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TANDEM SCANNING REFLECTED LIGHT MICROSCOPY OF PRIMATE ENAMEL

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Abstract

Studies of the cross sectional packing arrangements of primate enamel prisms have been used in a number of recent studies in attempts to determine their taxonomic utility. Credibility of the results has been greatly influenced by the methods employed to examine enamel prism packing patterns and also by the limited sampling. We report here the use of a technique for the non destructive examination, in depth, of enamel prism packing patterns in modern and fossil primate teeth which has considerable advantages over any others so far used, and the preliminary results of a survey of enamel structural diversity in the Order Primates. The phylogenetic implications of these findings are also discussed.

A novel microscope, the Tandem Scanning Reflected Light Microscope (TSM) has been used. This instrument has allowed these data to be obtained non destructively which has permitted the inclusion of rare fossil primates in this survey. The technique has many advantages relating to the interpretation of the results as the specimens are not etched or otherwise prepared. Primates exhibit all three major prism packing arrangements known for recent mammals. The distribution of these permits the recognition of haplorhine from strepsirhine primates and also cercopithecoid monkeys from other catarrhines.

KEYWORDS: Primates; fossils; enamel; prisms; Hominoidea; Cercopithecoidea; Ceboidea; Haplorhini; Strepsirhini; Confocal scanning light microscopy.

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Introduction

The vast majority of fossil primate and hominid material available for scientific study consists of dental and skeletal specimens, both of which are formed of the hard tissues of the body. Teeth are covered by enamel which is the most highly mineralized tissue formed in mammalian biological systems; so highly mineralized that it is no exaggeration to describe the enamel in all of our mouths as being already "fossilized". It is because of this fact that teeth are preferentially preserved in the fossil record and more commonly found in excavations of palaeontological material. Thus a large proportion of the primate and hominid fossils found are in the form of isolated teeth. Although some remarkable fossil sites do preserve the soft tissues of Primates, for example Messel in Germany, this is a rare occurrence. In fact, a number of sites, such as Pasalar in Turkey (a middle Miocene site) have so far yielded only dental remains of primates (Andrews and Tobien, 1977). Although such sites provide an abundance of dental specimens, the extent to which their external morphology can be used to inform us about the course of primate evolution is limited.

It is clearly necessary to extract the maximum amount of information from the skeletal and dental remains which are available to us. The contribution of microscopic studies to our understanding of primate and human evolution is already evident. Work on the patterns of dental microwear, observed by replicating worn tooth surfaces, may yield evidence as to the actual food items included in the diet of extinct species (Walker et al., 1978) Replicas of bone surfaces provide information about the patterns of bone growth in Plio-Pleistocene hominids (Bromage, 1986). Both of these methods are non-destructive and non-invasive and can be safely applied to even the most valuable fossil specimens.

There is a further area to which microscopic studies of internal structure can contribute significantly. The enamel of primate teeth is divided into structural units known as rods (American) or prisms (English) which are about 6-7 μm in diameter and which extend from the enamel-dentine junction (inside the tooth) to the tooth surface. The cross sectional shape of these prisms varies among mammals (Boyde, 1964) and

among Primates (Shellis and Poole, 1977; Shellis, 1984; Boyde and Martin, 1982; 1983; 1984a,b; Gantt et al.,1977; Gantt, 1979; 1980; 1983; Vrba and Grine, 1978a,b) and the enamel prism packing patterns may provide information about the relationships of fossil species to one another and to living forms.

When viewed in longitudinal section the prisms are seen to be marked with transverse bands at regular intervals, and these provide information about the way in which the tooth grew. The value of such incremental data in interpreting the development of enamel thickness has been shown by Martin (1983; 1985; Martin and Boyde, 1984). Doubts have been expressed as to whether these features, generally observed by SEM might in fact be artefacts and the ability to examine them in layers of the enamel which have not been subjected to specimen preparation allows this controversy to be resolved, (Warshawsky, 1984).

Obtaining data on enamel prism packing patterns and incremental features has previously necessitated destructive methods to get inside the tooth surface (Shellis and Poole, 1977; Shellis, 1984; Gantt et al., 1977; Vrba and Grine, 1978a,b; Boyde and Martin, 1982; Martin, 1985). While this can undoubtedly be justified for small samples of teeth that belong to well represented fossil species, we believe that we should try to discover less destructive, or ideally non-destructive, methods to obtain the same information. This would mean not only that we would not have to damage any fossils, but also that we could collect data from a larger number of specimens. The purpose of the present paper is to report our significant successes in this area using a direct view confocal scanning optical microscope.

Materials and Methods

Materials

In the present study we have used permanent teeth in situ in skulls and mandibles as well as isolated teeth. The material came from the collection of the Department of Anatomy, University College London (UCL), the British Museum of Natural History (prefix M) and from private collections. The following is a list of the species we had studied in the TSM prior to the end of 1985. The numbers in brackets after each species are the number of individuals and the number of teeth respectively.

Hominoidea: H. sapiens (>50, >50), **Gorilla gorilla** (5, 15), **Pan troglodytes** (4, 14), **Pongo pygmaeus** (6, 19), **Hylobates lar** (2, 6).

Cercopithecoidea; Cercopithecus sabaeus (1, 4), **Papio papio** (2, 6), **Cercocebus aterrimus** (2, 4), **Mandrillus sphinx** (1, 3), **Colobus polykomos** (1, 3).

Ceboidea: Cebus sp. (1, 2), **Lagothrix** sp. (1, 3), **Pithecia** sp. (1, 3), **Alouatta** sp. (1, 2), **Leontopithecus** sp. (1, 2), **Callicebus** sp. (1, 3), **Callithrix** sp. (1, 2).

Tarsioidea: Tarsius spectrum (1, 3).

Lemuroidea: Lemur catta (1, 4), **Propithecus coronatus** (1, 4), **P. diadema** (1, 3), **Cheirogaleus milii** (1, 3), **Daubentonia madagascarensis** (1, 5).

Fossil material: Kenyapithecus africanus (1, 2), **Dendropithecus macinnessi** (1, 1), **Paranthropus boisei** (2, 2), **Limnopithecus legetet** (1, 1), **Procunsul major** (1, 1), **Sivapithecus sivalensis** (1,1), **S. punjabicus** (1, 1), **S. darwini** (2, 2), **S. alpani** (2, 2), Kazinga Incisor (1, 1).

Methods

The principal method employed in this study is Tandem Scanning Reflected Light Microscopy. The Tandem Scanning Reflected Light Microscope (TSM) is a type of confocal scanning light microscope which allows the observation of a real time image of internal structure (Petran et al., 1985).

The TSM may be used to examine teeth without any specimen preparation. It simply requires that the specimen be linked to the objective lens by a suitable immersion medium. We have used microscope immersion oil (refractive index 1.51).

The Tandem Scanning Reflected Light Microscope (TSM)

Reflecting light microscopes are generally used to provide information about the surface of a specimen. Although some of the incident light does penetrate the specimen and does get reflected back to the operator in a conventional reflected light microscope, the information from internal layers is "swamped" by the information reflected from the specimen's surface and the same internal reflections. When we examine a tooth using a standard reflected light microscope we are unable to see any details of internal structure by looking at the specimen from the outside. It is also very difficult to make a good image of the surface of the specimen unless it is coated with metal as is normal in SEM practice.

