Fluoride and organic weak acids as modulators of microbial physiology

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Abstract

Fluoride is widely used as an anticaries agent in drinking water and a variety of other vehicles. This use has resulted in major health benefits. However, there are still open questions regarding the mechanisms of anticaries action and the importance of antimicrobial effects in caries reduction. Fluoride acts in multiple ways to affect the metabolism of cariogenic and other bacteria in the mouth. F⁻/HF can bind directly to many enzymes, for example, heme-containing enzymes or other metalloenzymes, to modulate metabolism. Fluoride is able also to form complexes with metals such as aluminum or beryllium, and the complexes, notably AlF₄⁻ and BeF₃⁺H₂O, can mimic phosphate with either positive or negative effects on a variety of enzymes and regulatory phosphatases. The fluoride action that appears to be most important for glycolytic inhibition at low pH in dental plaque bacteria derives from its weak-acid properties (pKₐ = 3.15) and the capacity of HF to act as a transmembrane proton conductor. Since many of the actions of fluoride are related to its weak-acid character, it is reasonable to compare fluoride action to those of organic weak acids, including metabolic acids, food preservatives, non-steroidal anti-inflammatory agents and fatty acids, all of which act to de-energize the cell membrane by discharging ΔpH. Moreover, with the realization that the biofilm state is the common lifestyle for most microorganisms in nature, there is need to consider interactions of fluoride and organic weak acids with biofilm communities. Hopefully, this review will stimulate interest in the antimicrobial effects of fluoride or other weak acids and lead to more effective use of the agents for disease control and other applications.

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Keywords: Fluoride; Organic weak acid; Dental caries; Enzyme modulator; Membrane physiology

Contents

1. Introduction ................................................................................ 494
2. New information on physiological actions of fluoride on microorganisms ........................................ 495
   2.1. Actions dependent on direct binding of F⁻/HF .................................................. 495
   2.2. Metal complexes and effects on phosphoryl transfer ........................................... 499
   2.3. Comparison of actions of fluoride as a transmembrane proton transporter with those of organic weak acids .................................................. 500
3. Weak-acid effects on cells in biofilms ................................................ 504
   3.1. Sensitization of biofilms to acid damage ......................................................... 504
   3.2. Effects of fluoride on multi-organism biofilms of oral bacteria ........................... 505
   3.3. Concentration and distribution of fluoride in dental plaque and other biofilms .... 506
4. Weak acids and dental caries ......................................................... 506
5. The future .................................................................................. 507

Acknowledgements .......................................................................... 507

References .................................................................................. 507

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1. Introduction

In a previous review [74], the multiple ways known at the time for fluoride to alter the physiology of microbial cells were considered. This review is an update with orientation to new information and changed views of the last 7–8 years. The major ways for fluoride to affect microbial cells include direct inhibition by F⁻ or HF of enzymes such as enolase, urease or catalase, through effects of aluminofluoride or berylliumfluoride complexes on enzymes such as nitrogenases or regulatory phosphatases, and as a result of physiological disturbances derived from the capacity of HF to act as a transmembrane proton transporter to discharge ΔpH across the cell membrane. A major theme of this review is the antimicrobial actions of fluoride on cariogenic bacteria. Human exposure to fluoride is very much related to its use as an anticaries agent. For reducing caries in individuals and in populations, fluoride is incorporated into drinking water at levels of some 0.5–1.0 ppm (26.3–52.6 μmol ml⁻¹) or into food items, such as salt, or into a variety of oral health products, especially toothpastes and mouthwashes. At this time, fluoride is the most effective agent available for control of dental caries. Although there is active debate on the level of fluoride that should be added to drinking water, there is almost no debate about the effectiveness of fluoride as an anticaries agent. A recent study of fluoride safety and effectiveness by an expert panel [63] led to strong support for continued and more widespread water fluoridation to reduce caries. Some countries in recent years have reduced water fluoridation, but others have increased it.

Human ingestion of fluoride results in effects not just on oral microorganisms but on the entire microbiota of the body, especially on that of the urogenital tract, since fluoride is excreted mainly in the urine and only in smaller amounts in the feces and saliva. Moreover, when fluoride is added to vehicles such as water, it enters the entire biological community through multiple products containing fluoridated water, through sewage and wastewater into soil and water. Plants such as the tea plant concentrate fluoride from natural sources. In fact, there are multiple dietary sources for humans and other animals. In some areas of the world, such as the American Southwest, levels of fluoride in the water supply may be very high, so fluoride effects on the biological community can be accentuated. In fact, the initial use of fluoride as an anticaries agent derived from observations of low levels of caries in areas with high levels of fluoride in drinking water. The natural levels of fluoride in water may be so high that steps have to be taken to de-fluoridate drinking water to prevent severe fluorosis of teeth and bone. However, this problem occurs only in restricted regions of the world. Some manufacturing processes, such as aluminum production, result in high levels of fluoride being released into the environment. There have been major efforts made to reduce fluoride-containing industrial emissions, but still, in highly industrialized areas, there may be high levels of contamination of soil and water [60]. Again, exposure of microorganisms to fluoride is not confined to the human mouth, although much of our knowledge of effects of fluoride on microbes derives from studies of oral bacteria.

There are concerns about fluorosis of the teeth related to fluoride ingestion in drinking water. Except for extreme situations of water with high natural levels of fluoride, the concerns are primarily cosmetic ones. Still, some municipalities have reduced levels of fluoride in drinking water below the recommended level of 1 ppm (52.6 μmol ml⁻¹), generally to about 0.5–0.7 ppm. On the other hand, there is also concern that the eradication of caries is far from complete. In fact, in terms of the total world caries burden, there may actually be an increase. Even in the industrialized countries, there is a subpopulation of some 20% of the total with very high caries rates [38,108]. Then, more people are retaining their teeth into old age when gums recede to expose susceptible root surfaces. The pattern of the disease is changing to a lifelong one instead of one primarily affecting younger people. The net result is that there is now need for better knowledge of the anticaries actions of fluoride to allow for more effective use of fluoride, possibly by combining it with other active agents.

The nature of the anticaries actions of fluoride is still not entirely clear [107,113]. Fluoride can act to enhance remineralization in the cyclic demineralization–remineralization associated with acidification and alkalinization of dental plaque. Fluoride incorporation into tooth mineral may result also in reduced demineralization since fluoroapatite is somewhat less acid-soluble than is hydroxyapatite. However, it is well known that the fluoride important for caries reduction is that in plaque, although it appears that fluoride from dissolved enamel or from fluoride-containing varnishes may enter into plaque and become part of the total [97,100]. Moreover, this released fluoride can inhibit acid production by plaque bacteria [44]. Still, for full benefit, fluoride exposure should be daily so that plaque levels of fluoride remain high. There is ongoing research on sustained-release devices for supplying introral fluoride and improving effectiveness. Fortunately, fluoride is concentrated by dental plaque to levels of some 0.1–0.5 μmol g⁻¹ plaque, wet weight, or about 100 times the level in saliva [33]. A recent paper by Vogel et al. [115] presents information on levels of fluoride in saliva, plaque and plaque fluid after rinsing with solutions containing NaF or monofluorophosphate. It also gives references to past literature on fluoride levels in plaque. Fluoride can be retained in plaque even after it has been largely cleared from saliva, mainly in the bacteria rather than plaque fluid. Concentration in the short term can involve formation of calcium fluoride crystals, but in the longer term is most likely due to bacterial metabolism, which results in the cytoplasm of cells being less acid than the environment in plaque. The tendency, then, is for a weak acid such as fluoride to be concentrated within metabolically active
cells able to maintain \( \Delta \text{pH} \) with the cytoplasm alkaline relative to the environment. Fluoride can be made to move into and out of streptococcal cells simply by manipulating the external pH and \( \Delta \text{pH} \) across the cell membrane [114]. The levels of fluoride in plaque are sufficient to have significant effects on bacterial metabolism, especially when the pH in plaque is reduced to minimal values of somewhat below 4.0. Moreover, dental plaque is a biofilm, and it has been found [91] that fluoride levels are elevated near the surface of plaque exposed in the mouth. This also is the region of the biofilm where metabolism of ingested sugars or those released from salivary glycoproteins is most active. Thus, the fluoride level is high in the regions of most active glycolysis and acid production. Overall, it appears that the anticaries action of fluoride may involve both effects on tooth mineral and antimicrobial effects. The review by van Loveren [113] in a recent issue of Caries Research devoted to fluoride and dental caries emphasizes the need for more research on the antimicrobial effects of fluoride and their contribution to caries prevention.

