THIRD SESSION

S. Y. Ericsson, moderator
Enzymes and Dietary Factors in Caries

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No explanation of the mechanism of dental caries initiation can defy the laws of thermodynamics or the physicochemical principles that have been carefully presented during this workshop. Solubility considerations and diffusion through the surface integuments and external layers of enamel can be considered of prime importance in any consideration of mechanism. In any such endeavor, however, it is essential to clearly define the system and to carefully consider the validity of various assumptions and simplifications that are implicit in such analyses. For example, a chemist considering the energy required to carry out a commonplace reaction such as the phosphorylation of glucose would correctly certify that the likelihood of such a reaction occurring to any extent at 37°C was small. But the phosphorylation of glucose can and does occur at such temperatures; the problem is an inappropriate description of the system and not a negation of the thermodynamic principle. The presence of complex organic molecules, even in small amounts, apparently can cause the system to behave in extraordinary ways.

For the purposes of this discussion, it is necessary to shift our focus from the pure mineral phase of tooth enamel to that of the fraction of the enamel in association with organic material. The experimental justification for this will become apparent later in the discussion. The organic material, often referred to as the matrix of the mineralized tissue, is often neglected in considerations of dissolution occurring during dental caries, since it is present as a quantitatively minor aspect of the total tissue. This matrix, however, apparently has a great deal to do with the early mineralization of tissues; it determines the orientation and perhaps the form of the crystallization and possibly forms the ionic environment for initiation of the mineralization process itself.

Phosphoproteins

Ion binding to the macromolecules of matrix does not conform to early models in which the macromolecule is a hard sphere with charge distributed uniformly on the surface. It is reasonably clear that ion binding, because of the discrete and specific charge distribution at specific sites on a macromolecule, is quite specific and in most cases can be intimately related to the metabolic function of the macromolecule. Binding of calcium to protein molecules is not unique to the macromolecules of teeth and bones. Extensive binding of calcium occurs with the milk protein casein, the egg yolk protein phosphovitin, and several other proteins, including the matrix proteins of bone, dentin, and enamel. These proteins have much in common, particularly regions of high polarity created by groups of acidic amino acids and the presence of a significant percentage of phosphorylated amino acids.

Casein probably has been the most thoroughly studied of the phosphoproteins. Sequence studies on a tryptic alpha-casein phosphopeptide by Österberg1 indicated the presence of seven phosphate groups within a peptide chain of 15 amino acid residues. Leaver and Shuttleworth2 reported the presence of organic phosphorus in proteins from ox bone and human dentin. Glimcher, Friberg, and Levin,3 studied proteins of embryonic and bovine enamel and found that both contained relatively large amounts of organic phosphorus. Apparently only phosphovitin contains significantly more phosphorus than the enamel proteins. Like the other phosphoproteins, most of the organic phosphorus appear to be present as serine phosphate. Veis and Perry4 reported the isolation of a phosphopeptide from dentin; this phosphopeptide is high in serine phosphate and apparently is similar in amino acid com-
position to the other phosphoproteins discussed thus far.

Mineral binding to these phosphoproteins seems to be considerably greater than the presence of the acidic or phosphated amino acids would suggest. There appears to be a cooperative binding of cations, especially calcium, which is held extremely tenaciously. Removal of some of the phosphate however, greatly reduces the affinity for calcium and the mineral is lost (Yamauchi, Takemoto, and Tsugo5). This is essentially a demineralization process. The enzyme that is responsible for the specific cleavage of phosphate from phosphoproteins is phosphoprotein phosphatase. Enzymes with this specificity have been isolated from carious dentin, bacterial plaque, and saliva (Mäkinen6) and also have been reported to potentiate the solubility of tooth enamel in vitro (Paunio, Mäkinen, and Scheinin7 and Kreitman et al8).

As a result of the somewhat more complex idea of tooth enamel as a mixed system containing mineralized matrix phosphoprotein in addition to the hydroxyapatite phase, some important considerations have been uncovered. These considerations may aid in the interpretation of some of the well-known facets of dental caries experimentation and control.

