

## COMPOSITION AND METABOLISM OF THE EXTRACELLULAR MATRIX IN THE PERIODONTAL LIGAMENT OF IMPEDED AND UNIMPEDED RAT INCISORS

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**Summary**—Potential differences were studied in the composition and metabolism of the connective tissue components (collagen, non-collagenous proteins and glycosaminoglycans) of the periodontal ligament (PDL) as a result of alterations in the rate of eruption. The amount of collagen, glycosaminoglycan (GAG) or the water content of the tissue was the same in the unimpeded and impeded situation. The only difference was in the amount of structural non-collagenous proteins: 33 per cent increase in the unimpeded PDL. The rate of turnover of insoluble collagen, structural non-collagenous proteins and the sulphated GAG, studied *in vivo* by administration of [ $^3\text{H}$ ]-proline or [ $^{35}\text{S}$ ]-sulphate, did not differ significantly in both situations: their extremely short half-lives were respectively 9.7, 3.5 and 1.7 days. Increase in the rate of eruption and hence in the remodelling process does not result in alterations in the metabolic activity of the PDL components. Their metabolism is probably already at maximal rate and may permit the more rapid rate of eruption when the opposing force of the antagonists is diminished during the non-occlusal state of the incisors.

### INTRODUCTION

It has been proposed that the periodontal ligament (PDL) plays a crucial role in generating the tooth-eruptive force. Individual components of the PDL suggested as the force producers are the fibroblasts because of their mobility, the collagen fibres because of their contraction and the ground substance because of its high hydrostatic pressure reviewed (by Moxham and Berkovitz, 1982).

Eruption rates of rodent incisors can be increased markedly by rendering them unimpeded and potential differences in composition and metabolism of the connective-tissue components can then be studied. This has been done biochemically in the guinea-pig incisor PDL by Slootweg (1976), who found no difference in the amount or metabolism of collagen but an increase in amount and a slight decrease in turnover rate of the structural non-collagenous proteins. From autoradiographic data, Beertsen and Everts (1977) deduced a slightly-faster turnover rate of [ $^3\text{H}$ ]-proline-labelled substances in the PDL of unimpeded mouse incisors. Rippin (1976, 1978), by autoradiography in young and old rat molars, found increased protein metabolism in the coronal region of hypofunctional ligaments. Shore, Moxham and Berkovitz (1982) reported that few ultrastructural changes could be observed in the PDL of rat incisors, i.e. a decreased amount of ground substance.

As it is conceivable that alterations in the rate of remodelling of the PDL would be accompanied by changes in composition and metabolism of its components, we studied the amount and the turnover rate of collagen, non-collagenous proteins and glycosaminoglycans (GAG) in the PDL.

### MATERIALS AND METHODS

Two groups of female Wistar rats (average wt 200 g; TNO, Zeist, The Netherlands) were used: one

(37 rats) with impeded eruption of the incisors and one (27 rats) with unimpeded eruption. The unimpeded state was obtained by trimming the mandibular and maxillary incisors every two days during 15 days with a rotating carborundum disc under ether anaesthesia.

After overnight fasting, 6 animals of each group were supplied by the tail vein with 1 mCi L[2,3- $^3\text{H}$ ]-proline (New England Nuclear, Dreieichenhain, West Germany); trimming of the incisors was continued for the duration of the experiment. To the rest of the animals in each group, 0.5 mCi  $\text{Na}_2^{35}\text{S}\text{O}_4$  (The Radiochemical Centre, Amersham, England) was given by intraperitoneal injection. Rats were killed at various times by a lethal dose of sodium pentobarbital (triotal, Gist-Brocades, Delft, The Netherlands), the lower and upper incisors were dissected out by careful removal of surrounding alveolar bone and the periodontal ligaments were scraped from the roots. The labial parts of the ligaments were not used. For GAG isolation, PDL from two rats were pooled, whereas for collagen the tissue of a single rat was analysed.

#### *Extraction and isolation of glycosaminoglycans*

The tissue was washed twice with water for 10 min at 4°C. The sediments were dehydrated in several changes of acetone and dried in an oven at 35°C. Digestion of the tissue with Proteinase K (Merck, Darmstadt, West Germany) and isolation of the GAG was performed as described by Smith *et al.* (1980), replacing the extraction of trichloroacetic acid with chloroform by a dialysis step. The recovery of the GAG in this isolation procedure was about 80 per cent.

