

From:
The Institute of Anatomy,
University of Bergen,
Norway

A QUANTITATIVE ANALYSIS OF THE NUMERICAL DENSITY AND THE DISTRIBUTIONAL PATTERN OF PRISMS AND AMELOBLASTS IN DENTAL ENAMEL AND TOOTH GERMS

II. SERIAL ETCHING OF DENTAL ENAMEL

by

GISLE FOSSE

INTRODUCTION

The method of preparing thin ground sections of dental enamel causes loss of appreciable amounts of tissue. To avoid this one of two methods has been employed:

1. Serial surface grinding of undecalcified enamel (*Fosse, 1964; Freiberg, 1939; Schnizer, 1925; Yosida, 1938*).
2. Serial sectioning of decalcified mature enamel (*Bödecker, 1927; Chase, 1927; Helmcke, 1964; Sognnaes, 1948*).

By the first method it is usually not possible to prepare series with a lower interproximate distance than $10\ \mu$. This is too great a distance to make possible the recognition of individual prisms in successive ground planes.

It is very difficult to decalcify mature enamel so that the organic stroma is left intact and morphologically undisturbed. Therefore the second method may hardly become a routine laboratory technique.

The purpose of this paper is to present a method by which series of etched plane enamel surfaces with a low and relatively constant interproximate distance are obtained.

The method was developed and employed by the present author to supplement the lapping capacity of the machine described in part I.

MATERIAL AND METHODS

The specimen of undecalcified enamel to be examined was embedded in a plastic column (cf. I, and *Fosse*, 1964), where it was positioned so that the direction of the intended series was orientated perpendicularly to the upper plastic surface which was ground down to the first plane of the series.

The etching of the exposed enamel on top of the column was carried out by immersing this surface in a given concentration of acid, preferably HNO_3 . After a given number of seconds the action of the acid was interrupted by dipping the specimen in fresh water. The etched surface was stained in Harris haematoxylin for 30 seconds. The procedure described above could be repeated at least 30 times in one series with the planarity of the etched enamel surfaces remaining fully satisfactory for microphotography.

Prior to microscopic examination the etched and stained surface might be covered by water and a cover glass to eliminate blurring of the image when using incident light.

Testing of method*Measurements of etching depth in test series*

For each of 14 test series the focal depths of the consecutive etched enamel surfaces were determined by the micrometer screw reading of the microscope (*Abbé*, 1880; *Brattgård*, 1954; *Hallén*, 1956, and *Lange & Engström*, 1954). The interval of the micrometer screw of the Ortholux microscope used by the present author corresponded to 0.98μ . The values in microns have been corrected accordingly.

Prior to the measurement of the depth of the individual etched surfaces in each series, the upper surface of the plastic column was made plano-parallel to the base in the lapping machine. The rim of the basal flange was provided with two diametrically opposite conical grooves. The planed base was then placed on an object slide. Heated Kerr resin was applied to both ends of the slide and at the insulated rim and grooves of the flange. After hardening, the resin which adhered to the glass constituted a replica of the flange rim. After removal, the column might thus be replaced in the same position on the slide. The slide was then glued permanently to the stage.

To ensure that the depth of the enamel surface was always measured along the same vertical line in one series a point on the plastic surface in the immediate vicinity of the exposed enamel was selected and marked by graphite. (The plastic and the graphite were not attacked by the acid). A second imaginary point was then selected on the enamel surface. The exact position

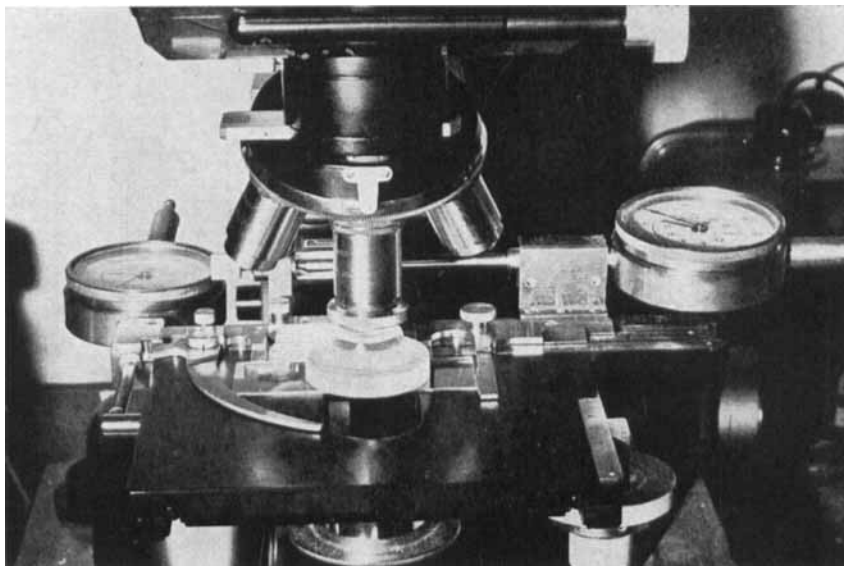


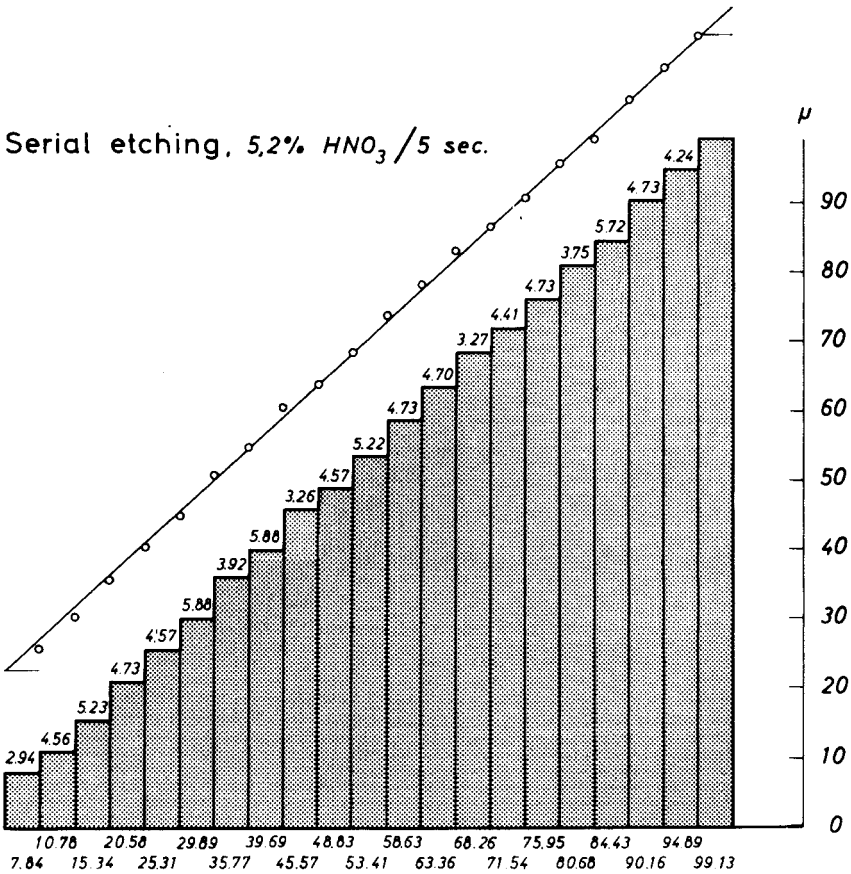
Fig. 1. The microscope stage with two microcaters for the recording of horizontal stage movements. A plastic column containing a specimen is mounted on a slide.

of this point or vertical axis of the series in relation to the reference point on the plastic surface was determined by the stage microcaters illustrated in Fig. 1.

The depth measurements were always carried out with a dry enamel surface, which means that the enamel was not covered by water or coverglass during the measurements.

Each depth measurement was repeated 3 times and consisted of 6 recordings of the micrometer screw reading; three for the point on the plastic surface and three for the imaginary point on the enamel. From these recordings three values for the depth of the enamel surface were calculated and the mean of these constituted an estimate of the actual depth. The difference between two such means from two consecutive etchings was thus the estimated thickness of the enamel layer dissolved by the last etching. Finally the mean of all these differences was calculated for the whole series.

Fig. 2 is a graphical representation of one of these test series. The graph consists of two parts. The lower part of the graph is a histogram illustrating the measured consecutive depths of the enamel. At the bottom of each column the depth in microns is given. The difference between this depth and that of the next etching to the right, is given at the top of each column.

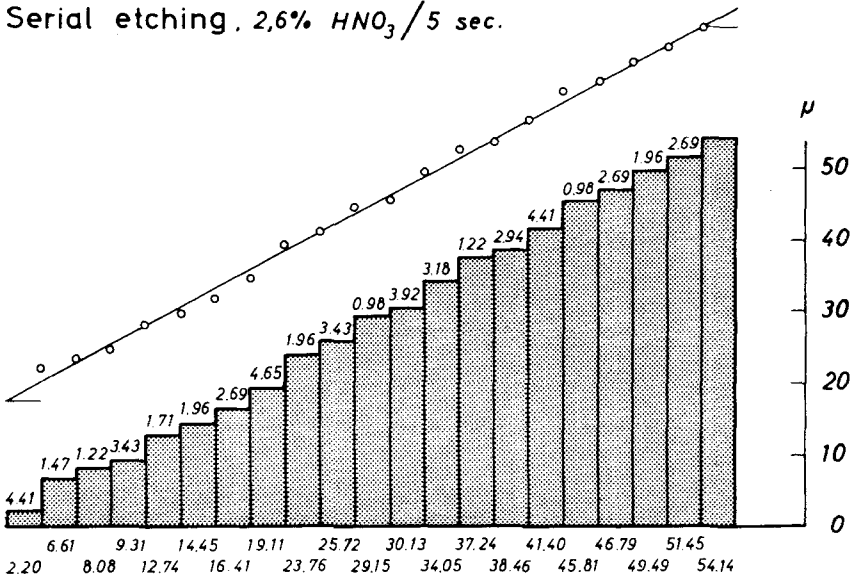


20 etchings = total descent of enamel surface 91.29 μ

Fig. 2. A graphical representation of the descent of a plane enamel surface by periodic etching with 5.2 % HNO_3 for 5 seconds. The mean etching depth was 4.56 μ.

