after use of fluoride-containing toothpastes or other fluoridated products. Fluoride tends to be retained at elevated levels in plaque for long periods after exposure, presumably because plaque bacteria maintain at least some ΔpH across the cell membrane with the interior alkaline relative to the environment. This ΔpH then acts to increase fluoride uptake and retention by bacteria (13). Fluoride does act to diminish ΔpH but not to bring about complete discharge of the proton-motive force across the membrane of metabolizing cells.

A major question regarding fluoride effects on urease activity *in vivo* is whether the levels of fluoride in plaque are inhibitory. A reasonable answer based on the data presented here is that fluoride would be highly inhibitory at low plaque pH values, especially at pH 4, which is close to minimal values assessed *in vivo*. Of the three organisms tested, *S. epidermidis* was found to be the most resistant to environmental acidification or fluoride inhibition of urease activity. However, it

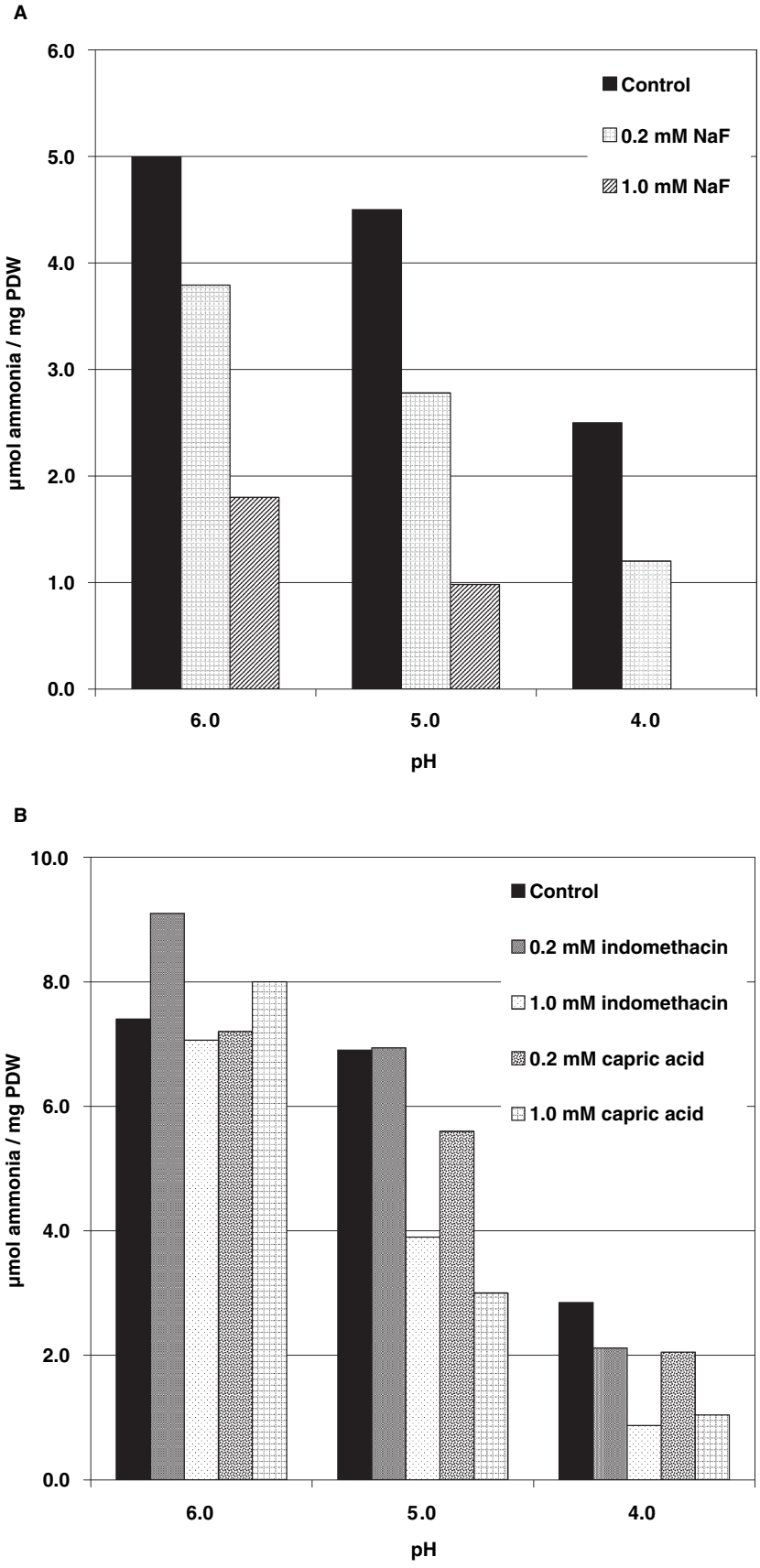


Fig. 6. Inhibition of ammonia production by human dental plaque by A) fluoride or B) organic weak acids, at constant pH values over 2 h. Mean values of two separate assays are presented.

seems that this resistance may be based mainly on the high ureolytic capacity of the organism, which would diminish any tendency to lower the cytoplasmic pH value. For *S. salivarius* and *A. naeslundii*, 0.1 mM fluoride had major inhibitory effects at pH 5 and nearly eliminated urease activity at pH 4. Urease activity of intact cells of these organisms was more sensitive to acidification than was that of intact *S. epidermidis* cells. Again, the difference is likely related to the very high ureolytic activities of *S. epidermidis* cells, which would counteract cytoplasmic acidification at lower environmental pH values and diminish acid inhibition of the enzyme. The behavior of freshly harvested dental plaque was more similar to that of *S. salivarius* or *A. naeslundii* than to that of *S. epidermidis*.

Part of the pH dependence of fluoride inhibition of urease in intact cells may arise from increased intracellular accumulation of fluoride by cells in acidified environments (9, 25). Thus, the urease in the cytoplasm would be exposed to higher levels of fluoride as the environmental pH was decreased.

The results of other studies, particularly those of Todd & Hausinger (23), along with our own results, have demonstrated that direct fluoride inhibition of urease enzyme is enhanced by acidification. The acid sensitivity of fluoride inhibition of urease activity of intact bacterial cells is therefore complex, involving both enhanced uptake of fluoride and enhanced sensitivity of the enzyme in acidified cytoplasm.