#### *Extraction and isolation of collagen*

The tissue was washed with water at 4°C for 30 min and subsequently extracted with 1 M NaCl in 0.05 M

Table 1. Composition of PDL from rat incisors with impeded and unimpeded eruption

	Unimpeded		Impeded		Significance of difference
	mean $\pm$ SD	(n)	mean $\pm$ SD	(n)	
Wet weight (mg)	9.6 $\pm$ 2.3	(27)	8.9 $\pm$ 1.1	(3)	NS
Percentage dry weight/wet weight	26.5 $\pm$ 1.1	(6)	27.2 $\pm$ 0.8	(6)	NS
Microgrammes of collagen/mg dry weight	224.0 $\pm$ 15.7	(6)	219.7 $\pm$ 5.1	(6)	NS
Percentage NaCl soluble collagen	3.6 $\pm$ 0.6	(6)	3.6 $\pm$ 0.6	(6)	NS
Microgrammes of non-collagenous proteins/mg dry weight	436 $\pm$ 21	(6)	378 $\pm$ 31	(6)	0.05 < <i>p</i> < 0.025
Microgrammes of H <sub>2</sub> O soluble protein/mg dry weight	201 $\pm$ 27	(6)	200 $\pm$ 33	(6)	NS
Microgrammes of structural non-collagenous protein/mg dry weight	235 $\pm$ 30	(6)	177 $\pm$ 44	(6)	0.025 < <i>p</i> < 0.0125
Microgrammes of uronic acid/mg dry weight	1.36 $\pm$ 0.33	(21)	1.26 $\pm$ 0.06	(31)	NS

\*According to Student's *t*-test.

tris-HCl pH 7.5 at 4°C for 24 h. The insoluble collagen was extracted with 5 per cent trichloroacetic acid at 90°C for 30 min (a modification of the method of Fritch, Harkness and Harkness, 1955). Almost no collagen (less than 5 per cent) was left in the sediments. After centrifugation (30 min, 40,000 *g*), the supernatant containing the collagen and the sediment containing the structural non-collagenous proteins were lyophilized and subsequently hydrolysed in 6 M HCl at 135–140°C for 3.5 h.

#### Quantitative analyses

Uronic acid was determined according to the method of Bitter and Muir (1962). <sup>35</sup>S-radioactivity was measured in a Philips Liquid Scintillation Apparatus. Radioactive samples were mixed with 10 ml Scintillator 299 (Packard Instrumental Company, Brussels, Belgium). The specific radioactivity of the GAG is defined as <sup>35</sup>S-dpm per  $\mu$ g uronic acid. The specific radioactivity of hydroxyproline and proline was determined by a modification of the method of Rojkind and Gonz  les (1974). Hydroxyproline was determined as described by Guis, Sloomweg and Tonino (1973), proline according to Summer and Roszel (1965). Total protein was measured by the ninhydrin method of Rosen (1957) in which a correction was made for the smaller colour yield of collagen (84.3 per cent; van Amerongen, Lemmens and Tonino, 1983). The half-life of the components of the extracellular matrix was determined by the decrease of their specific radioactivity with time after reaching maximal incorporation of the radioisotope. Linear regression curves of log-transformed specific radioactivities against time were calculated. Linearity of the regression curves was tested by means of analyses of variance.

#### RESULTS

Trimming the incisors caused the rate of eruption to increase from 2.1 to 4.2 mm per week for the upper incisors and from 2.8 to 4.8 mm per week for the lower incisors. This increase did not result in any clear changes in the biochemical variables studied for this connective tissue (Table 1). Differences in the composition of the PDL during impeded or unimpeded eruption were minor. The amount of tissue that could be dissected from the incisors, as well as

the water content of the tissue, was similar in both situations. The total amount of collagen and that of salt-soluble collagen was also the same. With relation to the ground substance, the amount of uronic acid containing GAG which could be isolated from the PDL of impeded or unimpeded incisors showed no statistically significant differences. The only difference was in the amount of non-collagenous proteins: unimpeded PDL contained about 15 per cent more of these proteins than the impeded PDL. This difference was just significant (Table 1). Of the non-collagenous protein, the amount of the H<sub>2</sub>O-soluble proteins (mainly blood proteins) was the same in both situations whereas a significant difference was found for the insoluble structural non-collagenous proteins: 33 per cent more of these proteins were found in the unimpeded state.

To estimate the half-lives of the PDL matrix components, linear regression lines of log values of specific radioactivities against time were calculated. There were no significant deviations from linearity ( $\alpha = 0.05$ ). The results of this study of the dynamics of the connective-tissue components are given in Tables 2 and 3. There was no significant difference in the half-lives of insoluble collagen, structural non-collagenous proteins or sulphated GAG between the impeded and unimpeded incisors. There were no differences in the levels of the specific radioactivities when the components from the PDL of impeded and unimpeded incisors were compared. The maximal incorporation of <sup>35</sup>(S)O<sub>4</sub><sup>2-</sup> into the GAG of the PDL occurred after 2 h, decreasing after that time. After 16 h, the blood activity was less than 0.1 per cent of that injected. The incorporation of [<sup>3</sup>H]-proline into collagen and the non-collagenous proteins of the PDL was maximal after 3 days while in blood after 24 h less than 0.02 per cent of <sup>3</sup>H-label could be detected.