According to the same scale, the oblique straight line at the top of the diagram had an angle coefficient of 4.55. This value corresponded to the numerical value in microns of the mean thickness of the dissolved enamel layers. The distribution of the recorded etching depths generally conformed to the course of this line.

Fig. 3 is a graphical representation of a new series, where the concentration of the acid was 2.6 %, the etching time being 5 secs. The mean thickness of the etched layers was 2.59 μ. The recorded values deviated more from the mean than in the former test series. This, undoubtedly, was mainly



20 etchings = total descent of enamel surface 51.94 μ

Fig. 3. A graphical representation of the descent of a plane enamel surface by periodic etching with 2.6% HNO₃ for 5 seconds. The mean etching depth was 2.59 μ.

due to the fact that depth measurements in this particular series were carried out only twice for each etching. Still there was an obvious conformity between the measured values and the straight line representing the mean rate of descent of the enamel surface.

Fig. 4 represents a series where 1.3% HNO₃ was applied in 5 secs. for each etching. The mean thickness was 1.67 μ. By the third etching, a negative difference was recorded, as will be seen in the histogram. This seems to be caused by the inherent error of measurement which will be dealt with in the next chapter.

Error in depth determination

Since the depth of the etched enamel surface was to be measured by the micro-adjustment screw of the microscope it was desirable to use optical equipment that gave the smallest possible depth of field to reduce the error of measurement (Abbé, 1880; Brattgård, 1954; Lange, 1954). The most powerful objectives with oil-immersion would thus be preferable. However,

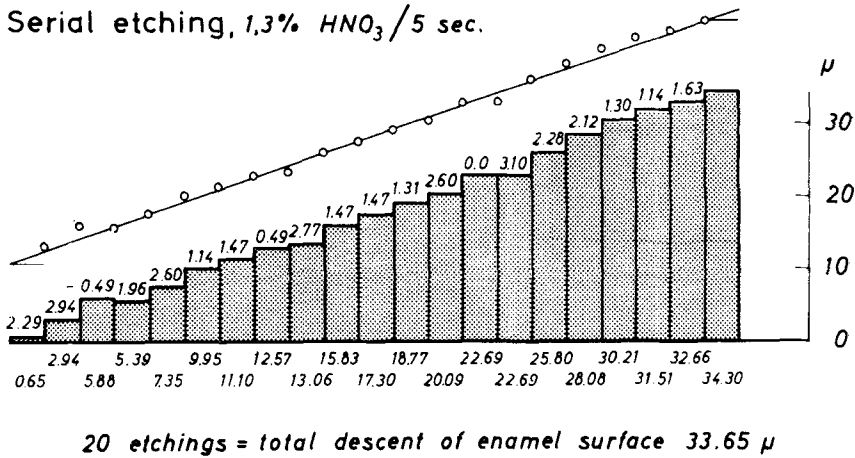


Fig. 4. A graphical representation of the descent of a plane enamel surface by periodic etching with 1.3 % HNO₃ for 5 seconds. The mean etching depth was 1.67 μ.

since the oil necessitated time consuming cleansing of the enamel surface before each etching, and might also influence the action of the acid on the enamel, the use of an oil-immersion objective had to be abandoned.

The author tested the accuracy of focusing by the 45× objective and the 25× objective and compared the variance of the micro-adjustment screw recordings for each of them in 12 test series each comprising 10 focusings of the same surface (polished plastic). The micrometer screw was always turned the same way to bring the surface into focus.

The variance of focal depth was calculated from the formula:

$$\text{Var } x_i = \frac{n \sum_{i=1}^n x_i^2 - \left(\sum_{i=1}^n x_i \right)^2}{n(n-1)}$$

where n signifies 10.

The variances for each series, are listed in Table I, those for the 25× objective in the left column, those for the 45× objective in the right.

These experiments seemed to indicate no significant advantage in using the 45× objective and, since it was desirable to photograph the greatest possible area of the etched surface, the 25× objective was chosen for the depth measurements.

Table I

Comparison between the variances of focusing by the Leitz 25/0,50 and 45/0,65 objectives; each of the twelve series in the two columns consisted of 10 focusings

	Var ₂₅	Var ₄₅
	0,96	0,64
	0,24	0,72
	1,38	1,44
	0,32	0,16
	0,27	0,78
	1,35	1,03
Mean	0,75	0,79

In each series of consecutive etchings the depth of the enamel surface for each etching was expressed as the difference between the focal reading of the plastic surface and that of the enamel surface. The determination of this difference or depth was generally carried out three times. Each depth determination thus consisted of 6 micrometer screw recordings giving three values for the actual depth.

The overall variation of the measured depth in the one series was expressed by the formula

$$\text{Var}_{d_{ij}} = \frac{n_i \sum_{i=1}^k \sum_{j=1}^{n_i} d_{ij}^2 - \sum_{i=1}^k \left[\sum_{j=1}^{n_i} d_{ij} \right]^2}{n_i (N - k)}$$

The depth of the etched surface is expressed by d. The index i signifies the ordinal number of the etched surface whose depth has been measured. k is thus the maximum number of etched and measured surfaces in one series.

The index j signifies the ordinal number of the measurement for one particular surface. The maximum number of measurements is signified by n which is constant throughout the series and generally equalled 3.

N is the total number of depth measurements, i.e. $n \cdot k$ or $\sum_{i=1}^k n_i$.

From the variation expressed by the formula above, the standard error of the mean depth of each etching, S.E. \bar{d}_i , which equals $\sqrt{\frac{\text{Var}_{d_{ij}}}{n}}$, was cal-

culated. The symbol n generally equalled the value 3 since the measurements were usually carried out three times.

The standard error of the difference between two consecutive enamel depths, viz. the thickness of the dissolved enamel layer, was thus equal to $\pm\sqrt{2} \cdot \text{S.E.}\bar{d}_i$ and is symbolized by $\text{S.E.}\bar{d}_{i+1}-\bar{d}_i$.

With a probability of 95 % the error of the calculated mean thickness of the enamel layers dissolved throughout the series is expressed as $2 \cdot \text{S.E.}\bar{d}_{i+1}-\bar{d}_i$.

In Table II are listed the experimental conditions for the different series, the number of etchings in each series (k), the mean thickness of the dissolved enamel layers for each series ($\bar{d}_{i+1}-\bar{d}_i$), the serial variations of measured etching depths ($\text{Var}_{d_{ij}}$) and, in the last column, the error of the mean thickness of the dissolved enamel layers through each series ($2 \cdot \text{S.E.}\bar{d}_{i+1}-\bar{d}_i$). The latter may be compared with the calculated mean thickness of the dissolved enamel layers, $\bar{d}_{i+1}-\bar{d}_i$, in the respective series. Experiments no's 3, 12 and 13 are the ones illustrated graphically by Figs. 2, 3 and 4.

Table II

Serial surface etching of enamel by different combinations of periodic etching time and concentration of acid; for each series is given the combination, the number of etchings, the mean thickness of the dissolved enamel layers, the variance of depth determination by measurement and the error of the calculated mean thickness of the dissolved enamel layers

Experiment no.	Acid. conc. seconds	k	$\bar{d}_{i+1}-\bar{d}_i$	$\text{Var}_{d_{ij}}$	$2 \cdot \text{S.E.}\bar{d}_{i+1}-\bar{d}_i$
1	5,2 % HNO ₃ /5	19	5,22 μ	0,871	1,520 μ
2	»	20	4,56 μ	0,635	1,298 μ
3	»	20	4,56 μ	0,655	1,322 μ
4	»	20	4,07 μ	0,952	1,588 μ
5	»	19	3,90 μ	1,032	1,654 μ
6	2,6 % HNO ₃ /10	20	4,60 μ	0,735	1,396 μ
7	»	20	4,50 μ	0,376	0,998 μ
8	»	20	4,20 μ	0,738	1,400 μ
9	2,6 % HNO ₃ /5	21	2,97 μ	0,716	1,380 μ
10	»	20	2,85 μ	0,722	1,384 μ
11	»	19	2,72 μ	0,610	1,272 μ
12	»	20	2,59 μ	0,956	1,956 μ
13	1,3 % HNO ₃ /5	20	1,68 μ	0,873	1,526 μ
14	0,1 % HCl/10	9	1,04 μ	0,456	1,100 μ

RESULTS

The Figures 5, 6, 7 and 8 represent numbers 3, 4, 5 and 6, respectively, in a continuous series of 26 etched surfaces with a mean interproximate distance of 1.30μ . The total length of this series was 33.80μ . Figures 9, 10, 11 and 12 are numbers 23, 24, 25 and 26, respectively, in the same series.

Individual prisms may be traced through the series. Five separate prisms were marked in the first photograph and followed through the series until the last surface represented by Fig. 12. The relative positions of the prisms were found to change considerably through the intervening 29.90μ .

