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The Effect of Fluoride on Treponema denticola

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THE EFFECT OF FLUORIDE ON TREPONEMA DENTICOLA

by

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VITA

Carrie A. Norton Hughes was born Carrie Ann Norton in Fort Atkinson, Wisconsin, on October 27, 1960, to Emmett W. and Mary Ann Norton.

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She is co-author of the following publications:

Papers:

Collins, M. L. P. and C. A. N. Hughes. 1983. Identity of succinate dehydrogenase in chemotrophically and phototrophically grown Rhodospirillum rubrum. Arch. Microbiol. 136:7-10.

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LIST OF ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
%C	bis (N,N-methylenebisacrylamide) conc.
°C	degree centigrade
CE	cell extract
cm	centimeter
d_2H_2O	deionized distilled water
diazo-NDS	7-diazonium-1,3-naphthylene disulfonate
EC	Enzyme Commission
EDTA	ethylenediamine tetraacetic acid
Fig.	figure
g	gram
h	hour
in	inch
IU	International Unit
kV	kilovolt
K	thousand
l	liter
lb	pound
M	molar
mA	milliamp
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
µl	microliter
MW_r	relative molecular weight
N	normal
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NDS	7-amino-1,3-napthylene disulfonic acid
nm	nanometer
OD	optical density
pI	isoelectric point
P_i	inorganic phosphate
PAGE	polyacrylamide gel electrophoresis
pNP	p-nitrophenol
pNPP	p-nitrophenyl phosphate
SDS	sodium dodecyl sulfate
%T	acrylamide concentration (conc.)
TEMED	N,N,N',N'-tetramethylethylenediamine
U	unit
UV	ultraviolet
V_e	elution volume
V_o	void volume
V_t	total bed volume

VIS	visible
v/v	volume (ml) solute/volume (100 ml) total
W	watts
w/v	weight (g) solute/volume (100 ml) total

INTRODUCTION

Treponema denticola is a common inhabitant of the human oral cavity. This gram-negative anaerobic spirochete is a member of a group of fastidious microorganisms which are associated with subgingival dental plaque. A strong correlation has been made between the percentage of spirochetes within a given subgingival plaque sample and clinical evidence of periodontal disease at the site from which the plaque samples were obtained (Loesche and Laughon, 1982). An increase in subgingival treponemes is associated with an increase in dental plaque index, gingival exudate, gingival index, bleeding tendency, and pocket depth, in addition to a loss of connective tissue attachment (Listgarten and Levin, 1981).

In healthy sites, treponemes are present in relatively few numbers in subgingival plaque; their numbers increase dramatically in sites affected by periodontal disease. Listgarten and Levin (1981) report that only spirochetes appear to be positively correlated with probing depth. Armitage et al. (1982) also found that subgingival spirochetes increased significantly when pocket depth and attachment loss exceeded 3 mm. While they did not identify the spirochetes, they noted that spirochetes were present in healthy sites, but only in low percentages. Although these studies have not provided direct evidence that spirochetes are an etiological agent of periodontal disease, they do demonstrate that the periodontal pocket provides a uniquely suitable ecological niche for spirochete growth.

Listgarten and Hellden (1978) determined the relative distribution of bacteria at clinically healthy and periodontally diseased sites within the same individual and reported that whereas spirochetes accounted for less than 2% of the total microscopic flora at the healthy sites, their proportions increased from 25 to 58% within the diseased sites. Schultz-Haudt et al. (1954) examined subgingival plaque taken from either healthy or periodontally involved patients and reported a 17% increase in the numbers of spirochetes in samples from patients with periodontal disease. Spirochetes were found to compose about 45% of the microscopic count in subgingival plaque samples removed from sites associated with adult periodontitis (Loesche et al., 1985). Slots et al. (1979) examined the pretreatment plaque of periodontitis patients and reported that the spirochetes comprised 30% of the total microscopic flora. Following treatment, the spirochetes were reduced to nondetectable levels in 50% of the pockets.

Recently, Simonson et al. (1988) provided evidence of a positive relationship between a specific spirochete, T. denticola, and severe periodontitis. The spirochete was present at a significantly elevated level in plaque samples collected from deep-pocket sites of severe periodontitis patients. Cheng et al. (1985) had previously studied the serological heterogeneity among the oral isolates of T. denticola and indicated that some serotypes may be more related to disease than others. These results should aid future studies related to detecting differences in disease associations for various spirochete serotypes.

An important factor in periodontal disease is bacterial plaque. The destructive nature of the periodontitis lesion can be maintained only

in the presence of subgingival plaque bacteria (Slots, 1979; Van Palenstein Helderman, 1981). Current periodontal therapy is directed toward the removal of subgingival bacterial deposits and the control of bacterial regrowth by controlling supragingival plaque. These goals are usually achieved by means of scaling and root planing, local or systemic administration of antibiotics, periodontal surgery where indicated, instruction in oral hygiene procedures, and periodic maintenance. Unfortunately, these methods require either considerable patient contact time or written prescriptions for the drugs used. It is important that, in order to be considered effective, a method must be shown to either control or reduce essentially all clinically detectable plaque, prevent gingivitis, periodontitis or caries, or alter bacterial components which are known to be essential for disease development. Chemotherapeutic agents will undoubtedly play a role in the future for plaque control (Kornman, 1986).

Despite the evidence of a relationship between gingivitis and plaque, and the spirochetes therein, only a few specific therapeutic agents are capable of controlling plaque formation. Chlorhexidine gluconate, benzethonium chloride, or other bisbiguanides may be used to reduce plaque and gingivitis (Eriksen and Gjermo, 1973; Flotra et al., 1971; Lobene, 1979). However, the lengthy period for treatment is associated with a staining of the teeth and the accumulation of calculus. In addition, the effectiveness of these antibacterial agents for periodontopathic bacteria remains questionable. A more effective means for the treatment of periodontal disease calls for the control, if not the elimination of the specific pathogens and their effects on the host

(Socransky et al., 1982). In order to achieve these means, more must be known about the pathogens involved, especially their involvement in the production of periodontal disease and their responses to the agents used for periodontal treatment.

The use of fluoride in preventive dentistry is widespread. In terms of dental caries prevention, fluoride represents one of the most successful agents in all medicine. Fluoride appears to have several modes of action in the prevention of tooth decay. Fluoride has been found to decrease tooth enamel solubility (Brown et al., 1977), to augment enamel remineralization (Silverstone, 1972), and to interfere with the growth and metabolism of oral bacteria (Kleinberg et al., 1977). However, the mechanism of action of fluoride is still not entirely understood.

Fluoride, in its ionic form, is one of the most reactive elements found in the periodic table. The high electronegativity of the F atom often has a profound effect on the properties of molecules in which F occurs. This extreme reactivity may account for the large number of potential mechanisms for the action of fluoride.

The level of fluoride in saliva has been found to be low (0.02 μg/ml; Shannon et al., 1973). However, fluoride concentrations in plaque have been found to range from 0 to 60 μg/ml (Hardwick and Leach, 1962). In 1959, Konig reported the inhibition of plaque formation in rats receiving stannous fluoride applications. Other studies using stannous fluoride rinses have shown significant reductions in plaque (Svantun et al., 1977; Tinanoff et al., 1980). A dose-related decrease in the number of subgingival motile bacteria and spirochetes, in addition to a

reduction in the bleeding index score has been shown following subgingival irrigation with a stannous fluoride solution (Mazza et al., 1981; Boyd et al., 1985). These studies do not differentiate between species of spirochetes. Studies have also shown that brushing with toothpaste containing stannous fluoride reduced both plaque and gingivitis (Bay and Rolla, 1980). Newbrun et al. (1984) also reported on the antimicrobial activities of sodium fluoride on selected periodontal pathogenic microbes. Various species of periodontopathic bacteria belonging to the genera of Actinomyces, Capnocytophaga, Eikenella, Fusobacterium, Actinobacillus, and Bacteroides have been shown to be inhibited by fluoride at concentrations of 128 to 256 μg/ml (Mandell, 1983; Yoon and Newman, 1980). However, spirochetes were not included in these studies. Recently, Kay and Wilson (1988) reported on the in vitro susceptibility of forty strains of subgingival plaque bacteria to amine fluorides. Their findings suggest that amine fluorides may be useful in the treatment or prophylaxis of plaque-related diseases. Unfortunately, this recent survey also failed to include strains of periodontopathic spirochetes. The accumulation of fluoride in dental plaque may provide enough fluoride to produce an inhibitory effect on the resident plaque bacteria. Studies on the effect of fluoride on periodontopathic bacteria are extremely limited and investigations on the in vitro action of fluoride on T. denticola are lacking.

The effect of fluoride on enzymatic regulation of the cariogenic bacteria, such as the oral streptococci, has been reviewed (Hamilton, 1977). These organisms derive their energy from the degradation of sugars. Studies indicate that fluoride has several sites of action: one

at enolase and one at some site prior to glucose-6-phosphate formation (Hamilton, 1977). However, the mechanisms by which the clinical effect is produced are still very much debated. Information on the effect of fluoride on the enzymatic regulation of the periodontopathic bacteria is non-existent.

In contrast to the carbohydrate metabolism predominant in the cariogenic bacteria, amino acid fermentation is dominant in T. denticola (Blakemore and Canale-Parola, 1976; Hespell and Canale-Parola, 1971). Some amino acids, such as L-cysteine, L-serine, and L-alanine, are fermented by pathways that involve pyruvate as an intermediate. Other amino acids, such as arginine, are dissimilated by pathways that do not involve pyruvate. Although T. denticola possesses the enzymes needed for glucose fermentation via the Embden-Meyerhof (EM) pathway, glucose does not serve as the primary substrate when the organism is grown in the presence of both glucose and amino acids (Hespell and Canale-Parola, 1971). Furthermore, T. denticola uses a clostridial-type cleavage to metabolize pyruvate derived from carbohydrates via the EM pathway or from amino acids. This anaerobic spirochete possesses a number of different metabolic pathways that enable it to derive energy (ATP) for its growth from a wide range of substrates. The metabolic flexibility of T. denticola is probably one of the factors that allows it to compete, survive, and thrive in the oral cavity.

Preliminary work by Hughes and Yotis (1986) has revealed that the T. denticola 33520 nonspecific acid phosphatase is reduced in the presence of fluoride. Nonspecific acid phosphatases (orthophosphoric monoester phosphohydrolases: EC 3.1.3.2) have been purified or partially

purified from microorganisms. In general, nonspecific acid phosphatases hydrolyze a variety of phosphate esters, are inhibited by fluoride, and have an acidic optimum pH for activity (Morton, 1965). The term "phosphatase" is a general name applied to all enzymes which catalyze the hydrolysis of an ester or anhydride bond to phosphorous in phosphorylated metabolites, while the term phosphomonoester is usually limited to the phosphatases specific for phosphomonoesters. In gram-negative bacteria, most of these phosphatases are believed to be located between the inner (cytoplasmic) cell membrane and the outer wall or membrane--in the periplasmic region. Many of the same enzymes are often exoenzymes in gram-positive bacteria (Heppel, 1971). The periplasmic enzymes, a term coined by Mitchell (1961), are selectively released by procedures that do not release any internal proteins, such as by osmotic shock and the formation of spheroplasts with lysozyme and ethylenediamine tetraacetic acid (EDTA)(Heppel, 1971). Further evidence for the presence of periplasmic enzymes is provided by the fact that the activity of these enzymes can be measured with intact cells (Brockman and Heppel, 1968; Torriani, 1968). Intact cells are able to efficiently hydrolyze phosphate ester and diester substrates, which are not usually transported into the cell.

In the osmotic shock procedure, gram-negative bacteria are first suspended in a concentrated solution of sucrose in the presence of EDTA, which causes an increase in cell permeability and releases portions of the cell wall lipopolysaccharide into the medium. In these plasmolyzed cells, the cytoplasmic membrane limits the condensed cytoplasm, and the rigid cell wall retains the shape of the cell. When the treated cells are subjected to an abrupt decrease in the environmental osmotic

pressure, the cells swell rapidly and a small amount of cellular protein is released. Only the rigid wall prevents the cell from bursting (Heppel, 1971).

Spheroplasts are made by the treatment of gram-negative bacteria with a combination of lysozyme and EDTA. The outer wall structure of the cell is weakened in the spheroplast, but in contrast to protoplasts of gram-positive organisms treated with lysozyme, they retain some of the outer wall components. The cytoplasmic membrane becomes the outermost boundary between the cytoplasm and the environment over large areas of the spheroplast surface (Heppel, 1971), and it bursts when added to distilled water.

Studies of E. coli had established that several phosphatase enzymes were present (Rogers and Reithel, 1960; Von Hofsten and Porath, 1962). Alkaline phosphatase (EC 3.1.3.1), originally purified and characterized by Garen and Levinthal (1960), hydrolyses a variety of phosphate esters as well as pyrophosphate bonds. Anraku (1964) purified a cyclic 2',3'-nucleotide phosphodiesterase that also hydrolyzed 3'-nucleotides. A 5'-nucleotidase activity was discovered by Neu and Heppel (1965) and purified by Neu (1967). In addition to hydrolyzing 5'-nucleotides, this enzyme also acts on nucleotide diphosphosugar compounds.

Dvorak et al. (1967) described at least three different enzymes that could hydrolyze p-nitrophenyl phosphate at acidic pH in the shock fluid of E. coli. The acid hexose phosphatase and cyclic phosphodiesterase were purified to homogeneity. A nonspecific acid phosphatase, with activity towards a wide variety of phosphate esters, was found to resist purification and was only partially characterized. Dassa et al.

(1980) identified another acid phosphatase in E. coli that is optimally active at pH 2.5 in vitro and is relatively specific for phosphomonoesters possessing numerous phosphoanhydride bonds.

Studies by Kier et al. (1977a) revealed the presence of three periplasmic phosphatases in Salmonella typhimurium: a cyclic 2',3'-nucleotide phosphodiesterase, an acid hexose phosphatase, and a nonspecific acid phosphatase. No evidence was found for the existence of an alkaline phosphatase. All three phosphatases could be measured efficiently in intact cells; however, they were not readily released by osmotic shock procedures.

The location of these periplasmic enzymes allows them to hydrolyze phosphate esters to inorganic phosphate and the corresponding alcohol outside of the cytoplasmic membrane, which is considered to be the cell's main permeability barrier. Bacteria are known to be relatively impermeable to phosphate esters that are not actively transported (Lichtenstein et al., 1960; Kasahara and Anraku, 1974). Such non-transported substrates must first be dephosphorylated for subsequent metabolism. Dephosphorylation is also associated with intracellular or extracellular movement of metabolites. The primary evidence for the periplasmic location of the Salmonella phosphatases is the ability of intact cells to efficiently hydrolyze phosphate ester and diester substrates (Kier et al., 1977a). Similar observations on the hydrolysis of substrates by E. coli phosphatases were made by Brockman and Heppel (1968) and by Torriani (1968). Therefore, the periplasmic phosphatases could act as scavenging enzymes, hydrolyzing nontransportable phosphate esters into components that could then be transported and utilized by the cell. Phosphatase

activity could supply many types of nutrients, depending on the esters' organic moiety, in addition to phosphate.

The isolation of Salmonella mutants lacking nonspecific acid phosphatase has helped to define the physiological role of the periplasmic enzyme (Kier et al., 1979). When phoP and phoN mutants, which lack nonspecific acid phosphatase, were forced to use certain phosphomonoesters as phosphate sources, the mutant strains grew slower than the wild-type bacteria. These observations suggest that in wild-type cells, nonspecific acid phosphatase serves as a beneficial, although not essential, scavenging enzyme which facilitates the utilization of various metabolites present in the environment as phosphomonoesters.

Spirochetes manifest themselves in subgingival plaques associated with periodontal disease, where they account for 35 to 55% of the microscopically detectable flora. They localize on the outer surface of the plaque, where they are in intimate contact with the sulcular or pocket epithelium, or both (Loesche and Laughon, 1982). Olsen (1984) has shown in electron microscopic studies that T. denticola adheres to human epithelial cells in vitro. In vivo, spirochetes have been found to invade the tissue in acute necrotizing ulcerative gingivitis (Listgarten, 1965) and in periodontitis (Frank, 1980). Points of entry for oral bacteria appear to be the underside of desquamating cells or through ulcerations in the pocket wall. The mechanism of bacterial penetration into the tissue is not understood (Newman, 1984). The possibility exists that intracellular spaces may be initial portals of entry for bacteria into the epithelium and/or into the connective tissues. Other unknown factors, such as activity of hyaluronidase, phospholipase, or other

enzymes produced by bacteria, may also operate to permit invasion of bacteria toward the basal layer and eventually into the connective tissue (Bulkacz et al., 1982).

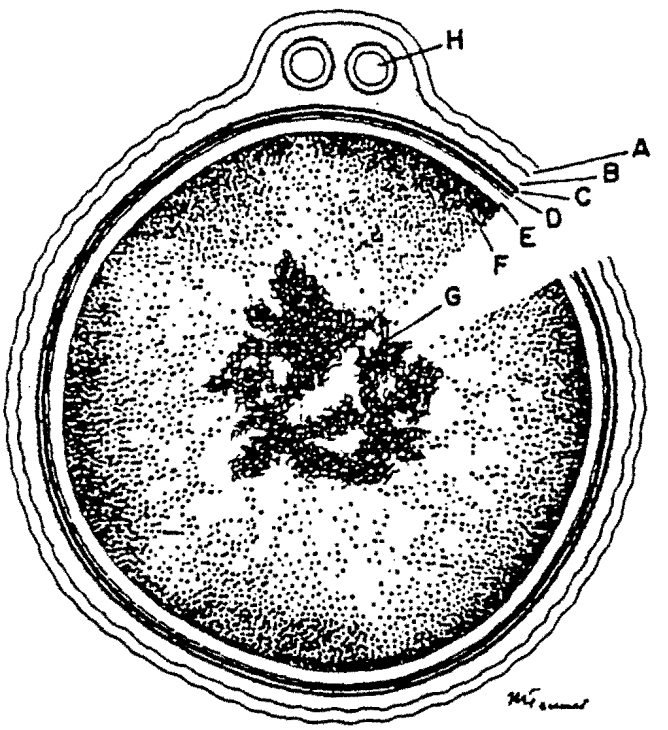
Among the oral spirochetes, T. denticola has been most studied (Canale-Parola, 1977; Loesche and Laughon, 1982; Harwood and Canale-Parola, 1984). In the gingival plaque environment, T. denticola has access to finite amounts of proteins, peptides, and amino acids. The metabolic diversity of the organism enables it to survive in the competitive environment of the gingival sulcus (Canale-Parola, 1977). The number and types of intra- and extracellular enzymes elaborated by this organism should reflect the nutritional requirements of the cell, since these enzymes would be needed to provide peptides or amino acids for the growth of the cells. The possible significance of Treponema enzymes as potential virulence factors in the development of periodontitis has been emphasized (Fiehn, 1986; Uitto et al., 1986). The organism is proteolytic; it hydrolyzes gelatin (Holdeman, 1977), dissolves fibrin (Nitzan, 1978), produces a collagenolytic enzyme (Makinen et al., 1986), and possesses a trypsin-like activity against benzoyl-arginine-naphthylamine (Ohta et al., 1986). Makinen et al. (1987) also report the presence of proline iminopeptidase activity in T. denticola. In addition, treponemes require a complex growth medium. They cannot synthesize or elongate fatty acids and rely upon those present in media or tissue for growth (Johnson, 1977; Livermore and Johnson, 1974).

The trypsin-like activity of T. denticola is a potentially pathogenic mechanism. Proteolytic activity may have a direct effect upon the junctional epithelium, as trypsin has been shown in vitro to disrupt

cell-cell and cell-substratum adhesins (Britch and Allen, 1980). Birkedal-Hansen et al. (1975) reported that trypsin activated latent gingival tissue collagenase by destruction of a collagenase inhibitor in serum. Trypsin is also known to activate the alternate pathway of complement fixation, causing the release of leukotactic factors (Ward et al., 1973). Furthermore, the oral treponemes have been surveyed for their ability to produce enzymes that are potentially destructive to host tissue (Laughon et al., 1982). Strains of T. denticola and T. vincentii have been found to produce C8 esterase, acid phosphatase, and phosphoamidase activities. These activities, singly or concertedly, could be responsible for significant damage to the peridontium.

The ultrastructure of spirochetes has been reviewed (Holt, 1978). This structure is diagrammatically depicted in Fig. 1. The most external layer of the cell is known as the outer sheath. This unit membrane may function as a primary permeability barrier. The region between the outer sheath and the outer layers of the protoplasmic cylinder is the equivalent of the periplasmic space in other gram-negative cells. The protoplasmic cylinder consists of the cell wall, cytoplasmic membrane and enclosed cytoplasmic contents. The cell wall-cytoplasmic membrane is characteristic of other gram-negative bacteria.

Fig. 1. A diagrammatic representation of a transverse section of a typical spirochete depicts the various cell layers. The outermost layer, the outer sheath (A), envelopes the cell. Situated in the electron-opaque periplasmic region (B) are the axial fibrils (H). The layers of the protoplasmic cylinder consist of the outermost helical lipoprotein layer (C), a peptidoglycan layer (D), and internal to the peptidoglycan, the innermost layer of the cell, the cytoplasmic membrane (E). The cytoplasmic region contains ribosomes (F) and a centrally disposed nuclear region (G)(taken from Holt, 1978).


H
A
B
C
D
E
F
G

The specific aims of this research are:

a) to examine the effect of fluoride on the growth of T. denticola;

b) to attempt to localize the p-nitrophenyl phosphate acid phosphatase of T. denticola;

c) to characterize partially the fluoride-sensitive p-nitrophenyl phosphate acid phosphatase of T. denticola.

