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Uptake of ^{32}P-labelled Phosphate into Developing Rat Incisor Enamel

Colin Robinson, Christopher R. Hiller, and John A. Weatherell

Biological Research Unit, School of Dentistry, University of Leeds

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To determine the sites at which ions were incorporated into the forming enamel of the rat incisor, the incorporation of ^{32}P-labelled phosphate has been studied *in vivo* at different time intervals after intraperitoneal injection with the ^{32}P and *in vitro*. The enamel from the lower incisor teeth was dissected into a series of about twenty pieces and the variation in specific activity (S.A.) from the root apex to the mature tissue determined. In general, S.A. was high in the early forming enamel falling towards the maturing tissue. Ten minutes after injection, this fall was interrupted by one or sometimes two areas of relatively high S.A. One occurred at the beginning of the maturation zone and persisted until the enamel was worn away by attrition at the incisal tip. This seemed to reflect the acquisition of phosphate by accretion during enamel maturation. The second S.A. peak, which occurred in the earlier forming enamel, tended to appear at short intervals after injection and was rarely observed when the period after injection was extended to 24 h. A S.A. peak at the same site was produced in the forming enamel when incisors were incubated *in vitro* in ^{32}P phosphate. This second peak therefore seemed to reflect some change in the tissue's physical or chemical properties rather than cellular activity. No S.A. peak was obtained *in vitro* in the maturing tissue.

Key words: Rat incisor — Enamel — Microdissection — Phosphate — ^{32}P incorporation.

Introduction

Histological descriptions of the continuously-forming rat incisor defined two stages of enamel development (Marsland, 1951, 1952). During the first stage, the organic matrix is formed and partially mineralised (Frank and Sognnaes, 1960), the tooth growing in the direction of its long axis and the enamel increasing in thickness. At or after the point where the enamel attains its full thickness, a transition occurs from the so-called stage of formation to the second stage, often referred to as the stage of maturation. This is marked by an abrupt increase in the opacity of the enamel which, according to microradiographic and autoradiographic evidence, is associated with a fairly sharp rise in mineral content (Suga and Murayama, 1965; Hammarström, 1967).

An attempt has been made in the present paper to determine the way in which mineral ions are incorporated into these histologically defined zones. This has been done by measuring the distribution of ^{32}P-labelled phosphate in developing enamel, after its incorporation *in vivo* and *in vitro*.

For reprints: Dr. Colin Robinson, Biological Research Unit, School of Dentistry, University of Leeds, Blundell Street, Leeds, LS1 3EU, England.

Materials and Methods

Uptake of ^{32}P in vivo

250 μc/kg body weight of ^{32}P-phosphate (in 0.1 ml 0.1 M phosphate buffer, pH 7.4) was injected intraperitoneally into young Wistar rats, 200–400 g body weight. The animals were later killed by chloroform and the ^{32}P activity of the incisor enamel determined as described below.

Preparation of Samples

The rats were killed at intervals varying from 10 min to 31 days after injection of the ^{32}P. The mandibles were removed and the lower incisors rapidly dissected out, care being taken to avoid damaging the soft enamel of the developing region. The enamel surface was wiped firmly with paper towel to remove adhering soft tissue.

After removing the tooth from the jaw, some of the enamel quickly dried out to form an opaque region with a sharply defined boundary. This seemed to correspond to the V-shaped boundary visible in ground sections of rat incisors (Hiller, 1971). Its position probably also coincided with the beginning of the zone of maturation as defined by Reith (1961); see also Fig. 1. Moving from root to tip, the transition from translucent to opaque enamel across the boundary seemed to coincide with a fairly abrupt increase in mineral content (unpublished data) and, in the present work, the boundary has been used as a histological reference point.

Sampling was carried out by cutting through the enamel to the underlying dentine at 0.5 mm intervals from the root apex to the hard enamel of the maturing region. The particles of enamel separated by the scalpel cuts could then be prised gently from the tooth (Figs. 1 and 2). They appeared to separate cleanly from the harder underlying dentine and analysis showed that they contained no detectable hydroxyproline (Robinson and Weatherell, unpublished). The samples were therefore essentially free from dentinal contamination. When it was necessary to sample the hard mature enamel towards the incisal tip (see Fig. 8) the teeth were first ground to a longitudinal section about 100–150 μ in thickness. Particles of softer enamel were then dissected out using a scalpel, and the harder mature tissue using the acid etching technique of Weatherell *et al.* (1966).

