# Fluorescence in dissolved fractions of human enamel

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Fluorescence induced by laser light is useful in early detection of enamel caries. The present work studied the fluorescence emission pattern in dissolved human enamel and in different molecular weight fractions obtained after gel chromatography or dialysis followed by ultrafiltration. For comparison, solutions of synthetic hydroxyapatite and bovine enamel were used. When the entire emission and excitation spectra of the corresponding excitation and emission wavelengths in the solutions of human enamel and bovine enamel were compared, no distinct differences were found between the solutions. With excitation at 375 nm, emission peaks were found at 460 and 560 nm, indicating the presence in human enamel solution of two different chromophores, unevenly distributed over the molecular weight fractions. The 460-nm and the lower 560-nm fluorescence peaks seem to be derived from both organic and inorganic components. The inorganic substances contributing to both the 460-nm and 560-nm peaks were incorporated in complexes. The 560-nm emission peak elucidates part of the basis of the laser fluorescence method. □ *Chromophores; inorganic/organic components; laser light*

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As early as 1928, Benedict reported that fluorescence from dental enamel and dentin can be obtained by excitation with light in the visible and in the ultraviolet (UV) range (1). Fluorescence in dental hard tissue has since been studied by several researchers. It is still not known, however, to what extent this fluorescence occurs in the organic or inorganic components of the teeth.

Armstrong (2) suggested that the fluorescence in human dentin may originate from inorganic complexes with some organic components. Foreman (3) found two fluorophores in human dentin, one resembling the amino acid tryptophan and the other of unknown identity.

Spitzer & ten Bosch (4) concluded that the organic component is responsible for most of the fluorescence of the enamel, furthermore, it has been demonstrated that synthetic hydroxyapatite and enamel emit fluorescence in the visible range when excited by UV laser light (5). In the work of Booij & ten Bosch (6) a fluorescing component of bovine enamel was identical with the dipeptide dityrosine. Studies of the microstructure of mineralized dental tissues in UV light have shown that in most cases an inverse relationship exists between the intensity of fluorescence and the degree of mineralization (7).

Fluorescence induced by laser light was used by Bjelkhagen et al. (8, 9), Sundström et al. (10, 11), and Hafström-Björkman et al. (12) in the early detection of enamel caries. This method is sensitive in the detection of incipient human enamel lesions and has therefore aroused further interest with regard to the identity of the components of the enamel involved in the fluorescence. For that reason it was decided to study the fluorescence of dissolved human enamel and different molecular weight fractions of dissolved human enamel. For comparison, solutions of synthetic hydroxyapatite and bovine enamel were used.

## Materials and methods

Intact premolar teeth from teenagers living in a non-fluoridated area in Stockholm were

extracted for orthodontic reasons before the patients had reached the age of 16 years. It was not known whether the patients had participated in a fluoride-rinsing program. The teeth were kept in a small box with a wet cotton roll. The enamel of teeth from different patients was ground to powder, which was dissolved in a 0.5 M (ethylenediaminetetraacetic acid (EDTA), 4 M guanidine–HCl mixture, pH 8, in accordance with the method described by Booij & ten Bosch (6). Surplus of undissolved enamel remained, but no noticeable precipitate was seen. The following centrifugation removed the undissolved enamel and the solution was used immediately.

In an attempt to separate the small inorganic molecules, dissolved human enamel was gel-filtered on a 1.5 × 53 cm Sephadex G-10 (Pharmacia) column eluted with 0.05 M $NH_4HCO_3$, pH 8.3. The molecular weight range of the column was 0–700. The absorption at 280 nm was monitored. The cell had an optical pathlength of 10 mm, and the Uvicord (Pharmacia, UV-1 single-path monitor) was set at 0.5 for all measurements. The size of the fractions (Pharmacia, Frac 100 Fraction collector) was 2 ml. The void volume was measured with Blue dextran 2000.