Our model of the microscope is shown in Figure 1. The light passing through the microscope is intercepted by an aperture disc containing a very large number of holes, each of which is at a unique radial distance from the centre of the disc, with the exception of its matching partner on the opposite side of the same diameter. This aperture disc, or Nipkow wheel, is located at the intermediate image plane of the objective lens which thus images the apertures in the focussed plane in the specimen. Light which is reflected from the focus plane is imaged by the objective back into the plane of the Nipkow disc, but on the opposite, observation, side. The arrangement of mirrors ensures that the pattern of holes on one side of the disc exactly images that on the opposite side.

Light which is reflected from out of focus planes is largely intercepted by the solid parts of the disc, so that high contrast is only obtained for portions of the specimen lying in the plane of focus. Thus the TSM is able to select a given focus plane deep to the surface of the specimen, eliminating the interference caused by reflections in the more superficial and the deeper layers of the specimen, which makes ordinary reflected light microscopes useless in the fields of investigation which we are considering here. Figure 2 is a simplified ray diagram which shows how this works.

This system means that, given a good high magnification objective lens of high numerical

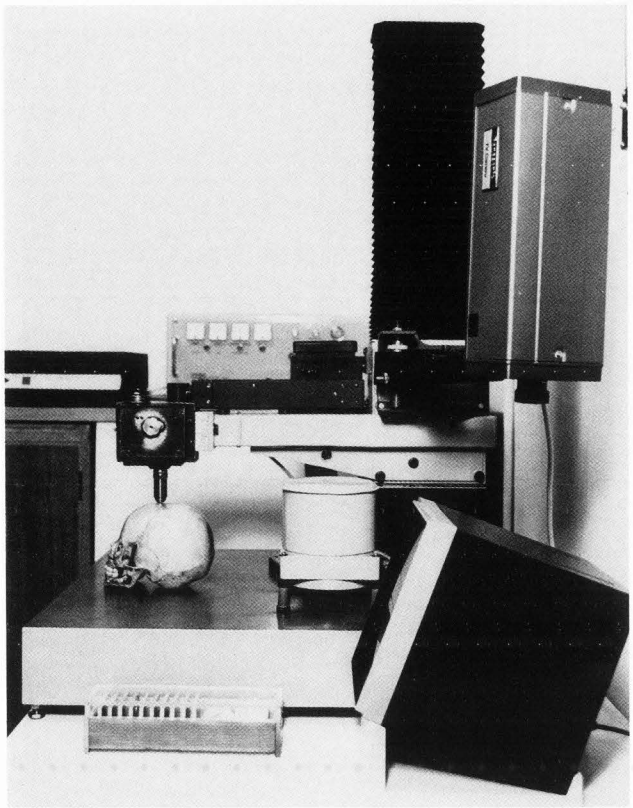


Figure 1. Photograph of the Tandem Scanning reflected light Microscope at UCL.

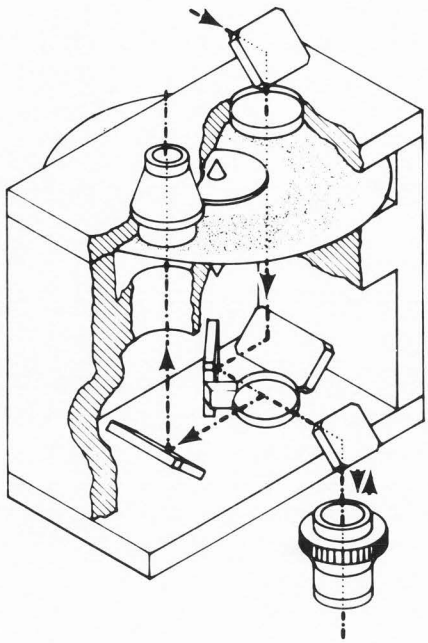


Figure 2. Diagram showing the construction of the TSM head. Light enters top, reflected by a mirror to pass a field lens placed close to the 4 inch, 1% transmissive aperture disc, with 17,600 approx. 30 micron diameter holes in a pseudohexagonal array on Archimedean spirals. Light passing the disc is reflected twice before passing a beam splitter: then is reflected downwards to enter the 160 mm tubelength RMS objective. Light reflected in the same specimen passes back through the same lens, off the same final mirror, to be reflected by the beam splitter; thence one more reflection before reaching the observation side of the disc. Light only reaches the instantaneously lit patches in the focussed-on plane from apertures matching one for one those on the eyepiece side; and only light from that plane can return through the disc. Other light hits solid portions of the disc. The last optical component is a Ramsden type eyepiece used to observe the image in the scanning disc.

aperture, the observer sees instantly a sharply focussed image from a layer of the specimen equivalent to a vertical thickness of =< 1 µm. The ability of the TSM to produce optical sections is particularly valuable as it means that we can form an image of the surface of the specimen and then, using the fine focus control, move the plane of the optical section below the specimen's surface. The depth to which we can penetrate and form images depends both on the clarity of the specimen and on the physical characteristics of the lenses. With relatively clear materials (and we include fossil bones and teeth in this category), we can form images up to 200 µm below the specimen's surface. The image can be viewed directly by looking down the eyepiece. Records can be made by taking still photographs or by using a TV camera and video tape. Optical sections can then be linked in three dimensions by using a computer which processes photographs of successive optical slices. By rapid adjustment of the fine focus control the operator is able to build a mental image of a 3-D structure.

The depth of field can be increased by through focussing whilst recording a photographic image, Boyde (1985a). The same procedure repeated along two focussing axes can generate stereoscopic pairs (Boyde, 1985b).

Enamel microstructure. The main thrust of our initial work with the TSM has been in determining its potential for the taxonomic assessment of fossil primates. This has concentrated on documenting the enamel prism packing patterns in a variety of fossil specimens. It is clear that an absolute interpretation of our findings will require a thorough knowledge of the enamel prism packing patterns in every species of living primate. Our initial survey of the primate order indicates a number of key branching points in primate evolution at which enamel structure has been modified. These results already have potential for addressing the relationships of some fossil primates of uncertain affinities and we would expect to find further differences when a more complete study of enamel structure in living Primates is completed.

TSM Protocol. We attempted to determine the common prism packing pattern in each species. We did not keep a **detailed** record of how much tissue we studied in each tooth but worked to a routine. We used a 100/1.3 oil immersion objective giving a field circle area of 13,300 μm^2 equal to roughly

330 Pattern 3 prisms. At each site we focussed through the surface layers to a depth usually greater than 40 µm, or more than 30 µm of prismatic enamel. As the depth of field is less than one micron, this represents roughly 10,000 "samples" per location. We moved to ten or more locations per tooth and examined two to four teeth from either the maxilla and mandible. We used the buccal surfaces of the first permanent molars, the second permanent molars, the second premolars, the first premolars and the third molars in that general order of preference, depending upon the degree of preservation of the individual teeth in the individual specimens. This level of surveying could be completed in one hour, perhaps 30 mins in a favourable case, excluding the time necessary for photographic recording.

Results

Enamel prism packing patterns in primates fall into three major categories (Boyde, 1964; Boyde and Martin, 1982; Shellis and Poole, 1977; Shellis, 1984). In Pattern 1 enamel the prism boundaries are complete and enclose circular prisms (rods). In Pattern 2 enamel the portion of the prism boundary away from the biting surface of the tooth is open, giving a horseshoe kind of pattern. In this pattern of enamel the open end of the horseshoe faces the closed end of the prism boundary in the next row. In Pattern 3 enamel the prism boundary is similarly open at one end, but in this case the open end faces the space in between two prism boundaries in the row below (Boyde, 1964).