Measurement of Radioactivity

Each enamel sample was fixed with nail varnish to the surface of a 3 cm diameter planchette and the ^{32}P activity counted as from a point source, using a Nuclear Chicago automatic gas-flow system. Each sample was counted for a minimum of 10,000 counts. The ^{32}P counts were expressed as specific activity (S.A.); i.e. counts/min/μg P. Accuracy: $\pm$ 5% Standard deviation on 10,000 counts.

Determination of Phosphorus

The small samples of enamel (10–100 μg in weight) were removed from the planchettes with acetone and dissolved in 5 ml 0.167% perchloric acid. The phosphate content of each solution was determined by the spectrophotometric method of Chen, *et al.* (1956) using standard solutions of KH_2PO_4. Accuracy: $\pm$ 1% Standard deviation.

Uptake of ^{32}P in vitro

Incisor teeth, removed from the mandibles of rats which had received no injection of ^{32}P, were placed in ^{32}P-labelled phosphate buffer (10 μc/ml ^{32}P in 0.1 M phosphate, pH 7.4) for periods of 1, 10, 30 min and 1, 2, 4 and 24 h. On removal from this solution, the teeth were washed for about 2 h in running tap water to remove unbound isotope. The enamel was then dissected into pieces and the S.A. of the pieces determined as described above.

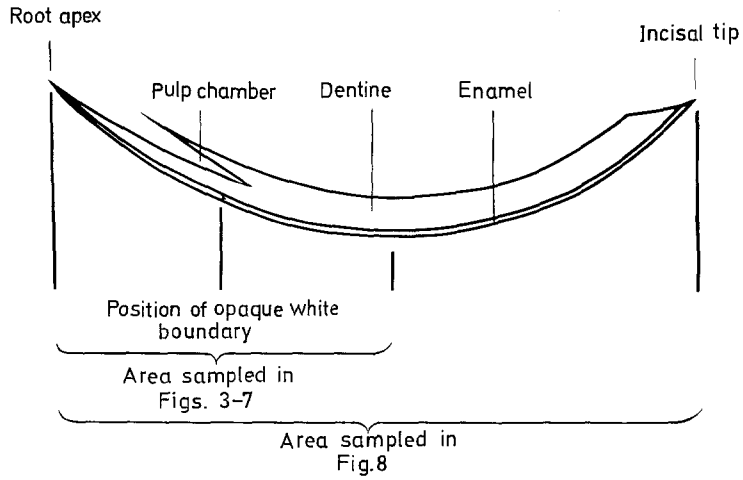

Fig. 1. Diagram of a rat lower incisor section showing areas sampled in this study. The position of the opaque boundary mentioned in the text is indicated

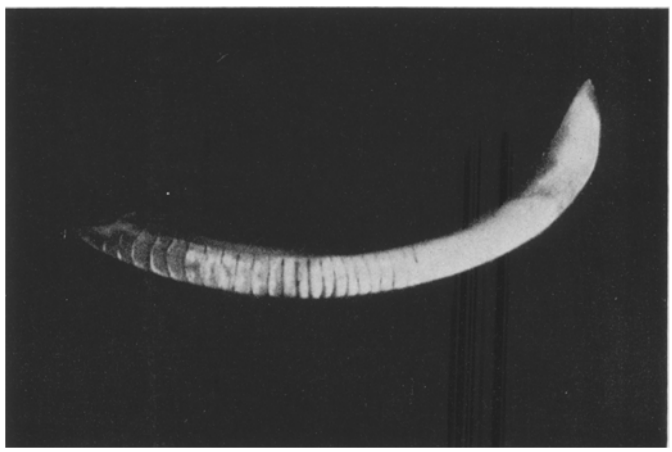

Fig. 2. Lower incisor, illustrating method of dissection. Cuts were made through the enamel to the underlying dentine at intervals of approximately 0.5 mm, to separate a series of enamel particles extending from the root apex to the hard maturing region of the tissue, $\times 4$