Two rows of prisms were likewise marked and traced in order to demonstrate their altered position.

The etched surfaces in this series were planoparallel to the amelodentinal junction at the cingulum of a human permanent canine. The distance between the last plane and the junction was approximately 45μ .

Figs. 13, 14 and 15 represent the fifth and the last two surfaces in a series of 20 etchings with an interproximate distance of 1.51μ . The total length was thus 30.38μ . Five groups, each consisting of 3 prisms, were traced through this series. The last plane represented by Fig. 15 was located parallel to, and approximately 10μ from, the amelodentinal junction.

Unless the prism rods follow a straight course with only slight changes in relative position, such as is obviously the case in the outer regions of the enamel mantle, it does not seem possible to recognize individual prisms from one surface to the next if the distance between the surfaces exceeded 2μ . This was especially so near the amelodentinal junction in human permanent teeth where the prisms may follow an especially tortuous course.

Figs. 16, 17, 18 and 19 represent the fourth and the last three planes in a series towards the amelodentinal junction at the palatal surface of a human canine. The interproximate distance was 2.45μ and the total length of the series was 39.2μ . Although rather difficult, it was possible to follow the prisms in the lower left-hand part of the photos, whereas the wavy rods at the border of the exposed dentin were not recognizable from one plane to the next.

Figs. 20 and 21 represent two consecutive planes with an interproximate distance of 5μ . Even though the course of the rods was quite regular and their relative position fairly constant, the individual prisms may not be recognized from the first photo to the next. These photomicrographs represent regions from the labial surface near the incisal edge of a deciduous central incisor. They were planoparallel to the amelodentinal junction which was exposed 10μ from the etched surface depicted in Fig. 21. The total length of this series was approximately 100μ .

In all the four series presented above the acid was allowed to act for 5 seconds. In the first two series 0.5 % HNO_3 was used, in the third 1.5 % HNO_3 , and in the fourth 5.2 % HNO_3 .

DISCUSSION

Table II shows that similar experimental conditions yield different mean thicknesses of dissolved enamel layers. Since in each experiment different specimens of enamel were used, this is probably caused by different degrees of mineralization.

The error in depth determination would have been smaller if more accurate measuring instruments could have been used, cf. *Hallén* (1956).

It is also obvious that the real thickness of the dissolved enamel layers was not constant throughout each series. Apart from the probability that the degree of mineralization varied even through small distances of the enamel, the equipment used by the author did not ensure a constant etching time. A device to control the etching time accurately is therefore to be recommended.

The standard errors of the mean do not seem to increase or decrease systematically with the mean thickness of the dissolved enamel layers. This means that the real variations in thickness of the dissolved enamel layers are fairly independent of the etching time and the concentration of acid.

Considering the error of measurement, the probable error of small variations in temperature, the error caused by varying degree of mineralization and the error caused by insufficient control of the etching time, the regularity of the recorded rate of enamel surface descent by serial surface etching seems quite amazing to the author. Consequently, future perfecting of the etching technique seems justified.

The constancy of surface planarity was perhaps the most unexpected quality in these experiments.

Lamellas offered the greatest resistance to the acid. It was usually possible, however, to remove such projecting structures from the surface by gentle use of a soft brush dipped in toluol. This was done to remove the lamella seen in the lower right-hand corner of Fig. 20. Its height had been reduced in the next surface, represented by Fig. 21.

The difference in level between the prism core proper and the interprismatic substance remained constant throughout each series.

The rims of the exposed enamel area, surrounded by the plastic, seemed to resist the acid more strongly than the other parts. This was corrected by planing off the upper plastic surface in the lapping machine. The thickness of the plastic layer thus removed must be recorded in order to obtain a

knowledge of the final length of the series. This technique was not applied in the test series nor in the other series whose surfaces have been photographically presented in this paper.

The author has not, as yet, tried to determine the maximal length of one series before the planarity of the enamel is lost. The longest series prepared with no perceptible reduction of planarity was 104 μ with an interproximate distance of 5.22 μ .

These studies are being continued and a series of papers will appear in forthcoming issues of this journal.

SUMMARY

The author described a method by which it was possible to photograph continuous series of etched and stained enamel surfaces with a constant interproximate distance. This distance was determined by the concentration of the acid and by the length of the etching time.

By photomicrographs it was demonstrated that individual prisms might be followed through a given series. The course of such prisms might thus be reconstructed.

The etched enamel surfaces remained plane throughout the series. The interproximate distance remained practically constant for each series and could vary from 1 to 7 μ according to the concentration of acid and the length of periodic etching time chosen.

The maximum total length of such series prepared by the author was 104 μ .

The author measured the interproximate distance in 14 test series with different etching time and concentration of acid. It was found that the double standard error of the mean thickness of dissolved enamel layers varied from 1.956 μ to 0.998 μ for these test series. This variation did not seem to be dependent on the etching time or the concentration of acid. The measurements were carried out by an ordinary Leitz Ortholux microscope and by recording the focal depth of the consecutive etched enamel surfaces.

RÉSUMÉ

ATTAQUE EN SÉRIE DE L'ÉMAIL HUMAIN PAR UN ACIDE

L'auteur décrit une méthode permettant de photographier des séries continues de surfaces d'émail attaquées par un acide et ayant subi une coloration, la distance interproximale restant constante. Cette distance était déterminée par la concentration de l'acide et par la durée de l'attaque par l'acide.

A l'aide de microphotographies, on a pu mettre en évidence le fait qu'on peut suivre différents prismes pendant toute une série, ce qui permet de reconstituer le parcours de ces prismes.

Les surfaces d'émail attaquées restaient planes au cours de toute la série. Les distances interproximales restaient pratiquement constantes pour chaque série et pouvaient varier de 1 à 7 μ suivant la concentration de l'acide et la durée de la période d'attaque que l'on avait choisies.

La longueur maximum totale des séries que l'auteur a ainsi préparées était de 104 μ .

L'auteur a mesuré la distance interproximale dans 14 séries expérimentales faites en utilisant différentes durées d'attaque et différentes concentrations d'acide. Il a mis en évidence que le double de l'erreur standard de l'épaisseur moyenne des couches d'émail dissoutes variait de 1,956 μ à 0,998 μ pour ces séries. Cette variation ne semblait pas dépendre de la durée de l'attaque ni de la concentration de l'acide. Les mesures ont été faites au moyen d'un microscope Ortholux ordinaire et en enregistrant la profondeur de foyer sur les surfaces consécutives d'émail attaquées par l'acide.

ZUSAMMENFASSUNG

SERIENÄTZUNG VON ZAHNSCHMELZ

Der Verfasser beschrieb eine Methode wodurch es möglich wurde, ganzen Serien von geätzten und gefärbten Schmelzflächen mit einem konstanten gegenseitigen Abstand zu photographieren. Dieser Abstand war durch die Stärke der Säure und die Länge der Ätzungszeit bestimmt.

Die Mikrophotographien demonstrierten dass einzelne Prismen durch eine solche Serie gefolgt werden könnten.

Die geätzten Schmelzflächen blieben flach und eben durch die ganze Serie. Der Abstand zwischen den einzelnen Flächen blieb ganz konstant für eine Serie, und konnte variiert werden von 1 μ bis zu 7 μ , abhängig von Säurekonzentration und Länge der periodischen Ätzungszeit. Die maximale und totale Länge einer Serie war 104 μ .

Der Verfasser mass den Abstand zwischen den einzelnen Flächen in 14 Prüfungsserien wo die Zeit der periodischen Ätzung und die Konzentration der Säure verschieden waren. Es wurde gefunden, dass der doppelte S.D. des mittleren Abstandes variierte von 1,95 μ bis zu 0,998 μ in diesen Serien.

Diese Variation schien nicht von der Ätzungszeit oder der Säurekonzentration abhängig zu sein.

Die Messungen wurden durch ein Standard Ortholux Mikroskop ausgeführt und die Fokustiefe der aufeinander folgenden geätzten Flächen wurde abgelesen.

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Adress:

