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The pH-Dependent Liberation of Phosphate from Human Dental Enamel and Dentine by Ammonium Sulphate*

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Commercial *E. coli* alkaline phosphatase preparations were found to liberate phosphate from powdered human dental enamel and dentine. Chemical analysis and chromatographic procedures revealed that the commercial enzyme preparations were able to liberate phosphorus due to their high content of ammonium sulphate. The rate of the reaction was pH-dependent, most phosphate being released at pH values close to 7.0. The reaction was not affected by EDTA, Mg^{2+} ions, and various enzyme inhibitors. The results showed that the "enzymic" liberation of phosphate from the tissues mentioned and reported by different laboratories is at least in part due to ammonium sulphate often present in commercial enzyme preparations, rather than to the action of specific enzymes.

The probable chemical events occurring in molecular disintegration of dental enamel structure can be classified according to two main theories: 1) dissolution of inorganic constituents by acids, 2) hydrolysis of organic constituents by enzymes. A combination of these and other effects, such as chelation, should also be considered. Results obtained in this laboratory [1] indicate that preparations of subtilopeptidase A (EC 3.4.4.16), or components present in them, are capable of liberating phosphorus from human dental enamel powder. This suggests that a certain fraction of dental enamel is attacked by the enzyme preparation involved (or by some impurities). Later, Kreitzman, Irving, Navia and Harris [2] have described the phosphate-releasing activity of *E. coli* alkaline phosphatase preparations.

The purpose of this paper is to offer the results of our investigations on the action of a compound discovered in alkaline phosphatase (EC 3.1.3.1) preparations, obtained from *E. coli*, on human dental enamel in liberating phosphorus. This paper also provides further information concerning the liberation of phosphorus from human dental enamel by preparations of subtilopeptidase A. We have been able to confirm most of the results of our earlier experiments

* Part of the results were presented at the 16th ORCA Congress, Stockholm, July 1969, and a preliminary note has been published elsewhere.[3]

and also most of those made independently of us by Kreitzman *et al.*,[2] although the latter school assumes that a phosphoprotein phosphatase (EC 3.1.3.16) is involved, while our results [3] have indicated some other factor. We have now been able to show that one such factor is the ammonium sulphate, present in commercial enzyme preparations of *E. coli*.

MATERIAL AND METHODS

1. *Substrates.* All of the *N*-L-aminoacyl-2-naphthylamines used in this study were obtained from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.), and all naphthyl esters of fatty acids were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

2. *Enzyme preparations.* Subtilopeptidase A (Grade VIII, from *Bacillus subtilis*, and *E. coli* alkaline phosphatase [Type III-S]) were obtained from Sigma Chemical Company.

3. *Other chemicals.* All other chemicals used in this study, unless otherwise stated, were purchased from E. Merck AG (Darmstadt, Germany).

4. *Human dental enamel.* Powdered human dental enamel, so-called UE enamel, and dentine of defined particle size (usually 200—270 mesh) were used in this study. The preparation of these materials was carried out as described in our earlier paper.[1]

5. *Molecular exclusion chromatography.* For the fractionation of *E. coli* alkaline phosphatase, usually 0.75 ml of the commercial enzyme suspension was applied on a Sephadex® G-200 column. When subtilopeptidase A was studied, 80 mg of the commercial preparation was dissolved in 5 ml of cold (+4°C) 0.02 M tris-HCl buffer, pH 7.2. The resultant protein solution was applied on a Sephadex G-100 column. In performing all of the fractionations the instructions given by the supplier of the gels (Pharmacia Fine Chemicals, Uppsala, Sweden) were followed. More details are given with each experiment later.