Results

Uptake of ^{32}P in vivo

The distribution of ^{32}P was measured in the enamel of teeth from over 50 rats killed at various times after injection of the isotope, as described above. Figs. 3–7 show the variations in S.A. along the enamel of incisors from animals killed 10 min, 2 h, 4 h, 8 h and 24 h after injection. There was considerable variability but certain consistent patterns emerged. It can be seen, moving from the root apex towards the maturing enamel, that as the developing enamel increased in

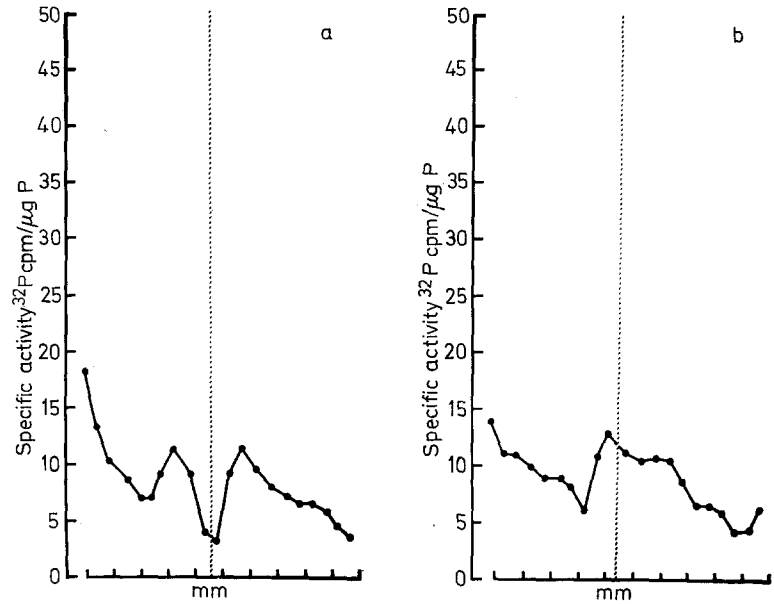

Fig. 3. a Two peaks occurred in the enamel from 5 out of the 10 animals killed 10 min after injection. b A single peak was found in 5 out of the 10 animals

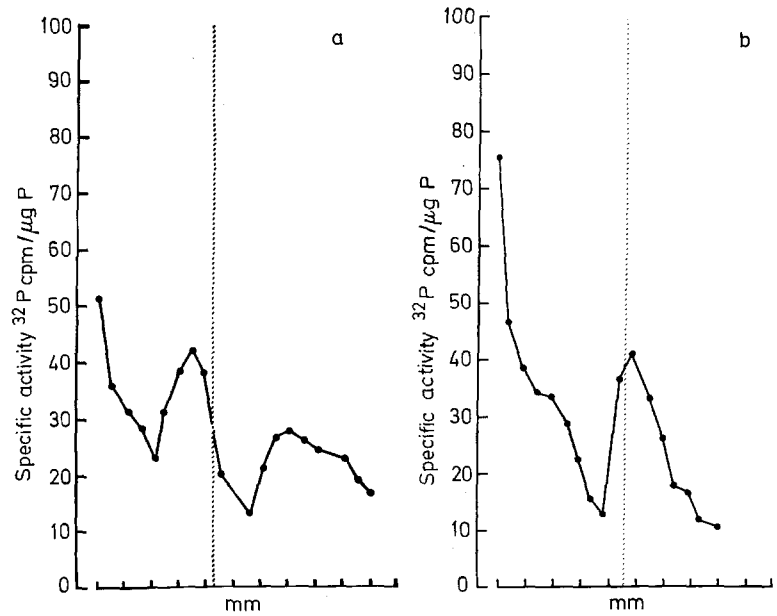

Fig. 4. a Two peaks occurred in the enamel from 5 out of the 10 animals killed 2 h after injection. b A single peak was found in 5 out of the 10 animals

