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Isolation, Characterization, and Inhibition Kinetics of Enolase from *Streptococcus rattus* FA-1

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One aspect in a broad spectrum of possible mechanisms of cariostatic reactions of fluoride is its interaction with the metabolism of oral bacteria. Information on the mechanisms and kinetics of fluoride inhibition of essential enzymes of the glycolytic pathway of the relevant bacteria is lacking. In this work, the isolation and purification of enolase from *Streptococcus rattus* and its characterization are described. The enzyme has been isolated in a monomeric (22 kilodaltons) and dimeric (49 kilodaltons) form. The K_m for 2-phosphoglycerate is 4.35 mM. Fluoride inhibition kinetics have competitive character, while phosphate in concentrations above 2 mM and in the presence of 0.5 mM fluoride alters the inhibition kinetics from competitive to noncompetitive. Without fluoride, 2 mM phosphate has a slight stimulatory effect on the enzyme. Monofluorophosphate has a noncompetitive inhibiting effect on the enzyme. This finding suggests that the effect of phosphate may be due to an additional binding of fluoride to the enolase, resulting in a conformational change of the enzyme.

The glycolytic enzyme enolase (EC 4.2.1.11) catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate, which is an important metabolic intermediate. The bacteriostatic effect of fluoride in the dental plaque is claimed to be due to the inhibition of enolase by fluoride (11). Most of the experiments described in the literature were carried out, however, with eucaryotic enolases (7, 18, 22) and relatively high millimolar concentrations of fluoride (14, 15). In dental plaque, the concentration of unbound fluoride is lower by 3 orders of magnitude and is only between 2 and 8 μM (1, 13). Bunick and Kashket (6) were the first to study the influence of submillimolar (50 to 100 μM) fluoride concentrations on the kinetics of enolase isolated from *Streptococcus salivarius* ATCC 25975, *Streptococcus sanguis* H7PR3, and *Streptococcus mutans* IB1600. They showed that the enzyme in the presence of 500 mM phosphate is inhibited by fluoride concentrations as low as 50 μM (1 ppm [1 mg/kg]). Additional confirmation of these data or comparison with other strains of oral streptococci, as well as evidence for inhibition by monofluorophosphate, is lacking so far. In our attempt to evaluate the mechanisms of the cariostatic action of low concentrations of fluoride and monofluorophosphate on the metabolism of oral streptococci, studies were carried out with enolase isolated from *Streptococcus rattus* FA-1. *S. rattus* strains display genotypic and serotypic uniformity, in contrast to those of *S. mutans* (10, 12, 16, 17). For this reason, a new method of isolation and purification of the enzyme had to be developed.

MATERIALS AND METHODS

Bacteria and growth conditions. Lyophilized *S. rattus* FA-1 (Behring-Werke, Marburg, Federal Republic of Germany) was grown for 24 h at 37°C (9) in a medium containing 29 g of thioglycolate bouillon per liter, 20 g of glucose (Merck, Darmstadt, Federal Republic of Germany) per liter, 5 g of mannitol (EGA, Steinheim, Federal Republic of Germany) per liter, 2.4 g of lactalbumin (Sigma, Munich, Federal Republic of Germany) per liter, and 0.1 g of $NaHCO_3$ per liter. The bacteria were harvested in the stationary phase of growth (centrifugation at 7,000 × *g*) and were washed with a 0.15 M NaCl solution. The yield was 2 g/liter (wet weight).

Crude sonic extract. After being washed and centrifuged, the pellet was suspended in a solution containing 200 mM Tris hydrochloride buffer (pH 7.8), 24% polyethylene glycol (M_W, 200), and 1 mg of lysozyme per ml; it was shaken at 37°C for 30 min and then centrifuged at 8,000 × *g*. The pellet was suspended in 5 volumes of 50 mM Tris hydrochloride buffer (pH 7.8) containing 25 μM sodium-*p*-tosyl-L-lysine-chloromethylketone, 25 μM sodium-*p*-tosyl-L-phenylalanine-chloromethylketone (Serva, Heidelberg, Federal Republic of Germany), 5 mM DL-dithiothreitol (Sigma), and 10 μg of DNase per ml. The suspension was then sonicated at 0°C for 10 min at intervals of 0.5 min. Cell debris was removed by centrifugation.