The larger molecules in fractions 10–20 of another gel-filtered human enamel solution were further concentrated by ultrafiltration with a 3500-MW millipore filter. For comparison, another human enamel solution was separately dialyzed twice against 0.005 M Tris buffer for 8 h at room temperature with 3500 MW as the cut-off size of the membrane and then ultrafiltered with a 3500-MW filter. To fractionate a human enamel solution further, dialysis and subsequent ultrafiltration through different millipore filters were performed. Fractions with MW of >3500 (the *total* first fraction), 3500–10,000, 10,000–30,000, and >30,000 were obtained. Each fraction was diluted with the dialyzed solvent to give 2 ml of test liquid. The various ultrafiltration fractions (>3500, 3500–10,000, 10,000–30,000, and >30,000 MW) were submitted to electrophoresis.

Several solutions were prepared to serve as controls to these enamel solutions.

Fine-grain inorganic synthetic hydroxyapatite powder (Biochemical BDH) was dissolved in the same solvent. Furthermore, six different solutions of $CaCl_2$ in the solvent EDTA guanidine-HCl with Ca concentrations of 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 M were made up. Finally, the solvent itself and one of its components, 0.5 M EDTA, were also subjected to the fractionation procedures.

All these solutions were scanned separately from 390 to 600 nm, using excitation at 375 nm in a fluorimeter (Perkin-Elmer LS-2). The cell had an optical pathlength of 1.5 mm. As zero reference for non-dialyzed solutions the solvent EDTA guanidine-HCl was used. The dialyzed solvent was used as the zero reference for all the dialyzed and ultrafiltered solutions.

Bovine enamel from molars of 2-year-old cattle was dissolved by means of the same procedure as described for human enamel. To half of the dissolved bovine enamel sodium fluoride was added corresponding to 0.14% F in the solution, which corresponds to the F concentration in human enamel. The two solutions and a human enamel solution were dialyzed independently as previously, followed by separate ultrafiltrations to obtain fractions with MW of 3500–10,000, 10,000–30,000, and >30,000. Fluorimetry of these solutions was done with a spectrofluorophotometer (Shimadzu RF-540). The cell had an optical pathlength of 4.4 mm. The emission spectra were scanned at different excitation wavelengths: 293, 315, 375, 410, 455, and 488 nm. The excitation spectra were scanned at different emission wavelengths: 340, 400, 455, 520, and 560 nm. Controls were as described.

Ca concentration (mg/l) was measured with the addition of 0.1% lanthanum chloride, using an atomic absorption spectrophotometer (Pye Unicam SP9). The detection limit for Ca was 0.08 mg/l.

## Results

Fluorescence emission spectra of total human enamel and synthetic hydroxyapatite solutions are shown in Fig. 1. The void vol-

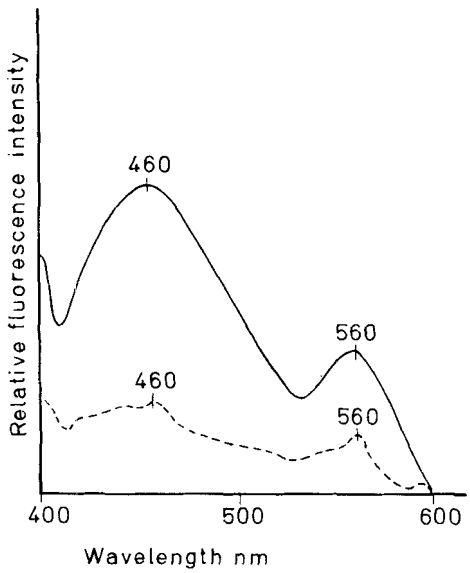


Fig. 1. Fluorescence spectra of dissolved human enamel (——) and synthetic hydroxyapatite (– – –) with excitation at 375 nm.