It is hoped that these efforts to elucidate the fluoride sensitivity of T. denticola will lead to an understanding of the effect of fluoride on oral spirochetes so that the potential efficacy of fluoride as a preventive measure for periodontal disease may be considered.

MATERIALS AND METHODS

Organism and culture conditions. T. denticola ATCC 33520 and 33521, originally isolated from subgingival plaque taken from periodontally diseased sites, were used in these investigations. Strain 10 and a pectinolytic spirochete, strain P4, isolated from human subgingival plaque (Weber and Canale-Parola, 1984) were provided by E. Canale-Parola, University of Massachusetts, Amherst.

Treponemes were cultured at 37°C in a prereduced basal medium with appropriate supplements (Livermore and Johnson, 1974), which included 10% (vol/vol) heat inactivated fetal bovine serum (GIBCO Laboratories, Life Technologies, Inc., Chagrin Falls, OH). Treponemes were also cultured at 37°C in GM-1 broth in an N_2 atmosphere. The GM-1 medium was prepared as described by Blakemore and Canale-Parola (1976), except that it included 1.7% (v/v) heat-inactivated fetal bovine serum. GM-1 medium was modified by the addition of 0.3% pectin (weight/vol, final concentration) for the culture of pectinolytic strain P4.

For studies of enzyme levels of cells grown on poor carbon and phosphorous sources, the reduced glucose and phosphorous GM-1 medium (C^- P^-) was used. This medium contained per liter: pancreatic digest of casein (Bacto-Tryptone), 8.5 g; papaic digest of soybean meal (BBL Phytone Peptone), 1.5 g; sodium chloride, 5.0 g; yeast extract (Difco-Bacto), 5.0 g; sodium thioglycolate, 0.5 g; L-cysteine hydrochloride, 1.0 g; 0.2% thiamine pyrophosphate, 3.0 ml; 10% (w/v) $NaHCO_3$, 5.0 ml; fetal bovine serum (Gibco), 17.0 ml; volatile fatty acid solution, 5.0 ml. The

volatile fatty acid solution consisted of 0.5 ml each of isobutyric, DL-2-methylbutyric, isovaleric, and valeric acids dissolved in 100 ml of 0.1 N KOH. C^+P^- medium consisted of the basal C^-P^- medium with the addition of 1.25 mg glucose per ml. C^-P^+ medium consisted of C^-P^- medium with the addition of 1.25 mg K_2PO_4 per ml. C^+P^+ medium consisted of the basal medium with the addition of glucose and K_2HPO_4. The pH of each medium was adjusted to 7.4 with KOH.

T. denticola stock cultures were maintained by storage in fresh medium with 10% (v/v) glycerol at -70°C. Stock cultures were thawed and distributed to fresh media for growth as needed.

Growth curve measurements. Inocula for growth curve studies were generally prepared from early-logarithmic-phase (24 h) cultures by centrifuging cultures at 18,000 x g for 30 min at 4°C and gently resuspending the cell pellet in fresh medium to a cell density that ranged in optical density from 0.070 to 0.100. The inoculum consisted of 0.25 ml (1×10^8 to 2×10^8 cells per ml) per 5 ml of medium in a butyl-rubber stoppered Klett-Summerson tube. After incubation at 37°C, duplicate culture tubes were removed at designated time intervals and measured turbidimetrically by means of a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., Inc., New York) fitted with a 660-nm filter. Cell growth yields were determined by direct cell counts, with a Petroff-Hausser counting chamber under a phase-contrast microscope. A standard curve relating the colorimetric readings to direct cell counts was prepared.

API ZYM system. The commercially available API ZYM system (Analytab Products, Plainview, NY) was used as described by Laughon et al. (1982) to visually assay for the following 19 enzyme activities (with the corresponding substrates): alkaline phosphatase (2-naphthylphosphate), C4 esterase (2-naphthylbutyrate), C8 esterase lipase (2-naphthylcaprylate), C14 lipase (2-naphthyl-myristate), leucine aminopeptidase (L-leucyl-2-naphthylamide), valine aminopeptidase (L-valyl-2-naphthylamide), cystine aminopeptidase (L-cystyl-2-naphthylamide), trypsin (N-benzoyl-DL-arginine-2-naphthylamide), chymotrypsin (N-glutarylphenylalanine-2-naphthylamine), acid phosphatase (2-naphthylphosphate), phosphoamidase (naphthyl-AS-BI-phosphodiamide), α-galactosidase (6-bromo-2-naphthyl-α-D-galactopyranoside), β-galactosidase (2-naphthyl-β-D-galactopyranoside), β-glucuronidase (naphthyl-AS-BI-β-D-glucuronide), α-glucosidase (2-naphthyl-2-D-glucopyranoside), β-glucosidase (6-bromo-2-naphthyl-β-D-glucosaminide), N-acetyl-β-glucosaminidase (1-naphthyl-N-acetyl-β-D-glucosaminide), α-mannosidase (6-bromo-2-naphthyl-2-D-mannopyranoside), and α-fucosidase (2-naphthyl-α-L-fucopyranoside).

Spirochetes were harvested in the stationary phase of growth by centrifugation and resuspended in sterile saline (0.85% NaCl) to 200 Klett units. Sterile test solutions (NaF, SnF_2, $SnCl_2$, and NaCl) were added to cell suspensions as desired. Binding was allowed to occur for 1 h at 24 ± 1°C. The API ZYM strips were activated by adding 60 μl of bacterial suspension to each microcupule and incubated aerobically for 4 h at 37°C. Controls consisted of: 0.85% NaCl, NaF, SnF_2, $SnCl_2$, or NaCl in the absence of cells. All controls were found to have no measurable enzyme activity.

Enzyme assays. Nonspecific acid phosphatase activity was measured by the method of Bessey et al. (1946). The reaction mixture contained: 0.2 ml cell material (whole cells or cellular fractions in 0.2 M sodium acetate buffer, pH 4.8); 0.1 ml 8 mM p-nitrophenyl phosphate; 0.1 ml deionized distilled water. The reaction mixture was incubated for 60 min at 37°C, and the reaction was stopped with the addition of 0.8 ml 0.03 N NaOH. The resulting color change was measured at 405 nm (maximum wavelength for p-nitrophenol) with the observed intensity of color formed proportional to acid phosphatase activity. The standard used in these assays was p-nitrophenol. Activity was reported as nanomols p-nitrophenol released per mg soluble protein, BSA equivalents.

When the nonspecific acid phosphatase was assayed with different phosphate esters, the reaction mixture (0.4 ml) contained: 2.5 μmols of the substrate; 0.15 ml 0.2 M acetate buffer (pH 4.8) with 1 mM $MgCl_2$ and 0.5 mM $CoCl_2$; 0.10 ml distilled water; and enzyme. The assays were initiated by addition of substrate and conducted at 37°C. At various times after substrate addition, samples were removed and inorganic phosphate was determined (Chen et al., 1956). All substrates were used as the sodium salt. Controls included substrates and reagents in the absence of enzyme treated in an identical manner. Control values were subtracted from those generated by samples to yield the values given in the tables and figures in Results.

ATPase activity was measured by the liberation of P_i as described by Kubak and Yotis (1981). The reaction was started by the addition of whole cells to 1 ml of reaction mixture containing 100 mM KCl, 4.0 mM

$MgCl_2$, 2.5 mM ATP (vanadate-free) and 50 mM Tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 6.5). After 30 min at 37°C, the reaction was terminated by the addition of 0.5 ml of 1.5 M $HClO_4$. The tubes were centrifuged in a Fisher micro-centrifuge (model 235A; Lexington, MA) for 5 min at 4°C and the supernatant was assayed for release of P_i by the method of Chen et al. (1956). Values were obtained from a standard curve, following absorbance readings at 820 nm. The controls included the reaction mixture in which the sample was added after addition of perchloric acid. All control samples and reactants were exposed to similar conditions as the test samples. Control values were subtracted from sample values to yield those given in the results tables and figures.

Hexokinase (EC 2.7.1.1) activity was measured using an NADP-linked assay (Joshi and Vagannathan, 1966), which measured the increase in NADPH absorbance at 340 nm. The assay mix contained: 0.1 M potassium phosphate buffer (pH 7.5) with 20 mM $MgCl_2$, 1.5 ml; 81 mM ATP, 0.1 ml; 11 mM NADP, 0.1 ml; H_2O, 1.1 ml; 2 U glucose 6-phosphate dehydrogenase; 300 mM glucose, 0.1 ml; and 0.1 ml sample containing enzyme activity. Hexokinase, derived from Baker's Yeast, type F-300, sulfate-free, was used as a standard.

Analytical procedures. Soluble protein was measured by the method of Lowry et al. (1951). Reagents for the protein assay were as follows: Reagent A, 2% Na_2CO_3 in 0.1 N NaOH; Reagent B, 0.5% $CuSO_4 \cdot 5H_2O$ in 1% (w/v) sodium tartrate; Reagent C, 50 ml Reagent A mixed with 1 ml Reagent B; Reagent D, 1 N Folin and Ciocalteau's phenol reagent. For the assay, 1 ml of Reagent C was added to a 0.2 ml volume of sample or standard and

allowed to stand for at least 10 min. Then, 0.1 ml Reagent D was added to the assay and mixed immediately. Following an incubation at 24 ± 1°C for 30 min, the absorbance of the reaction product was measured at 500 nm. Bovine albumin (98-99% pure) was used as the standard.

Phosphate was assayed by the method of Chen et al. (1956) at 820 nm. K_2HPO_4 was used to construct a standard curve.

Glucose was measured by a method which is a modified version of that of Raabo and Terkildsen (1960). Briefly, 2.5 ml of combined enzyme-color reagent solution was added to 0.25 ml of sample and mixed thoroughly. The combined enzyme-color reagent solution consisted of (per 100 ml): 500 IU glucose oxidase (Aspergillus niger); 100 Purpurogallin U Peroxidase (Horseradish); 4 mg o-dianisidine dihydrochloride. Following incubation for 30 min in a 37°C water bath, absorbance was measured at 446 nm. α-D-glucose was used as a standard.

Spectrophotometric determinations were measured on a Gilford (Ciba Corning Diagnostics Corp., Gilford Systems, Oberlin, OH) Response UV-VIS scanning spectrophotometer.

Cell fractionation. Cells were harvested at desired phases of growth, pelleted by centrifugation for 25 min at 5,000 x g, washed with cold 10 mM Tris-HCl (pH 7.3) with 30 mM NaCl, and resuspended in the same buffer for enzyme assays. The washing procedure did not release detectable amounts of the enzymes into the supernatant solution.

Cell extracts were prepared from washed spirochetes that had been resuspended in 10 mM Tris-HCl (pH 7.3) containing 4 mM dithiothreitol. The whole cell suspensions were flushed with N_2 (Medical Grade; AIRCO,

Murray Hill, NJ) and passaged twice through the French pressure cell (American Instrument Co., Inc., Silver Spring, MD) at 16,000 lb/in^2. The resulting disrupted cell suspension was labeled "cell extract".

The procedures outlined by Dassa and Boquet (1981) and Dvorak et al. (1967) were used with some modifications to obtain material from the different compartments of T. denticola. Cultures of T. denticola 33520 were harvested by centrifugation (5,000 x g, 25 min, 5°C) and washed twice with 50 mM Tris-HCl (pH 7.8) containing 30 mM NaCl. Cell pellets were suspended in 50 mM Tris-HCl (pH 7.8) containing 30% (w/w) sucrose and 1 mM EDTA at a concentration of 1 g wet wt/40 ml at 24 ± 1°C. The mixture was stirred for 10 min and centrifuged. The supernatant was discarded and the plasmolyzed cells were subjected to a sudden osmotic transition by rapid dispersal in cold water (40 ml/g wet wt). The mixture was stirred for 10 min and centrifuged in the cold. The shocked bacteria were pelleted and retained for future manipulation. The supernatant "shock fluid" was concentrated to dryness by lyophilization. The dry powder was dissolved in a volume of distilled water equivalent to one fortieth of the original volume.

The shocked bacteria were resuspended in 50 mM Tris-HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 0.2 mg/ml DNAse I, 0.2 mg/ml pancreatic RNAse A and 2 mM $MgCl_2$, and passaged three times through the French pressure cell at 16,000 lb/in^2. The unbroken cells were removed by centrifugation and the supernatant was retained for further centrifugation at 300,000 x g for 2 h at 5°C. The resulting supernatant was decanted and labeled "cytoplasmic material." The pellet or "envelope fraction" was resuspended in buffer.

Anion exchange chromatography. Anion exchange chromatography was performed as described by Kier et al. (1977a). DEAE-cellulose (Sigma, medium mesh, 0.98 mequiv/g) was equilibrated with Tris-salts buffer containing 5 mM Tris-HCl (pH 7.4) with 1 mM dithiothreitol (buffer A). The column (0.8 x 60 cm) was washed extensively with the same buffer. Sample was applied to the column and eluted with 100 ml of buffer A followed by a linear gradient of NaCl in buffer A. The linear gradient was begun with 50 ml of buffer A in the mixing vessel and an equal volume of buffer A containing 0.2 M NaCl in the reservoir. Each of the 2.5 ml fractions (collected from the beginning of sample application) was assayed for nonspecific acid phosphatase activity and protein, as previously described.

Sephadex chromatography. Methods for gel filtration chromatography were based on those described by Weppelman et al., (1977). A Sephadex G-100 superfine (Pharmacia Inc., Piscataway, NJ) column (2.5 by 23 cm; total bed volume (V_t) = 113 ml) was equilibrated in and eluted with 50 mM Tris-hydrochloride buffer, pH 7.4, containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM $CoCl_2$, and 0.5 M KCl. The KCl was present to prevent binding of nonspecific acid phosphatase to Sephadex. Calibration proteins (Pharmacia) included; ribonuclease A, 13,700; chymotrypsinogen A, 25,000; ovalbumin, 43,000; aldolase, 67,000; and blue dextran, 2,000. The calibration sample contained 2 ml of equilibration buffer with 10% (w/v) glycerol and the following: 5 mg ribonuclease A, 1.25 mg chymotrypsinogen A, 5 mg ovalbumin, and 5 mg aldolase. The elution volume (V_e) of the standard proteins was determined by measuring optical absorbance at 254 nm. The

blue dextran elution volume (V_o) was determined by measuring absorbance at 262 nm. Partially purified enzyme preparations (750 μg protein) were mixed with elution buffer containing 10 % (w/v) glycerol (2.0 ml total volume), applied to calibrated columns, and eluted as described above.

Sephadex G-10 was also used in an attempt to concentrate enzyme activity in the initial "soluble contents". Briefly, 3.0 g of dry Sephadex G-10 was added to 10 ml of soluble contents and allowed to swell with rocking at 5°C. The swelled beads were removed by centrifugation (5,000 x g; 15 min, 5°C) and the supernatant was retained for enzyme and protein analysis as described earlier. The pelleted Sephadex G-10 beads were resuspended in 10 mM Tris-HCl (pH 7.4) with 0.5 M KCl, transferred to a Bio-Rad disposable column (15 ml) and allowed to settle. The buffer was allowed to drain from the column and the resulting eluate was analyzed for enzyme activity and protein content.

Diazotization. The reagent, 7-diazonium-1,3-naphthylene disulfonate (diazo-NDS) was prepared by the method of Pardee and Watanabe (1968) as follows: a 55 mg amount of 7-amino-1,3-naphthylene disulfonic acid (NDS; Aldrich Chemical Co., Inc., Milwaukee, WI) was dissolved in 3.5 ml H_2O with 0.05 ml concentrated HCl and was cooled on ice-salt; then 0.25 ml of 0.5 M $NaNO_2$ solution was added at -3°C. After 30 min, 2 ml of the diazo-NDS was added to a 7 ml suspension of washed stationary phase T. denticola. After incubation for 1 h at 24 ± 1°C, the cells were washed by centrifugation. Shocked cells were treated in the same manner and subsequently disrupted by French pressure cell passage to obtain cell

"envelope" and "cytoplasmic" fractions. Controls contained all components except NDS.

Spheroplast preparation. Spheroplasts were prepared following the method of Collins et al. (1980). Harvested cells (mid- to late-exponential growth) were washed twice in 1 mM Tris-HCl (pH 7.3) and resuspended to 1.67 OD_{680} in 20% (w/w) sucrose prepared in the same buffer (Tris-sucrose). This suspension was stirred at 24 ± 1°C for 10 min followed by the 1/10 volume additions of lysozyme (EC 3.2.1.17; 6.4 mg/ml in Tris-sucrose) and EDTA (20 mg/ml in Tris-sucrose). The suspension was stirred at 24 ± 1°C for 20 min after each addition. The osmotically-sensitive spheroplasts were collected by centrifugation, washed once in Tris-sucrose and resuspended in the same buffer. Pelleted spheroplasts were lysed by the addition of ice cold distilled water. The controls included bacteria incubated in the absence of lysozyme, EDTA, or both.

Electron microscopy. The acid phosphatase histochemical methods of Okabayashi et al. (1974) were followed. Early stationary (60 h) phase cultures of T. denticola grown in C^-P^- medium were harvested and washed. The cells were resuspended in 0.2 M acetate buffer (pH 4.8) with 1 mM $MgCl_2$ and 0.5 mM $CoCl_2$. The incubation mixture contained the following: cell suspension, 0.4 ml; H_2O, 0.1 ml; 4 mg/ml $Pb(NO_3)_2$, 0.1 ml; 8 mM p-nitrophenyl phosphate, 0.2 ml. Following incubation for 1 h at 37°C, the cells were pelleted, washed, and processed for electron microscopy.

Samples were prepared for electron microscopy by the methods of Olsen et al. (1984). Washed cell pellets were immediately fixed with 2%

glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) with 0.1 M sucrose for 1 h at 24 ± 1°C and then for 16 h at 4°C. Cacodylate buffer (0.15 M; pH 7.4) was used for 2-5 min rinses. Post-fixation was done with 1% osmium tetroxide in cacodylate buffer for 2 h at 24 ± 1°C. Following 2-5 min buffer rinses, the pellets were dehydrated in a graded series of acetone and embedded in Epon 812 plastic at 60°C for at least 24 h. Ultrathin sections (silver-gold) were made on an LKB Ultramicrotome with a diamond knife, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 transmission electron microscope. Controls for the histochemical methods included: cells reacted in the absence of $Pb(NO_3)_2$; cells reacted in the absence of substrate; cells treated with fluoride (NaF) prior to the complete histochemical reaction; and cells in the absence of the histochemical reaction.

Polyacrylamide gel electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was performed as described by Lam et al. (1978) and Maurer (1971). The separation gel stock solutions consisted of: (a) separation gel buffer (pH 4.3) per 100 ml: 48.0 ml 1 N KOH, 17.2 ml glacial acetic acid and 4.0 ml TEMED; (b) acrylamide stock solution per 100 ml; 30.0 g acrylamide and 0.8 g bis-acrylamide; (c) d_2H_2O; (d) 0.28 g ammonium persulfate per 100 ml. The separation gel (7.7 %T, 2.6 %C) was prepared by mixing the above stock solutions in the ratio (a:b:c:d) 1:2:1:4.

The stacking gel stock solutions consisted of: (a) stacking gel buffer (pH 6.7) per 100 ml: 48.0 ml 1 N KOH, 2.87 ml glacial acetic acid,

and 0.46 ml TEMED; (b) acrylamide stock solution per 100 ml: 10.0 g acrylamide and 2.5 g bis-acrylamide; (c) 4.0 mg riboflavin per 100 ml; (d) d_2H_2O. The stacking gel (3.1 %T, 2.0 %C) was prepared by mixing the above stock solutions in the ratio (a:b:c:d) 1:2:1:4. The stacking gel was photopolymerized in the presence of a 14-Watt fluorescent lamp (Canalco, Rockville, MD).

The electrode buffer stock solution for this system consisted of 31.2 g β-alanine per liter adjusted to pH 5.0 with acetic acid. The stock buffer was diluted to 10% in aqueous solution. The gel was pre-electrophoresed at 50 mM for 1-2.5 h at 5°C prior to loading samples. The loaded gel was then run at 20 mA for 17 h at 5°C with the polarity reversed (top (+) and bottom (-)). Pyronine Y (1 mg/ml) was used to mark the advancing front of the gel.

Two-dimensional gel electrophoresis was performed as described by Iborra and Buhler (1976). The first dimension (thin-layer isoelectric focusing, pH 3.5-5.2) was conducted in an LKB (LKB-Produkter AB, Bromma, Sweden) Multiphor unit. The unpolymerized mixture, consisting of 10 ml of an acrylamide (30%)-bis-acrylamide (0.8%) solution; 7 ml of 87% (v/v) glycerol; 1.5 ml of LKB Ampholine pH 3.5-5.0; 1.5 ml of LKB Ampholine pH 4.0-6.0; and 40 ml distilled water, was degassed and poured between two glass plates separated by a 1 mm thick rubber gasket. After polymerization, one glass plate was removed and the gel plate was lowered onto the cooling plate of the unit. Small plastic frames placed on the gel surface were used as sample reservoirs. Isoelectric point markers (U. S. Biochemical Corp., Cleveland, OH) consisted of acetylated cytochrome c at various pI: 4.1; 4.9; 6.4; 8.3; 9.7; and 10.6. Electrode solutions

consisted of: anode, 1 M H_3PO_4; cathode, 2% (v/v) ampholine pH 5-7. Focusing was conducted for 2 h at a constant power of 30 W. After focusing, the gel plate was frozen on ice. Strips containing samples were carefully excised and used in the second dimension or processed for enzyme activity (see below). In addition, consecutive slices (5 mm) of the focused gel were placed in boiled, degassed distilled water for 2 h and measured to determine the pH gradient of the gel.