Chromatographic fractionation. From the remaining supernatant, the main enzymatic activity was precipitated at an ammonium sulfate saturation between 30 and 80%. The preparation dissolved in 50 mM Tris hydrochloride buffer (pH 7.8) was fractionated at 4°C on a DEAE-fractogel TSK 650 M column (Merck) by using for the elution a stepwise NaCl gradient (50 to 700 mM in 50 mM Tris hydrochloride buffer, pH 7.8). The enzymatic activity was detected in the 80 and 100 mM NaCl fractions.

Molecular weight determination. The enzyme was precipitated from the eluates at 80% ammonium sulfate saturation; both precipitates were codissolved in buffer and were purified on a fractogel TSK HW-50 (F) gel permeation column (Merck) equilibrated with the same buffer. For the determination of the molecular weights of the two enolase fractions, the column was calibrated with bovine serum albumin (M_W, 67,000), ovalbumin (M_W, 43,000), chymotrypsinogen (M_W, 25,000), ribonuclease A (M_W of the dimer, 27,000) (all the above were obtained from Pharmacia, Freiburg, Federal Republic of Germany), and rabbit muscle enolase (M_W of the monomer, 50,000; Sigma).

Enolase assays. (i) Assay a. The enolase activity was determined by measuring the transformation of NADH · H^+ to NAD^+ according to the following reactions (4):

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$$\text{2-phosphoglycerate} \xrightarrow{\text{enolase}} \text{phosphoenolpyruvate} + H_2O \quad (1)$$

$$\text{phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP} \quad (2)$$

$$\text{pyruvate} + \text{NADH} \cdot H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + NAD^+ \quad (3)$$

The reactions were performed at 37°C in 100 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Serva), pH 7.0, containing 3.3 mM $MgSO_4$, 0.2 mM NADH, 0.3 mM 2-phosphoglycerate (Sigma), 1.2 mM ADP, 10.3 IU of lactate dehydrogenase per ml, and 2.7 IU of pyruvate kinase (Serva) per ml. The reaction was started by adding 0.1 ml of the test solution containing enolase. The decrease of the extinction at 340 nm was recorded. The enolase activity was expressed in international units, 1 IU being the enzyme activity transforming 1 μmol of substrate per min.

(ii) Assay b. For the kinetic studies, a single enzyme assay system was used (see reference 8; system described was modified), involving only the transformation of 2-phosphoglycerate to phosphoenolpyruvate by enolase (reaction 1), thus avoiding interactions of the effectors with other enzymes. The reactions were performed at 37°C in 100 mM HEPES buffer (pH 7.0) containing 10 mM $MgSO_4$ and different concentrations of 2-phosphoglycerate (9 to 35 mM) and of the effectors, fluoride (as NaF), phosphate (as KH_2PO_4), and monofluorophosphate (as Na_2PO_3F). The reaction was started by adding 0.1 ml of the test solution containing enolase. The increase of the extinction at 240 nm (formation of phosphoenolpyruvate) was recorded. The enolase activity was expressed as δE_{240nm}/min, with 0.1 δE_{240nm}/min corresponding to the transformation of 0.266 μmol of substrate per minute (20). Each datum point given in the figures is the mean value of five measurements. In the Lineweaver-Burk plots, linear regressions were obtained, considering the statistical weight of the individual datum points (increasing standard deviations with increasing substrate concentrations).

Protein assay. The determination of the protein in the probes was performed by using the Coomassie protein assay reagent (Pierce Chemical Co., Rockford, Ill.) and the Bradford procedure (5).

Polyacrylamide gel electrophoresis. Monitoring of the purity of the enolase and molecular weight determinations were also performed by native polyacrylamide gel electrophoresis by using an acrylamide concentration of 12% and a ratio of acrylamide-bisacrylamide of 37.5:1. Marker proteins were bovine serum albumin, ovalbumin, and chymotrypsinogen; gel staining was performed by using Coomassie blue.

