Micro-determination of phosphate in enamel biopsy samples using the malachite green method

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A simple and sensitive malachite green method was adapted for the determination of phosphate in enamel demineralizing solutions. Comparison with other methods indicates that malachite green was about 60 times more sensitive than Fiske and Subbarow's method and 10 times more than that of Chen et al. The high sensitivity of this method allows very small biopsy samples to be used. The linearity is good between the tested range of 0.025–3.0 mM P. The relative standard deviation was low and amounted to 1.4% in duplicate determinations. No statistical differences in phosphate concentration of enamel biopsy samples determined by the three methods were found. Fluoride concentration up to 80 μg/ml did not interfere with phosphate determined by the malachite green. For sampling enamel, the acid-etch technique was used, and the fluoride concentration at successive demineralization runs was monitored using fluoride-ion electrode. □ *Enamel micro-samples; fluoride; phosphate analysis*

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Determination of the phosphate concentration in enamel biopsy is of fundamental interest in caries research. Although phosphate has been determined in biological materials by several methods, two of them, namely those of Fiske–Subbarow (1) and Chen et al. (2) are widely used in enamel biopsy samples obtained either by abrasion (3, 4) or acid-etch (5–7). Enamel biopsy is a valuable method for studying the enamel mineral distribution and the fluoride uptake from topical agents. However, in some enamel biopsy procedures only micro-amounts of enamel are available for analysis, as in the cases of in vivo determination of enamel solubility, sampling of the outer 1–2 μm of enamel delimited area, and surface etching of enamel in rats. A sensitive analytical procedure for determination of phosphate in enamel biopsy samples is thus required. Many workers have reported that the Fiske–Subbarow method (1) is less sensitive than others (2, 8–10). Although the method of Chen et al. (2) is quite sensitive, it is a lengthy procedure. Itaya & Ui (8) proposed a method for the determination of phosphate based on the formation of phosphomolybdate–malachite green complex. This was reported to be 30 times more sensitive than Fiske–Subbarow's method. Several modifications of the malachite green method have been introduced. Kallner (11) adapted the malachite green method for determination of phosphate in serum and urine by a one-step procedure.

The aim of the present study was a) to adapt the malachite green method for determination of enamel phosphate dissolved in acid solutions; b) to compare the malachite green method with two of the widely used methods for determination of phosphate in enamel biopsy samples; and c) to examine the feasibility of the method for assessing fluoride distribution in the enamel.

Materials and methods

Preparation of teeth for demineralization using the window technique

Nineteen sound, extracted human premolars were stored in deionized water containing thymol at 4°C. At the time of use the teeth were cleaned with a rotating rubber

cup and deionized water and blotted dry with tissue paper. A window of 12.6 mm^2 was made on the buccal surface, using Scotch tape (3M Scotch pressure-sensitive tape). The teeth were then covered with nail varnish, leaving behind a well-defined area. The enamel was then exposed for successive etching, using a cotton pellet saturated with 0.1 ml of 0.5 N $HClO_4$. In group A the enamel was etched for periods of 15, 15, 15, 30, and 30 sec, respectively. In group B the acid was allowed to be in contact with the enamel for periods of 5, 5, 5, and 15 sec, respectively. Immediately after etching the solution was buffered by directly pipetting onto the test area 0.4 ml of citrate buffer (0.5 M, pH 5.2), followed by 0.5 ml deionized water. Residual solution left on the tooth surface was aspirated with micro-sampling pipettes and small F-free pieces of filter paper and then transferred to the original sample. The thickness of enamel layers removed was calculated, using the amounts of calcium and phosphate dissolved in the demineralizing solutions, details of which have previously been described (12).

Analysis

Phosphate analysis. Aliquots were analyzed for phosphate by the malachite green method. For comparison, parallel determinations were carried out using the Fiske–Subarrow (1) and Chen et al. (2) methods. Malachite green reagent, prepared in accordance with Kallner (11) was used. The method of Chen et al. (2) was performed as described in the original paper with the exception that no deproteinizing agent (trichloroacetic acid) was added to the reagent. The Fiske–Subbarow procedure was carried out as described in Sigma Technical Bulletin No. 670 (1978) except that trichloroacetic acid was again omitted. Analytical-grade reagents and bidistilled water were used in preparing the reagents and standards. Dried KH_2PO_4 was used to prepare phosphate stock solution. Serial dilutions were made to obtain 0.025–3.0 mM P standards.