The main thrust of the present investigation was to provide some evidence as to the distribution of these enamel prism packing patterns in taxonomic groupings of primates. We were particularly concerned to try to discover differences in enamel prism packing patterns at familial, superfamilial and subordinal levels. The results are therefore presented taxon by taxon. The possible phylogenetic significance of the results together with a comparison of our findings with previous studies are presented in the discussion section of this paper.

Hominidae

We have examined enamel prism packing patterns in the species **Homo sapiens** (Fig 3) and **Paranthropus boisei** (Fig 4). In both cases, the outer layer of enamel was prism-free to a depth of about 6-10 µm from the unworn tooth surface. Deep to this layer a very thin (generally a few microns) layer was often encountered in which Pattern 1 prisms (Boyde, 1964) occurred closest to the prism free surface layer and these then became Pattern 3. The depth at which the transition from Pattern 1 to Pattern 3 occurred was variable from one prism to its neighbour so that in the subsurface regions a mixture of Patterns 1 and 3 enamel were often encountered. In all cases, Pattern 3 prisms predominated once the optical sectioning had proceeded more than 15 µm below the specimen surface. The patterns seen in Hominidae were also encountered in the middle Miocene taxon **Sivapithecus**.

Figures 3 to 29 are photographs taken with the TSM looking through (mostly) mid-lateral buccal surfaces of permanent molar teeth (Figs 3, 4, 6-27 and 29): Fig 3 looks through a cut surface: Fig 28 looks at a "polished" surface. The photographs were recorded using an Olympus OM2 back with automatic metering, using a 50 mm lens focussed at infinity placed over the TSM eyepiece. The film used was Ilford FP4, except for Figs 8 - 10 which are printed from colour negative film of the same speed (ASA 125). The objective used was a 100/1.3 oil immersion lens. The trimmed field width of each frame is 80 µm (except Fig 5).

Figures 3 to 14.

3. Homo sapiens: DI^2.
4. Paranthropus boisei: M_1.
5. Gorilla gorilla: M^2. Longitudinal section. 10X oil objective. FW = 800 µm.
6. Cercopithecus sabaeus: M_2.
7. Cercopithecus sabaeus: I_1.
8. Papio papio: M^1.
9. Papio papio: M^1.
10. Mandrillus sphinx: M^1.
11. Mandrillus sphinx: M^1.
12. Mandrillus sphinx: M^1.
13. Callicebus sp.: M^1.
14. Pithecia sp.: M_1.

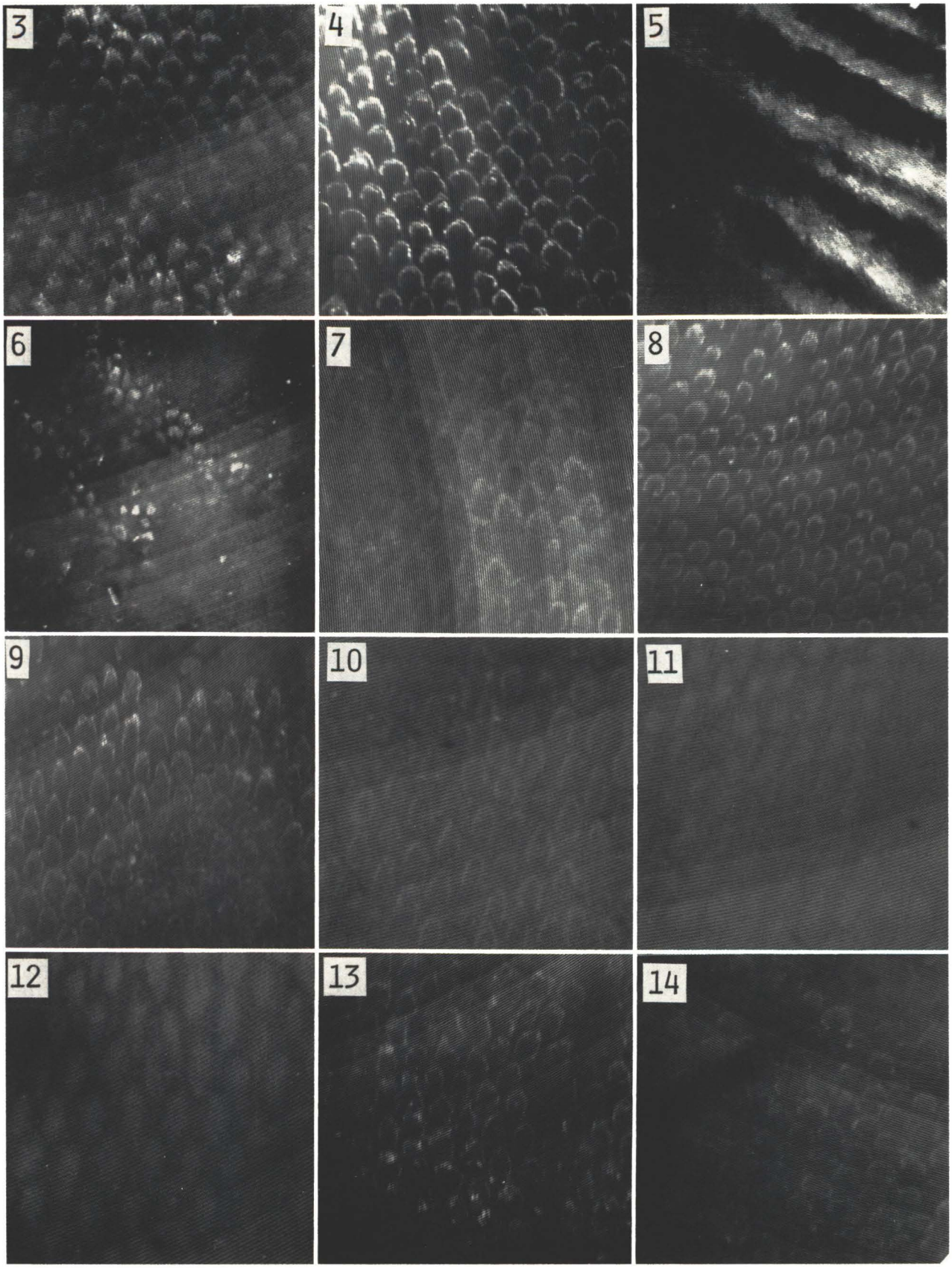
Field width (Figs. 3, 4, 6-29) = 80 µm.

Gorillidae

We have examined teeth from **Gorilla gorilla** (Fig 5) and **Pan troglodytes**, concentrating on the molar teeth. In both taxa the same overall pattern emerged. The prism-free surface layer was of the same thickness as found in Hominidae and at all locations on the tooth. This layer was underlain by Pattern 1 enamel but whereas in Hominidae this was of very limited thickness in the Gorillidae it was of substantial thickness. Over most of the tooth crown the Pattern 1 prisms were the only ones encountered at depths up to about 100 µm. However, towards the tooth cervix where the enamel is thinner (Martin, 1983) the Pattern 1 layer was less thick and could be determined to overlie Pattern 3 enamel. On the basis of work on longitudinal sections (Martin, 1985), developing enamel surfaces (Boyde and Martin, 1982) and deep, mature enamel (Shellis and Poole, 1977) it is clear that the predominant prism packing pattern in the deep enamel of Gorillidae is Pattern 3. Martin (1983) proposed that the Pattern 1 layer represented a region of slowed down enamel secretion which was found at a relatively constant distance from the enamel dentine junction. Given the distribution of enamel thickness over the tooth crown this would mean that the Pattern 1 layer would be of greatest thickness high up the tooth where the enamel was thickest and would become less thick cervically. This means that towards the tooth cervix, Pattern 3 grades through Pattern 1 to prism-free enamel in the same way as is seen in Hominidae.