**Distribution of Organic Material in Surface Enamel**

Although several laboratories have worked with the proteins isolated from mineralized tissues, the precise localization of much of this protein is still a mystery. Some observations of rodent teeth extracted with the organic solvent ethylenediamine are suggestive when considered in the context of similar teeth exposed to solutions containing phosphoprotein phosphatase. As far back as 1954, Williams and Irvine9 reported that ethylenediamine was an ideal organic extraction solvent because it apparently attacked the organic matrix of bone with a minimum of erosion and dislocation of gross inorganic particulates. Various studies from Losee’s laboratory10,11 using bone or dentin, demonstrated the optimum conditions for extraction of the organic material, with the subsequent production of “anorganic” bone and dentin.

This material has been used clinically in a variety of ways and with considerable variation in reported utility. Extraction of the molars of rats with ethylenediamine (McCaslin12) resulted in an essentially “anorganic” tooth which apparently contained small voids that were visible at low magnification, particularly after staining with ammonium purpureate (Fig 1). Damage to the surface of a tooth so treated suggested that the extracted tooth had a surface shell layer with a rather uniform void beneath the surface. A gouge, such as the one in Figure 2, would have been expected to produce a ragged deformation; however, apparently what was produced was a flat plane of subsurface material just beneath the surface. In conjunction with other data (which will be described subsequently) this was interpreted to represent an extraction of organic material present in pockets in the surface enamel and as a discrete layer immediately below the surface. This also would suggest that the concentration of organic material at certain sites in the surface enamel is much higher than is generally recognized. Calculations of percentage of organic material are generally made assuming a uniform distribution of organic material throughout the enamel; this assumption is known to be incorrect for many components of enamel, including nitrogen.

**Enzyme Treatment of Enamel**

When preparations containing phosphoprotein phosphatase activity are incubated with rat molars, the enamel undergoes change. There is some apparent subsurface destruction of the enamel, but the destruction, at least in the early stages, is of a focal nature. Figure 3 shows a tooth incubated

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**Figure 1.** Buccal enamel surface of rat molar after ethylenediamine treatment and staining with ammonium purpureate illustrates surface voids.
with the preparation for three hours; there are focal areas of subsurface destruction which are visualized by the staining pattern with ammonium purpurate. When specimens are incubated for longer periods (for example, nine hours) the destruction is still below the surface.

Brief ultrasonic treatment, however, removes the enamel that is apparently undermined by the incubation procedure. In Figure 4, which is a scanning electron micrograph of the height of contour of such a treated tooth, the external enamel surface, the lesion edge, and the subsurface enamel structure can be seen. The pattern of damage is suggestive of a focal attack at sites in the surface enamel; these sites apparently contain substrate for the enzyme. Several of the lesions, however, give the appearance of coalescence beneath the surface, perhaps extending across large segments of the surface and undermining wide areas of surface enamel. This is supported further by the rather uniform flat subsurface layer uncovered after enzyme treatment. If the appearance of these teeth after ethylenediamine treatment and the suggestion that there is an accumulation of organic material in such locations and patterns are recalled, it is possible to postulate that much of the substrate for the enzyme is distributed in patches in the surface enamel and in a layer beneath

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**TABLE**

<table>
<thead>
<tr>
<th>Description</th>
<th>Mean Caries Score ± Standard Error</th>
<th>% Control</th>
<th>No. of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIT No. 200 Diet</td>
<td>16.7 ± 0.873</td>
<td>. . .</td>
<td>26</td>
</tr>
<tr>
<td>MIT No. 200 + NaF Reinfected</td>
<td>13.3 ± 1.147</td>
<td>80%</td>
<td>26</td>
</tr>
<tr>
<td>MIT No. 200 + NaF Nonreinfected</td>
<td>7.3 ± 1.261</td>
<td>44%</td>
<td>26</td>
</tr>
</tbody>
</table>

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**Fig 2.**—Damaged enamel surface after ethylenediamine treatment suggests flat subsurface plane after removal of organic matter.
the first 7 to 8 micrometers (μm) of surface enamel. Such an observation could explain the early penetration beneath the surface, during caries initiation, of bacteria and bacterial products necessary for further destruction of the enamel.

Heat-inactivated control teeth show none of this destruction (Fig 5). Ethylenediamine-treated teeth are apparently not damaged further by the enzyme preparations. This suggests that the organic component of the enamel is necessary for the enzymatic destruction. Consideration of a role in the initial caries process for an enzyme such as phos-
phatase also could aid in understanding the mechanism of action of dietary phosphate in inhibiting dental caries.