The current need for updating information on the antimicrobial effects of fluoride is related to new data developed over the past 7–8 years and to the growing acceptance of the view that the anticaries action of fluoride is complex and includes antimicrobial effects. In fact, the antimicrobial effects may be the major anticaries effects. It has become apparent that organic weak acids also have anticaries actions, as described, for example, in a recent paper by Davis et al. [32] on reduction of caries in rats by use of benzoate or fluoride. They present the view that food-preservation weak acids, such as benzoate and sorbate, may be responsible for some of the reductions in dental caries of the past few years in countries where the preservatives are widely used. Annual consumption of benzoate in the USA has risen steadily, and in 1997, the estimate for average daily intake from carbonated beverages alone was 662 mg Na benzoate per person. Ingestion of other weak-acid preservatives, such as sorbate, has risen as well. In addition, fatty acids such as lauric acid have been found to be effective not only for reducing acid tolerance of oral streptococci [49] but also for reducing dental caries in rats [50]. Fatty acids are known to be proton conductors across cell membranes and may act in a flip-flop manner to move protons back and forth in response to changes in pH differences across the membrane [118]. In this review, the actions of organic weak acids as transmembrane proton conductors will be considered briefly in relation to the similar action of fluoride. Clearly, the human microbiota is being subjected to a considerable and increasing weak-acid stress associated with use of fluoride, food-preservative weak acids, and weak-acid anti-inflammatory agents.

This review is oriented mainly to microbial physiological aspects of the actions of fluoride and other weak acids. The authors are microbial physiologists. However, a good bit of the impetus to write the review comes from the need to come to terms with the widespread use of fluoride to control oral disease and of organic weak acids to preserve foods and other commodities against microbial spoilage. The inhibitory effects of metabolic weak acids, notably lactate, acetate and formate, have been considered by others, for example, by Carlsson and Hamilton [22], Russell and Diez-Gonzalus [92] or Dashper and Reynolds [29]. These metabolic acids can have major inhibitory effects, especially in crowded conditions such as those in biofilms. However, their capacities to act as transmembrane proton conductors or as enzyme inhibitors are much less than those of fluoride, and levels above about 20 mM are generally required for significant inhibitory effects.

2. New information on physiological actions of fluoride on microorganisms

2.1. Actions dependent on direct binding of \( F^-/HF \)

The most direct mode of action for fluoride involves binding of \( F^- \) or HF to specific sites of enzymes or other proteins, for example the heme components of a variety of enzymes. In many instances, \( F^- \) binds to sites that would normally bind \( \text{OH}^- \) and subsequently a proton, in a very general way similar to the binding of \( F^- \) to apatites in teeth with resultant formation of fluorapatites instead of the normal hydroxylapatites. For many proteins, the nature of fluoride binding is known in some detail. For example, Neri et al. [81] found that arginine in the distal cavity structure is a determinant for fluoride binding for peroxidases and that binding involves a strong hydrogen bond between the guanidinium group and \( F^- \). The distal histidine residue of the site was stabilizing for the binding, probably because of hydrogen bonding through a water molecule to fluoride with acceptance of a proton.

Fluoride inhibition of enzymes and regulatory proteins may be important physiologically for intact organisms. For example, enolase inhibition is considered to be important in fluoride inhibition of glycolysis by intact cells, and this inhibition can occur at low fluoride concentrations down to the micromolar level in acidified environments. Then, fluoride inhibition of catalase has been shown to be important in compromising the capacities of intact bacteria or bacteria in mixed communities in acid environments to cope with oxidative damage from hydrogen peroxide. A generality is that fluoride binding is enhanced by acidification. Binding of fluoride or of fluoride–metal complexes to proteins has been very useful for crystallization of various forms of proteins or for determining mechanisms of action. However, there is question with many of these actions about whether or not they are purely laboratory phenomena, helpful to biologists but not very pertinent to the daily lives of microorganisms. At this time, the question does not have a clear or uniform answer, but comments about physiological importance follow. A summary
of studied interactions of fluoride with proteins is presented in Table 1.

2.1.1. Enolase inhibition

Although the finding that fluoride inhibits enolase goes back to the initial studies of Warburg and Christian [116], there are still open questions about the actual nature of inhibition. The effect appears to be due to F⁻ itself without need for complex formation with Al or Be and can occur at low levels of fluoride. In their initial studies, Warburg and Christian found that Mg²⁺ and phosphate also were important for inhibition and they formulated a modulus for predicting inhibition based on the concentrations of three key agents, F⁻, Mg²⁺, and phosphate. Inhibition has been found [45] to occur at micromolar fluoride concentrations in the presence of 5 mM inorganic phosphate and 2 mM Mg²⁺. Kᵢ values were found to vary for enzymes isolated from a group of oral bacteria (Actinomyces naeslundii, Lactobacillus rhamnosus, Streptococcus mutans, Streptococcus salivarius and Streptococcus sanguis). The range of variation was from 16 to 54 mM fluoride. These results and those of previous studies [14] indicated that there is no constant relationship between sensitivities of enolases of different organisms to fluoride or to acid and the acid tolerance of glycolysis by intact cells of the organisms.

The standard view of reversible inhibition of enolase by fluoride was challenged by the findings of Curran et al. [27], who found that inhibition of the enolase of S. mutans was actually irreversible, of the type investigated previously for P-ATPases [80] and subsequently for bacterial ureases [111]. The work on fluoride inhibition of P-ATPases, which become phosphorylated during their catalytic cycle, has been mainly with mammalian cells and tissues. However, the same type of inhibition should occur for bacterial P-ATPases. In addition, Yamasaki et al. [119] have recently found that the Mg²⁺/F⁻/Ca²⁺-ATPase complex is remarkably stable and resistant to detergent denaturation. Curran et al. described the inhibition of enolase as quasi-irreversible because it was possible to reactivate the fluoride-inhibited enzyme with high levels of the substrate 2-phosphoglycerate. This latter finding allowed for reasonable interpretation of previous findings for oral streptococci [6] that fluoride inhibition of glycolysis by intact cells resulted in build-up in the cytoplasm of 2-phosphoglycerate but no loss of pools of phosphoenolpyruvate. In the intact cell, build-up of the substrate would act to reverse fluoride inhibition of enolase and allow for continued production of phosphoenolpyruvate. However, as described below, fluoride acts to cause acidification of the cytoplasm of cells in acid environments, and this acidification is highly inhibitory for glycolytic enzymes, including enolase [5]. Thus, fluoride inhibition of enolase in intact cells may be due more to cytoplasmic acidification than to binding of fluoride to the enzyme.

Kustrzeba-Wojcicka and Golczak [66] have carried out a study of fluoride inhibition of enolase isolated from Candida albicans. They found that fluoride inhibition was non-competitive when phosphate was not added to the assay mix but competitive in the presence of phosphate. Also, Mg²⁺ was the most potent activator compared with Mn²⁺ or Zn²⁺. Thus, the Candida enolase responded to fluoride in a manner similar to many other enolases, although the issue of pseudo-irreversibility was not addressed in this study. The reader is referred to Section 2.1.3 for details of another example of fluoride binding leading to complete inhibition of a metal–cation-activated enzyme.