The working reagent was prepared immediately before use by mixing four parts of the malachite green reagent with one part of 5 M urea solution. 2.5 ml of this working reagent was added to 0.02 ml of etchant solution, containing the dissolved enamel, in a disposable polystyrene cell. After 60 min the absorbance was read against water reagent blank at 630 nm, using a double-beam spectrophotometer (Beckman Model 24). All phosphate values were obtained from duplicate determinations unless otherwise stated.

Fluoride analysis. The fluoride concentration was measured with a fluoride-ion selective electrode (Orion model 96-09 and Orion model 801 digital pH/mV meter). A calibration curve was constructed from standards containing 0.05, 0.1, 0.5, and 1.0 ppm F, prepared in citrate buffer in the same manner as the samples.

Calcium analysis. The calcium concentration was determined with an atomic absorption spectrophotometer (Pye Unicam SP 190). Before the measurement, aliquots were diluted with 5% lanthanum chloride to eliminate phosphate interference.

Statistical analysis. To determine the error of the method, duplicate measurements were made on the demineralizing solutions. The standard deviation was calculated from the differences between duplicate readings (d) according to the following formula:

$$SD = \sqrt{\frac{\Sigma d^2}{2N}}.$$

A series of 33 samples were analyzed for phosphate, using the malachite green, Chen et al. (2), and Fiske–Subbarow (1) methods. Statistical differences between these methods were evaluated with the unpaired *t* test.

Results

Phosphate results

Stability of the color reaction. Fig. 1 shows that the color reaction was stable after 60 min for the malachite green reagent. Storage up to 24 h at 4°C had no appreciable effect on the absorbance results of the malachite green and slightly increased them in the Chen et

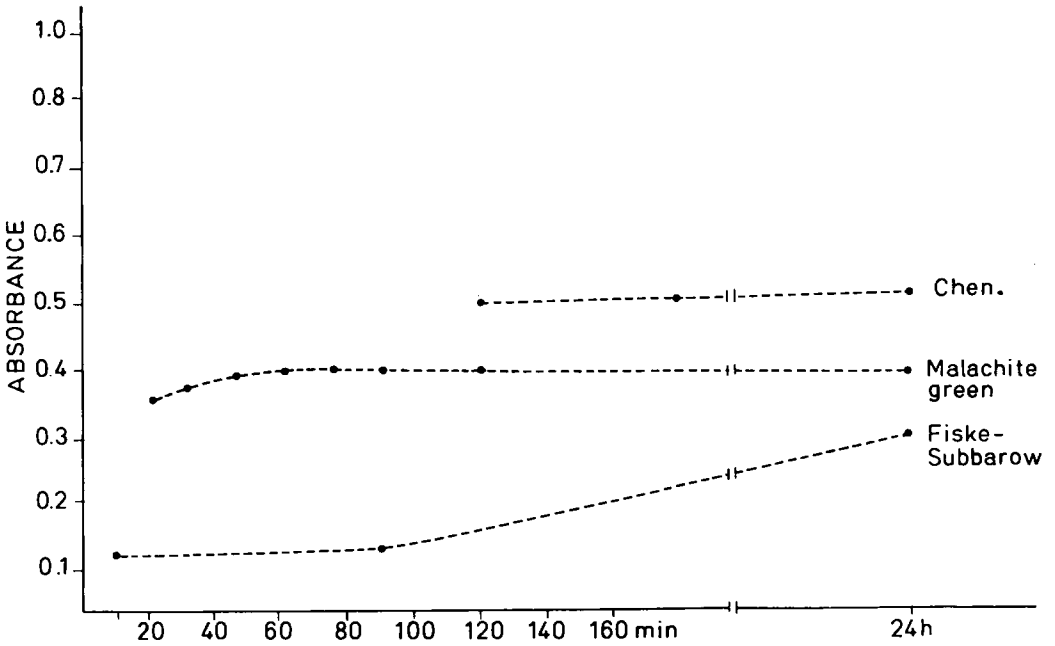

Fig. 1. Development and stability of color reaction using the malachite green, Chen et al. (2), and Fiske & Subbarow (1) methods.

al. (2) method, whereas in the Fiske–Subbarow method (1) the color intensity increased considerably.