RESULTS

Isolation and purification of enolase. The procedure for isolation and purification of enolase is summarized in Table 1. The yield in purified enolase was 22 μg/1 g (wet weight) of bacteria (12% of the crude activity, sum of monomer and dimer) with a specific activity of 1,118 IU/mg (dimer) and 918 IU/mg (monomer, assayed as dimer). Accompanying activities of pyruvate kinase were completely separated from the enolase during the ion-exchange chromatography (DEAE-fractogel), while the purified enolase retained 7% of phosphoglycerate mutase activity. Phosphatase activity was not detected. The purification procedure described here permits working under magnesium-free conditions and, hence, the separation of a monomer and a dimer of the enzyme by ion-exchange chromatography (Fig. 1). The dimer was eluted from the DEAE-fractogel column at a NaCl concentration of 80 mM, and the monomer was eluted at 100 mM. These results were confirmed by gel permeation chromatography (Fig. 2), which also yielded two separate protein fractions containing enolase activity with an apparent molecular mass of 22 ± 3 kilodaltons (kDa) for the monomer and 49 ± 5 kDa for the dimer, and also by polyacrylamide gel electrophoresis. Figure 3 shows the band of the monomer near the 25-kDa marker protein band and the band of the dimer close to the 43-kDa marker protein band. The monomer appears in the electropherogram to be slightly heavier than 25 kDa because the marker protein has a higher charge-to-molecular-size ratio than the enolase monomer does, thus wandering faster through the gel.

TABLE 1. Summary of procedures for enolase purification (the activities were determined by using assay a)

Source of enolase	IU/ml	IU total	Protein concn (mg/ml)	IU/mg of protein	Purification (fold)	Yield (%)
Crude sonic extract	18.4	275	3.1	5.9	1.0	100
NH_4SO_4 precipitation	17.5	175	2.5	7.0	1.2	64
Fractogel-DEAE						
Monomer	6.1	61	0.055	110.9	18.8	35
Dimer	5.0	50	0.037	135.7	23.1	18
Fractogel TSK						
Monomer	10.5	16	0.011	918	156.0	6
Dimer	11.6	18	0.010	1,118	190.0	6

Kinetic parameters of the enolase. For the kinetic studies, both enolase fractions were used. Since the monomer associates to the dimeric form in the presence of magnesium ions, there was no difference in the kinetic properties of the two fractions. *S. rattus* FA-1 enolase was inhibited competitively by fluoride and had a K_m value of 4.35 mM for 2-phosphoglycerate (Fig. 4). The fact that the K_m of *S. rattus* FA-1 enolase is different from the K_m of the enzyme isolated from *S. mutans* strains (6) is not surprising, since the values of the Michaelis constants for a given enzyme vary widely depending on the isolation conditions and the organism. The kinetics of the enolase reaction were drastically altered by fluoride concentrations as low as 50 μM. Increasing the

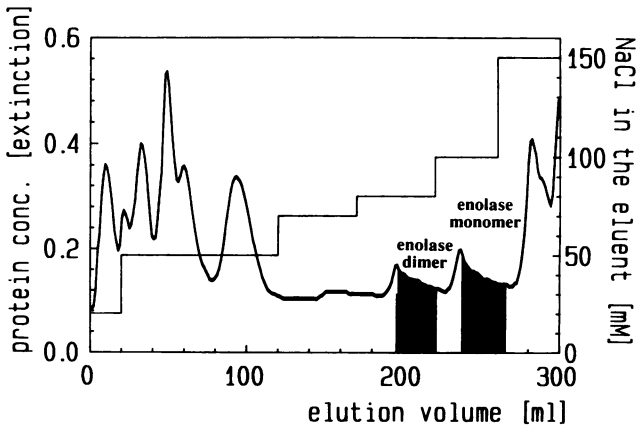

FIG. 1. Isolation of *S. rattus* FA-1 enolase by ion-exchange chromatography on fractogel TSK DEAE-650. The extinction was measured at 280 nm. Areas marked in black indicate the fractions containing the monomer and the dimer of the enzyme.