Figs. 3—7. Variation in specific activity of ^{32}P in enamel pieces dissected in a series extending from root apex to the maturing region of enamel from animals killed at various times after injection of the isotope. Specific activity is plotted against mm. along the tooth. The hatched lines mark the position of the opaque boundary in the enamel (see text). Each figure shows a curve representative of the results obtained

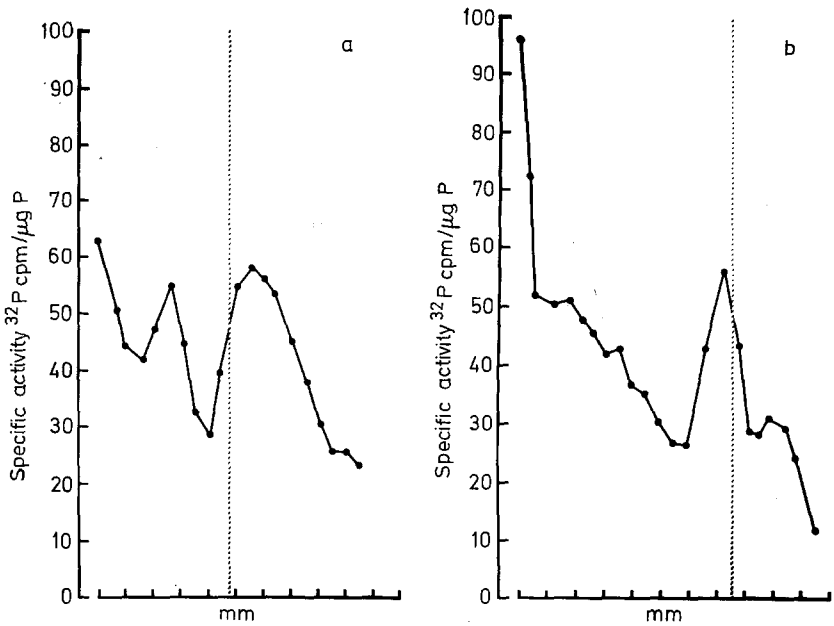

Fig. 5. a Two peaks occurred in the enamel from 4 out of the 9 animals killed 4 h after injection. b A single peak was found in 5 of the 9 animals

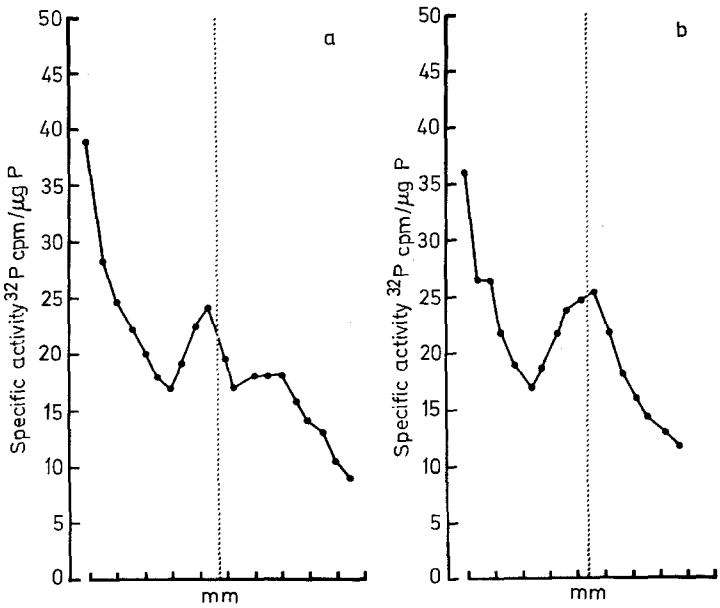

Fig. 6. a Two peaks occurred in 1 of the 4 animals killed 8 h after injection. b A single peak was found in 3 of the 4 animals