The analysis of the second dimension (SDS-PAGE) was conducted in a Hoefer (Hoefer Scientific Instruments, San Francisco, CA) vertical electrophoresis unit. The separation gel consisted of 8% acrylamide, 0.25 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel consisted of 5% acrylamide, 0.25 M Tris-HCl (pH 6.8), and 0.1% SDS. The electrophoresis buffer consisted of 0.6% Tris base, 2.88% glycine, and 0.1% SDS. Electrophoresis was conducted at a constant current of 30 mA at 4°C until the tracking dye reached the bottom of the gel.

Molecular weight standards included; bovine albumin, 66,000; egg albumin, 45,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; and α-lactalbumin, 14,200. The electrophoretic mobility of each known protein was plotted against a logarithmic scale of molecular weights. The electrophoretic mobility of each band observed in PAGE was measured and its relative molecular weight value (MW_r) was estimated from the plot of molecular weight standards. The protein bands were referred to by the estimated molecular weight multiplied by 10^{-3} and followed by the letter K (Ames, 1974).

Visualization of nonspecific acid phosphatase and protein in gels. Enzyme activity was detected on gels by a post-incubation capture method described by Uriel (1971). Following electrophoresis, the gel was removed from the cassette and allowed to equilibrate for 2 h at 37°C in 0.2 M sodium acetate buffer (pH 4.8). The gel was removed from the buffer, the substrate solution: 5 mg of naphthol AS-BI phosphoric acid in 20 ml of 0.2 M acetate buffer (pH 4.8), was applied to the gel surface, and the gel incubated for 2 h at 37°C. Excess substrate solution was removed from the gel and the coupling solution, consisting of 10 mg of Fast Garnet GBC diazonium salt in 10 ml 0.2 M acetate buffer (pH 4.8), was applied. When the activity band reached the desired intensity (0.5 to 1.5 h) the gel was transferred to a 2% acetic acid solution and allowed to destain. Acid phosphatase (EC 3.1.3.2.), type IV from Potatoes, was used as an enzyme control.

Gels stained for nonspecific acid phosphatase were subsequently stained for proteins using Coomassie Blue R250 (Eastman Kodak Co, Rochester, NY). The staining solution consisted of: 1.25 g CB R250, 230 ml methanol, 230 ml d_2H_2O and 40 ml acetic acid. Gels were destained, using a solution consisting of 1500 ml ethanol and 500 ml acetic acid diluted to 5 L with d_2H_2O. When gels were sufficiently destained they were soaked in destaining solution containing 2% (v/v) glycerol for 1 h with shaking. The gels were transferred to a piece of 3 mm Whatman filter paper cut slightly larger than the gel and dried under vacuum on a Hoefer Slab Gel Dryer (model SE1150) for 4 h at 80°C.

Statistical analyses. Results were analyzed by Student's t test (Sokol and Rohlf, 1981). The computer software program, Sigma-Plot, version 3.1 (Jandel Scientific, Sausalito, CA), was used for linear regression analyses.

Chemicals, reagents, and proteins. Whenever possible, chemicals of analytical reagent (AR) quality were used. Unless otherwise noted, all chemicals, reagents, and proteins were obtained from Sigma Chem. Co., (St. Louis, MO), or were of analytical grade.

RESULTS

I. EFFECT OF FLUORIDE ON THE GROWTH OF T. DENTICOLA.

A. Effect of sodium fluoride on the growth of T. denticola.

Experiments were conducted to determine whether fluoride had an effect on the growth of T. denticola ATCC strains 33520 and 33521, and strain 10 (Table 1). The action of NaF on the growth of T. denticola was determined by measuring absorbance and by actual cell counts. A standard curve relating the colorimetric readings to direct cell counts was prepared. Cell viability was judged by motility concurrently with actual cell counts. A typical growth curve was generated (Fig. 2). Cells grown in the presence of 10 μg of fluoride per ml had a growth profile similar to the control. Cells grown in the presence of 20 μg of fluoride per ml had an increased generation time or lag phase and a decreased growth rate or cell yield. Most of the growth parameters were not significantly different from the controls. Growth was completely inhibited by 40 μg of fluoride per ml. All three strains tested behaved in a similar manner.

B. Effect of stannous fluoride on the growth of T. denticola.

Since SnF_2 has been reported to have more actions against oral microorganisms in vitro than has NaF (Tinanoff et al., 1983), the effect of stannous fluoride on the growth of T. denticola 33520 in medium GM-1 was also studied (Table 2). When 5 μg of fluoride per ml was combined with 15.6 μg of stannous ions (as SnF_2) per ml, significant suppression of growth was observed. Growth was completely inhibited by fluoride at

Table 1. Effect of NaF on the growth parameters of T. denticola

F^- Concn (μg/ml)	Growth Parameter: Generation Time (h)	Growth Rate (gen per h)	Maximum Cell Yield (per ml)	Time Lag Constant (h)
strain 33520				
0	9.4 (3.2)[a]	0.037 (0.015)	4.0×10^8 (1.2×10^8)	4.3 (3.3)
10	9.9 (3.2)	0.035 (0.015)	3.6×10^8 (0.6×10^8)	6.0 (4.3)
20	21.8 (6.0)	0.015 (0.005)	1.9×10^8 (1.2×10^8)	12.7 (5.2)
40	∞	0	0	∞
strain 33521				
0	10.9 (2.6)	0.030 (0.007)	7.8×10^8 (3.6×10^8)	0
10	13.5 (3.4)	0.024 (0.006)	6.7×10^8 (2.2×10^8)	0
20	18.2 (5.0)	0.018 (0.006)	4.3×10^8 (1.7×10^8)	13.3 (3.4)
40	∞	0	0	∞
strain 10				
0	14.7 (0.7)	0.020 (0.001)	6.1×10^8 (1.6×10^8)	0
10	14.3 (0.3)	0.021 (0.000)	4.1×10^8 (0.2×10^8)	0
20	19.9 (3.7)	0.015 (0.002)	4.2×10^8 (2.7×10^8)	16.0 (23)
40	∞	0	0	∞

a Values are reported as the mean of at least 3 separate experiments. The standard deviation is in parentheses. Underlined values indicate a level of significance $P \leq 0.01$.

Fig. 2. Growth curve of T. denticola 33520. Direct cell counts of T. denticola were determined with a Petroff-Hausser counting chamber under a phase-contrast microscope. Values are reported as the mean ± standard deviation of at least three separate experiments. Open symbols, typical growth curve; filled symbols, growth curve of cells grown in the presence of 40 μg F^-/ml (NaF source).

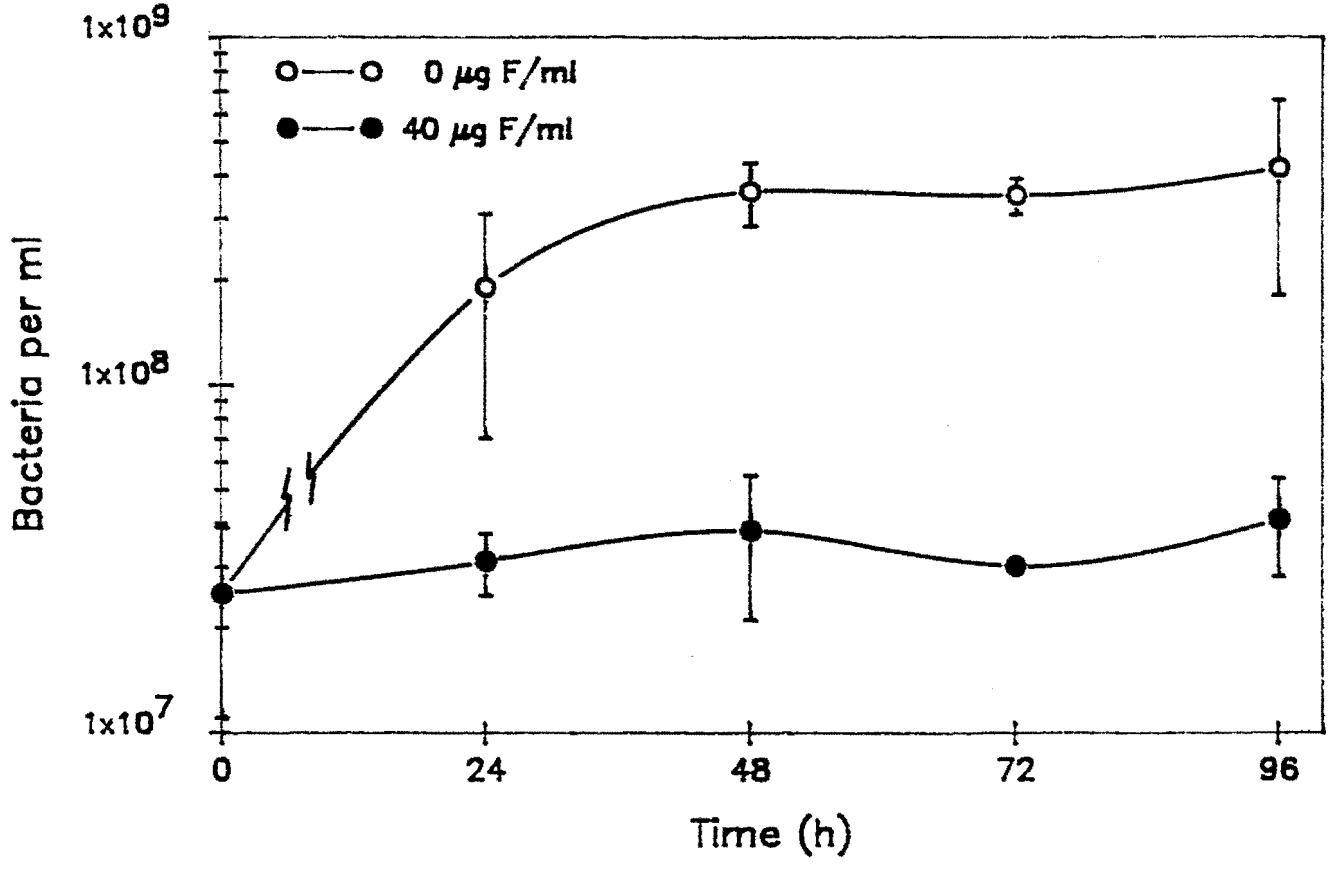

1x10^9
1x10^8
1x10^7
Bacteria per ml
o—o 0 µg F/ml
●—● 40 µg F/ml
0
24
48
72
96
Time (h)

Table 2. Effect of SnF_2 on the growth of T. denticola ATCC 33520

Concn (μg/ml) F^-	Concn (μg/ml) Sn^{++}	Growth Parameter: Generation Time (h)	Growth Rate (gen per h)	Maximum Cell Yield (per ml)	Time Lag Constant (h)
0.0	0.0	10.0 (3.3)[a]	0.034 (0.016)	6.4×10^8 (3.0×10^8)	0
1.0	3.1	8.3 (2.1)	0.038 (0.010)	3.9×10^8 (1.2×10^8)	0
2.5	7.8	7.7 (0.3)	0.039 (0.002)	3.3×10^8 (1.2×10^8)	0
5.0	15.6	16.2 (6.9)	0.021 (0.009)	1.3×10^8 (0.9×10^8)	6.4 (6.1)
10.0	31.2	∞	0	0	∞
20.0	62.4	∞	0	0	∞
0.0	1.95[b]	15.0 (nd)[c]	0.020 (nd)	6.8×10^8 (nd)	4.5 (nd)
0.0	3.9	15.3 (nd)	0.020 (nd)	6.1×10^8 (nd)	4.5 (nd)
0.0	7.8	19.4 (4.0)	0.016 (0.002)	5.1×10^8 (0.8×10^8)	4.0 (4.0)
0.0	15.6	24.1 (1.1)	0.012 (0.000)	2.0×10^8 (1.1×10^8)	5.0 (5.0)
0.0	31.2	∞	0	0	∞
0.0	62.4	∞	0	0	∞

a Values are reported as the mean of at least 3 separate experiments. The standard deviation is in parentheses. Underlined values indicate a level of significance $P \leq 0.01$.

b $SnCl_2 \cdot 2H_2O$ prepared to be equal in Sn content to that of SnF_2.

c Not done.

10 and 20 μg/ml. Stannous ions alone at 7.8 μg/ml (added as $SnCl_2 \cdot 2H_2O$) were also found to significantly reduce T. denticola growth. The inhibitory nature of the stannous ions observed in these experiments is not entirely understood.

c. Effect of sodium counter ion (Na) on the growth of T. denticola.

The effect of the counter ion (Na^+) present in sodium fluoride was investigated. As shown in Table 3, the addition of sodium chloride (as a control for Na^+) to the growth medium of T. denticola 33520 did not inhibit the growth of the organism. In agreement with previously published results (Hughes and Yotis, 1986), F^- inhibits the growth of the organism at concentrations greater than 20 μg/ml.

II. ENZYME PROFILE OF T. DENTICOLA.

Much of the work on the mechanism of fluoride action has centered on the effect of the inhibitor on enzymes. Therefore, the API ZYM system was used as a rapid means of surveying enzyme activities present in T. denticola. Whole cells from stationary phase cultures of T. denticola 33520, strain 10, and a pectinolytic spirochete, strain P4, were assayed for enzyme activities using the API ZYM system. All three strains consistently demonstrated acid phosphatase, C8 esterase lipase, and phosphoamidase activities (Table 4). In addition the T. denticola strains demonstrated trypsin, chymotrypsin, and α-galactosidase activities. Only T. denticola 33520 displayed leucine aminopeptidase activity. When T. denticola 33520 was incubated in the presence of sodium fluoride or stannous fluoride (5 μg/ml F^-) for 1 h and then assayed in

Table 3. Effect of NaF and NaCl on the growth of T. denticola 33520

Concn (μg/ml) F^-	Concn (μg/ml) Na^+	Growth Parameter: Generation Time (h)	Growth Rate (gen per h)	Max Cell Yield (per ml)	Time Lag Constant (h)
0	0.0	10.6	0.028	8.1×10^8	0
10	12.1	10.1	0.030	9.5×10^8	0
20	24.2	19.3	0.016	2.9×10^8	4
40	48.4	∞	0.000	0	∞
80	96.8	∞	0.000	0	∞
100	121.0	∞	0.000	0	∞
160	193.6	∞	0.000	0	∞
0	12.1[a]	9.7	0.031	9.5×10^8	0
0	24.2	10.0	0.030	9.5×10^8	0
0	48.4	10.0	0.030	1.0×10^9	0

[a] Added as NaCl, prepared to be equal in Na content to that of NaF.

Table 4. Enzymatic activities of selected oral spirochetes

Enzyme	Control[a]	*Treponema denticola* ATCC 33520	*Treponema denticola* strain 10	Pectinolytic strain P4
Acid phosphatase	0[b]	5	5	2
Trypsin	0	2	5	0
Phosphoamidase	0	2	1	3
Chymotrypsin	0	2	0	0
Leucine aminopeptidase	0	3	0	0
C8 Esterase lipase	0	3	3	1
α-Galactosidase	0	1	1	0
Alkaline phosphatase	0	0	0	0
C4 Esterase	0	0	0	0
C14 Lipase	0	0	0	0
Valine aminopeptidase	0	0	0	0
Cystine aminopeptidase	0	0	0	0
β-Galactosidase	0	0	0	0
β-Glucuronidase	0	0	0	0
α-Glucosidase	0	0	0	0
β-Glucosidase	0	0	0	0
α-Mannosidase	0	0	0	0
α-Fucosidase	0	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0	0

[a] Control included 0.85% NaCl in the absence of cells.

[b] Enzyme activities were recorded on a scale of 0 (low) to 5 (high) by comparison with the color chart provided by the manufacturer.

the API ZYM system, acid phosphatase activity was decreased (Table 5). Whole cells incubated in the presence of stannous ions alone demonstrated a decreased α-galactosidase activity. Trypsin activity was not affected by the presence of fluoride at the concentrations used in these experiments. Chymotrypsin, leucine aminopeptidase, and C8 esterase lipase activities were unaffected.

III. CHARACTERIZATION OF T. DENTICOLA 33520 NONSPECIFIC ACID PHOSPHATASE.

A. Effect of nutrient limitation on enzyme expression.

The synthesis and expression of acid phosphatases has been shown to be stimulated by P_i limitation (Kier et al., 1977b; Dassa et al., 1982). Therefore, T. denticola was grown under a variety of nutrient limitations in order to investigate the expression of nonspecific acid phosphatase. The media used were modifications of the complex medium, GM-1, normally used to cultivate the organism. The ingredients of the trypticase soy broth component of the GM-1 medium were added individually in order to control the addition of carbon (glucose) and P_i (dipotassium phosphate). The pH of each reduced medium was adjusted to pH 7.4 with KOH. Exogenous glucose concentrations were reduced greater than 90%, while exogenous P_i concentrations were reduced 68% (Table 6). The nonspecific acid phosphatase S. typhimurium has been shown to be limited by the presence of 0.075 mM phosphate (0.007 mg/ml) in the growth medium (Kier et al., 1977b). No clear differences were observed in the growth curves of T. denticola grown in the four different media (Table 7). When cells at various stages of growth in the four media were assayed, no clear differences

Table 5. Effect of fluoride on the enzymatic activities of T. denticola 33520

Enzyme	Intact Cells plus			
	0 μg/ml (F or Sn)	5 μg/ml F (NaF)	5 μg/ml F (SnF_2)	15.6 μg/ml Sn ($SnCl_2 \cdot 2H_2O$)
Acid phosphatase	5[a]	1	0	3
Trypsin	5	5	5	1
Phosphoamidase	3	3	2	3
Chymotrypsin	1	1	1	1
Leucine aminopeptidase	2	2	2	2
C8 Esterase lipase	3	3	3	3
α-Galactosidase	1	1	0	0
Alkaline phosphatase	0	0	0	0
C4 Esterase	0	0	0	0
C14 Lipase	0	0	0	0
Valine aminopeptidase	0	0	0	0
Cystine aminopeptidase	0	0	0	0
β-Galactosidase	0	0	0	0
β-Glucuronidase	0	0	0	0
α-Glucosidase	0	0	0	0
β-Glucosidase	0	0	0	0
α-Mannosidase	0	0	0	0
α-Fucosidase	0	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0	0

[a] Enzyme activities as described in preceding table. Controls included: 0.85% NaCl, 5 μg/ml F^- (NaF), 5 μg/ml F^- (SnF_2), or 15.6 μg/ml Sn^{++} ($SnCl_2 \cdot 2H_2O$) in the absence of cells. All controls were found to be negligible for enzyme activity.

Table 6. Chemical analysis of culture media containing reduced levels of carbon and phosphate

	average[a] mg glucose per ml[b]	average[a] mg phosphate per ml[c]
C^-P^-	0.0	0.108 ± 0.014
C^-P^+	0.0	0.320 ± 0.005
C^+P^-	1.35 ± 0.00	0.094 ± 0.012
C^+P^+	1.60 ± 0.08	0.301 ± 0.016

Reduction in glucose concentration: 100 % REDUCTION

Reduction in phosphate concentration: 67.5 % REDUCTION

[a] Average value from two separate samples.

[b] As determined by glucose oxidase assay.

[c] As determined by P_i determination, method of Chen et al. (1956).

Table 7. Growth of T. denticola 33520 in media containing reduced levels of glucose and phosphate

Medium[a]	Stage of growth (hours) 0	16	48.5	63
C^-P^-	$9.1x10^7$ ($0.5x10^7$)	$2.0x10^8$ ($0.9x10^8$)	$4.9x10^8$ ($1.8x10^8$)	$8.0x10^8$ ($3.4x10^8$)
C^+P^-	$9.1x10^7$ ($0.5x10^7$)	$1.9x10^8$ ($0.6x10^8$)	$5.4x10^8$ ($1.9x10^8$)	$8.7x10^8$ ($2.8x10^8$)
C^-P^+	$9.1x10^7$ ($0.5x10^7$)	$2.8x10^8$ ($0.7x10^8$)	$5.5x10^8$ ($1.2x10^8$)	$7.4x10^8$ ($3.0x10^8$)
C^+P^+	$9.1x10^7$ ($0.5x10^7$)	$2.7x10^8$ ($0.8x10^8$)	$4.8x10^8$ ($2.0x10^8$)	$9.3x10^8$ ($2.2x10^8$)

[a] An overnight culture grown in C^-P^- medium (20 ml) was used to inoculate 200 ml of each of the various media with reduced levels of exogenous carbon and phosphate.

[b] Growth was monitored by optical absorbance and direct cell counts. Values represent average ± standard deviation of three separate experiments (in parentheses).

were observed in acid phosphatase activity (Fig. 3). These results also indicate that acid phosphatase activity is greatest in stationary phase cells.

To determine whether or not the amounts of soluble protein measured at each phase of T. denticola growth were similar, whole cell suspensions from early-, mid-, and late-log phases of growth were adjusted to the same optical density and assayed for soluble protein as previously described. No significant differences were observed in the soluble protein contents of the three cell suspensions (Table 8). Therefore, the term used to describe enzyme activity, nmol pNP released per mg soluble protein, is logical.

B. Effect of pH on nonspecific acid phosphatase of intact cells of T. denticola.

To determine the dependence of enzyme activity on pH, intact early stationary-phase cells were assayed for nonspecific acid phosphatase activity over a range of pH values. Two optima were observed (Fig. 4). One pH optimum between 4.6 and 4.8 was quite distinct. Another broader pH optimum centered at about pH 6.2 suggests the presence of another enzyme in whole cells able to utilize pNPP as a substrate. These results support the choice of pH 4.8 for the enzyme assay. Enzyme activity at pH 6.2 was not investigated further.

