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An evaluation of an in vivo enamel acid etch biopsy technique for fluoride determination

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SUMMARY

An in vivo *acid etch biopsy method was developed by Hotz* et al *(1970) to determine fluoride uptake and retention in the outer 5 μm to 15 μm of enamel. This technique was evaluated by means of scanning electron microscopy and planimetry. Because of the difficulty experienced in controlling the areas etched, a modification was introduced by defining the biopsy areas with annular adhesive discs. This modified technique was evaluated and the reliability of the method confirmed using a surface roughness testing instrument.*

OPSOMMING

'n In vivo *suur-geëtste biopsie metode is deur Hotz* et al *(1970) ontwikkel om die opneming van fluoried in die buitenste 5 μm–15 μm van glasuur te bepaal. Hierdie tegniek is deur middel van skandeerelektronmikroskopie en oppervlakmeting ondersoek. Omdat dit moeilik is om die geëtste gebiede te kontroleer, is die metode gewysig en die biopsiedele met annulêre kleefdiskusse gedefineer. Hierdie gewysigde tegniek is ondersoek en die betroubaarheid van die metode is met 'n oppervlaksgrofheidtoetsinstrument bevestig.*

INTRODUCTION

The inverse relationship between the fluoride content of enamel and the incidence of dental caries is well documented (Armstrong and Brekhus, 1938; Jenkins and Speirs, 1953; Brudevold, Gardener and Smith, 1956). The regulation of the fluoride content of the public water supplies to 1,0 ppm is the most safe, practical and effective means of preventing dental decay (Doherty, 1968). This preventive measure is unfortunately not generally accepted nor applied and therefore topical fluoride therapy at present plays an important part in combating dental decay.

There is divergence of opinion about the minimal fluoride concentration in the outermost layers of enamel required to inhibit the onset of dental caries, but levels ranging between 700 ppm to 2 000 ppm of fluoride have been suggested (Englander, 1968). In evaluating the efficacy of caries preventive measures involving fluoride therapy, it is important to be able to assess the fluoride uptake and retention by tooth enamel.

Various techniques have been developed to determine the fluoride content of enamel in extracted teeth. Mühlemann, Schait and König (1964) etched surface and subsurface layers of enamel from intact crowns of extracted teeth for fluoride analysis by immersing the teeth in 2N perchloric acid. They determined the depths of the layers etched by measuring the diameter of the crowns of the teeth with a high-precision micrometer before and after etching. Weatherell and Hargreaves (1965) applied single spots of varnish on the enamel surface of a tooth before and after each successive exposure to 6N perchloric acid. A section of the tooth cut through the row of varnish spots provided a histological record showing directly the thickness of each layer removed. The same authors (1966) used this technique to determine the fluoride distribution pattern in enamel of deciduous and permanent teeth. They found that the fluoride concentration followed a negative exponential curve i.e. the fluoride concentration was maximal in the outermost layers and fell in a characteristic curve to a plateau in the deeper regions of the teeth. This finding confirmed the reports of Jenkins and Speirs (1953) and Brudevold *et al* (1956) regarding the fluoride distribution in enamel. Vrbic, Brudevold and McCann (1967) cut blocks of enamel of known surface area from human teeth and mounted them on plastic rods with sticky wax in such a way that only the external surfaces were exposed. Four layers of enamel were etched successively by immersing them in perchloric acid and this allowed them to determine the fluoride concentrations in the outermost 50 μm of enamel.

In most of these earlier studies, fluoride was determined in the etching solutions by a method developed by Wharton (1962) which involved gaseous diffusion of hydrofluoric acid followed by spectrophotometry. With the advent of the lanthanum fluoride selective fluoride ion electrode (Frant and Ross, 1966), fluoride could be measured directly and more rapidly. McCann (1968) determined the fluoride content of mineralized tissues using the fluoride ion electrode and found that this method was ideally suited to the determination of fluoride concentrations of etched layers of intact tooth enamel in extracted teeth. Another technique for *in vitro* use was designed by Cutress (1972). His surface enamel sampling and analyzing apparatus enabled him to analyze thin layers of enamel for carbonate, fluoride and other inorganic constituents.