Pongidae

The arrangement found in **Pongo pygmaeus** resembled that seen in Gorillidae in as much as that there was layer of considerable thickness of Pattern 1 enamel although it differed in that this layer was more restricted to portions higher up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the crown than had been the case for the Gorillidae. According to Martin (1983, 1985) this is due to the fact that a greater proportion of orang utan enamel is fast formed (i.e. Pattern 3) than is the case in Gorillidae. Consequently, the Pattern 1 layer is less thick at any point on the tooth crown and extends less far down the tooth crown than is the case in chimp and gorilla.

Hylobatidae

Hylobates lar samples exhibited the same arrangement of prism packing patterns as was found in Hominidae and in **Sivapithecus**, namely Pattern 3 extending to within a few microns of the tooth surface.

Cercopithecidae

A considerable variety of arrangements were found within this family and are therefore detailed according to groups of species and genera which exhibited similar arrangements. This survey has by no means sampled the entire spectrum for this diverse family and any conclusions must therefore be somewhat tentative, but the variation encountered offers considerable promise for taxonomic studies based on enamel. The main variations found differed in terms of the thickness of the prism-free layer and the relative proportions of Patterns 1, 2 and 3 enamels. Unfortunately, few data are presently available to relate prism packing patterns to formation rates and it may be unwise to assume that the correlations found among hominoids should apply to the Old World monkeys.

Cercopithecus sabaeus

Molar teeth from this species were found to be covered with a thin layer of prism-free enamel which ranged in thickness from 4 µm to 14 µm. Deep to this was a very thin region in which Pattern 1 prisms were found and these often had a solid appearance (Figure 6), i.e. the prism boundary was not distinct from the prism head. Deep to this layer was Pattern 3 enamel (Figure 7) which could be seen, by focussing up and down, to be strongly decussating. In any field some prism boundaries were very elongate while others were very broad which reflects oblique sectioning of the prisms resulting from the decussation. If the correspondence between prism packing patterns and rate of secretion found in hominoids applies also to cercopithecoid enamel then these observations would mean that all of the enamel was fast formed.

Papio papio

Molar teeth of this species were overlain with a prism-free layer which ranged in thickness from 6 µm to 20 µm. This layer graded into Pattern 1 enamel (Figure 8) and then into Pattern 2 and Pattern 3 enamel (Figure 9). Considerable portions of both Patterns 2 and 3 were found in this species, sometimes interspersed with one another and sometimes in clear fields of one or other type.

Mandrillus sphinx

The prism-free layer in this species is very thin and is underlain by a layer of Pattern 1 prisms (Figure 10) and deep to that layer is Pattern 2 and Pattern 3 enamel. Some optical sections show very clear Pattern 2 enamel (Figure 11) but layers immediately above and below these show Pattern 3 enamel (Figure 12).

Cercocebus aterrimus

This species was found to have a thin layer of prism-free enamel overlying some Pattern 1 enamel. Deep to these surface layers the enamel was often Pattern 3 with prisms widely spaced, i.e. enamel with a large interpit phase component, but there was also Pattern 2 enamel.

Colobus polykomos

In this species the Pattern 1 surface layer was found to be two to four microns thick and was underlain by a mixture of Pattern 2 and 3 enamel.

Cercopithecoid enamel may therefore often be distinguished from hominoid enamel by the far greater frequency with which Pattern 2 enamel is found together with Pattern 3 enamel. This was true for all of the species studied here except for **C. sabaeus** which had no significant amount of Pattern 2. None of the Old World monkeys showed a significant thickness of Pattern 1 enamel which might suggest that they all form their enamel relatively quickly compared to the extant great apes.

Cebidae

Callicebus

The subsurface enamel in this taxon contained a mixture of Pattern 2 and Pattern 3 enamel with a predominance of Pattern 3 (Figure 13).

Pithecia

Immediately deep to the surface layer of prism-free enamel was a layer of Pattern 1 enamel which overlies Pattern 3 enamel which, in this taxon, was a very tight horseshoe arrangement (Figure 14).

Alouatta

The prism-free layer in this taxon was about 8 µm thick and was underlain by Pattern 1 enamel. The enamel deep to these superficial layers was Pattern 3 (Figure 15).

Cebus

This genus had Pattern 1 enamel close to the tooth surface which was underlain by Pattern 3 enamel (Figure 16).

Lagothrix

In this species, the prism-free surface layer was about 10 µm thick and was underlain by a considerable amount of Pattern 1 enamel (Figure 17). The enamel deep to these superficial layers was difficult to image but appeared to be Pattern 3.

Callitrichidae

Callithrix

The subsurface enamel in this species was found to be well defined Pattern 2 enamel (Figure

18). Boyde and Martin (1982) have previously reported enamel in this genus to be strongly decussating Pattern 1 enamel so there would appear to be a degree of variability in enamel prism packing patterns in this genus.

Leontopithecus

Molar teeth of this taxon had a prism-free surface layer of about 16 µm thickness underlain by a thin layer of Pattern 1 enamel. Deep to the superficial enamel was Pattern 2 enamel with areas of Pattern 3 enamel (Figure 19). The enamel decussation was very marked when through focussing and showed a sinusoidal pattern from cusp to cervix.

Tarsiidae

Tarsius

In the example of this taxon which we examined, it was possible to focus all of the way through from the tooth surface to the dentine. the enamel was thin, about 100 µm, but even so this was remarkable and might offer an interesting way to examine the enamel dentine junction by visualizing it intact. In the specimen which we studied, we found Pattern 1 enamel just deep to the tooth surface but throughout the rest of the thickness we found Pattern 3 enamel (Figure 20) all the way through to the enamel-dentine junction. This finding contrasts with an SEM study by Grine (see Martin, Boyde, and Grine, 1988) in which only Pattern 1 enamel was found. It is presently unclear how to account for this discrepancy in our findings unless the layer sampled by Grine was very superficial or there is considerable inter or intraspecific variation in enamel prism packing Patterns in tarsiers. We must await a study of further material which can be accurately identified at the species level to resolve this important question for this pivotal taxon.

Lemuridae

Lemur catta

In this species there is thin prism-free surface layer, deep to which there is non-decussating Pattern 1 enamel extending to the maximum depth visible by the TSM. The prism outlines are circular (cf. **Propithecus**).

Cheirogalidae

Cheirogaleus milii

In the specimen of brown mouse lemur which we examined it was very difficult to obtain sufficient contrast to determine enamel prism packing patterns deep to the tooth surface. All of the prisms appeared to be Pattern 1 and some appeared "solid" while in other regions we found Pattern 1 prisms with complete prism boundaries which were distinct from the prism heads. The prisms in this taxon were all very small. We found no evidence of either Pattern 2 or Pattern 3 enamel.

Indriidae

Propithecus coronatus and Propithecus diadema

Teeth from these species had a prism-free surface layer of enamel which was about 10 µm to 12 µm thick. Deep to these superficial layers the prisms were very closely approximated which makes it difficult to decide which packing pattern is seen. We have convinced ourselves that there is Pattern 1 at the tooth surface with Pattern 2 (Figure 21) and Pattern 3 deep to that. In an upper canine, we found very clear Pattern 3 (Figure 22) which went back to Pattern 1 **deep** to that layer. When focussing up and down through the enamel strong prism decussation was evident with well marked left and right going zones about ten prisms wide. The prisms were all in very close contact which, as noted above, made recognition of packing patterns difficult but there was a tendency for prisms to line up in cuspal to cervical columns resembling Pattern 2.