C. Effect of fluoride on nonspecific acid phosphatase.

To determine whether there was a correlation between the level of fluoride required to suppress the growth of T. denticola and the

Fig. 3. Effect of exogenous carbon and phosphate on the expression of nonspecific acid phosphatase. An overnight culture of T. denticola 33520 grown in C^-P^- medium (20 ml) was used to inoculate 200 ml of each of the various media. At various times, samples were removed and assayed for nonspecific acid phosphatase activity as described in the text. Values represent the average ± standard deviation of at least three separate experiments.

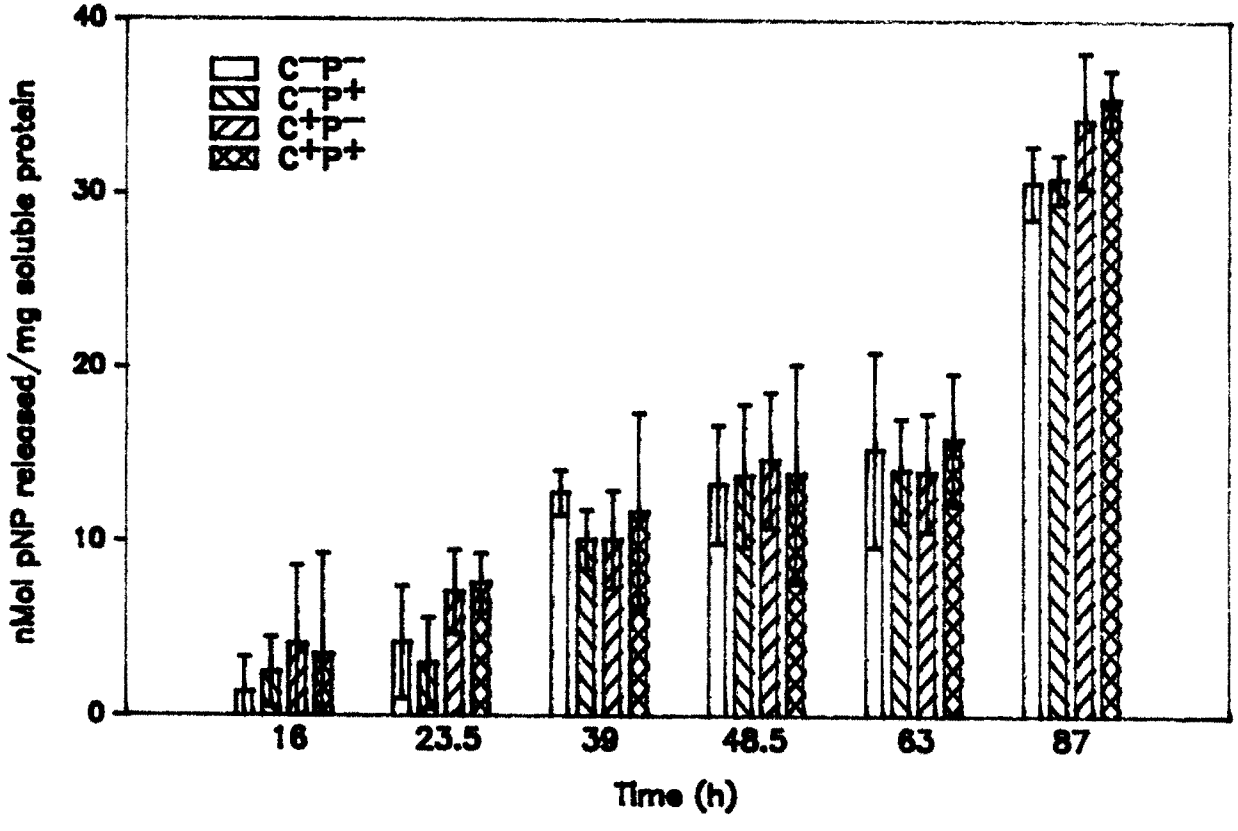

C⁻P⁻
C⁻P+
C+P⁻
C+P+
nMol pNP released/mg soluble protein
40
30
20
10
0
16
23.5
39
48.5
63
87
Time (h)

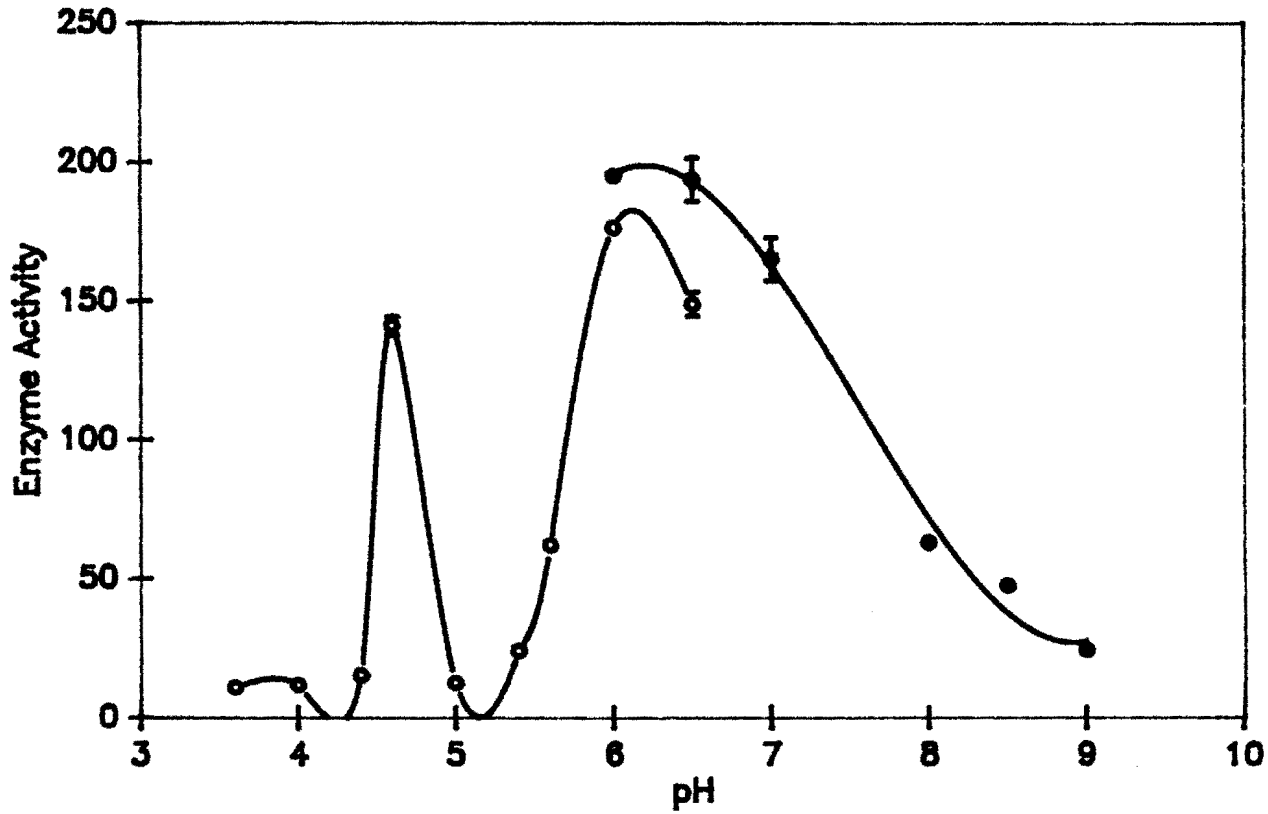
Table 8. Measurement of soluble protein in whole cell suspensions of T. denticola 33520

Stage of growth (age)	µg soluble protein / ml[a]	P value[b]
Early-log (22 h)	354 ± 25	----
Mid-log (46 h)	384 ± 14	0.28
Late-log (70 h)	398 ± 14	0.17

[a] Soluble protein of cell suspensions adjusted to 160 Klett Units was measured by Lowry assay, as described in materials and methods. Values are reported as the mean ± standard deviation of triplicate samples.

[b] As determined by Student's t test (Sokol and Rohlf, 1981).

Fig. 4. Profile of nonspecific acid phosphatase in whole cells as a function of pH. T. denticola 33520 was grown to early stationary phase, washed and assayed. Enzyme activity is expressed as nmol p-nitrophenol released per mg soluble protein. Values represent average ± standard deviation of triplicate samples. Open symbols, 0.1 M sodium acetate buffers; filled symbols, 0.1 M Tris-hydrochloride buffers.


250
200
150
100
50
0
Enzyme Activity
3
4
5
6
7
8
9
10
pH

concentration at which fluoride (as NaF) inhibits the nonspecific acid phosphatase of T. denticola, fluoride (0-200 μg/ml) and substrate were added to washed, early stationary phase (60 h) cells of T. denticola and assayed for nonspecific acid phosphatase. Inhibition of nonspecific acid phosphatase was observed with greater than 20 μg/ml (1 mM F^-) (Fig. 5). It was also noted that approximately 40 min was necessary to observe enzyme inhibition at the lower concentrations of fluoride.

D. Effect of ions on nonspecific acid phosphatase in intact cells.

Although only 2-3% of the fluoride in plaque appears to be ionized (Jenkins et al., 1969), the remaining fluoride in plaque is thought to be bound either to inorganic components, such as Ca^{++}, Mg^{++}, phosphate and other ions (Gron et al., 1969; Singer et al., 1970; Birkeland and Rolla, 1972), or to bacteria (Jenkins et al., 1969). The bound fluoride may or may not exert an action in the system. In order to examine any possible interaction between fluoride and Mg^{++} in the system, whole cells were assayed for enzyme activity in the presence or absence of Mg^{++}. In addition, the effect of 20 and 100 μg F^-/ml was examined in the presence or absence of Mg^{++} in the system. The absence of Mg^{++} in the buffer system did not significantly affect enzyme activity. In addition, the presence of Mg^{++} did not affect the ability of F^- to inhibit enzyme activity (Fig. 6). These results also indicate that greater than 40 min are needed for optimal release of product (pNP) in the enzyme assay, confirming that the standard assay procedure of 60 min is reasonable.The effect of other divalent cations (Mg^{++}, Mn^{++}, Co^{++}, Ca^{++}, or Zn^{++}) on nonspecific acid phosphatase was also evaluated. When metal ions were added to whole cell

Fig. 5. Effect of fluoride concentration on nonspecific acid phosphatase in intact T. denticola 33520. Fluoride, as NaF, was added to stationary phase cells at time zero. At 30 and 60 min, samples were removed and assayed for nonspecific acid phosphatase. Enzyme activity represents nmol p-nitrophenol released per mg soluble protein.

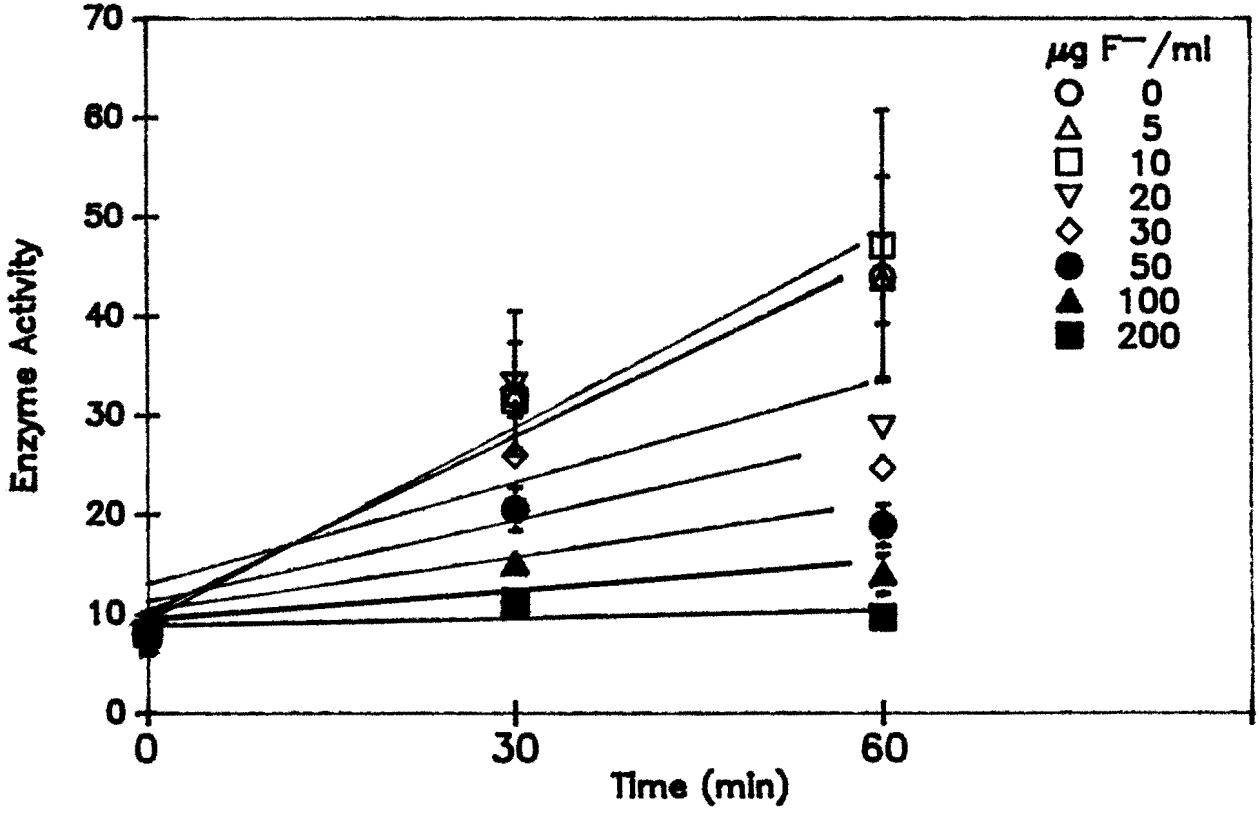
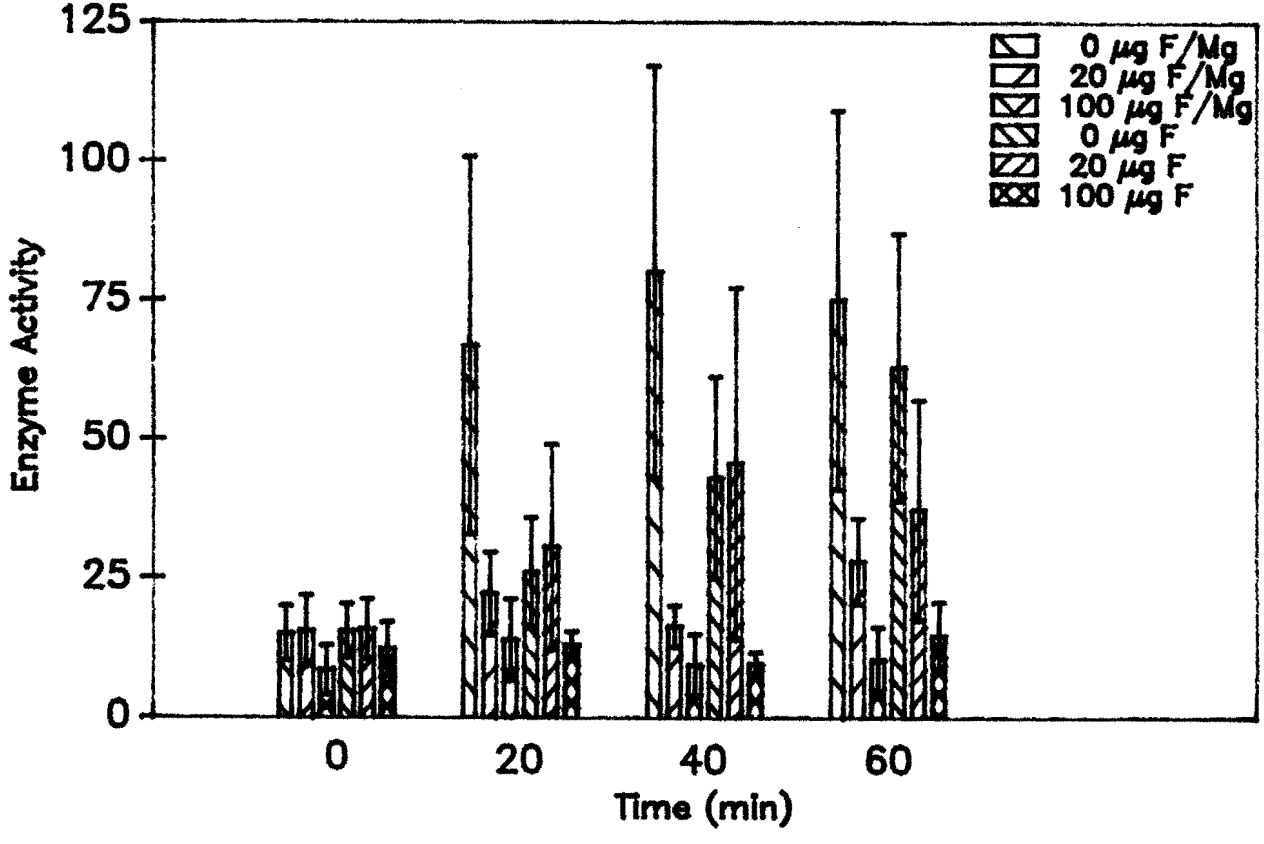

70
60
50
40
30
20
10
0
Enzyme Activity
0
30
60
Time (min)
µg F⁻/ml
0
5
10
20
30
50
100
200

Fig. 6. Effect of fluoride and magnesium ions on nonspecific acid phosphatase in intact cells. Stationary phase T. denticola 33520 was assayed for enzyme activity in the presence or absence of 1 mM $MgCl_2$ in the assay buffer. Fluoride, as NaF, at varying concentrations was also added to the system at time zero. At various times, samples were removed and assayed for nonspecific acid phosphatase activity. Enzyme activity represents nmol p-nitrophenol released per mg soluble protein. Values represent the average ± standard deviation of at least three separate experiments.


125
100
75
50
25
0
Enzyme Activity
0
20
40
60
Time (min)
0 µg F/Mg
20 µg F/Mg
100 µg F/Mg
0 µg F
20 µg F
100 µg F

suspensions, in addition to the standard assay components, no stimulatory effects were observed (Table 9). Exceptions were Mg^{++} and Ca^{++} at rather high (500 mM) levels, which appeared to double activity. Zn^{++} appeared to inhibit enzyme activity, albeit slightly.

E. Michaelis constant for nonspecific acid phosphatase.

Kinetic parameters for the hydrolysis of pNPP by intact cells were determined by measuring the release of pNP at various times and concentrations. The initial slope of a curve which represents the release of pNP as a function of time was calculated to determine reaction velocity. The apparent K_m and K_i for sodium fluoride were determined by the method of Lineweaver and Burk (1934), as shown in Fig. 7. Fluoride appears to be a noncompetitive inhibitor of the enzyme in intact cells, with an apparent K_i of 0.3 mM.

F. Effect of fluoride on nonspecific acid phosphatase in cell extracts of T. denticola.

In an effort to understand enzyme activity at a level beyond that of the intact cell, so that the nonspecific acid phosphatase could be isolated and characterized, whole cells of T. denticola 33520 were disrupted with the French pressure cell, as outlined in Materials and Methods, and the resulting cell extract (CE) was assayed for nonspecific acid phosphatase in the presence and absence of fluoride (Table 10). The addition of 20 and 40 μg/ml F^- (as NaF) significantly decreased nonspecific acid phosphatase in disrupted cell suspensions. The addition of 10 μg/ml F- (as SnF_2) induced an 18% decrease in nonspecific acid

Table 9. Effects of ions on nonspecific acid phosphatase of intact cells of T. denticola 33520[a]

Addition	Concn in assay mix (mM)	Relative enzyme activity substrate pNPP[b]
None		1.0
Mg^{++}	0.1	0.8
	1.0	0.8
	5.0	0.8
	10.0	1.2
	100.0	0.7
	500.0	1.9
Mn^{++}	0.1	0.8
	1.0	0.6
	5.0	0.8
	10.0	0.9
	100.0	0.9
	500.0	1.0
Co^{++}	0.1	1.0
	1.0	0.9
	5.0	1.0
	10.0	1.3
	100.0	1.2
	500.0	1.4
Ca^{++}	0.1	1.0
	1.0	1.0
	5.0	0.6
	10.0	1.4
	100.0	1.4
	500.0	2.0
Zn^{++}	0.1	0.9
	1.0	0.9
	5.0	0.2
	10.0	0.4
	100.0	0.6
	500.0	0.5

a ATCC 33520 was grown to early stationary phase in GM-1 medium for assay.

b Activity measured by release of p-nitrophenol, as previously described.

Fig. 7. Lineweaver-Burk plots and Michaelis constants of fluoride-inhibited nonspecific acid phosphatase in intact cells. Assay mixtures contained early-stationary phase T. denticola 33520 and pNPP as substrate. The initial slope of a curve representing the release of p-nitrophenol as a function of time was calculated to determine reaction velocity.