An *in vivo* enamel biopsy technique, however, would be of greater value in assessing fluoride uptake and retention under intra-oral conditions. Candeli *et al* (1967) developed an *in vivo* enamel biopsy technique whereby enamel was separated mechanically from deciduous molars and the biopsied teeth repaired with amalgam. The fluoride content of the whole enamel specimen was determined which is a distinct disadvantage of this technique as the fluoride content of the superficial enamel layers is of prime importance.

Brudevold, McCann and Grφn (1968) developed an abrasive biopsy technique which enabled them to determine the fluoride concentration in the superficial layers of enamel. They used a felt cone impregnated with a silicon carbide glycerine slurry. A disadvantage of this method is that they assumed that a constant area was sampled and used this in calculating the depth of enamel removed. A method, very similar to the above, was introduced by Larsen, Kold and von der Fehr (1972). As a result of difficulties experienced with felt cones with regard to sampling and fluoride recovery, they introduced rubber cups which offered several advantages. For estimation of the depth of biopsy, it was again assumed that the biopsy area was approximately 40 mm^2. According to this assumption, they calculated that each 100 μg of biopsied material would correspond to an enamel layer of approximately 1 μm thick.

To overcome these shortcomings, Aasenden, Moreno and Brudevold (1972) modified the abrasive technique by standardizing the area from which the biopsy was taken. This made possible the accurate calculation of the sampling depth. Mellberg *et al* (1973) further modified the abrasive technique by controlling the biopsy area, the polishing speed and the pressure applied. This was achieved by using a specially constructed handpiece. Despite these modifications, the amount of enamel removed from a controlled area varied from 59,0 μg to 195,5 μg. Bruun (1973) slightly modified the abrasive technique by reducing the sample area which enabled him to do two biopsies on the same tooth. This made intradental control possible in comparative studies.

Hotz, Mühlemann and Schait (1970) adopted a different approach and introduced an acid etch *in vivo* enamel biopsy technique for determining the fluoride concentration of the outer 5 μm–15 μm of enamel.

Munksgaard and Bruun (1973) employed gas chromatography for *in vivo* and *in vitro* fluoride determinations in enamel solutions obtained with perchloric acid etching. Less than 1 ng of F could be detected with this sensitive analytical technique. As a result, they were able to reduce the biopsy surface area and time of etching considerably and up to six consecutive biopsies were etched from the same area within approximately 5 μm of the surface enamel.

The object of the present investigation was to evaluate some aspects of the acid etch biopsy method of Hotz *et al* (1970) and to attempt to reduce any shortcomings by modifying the technique.

MATERIALS AND METHODS

Freshly extracted, sound human maxillary incisors were used in this investigation. The teeth were cleaned and stored moist at −4°C until required.

1. *Evaluation of acid etch biopsy technique*

The biopsy technique developed by Hotz *et al* (1970) was used to obtain enamel samples for analysis. The labial surfaces of 30 lateral incisors were cleaned with a slowly-rotating bristle brush and pumice, washed well with tap water and dried with compressed air. A circular plastic adhesive disc (3M Co., No. 471), 3 mm in diameter, was placed on each tooth surface and carefully burnished to ensure good marginal adaptation. The discs and exposed adjacent tooth surfaces were coated with Copalite varnish (Cooley & Cooley). The varnish was allowed to set and the discs removed just prior to etching to expose the underlying unvarnished surfaces.

Special filter paper discs with adhesive backing were prepared and handled with a vacuum pipette. On to each of these, 10 μl of 2N perchloric acid was pipetted. The exposed area on each tooth was etched for 8 sec, while intermittent pressure, timed with a metronome, was exerted with the vacuum pipette to ensure controlled agitation of the etching solution. Immediately after etching, the exposed surface was swabbed with a second filter paper disc, 5 mm in diameter, to absorb residual perchloric acid. Both the discs were transferred to a plastic bottle containing 2 ml of TISAB (Total Ionic Strength Adjustment Buffer). Filter paper, which was free of calcium, was used and three control samples were prepared at the same time.

The solutions in the plastic containers were left for 3 days to reach equilibrium after which the samples and controls were diluted 25 times with deionized water. The calcium content of the diluted samples was determined by atomic absorption spectrometry (Zeiss PMQ II with flame attachment FA2).