6. *Determination of phosphorus liberation and phosphatase activity.* The ability of preparations of alkaline phosphatase and subtilopeptidase A to liberate phosphorus from human dental enamel powder was in principle tested as earlier described.[1] In subtilopeptidase A studies, powdered enamel (50 mg) was incubated for various periods of time (from 60 to 120 min) in a reaction mixture consisting of 0.25 ml enzyme solution and of 0.5 ml 0.02 M tris-HCl buffer, pH 7.2. When alkaline phosphatase was studied, the phosphorus liberating activity was usually determined in a reaction mixture consisting of 3 ml of 0.02 M tris-HCl buffer, pH 7.2, in which 5 μl of the commercial enzyme preparation was dissolved and to which the powdered enamel of defined particle size (100—140 mesh) was added in various amounts (from 5 to 100 mg). Later the volume of the reaction mixture was decreased to 0.75 ml without altering the amount of enzyme and enamel powder. Essentially similar results were obtained in both reaction mixtures, but the smaller ones were more rapidly handled in millipore filtration. For testing the phosphorus-liberating activity of fractions resulting from column chromatography, 0.25 ml aliquots of the fractions were mixed with 5 mg dentine or enamel powder and 0.5 ml 0.01 M tris-HCl buffer, pH 7.2. The reaction mixtures were incubated for 60 min at 37°C while being shaken. After this time the mixtures were rapidly passed through a millipore filter to stop the reaction and to remove all of the solid particles. Phosphate was then analyzed from the millipore filtrates according to the method of Fiske and Subbarow.[4]

The above method was used in the estimation of the hydrolysis of DL-*O*-serinephosphate and other phosphates. In this case the reaction mixture was composed of 0.1 ml 0.05 M glycine-NaOH buffer of varying pH values, of 0.05 ml 6×10^{-3} M $MgCl_2$ solution, of 0.1 ml aqueous substrate solution, and of 0.05 ml enzyme solution. The enzyme reactions were carried out for various periods of time (usually 60 min) and were stopped by adding 0.1 ml of 10 % trichloroacetic acid solution. The determination of phosphorus was then performed. Appropriate controls were included in all experiments.

7. *Determination of other enzyme activity.* The enzymic hydrolysis of α- and β-naphthyl esters was in principle assayed as earlier described, *i.e.* by preparing an azo-compound

from the liberated naphthol.[5] The enzymic hydrolysis of *N*-L-aminoacyl-2-naphthylamines was tested as earlier given.[6,7] Inorganic pyrophosphatase activity was assayed with sodium pyrophosphate (at 0.167 mM substrate concentration) in 5 mM tris-HCl buffer, pH 7.2, and in the presence of 1 mM $MgCl_2$. The reaction mixture and conditions were the same as earlier described.[1]

8. *Photo-oxidation.* Photo-oxidation in presence of methylene blue was in principle conducted according to a method described elsewhere, employing a Cremer Twiniode spotlight (2×800 W) as the light source.[8]

9. *Determination of total phosphorus.* The total phosphorus of protein samples was in principle assayed as suggested by Berenblum and Chain.[9]

10. *Determination of ninhydrin-positive compounds.* The fractions obtained from various chromatographic procedures were tested for ninhydrin-positive compounds according to the method described by Yemm and Cocking.[10]

11. *Determination of DNA.* The fractions resulting from column chromatographic steps were assayed for DNA according to the method of Burton.[11]

12. *Determination of polysaccharides.* The fractions resulting from column chromatography were tested for polysaccharides according to the method of Dreywood [12] and by taking the notations of Morris [13] into account.

13. *Determination of protein.* Protein concentration was determined using the Folin-Ciocalteu method.[14] Bovine serum albumin (crystallized and lyophilized Sigma Chemical Company) was used as a standard. The fractions resulting from column chromatography were tested for protein also by measuring the absorbance at 280 mμ. The absorption at 260 mμ was simultaneously determined. In these measurements as well as other spectrophotometric determinations the Hitachi Perkin Elmer spectrophotometer Model 139 was used.

14. *Determination of ammonia.* Ammonia was assayed using the phenol reagent-hypochlorite method of Searsy *et al.*[15]

15. *Determination of guanidino groups.* In these assays the Sakaguchi reagent was used as described by Gilboe and Williams.[16]

16. *Determination of ribose.* For the determination of RNA the orcinol test of Bial,[17] modified by Mejbaum,[18] was used.

17. *Amino acid analysis.* The samples to be studied on the Beckman Unichrom Amino Acid Analyzer were handled, hydrolyzed, and analyzed as described in the Beckman Instruction Manual for Beckman Unichrom Amino Acid Analyzer, 1966.

The hydrolysates resulting were also studied for their amino acid content by thin layer chromatography on Silica Gel as described by Randerath (see also Ref. 7).