2.1.2. Inhibition of enzymes involved in oxidative stress and protection against oxidative damage

Fluoride is known to bind to heme catalase in a pH-
sensitive manner, and this binding leads to inhibition of the enzyme, especially in acidified environments. The pH sensitivity has been interpreted in terms of binding of a proton along with $\text{F}^-$. Sulphide can be nearly as potent as $\text{F}^-$ for inhibition of bacterial catalases, e.g., that of *Staphylococcus aureus* [87]. At a low pH value of 4, major inhibition by sulphide occurs at levels as low as 0.025 mM Na$_2$S. This inhibition could affect organisms in environments in which sulphide is present, although often sulphide-rich environments are anaerobic. However, sulphide action can differ from fluoride action, for example, pseudocatalase of *Lactobacillus plantarum* was found [87] to be inhibited by fluoride but not by sulphide, even at low pH values.

Because a very wide variety of microorganisms have heme catalases, which are sensitive to F and HS in acid environments, this type of inhibition probably has major environmental consequences. However, many organisms, including oral streptococci and anaerobes such as *Treponema denticola*, have flavin peroxidases rather than heme peroxidases. The flavin enzymes are insensitive to fluoride [19].

Heme catalases are not commonly excreted enzymes, and therefore, their activities usually require that H$_2$O$_2$ penetrate the cell membrane. Fluoride inhibition then would require also that fluoride penetrate into cells. Fluoride inhibition of heme catalases of cells in acidified environments may be enhanced not only by greater binding of fluoride to the enzyme at low pH but also by greater penetration of fluoride into cells associated with increased ΔpH and enhanced formation of HF.

One of the major defense mechanisms in the human mouth against infectious diseases is the salivary peroxidase system. Salivary peroxidase catalyzes reduction of H$_2$O$_2$ and oxidation of thiocyanate, SCN$^-$, to form hypohiocyanite, OSCN$^-$; fluoride is a potent inhibitor of this enzyme in acid environments, as first demonstrated by Thibodeau et al. [109]. Peroxidases in phagocytes and in other parts of the body commonly catalyze formation of reactive halogen species such as hypochlorite or hypothiocyanite rather than heme peroxidases. These peroxidases, e.g., myeloperoxidase, are widespread among animals and represent major defense systems. They all are fluoride-sensitive because of being heme-based. Because the pH values in infected sites are generally in the acid range, significant inhibitory effects of fluoride on defense peroxidases should occur commonly. Recently Lenander-Lumikari et al. [67] showed that hypohiocyanite and fluoride could act additively to inhibit growth and glycolysis of *S. mutans* at pH 5.0 but not at 6.5. Thus, it appears that under some conditions, fluoride may not be antagonistic to peroxide-based defense systems. However, at still lower pH fluoride inhibition of the peroxidase enzymes would be expected to predominate over any additive action.

Pseudocatalase is an Mn$^{2+}$-containing enzyme produced by bacteria such as *L. plantarum*, which is inhibited by fluoride over a wide range of pH values [87]. In acidified environments, major inhibition occurs with F levels below 1.0 mM. Whittaker et al. [117] showed that enzymes from *L. plantarum* and *Thermus thermophilus* contain binuclear manganese clusters capable of redox dismutation of H$_2$O$_2$ with conversion of dimanganese([II][2,2] to dimanganese-([III][3,3]. Presumably fluoride binds to these metal complexes, and this bonding leads to inhibition.

Mn/Fe superoxide dismutase of bacterial cells has been found to be fluoride-sensitive but very high levels are required for inhibition [76,87,93]. Crystallization of enzyme from *Propionibacterium shermanii* has led to determination of a structure with compact tetramers and a conclusion that fluoride binding is similar to OH$^-$ binding.

Fluoride binds to and inhibits various heme oxidases. For example, the alternative oxidase, cytochrome bo, of *Escherichia coli* has a very high affinity for O$_2$. It predominates over the lower-affinity cytochrome bo heme-copper oxidase under microaerophilic conditions, say, when cultures are approaching stationary phase in moderately aerated conditions. Fluoride has been found [112] from studies of visible absorption and electron paramagnetic resonance (EPR) spectroscopy to bind primarily to ferric heme $d$ in a pH-sensitive manner. $K_d$ values ranged from about 20 mM at pH 6 to nearly 500 mM at pH 8. Ferric heme $b_{595}$ also took part in binding providing a low-affinity site.

Fluoride can also bind to and inhibit copper-based oxidases. A good example has been described recently by Fernandez et al. [39] for a multipotent polyphenol oxidase of the marine bacterium *Marinomonas mediterranea*. This organism produces a blue multicopper laccase and also a tyrosinase activated by sodium lauryl sulfate. The laccase was found to be fluoride-sensitive with a 50% inhibitor level of somewhat above 10 mM fluoride. They found that the laccase of *Pyricularia oryzae* had about the same fluoride sensitivity and that mushroom tyrosinase was more sensitive to fluoride inhibition. It is generally considered that fluoride interaction with copper enzymes involves only certain coordination sites, generally what is called type 2 Cu$^{2+}$ sites to which fluoride bonds in the blue oxidases. Fluoride appeared not to bind to type 1 Cu$^{2+}$ sites. The interactions of fluoride with type 2 Cu$^{2+}$ sites were reflected in changes in EPR spectra. The affinity for fluoride can be high in many proteins, including laccases, with stability constants in the high micromolar range. Thus, the interactions could be physiologically relevant for intact microbial cells. A variety of other copper enzymes such as galactose oxidase or Cu/Zn superoxide dismutases can also be inhibited by fluoride [72]. A novel fungal laccase described by Höff er and Schlosser [53] catalyzes oxidation of Mn$^{2+}$ to Mn$^{3+}$, and fluoride appears to inhibit by blocking intramolecular electron transfer from type 1 copper to type 2 and 3 copper which form a trinuclear structure capable of reduction of oxygen to water.
2.1.3. Urease inhibition

Although it has been known for decades that fluoride is a potent inhibitor of ureases, it is only recently that a more detailed view of this inhibition is emerging. Todd and Hausinger [111] found that fluoride inhibition of urease has similarities to fluoride inhibition of enolase in that inhibition increased with time leading to complete stoppage of enzyme action. The inhibition appeared to be pseudo-non-competitive. Their proposal is that fluoride inhibition involves two bound nickels in the enzyme, Ni-1 and Ni-2, and that fluoride binding is enhanced by substrate binding. Fluoride binds predominantly to the activated complex to form a urease-substrate-F complex or a urease-carbamate (product)-F complex. The inhibition is pH-sensitive and is enhanced in acid environments.

As described by Burne and Marquis [17] fluoride can inhibit alkali production by oral streptococci either by the urease system or by the arginine deiminase system. Inhibition of the former, in S. salivarius or A. naeslundii, involves mainly inhibition of the urease enzyme itself. Urea can enter bacterial cells passively, although there do appear to be urea transport systems in most urease-positive bacteria. In contrast, the enzymes of the arginine deiminase system of oral streptococci are essentially insensitive to fluoride except at extremely high concentrations [15]. Inhibition by fluoride of alkali production from arginine or arginine-containing peptides is interpretable mainly in terms of fluoride inhibition of membrane transport systems for substrate uptake. Certainly, transport systems energized by Δp would be fluoride-sensitive, as discussed in Section 2.3. Even those energized by ATP should be sensitive because ATP is depleted in fluoride-treated cells in acidified environments because of the increased energetic demand to maintain pH balance across the cell membrane.