The depth of etch was calculated by the following formula: Depth of etch (μm) =

$$\frac{\text{Weight of dissolved enamel}}{\text{Density of enamel} \times \text{Biopsy surface area}}$$

For the calculations it was assumed that the calcium content of human tooth enamel was 37,1 per cent (Retief *et al*, 1971), and that the density of enamel was

2,95 gm ml^{-1} (Manly and Hodge, 1939). The biopsy surface area of 7,065 mm^2 was calculated from the diameter of the disc.

The etched teeth were then prepared for examination in the scanning electron microscope (SEM). The crowns of the teeth were mounted on aluminium stubs with a colloidal graphite solution and coated with an approximately 500 Å thick layer of silver in a high vacuum evaporator equipped with a rotating stage (Edwards Coating Unit, Model E12E4). The specimens were viewed in a SEM (Cambridge Stereoscan S4) operated at 20 keV. The beam/specimen angle was varied to obtain a circular etched area. The surfaces were photographed at a low magnification (× 20) to include the whole etched area in the field of observation. The periphery and any irregularly etched areas as well as the floor of the etched surface were examined at higher magnifications.

The negatives taken at low magnification were used to make photographic prints such that their magnification was 50 times that of the specimen. The areas which were isolated for the biopsy were outlined with ink on the photographs. Where the periphery of this area was not clearly defined, it was delineated by extrapolating the curvature of the adjacent obvious margins. The margins of the regions within this area which were not etched were also outlined. As the surfaces of the prints were too smooth for planimetry to be carried out directly, it was found necessary to transfer the outlines onto tracing paper. The surface areas of the original biopsy area and the surfaces actually etched were determined by means of a planimeter.

2. *Modification of acid etch technique and evaluation of the modified method*

Annular adhesive discs with an outer diameter of 5 mm and an inner diameter of 3 mm were prepared. Twenty lateral maxillary incisors were cleaned as previously described and the annular discs placed on the labial surfaces (Fig. 1). The discs were again burnished carefully to ensure good marginal adaptation and the etching procedure carried out as before. The depth of etch was calculated for each specimen and the biopsy areas examined by SEM. The surface areas of the tracings were measured with a planimeter.

In addition, this modified technique was evaluated as follows. The labial surfaces of 10 central maxillary incisors were polished with 600 grit silicon carbide paper on a polishing machine (Kent Mk 2). Care was taken not to expose the underlying dentine, and specimens in which this occurred were discarded. The teeth were washed well in tap water, dried and the annular discs placed on the planar surfaces. The acid etch enamel biopsy technique was carried out and the depth of etch calculated after analysis of the samples.

The actual depths of etch on the smooth surfaces were determined by means of a surface roughness testing machine (Taylor-Hobson Talysurf Model 3). The machine was set on external datum and a stylus pressure of 100 mg employed. Surface profiles, magnified 20 times along the horizontal and 2 000 or 5 000 times in the vertical directions, were recorded for all specimens.

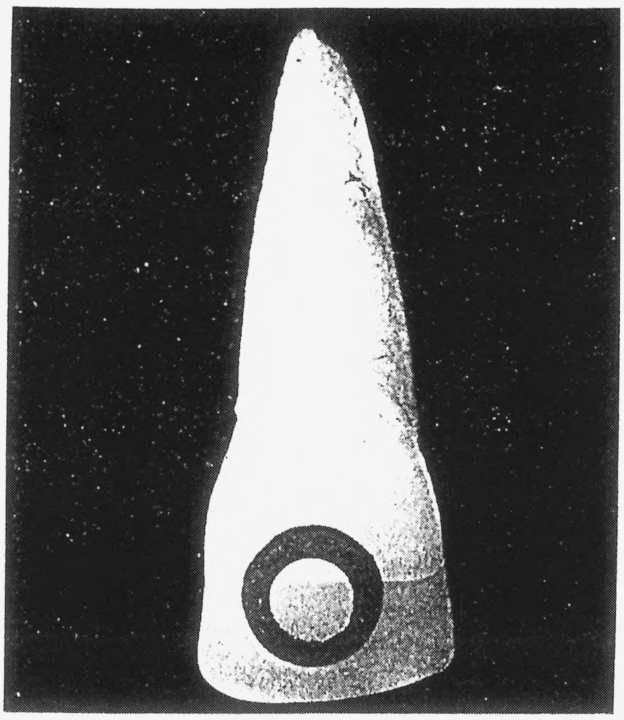

Fig. 1. Annular plastic adhesive disc on the surface of an incisor.