*The Institute of Anatomy,
University of Bergen,
Norway*

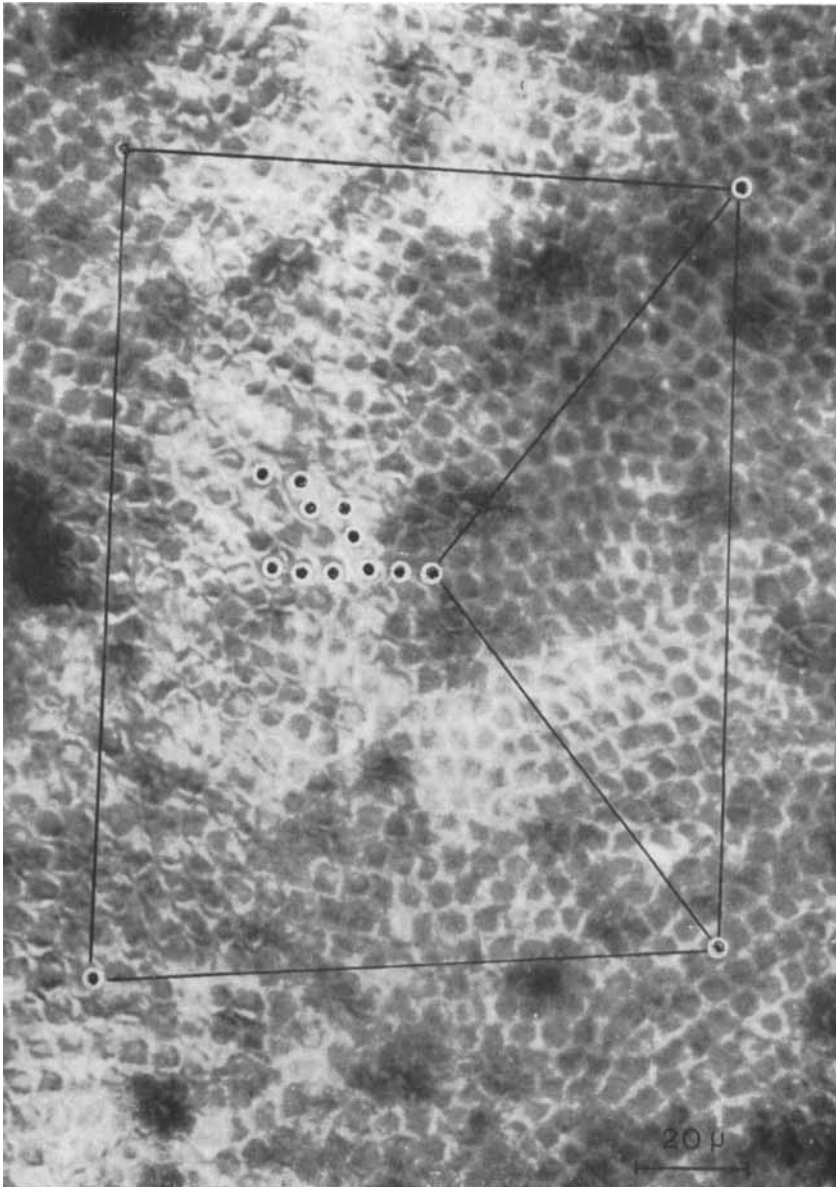


Fig. 5.

Figs. 5—8. The 3rd, 4th, 5th and 6th etched enamel surfaces of a series with a periodic etching of 5 seconds with 0.5 % HNO_3 . The mean interproximate distance between the surfaces of this series was 1.27μ . Four prisms near the corners of the photographed region have been marked and followed through the series. Two rows of prisms have also been marked and followed.

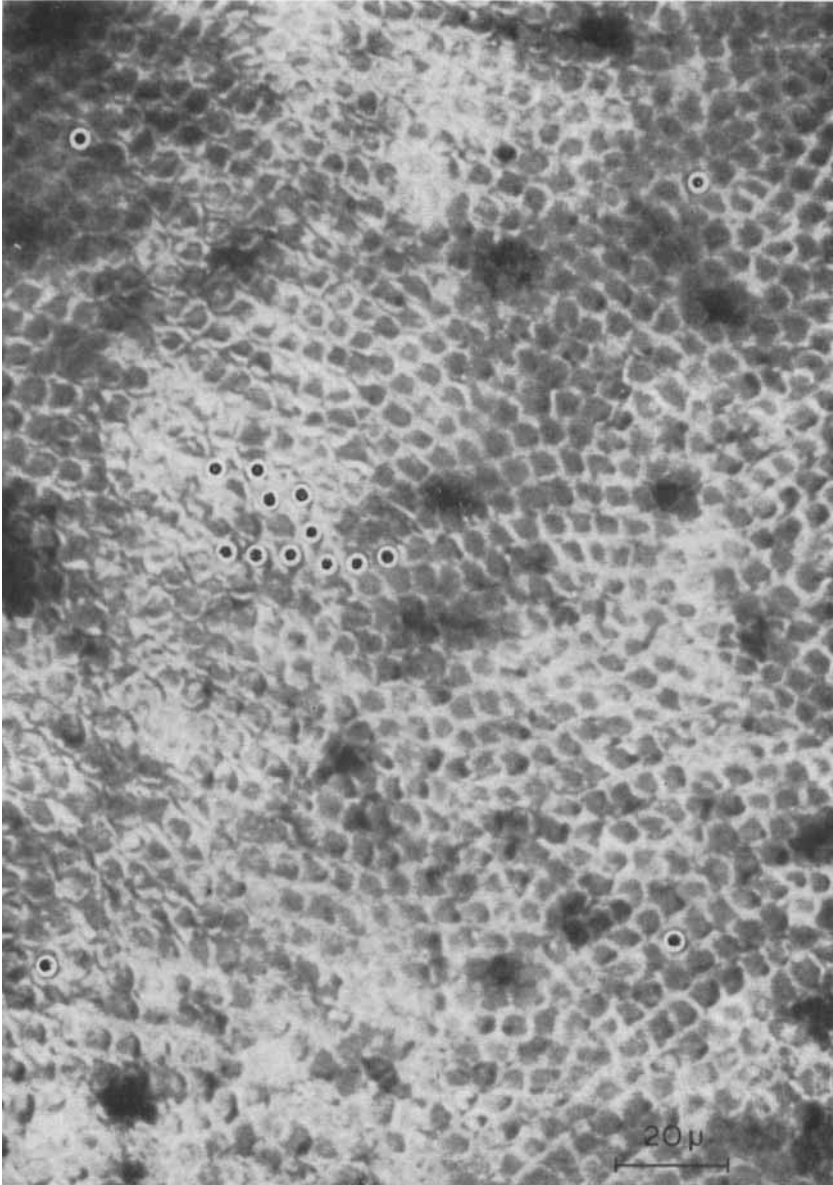


Fig. 6.

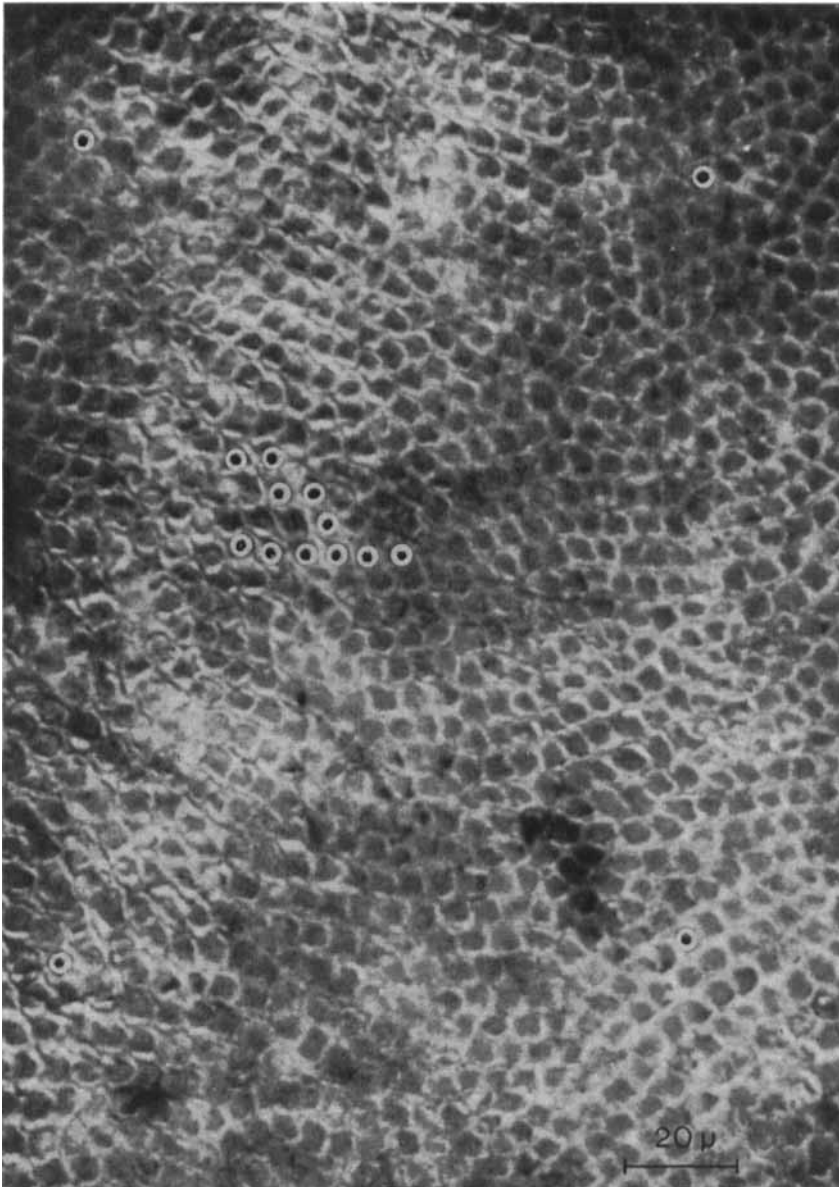


Fig. 7.