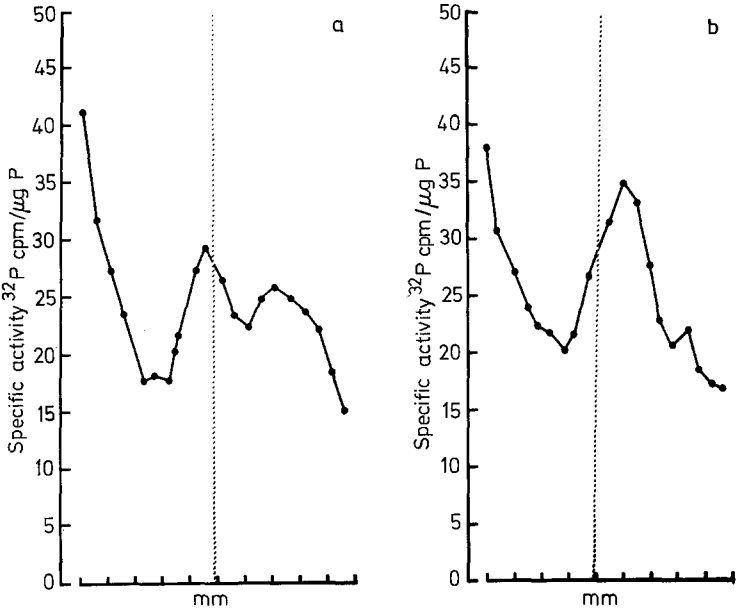

Fig. 7. a Two peaks were found in 1 out of the 10 animals killed 24 h after injection. b A single peak was found in 9 out of the 10 animals

thickness in the earlier region of formation, the S. A. invariably fell. The S.A. then rose again to produce one, or two, regions of high activity in the vicinity of the white opaque boundary. Finally, as mineralisation progressively increased, the S.A. decreased, falling to a minimum in the most highly mineralised regions of enamel. When the interval between injection and death was extended beyond 24 h, the region of highest S.A. moved towards the tip at about 0.6 mm per day (Fig. 8); a rate consistent with the histologically established rate of eruption (Devoto *et al.*, 1966).

At time intervals less than 24 h, the patterns of ^{32}P activity were variable. Some teeth showed one well-defined peak of high S.A. and others showed two. Two peaks were more frequently observed when the interval between injection and death was relatively short. For instance, about half the teeth from animals killed 10 min, 2 h and 4 h after injection, showed two peaks (Figs. 3a, 4a and 5a); half showed one peak (Figs. 3b, 4b and 5b). At 8 h after injection, only one from the 4 teeth examined gave a suggestion of two S.A. peaks (Fig. 6a). None of the 4 teeth examined 16 h after injection showed two clear peaks and, at 24 h after injection, only one out of 10 teeth examined showed two peaks (Figs. 7a and 7b).

In an attempt to clarify the picture, the positions of the peaks in the enamel were noted in relation to the position of the opaque enamel boundary previously described. Where two peaks were found, these tended to occur one at either side of the boundary. Whenever it was difficult to decide if there was one or two peaks, the region of high S.A. tended to straddle the boundary. The single regions of high S.A. found at intervals less than 24 h after injection occurred on or just

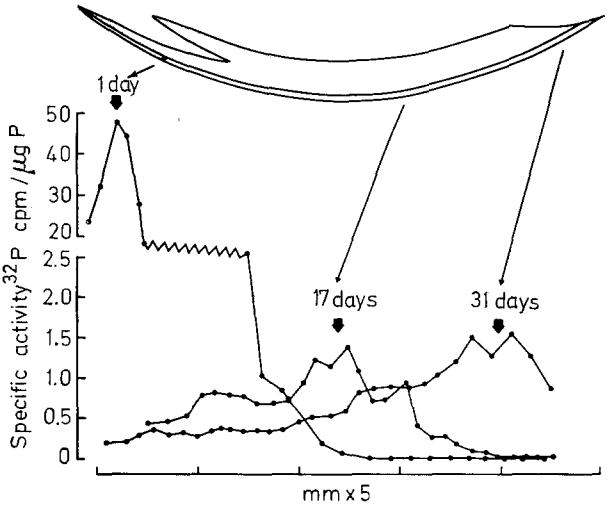

Fig. 8. A single peak of relatively high specific activity moved with time towards the tip of the enamel at a rate corresponding to a rate of eruption of 0.6 mm/day