2.1.4. Pyrophosphatases

Pyrophosphatases play central roles in metabolism of many microorganisms in recycling of pyrophosphate formed in biosynthetic reactions leading to release of Ppi from nucleotide triphosphates and are now thought to serve other physiological roles, including those in regulation of metabolism. Soluble pyrophosphatases have recently been grouped into three classes – A, a prokaryotic class, B, a eukaryotic class, and C, a class of fluoride-insensitive prokaryotic enzymes with apparently a different active-site structure. Class A and B enzymes are considered to be in family I, while class C is in family II [99]. Inhibition of enzymes in family I by fluoride is thought to involve formation of a magnesium-fluoropyrophosphate complex, and enzyme-substrate complexes stabilized by fluoride have been isolated by gel filtration [2]. The active-site structures of the enzymes are formed from 14–16 amino acids and three to four Mg$^{2+}$ ions with which fluoride can associate. Class C enzymes can be activated by Ca$^{2+}$ and Mn$^{2+}$, which have been found to be protective against fluoride inhibition for the enzyme from Methanococcus jannaschii [65]. In fact, it seems that class C enzymes are actually fluoride-sensitive but that transition-metal activators reduce inhibition. Class C pyrophosphatase of Bacillus subtilis was the first member of the class studied in detail. It is now known that the enzyme, which has a completely different amino acid sequence from the enzymes of family I, occurs widely in bacteria, including the oral bacteria Streptococcus gordonii and S. mutans [83]. The enzymes have specific requirement for Mn$^{2+}$ and so may be at least part of the basis for the Mn requirements of many bacteria for growth in that degradation of pyrophosphate is necessary for growth of most organisms. Fluoride inhibition of pyrophosphatases, even of the family I type, occurs at relatively high levels in the millimolar range [89]. The pattern of fluoride inhibition is indicative of fluoride replacing Mg$^{2+}$-bound water in the active site of the enzyme. Because of the high levels of fluoride required for inhibition, there is some question about how important inhibition would be for microorganisms living in the wild. However, in acid environments, fluoride can be concentrated by bacteria to these high levels [34], and pyrophosphate turnover could be a problem under some natural conditions.

Polyphosphates are common storage polymers in bacterial cells. Synthesis is catalyzed by pyrophosphate kinase, and degradation by pyrophosphate:AMP phosphotransferase and pyrophosphatase. Pyrophosphatase is formed during degradation. Bonting et al. [7] isolated the pyrophosphatase from Acinetobacter johnsonii and showed that it was completely inhibited by 2 mM NaF. The enzyme was active with pyrophosphate and triphosphate but not with larger polyphosphates. However, it appears that pyrophosphatase can play roles in polyphosphate metabolism and that fluoride inhibition may cause metabolic problems for cells in acid environments.

2.1.5. Other phosphatases

Fluoride can affect a variety of phosphatases. For example, Camacho et al. [20] found that, in contrast to most dUTP nucleohydrolases studied previously, the enzyme of kinetoplasts of Leishmania is remarkably sensitive to NaF and could be inhibited 73% by 0.2 mM fluoride. There was no stated need for co-ions such as aluminum, and inhibition may have been due simply to binding of fluoride, especially in view of the activation of the enzyme by Mg$^{2+}$ or Mn$^{2+}$. The product from dUTP is pyrophosphate, while that from dUDP is phosphate. Therefore the enzyme has similarities to an ATPase and a pyrophosphatase.

Fluoride has been found to be a potent inhibitor of phytase (myo-inositol hexakisphosphate phosphohydrolase) of Klebsiella terrigena with a $K_i$ value of 0.18 mM [43]. Phytases in general appear to be fluoride-sensitive, including the enzyme from Bacillus, which appears to have a different reaction mechanism [59]. Again, this action probably involves direct binding of fluoride rather
than of a metal complex containing fluoride. Meyer-Fernandez et al. [78] found that NaF inhibited strongly the activity of a metal complex containing fluoride. Trypanosoma cruzi, an enzyme able to catalyze dephosphorylation also of phosphoamino acids and phosphoproteins. Fluoride can inhibit other microbial protein phosphatases [94]. Again, inhibition is likely due to fluoride itself rather than a fluorometal complex. Inhibition of protein phosphatase may affect multiple functions in microbial cells because often functions are regulated through protein phosphorylation and dephosphorylation. For example, inhibition of HPr phosphatase of Bacillus thuringiensis can modify catabolite repression and synthesis of CryIVA protoxin [4].

2.1.6. Other enzymes

Chen et al. [23] found no time dependence for fluoride inhibition of aminopeptidases of Aeromonas proteolytica of the type found for urease or enolase. The $K_i$ for fluoride inhibition at pH 8.0 was 30 mM, but only 1.2 mM at pH 6.0. Only a single fluoride ion was bound. Subsequently, Harris and Ming [47] had similar findings for non-competitive fluoride inhibition of Zn-activated aminopeptidase of Streptomyces griseus. Moreover, they proposed that fluoride inhibition involved coordinated water and a different binding site from that for phosphate. Phosphate is also a non-competitive inhibitor and it binds differently to the crystalline enzyme than to the soluble form. $K_i$ values for fluoride inhibition ranged from 3.72 mM at pH 5.5 to 43.6 mM at pH 9.0. Again, there are questions about fluoride inhibition of the enzyme in intact cells. However, the organisms can generate acid, even though they are primarily aerobic, and fluoride inhibition could possibly occur in acidified environments.

So-called chemical rescue of glutamate dehydrogenase has been described by Hayden et al. [48] for an Asp165 $\rightarrow$ Ser mutant of Clostridium which had 100,000 times less activity than the wild-type enzyme. Activity was increased 1000-fold by NaF (1 mM), and the finding was interpreted in terms of bound $F^-$ providing negative charge at the position of the missing aspartate carboxyl.

NaF inhibits the Escherichia coli enzyme N-acetylmuramethine deacetylase in a non-competitive manner with $k_i = 3.4 \pm 0.1$ mM [55]. The inhibition is another example of F inhibition of metalloproteases and appears to involve F substitution for $OH^-$ or water as a reactive nucleophile for hydrolysis of the substrate. The data suggest that F$^-$ binds exclusively to the ES complex to form ESI.

2.2. Metal complexes and effects on phosphoryl transfer

The importance of metal-fluoride complexes, especially $\text{AlF}_3^-$ or $\text{BeF}_2^-$H$_2$O, as modulators of enzyme activities and in metabolic regulation was initially recognized by Sternweis and Gilman [101], when they found that fluoride activation of adenylate cyclase was associated with trace amounts of aluminum in the fluoride salts they were using. Subsequent studies showed that beryllium could be as effective as aluminum in serving as a partner for fluoride and that the effectiveness of the complexes could be related to their acting as mimics for phosphate [85]. The complexes have been extensively studied in terms of their abilities to affect phosphatase enzymes, such as F-ATPases. The complexes may stimulate rather than inhibit. For example, they stimulate adenylate cyclases of both prokaryotic and eukaryotic cells. Because the complexes can form at micromolar concentrations of Al or Be, it is felt that they may be relevant to the physiology of living cells, but the issue is not well resolved at this time. They have certainly been useful as tools in enzymology and regulatory physiology in helping to elucidate molecular mechanisms of action, and recently, for trapping response regulatory proteins in the active state by use of beryllium fluoride [20]. MgADP–aluminum–fluoride–acetate complexes appear to be involved in inhibition of acetate kinase of Methanosarcina thermophila, presumably by acting as transition state analogs to form abortive complexes at the enzyme active site [79].

There is evidence from an in vivo study that Al can reduce acidogenicity of dental plaque [82]. Moreover, Kleber and Putt [61] have reviewed the literature on aluminum and dental caries and came to the conclusion that there was a negative correlation between aluminum in the body and caries. They developed this view further by carrying out a clinical trial and described anticaries effects of rinses containing 500 ppm aluminum [62]. However, the results of a recent 3-year caries protection study [51] of 2087 school children in Denmark indicated that a toothpaste containing 1.77% KAl(SO$_4$)$_2$ was less effective in reducing caries than a positive control paste containing 0.83% NaFPO$_3$. A paste containing both fluoride and aluminum might have been more effective than ones with single agents, in keeping with the view that a large part of the anticaries action of fluoride involves antimicrobial effects.

There is very little information regarding effects of beryllium on caries. Beryllium poisoning of humans is well documented, especially in the beryllium industry and in many commercial applications, including use of beryllium-containing alloys in dental laboratories. However, the disease is mainly a pulmonary disease resulting from inhalation of the metal [64] and there is no clear view of a connection between beryllium levels in, say, teeth and caries.