RESULTS

1. *The phosphorus-liberating factor of* E. coli *alkaline phosphatase preparations.* The preliminary experiments indicated that the bacterial alkaline phosphatase preparations of *E. coli* were capable of liberating about 7.5 μg phosphorus from 100 mg of 100 – 140 mesh enamel powder (in 60 min, in a reaction mixture of 3 ml). More phosphorus was released from particles of smaller size, indicating that the grinding of enamel and dentine into smaller particles also produces more phosphate residues which are subject to the action of the factor involved. This increase in enamel solubility through reduction of particle size was continuously measured and the total phosphorus content of the enzyme preparations assayed. Only slightly more phosphorus was liberated from dentine than from enamel powder of the same particle size, indicating the existence of the same phosphorus-providing structural function in both of the tissues.

EDTA at concentrations ranging from 1.0×10^{-2} M to 5.0×10^{-5} M did not inhibit the phosphorus-releasing activity of *E. coli* alkaline phosphatase

preparations, nor did Mg^{2+} ions activate it, although both compounds retarded the dissolution of phosphorus by buffer (pH 7.2). Photooxidation in the presence of methylene blue (at 1 and 10 μM) did not affect the activity in any way, and phenylmethanesulphonyl fluoride (at concentrations ranging from 1.0×10^{-2} M to 5.0×10^{-5} M) did not inhibit the release of phosphorus by the enzyme preparation.

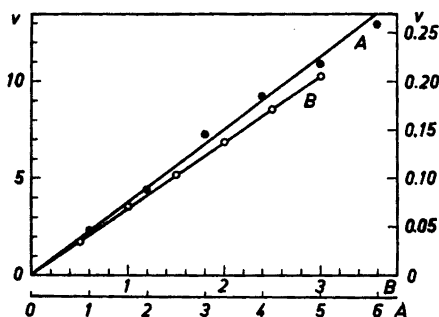

Fig. 1. Plot of velocity v (in μg phosphorus per ml) *versus* enzyme concentration in the action of the phosphorus-liberating component of *E. coli* alkaline phosphatase preparation on human dental enamel powder. Curve A: experiment with unfractionated alkaline phosphatase preparation (right-hand scale). Curve B: experiment with an enzyme preparation made by pooling the active fractions (290–340 ml) in the fractionation given in Fig. 4 (left-hand scale). For curve A the enzyme concentration is given in μg per 3 ml and for curve B in ml of the active pool per 3 ml. The volume of the reaction mixtures was 3 ml.

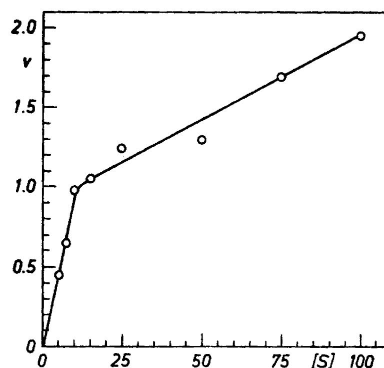

Fig. 2. The Michaelis-Menten plot of the velocity v (in μg phosphorus per ml) of the release of phosphorus from human dental enamel powder caused by a component present in *E. coli* alkaline phosphatase preparations (unfractionated) *versus* the "substrate" concentration [S] (in mg).

The rate of the reaction was proportional to the amount of the enzyme preparation, as shown in Fig. 1. The effect of "substrate" concentration is shown in Fig. 2. The effect of pH on the liberation of phosphorus is shown in Fig. 3. A fairly typical pH-dependence curve of enzyme activity was obtained, indicating the participation of a system with two successive ionizations.

When the alkaline phosphatase preparations of *E. coli* were fractionated on Sephadex G-200 columns, the activity towards typical phosphatase substrates was separated from that towards dental enamel (Fig. 4). The activity towards α- and β-naphthyl acetate seemed to coincide, or nearly coincide, with that releasing phosphorus from dental enamel. However, separate experiments showed that ammonium sulphate at concentrations ranging from 7

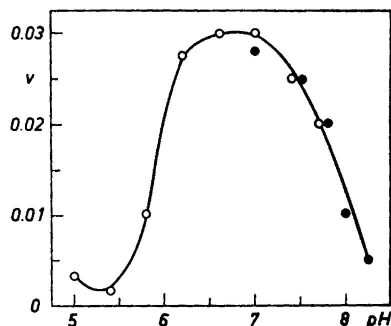

Fig. 3. Effect of pH on the rate of the liberation of phosphorus from human dental enamel powder by an alkaline phosphatase preparation (unfractionated) of *E. coli*. Open circles: reaction in 0.05 M β,β-dimethylglutarate-NaOH buffer. Solid circles: reaction in 0.02 M boric acid-borax buffer.