Daubentoniidae

Daubentonia madagascarensis

In a lower first molar of this taxon, we were unable to resolve the prism packing pattern of the enamel but using a low power objective we determined that there was a prism-free layer of about 3 µm thickness over an apparently unworn tooth surface and that the occusal surface had clearly marked Hunter-Schreger bands running across it.

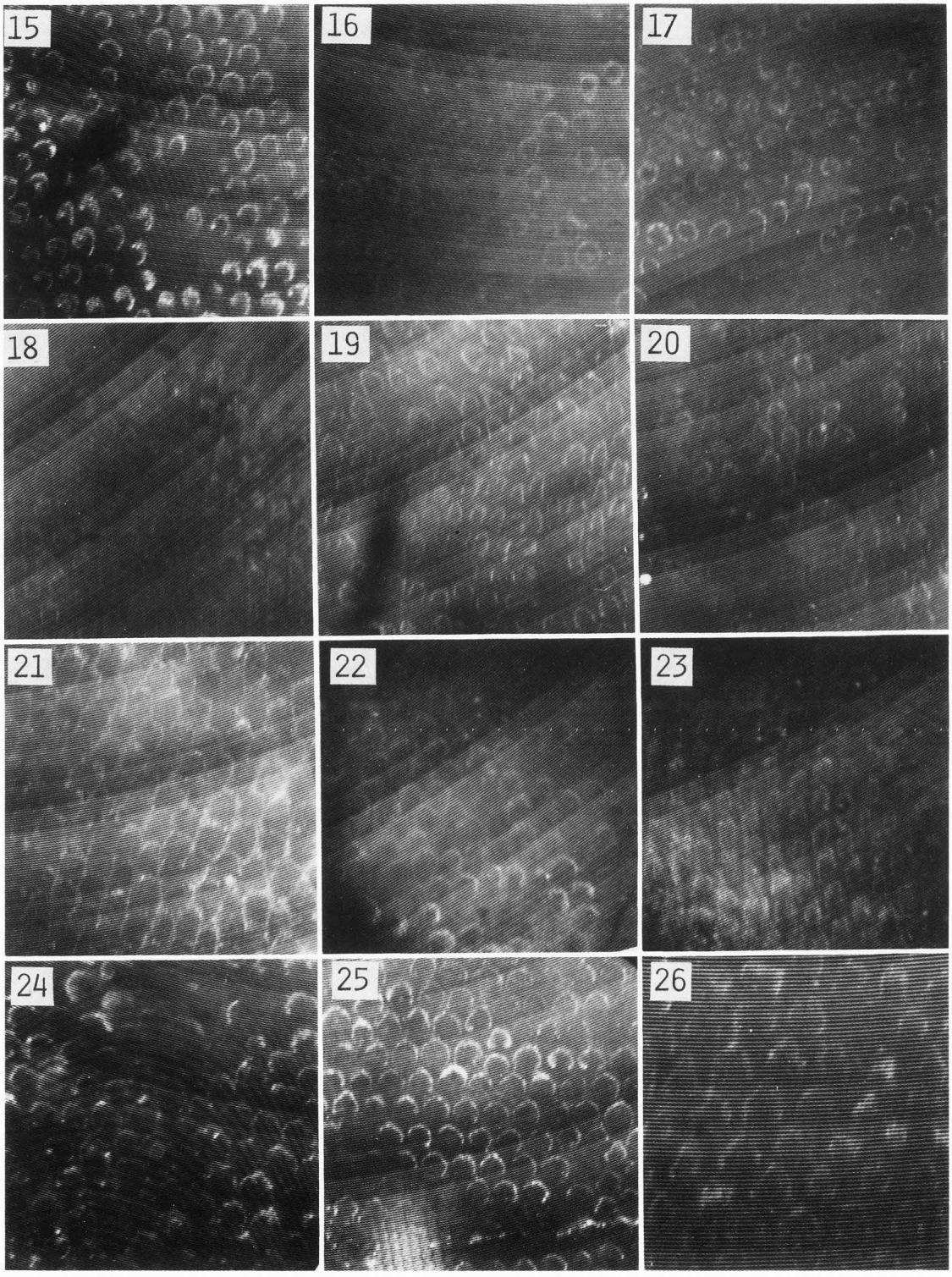
The lower incisor had a prism-free layer of about 10 µm thickness which was underlain by Pattern 2 enamel, which we saw very obliquely sectioned. This finding corresponds with the detailed SEM observations made by Shellis and Poole (1979). There is a tremendous amount of prism decussation with up to 90^{0} change of orientation between nearby rows. This decussation renders accurate determination of the prism packing pattern difficult. The enamel prism packing pattern in the deep enamel was mainly Pattern 2 (Figure 23) but there were some areas which appeared to be Pattern 3, although these could be focussed through to Pattern 2. There is a problem in recognising Pattern 2 from Pattern 3 enamel in very obliquely sectioned, decussating enamel but we feel sure that this species is characterized by Pattern 2 enamel as previously reported by Shellis and Poole (1977, 1979) for incisors and by Shellis (1984) in an upper molar, who also found that both molars and incisors have a layer of non-decussating Pattern 1 prisms just beneath the outer surface. In all areas, we encountered a large amount of interpit phase enamel.

Fossil specimens

Middle Miocene great apes

Kenyapithecus africanus (Holotype, M 16649)

We examined the buccal surfaces of the upper left M1 and P3 in this intact specimen. There was a thin prism-free layer, about 2 µm to 4 µm thick and deep to this a few microns thickness of Pattern 1. The enamel deep to these superficial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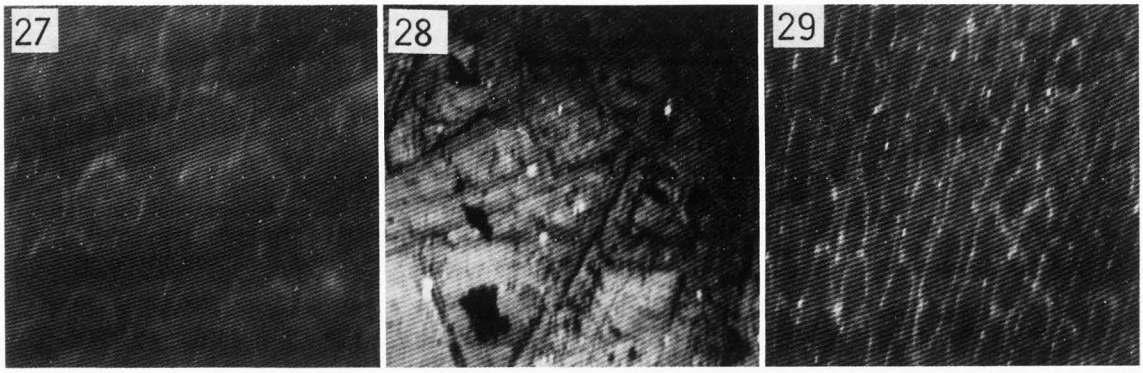

Figures 15 to 26

Field width = 80 μm.

15. **Alouatta** sp.: M^1.
16. **Cebus** sp.: M^1.
17. **Lagothrix** sp.: M^1.
18. **Callithrix** sp.: M^1.
19. **Leontopithecus** sp.: M^1.
20. **Tarsius spectrum:** M_1.
21. **Propithecus coronatus:** M^3.
22. **Propithecus coronatus:** M^3.
23. **Daubentonio madagascarensis:** I^1.
24. **Kenyapithecus africanus** M16649: M^1.
25. **Limnopithecus legetet** M14284: PM_4 (lower second premolar).
26. **Dendropithecus macinnessi** M14052: M^1.