The effectiveness of dietary phosphates in inhibiting dental caries in experimental animals and in humans has been reported emphatically and continuously for more than 40 years. Reviews devoted to this subject (Bibby and Averill,13 Nizel and Harris,14 and Gilmore15) attest to the reported effectiveness, particularly in animal model systems. Several aspects seem to be important with respect to the mechanism.

**Dietary Phosphates**

Nizel and Harris16 observed that the development of carious lesions in hamsters was affected by diets that contained foods produced in Texas as opposed to those produced in New England, and that this difference apparently was due to differences in the phosphate content of milk and corn from New England. This observation stimulated renewed interest in the caries-preventive activity of phosphate salts. Stralfors,17-20 also worked with hamsters and demonstrated a reduction in caries by use of trisodium phosphate and other salts, including calcium phosphates. McClure21,22 evaluated the cariostatic effect of disodium phosphates and sodium phytate in rats. All of the phosphate-supplemented groups experienced fewer lesions than their controls. Disodium phosphate (1.17%) and sodium phytate (1.41%) provided complete inhibition of lesions; di-ammonium phosphate gave about a 75% reduction.

Van Reen and Ostrum23 and Van Reen, Ostrum, and Barzinskas24 also studied rats and demonstrated the caries-inhibiting effect of 1.5% disodium phosphate and 1.5% dibasic calcium phosphate with either a purified diet or a diet of whole wheat flour. Of interest, in terms of mechanism, was the fact that no correlation could be determined between the capacity for buffering of the various salts used and their inhibition of the lesion. Harris, Nizel, and Walsh25 studied the cariostatic effect of five different phosphate anions on the teeth of rats. Orthophosphate, pyrophosphate, tripolyphosphate, hexametaphosphate, and trimeta(cyclic)phos-
phate all reduced the number of lesions compared with controls; however, the greatest reduction was obtained with trimetaphosphate. These investigators also reported an inverse correlation of cariostatic effects with buffering capacity.

From these and many other studies, it can be concluded that phosphates, particularly sodium trimetaphosphate, are particularly effective compounds for reducing dental caries in experimental animals, even in the presence of high sugar cariogenic diets. The effectiveness does not appear to be related to buffering capacity. Other suggestions in regard to mechanism have been made. König, Schait, and Mühlemann reported that after topical treatment with solutions containing phosphate, the enamel appeared to exhibit higher dissolution rates; they suggested that this might be due to the precipitation on the rat molar surfaces of a layer more soluble than the enamel itself. Therefore, a mechanism involving solubility reduction by phosphate is unlikely.

Two possible mechanisms of phosphate action have been considered recently. The specific inhibition (discussed earlier) of phosphatase enzymes by phosphates is well documented and is one possibility. Some observations by Pruitt, Jamieson, and Caldwell on the ability of phosphate compounds to elute proteins adsorbed onto enamel present another reasonable mechanism. The desorption effectiveness of phosphate solutions in order of decreasing potency was sodium trimetaphosphate, sodium dihydrogenphosphate, calcium phosphate, and water. Also of importance are the results of studies in which columns of enamel powder were pretreated with various phosphate salts. Only trimetaphosphate altered the adsorbing properties of the enamel. It was found that the adsorption of amylase to trimetaphosphate-treated enamel was drastically altered. After trimetaphosphate treatment, amylase was removed easily by water. This suggests that there is a preferential, strong binding of trimetaphosphate to the enamel surface. Because binding of proteins such as phosphatase, or perhaps cariogenic microorganisms, to the enamel surface is inhibited by trimetaphosphate, they are no longer available for enamel destruction.

**Dietary Fluoride**

Since the classic studies of Volker a great deal of experimental consideration has been given to the idea of a mechanism for fluoride action involving solubility reduction of tooth enamel. The ability of fluoride to enter the crystal lattice of hydroxyapatite to form fluorapatite, a crystal of lesser solubility, has been invoked as an explanation of the effectiveness of fluoride, particularly fluoride administered during developmental stages of tooth mineralization. Based on this idea we analyzed our data from experiments investigating enamel solubility, in enzyme preparations or buffer solutions, of teeth from fluoride-fed and fluoride-free animals.