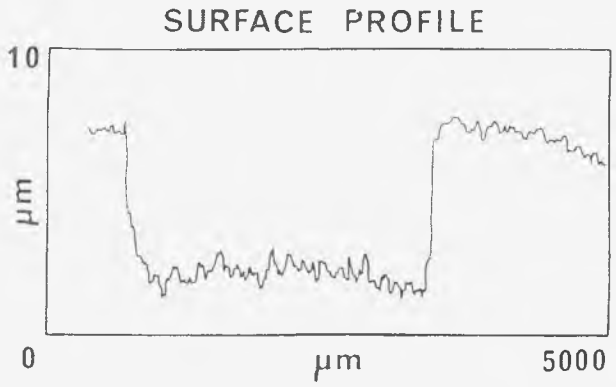

Fig. 2. Talysurf profile of etched area.

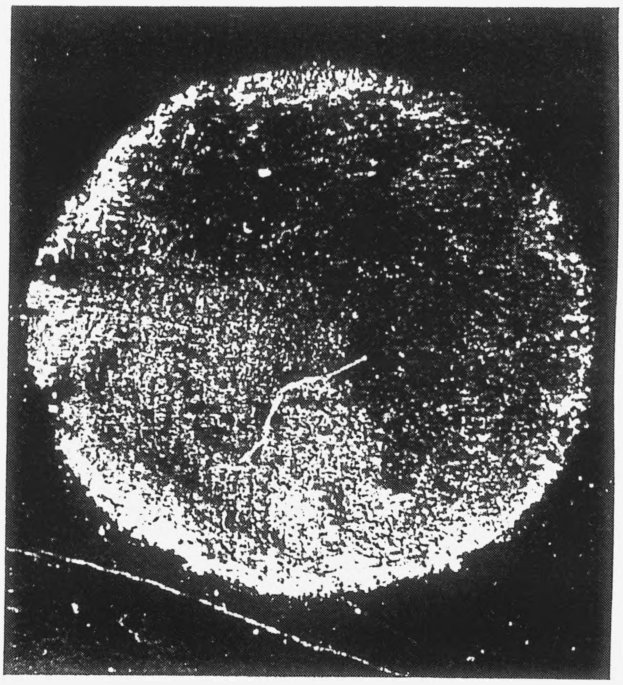

Fig. 3. Well defined biopsy area obtained with conventional technique. SEM x 20.

The stylus was traversed across the etched area including the margins on either side. Since the profiles of the floors of the etched areas were not regular (Fig. 2), it was necessary to approximate a mean straight line through the etched surface which was drawn parallel to the line between the marginal edges. The depth of etch was measured as the distance between the two parallel lines on the calibrated recording paper.

RESULTS

1. *Evaluation of acid etch biopsy technique, Hotz et al,* (1970)

The amount of enamel removed from the 30 teeth ranged from 93,2 μg to 209,4 μg with a mean of 161,3 μg (S.D. $\pm$ 31,3). The depth of etch calculated ranged from 4,4 μm to 9,8 μm with a mean of 7,5 μm and a standard deviation of 1,4 μm (Table I).

The photomicrographs obtained at low magnifications showed biopsied areas with well defined peripheries (Fig. 3) as well as with very irregular margins resulting from regions which were not etched (Fig. 4). Each area which was actually etched was expressed as a percentage of the area which had been isolated for the biopsy. These varied between 100 per cent and 31,6 per cent with a mean of 77,1 per cent and a standard deviation of 16,5 per cent. From these results and the corresponding weight of enamel removed during each biopsy, the depths of etch were recalculated and ranged from 5,8 μm to 13,8 μm with a mean of 10,1 μm and a standard deviation of 2,2 μm (Table I).

At higher magnifications some photomicrographs of the etched surfaces showed even etching patterns with classical prism-end structure (Fig. 5). In other cases islands of irregularly etched enamel were shown surrounded by apparently unetched surfaces (Fig. 6). In addition, perikymata were shown on some surfaces with the crests etched to a greater degree than the grooves between them (Fig. 7).