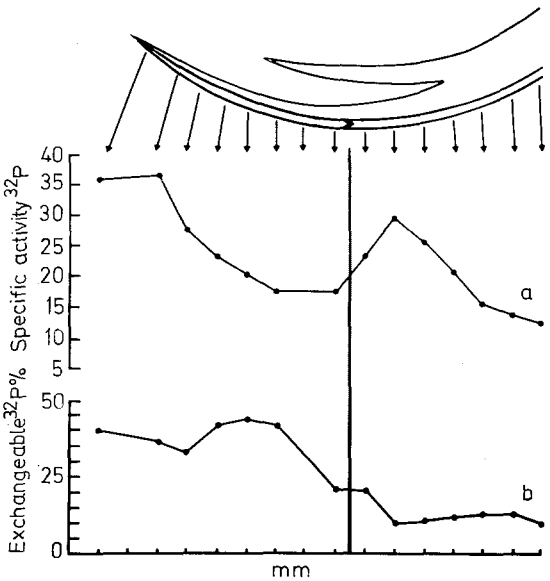

Fig. 9. a Variation in specific activity of ^{32}P in enamel from a rat killed 24 h after injection. b Percentage of ^{32}P counts removed from the enamel by incubating the pieces of labelled enamel in phosphate buffer for 24 h

distal to the boundary. After 24 h, the single region of high S.A. almost invariably occurred distal to the boundary.

The high S.A. proximal to the boundary decreased until, about 24 h after injection, only the more distal peak of high S.A. remained. This peak of relatively high S.A. persisted, moving towards the tip as the tooth erupted, until the enamel was lost through attrition at the biting edge.

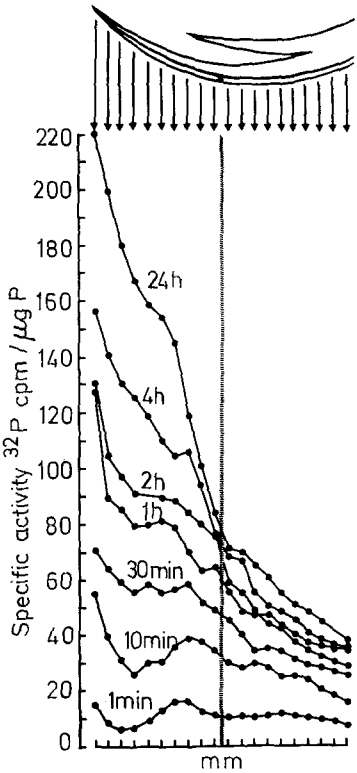

Fig. 10. Variation in specific activity of ^{32}P in enamel from teeth which had been incubated in ^{32}P-labelled phosphate buffer for various periods of time. The time of incubation is shown against each curve

Removal of ^{32}P from Enamel Labelled in vivo

To determine whether the ^{32}P taken up *in vivo* remained on the surfaces of non-growing crystallites or had been buried by subsequent accretion of mineral, an attempt was made to remove the ^{32}P label by back-exchange with unlabelled phosphate *in vitro*.

When particles of enamel which had been labelled with ^{32}P *in vivo* were placed in water or isotonic saline, a small amount of the ^{32}P label was washed out of the tissue. When particles were similarly incubated in isotonic phosphate buffer (0.1 M, pH 7.4), much more ^{32}P was removed from the tissue; presumably because some of the $^{32}PO_4$ exchanged with the unlabelled phosphate in the incubation solution.

The amount of ^{32}P removed varied from place to place in the enamel, more being lost from the earlier forming region than from the maturing enamel in the region of the white opaque boundary. The proportion of ^{32}P lost from enamel particles which had been labelled by injecting ^{32}P into the animal 24 h before death and then placed in a solution of "cold" phosphate buffer for a further 24 h is shown in Fig. 9. Forty per cent of the activity was removed from the forming region and 10% from the maturing region.

Uptake of ^{32}P in vitro

Freshly extracted (unlabelled) teeth were incubated in ^{32}P-labelled phosphate buffer (0.1 M, pH 7.4, 10 μc/ml) for periods of 1, 10, 30 min and 1, 2, 4, 24 h. They were then washed for 2 h and the enamel dissected as described above.