In these experiments (Fig 6) no solubility reduction was demonstrated, although sufficient fluoride was fed to the rat dams during the developmental period to result in a 50% reduction of caries in the pups. Fluoride had been discontinued when teeth erupted or shortly thereafter. Therefore, since preeruptive fluoride reduced caries in this system, it must be by some kind of preeruptive mechanism, and it was expected that sufficient fluoride was reaching the pups in some way to make these teeth less soluble. Five separate replications of this experiment, however, indicated no such solubility reduction. In fact, in two of the studies the solubility of the fluoride in teeth actually was greater than that of the controls. In one of these studies the difference was great enough to reach statistical significance (Clark and Kreitzman).

This was a disturbing finding, but a search

![Fig 6.—Results of four in vitro studies measuring release of isotopically labeled phosphate into incubation media under conditions of mild acid buffer (A, B, C, D), or phosphatase (A1, B1, C1, D1); control teeth are compared with teeth from rats supplemented with NaF (50 ppm F) during period of lactation.](http://jdr.sagepub.com)
of the literature revealed that there are isolated reports (Brudevold and McCann, Volker, and Jenkins, Armstrong, and Speirs) that human teeth from individuals in fluoride-rich areas are not measurably different in solubility than teeth from regions low in fluoride. It became important to determine, therefore, the mechanism of action of preeruptive fluoride in these experimental animals. If it is not mediated through solubility reduction, what alternative explanation is there for the observed reduction in caries?

To be certain that the fluoride did reach the pups, fluoride was injected into the pups during the period of tooth formation; the expected reduction in solubility was not demonstrated. The microbiologic aspect of dental caries suggests the possibility that some alteration in the transmissible flora, some change in the bacterial composition (perhaps even of the mother), would result in fewer caries in the pups. To test this hypothesis, fluoride was fed to the experimental animals as before until the time of tooth eruption, then the fluoride was discontinued and at the same time the fluoride group was separated into a fluoride control, and a group that was to be reinfected with feces from animals that had received no fluoride. The reinfected animals were the control group for the duration of the experiment. This design is similar to that used by Keyes and Jordan in the classic studies of the transmissible flora. Simply by reinfesting the animals with feces from control animals, much of the caries that had been reduced by the fluoride was restored, as shown in the table.

Subsequent experiments (Howell, Clark, and Kreitzman) demonstrated that pure strains of Streptococcus mutans are able to increase or reestablish caries levels to a varying degree, depending on the relative caries pathogenicity of the microorganism strain. When microorganisms are cultured in such a way that production of phosphatase is increased (that is, culture in a low-phosphate medium), the amount of caries produced by those microorganisms when reinfected into the fluoride models is increased.

A recent pilot study indicates that feeding of fluoride before eruption of the teeth in experimental animals drastically reduces the number of S. mutans that can be isolated from the teeth in periods after eruption of the teeth. This does not seem to be the case for several other organisms in the rodent oral cavity, for example, Streptococcus faecalis. The primary mechanism of fluoride action in caries reduction, at least in this experimental model, is mediated through a modification of the bacterial flora and not through a solubility reduction by formation of fluorapatite or more perfect crystals or similar mechanism. A recent report by Loesche, Murray, and Mellberg suggests similar findings in human populations.

Conclusions

We have come a long way indeed, in our recognition of the way in which dietary factors can interact to modify and reduce the incidence and severity of dental caries. No longer is it sufficient to proclaim sugar as the sole enemy without consideration of the fact that other dietary factors can, in large measure, interrupt the decay process despite the presence of large amounts of sugar. Effective dietary factors such as fluoride and phosphate challenge us to understand caries mechanisms in a broader way than the restrictive consideration of hydroxyapatite crystals alone. We must direct our consideration to the oral microflora and the other components of tooth structure that differentiate teeth from geologic samples of hydroxyapatite. We have at our command at least two powerful dietary agents that, at least in experimental systems, show great promise for control of caries. These also can provide greater insight into the mechanisms involved in caries. We must view the entire ecological system existing in the oral cavity and develop our models in conformance with this system.

References

5. Yamauchi, K.; Takeda, M.; and Tsugo, T.

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