2.2.1. F-ATPases

It has been known for many years that fluoride can inhibit F-ATPases of microorganisms, but it was only after the discovery of Sternweis and Gilman [101] that it became apparent that inhibition depended on trace amounts of aluminum in the F-ATPase preparations [104]. Recently, aluminum fluoride in the presence of MgADP has been used for crystallization of the F$_1$-ATP-
2.2.2. Sensor kinases and regulatory proteins

membrane. Proton permeability and dissipation ofpling effects of fluoride due to increased transmembrane transfer involving F-ATPases have to do with the uncoupling more likely that the major effects of fluoride on energy levels of aluminum required are micromolar, so there is a possibility that such inhibition would occur and that it could affect the physiology of the cells. However, it is more likely that the major effects of fluoride on energy transfer involving F-ATPases have to do with the uncoupling effects of fluoride due to increased transmembrane proton permeability and dissipation of ΔpH across the membrane.

2.2.2. Sensor kinases and regulatory proteins

Datta et al. [30] have used ADP-AlF₃ to investigate the crystal structure of the RecA protein from Mycobacterium tuberculosis. The fluoride-containing analog interacted in the P-loop of the protein containing the ATP binding site. Again, this example shows the usefulness of fluoro-complexes for determining protein structure and indicates that the complexes can generally affect regulatory phosphatases. However, whether or not inhibition of the regulatory phosphatases occurs in living cells is again an open question.

Similarly Cho et al. [24] were able to induce a state of persistent activation in CheY with use of BeF₃⁻ and to carry out detailed nuclear magnetic resonance structural analysis of the active regulator. The response regulator takes part in conveying signals from the membrane chemoreceptor for chemotaxis in E. coli to the flagellar motor. CheY is phosphorylated in the active form at aspartate 57, but the aspartyl-phosphate complex has a very short life. The complex with berylliofluoride is much more stable.

Yan et al. [120] have found that berylliofluoride can mimic phosphorylation for bacterial response regulators such as NtrC of Salmonella typhimurium. A constitutive mutant form of the sensor was known to have the capacity to hydrolyze ATP and activate transcription without being phosphorylated. ADP-BeF₃⁻, ADP-aluminofluoride or ADP-VO₃²⁻ were not inhibitors of ATPase activity. However, BeF₃⁻, but not aluminofluoride or vanadate, stimulated the ATPase activity. It appeared that berylliofluoride is an analog of aspartyl phosphate. Berylliofluoride activated chemotaxis, sporulation, osmosensing and nitrate/nitrite regulators – CheY, Spo0F, OmpR and NarL. With NtrC there was a threshold of fluoride concentration of about 1 mM, below which no activation occurred. Then maximal activation occurred between 5 and 20 mM with a fixed BeCl₂ concentration of 50 μM. Activation of ATPase activity could occur over a range of Be concentrations from 5 to 100 μM. Inhibition of activity occurred at still higher concentrations of more than 200 μM Be or 50 mM NaF, presumably because of ADP-berylliofluoride formation. It is difficult to assess whether or not these interactions with regulatory proteins are physiologically pertinent, although they certainly are biochemically pertinent.

2.2.3. Nitrogenase

BeF₃⁻ has been used for isolating stable, transition-state complexes of nitrogenases of bacteria, for example, that of Klebsiella pneumoniae. The enzyme was found [25] to be progressively inhibited in the presence of BeF₃⁻ and MgADP. Inhibition was fully, but slowly, reversible. It appeared that two BeF₃⁻ ions bound to each MoFeprotein of the enzyme and that the major complex also involved the Feprotein. Complexes of 2:1 and 1:1 [MoFeprotein:Feprotein] could be separated by FPLC gel filtration. Thus, bacterial nitrogenases are examples of yet another class of enzymes for which formation of metal-fluoride complexes has been of major help in the study of catalytic mechanisms. The inhibition of the enzyme occurred at low concentrations of Be. Again, there is question about the importance of such inhibition in living organisms. Quite possibly, the inhibition is mainly a laboratory phenomenon, albeit a very useful one.

2.3. Comparison of actions of fluoride as a transmembrane proton transporter with those of organic weak acids

There is ample documentation in the literature that fluoride in the form of HF acts to convey protons across artificial membranes or the membranes of living bacteria and to diminish ΔpH across the cell membrane, as reviewed previously [74]. This action has been demonstrated with simultaneous monitoring of intracellular pH and proton excretion [54]. The transport of protons into cells in acidified environments acts against the functioning of the F-ATPase to move protons out of the cytoplasm. Fluoride appears not sufficient to suppress completely the function of the F-ATPase. For example, for a set of oral bacteria ranging from A. naeslundii to Lactobacillus casei, acid tolerance was found [105] to be set primarily by the amount of F-ATPase activity per unit of biomass, or of membrane and also by the pH-activity profiles of the enzymes of the specific organisms. The enzyme of an organism with low acid tolerance, S. sanguis NCTC10904, had an optimal pH for activity above 7, whereas the enzyme of an acid-tolerant organism, L. casei, functioned optimally at a pH around 5.5. When fluoride was added to glycolyzing cells

ase from bovine mitochondria and determination of structure at 2 Å unit resolution [77]. Two of the three active sites of the enzyme were complexed with ADP-alumino-fluoride, while the third bound ADP and sulfate. The view is that this complex with all three active sites occupied is similar to the post-hydrolysis, pre-release-of-product stage of the catalytic cycle, or the stage at which energy transfer mainly occurs in ATP synthesis. Previously, Braig et al. [12] used aluminum trifluoride for crystallization and X-ray analysis of structures that mimic the transition state of the enzyme. It appeared that the presence of aluminofluoride caused only minor changes in the surrounding protein.
with excess glucose, there were initially reductions in acid tolerance directly related to fluoride levels [73]. However, above a fluoride concentration of about 4 mM, there was not much further decrease in acid tolerance. In effect, it appeared that the fluoride effect was saturable, a finding suggesting either a saturable carrier or some other restriction on fluoride movement into the cells possibly related to capacities of the cells to maintain ΔpH in the presence of high levels of fluoride. In addition, the hierarchy of acid tolerance among the organisms tested was not altered by fluoride. *L. casei* remained more acid-tolerant than *S. sanguis* even in the presence of 20 mM fluoride, apparently because the F-ATPase retained sufficient function to allow some glycolysis to continue.

Organic acids also are known to be transmembrane proton transporters in a manner at least superficially like that of fluoride. A major basis for inhibitory effects of food-preservation weak acids, such as benzoate and sorbate, or of metabolic acids, such as acetate and lactate, involves acidification of the cytoplasm of cells in acid environments. Acidifying of the cytoplasm then leads to inhibition of acid-sensitive enzymes, such as those of glycolysis [31]. The organic acids can also be direct inhibitors of many enzymes, but generally only at much higher concentrations than those at which major effects on ΔpH occur [103]. We have found [5] that weak-acid, non-steroidal, anti-inflammatory agents, such as indomethacin and ketoprofen, also can act as proton carriers to inhibit metabolism as do fluoride and food-preservation weak acids. In fact, indomethacin was found to be essentially as potent as fluoride for sensitizing glycolysis by intact cells to acidification. As indicated by the material presented in the next section, fatty acids have similar acid-sensitizing actions and also have potential as anticaries or anti-infection agents.

### 2.3.1. Comparison of effectiveness for acid sensitization and proton transport

It is well known that the inhibitory actions of fluoride for processes such as glycolysis of intact microbial cells are highly pH-dependent. At pH values around neutrality, inhibitory levels of fluoride for glycolysis are well above 10 mM, whereas at a pH value of 4, levels for complete inhibition are in the micromolar range. This pH dependence is important in relation to cariogenic bacteria and caries because the disease progresses only at acid pH values. Indeed, the difference is important generally for all organisms in acidified environments containing fluoride. Acid sensitization can be readily seen in standard pH-drop experiments in which cells are suspended in dense suspensions in a salt solution, commonly 50 mM KCl plus 1 mM MgCl₂, and given excess sugar. The cells degrade the sugar and lower the suspension pH to some minimum value at which they can no longer maintain a cytoplasmic pH compatible with the workings of glycolytic enzymes. If the suspension is then neutralized with alkali, a new round of glycolysis occurs. Thus, the glycolytic system appears not to be inactivated by the level of acidification, at least over a period of some hours. The final pH value attained in a pH-drop experiment is a good indicator of the acid tolerance of the cells, in this case for glycolysis.