The patterns of variation in S.A. along the enamel varied with time as shown in Fig. 10. Specific activity was highest in the forming region of the enamel and lowest in the fully mineralised mature tissue. A single peak of relatively high S.A. tended to develop, proximal to the white opaque boundary, and was most pronounced after 10 min incubation. After longer periods, during which ^{32}P S.A. increased in every region of enamel, this peak of S.A. became obscured and, after 24 h, S.A. decreased continuously from the developing tissue at the root apex to the mature enamel.

The single peak of S.A. occurred just proximal to the boundary of opaque enamel and therefore coincided in position with the region of *transient* high S.A. found *in vivo*.

Discussion

There were considerable variations in the distribution pattern of ^{32}P-labelled phosphate incorporated into rat incisor enamel *in vivo* and *in vitro* but two features were common to all teeth and both procedures: (a) the S.A. of the enamel invariably fell as the tissue increased in thickness during the earliest stages of development and (b) one or more regions of relatively high S.A. occurred during development.

The initial fall in S.A. from root apex towards the maturing tissue probably reflected a limited diffusion of tracer through an increasing thickness of enamel. It seems likely that the ^{32}P incorporated by this part of the enamel mainly reflected exchange with the enamel mineral, since a similar pattern was observed *in vitro* and since most of the isotope incorporated by this region *in vivo* could be easily removed by washing with unlabelled phosphate.

Several observations, on the other hand, suggest that much of the isotope taken up *in vivo* at or just after the boundary of opaque enamel had been firmly trapped within growing crystallites:

a) The ^{32}P in this region was relatively difficult to remove with unlabelled phosphate *in vitro*.

b) Chemical analysis (unpublished) and previous microradiographs (Suga and Murayama, 1965) showed that the mineral content of the enamel increased rapidly in this region.

c) Some of the ^{32}P persisted until removed by attrition at the incisal tip of the tooth; the observed fall in the S.A. peak between 1 and 17 days (Fig. 8) was due mainly to dilution of ^{32}P by the subsequent uptake of unlabelled mineral during maturation.

d) Incubation of unlabelled rat incisors *in vitro* without ameloblasts and in solutions of ^{32}P-labelled phosphate where nett apatite growth could not occur did not produce a S.A. peak in this region.

The S.A. peak found after *in vitro* incubation occurred at a different, earlier stage of development, proximal to the boundary of opaque enamel and apparently before the enamel had reached the stage of development when a rapid accretion of mineral occurs. It probably reflected a region where the crystallite surfaces

were particularly accessible to exchange. Perhaps it coincided with an area where some of the enamel matrix had been removed; a view supported by recent chemical analysis (C. Robinson, C. R. Hiller and J. A. Weatherell, unpublished results). After half-an-hour or so, the *in vitro* peak became obscured, probably because the activity of the adjacent forming enamel rapidly increased as the ^{32}P penetrated the matrix. It could be, however, that the composition and structure of the enamel was gradually altered as the incubation solution dissolved labile components out of the forming enamel.

This *in vitro* peak coincided in position with the second, transient region of high S.A. found in some of the shorter-term *in vivo* experiments so that, by analogy, this *in vivo* peak also probably reflected easy access of $^{32}PO_4$ to crystallite surfaces. As in the case of its *in vitro* analogue, the *in vivo* peak of S.A. in this region probably became obscured as more isotope diffused into the adjacent enamel. The S.A. of this apparently accessible region might possibly also reflect to some extent the rise and fall in the S.A. of the blood which, in the rat, is maximal at about 10 min after injection of the isotope.

Whether or not these explanations are correct in detail, the injected $^{32}PO_4$ ions seemed to be preferentially incorporated at *two* stages of enamel development, *viz.* just prior to and during maturation. Uptake proximal to the white opaque boundary, probably in transitional enamel between formation and maturation appeared to reflect merely the physico-chemical state of the tissue, i.e. its capacity to exchange with available ions and the $^{32}PO_4$ incorporated at this stage of development was probably not permanently bound within the enamel mineral. Uptake distal to the white opaque zone, on the other hand, appeared to reflect crystallite growth occurring during a fairly abrupt increase in mineral accretion associated with maturation. The $^{32}PO_4$ incorporated at this stage of development was thus more firmly bound within the growing crystallites.

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