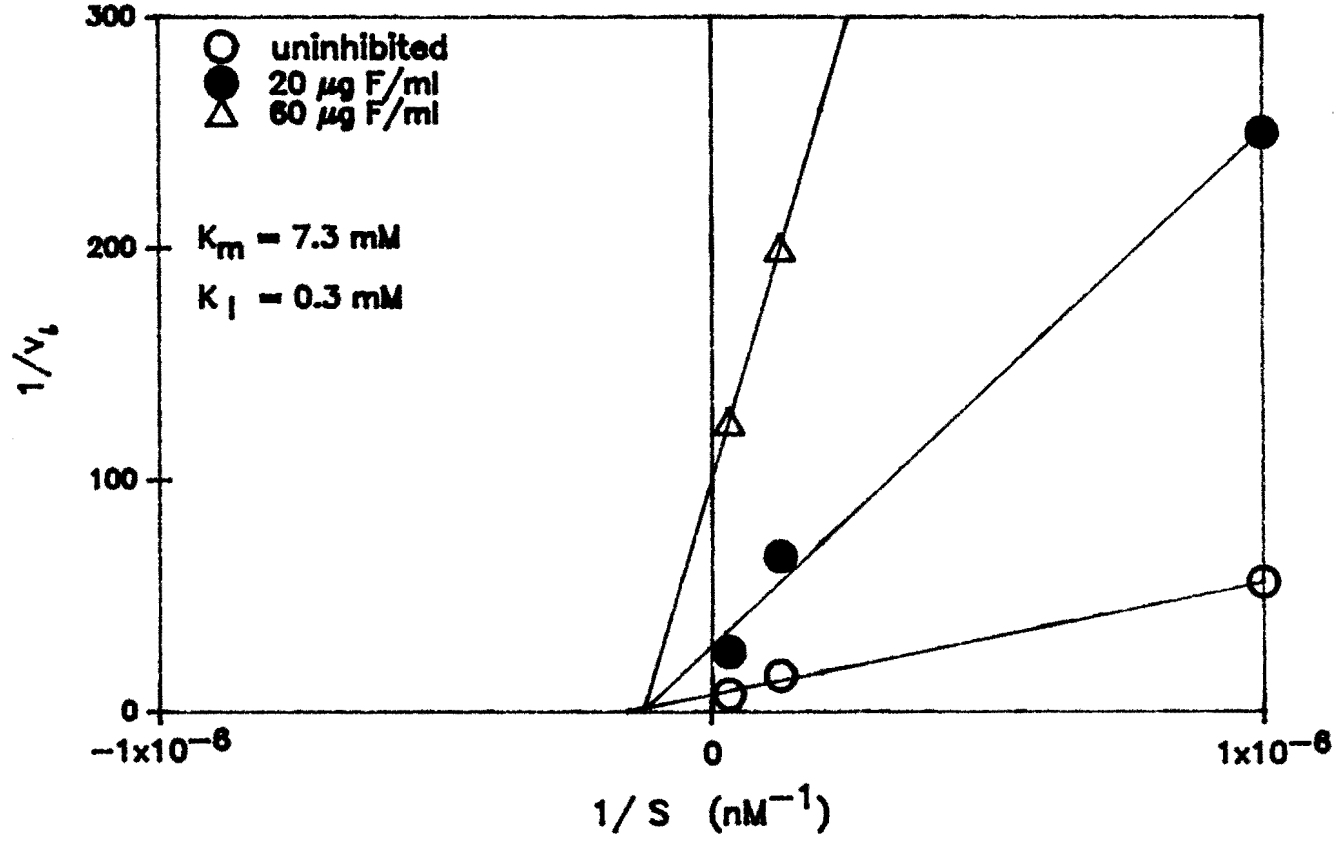

300
200
100
0
uninhibited
20 μg F/ml
60 μg F/ml
K_m = 7.3 mM
K_I = 0.3 mM
$1/v_i$
$-1x10^{-6}$
0
$1x10^{-6}$
1/ S (nM^{-1})

Table 10. Effect of fluoride on nonspecific acid phosphatase in cell extracts of T. denticola 33520

Sample	nmols p-nitrophenol released[a] mg soluble protein
CE[b]	23.1 ± 5.4
CE, boiled 15 min	1.5 ± 0.6
CE + 5 μg F^-/ml (SnF_2)	17.5 ± 8.2
CE + 10 μg F^-/ml (SnF_2)	14.3 ± 5.7
CE + 5 μg F^-/ml (NaF)	23.5 ± 9.2
CE + 10 μg F^-/ml (NaF)	18.9 ± 5.6
CE + 20 μg F^-/ml (NaF)	11.7 ± 2.3
CE + 40 μg F^-/ml (NaF)	10.3 ± 1.0

[a] Values reflect mean ± standard deviation of at least 3 separate experiments. Underlined values indicate a level of significance $P \leq 0.01$.

[b] CE = Cell extracts. Whole cell suspensions were passaged through the French pressure cell twice at 16,000 lb/in^2, as previously described.

phosphatase activity; however, the difference from the activity observed in the absence of fluoride was not significant. Higher concentrations of F^-, as SnF_2 solutions, were difficult to work with due to precipitation of the compound.

G. Nonspecific acid phosphatase in subcellular fractions of T. denticola.

Analysis of the cell fractionation scheme reveals that nonspecific acid phosphatase is present in all subcellular fractions of T. denticola 33520. This is summarized in Table 11. Although the nonspecific acid phosphatase of T. denticola seems to behave like the corresponding E. coli enzymes by exhibiting activity in intact cells (Neu and Heppel, 1964), the activity does not appear to be efficiently released by osmotic shock procedures. This inefficient osmotic shock release is also characteristic of S. typhimurium phosphatases (Kier et al., 1977a).

This does not necessarily mean that the nonspecific acid phosphatase activity found in osmotic shock "periplasmic contents" of T. denticola is due to osmotic shock damage of the cytoplasmic membrane and release of the enzyme from the cytoplasmic contents. In order to investigate this possibility, freshly prepared subcellular fractions were assayed for hexokinase activity, a cytoplasmically located enzyme. The osmotic shock fluid ("periplasmic contents") was not contaminated with hexokinase activity (Table 12). Therefore, the osmotic shock procedure does not appear to damage the cytoplasmic membrane and release proteins from the cytoplasmic contents.

Table 11. Nonspecific acid phosphatase measured in subcellular fractions of T. denticola 33520

Fraction[a]	Volume (ml)	Protein (mg/ml)	nmols pNP released[b] / mg soluble protein	Total Units ($\times 10^3$)
Whole cells[c]	480	3.3	43.6	69.0
Osmotic shock treated cells	35	28.5	20.2 ± 0.01	20.1
Osmotic shock fluid	15	1.0	23.2 ± 0.01	0.4
"Cytoplasmic" contents	32	14.0	7.7 ± 1.0	3.4
"Membrane" fraction	25	35.3	15.2 ± 0.4	13.4

[a] Fractions obtained from cell fractionation scheme, as previously described.

[b] Activity measured by the release of p-nitrophenol, as previously described.

[c] Represents washed cells, pelleted and pooled from 18,875 ml of cultured media.

Table 12. Hexokinase (EC 2.7.1.1) assay of the subcellular fractions of T. denticola 33520

Fraction[a]	Specific Activity[b]
Whole Cells	n.d.[c]
Shocked Cells	1.1 ± 0.01
French Pressure Cell Extract	2.2 ± 0.2
Osmotic Shock Fluid	0.7 ± 0.2
"Membrane" Fraction	n.d.
"Cytoplasmic" Contents	5.9 ± 0.3
Hexokinase standard (Sigma)	14,600 ± 265

[a] Fractions obtained from cell fractionation scheme, as previously described. All samples were freshly prepared.

[b] Nanomols of pyridine nucleotide change per min per mg soluble protein.

[c] n.d. = not detected.

Osmotic shock fluid preparations containing nonspecific phosphatase were also electrophoresed using native polyacrylamide gels and stained for enzyme activity (Fig. 8). Two separate bands exhibiting nonspecific acid phosphatase were detected in the shock fluids. These bands were found to have relative molecular weights of 53K and 40.5K. Two other protein bands, which did not exhibit nonspecific acid phosphatase, were observed following staining with Coomassie blue and had apparent MW_r of 24K and 21K. Because neither of these two proteins could be unambiguously identified as belonging to the nonspecific acid phosphatase, the possibility remains that either or both of the proteins may be subunits of nonspecific acid phosphatase.

In addition, when culture fluid supernatants were assayed for nonspecific acid phosphatase, negligible enzyme activity was detected (data not shown). This indicates that the enzyme is not an exoenzyme.

H. Effect of enzyme concentration on product formation.

To determine whether or not the enzyme activity under investigation was linear with respect to the amount of enzyme, increasing volumes of subcellular fractions containing nonspecific acid phosphatase were added to the assay mixture. The results are shown in Fig. 9. When both soluble contents (Fig. 9a, above) and membrane fraction (Fig. 9b, above) were assayed in increasing volumes, the amounts of product released remained linear. These results indicate that the assay used to determine enzyme activity is a valid assay system.

Fig. 8. Detection of nonspecific acid phosphatase after electrophoresis on native polyacrylamide gel. Lane 1, molecular weight markers; 2, osmotic shock fluid (300 μg protein); 3, osmotic shock fluid (300 μg protein); 4 through 9, acid phosphatase control, type IV from potatoes, decreasing concentrations; 10, molecular weight markers. In lanes 2-3, (*) denotes bands detected by acid phosphatase enzyme stain (see methods); (-) denotes bands detected only after protein staining.

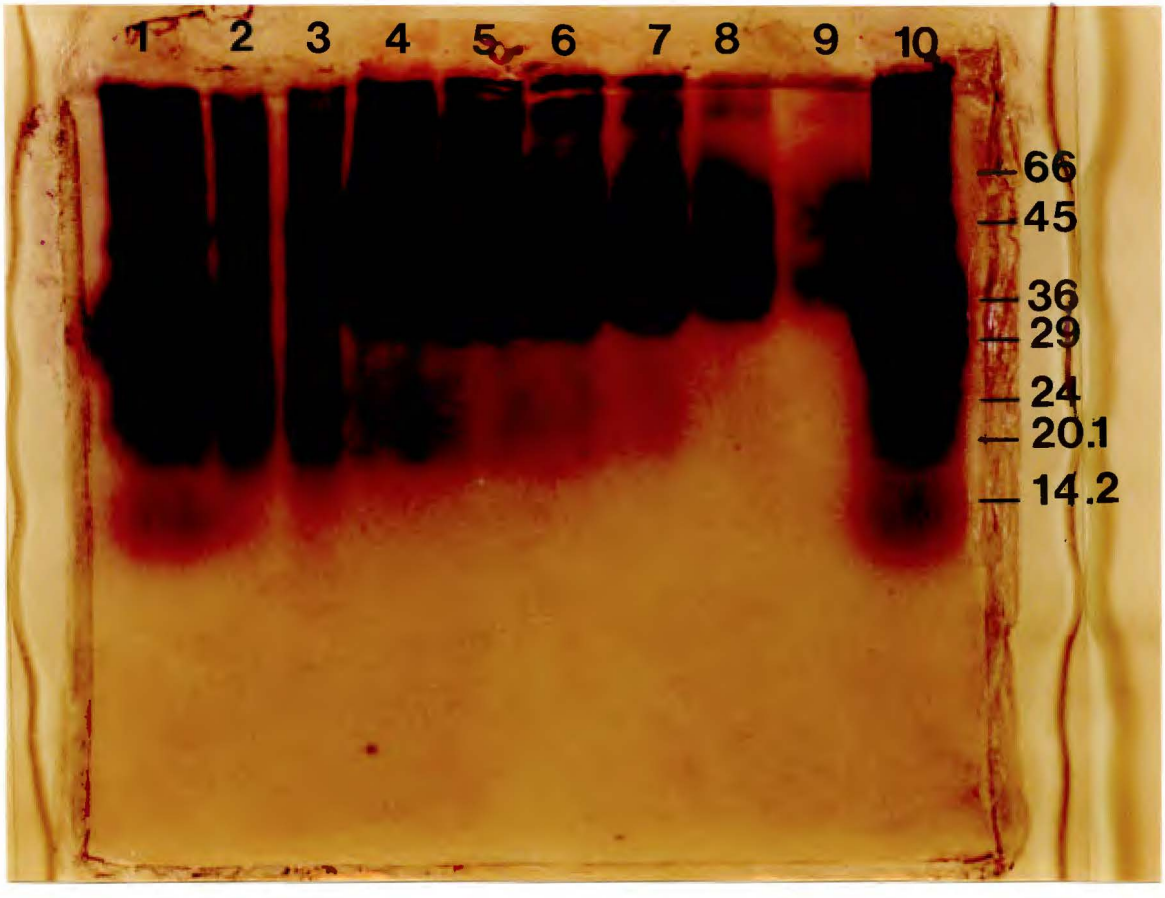
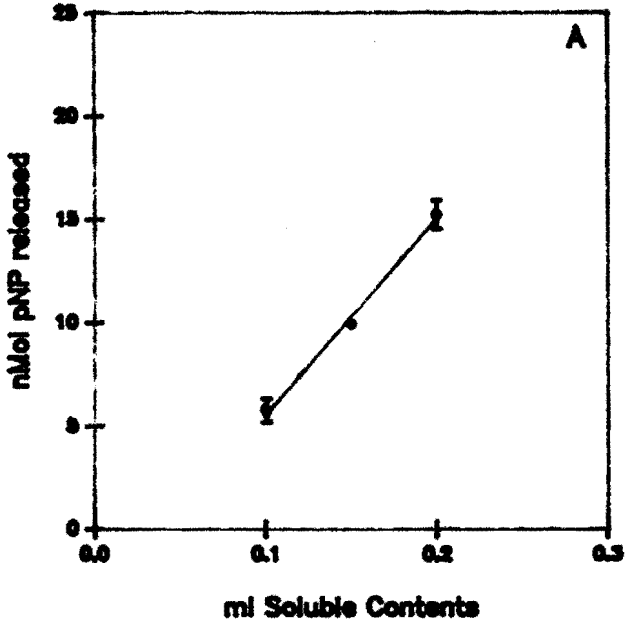
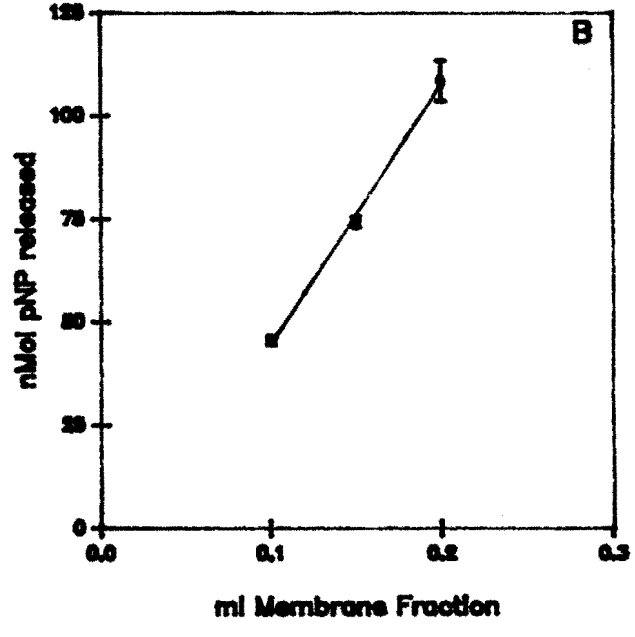

1
2
3
4
5
6
7
8
9
10
66
45
36
29
24
20.1
14.2

Fig. 9. Effect of enzyme concentration on product formation. Increasing volumes of designated subcellular fractions were added to the standard enzyme assay. Values represent average ± standard deviation of duplicate samples. (A) soluble contents. (B) membrane fraction.


A
nMol pNP released
0
5
10
15
20
25
0.0
0.1
0.2
0.3
ml Soluble Contents


B
nMol pNP released
0
25
50
75
100
125
0.0
0.1
0.2
0.3
ml Membrane Fraction

I. Assay of subcellular fractions for ATPase.

Various cell fractions, which exhibited nonspecific acid phosphatase with pNPP as a substrate, were also assayed for activity against ATP, another phosphate containing substrate (Table 13). ATPase was present in osmotic shock fluid, suggesting that the nonspecific acid phosphatase is able to utilize ATP as a substrate.

J. Anion exchange chromatography of T. denticola soluble contents.

The next step in the purification scheme of the nonspecific acid phosphatase involved anion exchange chromatography. The soluble T. denticola contents (3 ml; 14 mg protein per ml) were applied to a DEAE-cellulose column (0.8 x 60 cm) equilibrated with 5 mM Tris-HCl (pH 7.4) containing 1 mM dithiothreitol (buffer A). The column was eluted with the same buffer followed by a linear gradient of NaCl in buffer A. The linear gradient was begun with 50 ml of buffer A in the mixing vessel and an equal volume of buffer A containing 0.2 M NaCl in the reservoir, yielding a NaCl concentration ranging from 0 to 4 x 10^{-2} M. Each of the fractions (collected from the beginning of sample application) was assayed for nonspecific acid phosphatase activity with p-nitrophenyl phosphate as the substrate at pH 4.8. Fractions were also assayed for protein using the Lowry assay (Lowry et al., 1951). A typical DEAE-cellulose chromatography profile of the soluble contents, as consistently obtained from greater than five determinations, is shown in Fig. 10. Two major protein peaks are eluted early in the column, while another peak is eluted after the gradient has been applied. A major portion of the nonspecific acid phosphatase eluted with the first major protein peak. The

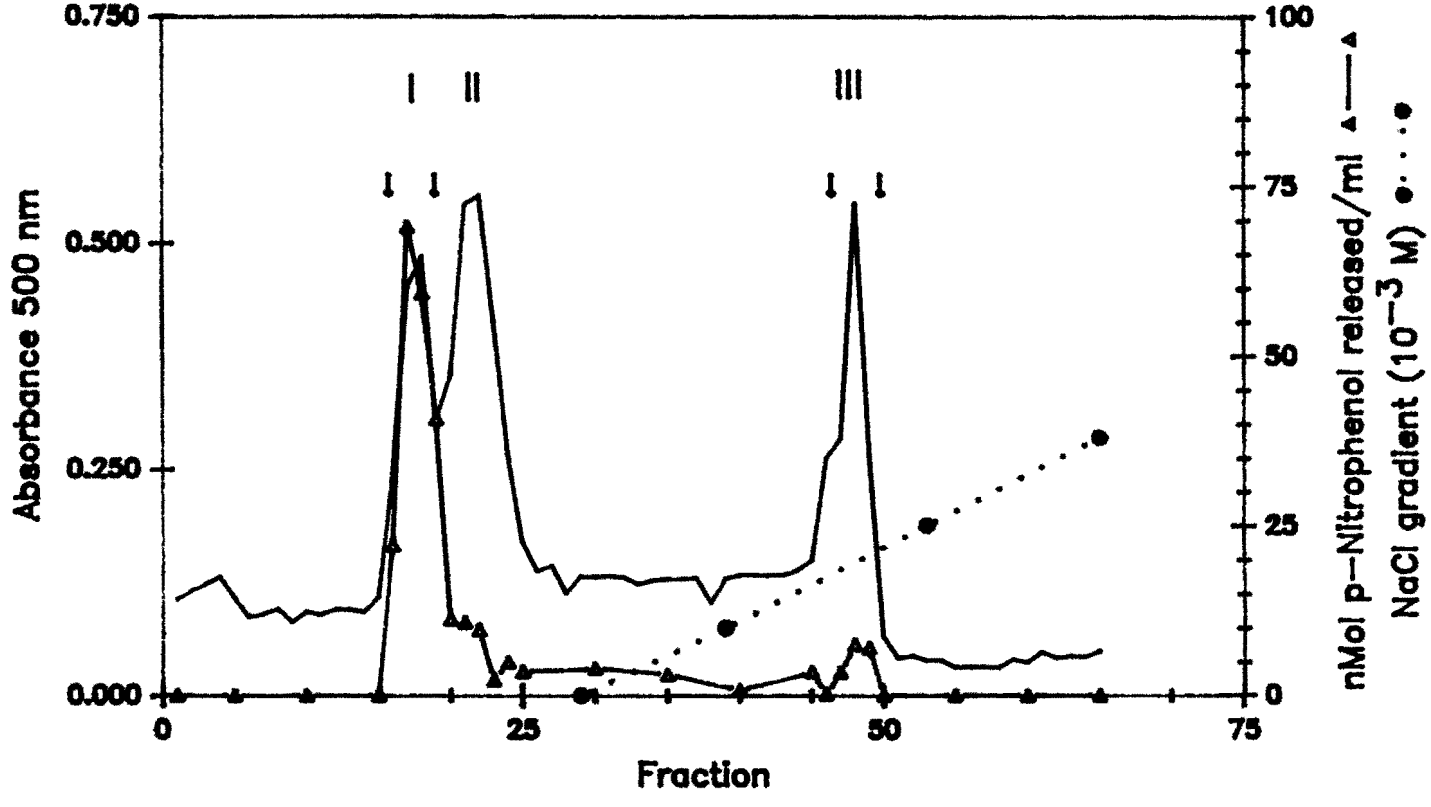
Table 13. Assay of subcellular fractions of T. denticola 33520 for ATPase

Fraction	ATPase Activity[b]
Whole Cells	29.1 ± 0.5
Shocked Cells	5.4 ± 0.4
French Pressure Cell Extract	11.3 ± 2.1
Osmotic Shock Fluid	62.7 ± 7.4
"Membrane" Fraction	12.7 ± 0.5
"Cytoplasmic" Contents	10.9 ± 0.9

[a] Osmotic shock and cell fractionation procedure as previously described.

[b] ATPase activity measured as μg P_i released per mg soluble protein. Values represent average ± standard deviation of duplicate samples.

Fig. 10. DEAE-cellulose chromatography of T. denticola 33520 nonspecific acid phosphatase. A solution of 3 ml (14 mg protein per ml) of soluble contents prepared as described in the text was applied to a DEAE-cellulose column (0.8 x 60 cm) equilibrated with buffer A. The column was eluted with 100 ml of buffer A followed by a linear gradient of NaCl in buffer A from 0 to 4 x 10^{-2} M. Each of the 2.1 ml fractions (collected from the beginning of sample application) was assayed for nonspecific acid phosphatase. The arrows indicate the inclusive fractions pooled for further analysis. This is a typical chromatographic profile, as obtained from greater than five DEAE-cellulose columns prepared in a similar manner.