Another characteristic of dental plaque that could affect the fluoride sensitivity of urease activity is the nature of its biofilm. However, biofilms of *S. epidermidis* or *S. salivarius* were nearly as fluoride sensitive as suspensions. Biofilms of *A. naeslundii* did show increased fluoride resistance compared with cells in suspensions. *A. naeslundii* is noted for fluoride resistance, and possibly very slow ureolysis of *A. naeslundii* biofilms may preclude pronounced fluoride effects when cytoplasmic acidification can occur without involvement of a weak acid.

Fluoride inhibition of urease is similar to fluoride inhibition of enolase in being quasi-irreversible (13). Todd & Hausinger (23) found that fluoride binding associated with nickel binding in the active site of urease of *K. aerogenes* resulted in progressively decreased values for V_{max} and increased values for K_M until activity was completely inhibited. However, fluoride could slowly dissociate from its

complex with urease, the enzyme slowly becoming reactivated. The differences in responses of intact cells of *S. salivarius* and intact cells of the other two test organisms to fluoride appear to be due mainly to differences in the physiology of the cells rather than to the enzymes, presumably due to the capacity of the cells to maintain ΔpH across the cell membrane and resist cytoplasmic acidification.

In our work, organic weak acids were used in attempts to separate direct effects of fluoride involving binding to the nickel-binding site of urease and indirect effects related to cytoplasmic acidification. The organic weak acids chosen, indomethacin and capric acid, are not direct inhibitors of urease enzyme. However, they were effective for inhibiting ureolytic activities of intact cells of all three organisms used. Clearly, cytoplasmic acidification is important for inhibition of ammonia production by intact cells, including those in biofilms. Somewhat higher concentrations of the organic acids compared with fluoride were required for major effects. The conclusion, however, is that both direct urease inhibition and indirect inhibition due to cytoplasmic acidification play important roles for intact cells. Clearly, the fluoride effects are more complex than those due to the organic weak acids. Still, human exposure to weak acids, especially benzoate and sorbate in foods, which has increased greatly in recent years, may well have effects on the ureolytic activities of oral bacteria in the human mouth. For oral diseases such as caries, inhibition of ureolysis would not be desirable, but for other diseases in which ammonium acts as an inflammatory agent, inhibition may be beneficial.

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