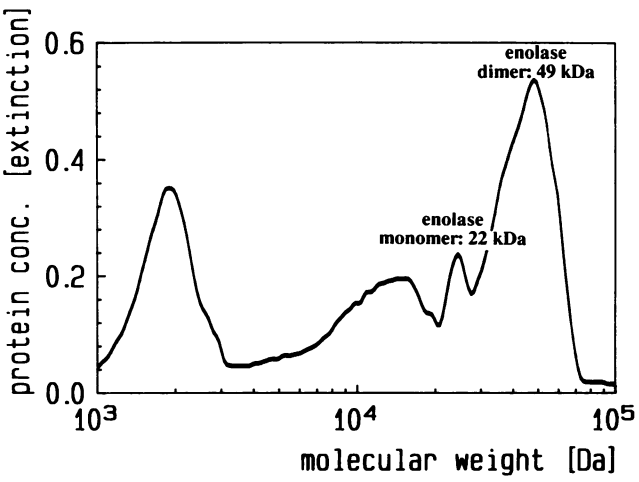

FIG. 2. Purification and molecular weight determination of the *S. rattus* FA-1 enolase by gel permeation chromatography on fractogel TSK HW-50. The extinction was measured at 280 nm.

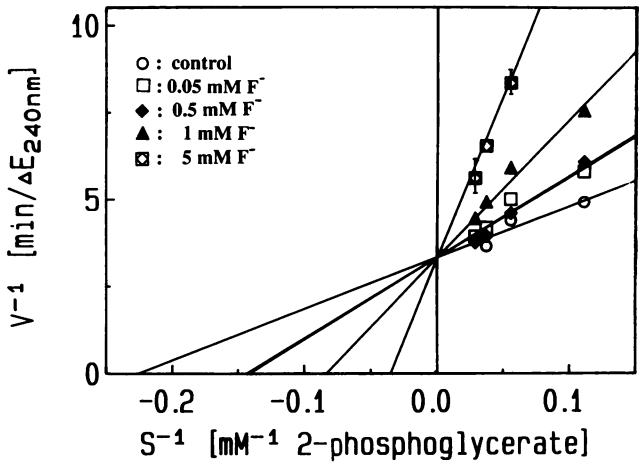

FIG. 4. Inhibition kinetics of *S. rattus* FA-1 enolase by fluoride. Tests were performed at 37°C with assay b. Data are presented as a Lineweaver-Burk plot.

fluoride concentration to 500 μM only slightly affected the kinetics of the reaction, while higher concentrations distinctly reduced the velocity, with a K_i of 0.85 mM. Phosphate had only a moderate effect on the kinetics of the reaction (Fig. 5 and 6). At 1 mM there was no significant difference relative to the control without phosphate; 2 mM seemed to have a slightly stimulating effect (Fig. 6). A moderate inhibiting effect was manifest at higher phosphate concentrations. In contrast to the individual ions, the inhibition of the enzyme reaction was enhanced significantly by mixtures of phosphate and fluoride (Fig. 5). The synergistic effect became obvious when only 1 mM phosphate was added to 500 μM fluoride. At phosphate concentrations higher than 1 mM, the linear fit of the datum points gave some evidence for a change of the type of inhibition kinetics from competitive to noncompetitive (Fig. 5). The type of inhibition by monofluorophosphate derived from the linear fits seems to be noncompetitive (Fig. 7). The inhibiting capacity of monofluorophosphate exceeded that of fluoride and even that of the fluoride-phosphate mixtures. This is supported by the fact that while monofluorophosphate-containing fluoryl and phosphoryl residues in a molar ratio of 1:1 showed noncompetitive kinetics with a K_i of 0.54 mM, noncompetitive inhibition was established only when fluoride and phosphate were present in a molar ratio of higher than 1:4, with a K_i for fluoride of 0.65 mM. The inhibition constants of fluoride, fluoride-phosphate, and monofluorophosphate were 0.85, 0.65 (fluoride), and 0.54 mM, respectively.