2. *Evaluation of the modified technique*

The weight of enamel removed during the biopsy of the 20 teeth ranged from 89,2 μg to 171,6 μg with a mean of 144,2 μg (S.D. $\pm$ 19,6). The depth of etch ranged from 4,2 μm to 8,0 μm with a mean of 6,7 μm and a standard deviation of 0,9 μm (Table I).

The majority of the etched areas had well defined margins (Fig. 8). A higher magnification of this specimen showed irregularly etched perikymata adjacent to an area which did not show a characteristic etching pattern (Fig. 9). In a small number of these specimens, the etched area extended beyond the margins of the exposed area (Fig. 10).

The etched areas expressed as percentages of the delineated areas ranged from 105,0 per cent to 96,0 per cent with a mean of 98,9 per cent and a standard deviation of 2,2 per cent. The range of the recalculated depths of etch was between 4,0 μm and 8,2 μm with a mean of 6,8 μm and a standard deviation of 1,0 μm. These results, compared with those obtained with the conventional technique are summarised in Table I.

Table I: Summary of results obtained with conventional and modified biopsy techniques

	Number of specimens n	Mean weight of enamel removed $\pm$ S.D. μg	Mean calculated depth of etch $\pm$ S.D. μm	Mean percentage area etched $\pm$ S.D.	Mean recalculated depth of etch $\pm$ S.D. μm
Biopsy Technique Hotz *et al* (1970)	30	161,3 $\pm$ 31,3	7,5 $\pm$ 1,4	77,1 $\pm$ 16,5	10,1 $\pm$ 2,2
Modified Biopsy Technique	20	144,2 $\pm$ 19,6	6,7 $\pm$ 0,9	98.9 $\pm$ 2,2	6,8 $\pm$ 1,0



Fig. 4. Biopsy area showing irregular margins and unetched regions. SEM x 20.

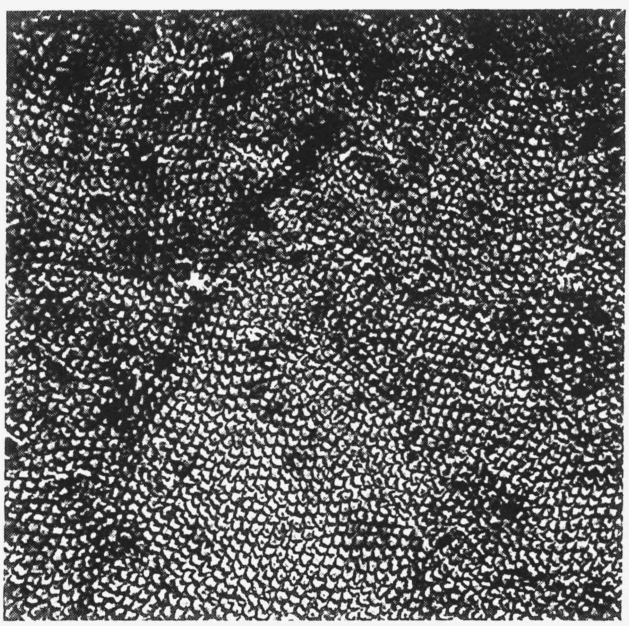

Fig. 5. Regularly etched surface showing characteristic prism-end structure. SEM x 200.

Table II: Calculated and measured depths of etch obtained with modified biopsy technique

Specimen number	Calculated depth of etch μm	Measured depth of etch μm
1	6,5	5,9
2	9,7	8,7
3	8,3	8,4
4	9,8	10,0
5	4,5	4,8
6	4,0	6,0
7	10,3	9,0
8	9,4	8,3
9	9,8	9,1
10	8,6	7,8
	Mean 8,1 S.D. ± 2,3	Mean 7,8 S.D. ± 1,7