Figures 27 to 29

27. **Proconsul major** M32237: M_3 lingual entoconid.
28. Kazinga incisor, surface image.
29. Kazinga incisor, focus 8 μm below surface.

layers was all Pattern 3 (Figure 24) so that the distribution of enamel prism packing types could not be distinguished from the patterns seen in hominids, **Sivapithecus** and in gibbons. If the enamel is thick, as appears to be the case, then this species shows no evidence of anything other than fast formed enamel.

The same distribution of enamel prism packing patterns was also seen in four species of **Sivapithecus, S. sivalensis, S. punjabicus, S. darwini** and **S. alpani** (BP 17) and in a specimen of "**Ouranopithecus**" as well as in a specimen of **Dryopithecus fontani** (IPS 68) although the enamel in this latter species was very impenetrable.

These finding are significant in so far as they relate to the rate of enamel formation which has been shown to correlate with prism packing patterns in hominoids (Martin, 1983, 1985; Martin and Boyde, 1984). In the absence of precise enamel thickness measurements it is impossible to be sure of the phylogenetic position for these taxa implied by these results. It is important however that all of the middle Miocene taxa which we have examined, **Sivapithecus, Kenyapithecus, Dryopithecus,** and "**Ouranopithecus**" have entirely fast formed enamel. This finding tends to confirm the interpretation of the evolution of hominoid enamel thickness proposed by Martin (1983, 1985) in as much as that the condition predicted for the common ancestor of the great ape and human clade is displayed by all known middle Miocene hominoid taxa which have been argued on other grounds to be more closely related to all great apes and humans than are the gibbons (Martin, 1986).

Early and middle Miocene catarrhines of uncertain affinities

Teeth assigned to **Proconsul major, Proconsul nyanzae, Proconsul africanus, Limnopithecus legetet** (Figure 25), and **Dendropithecus macinnessi** (Figure 26) all displayed the distribution of patterns described above for hominids, gibbons and middle Miocene great apes. This finding also tends to confirm that the relatively great thickness of Pattern 1 enamel which has been reported in great apes is a specialized characteristic which, at present, appears to be unique to them. In other words, no Miocene hominoids or catarrhines show any evidence for a reduction of enamel thickness by the mechanism of a reduced secretory rate.

The only variant in enamel prism packing patterns seen in the early Miocene taxa was found in **Proconsul major** which differed from other hominoids in an interesting, unexpected and unique way. In this taxon, the Pattern 3 prisms were of significantly larger size than have been encountered in any other taxon (Figure 27). Since this implies that its enamel secreting cells, the ameloblasts, must also have been large this is a surprising finding whose significance awaits further investigation. If further specimens of the same species show the same specialization then it would be unlikely that **P. major** could be ancestral to any other known hominoids which have normal size prisms. The finding is particularly surprising as prism packing patterns do not appear to change in size with animal size. In all other taxa with Pattern 3 prisms, ranging in size from gibbons to elephants, the prism areas are the same size.

Oreopithecus bambolii (IGF 10885)

This enigmatic Miocene primate has been considered a hominid, a hominoid and a cercopithecoid in various studies. Recently, the latter two interpretations have enjoyed favour and we were interested to determine whether it might show the high degree of Pattern 2 enamel which apparently characterizes Old World monkeys. The prism free layer was of very variable thickness, ranging from a few microns to 30 μm thick. This layer was underlain by a thin layer of Pattern 1 enamel and then by Pattern 3 enamel. In our

survey of much of the tooth we found no Pattern 2 enamel. As Pattern 2 enamel appears likely to be a shared derived feature of cercopithecoids this finding does not mean that **O. bambolii** is a hominoid as it resembles hominoids in what is probably a shared primitive characteristic. Neither does it lend any support to the interpretation of **Oreopithecus** as a cercopithecoid.

Discussion

The present study confirms earlier work with other methods which has indicated that many, if not most, primate (and other mammalian) species have a thin layer of Pattern 1 enamel over the whole tooth surface. This is usually of about 10-20 μm thick and represents the end of the secretory life span of the ameloblasts (the enamel forming cells) during a period in which they secrete enamel relatively slowly. However, most of the strepsirhine primates which we have studied (sampling the families Indriidae, Lemuridae and Lorisidae) have Pattern 1 enamel throughout the enamel thickness, the exceptions being **Propithecus** and **Daubentonia.** In the haplorhine primates we have studied (sampling the families Tarsiidae, Callitrichidae, Cebidae, Cercopithecidae, Hylobatidae, Pongidae, Gorillidae and Hominidae) we have found that the outer layer of Pattern 1 enamel is of variable thickness but is always underlain by an abundance of Pattern 3 enamel. In the old world monkeys (Cercopithecidae) this is mixed with large areas of Pattern 2 enamel - which makes it very easy to recognise monkey enamel from hominoid enamel. In all groups, the thickness of the outer Pattern 1 layer varies from species to species. This suggests that their enamel forming cells slow down secretion for different periods of time. The length of this slowing down period has already been shown to be especially significant in relation to enamel thickness in the hominoid primates (Martin, 1985) and when thoroughly studied may be of value in other groups.

Comparison with SEM studies of etched enamel

A provocative stimulus to recent work on primate enamel was provided by Gantt et al. (1977) who claimed to have discovered a dichotomy of enamel prism packing patterns among living hominoids. They reported that **Pongo**,(the orang-utan), **Gorilla** and **Pan** (the chimpanzee) displayed Pattern 1 enamel (in molars) while humans had Pattern 3 enamel.

A fossil specimen belonging to the species "**Ramapithecus**" **punjabicus** subjected to the same preparative method was found to share the human pattern and, therefore, held to represent a hominid. The ability to recognise a hominid fossil from even a small dental sample received considerable acclaim The method that Gantt et al. used was to etch the teeth in 10% Hydrochloric acid (HCl) for 2.5 minutes to remove the overlying prism-free surface layers and "etch-up" the prism patterns. In earlier publications,Boyde (1964) and Shellis and Poole (1977) had noted a distinction between the structure of the outermost enamel layer and the deeper enamel. Vrba and Grine (1978a, 1978b) set out to validate Gantt et al's results using the same technique applied to a sample of great ape and human teeth, and also to a specimen of **Australopithecus.** They were, however, unable to do so: they found no evidence for a dichotomy in enamel prism packing patterns in hominoids, but rather that all great apes and humans displayed the same arrangement: Pattern 3 enamel.

Boyde et al. (1978) published the details of their earlier study of the effects of various etchants on 70% ethanol preserved, wet enamel (Boyde, Jones and Reynolds, 1972). They showed that 10% HCl would be liable to produce etching effects which would render interpretation of the enamel prism packing patterns difficult. They recommended a milder etching regime, such as 0.5% H_3PO_4 for wet (recent, modern unfixed, not dried) teeth and mild HCl for fossil teeth (in which there would be little organic component to exploit). An earlier study by Boyde (1964) was not prone to these problems as he studied the developing enamel surface in its natural state following the removal of cell debris. A further point was that his study also examined the three-dimensional arrangements of the prisms and revealed that any two-dimensional analysis would be liable to over-simplification of the true situation. These points were adapted by Gantt (1980) to discredit Vrba and Grine's comments on his findings. The issue was, subsequently, clarified by study of the developing enamel surface in hominoids by Boyde and Martin (1982; 1983; 1984a). This work concentrated on the three dimensional analysis of enamel prism packing patterns and was not subject to any etching problems as the enamel surface was examined without etching. The study concurred with the findings of Vrba and Grine that Pattern 3 enamel prism packing patterns was the predominant arrangement of the prisms in hominoids. It concluded that the discrepancy between the results obtained by Gantt et al. and Vrba and Grine could be accounted for by the fact that the great apes have a relatively thick surface layer of Pattern 1 enamel, much more than in humans.