0.750
0.500
0.250
0.000
Absorbance 500 nm
I
II
III
100
75
50
25
0
nMol p-Nitrophenol released/ml
NaCl gradient (10^{-3} M)
0
25
50
75
Fraction

nonspecific acid phosphatase fractions from the DEAE-cellulose column were pooled and reserved (frozen) for further analysis.

K. Fluoride sensitivity of the DEAE-cellulose nonspecific acid phosphatase chromatographic fractions.

The pooled fractions from the DEAE-cellulose column were assayed for the presence of fluoride-sensitive acid phosphatase. Pooled fractions, in addition to unchromatographed soluble contents, were incubated in the presence or absence of varying concentrations of sodium fluoride for 1 h and then assayed for nonspecific acid phosphatase activity. The results of these experiments are shown in Table 14. DEAE-cellulose peak I exhibited fluoride-sensitive nonspecific acid phosphatase activity. This enzyme activity is enriched over that of the starting (soluble contents) material. DEAE-cellulose peaks II and III exhibited much lower enzyme activities when compared to peak I enzyme activity (8 and 3%, respectively).

L. Purification of nonspecific acid phosphatase.

The pooled nonspecific acid phosphatase fractions from the DEAE-cellulose column resisted further purification. Sephadex columns were prepared and calibrated for gel filtration chromatography. A typical calibration curve is shown in Fig. 11. However, when the pooled DEAE-cellulose fractions containing nonspecific acid phosphatase activity were applied to Sephadex columns, enzyme activity was not detected in the eluate fractions. To investigate whether the sample was too dilute for detection, a concentration step was employed to increase the activity

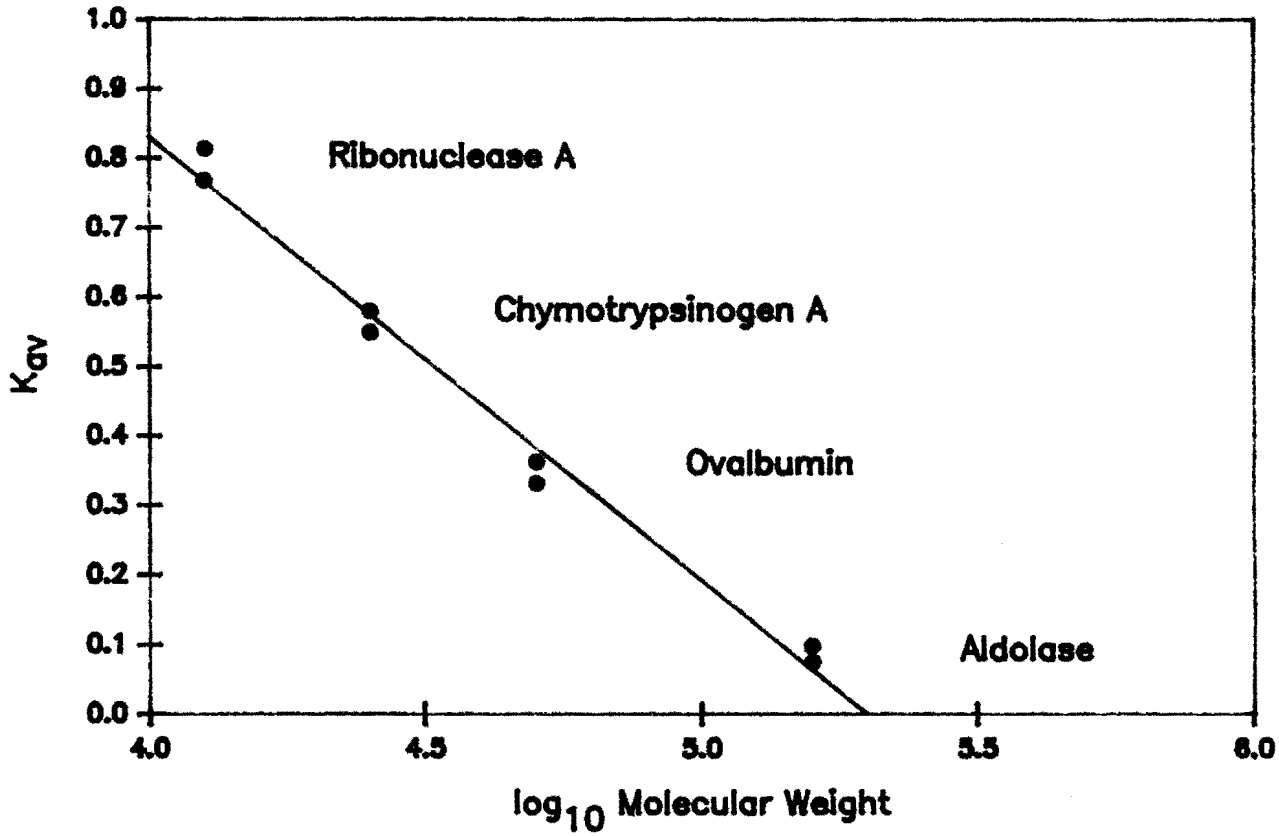
Table 14. Effect of fluoride on nonspecific acid phosphatase isolated by DEAE-cellulose column chromatography

Sample		nmol p-Nitrophenol released[a] mg soluble protein
Soluble Contents	+ 0 μg F^-/ml	7.6 ± 1.0
	+ 20 μg F^-/ml	5.4 ± 0.8
	+ 100 μg F^-/ml	4.3 ± 1.1
DEAE-cellulose peak I	+ 0 μg F^-/ml	155.0 ± 0.4
	+ 20 μg F^-/ml	10.2 ± 6.0
	+ 100 μg F^-/ml	6.3 ± 2.0
DEAE-cellulose peak II	+ 0 μg F^-/ml	11.7 ± 0.01
	+ 20 μg F^-/ml	n.d.
	+ 100 μg F^-/ml	n.d.
DEAE-cellulose peak III	+ 0 μg F^-/ml	4.8 ± 0.6
	+ 20 μg F^-/ml	n.d.
	+ 100 μg F^-/ml	n.d.

[a] Activity measured by release of p-nitrophenol as previously described. Values represent average ± standard deviation of duplicate samples.

n.d. = nondetectable levels

Fig. 11. Selectivity curve for molecular weight markers on Sephadex G-100. Values of K_{av} were calculated from the peak elution positions of the standards chromatographed on a Sephadex G-100 superfine column as described in the Materials and Methods.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
K_{av}
Ribonuclease A
Chymotrypsinogen A
Ovalbumin
Aldolase
4.0
4.5
5.0
5.5
6.0
$\log_{10}$ Molecular Weight

in the "soluble contents". Dry Sephadex G-10 beads were added to a volume of "soluble contents" and allowed to swell. The swelled beads were removed and the remaining supernatant was assayed for enzyme activity (Table 15). The concentrated "soluble contents" were not enriched for enzyme activity despite an overall reduction in the volume of the "soluble contents". When the beads were washed with buffer containing 0.5 M KCl, the nonspecific acid phosphatase activity present in the eluate was concentrated only two-fold. Although the concentration step cannot be considered successful, these findings indicate that the presence of KCl is necessary to prevent the binding of the nonspecific acid phosphatase to Sephadex. Data for the partial purification of the nonspecific acid phosphatase are presented in Table 16.

M. Substrate specificity of partially purified nonspecific acid phosphatase.

In order to define the substrate preference of the nonspecific acid phosphatase, partially purified enzyme preparations were assayed for activity against a variety of phosphate esters, phosphodiesters, and of inorganic pyrophosphate. Table 17 presents the activities of the enzyme towards these substrates. Differences are observed when the Treponema nonspecific acid phosphatase is compared with the analogous Salmonella (Weppelman et al., 1977) and E. coli enzymes (Dvorak et al., 1967). Under the experimental conditions used here, fewer substrates are hydrolyzed by the T. denticola enzyme. Inorganic pyrophosphatase activity is present in the partially purified nonspecific acid phosphatase from

Table 15. Sephadex G-10 treatment of the soluble contents of T. denticola 33520

Treatment[a]	nmol pNP released[b] / mg soluble protein
Soluble contents	5.4 ± 0.6
Supernatant, Sephadex G-10 treatment	4.7 ± 1.5
0.5 M KCL eluate, Sephadex G-10 beads	11.7 ± 0.7

[a] Treatments as described in materials and methods.

[b] Activity measured by release of p-nitrophenol as previously described. Values represent average ± standard deviation of duplicate samples.

Table 16. Purification of nonspecific acid phosphatase

Purification Step	(a) Volume (ml)	(b) Protein (mg/ml)	(c) $\frac{\text{nmol pNP}}{\text{mg sol. protein}}$	$d=a \cdot b \cdot c$ Total Units ($\times 10^3$)	$e=c_1/c_0$ Fold Purification	$f=d_1/d_0$ Yield (%)
Whole cells[a]	30	3.3	43.6	4.3	---	---
Shocked cells	2.2	28.5	20.2	1.3	1	100
"Soluble" contents	2.0	14.0	7.65	0.21	0.38	16
DEAE-Cellulose chromatography	7.0	0.5	155.0	0.54	20.3	41.5

[a] pertains to 2 ml of cytoplasmic contents applied to DEAE-cellulose column back calculated 1/16 of volume from original.

Table 17. Relative activity of nonspecific acid phosphatase towards various substrates

Substrate	Relative Enzyme Activity[a]
Glucose 6-phosphate	<0.01
α-D-Glucose 1-phosphate	5.00
6-Phosphogluconate	<0.01
Ribose 5-phosphate	<0.01
DL-α-Glycerophosphate	<0.01
2'-AMP	<0.01
3'-AMP	<0.01
5'-AMP	<0.01
5'-dAMP	0.25
3'-UMP	<0.01
5'-UMP	<0.01
ADP	<0.01
ATP	2.00
UDP	0.50
UTP	<0.01
Inorganic pyrophosphate[b]	8.25
α-Naphthyl phosphate	<0.01
Cyclic 2',3'-AMP	<0.01
Cyclic 3',5'-UMP	<0.01
Cyclic 3',5'-AMP	<0.01
Cyclic 3',5'-GMP	<0.01
bis-pNPP	<0.01
Fructose 1,6-diphosphate	9.00
Phospho-L-arginine	<0.01
Phospho-L-serine	<0.01
pNPP	1.00

[a] Assays were conducted using the standard assay buffer, 2.5 μmol substrate, and 50 μl of partially purified enzyme.

[b] Values of units with inorganic pyrophosphate as a substrate have been divided by two because two phosphate molecules are released for every pyrophosphate bond cleaved.

T. denticola. This activity was observed in the Salmonella enzyme, but was not observed in the partially purified E. coli preparation.

N. Effect of pH on partially purified nonspecific acid phosphatase.

In order to ascertain the dependence of the partially purified nonspecific acid phosphatase activity on pH, the enzyme preparation was assayed for nonspecific acid phosphatase at a range of pH values. Enzyme activity was observed to have an optimum at about pH 4.0 when pNPP serves as a substrate (Fig. 12).

O. Thermal inactivation of partially purified nonspecific acid phosphatase.

Since the thermolability of nonspecific acid phosphatase has been shown to vary from source to source of enzyme (Weppelman et al., 1977; Kier et al., 1979; Dassa and Boquet, 1985), the thermolability of the partially purified enzyme from T. denticola was investigated. When partially purified enzyme was incubated at 60 °C for up to 1 h, enzyme activity toward pNPP was not affected (Fig. 13). However, when partially purified enzyme was incubated at 90 °C for the same length of time, the enzyme lost 27% of its activity.

P. Kinetic parameters of partially purified nonspecific acid phosphatase.

Despite the indication that fluoride is a noncompetitive inhibitor of acid phosphatase in the undefined environment of the intact cell, it was important to investigate the effect of fluoride on enzyme activity in

Fig. 12. Profile of partially purified nonspecific acid phosphatase as a function of pH. Enzyme activity represents nmol p-nitrophenol released per mg soluble protein. Values are expressed as average ± standard deviation of duplicate samples. Open symbols, 0.1 M sodium acetate buffers; filled symbols, 0.1 M Tris-hydrochloride buffers.

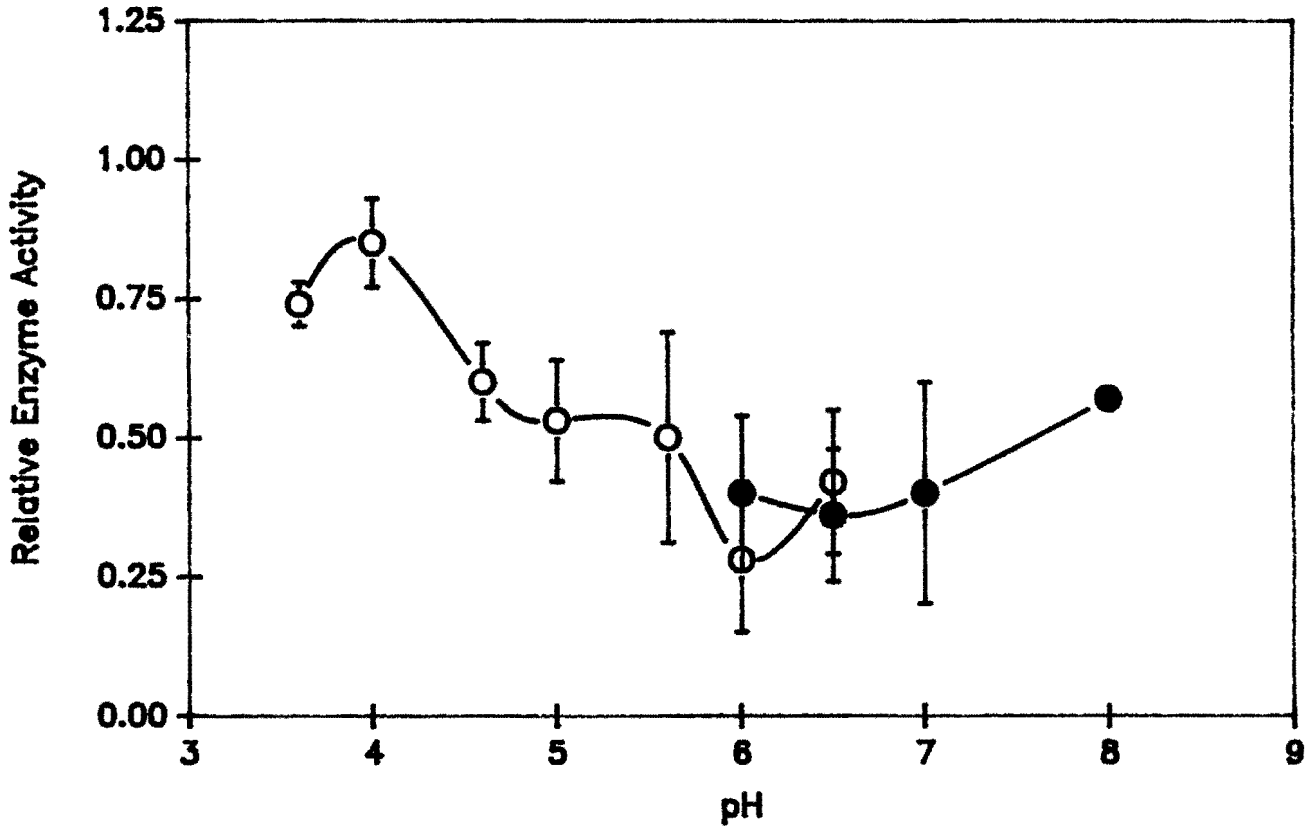
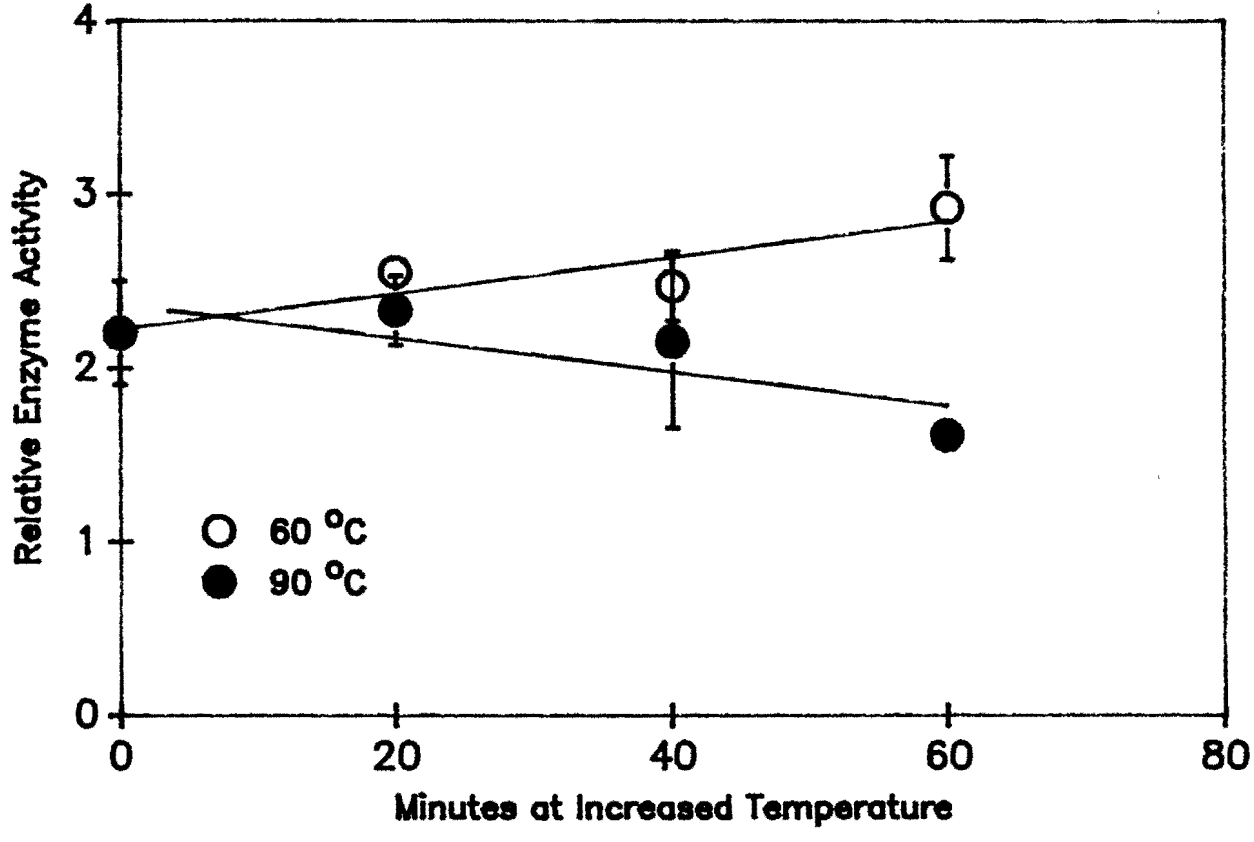

Relative Enzyme Activity
1.25
1.00
0.75
0.50
0.25
0.00
3
4
5
6
7
8
9
pH

Fig. 13. Thermal inactivation of nonspecific acid phosphatase. Samples of partially purified nonspecific acid phosphatase (0.1 ml) in 0.2 ml 0.2 M sodium acetate, pH 4.8, were kept on ice at 0 °C or incubated at 60 °C (open symbols) or 90 °C (closed symbols) for various times and returned to 0 °C. After samples were at 0 °C for 25 min, they were assayed with pNPP as substrate. Enzyme activity is expressed as nmol p-nitrophenol released per mg soluble protein. Values represent average ± standard deviation of duplicate samples. This a typical profile, as observed with two separate experiments.


Relative Enzyme Activity
4
3
2
1
0
60 °C
90 °C
0
20
40
60
80
Minutes at Increased Temperature

a more defined, partially purified preparation. When the partially purified enzyme preparation was incubated in the presence of fluoride and analyzed, mixed results were obtained (Fig. 14.). Fluoride appears to inhibit enzyme activity at high concentrations (100 μg F^-/ml); yet, it appears to activate enzyme activity at lower concentrations (20 and 60 μg F^-/ml). The mechanism of inhibition appears to be that of mixed inhibition (Cornish-Bowden, 1979).

Q. Polyacrylamide electrophoresis of nonspecific acid phosphatase.

Two-dimensional gel electrophoretograms of the soluble contents and the partially purified enzyme preparation are shown in Fig. 15. When the first dimension (thin-layer isoelectric focusing) was stained for nonspecific acid phosphatase, areas of enzyme activity were detected in the acidic pH range of 3.5 to 4.0. Following the second dimension (SDS-PAGE), gels were stained for protein and examined. At least eight proteins were detected in the soluble contents ranging in relative molecular weight from 15K to 40K (Fig. 15a). When partially purified enzyme was electrophoresed under identical conditions, three proteins were detected with relative molecular weights of 33K, 24K, and 14K (Fig. 15b). These three proteins co-migrated with three proteins detected in the soluble contents.

R. Localization of nonspecific acid phosphatase.

One approach to protein localization of periplasmic enzymes involves the use of reagents incapable of penetrating the bacterial permeability barrier, the cytoplasmic membrane (Pardee and Watanabe, 1968).

Fig. 14. Typical Lineweaver-Burk plots of fluoride-inhibited nonspecific acid phosphatase activity in partially purified enzyme preparation. Assay mixtures contained partially purified enzyme and pNPP as substrate. The initial slope of a curve representing the release of p-nitrophenol as a function of time was calculated to determine reaction velocity. The plots were drawn from data obtained in two experiments.