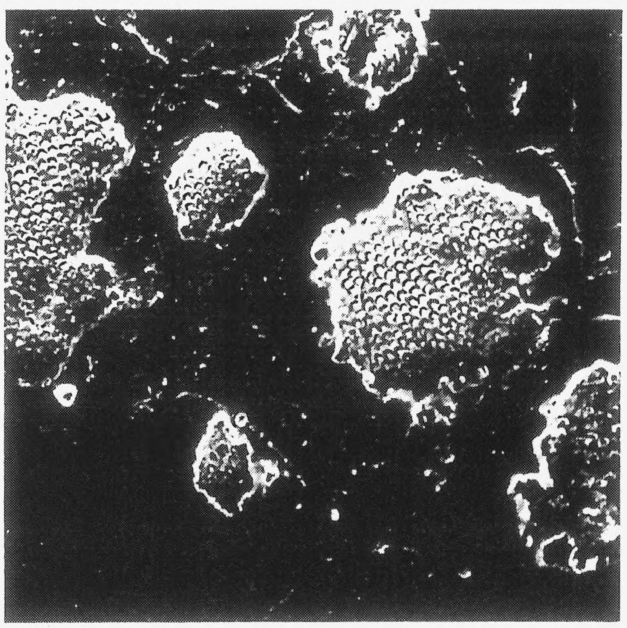

Fig. 6. Islands of etched enamel surrounded by unetched areas. SEM x 200.

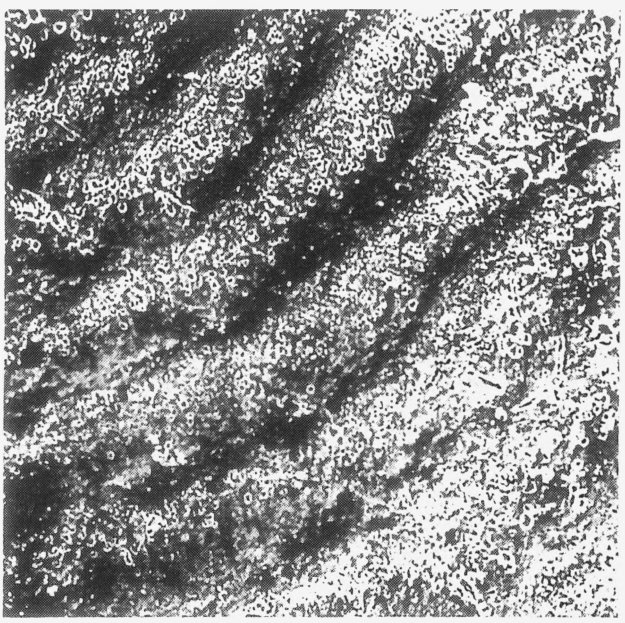

Fig. 7. Etched area showing differential etching of perikymata. SEM x 100.

Most of the specimens showed well defined margins at higher magnifications (Fig. 11). The etched surface of this specimen was regularly and characteristically etched. Exposed prism-ends were seen in a higher magnification (Fig. 12).

The depths of etch of the 10 specimens measured from the Talysurf profiles are compared with the calculated depths obtained with the modified biopsy technique in Table II.

DISCUSSION

Trisodium citrate has been used in TISAB to complex aluminium in order to liberate fluoride bound to aluminium so that total fluoride may be determined (McCann, 1968; Brudevold *et al*, 1968; Iizuka, Akiba and Nakayama, 1970). Retief *et al* (1971) showed that human enamel contained appreciable amounts of aluminium and iron. According to Harwood (1969), these

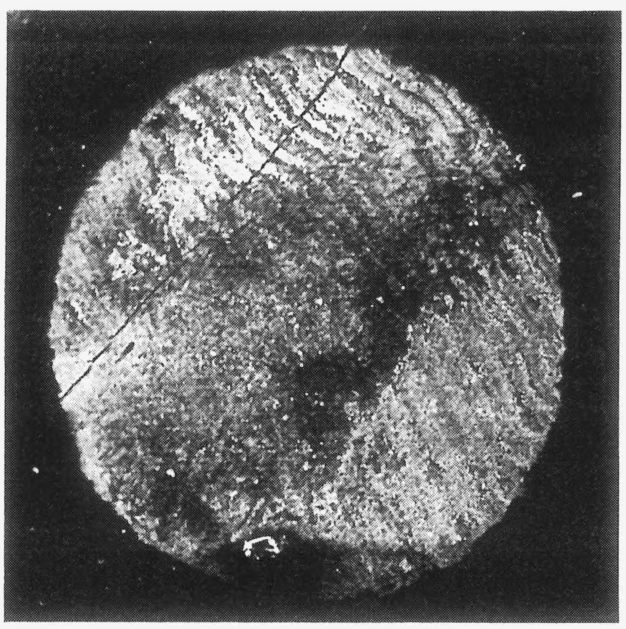

Fig. 8. Well defined biopsy area obtained with modified technique. SEM x 20.