The situation for hominoids was later accepted to be a predominance of Pattern 3 by Gantt (1983). Gantt continued to believe that a dichotomy between apes and hominids existed whereby apes had Pattern 3a enamel and humans had Pattern 3b enamel - subtypes of Pattern 3 as indicated in Figure 1 of Boyde (1964). Neither Vrba and Grine nor Boyde and Martin found any evidence to support this conclusion. The reasons for the different findings of Vrba and Grine and Gantt et al. were further clarified in studies of enamel thickness in relation to incremental formation rates (Martin and Boyde, 1984; Martin, 1985) which suggested the outer layer of great ape enamel was formed more slowly than the deeper layers and that this "slowed down" region was characterized by Pattern 1 enamel.

A number of problems have bedevilled the study of enamel prism packing patterns. These include:-

1 Inconsistencies in the positions and depths at which enamel microstructure was examined.

2 Inadequate knowledge of within species

variation - simply overcome and inexpensively in terms of numbers of specimens using the TSM.
3 Inadequate sampling of taxa within familial and superfamilial groups - easy using museum skulls.
4 Etching "artefacts" and wholesale destruction of valuable tissue which could have been sampled "in depth" using TSM.
5 Lack of stereoscopic analysis, in particular the question of whether one is viewing a) in an undefined direction, b) along the local long axis, c) as if from a developing enamel surface, or d) from the real surface of the tooth.

Dental microwear

In the present paper we have concentrated upon the advantages presented by the TSM in examining sub-surface structure. However, we have also been impressed with the high contrast images obtained from the surface relief. This potential of the new method could well be exploited in the study of dental microwear patterns for the purpose of reconstructing the diet of fossil species. At present, we are unable to separate the influence of internal structure on wear patterns from that of dietary items. Consequently, such studies have assumed that all mammalian enamel reacts in the same way to any given food types. Equivalence of microwear patterns is, therefore, taken as evidence for identity of dietary adaptations. Given the variety of enamel structure patterns among primate and mammalian enamels (Boyde, 1964; 1978; Boyde and Martin, 1982; 1984) and the fact that some of these have already been shown to have significantly different mechanical properties (Boyde, 1976; Boyde and Fortelius, 1986; Koenigswald, 1980) (especially among and between rodent and human enamels), it seems likely that structural influences may be confusing the picture of dental microwear and consequently of ancestral diets.

The TSM produces excellent images of the microwear patterns on the surfaces of wear facets on fossil teeth quickly and easily, with much higher contrast than any normal reflection microscope. By taking a number of optical slices through the microwear features and into the enamel below we can determine how far the wear patterns are influenced by underlying microstructure.

Fossils

How does this help us with the interpretation of fossil specimens? There has been considerable debate as to whether the omomyid fossil primates are related to the tarsiers and the higher primates more closely than are the adapid primates. The difference in enamel prism packing patterns in Strepsirhine and Haplorhine primates should allow this method to contribute to the resolution of their affinities. If omomyid primates were found to have Pattern 3 enamel and adapid primates to have Pattern 1 enamel this would lend considerable support to the interpretation of omomyids as the earliest haplorhines.

We have examined enamel structure in many fossil species from the Miocene and Plio-Pleistocene. All of the early Miocene taxa from Kenya which we have so far studied (**Limnopithecus legetet, Dendropithecus macinnessi, Proconsul africanus, Proconsul nyanzae,** and **Proconsul major**) have Pattern 3 enamel (Figs 25 & 26). One exceptional variant has been discovered in **Proconsul major** which has Pattern 3 prisms of about twice the cross-sectional area of Pattern 3 prisms in any other species (Fig 27). This is particularly surprising in view of the fact that animals of very different sizes (e.g. elephants and gibbons) have Pattern 3 prisms of the same size. This implies that **Proconsul major** had developed very large enamel forming cells. The significance of this result is hard to determine as it is a unique pattern at present. However, it is unlikely that any other hominoid species with normal size Pattern 3 prisms could be directly descended from such a specialized form.

Among middle Miocene species examined (**Sivapithecus sivalensis, Sivapithecus punjabicus** (formerly **"Ramapithecus" punjabicus**), **Sivapithecus alpani, Sivapithecus darwini, Dryopithecus fontani,** and **Kenyapithecus africanus**) all have Pattern 3 enamel which extends close to the tooth surface. This is important as these forms show dental and postcranial specializations which indicate that they are more closely related to living great apes and humans than are the gibbons, Martin (1985) has already discussed the relationship between enamel thickness and prism packing patterns in hominoids. This finding means that none of these middle Miocene forms show an indication of a secondary reduction in enamel thickness.

One of the more mysterious aspects of the fossil record, so far as Palaeoanthropology is concerned, is the absence of any fossil evidence relating to the ancestry of the African great apes, the gorilla and the chimpanzee.

There are many stages of human evolution for which we have fossil evidence from South and East Africa, from Java, China and many European sites. However, in spite of the amazing quantity and quality of fossil material from East Africa which has been accumulated since the 1930s, we still have no direct fossil evidence for African ape evolution. This, in spite of the fact that the Kenyan fossil record extends from the present to the lower Miocene with decreasing numbers of gaps in time as research progresses.

The best claim for a fossil African ape is an incisor tooth from Kazinga near Lake Edward in Uganda which is of lower Pleistocene age. On the basis of its external morphology it was thought that this specimen could belong either to an ungulate (such as a member of the horse family) or to a fossil African ape. Von Bartheld et al. (1970) recognised that these two possibilities could be distinguished on the basis of enamel microstructure, because ungulates have entirely Pattern 2 enamel. They polished and etched a portion of the tooth but had difficulty in determining the enamel prism packing pattern. In the few spots where they could see prisms, they decided that these were not in fact Pattern 2 and concluded that the incisor was that of the first recovered fossil gorilla.

We have recently had the opportunity to examine this specimen non-destructively using the TSM through the courtesy of Dr Adrian Kortlandt. We were easily able to determine the enamel prism

packing pattern in all parts of the tooth. In Figure 28 one can see images of the heavily scratched, "polished" area used by von Bartheld et al. We were able to focus through this scratched region to reveal a typical ungulate Pattern 2 arrangement of the prisms (Figure 29). This result means that the Kazinga incisor can no longer be taken as fossil evidence for the ancestry of the African apes. This negative result means that we still do not have any fossils relating to the evolution of the African apes, but it does illustrate the potential of the TSM.

Conclusion

The growing interest in the utility of enamel prism packing patterns results in part from the fact that such studies offer a potential way to determine the affinities of otherwise dubious fossil specimens. Enamel is so highly mineralized that it is virtually a fossil in vivo. It is little affected by diagenetic changes during burial and fossilization. The material may undergo chemical alteration, but is essentially unchanged structurally from life. As the enamel prism packing patterns reflect the past history of the positions and movements of the ameloblasts, studies of enamel microstructure in three dimensions offer the possibility to reconstruct cell arrangements and secretory behaviours in extinct species.