As shown in Fig. 1, fluoride at a concentration of 1 mM sensitized cells of *S. mutans* to acidification so that the final pH value was about 5.5, compared with about 3.7 for cells not exposed to fluoride. The other curves show...
that organic weak acids, here fatty acids, at a concentration of 1 mM had a similar sensitizing effect. Caprylic (C9) acid was not very potent for sensitizing, while nonanoic acid (C11) was actually more effective than fluoride. Thus, the length of the hydrocarbon chain of the fatty acid is important for potency. Fatty acids with longer chain lengths up to lauric acid (C12) were as potent as nonanoic acid. However, still longer-chain fatty acids had much lower potency. In other words, there is an optimal chain length for activity. As discussed below, the optimal chain length for potency of fatty acids could possibly be related to so-called flip-flop mechanisms for proton transport of the sort described for eukaryotic systems [57].

The methyl ester of capric acid was largely ineffective for acid sensitization of glycolysis (Fig. 1), and the small effect is likely due to penetration of hydrophobic regions of the membranes by the hydrocarbon chain of the ester. Thus, it appears that the carboxyl function is the key for potency. Esters of weak acids with larger fatty acid chains, e.g., the parabens (esters of p-hydroxybenzoic acid) are highly bactericidal, but their mechanisms of action are different from the weak acids [71]. They cannot act as proton transporters but can affect membrane structure, and butyl paraben has been shown to be a potent enzyme inhibitor, including for the phosphoenolpyruvate:sugar phosphotransferase system [70]. Paraben potency can be related to the fatty acid substituent – the larger the substituent, the more potent the paraben, but also, the lower the water solubility.

Fluoride and organic weak acids increase proton permeabilities of bacterial cells, roughly in proportion to their potencies to reduce acid tolerance of glycolyzing cells [35]. This increase in permeability then leads to increased ATP demand to move protons out through the F-ATPase. The net effect in a growing culture is lower growth efficiency in terms of biomass produced per unit of catabolite used. An example is presented in Fig. 2 for glucose-limited cultures of S. mutans GS-5 growing in a chemostat at various pH values. When the culture pH was around 7, 0.1 mM NaF had no effect on biomass concentration. As the growth pH was lowered, fluoride was progressively more effective for reducing growth yields. The reductions occurred presumably because more and more of the ATP produced from glycolysis was required for acid-base balance to maintain ΔpH across the cell membrane and was not available for growth processes. At a pH value of 5.8, there was complete washout of the culture with 0.1 mM NaF but reduction of yield in the unfluoridated culture of only about one third. However, at pH values below 5, the unfluoridated culture also washed out of the chemostat.

Sensitization of cells to acid conditions can affect multiple systems in bacteria. The effects are indirect and related to acid inhibition of the systems. However, the effects are important in the physiology of the organisms and their capacities to function in acidified environments. Examples include inhibition by fluoride and organic weak acids of alkali production from arginine in acidified cultures [16] or of respiration [88]. Inhibition of alkali production further sensitizes cells to acid inhibition and impairs their adaptive capacities. Inhibition of respiration reduces oxidative stress and damage and works oppositely to fluoride inhibition of catalase and other protective enzymes considered above.

Sensitization to acidification can result also in death of bacteria, as shown by Phan et al. [86]. Moreover, at lethal pH values, fluoride and organic weak acids were found to have significant effects related to transmembrane proton transport into cells leading to more rapid acid killing. In other words, even at these slowly lethal, low pH values, there appears to be some maintenance of ΔpH across the cell membrane that can be diminished by fluoride and organic weak acids. This same effect was demonstrated for cells in biofilms, as reviewed below.

2.3.2. Mechanisms of transmembrane proton transport

The flip-flop mechanism for transmembrane proton transport by fatty acids, which is considered important in the physiology of animal cells, is driven by ΔpH across cell or vesicular membranes [106]. Fatty acids added to cells initially associate with the lipid bilayer of membranes with their hydrocarbon chains inserted into the layer. If the pH outside is lower than that inside, external carboxyl groups become protonated, which causes them to flip to expose protonated carboxyls to the interior. Because the interior pH is higher, the carboxyl groups dissociate and release protons. They then can flop back across the membrane to repeat the transport process. Flip-flop will continue until ΔpH becomes zero. Eukaryotic cells also have specific transport systems for fatty acids, and so fatty acid transport is more complicated than just that involving the flip-flop mechanism. Flip-flop can result in net transport
of fatty acids into cells because molecules brought to the inside of the membrane then equilibrium with those in the cytoplasm. Generally for eukaryotic systems, the optimal chain length for the flip-flop mechanism is higher than that for sensitization of glycolysis of intact bacterial cells to acidification. This difference may be related to peculiarities of bacterial membranes compared with the membranes of eukaryotes, although much of the work with eukaryotes has been carried out with mitochondria [106] which have bacterial characteristics. Moreover, when we tested many of the variants of fatty acids highly effective for flip-flop proton transport in eukaryotes [56], they were not effective for sensitizing oral bacteria to acidification. Previously, with a series of food-preservative and anti-inflammatory weak acids, we attempted unsuccessfully to develop a modulus to relate acid-sensitizing potential to \( pK_a \), molecular size and octanol-water partition coefficient [5]. In effect, there are still fundamental questions regarding actual mechanisms for proton translocation. It is likely that no single modulus will be predictive because there seem to be multiple mechanisms for transmembrane proton transport. HF is such a small molecule that it should cross biological membranes through water channels, including aquaporins. In contrast, the larger acids with hydrophobic components associate strongly with hydrophobic components of cell membranes and other parts of the cell. Moreover, we have studied uptake and cell retention of capric acid and lauric acid, which have similar potency for sensitizing cells of \( S. \) mutans to acidification. We found that laurate is washed out of cells only with difficulty and is taken up even by permeabilized cells, albeit to only about 50% of the level for intact cells. In contrast, caprate is not taken up to the high levels found for laurate. Caprate is taken up mainly by intact cells, only minimally by permeabilized cells, and is easily washed out of intact cells. Clearly, there seem to be multiple mechanisms leading to the general antimicrobial effects of weak acids.

### 2.3.3. Inhibition of solute transport

This topic was considered briefly in relation to arginine uptake and metabolism. Another good example of fluoride acting as an inhibitor of a bacterial transport system is described by Byers et al. [18] for transport of N-acetylneuraminic acid by \( \text{Streptococcus oralis} \). NaF was found to be the most potent inhibitor tested apparently because of its capacity to reduce \( \Delta p \) and de-energize transport. Clearly, there are many such situations in the microbial world in which microbes in acidified environments need to transport solutes into the cytoplasm. Fluoride or other weak acids interfere with energization of transport either directly by dissipating \( \Delta p \)H, or less directly, by reducing ATP supply as the organisms try to meet the increased demand for proton excretion. Generally, inhibition of solute-transport systems is reversible and not lethal in the short term. However, the inhibition clearly affects the metabolic and adaptive capacities of the organisms, and in the long term, can be lethal.

#### 2.3.4. Inhibition of macromolecular synthesis and export

Fluoride and other weak acids can inhibit synthesis and export of macromolecules by mechanisms not well defined at this time. Probably inhibitory effects are related to loss of \( \Delta p \) across the cell membrane, which can have multiple secondary effects, including activation of hydrolytic enzymes associated with the cell wall and membrane, e.g., activation of autolysins and inhibition of protein export [58]. Protein export could also be affected by inhibitory actions of F-metal complexes on the ATPase components of transport systems. For example, ABC transport systems active in transmembrane movements of both small and large molecules are energized through ATP binding and hydrolysis by their ATPase components. In addition, any transport system dependent on \( \Delta p \) would be affected.