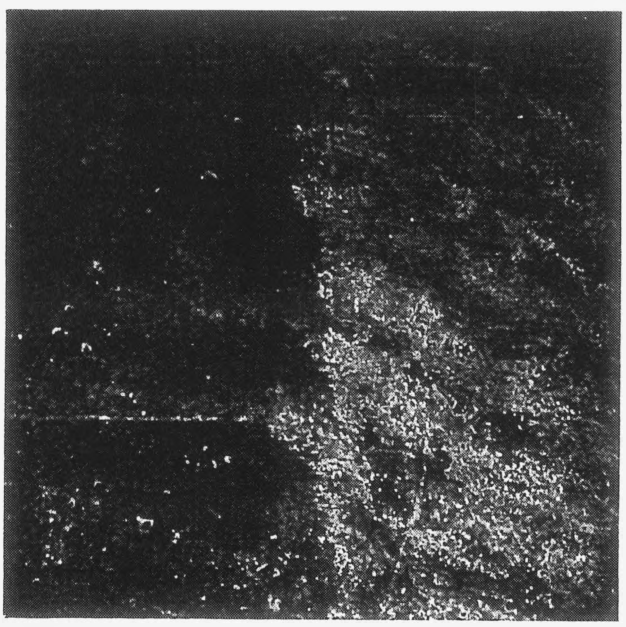

Fig. 9. Perikymata adjacent to uncharacteristic etched area. SEM x 50.

elements form complexes with fluorine which were more efficiently broken down to release fluorine by the incorporation of cyclohexanediamine-tetra-acetic acid (CDTA) instead of citrate in TISAB. This formulation was used in this study.

In the conventional biopsy technique the percentages of areas etched varied considerably. The observation that the controlled areas were not completely etched may be due to seepage of varnish underneath the adhesive disc because of poor adaptation to the tooth surface. The solvent in the Copalite varnish may also dissolve the adhesive backing at the periphery leading to further seepage of the varnish. Finally, during removal of the adhesive disc prior to etching, adhesive debris may remain on the surface. These factors may result in parts of the enamel surfaces being covered with a protective coating which will prevent them from being etched.

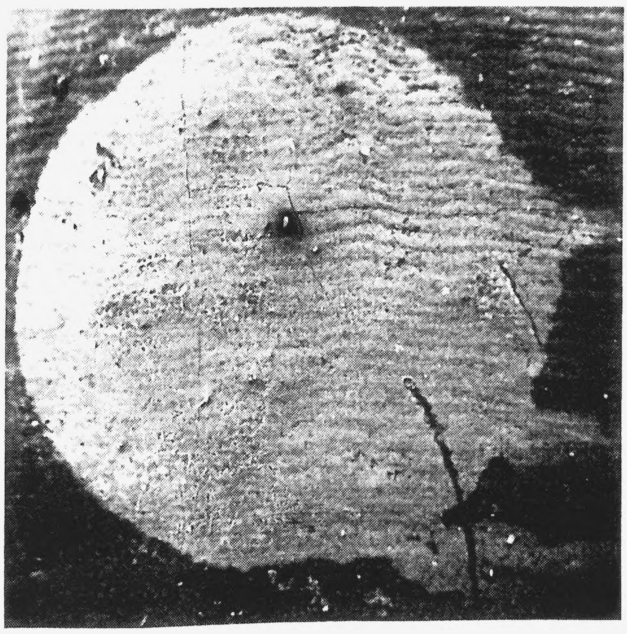

Fig. 10. Etching extending beyond margins of control area. SEM x 20.