Linearity and sensitivity. For this test a calibration curve for each method was constructed (Fig. 2). Each point represents the average of four determinations. A change in the absorbance of 0.1 is achieved by the introduction of 0.08 µg phosphate in standard solution as assayed by the malachite green, compared with 0.90 and 5 µg phosphate by the Chen et al. (2) and Fiske–Subbarow (1) methods, respectively. Thus the sensitivity of malachite green is about 60 times higher than the method of Fiske–Subbarow (1) and 10 times that of Chen et al. (2). For malachite green, the coefficient of correlation was better than 0.999, calculated from 93 duplicate values used to construct a standard curve with the concentration between 0.025 and 3.0 mM P (0.20–23.8 µM P in the final mixture) (Fig. 3).

Recovery of phosphate added to the etching solutions containing the sampled enamel ranged from 95% to 103% (Table 1).

Interfering substances

To study the validity of the malachite green procedure, diverse substances were added to different phosphate concentrations ranging between 0.25 and 1.0 mM. The following concentrations of added substances did not cause any disturbing change in color development: citrate buffer 2 M, acetate buffer 2 M, sodium chloride 0.15 M, zinc sulphate 1.54 mM, aluminum hydroxide 3.70 mM, and sodium fluoride at levels of 5.26 up to 526 mM. In all these tests 2.5 ml of malachite green reagent was added to 0.02 ml of the tested solutions.

Comparison with other methods

The statistical analysis showed that the relative standard deviation between dupli-

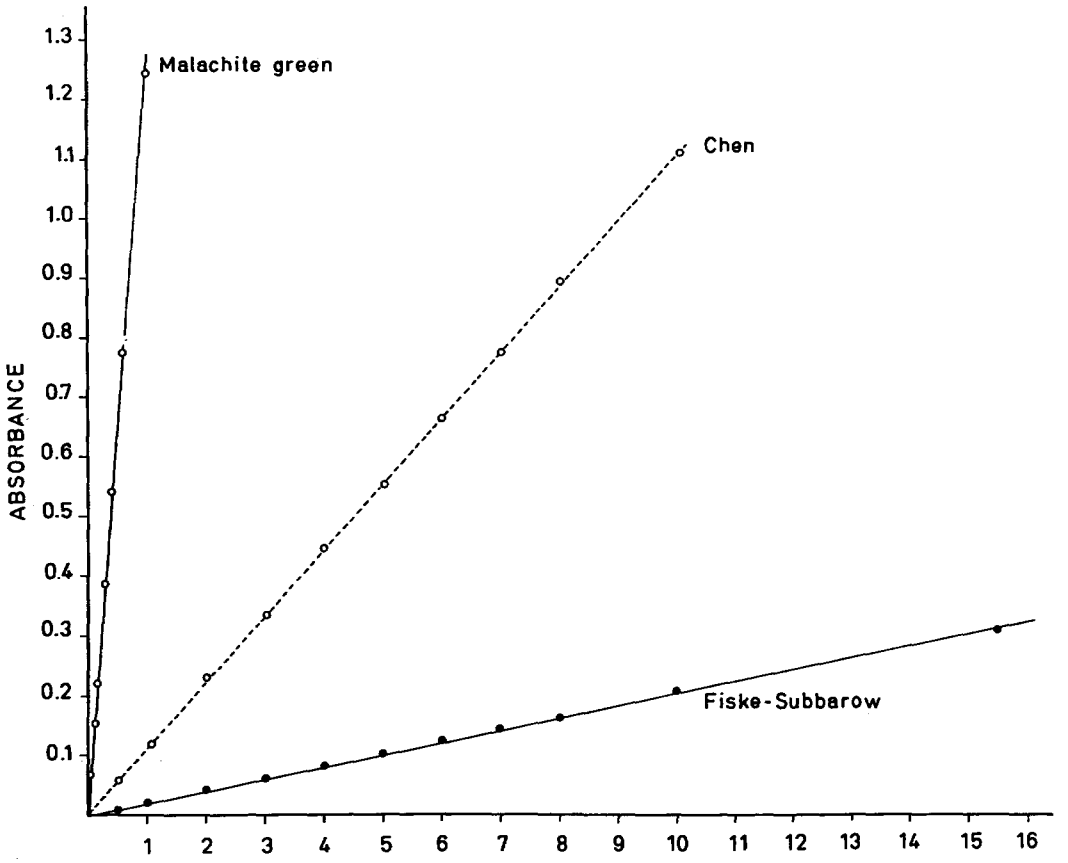

Fig. 2. Analytical sensitivity for phosphorus determination (using the three methods).