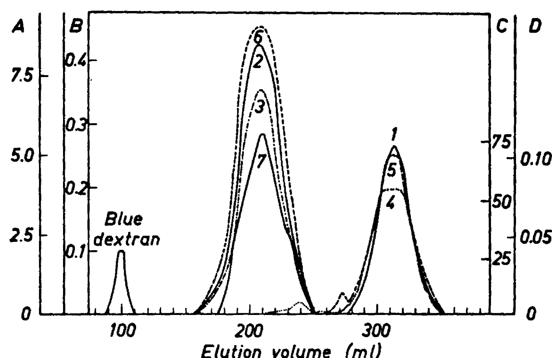

Fig.4. Molecular exclusion chromatography of the phosphorus-liberating factor of *E. coli* alkaline phosphatase preparation on a Sephadex G-200 column (57×2.5 cm). Sample: 0.75 ml of the commercial enzyme preparation containing 7.5 mg protein. Elution buffer: 0.01 M tris-HCl, pH 7.0. Hydrostatic pressure: 25 cm. Flow rate: 8.6 ml/h. Temperature: +2°C. Curve 1: phosphorus-liberating activity (in μg of liberated phosphorus per 0.75 ml; scale A). Curve 2: *p*-nitrophenyl phosphate (in absorbance; scale B). Curve 3: DL-*O*-serine phosphate (scale A). Curve 4: β-naphthyl acetate (in absorbance; scale D). Curve 5: α-naphthyl acetate (scale D). Curve 6: α-naphthyl phosphate (scale D). Curve 7: protein (Folin-Ciocalteu, in μg/ml; scale C). All estimations were carried out for every second fraction at the substrate concentration of 0.166×10^{-3} M (for testing the release of phosphorus from enamel, 100 mg of enamel powder was used).

mM to 3.5 M increased the "chromogenic potential" of the red-coloured azo-dye, resulting from the coupling between α- or β-naphthol and diazotized 4-amino-3,1'-dimethylazobenzene. Consequently, no true esterases evidently are involved.

None of the phosphorus-liberating fractions contained any, or at most only traces, of phosphorus, according to the total phosphorus assays. The peak in Fig. 4 representing the release of phosphorus was drawn after first subtracting the phosphorus values of blanks (caused by dissolution of enamel by buffer). Hence the x-axis can be considered to represent this dissolution and any phosphorus value above this axis describes the activity of the phosphorus-releasing factor.

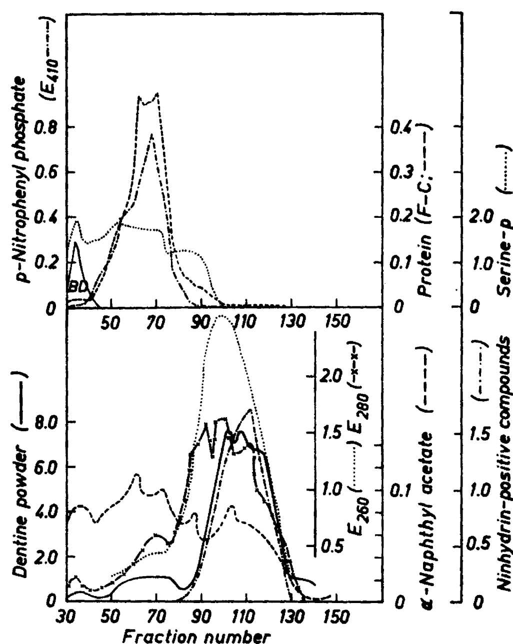

Fig. 5. Molecular exclusion chromatography of the phosphorus-liberating factor of *E. coli* alkaline phosphatase preparation on a Bio-Gel P-300 column (59.0 × 2.5 cm). Sample: 2.0 ml of the commercial enzyme preparation containing 40 mg protein. Elution buffer: 0.01 M tris-HCl buffer, pH 7.0. Hydrostatic pressure: 20 cm. Flow rate: 14 ml/h. Temperature: +1°C. F–C=Folin-Ciocalteu method (E_{500}); serine-P=serine phosphate. BD=Blue Dextran. For testing the phosphorus liberating activity, 5 mg dentine powder was used. Ninhydrin-positive compounds are given as E_{540}. Other details as for Fig. 4.