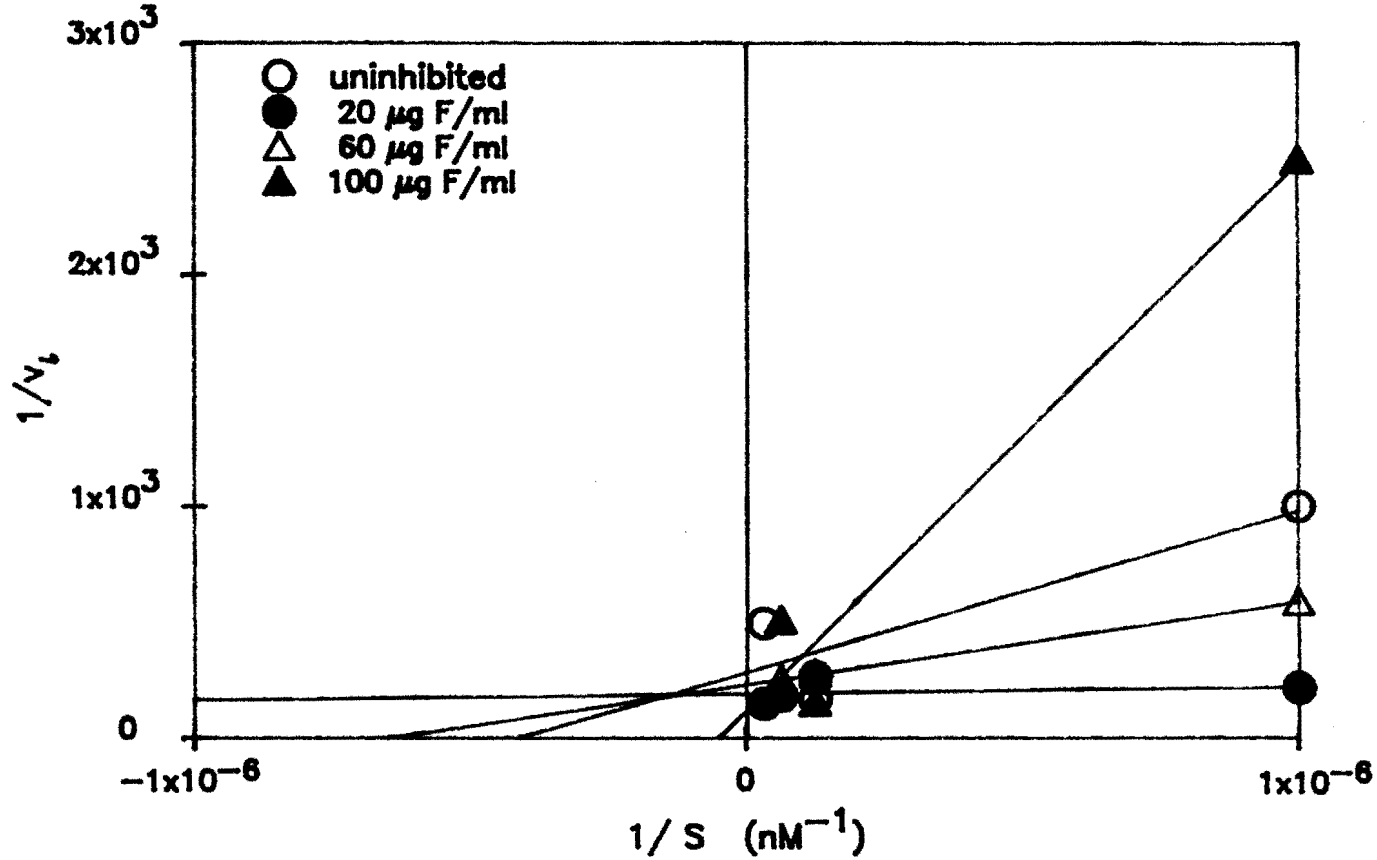
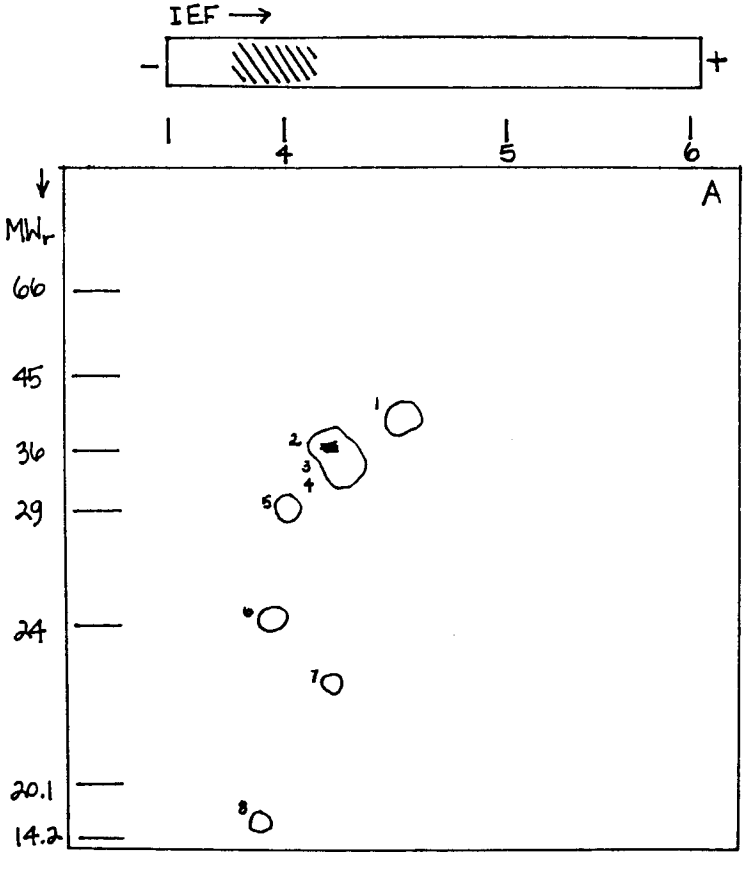
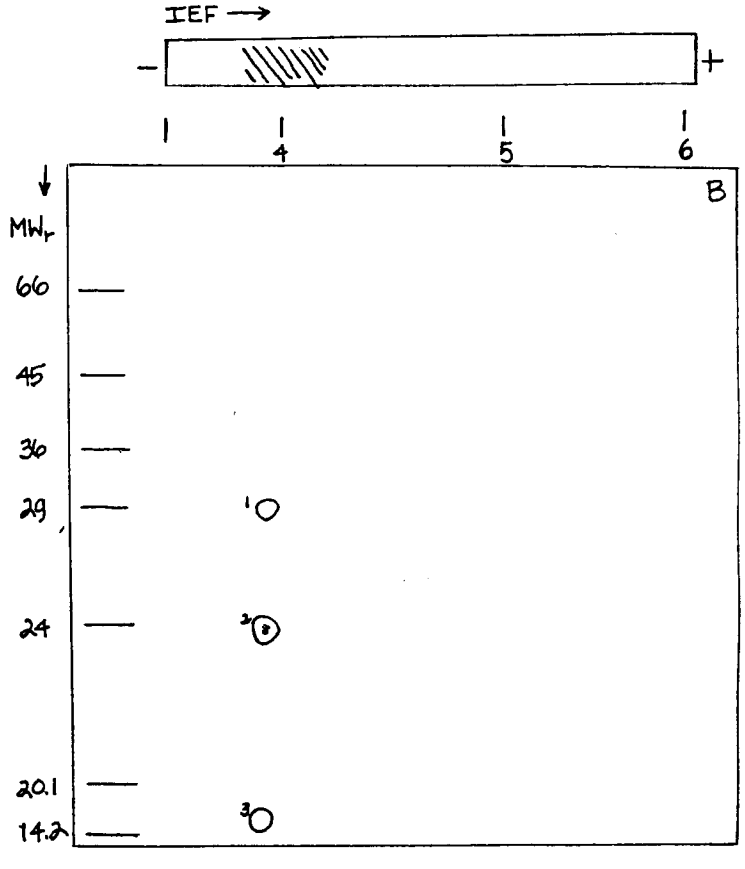

3x10^3
2x10^3
1x10^3
0
1/v
uninhibited
20 µg F/ml
60 µg F/ml
100 µg F/ml
−1x10^−6
0
1x10^−6
1/ S (nM^−1)

Fig. 15. Two-dimensional gel electrophoresis of T. denticola 33520 soluble contents and partially purified nonspecific acid phosphatase. The first dimension (basic thin-layer isoelectric focusing) was conducted with samples of (A) soluble contents (350 μg) and (B) partially purified enzyme (50 μg). The gel was cut into pieces and stained for enzyme activity as indicated in the text. The second dimension was conducted in the presence of SDS, and afterwards stained for protein. From the top to bottom of the figure the following are shown: a diagrammatic representation of the first dimension gel strip showing the region of enzyme activity; the pH distribution measured in the gel strip; the second dimension, after staining with Coomassie blue. Molecular weight standards, as described in the text, are represented to the left.


IEF →
−
+
1
4
5
6
A
MWr
66
45
36
29
24
20.1
14.2
1
2
3
4
5
6
7
8


IEF →
−
+
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B
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45
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29
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20.1
14.2
1
2
3

Enzymes inside the cytoplasmic membrane cannot be inactivated unless the cells are disrupted; enzymes on or outside the membrane can be inactivated. With one such reagent, 7-diazonium-1,3-naphthylene disulfonate (diazo-NDS), one can determine whether an enzyme lies inside the cell membrane or whether it is exposed to the external environment. Whole cells and osmotically shocked cells were treated with the reagent. The shocked cells were then disrupted to determine distribution of the enzyme in cell fractions. Based on color observations (not shown), diazo-NDS, an orange reagent, binds to the membrane portions of the cells. Diazo-NDS inhibits nonspecific acid phosphatase in whole cells and in shocked cells (Table 18). In diazo-NDS treated shocked cells that are further fractionated to "membrane" and soluble "cytoplasmic contents", the membrane-associated enzyme activity is inhibited, whereas the soluble activity is not. Therefore, diazo-NDS does not penetrate the cytoplasmic membrane of the cell. The increased activity in the diazo-NDS treated shock cell "cytoplasm" cannot be explained at this time. These results provide evidence that the nonspecific acid phosphatase is exposed to the periplasmic space of the organism.

Spheroplasts of T. denticola were also prepared in an effort to increase enzyme release and localize the enzyme. Treatment of whole cells with lysozyme resulted in "bulging" of the cells at their termini as visualized by phase-contrast microscopy. Treatment of the cells with EDTA resulted in "blebbing" of the outer sheath. Only cells treated with lysozyme and EDTA were observed to have both "bulging" and "blebbing". Nonspecific acid phosphatase is inefficiently released by lysozyme treatment, as evidenced by its presence in the supernatant of

Table 18. Effect of diazo-NDS on nonspecific acid phosphatase

Treatment and Fraction[a]	nmol pNP released[b] / mg soluble protein
Untreated, whole cells	24.0 ± 1.1
Diazo-NDS, whole cells	3.0 ± 2.4
Untreated, shocked cells	21.1 ± 1.4
Diazo-NDS, shocked cells	8.6 ± 3.6
Untreated shocked, French press, "membrane"	16.7 ± 0.01
Diazo-NDS shocked, French press, "membrane"	1.8 ± 0.8
Untreated shocked, French press, "cytoplasm"	18.2 ± 2.7
Diazo-NDS shocked, French press, "cytoplasm"	55.3 ± 9.2

a Diazotization and cell fractionation scheme as previously described.

b Activity measured by release of p-nitrophenol as previously described. Values represent average ± standard deviation of triplicate samples.

treated cells. The enzyme is also present in the supernatant of lysed spheroplasts (Table 19). EDTA-Tris treatment alone did not result in release of nonspecific acid phosphatase. If the nonspecific acid phosphatase is "free" in the periplasmic space, one would expect it to be released by this treatment. The inefficient release of the enzyme indicates that the protein is most likely associated with the protoplasmic cylinder, with exposure of the enzyme's active site to the periplasmic space.

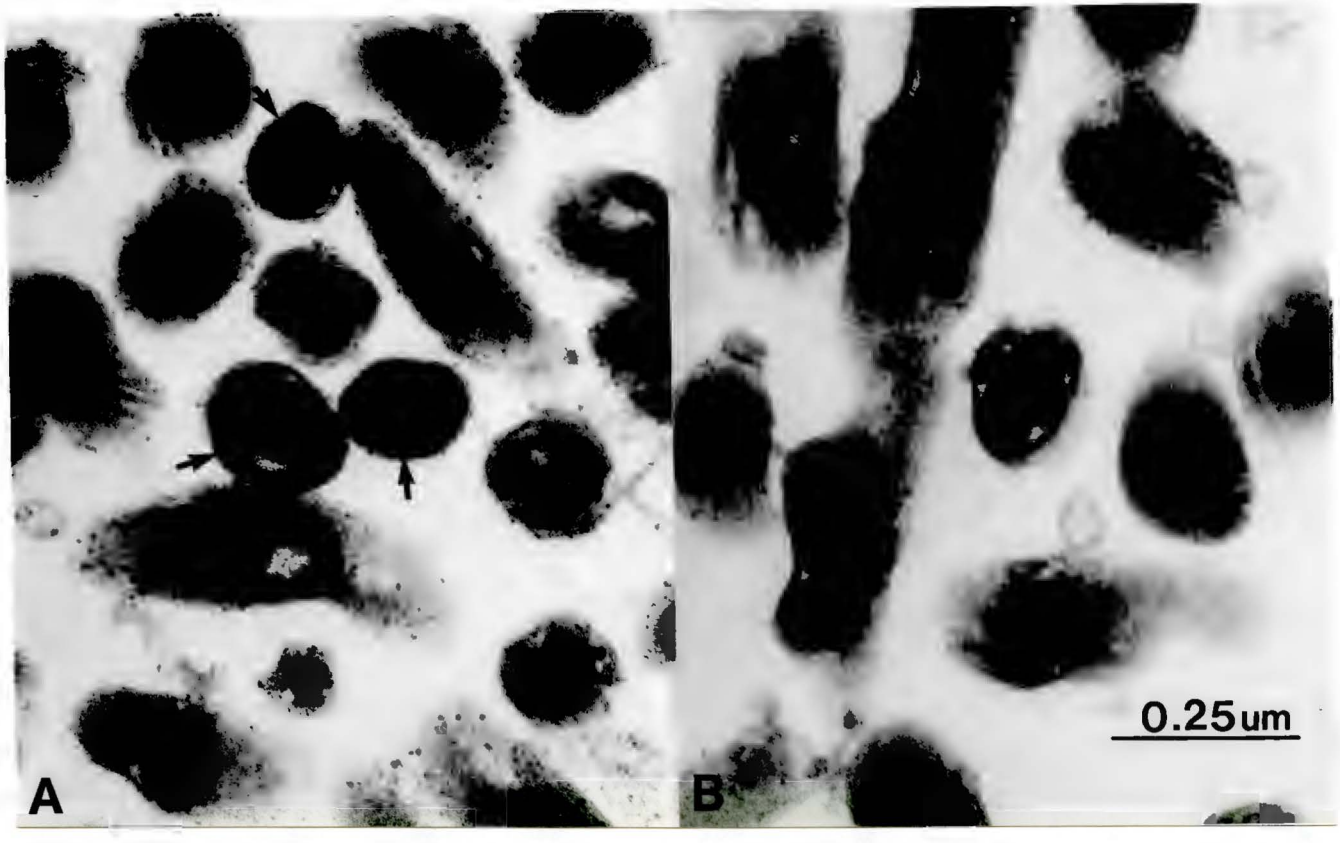
Histochemical staining was used to localize enzyme activity in whole cell preparations for electron microscopy. The hydrolysis of pNPP by the bacteria at an acidic pH, as evidenced by the precipitation of lead phosphate, appears to be at locations between the outer sheath and the protoplasmic cylinder of the organism (Fig. 16). This suggests a periplasmic location for the enzyme. However, when the thin-sections are not counterstained with lead citrate and uranyl acetate preceding microscopic examination, nonspecific lead precipitation is evident in controls. Extensive washing during the histochemical treatment did not alleviate this nonspecific binding. Therefore, the localization of nonspecific acid phosphatase in T. denticola using these histochemical methods, unfortunately, remains inconclusive.

Table 19. Nonspecific acid phosphatase in spheroplasts of T. denticola 33520

Preparation	nmol pNP released[a] mg soluble protein	% of original
Intact, washed T. denticola ATCC 33520	69.3 ± 13.0	100
Lysozyme spheroplasts, pellet	78.3 ± 4.0	112
Lysozyme spheroplasts, supernatant	0.0	0
Lysed spheroplasts, pellet	68.4 ± 12.2	99
Lysed spheroplasts, supernatant	115.9 ± 29.0	167
Lysozyme-Tris alone, pellet	66.5 ± 2.0	96
Lysozyme-Tris alone, supernatant	21.1 ± 0.01	30
Lysozyme-Tris, lysed, pellet	60.4 ± 19.3	87
Lysozyme-Tris, lysed, supernatant	0.0	0
EDTA-Tris alone, pellet	72.1 ± 14.4	104
EDTA-Tris alone, supernatant	0.0	0
EDTA-Tris, lysed, pellet	65.9 ± 17.1	95
EDTA-Tris, lysed, supernatant	0.0	0

[a] Activity measured by release of p-nitrophenol. Values are expressed as the average ± standard deviation of duplicate samples.

Fig. 16. Electron microscopic histochemistry of nonspecific acid phosphatase of intact T. denticola 33520. Arrows indicate deposition of lead phosphate, suggesting the location of nonspecific acid phosphatase. Photomicrograph approximately 100,000X magnification. (A) nonspecific acid phosphatase reaction (B) untreated cells.


A
B
0.25um

DISCUSSION

The effect of fluoride on the growth of the oral spirochete T. denticola has been studied. The fluoride concentrations used in these experiments are well within the fluoride levels known to exist in human dental plaque (Hardwick and Leach, 1962). Sodium fluoride, a simple ionic salt, was the preferred source of fluoride used in these experiments. It is readily available, easy-to-work with, and it is commonly used in dental preparations. Fluoride, as NaF, was shown to inhibit the growth of the T. denticola strains tested here (Table 1). The sodium counterion did not effect growth (Table 3). This was the first report in the literature that fluoride inhibits the growth of T. denticola (Hughes and Yotis, 1986).

Other fluoride compounds used topically in the oral cavity include stannous fluoride and monofluorophosphate. SnF_2 has been shown to have more effects than NaF against oral microorganisms in vivo (Andres et al., 1974) and in vitro (Tinanoff et al., 1983) than has NaF. The different effects of NaF and SnF_2 on oral bacteria have been attributed to: (1) the divalent cation, tin, interacting with the negatively charged plaque components to alter bacterial adhesion/cohesion (Skjorland et al., 1978); (2) the oxidation of thiol groups of bacterial enzymes by tin (Oppermann et al., 1980); (3) the alteration of bacterial metabolism due to uptake of tin by bacteria (Attramadal and Svantun, 1980; Tinanoff and Camosci, 1980); or (4) the naturally low pH of SnF_2 causing HF formation, which is reportedly more anti-bacterial than fluoride (Whitford et al., 1977).

When T. denticola was grown in the presence of either NaF or SnF_2, a greater inhibitory effect was observed with SnF_2 than with NaF (Table 1; Table 2). However, the SnF_2 was difficult to work with, in that it precipitated out of solution at very low concentrations (greater than 10 μg F^-/ml). Stannous ions alone, added as $SnCl_2$, were also observed to inhibit T. denticola growth (Table 2). Whether this Sn inhibitory effect is due to an oxidation of thiol groups of bacterial enzymes by tin, to an alteration of bacterial metabolism due to the uptake of tin, or to some other mechanism, is not understood at this time.

Since much of the work on the mechanism of fluoride action has centered on the effect of the inhibitor on enzymes, the same approach was taken with T. denticola. The API ZYM system served as a rapid and convenient method by which a battery of T. denticola enzymes could be screened for fluoride-sensitivity. A number of enzyme activities were consistently observed in the T. denticola strains including acid phosphatase, trypsin, phosphoamidase, C8 esterase lipase and α-galactosidase (Table 4). These enzyme profiles are generally consistent with the findings of Laughon et al. (1982), who surveyed twenty-three unspecified strains of T. denticola and T. vincentii. However, in contrast to the study of Laughon et al. (1982), alkaline phosphatase activity was not observed in the three oral spirochetes tested here. Fiehn (1986) also reported the absence of alkaline phosphatase activity in eight small-sized oral spirochetes. The pectinolytic strain, P4, exhibited only acid phosphatase, phosphoamidase and C8 esterase lipase activity. The enzyme profiles of the three strains of oral spirochetes observed here are also

consistent with the available information on the physiology of the spirochetes (Canale-Parola, 1977; Harwood and Canale-Parola, 1984).

Of the seven enzyme activities detected in T. denticola 33520 with the API ZYM system, only acid phosphatase activity was sensitive to the presence of 5 μg F^-/ml (Table 5). That the acid phosphatase was sensitive to fluoride was not surprising, since nonspecific acid phosphatases, in general, are inhibited by fluoride (Morton, 1965). Although acid phosphatases have not specifically been implicated as virulence factors in periodontal disease, they may play an important role in the metabolic diversity of T. denticola in the oral cavity. Cell-bound enzymes, if located on or near the cell surface, can hydrolyze substrates present in the periodontal pockets or in the tissues, if the cells have been invasive. This implies that the enzymes are always available to hydrolyze host components. In the case of nonspecific acid phosphatase, the enzyme would be available to remove phosphate groups from host-produced nontransportable phosphate esters, thereby allowing the hydrolyzed host components to be transported and utilized by the cell. The phosphatase activity could supply a variety of nutrients to the cell depending on the esters' organic moiety, in addition to phosphate.