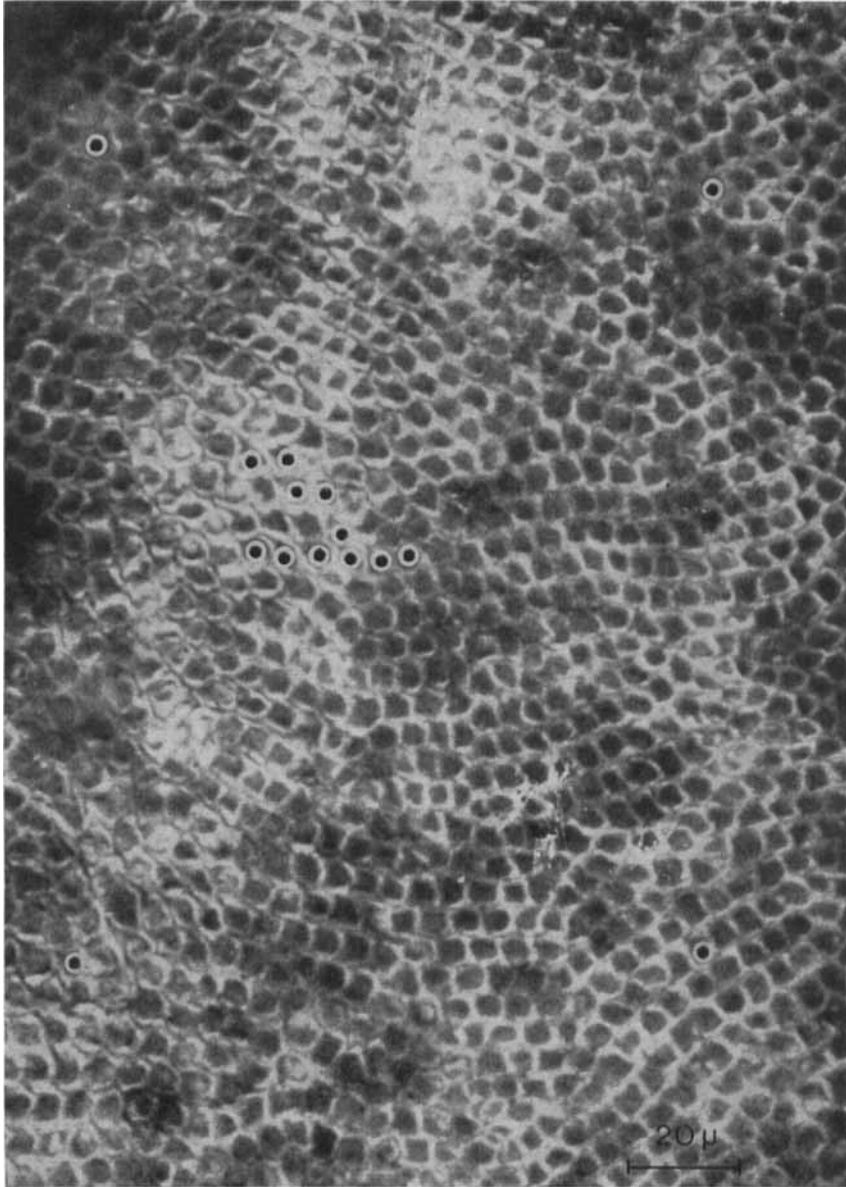


Fig. 8.

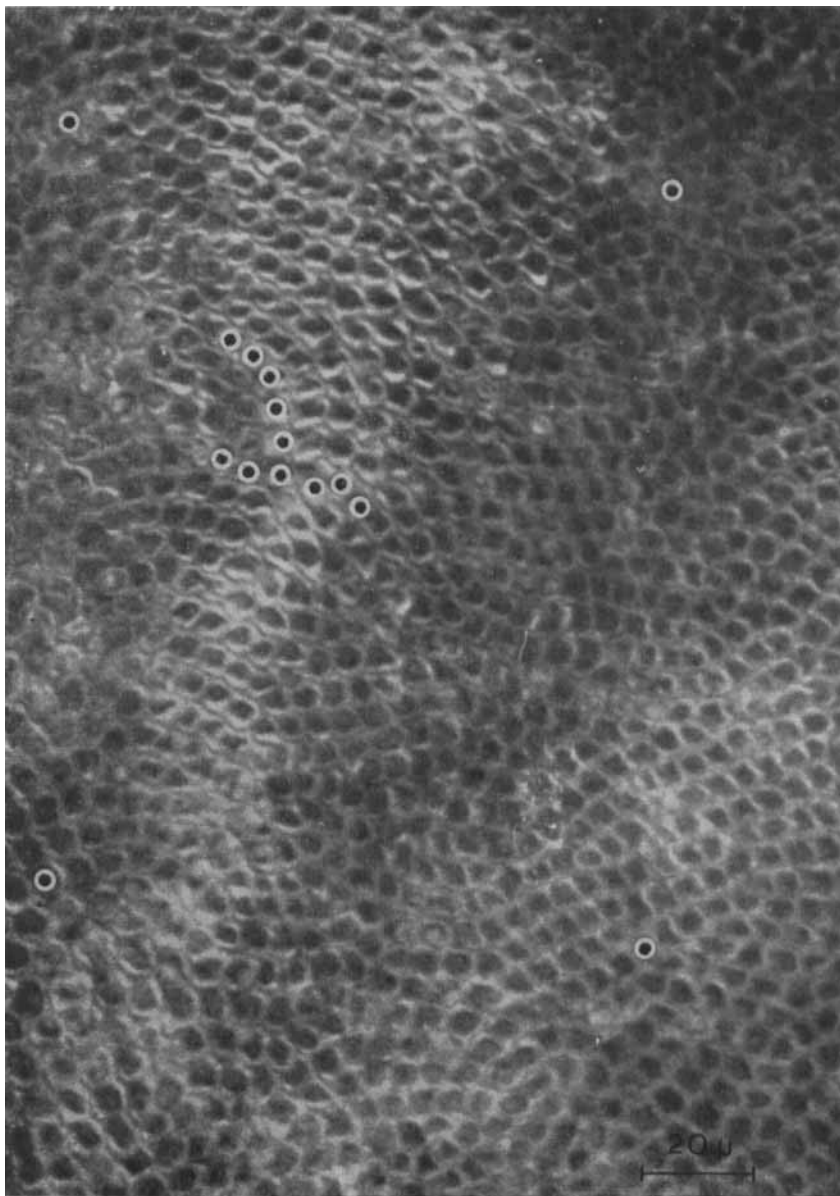


Fig. 9.

Figs. 9—12. The 23rd, 24th, 25th and 26th etched enamel surfaces of a series with a periodic etching of 5 seconds with 0.5 % HNO_3 . The mean interproximate distance between the surfaces of this series whose first surfaces are represented by figs. 5—8, was 1.27μ . The relative positions of the marked prisms have changed from the first to the last surfaces of this series, see figs. 5—8.

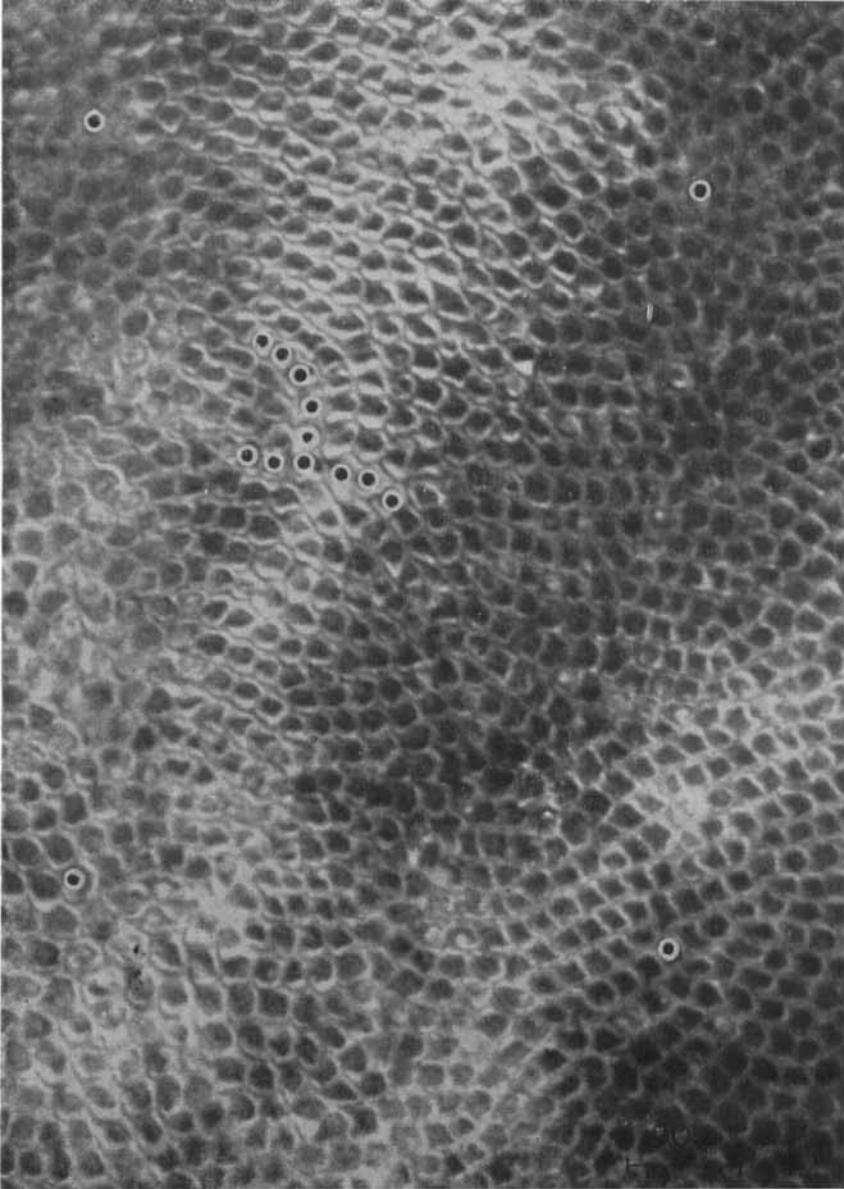


Fig. 10.