Fig. 5 presents the molecular exclusion chromatography of *E. coli* alkaline phosphatase preparation tested with dentine powder. The figure also gives the results obtained when measuring the absorption of the fractions at 260 and 280 mμ and when the fractions were tested for ninhydrin-positive compounds. A significant result was the constant coincidence of the phosphorus-liberating peak and the peak representing ninhydrin-positive compounds. No peak with ninhydrin was even seen in the elution volume of the essential alkaline phosphatase, containing almost all the protein applied on the column. The phosphorus-liberating peak did not coincide with those representing absorption at 260 or 280 mμ. No traces of polysaccharides or DNA were found in the fractions. Total phosphorus assays revealed that the 0.25 ml aliquots used in the activity assays contained practically no phosphorus between fractions 90 and 120. In this case as well, no measurable amounts of protein were found in fractions 90–120. The strong absorption at 280 and 260 mμ was caused by some component with absorption maximum at 258–260 mμ. Subsequent studies showed that this peak contained ribose (but not deoxyribose) which is evidently derived from RNA.

Further experiments showed that fractions in which the phosphorus-releasing factor was eluted reacted as guanidino groups with the Sakaguchi reagent and that these fractions also contained chemical groups providing ammonium ions (Fig. 6). However, the color resulting from the Sakaguchi

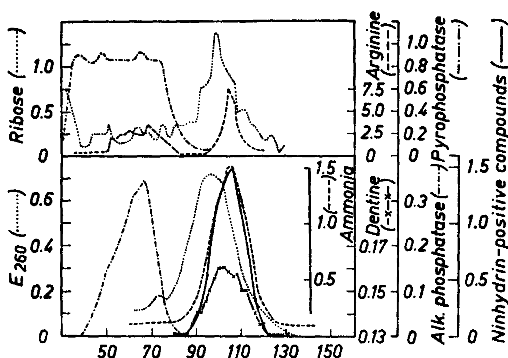

Fig. 6. Molecular exclusion chromatography of the phosphorus-liberating factor of *E. coli* alkaline phosphatase preparation on a Bio-Gel P-300 column showing the coincidence of the peaks representing liberation of phosphorus, ninhydrin-positive compounds, guanidino groups, and ammonia. Sample: 0.75 ml of the commercial enzyme preparation containing 15 mg protein. The amount of arginine and ribose is expressed in μg per 0.25 ml (=the volume of the aliquot taken from the fractions). Alkaline phosphatase was tested with *p*-nitrophenyl phosphate (E_{410}). For ammonia and ninhydrin-positive compounds the figures given refer to absorbance (E_{540} in both cases). The activity for pyrophosphatase is given as E_{700}. Other details as for Fig. 5.

reaction was more brown than reddish, an indication to the involvement of some other residues than those of arginine in the reaction.

It was also necessary to show that the peak representing the phosphorus-releasing factor does not display inorganic pyrophosphatase activity. The results shown in Fig. 6 demonstrate that inorganic pyrophosphatase activity was accompanied by the alkaline phosphatase itself. (The pyrophosphatase activity curve of Fig. 6 was purposely taken from an experiment carried out for a longer time to show the absolute absence of pyrophosphatase activity in fractions 90 – 120; hence the flattened curve.)

Experiments with gel columns showed that the fractions with phosphorus-releasing activity (90 – 120, Figs. 4, 5, and 6) became somewhat turbid during cold storage (appr. +2°C). However, the phosphorus-releasing activity was constantly encountered in the supernatant fraction (as well as the absorption at 260 mμ) when the turbid fractions were centrifuged.

The molecular weight of the factor was estimated by gel permeation chromatography on Sephadex G-10 to be very small (between the molecular weight of water and 100; the actual molecular weight was difficult to estimate because it was not known to what extent the possible hydration layer on the surface of the molecule increases its size). When the active fractions forming the peak were pooled, it was found that this preparation yielded, with

enamel powder, results essentially similar in all respects to the unfractionated enzymes. So, for example, the rate of the release of phosphorus by this peak was linear with "enzyme" concentration (Fig. 1).