Recent information presented by Cox et al. [26] for cultures of \( \text{Streptococcus sobrinus} \) 6715 growing exponentially in defined medium indicates that F in the growth medium has major effects on levels in the cells of various proteins with synthesis of some upregulated and that of others downregulated. The cells were labelled with \( \text{[35S]} \)methionine, and proteins were extracted with sodium dodecyl sulfate for separation by 2-D gel electrophoresis. In essence, there appeared to be a global response to fluoride of the types to acidification, oxidative stress or heat stress. Results of experiments in which the organism was grown in complex medium with or without fluoride indicated that fluoride strongly repressed production of the glucan binding lectin (GBL) of \( S. \) sobrinus. Inhibition was quickly reversed when the cells were washed and placed in fresh, fluoride-free medium. Losses of GBL resulted in diminished abilities of the cells to bind glucan T-200 and to be aggregated by the glucan. Growth in fluoridated medium also resulted in increased chain length of the streptococci. The novel conclusion of this work is that there may be a fluoride-stress response, but more work is needed to separate responses unique to fluoride from those to acidification.

More recent work from the Doyle laboratory [21] indicates that growth of \( \text{Streptococcus pyogenes} \) in the presence of fluoride results in cells with reduced capacities to adhere to buccal cells, collagen, fibronecetin and laminin. In addition, fluoride was found to be a competitive inhibitor of adhesion of cells to collagen and fibronecetin but not laminin. Subsequently, Thongboonkerd et al. [110] found that growth of \( S. \) pyogenes in defined medium with 5 mM NaF resulted in major changes in protein expression, especially decreases in proteins required for stress responses including antioxidants, glycolytic enzymes, transcriptional or translational regulators and proteins involved in proper folding of macromolecules. The previous finding that growth in the presence of fluoride resulted in decreased binding to fibronecetin was reflected by decreased expres-
sion of glyceraldehyde-3-phosphate dehydrogenase, a promoter of adherence.

2.3.5. Fluoride resistance

Perhaps the most impressive finding regarding the capacities of bacteria to adapt to fluoride is that, despite fluoridation of drinking water starting just after World War II and later use of fluoride in oral care products, major resistance to fluoride among bacteria isolated from the human mouth is not a problem. The main report of fluoride resistance in vivo is that of Brown et al. [13] with organisms isolated from xerostomic patients who were undergoing intensive fluoride prophylaxis against caries after radiation treatment for cancer, which leads to dysfunction of the salivary glands. Even though fluoride is known to be concentrated in plaque to some 100 times the salivary level and to be retained for long periods, still, selection for fluoride resistance does not seem common. This lack of selection may be related to the finding that fluoride is not generally bactericidal except for a few types of bacteria, for example those with highly active autolytic systems [68]. Fluoride can enhance lethality of other agents, such as acidification [86] or peroxides [87]. It seems this sort of sensitization must not be sufficient to allow for selection. Also, in the animal body, stresses from acidification or oxidative metabolism tend to be periodic rather than continuous, so any selection pressure is intermittent.

Fluoride-resistant strains of mutans streptococci can be isolated in the laboratory. In general, they are slowly growing organisms and not likely to be competitive in mixed populations. However, Sheng and Liu [96] were able to isolate fluoride-resistant strains of S. sobrinus 6715 with enhanced acidogenicity and acid tolerance by growing the organism in agar with progressively higher levels of fluoride up to 5.0 mM. Previously, Bowen et al. [8] had found that, when S. sobrinus 6715 colonized desalivated rats, more acidogenic and cariogenic organisms were selected. Desalivation of the animals results in an extreme situation and a high level of susceptibility to caries. It seems that in extreme situations there may be selective pressures favoring more acid-tolerant and fluoride-tolerant organisms but that normally the pressures are too intermittent for selection. Adaptation to fluoride has been demonstrated also by Guha-Chowdhury et al. [46] and by Hoelscher and Hudson [52].

3. Weak-acid effects on cells in biofilms

3.1. Sensitization of biofilms to acid damage

Fluoride and organic weak acids can sensitize cells in mono-organism and multi-organism biofilms to acidification in much the same way as cells in suspensions are sensitized. An example of the type of data we routinely obtain is presented below for biofilms of S. mutans UA159 (Fig. 3). The biofilms were grown on glass microscope slides as described by Phan et al. [86] in batch cultures with daily changes of complex tryptone–yeast-extract–sucrose medium. After about 5 days, the slides are covered
with a thick film of organisms. Then, on the day before carrying out a pH-drop experiment, the biofilms were fed with medium containing glucose instead of sucrose. The experimental procedure is then the standard one for pH-drop assays. The biofilms on slides are placed in a solution of 50 mM KCl and 1 mM MgCl$_2$, excess glucose is added, and the fall in pH is recorded. The pH drop in biofilms is slower than that with suspensions, and can take up to 3 h before the final pH is attained. The final pH is usually somewhat higher than for suspensions, but only by about 0.2–0.4 pH units. Basic information on acid-base physiology of biofilms of oral streptococci is presented by Byrne and Marquis [17]. As shown in Fig. 3, caprylic acid was actually more effective than NaF for sensitizing the biofilms to acidification, and so even for biofilms, a fatty acid can be as potent as or more potent than fluoride for sensitizing to acid inhibition of glycolysis. The other fatty acids were either equally effective as or somewhat less effective than fluoride. The major difference found between suspensions and biofilms is that caprylic acid is more effective as a sensitizer for biofilms than for suspensions.

Cells in biofilms and suspensions can be sensitized to acid killing by addition of fluoride or organic weak acids [86]. An interesting conclusion of the findings is that, even in lethally acidic environments, the bacteria must still have capacities to exclude protons and this capacity is compromised by weak acids. Suspensions of $S. sanguis$ NCTC10904 and $A. naeslundii$ ATCC19246 in the study cited were readily sterilized by acidification to pH values below 3.7 and 4.0, respectively. Cells in biofilms were killed somewhat more slowly than those in suspensions, and it was much more difficult to sterilize biofilms because of high levels of persister organisms. However, fluoride and organic weak acids were found to be effective not only for increasing the rate of killing in the biofilms but also for reducing levels of persisters.

Fluoride can act also to reduce adhesion of organisms such as $S. pyogenes$ [21] and so can inhibit formation of biofilms and other types of multi-cell aggregates.

In addition, Embleton et al. [37] found that amine and tin fluoride could inhibit adhesion of $S. sanguis$ in a parallel-plate, flow-cell system. They had found previously [36] that biofilms were less sensitive to amine fluorides than planktonic cells. Again, it appeared that fluoride could affect not only the physiology of biofilms but also their formation. In the latter study, the effects were somewhat complicated by the amine and tin constituents, which can also have antimicrobial effects. Fluoride itself is not generally lethal for cells of oral streptococci, but amine fluorides and tin can be. Amine fluorides also can inhibit glucosyltransferase in solutions at a concentration of 0.1 mM, but when the enzyme was surface-bound as it would be in a conditioning films, inhibitory concentrations were some 100 times greater [95].

Biofilms have been used to demonstrate a variety of fluoride effects, for example, that fluoride supplied to mult-organism biofilms in milk is effective [90] for reducing acid production by sensitizing the population to acid conditions and also in favoring development of a less aciduric microbiota. Biofilms formed on apatite enriched or not with fluoride were used by Li and Bowden, [69] to show that fluoride from the mineral phase can be solubilized to have major effects on $S. mutans$ or $A. naeslundii$ but almost no effect on the highly acid-tolerant $L. casei$. The more moderated effects of fluoride on $L. casei$ are probably related to the high levels of F-ATPase of the organism and the low pH optimum of the enzyme [73,105]. In effect, the organism has a high capacity to excrete protons brought into the cell with HF at low pH values. Recently, Balzar Ekenback et al. [3] have shown that both NaF and fluoride varnish reduced carbohydrate metabolism and acid production by $S. mutans$ biofilms on hydroxyapatite disks.