Trypsin-like activity, which has been implicated as an important determinant in the virulence of periodontopathic bacteria (Laughon et al., 1982), was unaffected by the levels of fluoride used in these experiments (Table 5). Of course, the possibility exists that T. denticola enzymic activities other than those detectable with the API ZYM system may be sensitive to fluoride. Other enzyme activities were not investigated.

The API ZYM is a semi-quantitative micromethod system designed for the detection of enzyme activities. The system consists of a series of microcupules containing dehydrated chromogenic substrates. The addition of aqueous sample to be tested rehydrates the components and initiates the reactions. Reactions are visualized after the addition of the detector reagents. Although the API ZYM system allows for the rapid and systematic study of nineteen enzymatic reactions, the system is not without its limitations. Since the substrates are chromogenic, the enzyme must react with the substrate to yield products that in turn will react with the detector reagents. For this reason, the assay is an indirect assay of enzyme activity. The final reaction product must be detectable by the human eye and comparable to a color chart provided by the manufacturer. Therefore, the assay is only semi-quantitative. Most importantly, the microcupule support provides contact between the sample and a typically insoluble substrate. If intact cells are placed in the microcupule and the substrate is insoluble, the possibility exists that the substrate may never reach intracellular enzymes, and that these enzyme activities may go undetected. Finally, the API ZYM system is limited to the study of only nineteen enzymatic reactions. Since bacteria contain more reactions than those detectable with the API ZYM system, many enzyme activities are overlooked. Nonetheless, the API ZYM system was useful as a preliminary enzyme assay system in these investigations.

An understanding of the role of nonspecific acid phosphatase in T. denticola appears tied to the fluoride-sensitivity of the organism. The physiological regulation of this enzyme will first be considered.

The nonspecific acid phosphatase of T. denticola described here is optimally synthesized in the stationary phase of growth (Fig. 3). It behaves like a member of a group of degradative enzymes, which include E. coli acid phosphatase (optimum pH 2.5; Dassa et al., 1982), Salmonella nonspecific acid phosphatase (Kier et al., 1977b), and proteases (St. John et al., 1978). This stationary phase induction suggests that the acid phosphatase is not required during balanced growth, and that it may be synthesized in response to a limitation in some nutrient. An attempt at various nutrient limitations was made with T. denticola; however, neither glucose or phosphate limitation was able to provoke an immediate enzyme expression. Under these experimental conditions then, it is concluded that exogenous levels of glucose and P_i, within the concentrations tested, do not regulate nonspecific acid phosphatase expression in T. denticola. Neu and Heppel (1965) also report that the level of acid phosphatase in E. coli appears to be unaffected by the concentration of P_i in the growth medium. This situation is different from that observed with the nonspecific acid phosphatase of Salmonella typhimurium, whose synthesis has been shown to be stimulated by a limitation in carbon, sulfur, phosphorous, or nitrogen availability (Kier et al., 1977b). A level of 0.075 mM phosphate repressed nonspecific acid phosphatase expression in S. typhimurium. It also differs from that observed with the acid phosphatase (optimum pH 2.5) of E. coli, whose synthesis has been shown to be derepressed by P_i starvation (Dassa et al., 1982).

Although the expression of T. denticola nonspecific acid phosphatase does not appear to be affected by glucose or phosphate limitation, it is important to remember that T. denticola is grown in a complex medium. The endogenous levels of phosphate in the phosphate-limited GM-1 medium was approximately ten fold higher than phosphate levels known to repress nonspecific acid phosphatase expression in S. typhimurium (Table 6). It is highly likely that, despite the reduction of glucose and phosphate levels in the medium (Table 6), the other components of the medium, particularly the yeast extract, serum, or fatty acids, provided nutrients, thereby relieving any limitations. Also, since glucose does not serve as the primary substrate when T. denticola grows in media containing glucose and amino acids (Hespell and Canale-Parola, 1971), it is not surprising that an effect by glucose on nonspecific acid phosphatase expression was not observed under these conditions. The effect of P_i just may not have been low enough to derepress the nonspecific acid phosphatase of T. denticola.

The fluoride-sensitive nonspecific acid phosphatase is measurable in intact cells of T. denticola (Table 5; Fig. 3). Most of the properties of the T. denticola nonspecific acid phosphatase measured in intact cells resemble those described for the corresponding Salmonella (Weppelman et al., 1977) and E. coli enzymes (Dvorak et al., 1967). The T. denticola enzyme in intact cells has an acidic pH optimum around 4.8 (Fig. 4), whereas the partially purified T. denticola enzyme exhibits a pH optimum in the vicinity of 4.0 (Fig. 12). Optimum pH values for the corresponding E. coli and Salmonella enzymes center around pH 5.0. Similarly, no metal ion requirements are detectable for the nonspecific

acid phosphatase of intact cells of T. denticola (Table 9) or of E. coli and Salmonella. In contrast, alkaline phosphatases are thought to be zinc metalloenzymes (Reid and Wilson, 1971). The partially purified T. denticola nonspecific acid phosphatase also appears to be thermostable at 60 °C (Fig. 13). Dassa and Boquet (1985) reported a similar thermal stability with the pH 2.5 acid phosphatase isolated from an E. coli strain which overproduces the enzyme.

The ability of fluoride to inhibit enzyme activity in the absence of a magnesium ion requirement was observed (Fig. 6). Since 97-98% of the fluoride in dental plaque is thought to be bound to inorganic components or to bacteria (Gron et al., 1969; Jenkins et al., 1969), a proposed mode of fluoride action involves the formation of a magnesium-fluoride complex, which functions as a magnesium removal mechanism. This proposed mode of fluoride action does not apply to the T. denticola nonspecific acid phosphatase system.

The nonspecific acid phosphatase of S. typhimurium, appears to be a dimer of two subunits of 26,000-molecular-weight polypeptide chains (Weppelman et al., 1977). This possibility cannot be ignored with the nonspecific acid phosphatase of T. denticola. Protein bands exhibiting nonspecific acid phosphatase in the molecular weight range of 53,000 have been observed on polyacrylamide gels (Fig. 8). Enzyme preparations subjected to SDS-polyacrylamide electrophoresis contained proteins in the molecular weight range of 24,000 (Fig. 15). These values are close to the values determined for the Salmonella enzyme. The possibility for dimerization in T. denticola exists. According to Weppelman et al. (1977), the fact that at least two, and possibly three of the Salmonella

phosphatases are oligomers may have some relationship to protection of the cytoplasm from phosphatase activity. Schlesinger et al. (1969) among others have performed experiments that indicate that active alkaline phosphatase is not present inside E. coli but that inactive subunits are synthesized in the cytoplasm and excreted into the periplasm, where they become active upon dimerization in the presence of zinc. In a similar manner, subunits of the T. denticola nonspecific acid phosphatase might be excreted into the periplasm where they could become activated by dimerization processes.

The partially purified enzyme of T. denticola is able to degrade a variety of phosphate containing substrates, including fructose 1,6-diphosphate, inorganic pyrophosphate, and ATP (Table 17). The acid phosphatase of the yeast, Rhodotorula rubra, was also observed to exhibit high activity with ATP as a substrate (Watorek et al., 1977). Mildner et al. (1976) suggested that this high activity was due, not to contamination by ATPase, but to the presence on the yeast acid phosphatase of a second active site exhibiting ATPase activity. The possibility of a second active site on the nonspecific acid phosphatase of T. denticola was not investigated. There does not appear to be any definite pattern in the substrate specificity of the nonspecific acid phosphatase of T. denticola which would allow further classification of the enzyme activity. Therefore, the activity remains identified as a nonspecific acid phosphatase.

The presence of this nonspecific acid phosphatase in T. denticola would be advantageous for the cell. It would be more efficient for the cell to have one enzyme capable of hydrolyzing a number of similar

substrates than to have several separate enzymes that would each hydrolyze only one particular substrate. This efficiency would allow the cell to channel its energies into other areas, such as synthesizing other proteins or utilizing other metabolites. In the competitive niche of the periodontal pocket, where many different species of bacteria vie for the same limited pool of nutrients, it is important for T. denticola to maintain a competitive edge. If the activity of a versatile nonspecific acid phosphatase allows T. denticola to be a more effective scavenger of available metabolites than other bacteria, then T. denticola will survive and flourish in the periodontal milieu.

The fluoride-sensitivity of T. denticola nonspecific acid phosphatase has been examined. In intact cells, fluoride, as NaF, appears to be a classical noncompetitive inhibitor of enzyme activity (Fig. 7). To paraphrase Cornish-Bowden (1979) on noncompetitive inhibition:

> It arose originally because the earliest students of inhibition, Michaelis and his collaborators, assumed that certain inhibitors acted by decreasing the apparent value of V, but had no effect on K_m. This effect would be an obvious alternative to competitive inhibition, and was termed "noncompetitive inhibition". It is difficult to imagine a reasonable explanation of such effects, however: one would have to assume that the inhibitor interfered with the catalytic properties of the enzyme, but that it had no effect on the binding of substrate. This might be possible for very small inhibitors... In general, it is best to regard noncompetitive inhibition as a special, and not very interesting case of mixed inhibition.

To say that fluoride inhibition of T. denticola nonspecific acid phosphatase is noncompetitive using data obtained from whole cells may be a bit presumptive, but it is a beginning. The milieu of the whole cell is a complex one. Any number of factors, whether it be ions, molecules, proteins, or some other factor in the intact cell environment, may have an impact on enzyme activity or conformation. Fluoride inhibition of the

partially purified enzyme is more difficult to categorize. Fluoride inhibition is no longer thought to be of a classical noncompetitive nature. Rather, it is a more complex, mixed inhibition (Fig. 14). In fact, fluoride appears to function as an activator of nonspecific acid phosphatase activity at low fluoride concentrations, while it appears to inhibit enzyme activity at high fluoride concentrations.

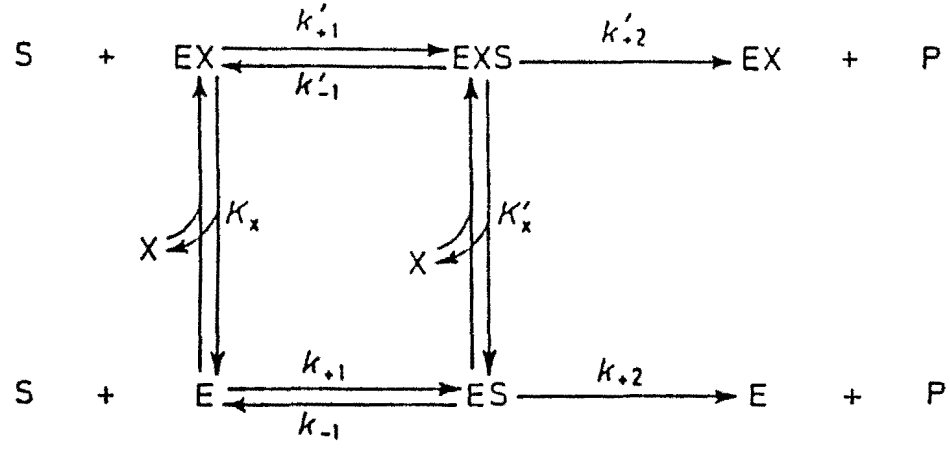
This activator-inhibitor function may be explained by the "general modifier mechanism" of Botts and Morales (1953), in which the term "modifier" is used as a general term that embraces both activators and inhibitors. This mechanism is not confined only to examples of activation, but it can also account for more complex types of inhibition. In Fig. 17 (taken from Botts and Morales), if

$$k_{+2} > k'_{+2} \text{ and } k_{+1}k_{+2}/(k_{-1} + k_{+2}) > k'_{+1}k'_{+2}(k'_{-1} + k'_{+2}),$$

then X is a hyperbolic inhibitor at all substrate concentrations; if the reverse inequalities are obeyed X is a hyperbolic activator at all substrate concentrations; if only one inequality applies, X is an inhibitor in one range of substrate concentrations but an activator elsewhere.

In a rather simplistic explanation, since fluoride inhibition of nonspecific acid phosphatase activity appears to be noncompetitive, fluoride would interact with the enzyme at some site other than the active site. Fluoride, at low concentrations, would interact with the enzyme to create a more favorable environment under which substrate would be able to bind, thereby activating enzyme activity. However, at higher concentrations, fluoride would interact with the enzyme, perhaps at a number of sites, to create conformational or allosteric changes which

Fig. 17. Scheme representing the "general modifier mechanism" of Botts and Morales (1953), where X is the modifier (taken from Cornish-Bowden, 1979).


S + EX
k'_{+1}
k'_{-1}
EXS
k'_{+2}
EX + P
X
K_x
X
K'_x
S + E
k_{+1}
k_{-1}
ES
k_{+2}
E + P

would not allow substrate to interact with the active site in a favorable manner, thereby inhibiting enzyme activity.

Granted, these are only mechanistic hypotheses for the action of fluoride on the partially purified nonspecific acid phosphatase of T. denticola. It is also conceivable that a determinant of enzyme activity important to the action of fluoride on the enzyme may have been lost in the purification scheme. This determinant may be an ion, molecule, or cofactor other than those investigated here. Effects of the local periplasmic environment on the enzyme, as well as the presence of other proteins, may also influence activity. Cofactors and enzyme may even be located in separate compartments of the cell.

Nonetheless, it is important to recognize the noncompetitive nature of fluoride inhibition of the nonspecific acid phosphatase of T. denticola. Fluoride has been shown to be a strong inhibitor of acid phosphatases from a variety of sources including: E. coli (pH 2.5; Dassa et al., 1982); Trypanosoma cruzi (Letelier et al., 1985); Drosophila (Feigen et al., 1980); Sporothrix schenckii (Arnold et al., 1987); and rabbit kidney cortex (Helwig et al., 1978). However, these investigators did not further characterize the inhibitory nature of fluoride. Other investigators have examined the inhibitory nature of fluoride more extensively to find that fluoride is a noncompetitive inhibitor of acid phosphatase in: the yeast, Schizosaccharomyces pombe (Dibenedetto and Mura, 1978); the potato (Kasho et al., 1982); Poa pratensis seeds (Lorenc-Kubis and Morawiecka, 1980); human skin epidermis (Makinen, 1985); and human seminal plasma (NagDas and Bhattacharyya, 1984). The finding then, that

fluoride acts as a noncompetitive inhibitor of T. denticola nonspecific acid phosphatase is not unusual.

Evidence has also been provided suggesting a periplasmic location for the nonspecific acid phosphatase in T. denticola. Admittedly, evidence for the location of the enzyme in the study is indirect. The primary evidence for the periplasmic location of the T. denticola nonspecific acid phosphatase is that the enzyme is inefficiently released in osmotic shock fluids and spheroplast preparations and that the enzyme is able to hydrolyze phosphate esters in whole cells (Table 11; Table 19). Similar observations on the hydrolysis of substrates by E. coli phosphatases (Brockman and Heppel, 1968; Torriani, 1968) and Salmonella phosphatases (Kier et al., 1977a) have been made.

The methods used here to study the periplasmic location of the nonspecific acid phosphatase are selective. The procedures, namely osmotic shock and the formation of spheroplasts with lysozyme and EDTA, do not release internal, cytoplasmic proteins. Another approach, utilizing the reagent diazo-NDS, inactivates proteins (enzymes) on or outside the cytoplasmic membrane, while proteins inside the permeability barrier are unaffected unless the cells are disrupted. Although the use of diazo-NDS in these experiments may have been helpful (Table 18), diazo-NDS is not the ideal reagent for locating proteins. It inactivates only a limited fraction of enzymes or transport systems (Pardee and Watanabe, 1968). Diazonium compounds can combine with several amino acid residues, primarily histidine and tyrosine (Horinishi et al., 1964). The charged sulfonate groups appear to prevent its penetration into the cell. A disadvantage to using diazo-NDS is that if a protein does not contain an

adequate number of histidine or tyrosine residues, diazo-NDS will not be bound and inactivation will not occur. The amino acid content of the nonspecific acid phosphatase of T. denticola is not presently known.

The electron microscopic histochemical localization of nonspecific acid phosphatase in T. denticola appears promising (Fig. 16). However, the reagent used to trap the P_i released by the enzymatic reaction may need to be altered. Lead salts were used here. Perhaps, calcium salts may be a more effective means of precipitating phosphate groups as they are released by the enzymatic reaction. Another possible method for the localization of nonspecific acid phosphatase in T. denticola involves the use of antibodies prepared against the enzyme. The anti-acid phosphatase antibodies could be teamed with colloidal gold in an electron microscopic technique designed to visualize the location of the protein in thin-sections of whole cells.

Certainly, the isolation of mutants altered in the expression of nonspecific acid phosphatase would help to define the physiological role of the enzyme in T. denticola. The isolation and study of T. denticola mutants resistant to fluoride may also provide information about the mechanism of action of fluoride in this organism. However, isolation of such mutants was not undertaken in these studies.

These studies have identified nonspecific acid phosphatase as a potential target for fluoride action in T. denticola. Fluoride inhibits the growth of the organism. Fluoride effects nonspecific acid phosphatase activity. These studies do not suggest that nonspecific acid phosphatase is the only target for fluoride action. The potential effects of fluoride on membrane potential, extracellular and intracellular pH, or

transport mechanisms were not considered in these studies. Other fluoride target molecules may exist in T. denticola.

A possible role for the periplasmically-exposed nonspecific acid phosphatase of T. denticola would appear to be that of a scavenging enzyme. Given that the phosphatase is inhibited by fluoride, presumably, it would be difficult for the organism to obtain utilizable phosphate-containing compounds in the presence of fluoride. Since there is no indication of alkaline phosphatase activity in T. denticola (Hughes and Yotis, 1986b), many phosphate-containing compounds may need to be degraded by a nonspecific acid phosphatase in order to be utilizable. When the activity of the acid phosphatase is inhibited, nutrients containing phosphate would not be degraded to a usable state. Without sufficient metabolites, the organism would not be able to survive, let alone thrive.

Although acid phosphatases have not specifically been implicated as virulence factors in periodontal disease, acid phosphatase may have a role as a potential measure for periodontal disease progression. Since acid phosphatases are detected in intact cells, it may be possible to test periodontal pocket samples for enzyme activity. High enzyme activity may indicate a potential disease site, with spirochetes in an active metabolic state. Low enzyme activity may indicate a waning spirochete population, with a return to periodontal health.

In a broader sense, given that spirochetes are present in large numbers in subgingival plaque associated with periodontal disease; then, based on the findings reported here, if fluoride is introduced to the periodontal pocket environment, T. denticola and other oral spirochetes

would not be able to compete or survive. Spirochete numbers would theoretically decrease and periodontal health would be restored. As a preventive measure, fluoride administration would reduce the accumulation of subgingival plaque bacteria, thereby reducing the incidence of periodontal disease.

SUMMARY

The effect of fluoride on the oral spirochete T. denticola has been studied. Fluoride, as NaF, was shown to inhibit the growth of all three strains of T. denticola studied. Cell growth was completely inhibited by 40 μg fluoride per ml.

The API ZYM system was used as a rapid means of surveying enzyme activities present in T. denticola. Of the seven enzyme activities detected in T. denticola 33520 with the API ZYM system, only nonspecific acid phosphatase was sensitive to 5 μg fluoride per ml.

The fluoride-sensitive nonspecific acid phosphatase of T. denticola 33520 was further characterized. The observed enzyme activity was optimally expressed in the stationary phase of growth. In addition, exogenous levels of glucose and P_i did not appear to regulate nonspecific acid phosphatase expression in T. denticola. The fluoride-sensitive nonspecific acid phosphatase that is measurable in intact cells has an acidic pH optimum around 4.8 and does not appear to have any metal ion requirements for activity. Fluoride, as NaF, appeared to be a classical noncompetitive inhibitor of enzyme activity in intact cells.

The nonspecific acid phosphatase was partially purified (twenty-fold) following anion exchange (DEAE-cellulose) chromatography. The partially purified enzyme exhibited a pH optimum in the vicinity of 4.0 and appeared to be thermostable at 60 °C. The partially purified enzyme was able to degrade a variety of phosphate containing substrates, including fructose 1,6-diphosphate, inorganic pyrophosphate, ATP, and

p-nitrophenyl phosphate. Fluoride inhibition of the partially purified nonspecific acid phosphatase was more difficult to categorize. Fluoride inhibition was no longer of a classical noncompetitive nature, but rather of a more complex, mixed type. At low fluoride concentrations, fluoride appeared to function as an activator of nonspecific acid phosphatase, while it appeared to inhibit enzyme activity at high fluoride concentrations.

Evidence that suggests a periplasmic location for the nonspecific acid phosphatase in T. denticola has also been provided. Intact cells are able to hydrolyze efficiently phosphate esters that are incapable of penetrating the cells cytoplasmic membrane. The enzyme is inefficiently released in osmotic shock fluids and spheroplast preparations. In another approach, which utilized the reagent diazo-NDS, nonspecific acid phosphatase on or outside the cytoplasmic membrane was inactivated, while proteins inside the permeability barrier were unaffected unless the cells were disrupted. The electron microscopic histochemical localization of nonspecific acid phosphatase in T. denticola was inconclusive.

A possible role for the periplasmically-exposed nonspecific acid phosphatase of T. denticola appears to be that of a scavenging enzyme. It is hoped that these efforts to elucidate the fluoride sensitivity of T. denticola will lead to an understanding of the effect of fluoride on oral spirochetes so that fluoride may be established as a preventive measure for periodontal disease.

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The dissertation is therefore accepted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy (Microbiology).

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