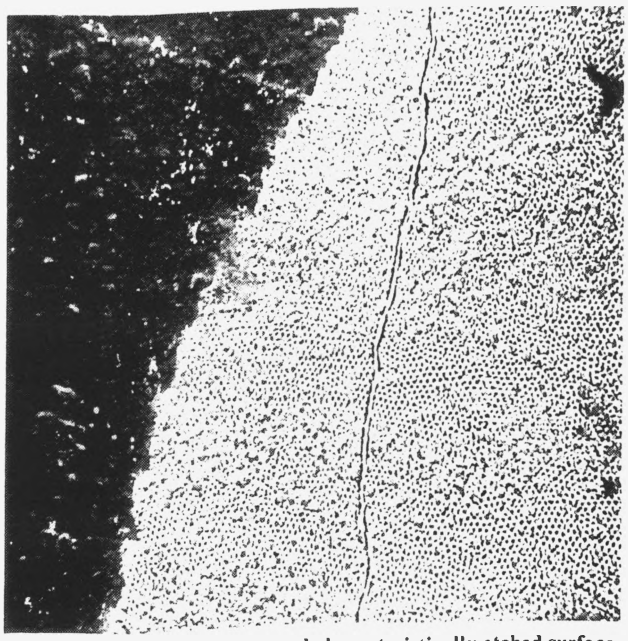

Fig. 11. Well defined margin and characteristically etched surface. SEM x 100.

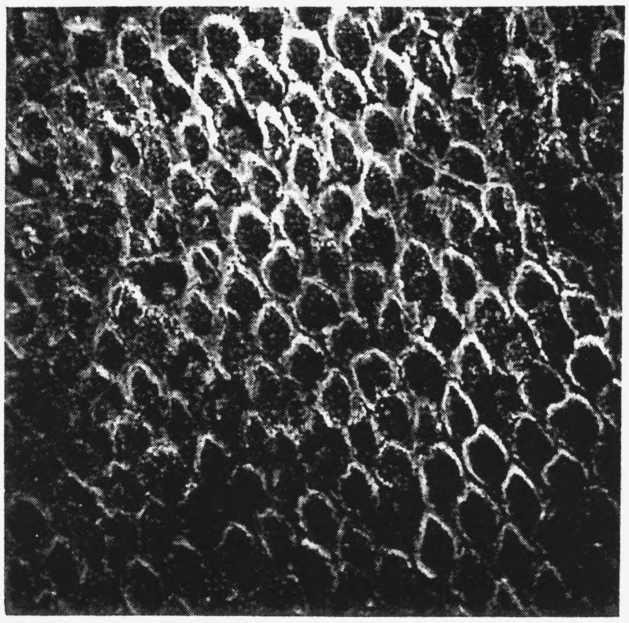

Fig. 12. Exposed prism-ends on etched surface. SEM x 1 000.

Using the modified technique the percentage area etched was never less than 96,0 per cent. Small, unetched areas were observed at the margins of the exposed areas which can only result from irregularities in the peripheries of the punched annular discs. In the conventional technique similar irregularities were obscured by the other factors described. In some cases, however, the etched area extended beyond the margins of the control area. This again may have resulted from the poor adaptation of the annular adhesive disc to the tooth surface. A marked improvement in the percentage of the control areas etched was obtained with the modified technique (Table I). The difference between the mean percentages of areas etched by the two techniques was statistically highly significant ($t = 5{,}46$; $P < 0{,}0001$).

There is a marked difference between the mean calculated and recalculated depths of etch in the conventional technique. This can be accounted for by the considerable reduction in the etched surface area which was taken into account in the recalculation. In view of the more complete etching of the exposed surfaces in the modified technique, this difference is much smaller. The depths of etch measured from the Talysurf profiles are very similar to those obtained by calculation (Table II). Statistical analysis showed that the difference between the means of the depths of etch as calculated and measured was not significant ($t = 0{,}314$). This confirms the reliability of the modified acid etch biopsy technique.

The technique developed by Hotz *et al* (1970) has been used to evaluate the efficacy of a topical fluoride treatment in increasing surface enamel fluoride content (Barbakow *et al*, 1973). This biopsy technique could be carried out with ease on posterior teeth. The modified technique using the annular discs is being used at present (Friedman *et al*, unpublished work) and a further advantage is that the area isolated on the tooth surface is more readily visible.

Weatherell, Robinson and Hallsworth (1972) reported that the fluoride concentration varied considerably from the incisal edge towards the cervical margin of upper incisors. An added advantage of the acid etch biopsy technique is therefore the small sampling area of 7,065 mm².

Hotz *et al* (1970) recognized the possibility of reprecipitation of free fluorides from the etching solution during the biopsy procedure. They believed that this problem was overcome by agitation of the demineralizing acid. This aspect was not investigated in the present study.

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