3.2. Effects of fluoride on multi-organism biofilms of oral bacteria

Bradshaw et al. [11] have assessed the effects of fluoride on a nine-organism, biofilm/planktonic community in chemostat culture with hog gastric mucin as major carbohydrate source and a dilution rate of 0.1 h$^{-1}$ (generation time of 6.9 h). Their conclusion, based on analyses of the effects of fluoride on pH drop after glucose feeding of biofilms grown in a constant-depth film fermenter, was that fluoride acted directly on glycolysis to reduce acidification of the communities. Final pH values after 10 successive feedings of the biofilms with glucose were 4.41 for the control cultures and 4.83 for those to which 0.53 mM fluoride was added. In addition, analyses of population shifts during biofilm development indicated that fluoride affected the ecology of the biofilm/planktonic community to reduce the enrichment for mutans streptococci that normally occurs with environmental acidification. The communities to which fluoride was added to reduce pH drop became dominated by Veillonella dispar and $A. naeslundii$ so that mutants streptococci made up less than 3% of the total microbiota. The information obtained with the complex biofilm/planktonic cultures supplements information obtained previously with multi-organism suspension cultures containing aggregates of multiple bacterial species in which Fusobacterium nucleatum appeared to be the major agent catalyzing aggregate formation [10]. These aggregates share many of the properties of biofilms, including enhanced resistance to antimicrobial agents.

Other types of multi-species consortial biofilms of microorganisms have been grown in various types of vessels at various flow rates and levels of complexity in terms of feeder cultures. A major advantage of biofilms for studies of consortia is that a diverse population can be maintained in chemostat, turbidostat or other controlled-flow systems. These mixed culture systems have been exploited especially in specialized disciplines in microbiology, for example in
bioremediation studies or in studies of dental plaque bacteria, as indicated above. Another type of system that has been used successfully for studies of fluoride effects includes an artificial mouth apparatus and involves formation of biofilms of dental plaque bacteria on the surfaces of enamel or dentine blocks [98]. In the system used, the major outcomes assessed were pH drop, softening of enamel due to acid-induced dissolution and lesion depth for enamel and dentine. Fluoride incorporated into the feed medium at a level of about 1 mM was highly effective in reducing pH drop and demineralization but did not bring about major shifts in the consortium population, which under the conditions of feeding with sucrose-containing medium was composed mainly of mutans streptococci and L. rhamnosus with no detectable A. naeslundii.

Another less readily controlled type of biofilm that has been very useful for research is that formed on intra-oral appliances. An example is the system developed by Giertsen et al. [42] in which acrylic appliances containing bovine enamel disks were worn in the mouth for 1-week periods by human subjects. Twice daily, the appliances were removed and dipped in various agents, including 26.3 mM NaF with or without 20 mM zinc acetate, with water as control agent. Then, the microbiota that developed on the disks were analyzed by standard culture, automated quantitative immunofluorescence and viability fluorescence staining. The treatments did not significantly lower total numbers of colonizing organisms. The fluoride treatments did lower numbers and relative numbers of streptococci in the biofilms. Numbers of mutans streptococci in the films were low, and fluoride did not act to reduce greatly their relative numbers. The general finding is that fluoride favors development of a less aciduric microbiota in mixed populations. However, to obtain this type of outcome, the conditions for film development must be such that there are periods of acidification sufficient to allow for selection of aciduric organisms in the untreated biofilms.

3.3. Concentration and distribution of fluoride in dental plaque and other biofilms

Fluoride is concentrated in dental plaque to levels of some 100 times salivary levels [33]. As shown by the data in Fig. 4, this same sort of concentration occurs for biofilms, here of S. mutans, grown on glass slides. It has been possible also to assess gradients of solutes in biofilms by freezing and sectioning the films and then analyzing specific chemicals in the sections. Robinson et al. [91] were able to show for plaque biofilms formed in the mouth on disks of enamel that fluoride levels were highest near the outer surfaces of the films and declined in the direction of the enamel surface. Levels of [14C]sucrose showed a similar pattern of change, while 32P-labelled phosphate was more concentrated in the more central regions of the films. Thus, it appears that fluoride is present in highest concentrations where the level of glycolysis would be expected to be greatest. When the enamel disks on which the plaque biofilms formed were pretreated with a 900 ppm (ca. 47 mM) solution of fluoride, the profile in the biofilms was modified so that there was a peak of fluoride near the biofilm–enamel interface [1]. Thus, it seemed that enamel had the potential to serve as a source of fluoride for plaque biofilms.

4. Weak acids and dental caries

The major debates about weak acids and dental caries revolve around views of the mode of anticaries action of fluoride. There is a tendency in the literature to downplay the role of antimicrobial actions of fluoride for anticaries action and to ascribe the major benefit of fluoride to its interactions with tooth mineral. The extreme of this tendency is seen in in vitro experiments on caries either with no bacteria present or with bacteria not generally considered to be cariogenic. However, it seems that there is more and more difficulty in maintaining this ‘mono-theistic’ view. Perhaps the strongest indicator that a multi-effect view is needed comes from the findings that organic weak acids have anticaries actions similar to those of fluoride. The results of recent studies of the effects of food-preservative weak acids and non-steroidal anti-inflammatory agents against rat caries point in the direction of an antimicrobial effect [9,32]. Also agents such as lauric acid have been shown [50] to be effective anticaries agents, even though they would not be expected to affect in any major
ways demineralization or remineralization. As with all subjects, there is need for additional research. However, the ways in which to proceed are clear based on current knowledge. Fluoride has specific effects on biological systems not shared with organic weak acids, mainly anti-enzyme effects due to fluoride binding or to binding of fluoride–metal complexes. Fluoride also has effects shared with organic weak acids, mainly those having to do with enhanced transport of protons across the cell membrane. It is these latter effects that seem to be most pertinent to the antimicrobial–anticaries properties of fluoride. Fluoride can even have an anticaries effect when added to sucrose in the diet [28,84] and can act in concert with other anticaries agents [40,41,121]. Moreover, fluoride appears to have important ecological effects on dental plaque in that it acts to reduce acidification and in the long run serves to select for a less acid-tolerant, less cariogenic microbiota, as suggested by, for example, Marsh [75].

Overall, it seems that the actions of fluoride most important for its antimicrobial–anticaries effects are those having to do with reduction in the acid tolerance of glycolysis by intact, cariogenic bacteria in plaque. As a result, acid production is stopped before the plaque pH drops to values leading to rapid demineralization. The caries process results from the glycolytic activities of non-growing bacteria at pH values below 5, which is approximately the minimum growth pH even for organisms such as the mutants streptococci. Any inhibitory effects fluoride may have on growth of plaque bacteria are not very pertinent to its anticaries action. Growth occurs during the pH-rise phases of the acidification–alkalinization cycles in plaque, whereas caries develops in the pH-fall phases. The potency of fluoride or of organic weak acids to inhibit glycolysis is very pH-dependent and increases sharply as pH declines. Thus, the weak acids seem to be ideal anticaries agents with minimal effects at higher, non-cariogenic pH values but major effects at cariogenic values.

5. The future

Hopefully, this review will facilitate research to gain a greater appreciation for the major roles played by weak acids in microbial physiology and gain more information on their actions, including information on mechanisms. In turn, more information should lead to more effective use of weak acids to control microbes in the environment, in disease processes and in manufactured goods. There is clearly a need for antimicrobial agents that do not select for antibiotic resistance. An example of a situation in which weak acids may be valuable might be in Helicobacter pylori infection. The organism depends on urease action for virulence and to survive in the acid environment of the stomach [102]. Fluoride is an effective inhibitor of the enzyme of intact cells, especially in acid environments, and could be used to reduce virulence. Of course, the use would have to take into account the sensitivities of cells lining the stomach to fluoride. However, this is only one example of very many in which fluoride and other weak acids may be useful. There is need also with fluoride use to combine the halogen with other agents to develop more effective antimicrobial regimens. The use of lower levels of fluoride would reduce side effects, e.g., fluorosis. Thus, combinations of fluoride and organic weak acids would allow for lower intake of fluoride but with the same desirable effects.

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