When it became evident that at least one of the compounds involved in the liberation of phosphorus is ammonium sulphate, separate experiments were carried out with pure ammonium sulphate to prove this. Molecular exclusion chromatography on Sephadex G-10, G-15, and G-25 revealed that the phosphorus-liberating activity of *E. coli* alkaline phosphatase preparations and that caused by ammonium sulphate coincided in all experiments. Consequently, the activity also coincided with ninhydrin-positive peaks and with those representing Sakaguchi-positive compounds. It was also found that the preparations liberating phosphorus and which derived from all chromatographic procedures did not contain any noticeable amounts of any amino acid. Instead, they contained up to 20 – 30 % SO_4^{2-} ions.

2. *The phosphorus-liberating factor of* Bacillus subtilis. The results obtained with subtilopeptidase A preparations of *B. subtilis* are now compactly summarized. The release of phosphorus by the protease preparations was far slower than that caused by preparations of *E. coli*. Molecular exclusion chromatography on Sephadex G-100 gel revealed two main protein peaks which roughly coincided with two phosphorus-liberating peaks in the chromatogram. Not all lots of the commercial subtilopeptidase A preparations were capable of releasing phosphorus from dental enamel. Thus the bacterial enzyme preparations of *B. subtilis* may or may not contain the factor involved, evidently depending on the procedure of the partial purification of the protease. In Fig. 7 results from a typical experiment with the protease are shown. The

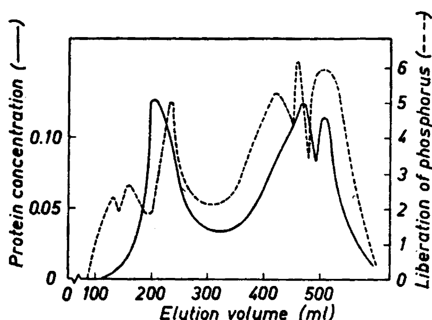

Fig. 7. Molecular exclusion chromatography of commercial subtilopeptidase A preparation on a Sephadex G-200 column (85.0 × 2.8 cm). Sample: 80 mg of preparation dissolved in 3 ml of 0.02 M tris-HCl buffer, pH 7.0. Elution buffer: 0.01 M tris-HCl, pH 7.0. Flow rate: 3.0 ml per h. Other details as for Fig. 4.

"total phosphorus" assays showed that the fractions did not contain any measurable phosphorus. (Separate experiments revealed that 10 mg amounts of the protease preparation contained practically no phosphorus.) The following three pools were prepared from the fractions: pool I (fractions from 90 to 190 ml), pool II (fractions from 190 to 300 ml) and pool III (fractions from 300 to 600 ml).

When the ability of the three pools to hydrolyze several synthetic peptidase and esterase substrates was studied, it was found that *N*-L-leucyl-2-naphthylamine was hydrolyzed at a considerable rate only by pool II, *N*-L-phenylalanyl-

2-naphthylamine by pools II and III, and α-naphthyl phosphate by all three pools. *p*-Nitrophenyl phosphate and all of the sugar phosphates used in earlier studies [19] were hydrolyzed by all three enzyme pools, but, because the phosphorus-liberating activity of these pools was seen to be very low, a more detailed presentation of the results will not be made. We conclude that the phosphorus-liberating effect of the protease preparations evidently has nothing to do with the peptidase and esterase activity, but is of more specific nature. It may be that different compounds are involved in the liberation of phosphorus by subtilopeptidase preparations than that demonstrated in alkaline phosphatase.

DISCUSSION

It was initially assumed in our studies that, were specificity of the enzymes known and were the enzymes seen to act on the tissues involved, important observations could be made on the subject. Our studies have shown that alkaline phosphatase itself does not catalyze the liberation of phosphorus from human dental enamel. Instead, the alkaline phosphatase preparations contain some other factor(s) capable of releasing phosphorus from enamel and dentine powder. We have earlier stated that the reaction accomplished by subtilopeptidase A preparations is most likely of enzymic nature and requires the action of somewhat specific components.[1] The involvement of a real enzyme can be suspected. More thorough studies are now being carried out with preparations of subtilopeptidase A.

On the basis of the numerous chemical analyses it can be suggested that most of the total ability of the commercial preparations of *E. coli* alkaline phosphatase to liberate phosphorus from human dental enamel and dentine is derived from ammonium sulphate, present in the enzyme preparation. We have offered this kind of an explanation even earlier [3] and we suggest that also the Worthington enzyme preparation used by Kreitzman *et al.*[2] contains ammonium sulphate which may lead to the same effect. The effect of ammonium and sulphate ions may increase the solubility of enamel in a way described by LaMer.[20]

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