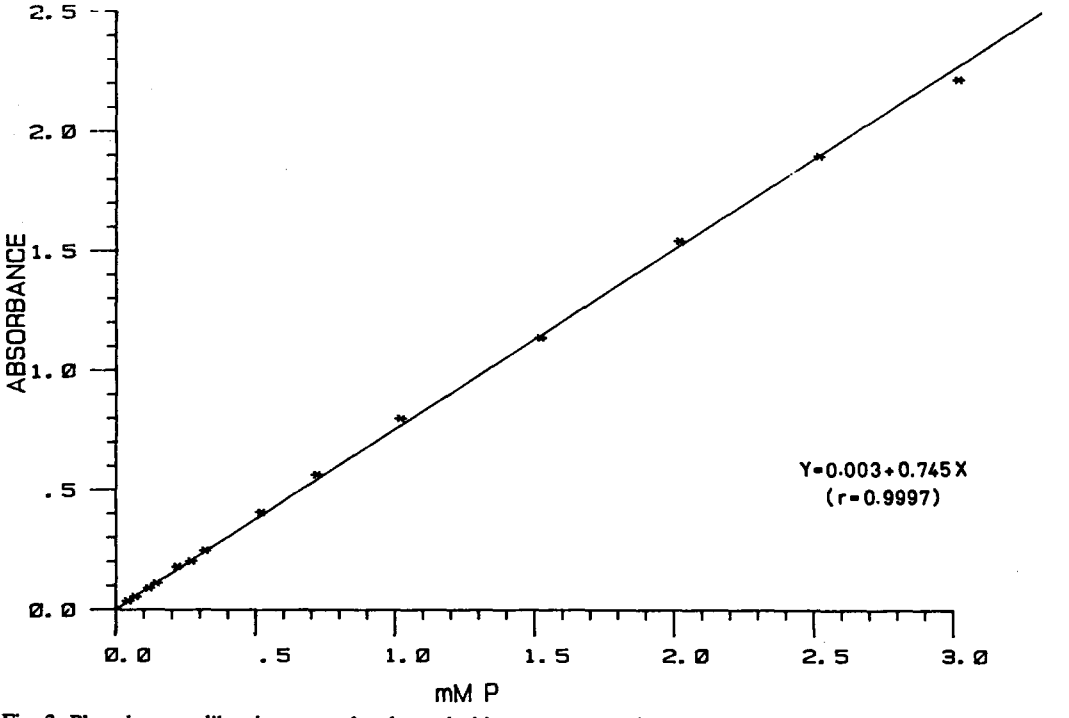

Fig. 3. Phosphorus calibration curve for the malachite green method.

Table 1. Recovery of phosphate added to the enamel dissolved in 0.5 N $HClO_4$

P added, µg/ml	P expected, µg/ml	P found, $\bar{x} \pm SD$ (n = 7)	Recovery, %
0	—	2.97 ± 0.733	—
3.88	6.85	6.53 ± 0.480	95
7.75	10.72	10.40 ± 0.418	97
15.50	18.47	18.24 ± 0.323	99
31.00	33.97	34.91 ± 0.302	103
77.50	80.47	80.61 ± 1.746	100

cates determined by the malachite green and Chen et al. methods was 1.4% (no. = 129) and 1.2% (no. = 33), respectively. The data obtained from the unpaired *t* test indicate no significant difference between the mean phosphate values assayed by the present method, Chen et al. (2), and Fiske–Subbarow (1).

Dissolution of mineral from enamel surface

Table 2 shows the average weight of enamel, depth of the etched enamel, and the fluoride concentration at successive enamel layers dissolved in 0.5 N $HClO_4$. The weight of enamel was calculated from calcium and phosphate data. The average rate of enamel removed in group A and B was 0.22 ± 0.03 and 0.38 ± 0.13 µm/sec, respectively (mean ± SD). The relative standard deviations of the depth and fluoride concentration in groups A and B calculated from cumulative enamel layers were as follows: for depth 19% and 21%, respectively, and for fluoride concentration 28% and 30%, respectively. A tendency to more consistent results was found in group A. The mean Ca/P weight ratio was 2.1 ± 0.08 (mean ± SD).