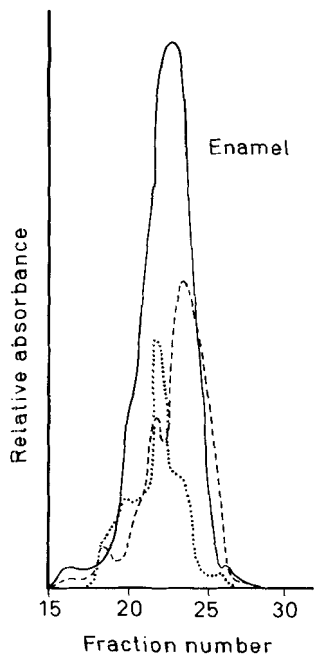


Fig. 2. Gel filtration of dissolved human enamel (——), its solvent, EDTA guanidine-HCl (– – –), and EDTA (. . .) on a 1.5 × 53-cm Sephadex G-10 column eluted with 0.05 M $NH_4HCO_3$, pH 8.3. Absorption at 280 nm. The Uvicord set to 0.5. Size of fractions, 2 ml.

ume of the column used for all gel filtration was measured to be about 34 ml, corresponding to fractions number 1–17. The gel-filtration diagrams of the human enamel and solvent solutions are shown in Fig. 2. Subsequent fluorimetry of the different gel-filtered fractions of the enamel solution showed no fluorescence.

When human enamel solution was dialyzed, the Ca concentration inside the membrane was 11.3 mg/l Ca, as compared with 3.5 mg/l in the second solution outside. So most of the Ca molecules did remain inside the membrane, probably in the form of the common large molecular Ca–EDTA complex. The dialyzed control solvent had a negligible amount of Ca.

The emission from different molar Ca concentrations in the solvent EDTA guanidine–HCl showed increased relative fluorescence intensities of 2–12 for the resulting higher 450-nm peak and 1–6 for the lower 560-nm peak corresponding to the increase of Ca molarity. No emission peaks were found in the gel-filtered and subsequently ultrafiltered larger molecular 10–20 fractions of the human enamel solution. The dialyzed and ultrafiltered human enamel solution, however, had an emission peak of high intensity at 450–470 nm and another emission peak of low intensity at 560 nm.

The emission spectra of the different fractions of the human enamel solution obtained by dialysis and ultrafiltration are shown in Fig. 3. These spectra were corrected to compensate for the dilutions made before the fluorimeter scannings. The ensuing electrophoresis of the various ultrafiltered fractions with MW of >3500, 3500–10,000, 10,000–30,000, and >30,000 showed traces of proteins in the >30,000 MW fraction and traces of protein in the 10,000–30,000 MW fraction.

When the entire emission and excitation spectra of the corresponding excitation and emission wavelengths in the solutions of human enamel, bovine enamel, and bovine enamel with added fluoride were compared, no distinct differences were found in intensities between the three solutions. When the different concentrated ultrafiltration fractions with MW of 3500–10,000, 10,000–

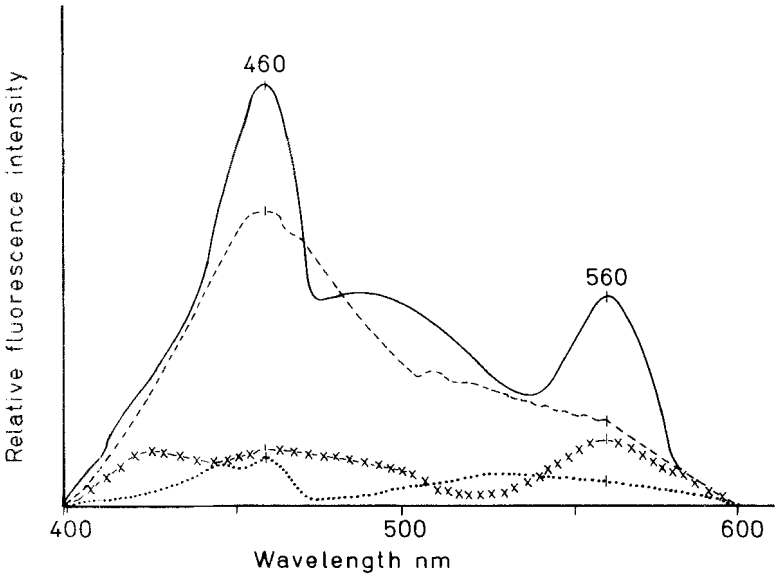


Fig. 3. Fluorescence spectra from dialyzed dissolved human enamel in different ultrafiltration fractions with excitation at 375 nm. The *total* first fraction >3500 MW (——), 3500–10,000 MW (. . .), 10,000–30,000 MW (– – –), and >30,000 MW (×××). The graphs are recalculated on the basis of observed filtration volume to have the same units on the intensity axis.