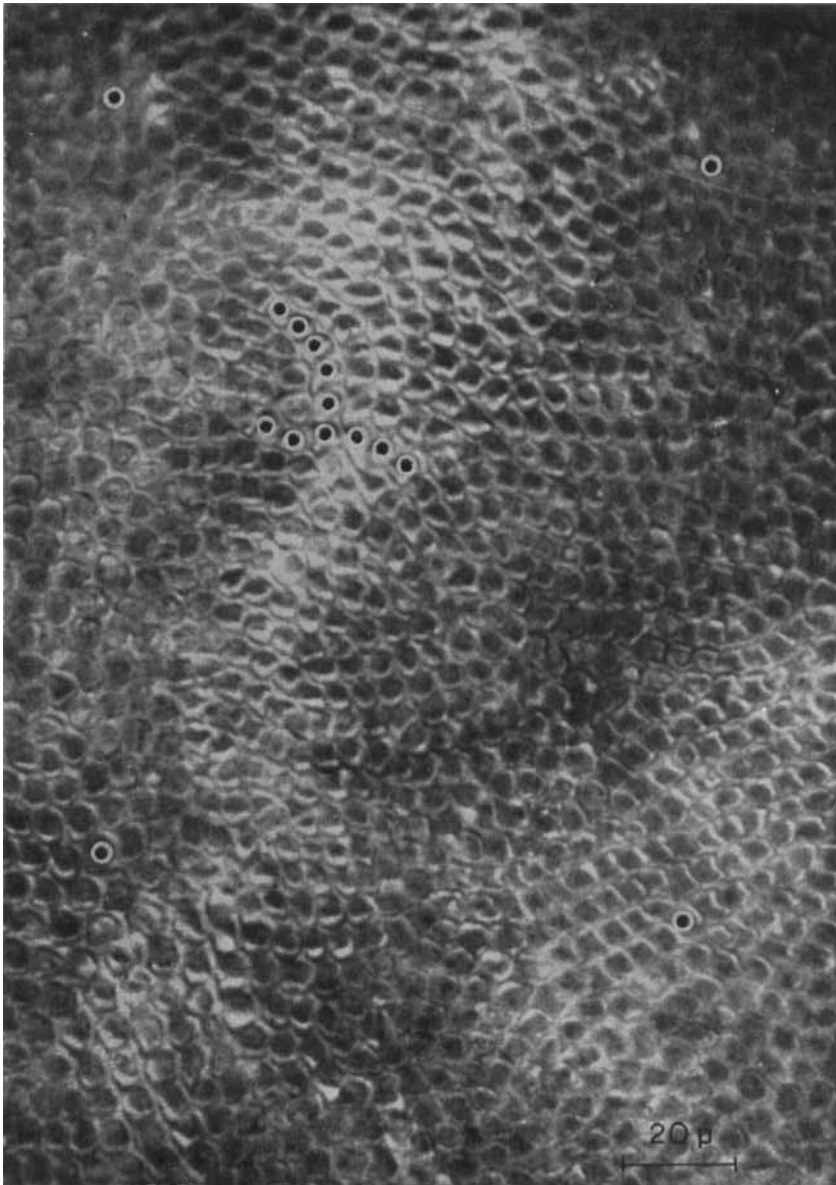


Fig. 11.

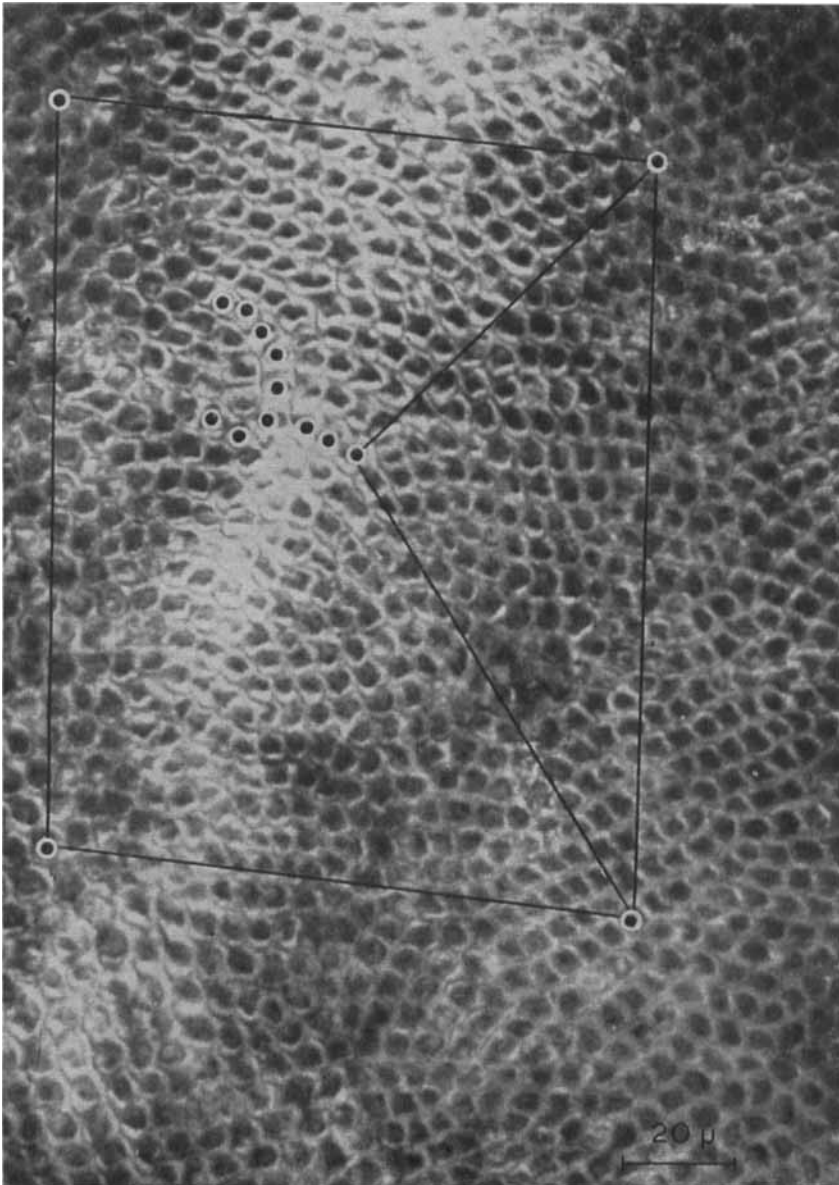


Fig. 12.

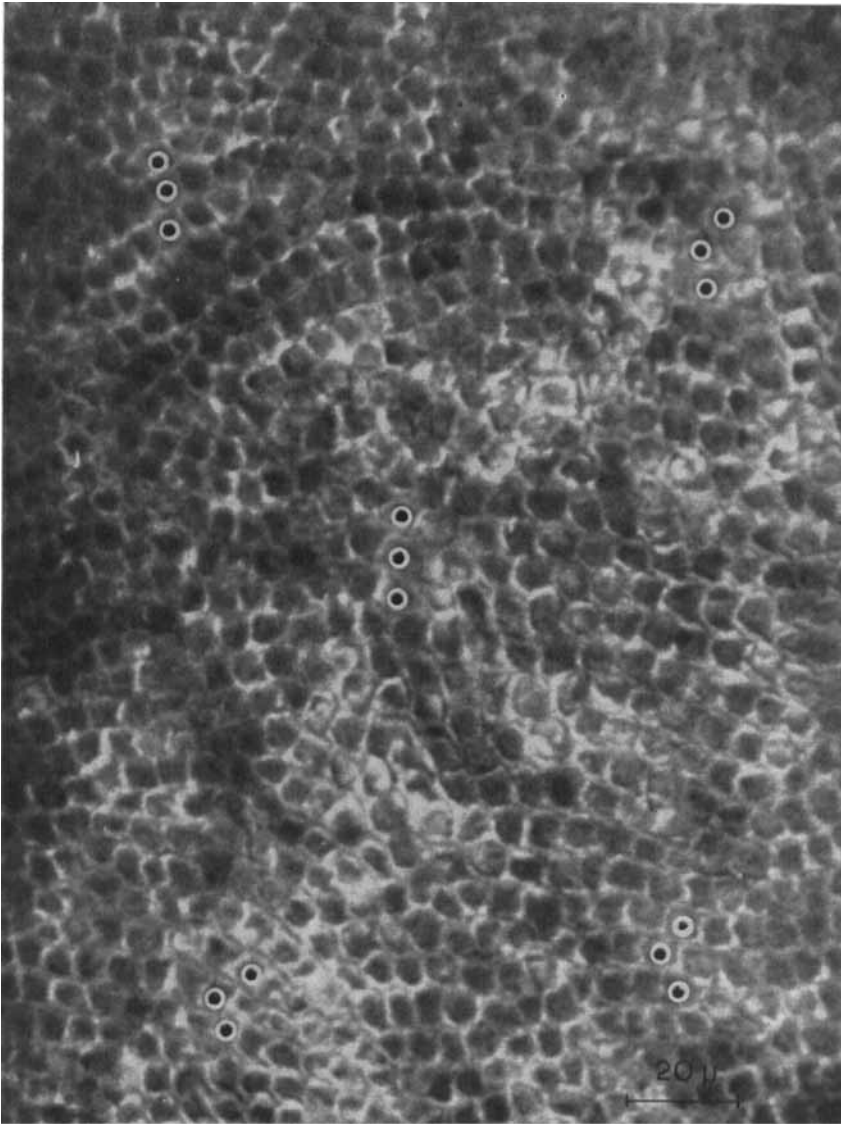


Fig. 13.

Figs. 13—15. The 5th, 19th and 20th enamel surfaces of a series with a periodic etching of 5 seconds with 0.5% HNO_3 . The mean distance between each pair of consecutive surfaces was 1.51μ . Five groups of prisms have been marked and followed through the series.