Discussion

The evaluation of F topical agents is frequently based on their reduction of enamel solubility and on enamel fluoride uptake and penetration in the surface enamel (for reviews, see Refs. 13, 14). From fluoride and phosphate and/or calcium, abraded or dissolved, the fluoride concentration at successive enamel depths can be calculated. Although phosphate dissolution may not give a true picture of apatite solubility in all circumstances, it appears to be a good indicator under acid conditions (15). Determination of both calcium and phosphate dis-

Table 2. Summary of data obtained from enamel surfaces dissolved in 0.5 N $HClO_4$

Layer no.	Acid exposure (sec)	No. of biopsies	Weight of enamel (µg), $\bar{x} \pm SD$	Layer thickness (µm), $\bar{x} \pm SD$	Fluoride concentration (ppm), $\bar{x} \pm SD$
Group A					
I	15	10	114 ± 29	3.1 ± 0.77	2153 ± 393
II	15	10	139 ± 28	3.7 ± 0.65	1604 ± 429
III	15	10	140 ± 25	3.8 ± 0.68	1383 ± 417
IV	30	10	210 ± 36	5.6 ± 0.97	1009 ± 410
V	30	10	218 ± 40	5.9 ± 1.07	783 ± 275
Group B					
I	5	9	69 ± 19	1.9 ± 0.51	2305 ± 562
II	5	9	86 ± 15	2.3 ± 0.39	1655 ± 543
III	5	9	88 ± 23	2.4 ± 0.49	1314 ± 430
IV	15	9	112 ± 23	3.0 ± 0.61	1084 ± 296

solution is indicated because, first, an artificially high phosphate value could be recorded if the enamel treated with fluoride agents that contain phosphate is not thoroughly washed before the etching procedure. This would indicate a greater enamel solubility than was in fact the case. Second, the formation of either calcium- or phosphate-rich coatings on enamel treated with fluoride agents may influence the calculated enamel depth (16).

It has been found that the malachite green method is 30 times more sensitive than the Fiske–Subbarow (8). The present procedure showed a 60 times higher sensitivity than Fiske–Subbarow—that is, twofold higher than that previously reported (8). This could be explained on the basis that the final volume of the mixture used in the present method was about half that of Itaya & Ui (8). Although certain studies indicate that fluoride influences the phosphate determination (10, 17, 18), others showed little or no influence (19–21). The present method indicates that fluoride had no influence on phosphate values up to 4.21 mM in the final mixture (80 μg F/ml).

As expected in this study, the F concentrations decreased with increasing thickness of the enamel. This is in accordance with the findings of others (14, 22, 23) (Table 2). Assuming that 0.03 μg F can be analyzed in 1 ml of demineralizing solution by direct measurement with an F-ion electrode and that 1 to 2 μm is removed from an enamel surface area of 12.6 mm^2, the enamel F concentration levels of about 800–400 ppm can thus be determined. The corresponding enamel weights removed at these depths are 37.2 and 74.4 μg, respectively. This indicates that the F and phosphate obtained from the present procedures are well above the limit required for analysis. Owing to the F gradient, a thinner enamel layer showed a higher F-level than the thicker layer (Table 2).

Table 2 also shows that the thickness of the enamel layer can be controlled by adjusting the length of time for acid–enamel contact. In group B, etching of the first 1–2 μm was attained by exposing the enamel to 0.5 N $HClO_4$ for 5 sec. The weight of biopsied enamel removed from the first etching run was in good agreement, after compensating for the sampling area, with the in vivo grinding method reported by Brudevold et al. (4). Nevertheless, a higher accuracy was obtained in present acid-etching than the grinding method (CV = 19% versus 30%). From the thickness of the enamel layer removed in group B (Table 2), the present findings indicate that two enamel layers can be etched off in vivo without exceeding the 5 μm depth, which is considered to affect the tooth surface physiologically and/or esthetically (24).

To utilize the acid-saturated cotton pellet technique (25) for etching successive enamel layers, some modifications are necessary to prevent re-precipitation of the dissolved fluoride, calcium, and other ions to the underlying enamel. Thus, in the current procedure the etched enamel surface was immediately washed with citrate buffer and then with deionized water. Citrate serves the role of preventing precipitation of fluoride as fluorapatite or calcium fluoride by complexing the calcium (26). The present findings show that malachite green offers many advantages over the other widely used methods in determination of phosphate in enamel samples.

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