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The studies reported here were conducted at UCL while Lawrence Martin held a post doctoral research training fellowship from the MRC. He left in August 1985 to join the Department of Anthropology, State University of New York at Stony Brook, Stony Brook, N.Y. 11794 U.S.A. (Tel. 516 632 7625).

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Discussion with Reviewers

A.D. Beynon: The authors quote a vertical depth of focus of about one micron with the TSM. Does this not depend upon the magnification and numerical aperture of the particular objective used and refractive index of the immersion medium?
Authors: The depth of field does depend upon the NA of the objective and its magnification in the TSM, but is much reduced compared with the use of the same objective in a conventional light microscope. The depth of field with a 100/1.3 objective is demonstrably less than one micron if there is appropriate fine structure to be seen: this is not the case with prisms viewed head on. The functional NA of the objective is related to the refractive index of the immersion medium.

A.D. Beynon: The authors are rightly cautious about extrapolating data on prism packing pattern and daily formation rates in the Cercopithecidae, since there is no direct correlation between prism packing patterns and daily incremental rates. Was it not possible using the TSM to measure the prism cross-striation repeat interval in the outermost 200 μm of the enamel in this family?
Authors: It hardly needs to be said that it is easy to measure the cross-striation repeat interval from a lateral view of the prisms if the tooth is cut or fractured. We hope that we will be able to measure and/or reconstruct this aspect of the prisms from a head on view, but have not yet developed the means for doing so.

R.P. Shellis: The authors have published several accounts of primate enamel structure but have not so far illustrated the sub-surface Pattern 1 layer of ape enamels. This evidence is at present available only with difficulty in Martin's thesis.
Authors: This layer is so often present in the teeth of so many mammals that we do not stop to document it. Illustrations are available in Gantt et al (1977) and Gantt (1979).

R.P. Shellis: Classification of prism packing patterns simply as 1, 2 or 3 sometimes seems to lead to a loss of specificity. For example, prism patterns referred to as Pattern 1 in Figs 8, 10, 16 and 17 show a mixture of open and closed profiles; the latter, by your definition, cannot be Pattern 1. Why do you not subdivide Pattern 3 into 3a, 3b, 3c (Boyde, 1964) any longer?
Authors: One may frequently or usually find some partially open boundaries in Pattern 1 enamel, and particularly where it is derived from a deeper Pattern 3 or 2 layer and where there must be an unsharp transition between the patterns. We have been working with an undefined extension of the definition of the patterns for many years. We have always been at pains to point out that the patterns are mixed together in any one tooth or field. A description that may be valid for any one cluster of seven prisms would frequently not apply across a larger field of view: as we point out here, we apply a description to the common prism packing pattern seen in a group of >300 prisms. Secondly, the distinctions between Patterns 1, 2 and 3 as we use them are relatively clear cut and can be made rapidly and reproduceably (and, we believe, also reliably!) by a human observer. The subtler distinctions between 3a, 3b and 3c should be integrated with a more refined description of the tissue organisation based upon mathematical morphology; to deal with groups of >>>10 prisms would sensibly and realistically involve the use of an image analysis machine. The first step in this direction would be to process the images to binary images in which the centre line of the prism boundary is skeletonised to a one pixel wide line. We await these developments in the near future and we believe that it is worth waiting for.

K.S. Lester: Given the suggestion by Krause and Carlson (Scanning Electron Microscopy/1986/IV/ pp. 1591-1607: presented at the New Orleans meeting in May 1986 in the same session as this paper) that large arc-shaped prisms represent the primitive condition in multituberculates with prismatic enamel ratherthan small circular prisms as has been proposed previously, do you suspect a similar evolutionary experience for **placental** mammals?
Authors: This is an important finding for multituberculates, but we doubt that it would apply to placental mammals for several reasons. Firstly, Pattern 1 enamel is the most commonly found pattern in placental mammals in the sense that it can be found to some extent in almost

every (studied) species. Secondly, Pattern 1 enamel appears, at least developmentally, to be a precursor of Patterns 2 or 3 in placental mammals. Thirdly, the earliest mammal-like reptiles with prismatic enamel have Pattern 1 enamel.

K.S. Lester: In the determination of enamel prism packing patterns and given the commonality of a regular hexagonal array, could it be said that the shape and extent of the prism outline is possibly the prime consideration and more significant than prism alignment?
Authors: We take it that the suggestion here is that the sub-varieties of Pattern 2 and of Pattern 3 might be more significant than the Pattern 2 - Pattern 3 distinction. We understand that some people have difficulty in distinguishing these patterns, but we find them to be easily recognise-able categories in most instances. The change in orientation of the hexagonal array from Pattern 2 to Pattern 3 enamel is of developmental significance and seems a good basis on which to divide up the continuum of enamel structure. We look forward to the acquisition of numerical data describing the extent of the prism outline, but it would seem that the difficulties involved in deriving large statistical samples, so long as the methods involve tedious semi-manual image analysis procedures, will mean that we will still have to wait for some years to have enough data upon which to base some sensible comment. The simple distinction of Pattern 2 vs 3 (combined with a relative description of the extent of the prism outline) can be obtained rapidly from samples of hundreds of thousands of prisms, at different sites, in numbers of teeth, at contrasting depths, in numbers of individuals, species, families etc.: the acquisition of this type of information is greatly accelerated via the use of the TSM.

D.G. Gantt: Why are there areas within the TSM images in which the prism boundary outlines cannot be recognised?
Authors: Because, in these regions, there are insufficient changes in refractive index to produce the reflections upon which the image formation is based. As regards deep, bulk enamel, these areas are rare but more common in fossil specimens, where BSE-SEM information would confirm the suggestion that an infilling of the prism boundary pore-gap has occurred. They are more common in sub-surface enamel at the region of transition to the prism-free surface zone. Here the explanation is that the biological developmental surface is not as flat as the objective lens field, and that not all ameloblasts lose their Tomes' processes at exactly the same instant.

D.G. Gantt: In Fig.11, there appear to be rows of material between some prisms and not others. How does this Pattern 2 compare with the Pattern 2 image in Fig. 21?
Authors: The close approximation of the prisms in Propithecus coronatus is an unusual aspect of enamel structure in this species. There appears to be little interprismatic (interpit phase) enamel. A real answer to your question must await the study of a developing enamel surface preparation.

D.G Gantt: In Fig. 14, do you consider this pattern to be different from that seen in Fig. 3?
Authors: Both figures show Pattern 3 enamel. In Figure 15 the horseshoes are very tightly packed and there is less interprismatic substance than in the human enamel.

D.G Gantt: In Fig. 16, are you describing the pattern to be 1 or 3; it appears to be 1?
Authors: As stated in the text Cebus has Pattern 1 enamel underlain by Pattern 3 enamel. The field of view in Figure 16 shows the surface layer of mainly Pattern 1 enamel.

D.G Gantt: To what depth can the TSM be used to determine enamel prism packing patterns?
Authors: It depends very much on the nature of the specimen. Fossil enamel often allows imaging of deeper enamel than does recent enamel in which the contrast formation at prism boundaries may be less. In some specimens one can image enamel prisms 100 µm below the sample surface, in others one runs out of contrast after only 30 µm.

D.G Gantt: In the study of fossil primates do you feel, or did you find, that image problems existed due to the effects of fossilization?
Authors: Fossilization in most cases appears to enhance prism boundary contrast at the same time as improving the clarity of the enamel by reducing light scattering. One of the great advantages of the TSM is that it is unnecessary to etch the enamel to image the prism packing patterns. It is the unpredictable reaction of fossilized enamel to acid etchants which has caused greatest problems in previous attempts to image enamel structure in fossil enamel.