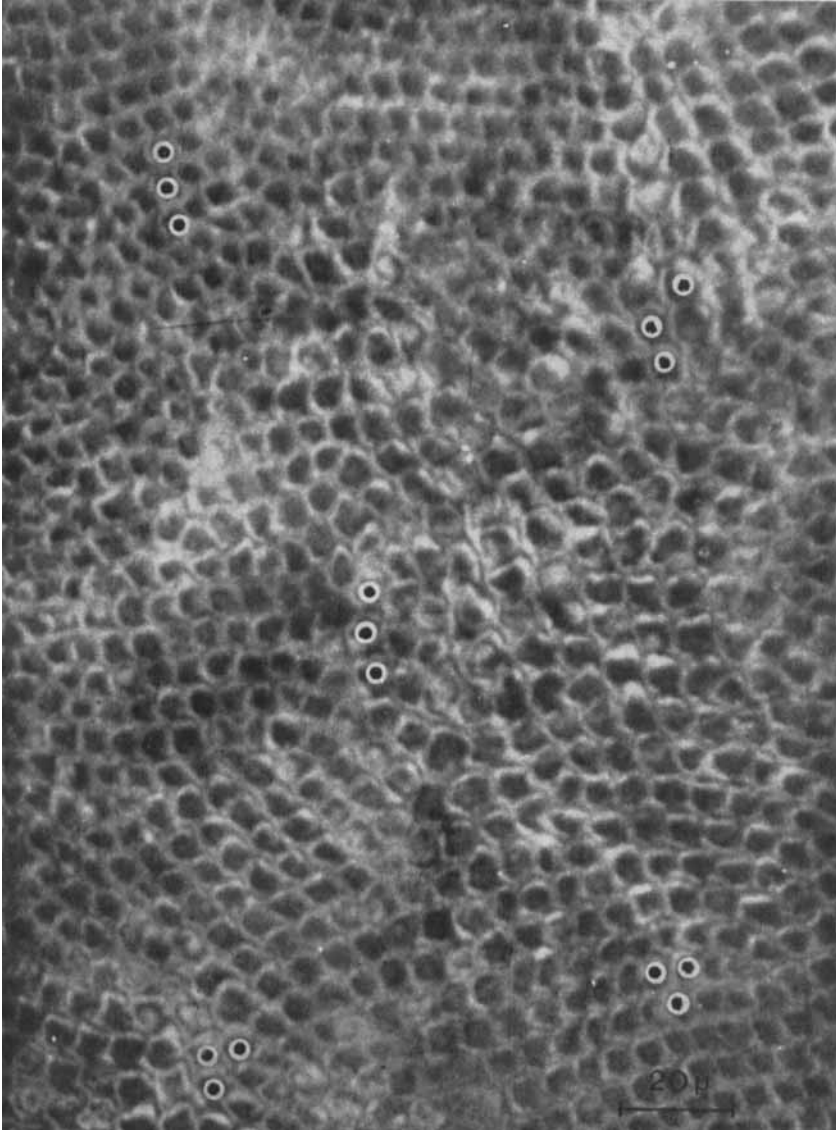


Fig. 14.

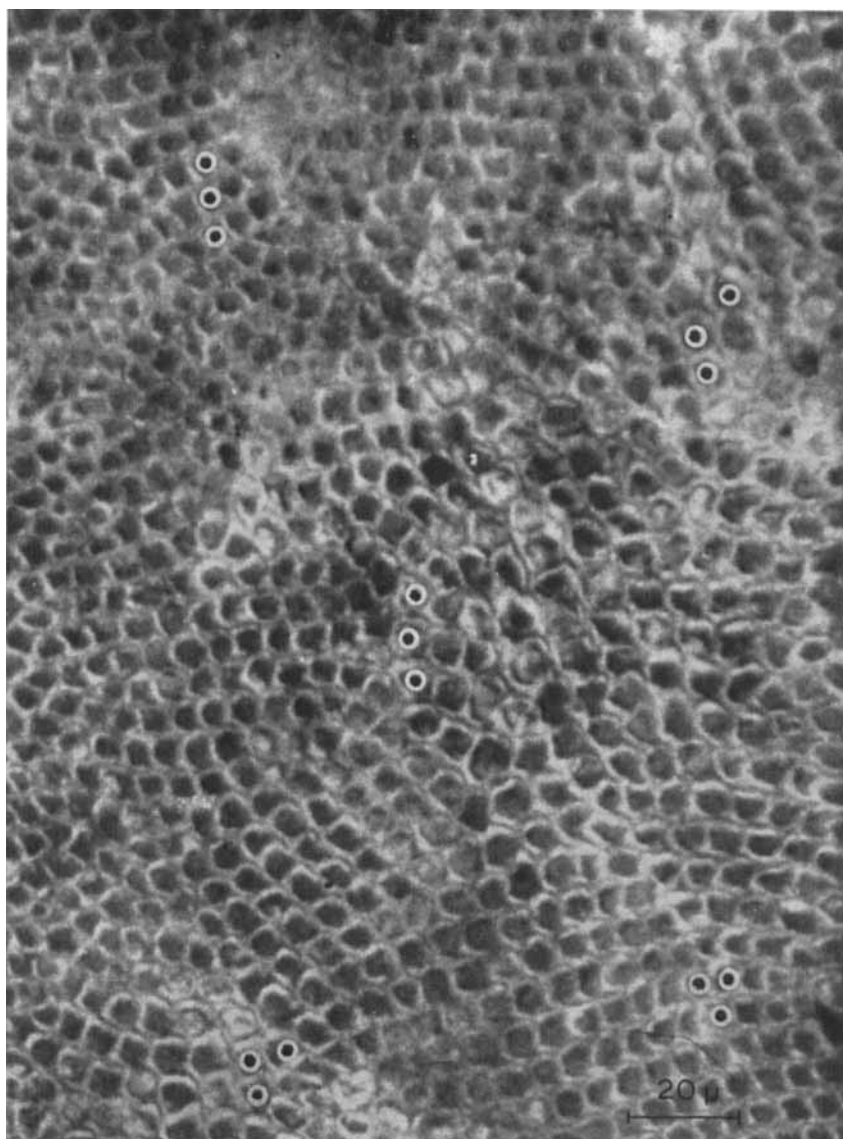


Fig. 15.



Fig. 16.

Figs. 16—19. The 4th, 14th, 15th and 16th enamel surfaces of a series with a periodic etching of 5 seconds with 1.3 % HNO_3 . Even though the interproximate distance was only 2.45μ , it was not possible to recognize individual prisms from one surface to the next.

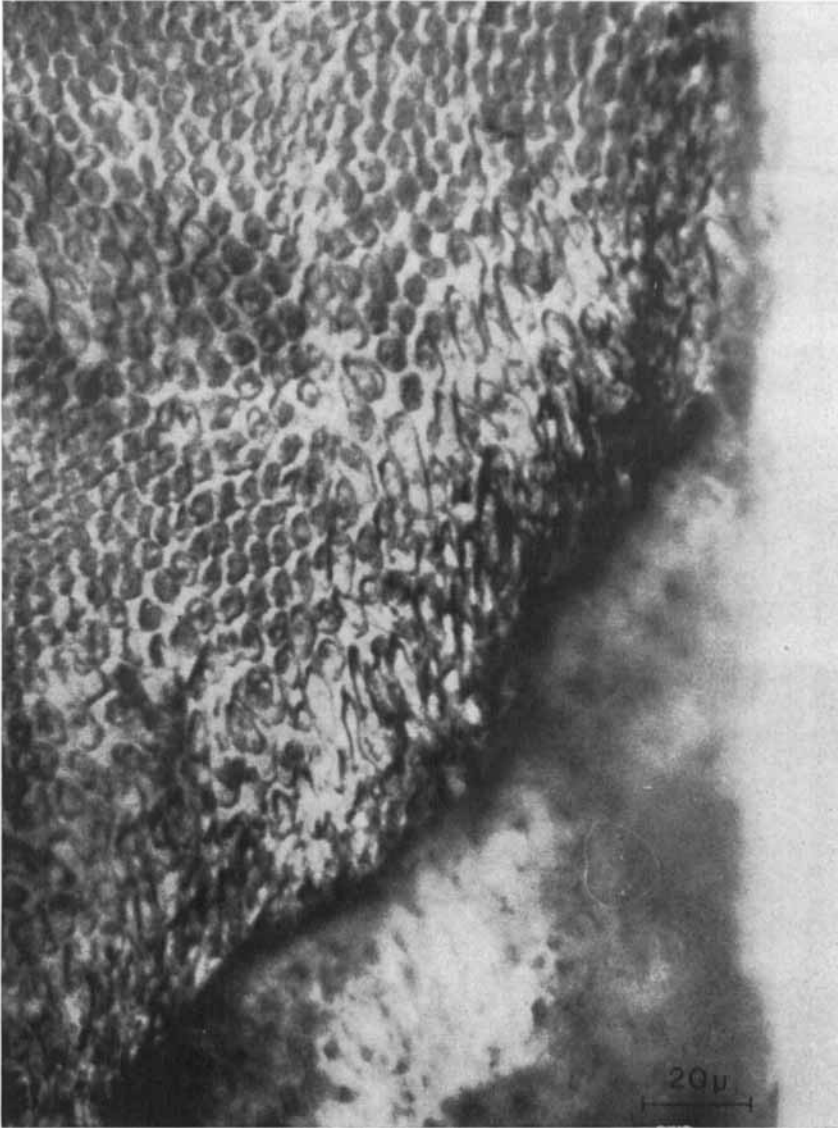


Fig. 17.