30,000, and >30,000 and their respective emission spectra for all the various excitation wavelengths were compared, the MW interval of 3500–10,000 gave the peaks with high fluorescence intensities. After calculation we found no Raman lines of water corresponding to the Raman lines of the different solvents used as zero references, and no interference with the measured peaks in the different emission or excitation spectra occurred.

## Discussion

Synthetic hydroxyapatite solution gave fluorescence peaks at 460 and 560 nm when excited at 375 nm. These inorganic chromophores were also most probably part of the origin of the corresponding 460- and 560-nm emission peaks in the human enamel solution. We suggest tentatively that they are Ca-EDTA complexes of considerable size, as was shown in the test in which the larger part of the Ca molecules remained inside the dialysis membrane. The remaining part of the two emission peaks would consequently consist of the fluorescence from organic molecules.

The research with laser fluorescence is focused on *enamel* fluorescence (8–12). It was therefore decided to try to separate the small inorganic from the large organic molecules in the dissolved human enamel. This was attempted even though it is difficult to purify the organic substance because of its low concentration in enamel. Gel filtration is the standard separation technique of molecules. This method was also used on bovine enamel by Fincham et al. (13), studying enamel proteins, and by Booij & ten Bosch (6) when analyzing a fluorescent compound. The gel filtration of the human enamel solution, the solvent sample, and EDTA gave nearly identical absorption diagrams. The peaks in these three diagrams corresponded to the molecular weight of EDTA, 372. When the fluorescence for each fraction of human enamel solution was scanned, the corresponding fraction from the separately gel-filtered solvent was used as the zero reference to eliminate the possible disturbance from the EDTA guanidine–HCl solvent. Nevertheless, neither of these fractions showed any fluorescence. Since, in contrast, the dialyzed and ultrafiltered molecules from the human enamel solution had emission peaks at 460 and 540 nm, the larger mol-

ecules must have been absorbed in the chromatographic bed in the stationary phase during gel filtration.

Consequently, as dialysis and ultrafiltration of the human enamel solution gave fruitful results, these methods were used to fractionate further the molecules in accordance with their molecular size. The relative fluorescence intensities at wavelengths of 460 and 560 nm of the *total* fraction of >3500 MW (Fig. 3) were found to be in good agreement with the sum of the relative fluorescence intensities at the corresponding wavelengths in the different fractions with MW of 3500–10,000, 10,000–30,000, and >30,000.

Only the excitation at 375 nm was used for the fluorescence spectra of the human enamel solution. In the summary of fluorescence peak positions by ten Bosch & Zijp (14) different excitation wavelengths in dentin were used. Therefore, in the comparison of human and bovine enamel solutions various excitation wavelengths were included, from excitation at 293 nm, commonly used for protein, to excitation at 488 nm, used in the laser fluorescence method (8–12). As expected, the highest fluorescence intensity was registered with excitation at 293 nm, deriving from the organic substances in the three different solutions. However, no distinct differences were found between the solutions.

In conclusion, with excitation at 375 nm, emission peaks were found at 460 and 560 nm, indicating the presence in human enamel solution of two different chromophores unevenly distributed over the molecular weight fractions. Only a part of the 460-nm and the lower 560-nm fluorescence peaks seem to be derived from organic substances, since the peaks recorded from the solutions contained a substantial contribution resembling dissolved synthetic hydroxyapatite. The inorganic substances contributing to both the 460-nm and 560-nm peaks are probably incorporated in complexes. The 560-nm emission peak elucidates part of the basis of the laser fluorescence method. This method can be used for initial caries diagnosis with excitation at 488 nm, a barrier filter at 540 nm, and the resulting tooth fluorescence visible at longer wavelengths than 540 nm, where enamel caries causes a loss in fluorescence (8–12). This was also reported by Naleway et al. (5) and Alfano & Yao (15). Thus, in a carious lesion there is less fluorescence above 540 nm. Loss of inorganic material will contribute to this decrease, apparently together with other factors (16).

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