DISCUSSION

The purification procedure for enolase from *S. rattus* described here (Fig. 3) yields pure enzyme with a specific activity of 918 IU/mg of protein (monomer fraction, assayed as a dimer) and 1,118 IU/mg of protein (dimer). Other purification procedures for enolase published so far have been developed for eucaryotic enzymes and include five or six purification steps. A method described by Bunick and Kashket (6) involves procedures such as acetone precipitation and heat denaturation, The method described here

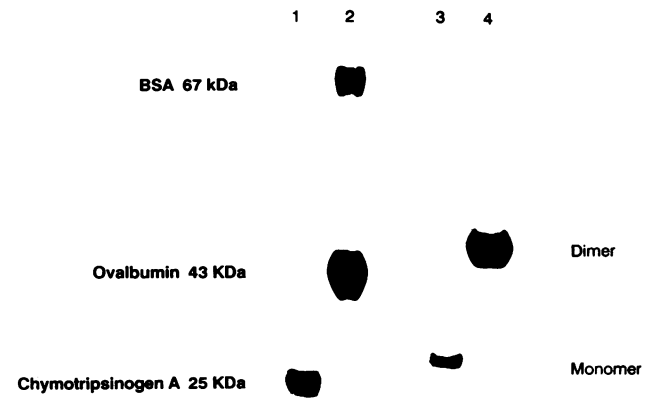

FIG. 3. Separation of the monomer and the dimer of the *S. rattus* FA-1 enolase by polyacrylamide gel electrophoresis (12% acrylamide; Coomassie blue staining). Lanes: 1, 40 μg of chymotrypsinogen A (25 kDa); 2, 40 μg of bovine serum albumin (BSA) (67 kDa)–50 μg of ovalbumin (43 kDa); 3, 25 μg of enolase monomer (22 ± 3 kDa); 4, 35 μg of enolase dimer (49 ± 5 kDa).

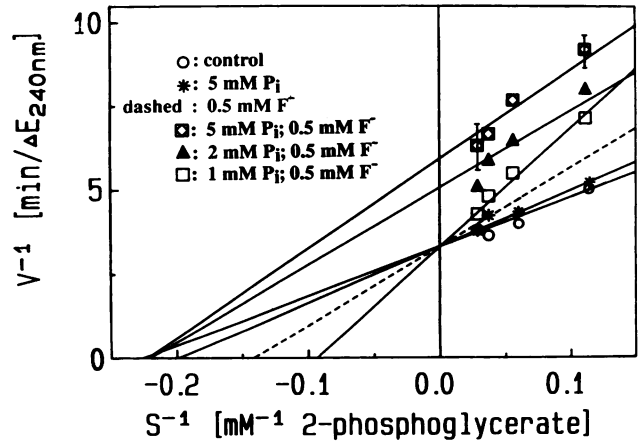

FIG. 5. Inhibition kinetics of *S. rattus* FA-1 enolase by phosphate and fluoride-phosphate. Tests were performed at 37°C with assay b. Data are presented as a Lineweaver-Burk plot. Standard deviations given are representative for the highest and lowest substrate concentrations.

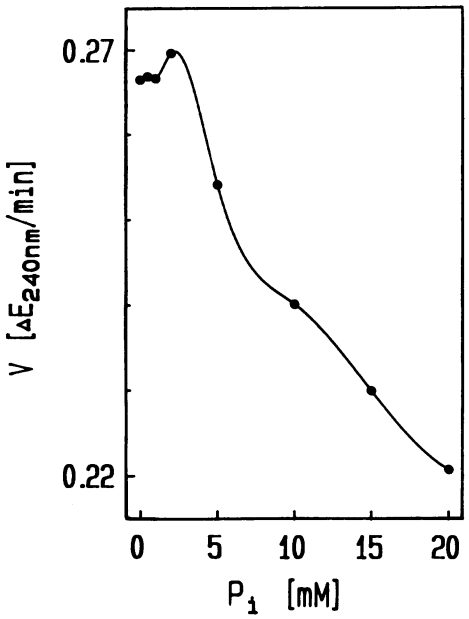

FIG. 6. Influence of increasing concentrations of phosphate on the velocity of the reaction catalyzed by enolase. Tests were performed at 37°C with assay b.