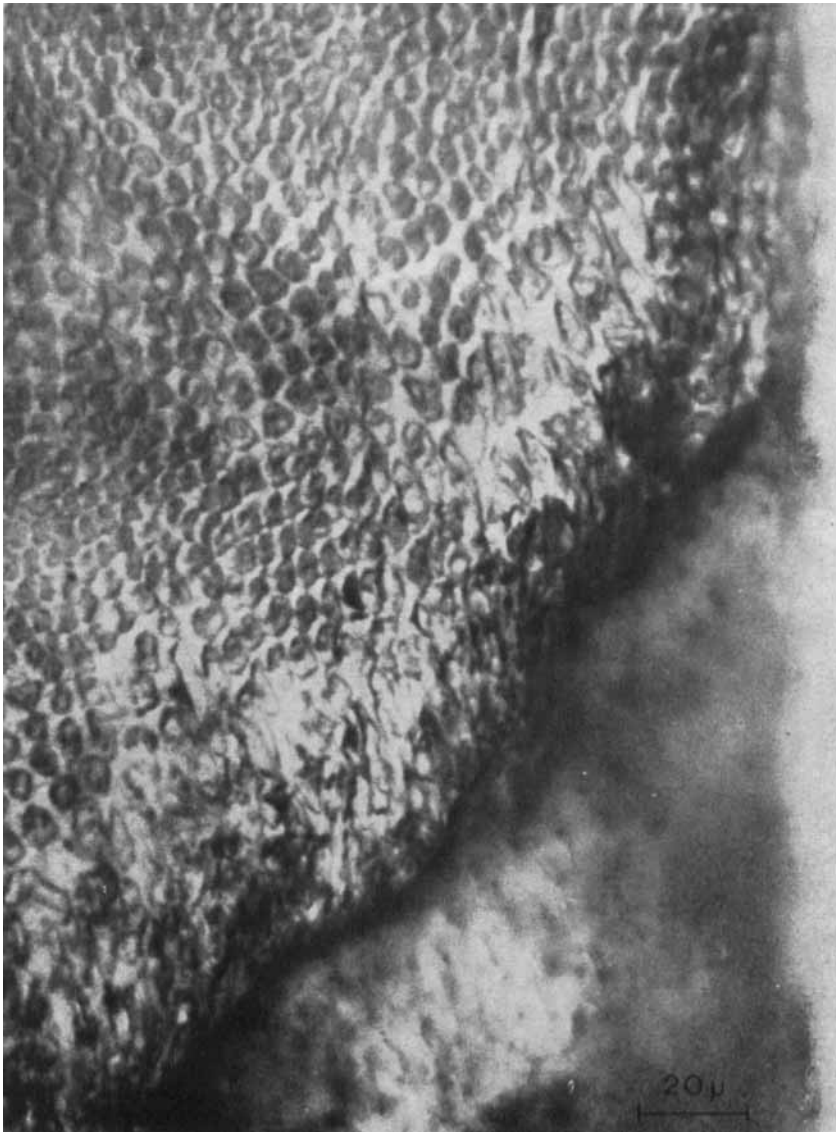


Fig. 18.

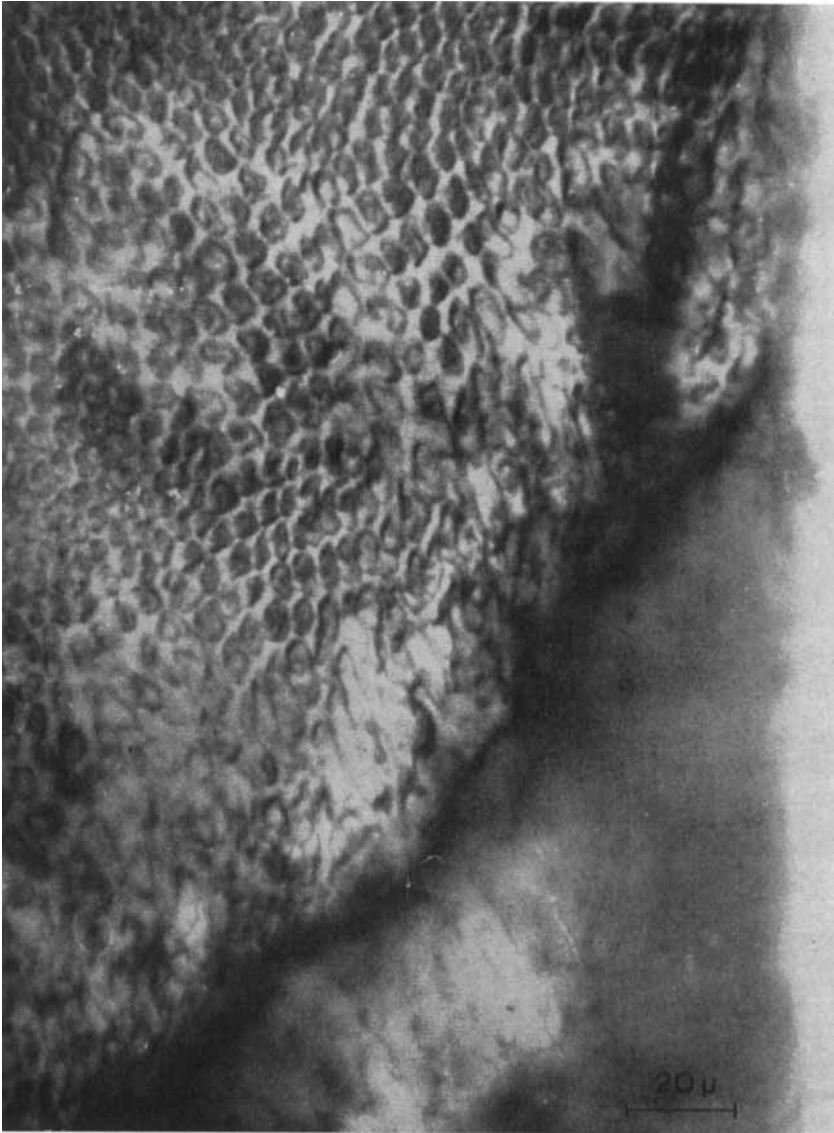


Fig. 19.



Fig. 20.

Figs. 20—21. The 16th and 17th enamel surfaces of a series with a periodic etching of 5 seconds with 5.2 % HNO_3 . The interproximate distance was 5μ . Even though the prism course is quite regular, individual prisms are not recognizable from one surface to the other.

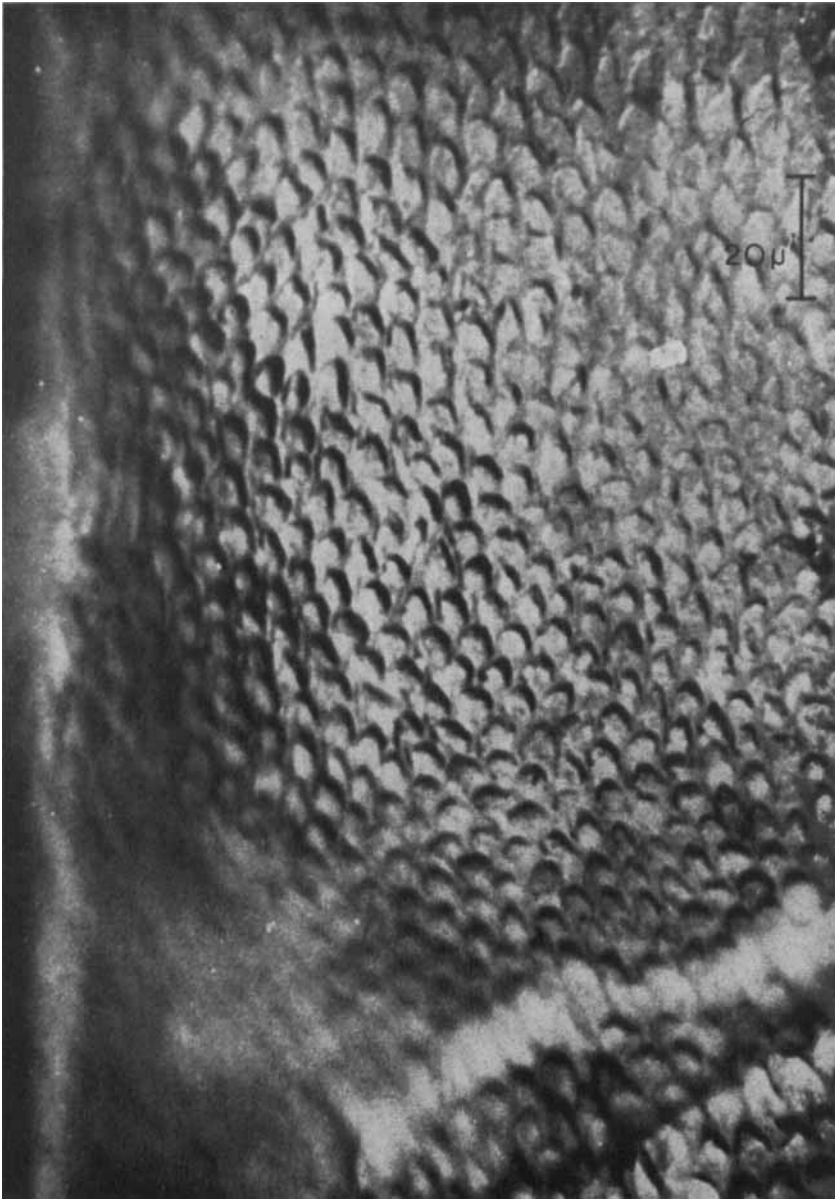


Fig. 21.