involves only three steps and is also well suited for processing small amounts of bacteria (1 g). Ion-exchange chromatographic fractionation of the crude precipitate in magnesium-free medium yielded two protein fractions showing enolase activity. Gel permeation chromatography on fractogel TSK revealed that the form which binds less tightly to the ion exchanger is the dimer and the stronger binding form is the monomer of the enzyme, i.e., its affinity to the ion exchanger decreases with increasing degree of association. Molecular mass determinations performed with enolase isolated from other organisms revealed apparent values of 46 and 92 kDa for the *Escherichia coli* (8, 20), 82 kDa for the rabbit muscle, and 67.3 kDa for the yeast enzyme (2). The *S. rattus* enzyme is significantly smaller (22 and 49 kDa).

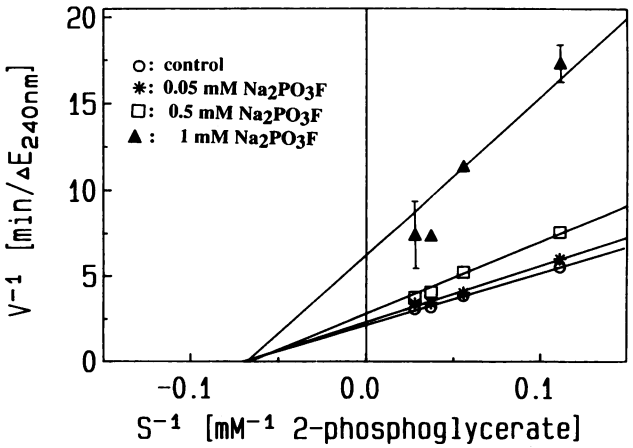

FIG. 7. Inhibition kinetics of *S. rattus* FA-1 enolase by monofluorophosphate. Tests were performed at 37°C with assay b. Data are presented as a Lineweaver-Burk plot. Standard deviations given are representative for the highest and lowest substrate concentrations.

Enolase has a central position in the streptococcal metabolism. Its inhibition by millimolar quantities of fluoride, which is extensively described in the literature, has grave consequences for the survival of these organisms. Although these concentrations exceed those in the buccal fluids and plaque by orders of magnitude, there is some new evidence that the inhibitory effect of fluoride should be considered as one of the aspects of the cariostatic reaction mechanisms (17, 19). Only Bunick and Kashket (6) studied the effect of the physiological concentrations of fluoride so far, reporting inhibition of enolase with 50 μM fluoride in the medium. Our data confirm their findings and indicate a competitive mechanism of inhibition, whereas studies with the enzyme isolated from rabbit muscle showed noncompetitive inhibition kinetics (3, 21). The negative effect of fluoride could be enhanced by adding phosphate (1 mM) to the medium, confirming also the findings of Bunick and Kashket (6), while phosphate without fluoride had, in concentrations greater than 2 mM, only a small inhibiting effect on the enzyme. Evidence for the alteration of the type of enolase inhibition kinetics from competitive to noncompetitive by increasing the phosphate concentration in the presence of a given fluoride concentration and for the noncompetitive effect of monofluorophosphate suggests that phosphate possibly causes an additional binding of fluoride to the enolase, resulting in a conformational change of the enzyme and, thus, in noncompetitive inhibition.

Enolase has been isolated from a variety of organisms (22), but little attention has been paid to the streptococcal enzymes, although these bacteria are among the main cariogenic factors in the buccal environment. The full elucidation of the mechanism of fluoride and phosphate inhibition of this enzyme is needed for a better understanding of the cariostatic action of fluoride.

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LITERATURE CITED

1. **Agus, H. M., P. S. H. Un, M. H. Cooper, and R. G. Schamschula.** 1980. Ionized and bound fluoride in resting and fermenting dental plaque and individual human caries experience. Arch. Oral Biol. **25**:517–522.
2. **Barman, T. E. (ed.).** 1969. Enzyme handbook, vol. 2. Springer-Verlag, New York.
3. **Bartholmes, P., H. Senkel, and H. Spranger.** 1987. Inhibition of glycolytic enzymes by fluoride ions. Deutsch. Zahnärztl. Z. **42**:916–919. (In German.)
4. **Bergmeyer, H. (ed.).** 1973. Methods of enzymatic analysis. Verlag Chemie, Weinheim, Federal Republic of Germany.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**:248–254.
6. **Bunick, F., and S. Kashket.** 1981. Enolases from fluoride-sensitive and fluoride-resistant streptococci. Infect. Immun. **34**:856–863.
7. **Bunick, F., and S. Kashket.** 1982. Binding of fluoride by yeast enolase. Biochemistry **21**:4285–4291.
8. **Danelly, H. K., and H. C. Reeves.** 1988. Purification and characterization of enolase from *Escherichia coli*. Curr. Microbiol. **17**:265–268.
9. **Duschner, H., H. Bergmann, and N. Psarros.** 1985. The effect of the extracellular pH on the accumulation of fluoride in *S. mutans*. Deutsch. Zahnärztl. Z. **40**:410–415. (In German.)
10. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331–384.

11. **Hamilton, I. R.** 1977. Effects of fluoride on the enzymatic regulation of bacterial carbohydrate metabolism. Caries Res. **11**(Suppl. 1):262–291.
12. **Ikeda, T., K. Ochiai, and T. Shiota.** 1979. Taxonomy of the oral *Streptococcus mutans* based on colonial characteristics and serological, biochemical and genetic features. Arch. Oral Biol. **24**:863–867.
13. **Jenkins, G. N., and W. M. Edgar.** 1977. Distribution and forms of F in saliva and plaque. Caries Res. **11**(Suppl. 1):226–242.
14. **Kanapka, J. A., and I. R. Hamilton.** 1971. Fluoride inhibition of enolase activity in vivo and its relationship to the inhibition of glucose-6-P formation. Arch. Biochem. Biophys. **146**:167–171.
15. **Kashket, E. R., and F. J. Bunick.** 1978. Binding of fluoride in oral streptococci. Arch. Oral Biol. **23**:993–996.
16. **Kilpper-Bälz, R., P. Wenzig, and K. H. Schleifer.** 1985. Molecular relationships and classification of some viridans streptococci as *Streptococcus oralis* and emended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int. J. Syst. Bacteriol. **35**:482–488.
17. **Nikiforuk, G. (ed.).** 1985. Understanding dental caries. S. Karger, Basel.
18. **Nowak, T., and P. J. Maurer.** 1981. Fluoride inhibition of yeast enolase. II. Structural and kinetic properties of the ligand complexes determined by nuclear relaxation rate studies. Biochemistry **20**:6901–6907.
19. **Psarros, N.** 1988. Interactions of fluoride with the metabolism of the oral cariogenic bacterium *Streptococcus mutans* FA-1 (*S. rattus*). *In* A. Intemann and C. Intemann (ed.), Wissenschaftliche Forschungsbeiträge: Biologie/Biochemie/Chemie, vol. 8. Gauting bei München. (In German.)
20. **Spring, T. E., and F. Wold.** 1971. The purification and characterization of *Escherichia coli* enolase. J. Biol. Chem. **246**: 6797–6802.
21. **Wang, T., and A. Himoe.** 1974. Kinetics of the rabbit muscle enolase-catalyzed dehydration of 2-phosphoglycerate. J. Biol. Chem. **249**:3895–3902.
22. **Wold, F.** 1971. Enolase, p. 495–538. *In* P. D. Boyer (ed.), The enzymes, vol. 5. Academic Press, Inc., New York.