#### DISCUSSION

Our results show that few biochemical changes occur in the PDL from rat incisors when these teeth are rendered unimpeded for a period of 15 days. Of the components that form part of this connective tissue *viz.* insoluble collagen, proteoglycans and structural non-collagenous proteins, only the last one was increased in amount (33 per cent) in the unim-

Table 2. Specific radioactivities (dpm  $^3\text{H}/\mu\text{g}$  hydroxyproline) of insoluble collagen from rat PDL

Time (days)	Unimpeded		Impeded	
	mean $\pm$ SD	(n)	mean $\pm$ SD	(n)
3	522 $\pm$ 19	(2)	516 $\pm$ 20	(2)
5	426 $\pm$ 9	(2)	520 $\pm$ 50	(2)
8	372 $\pm$ 17	(2)	359 $\pm$ 48	(2)

Specific radioactivities (dpm  $^3\text{H}/\mu\text{g}$  proline) of structural non-collagenous proteins from rat PDL

Time (days)	Unimpeded		Impeded	
	mean $\pm$ SD	(n)	mean $\pm$ SD	(n)
3	2141 $\pm$ 230	(2)	2003 $\pm$ 141	(2)
5	1359 $\pm$ 13	(2)	1708 $\pm$ 28	(2)
8	803 $\pm$ 22	(2)	742 $\pm$ 88	(2)

Specific radioactivities (dpm  $^{35}\text{S}/\mu\text{g}$  uronic acid) of sulphated GAG from rat PDL

Time (hours)	Unimpeded		Impeded	
	mean $\pm$ SD	(n)	mean $\pm$ SD	(n)
16	2170 $\pm$ 134	(3)	2175 $\pm$ 510	(4)
24	1572 $\pm$ 163	(5)	1563 $\pm$ 285	(11)
48	1278 $\pm$ 85	(5)	1256 $\pm$ 229	(6)
65	759 $\pm$ 143	(3)	750 $\pm$ 70	(5)
72	759 $\pm$ 148	(5)	745 $\pm$ 92	(5)

peded state. As shown by Slootweg (1976), no plasma proteins can be detected in this last fraction, which is heterogeneous and contains many components. One of these components is fibronectin (Connor, Aubin and Melcher, 1983). When the composition of PDL from rat incisors is compared with bovine incisors (Guis *et al.*, 1973) a different ratio of collagen/non-collagenous proteins is apparent: the PDL from rat incisors contains about 50 per cent less collagen/mg dry weight. This value has also been found by Guis (1976) and Slootweg (1976) for PDL of guinea-pig incisors. The difference in collagen content may therefore be accounted for by species and functional differences: permanent or limited-erupting teeth.

In the studies of turnover rate of the components no changes were found when the rate of remodelling was increased. The half-life of insoluble collagen of the PDL is similar to the values found by A.A.R. Taverne and G. J. M. Tonino (data not presented) and Orłowski (1978) for rat and Guis (1976) and Slootweg (1976) for guinea pig, whereas the values reported by Sodek (1977) indicate a much shorter half-life. However, this may be ascribed to his unconventional approach to study half-life of collagen by measuring the rate of the initial incorporation of [ $^3\text{H}$ ]-proline as hydroxyproline in insoluble collagen in contrast with our approach in which after reaching maximal incorporation the decrease of specific radioactivity of incorporated hydroxyproline with time is determined.

In the short-term experiments of Sodek (maximal 24 h), the synthesized and labelled tropocollagen molecules may not be entirely incorporated into functional mature fibres. Bienkowski (1983) showed that 10 to 40 per cent of newly-synthesized collagen is degraded intracellularly before it can be processed to collagen fibrils. It may be therefore that Sodek has looked mainly at the metabolism of non-functional collagen and in long-term experiments this problem will be less.

We have evidence from short-term labelling experiments (2 h incorporation; Tonino and van den Bos, data not presented) that in salt-insoluble collagen of the PDL a small, specific fraction is present with a much higher turnover rate than the rest of the collagen. If initial incorporation rates only are studied, this specific fraction will be the major contributor to turnover rates. On the other hand, conventional methods reflect the total collagen pool. Sodek (1977) argued in favour of the short-term experiments that the plasma proline pool is variable, that re-utilization of proline occurs (Jackson and Heiniger, 1975) and that in long-term experiments growth of the animals would disturb determination of half-lives. However, the large variation in plasma proline pool can be easily eliminated by a period of fasting and experiments in guinea pigs showed that newly-synthesized collagen has negligible specific radioactivity after 3 days, indicating relatively complete absence of proline recycling (Guis, 1976). Further, corrections for growth can be made as described by Zika and Klein (1971), Guis (1976) and Slootweg (1976). The conventional method of studying half-life of collagen can therefore be still valid and useful.

The more rapid turnover of structural non-collagenous proteins agrees well with the data of Orłowski (1978), Sodek (1977) and Slootweg (1976). Apart from the results of Orłowski (1976), only autoradiographic data on the turnover rate of sulphated GAG are available, all indicating a short half-life (Eccles, 1962; Baumhammers and Stallard, 1968). Our biochemical data indicate that of the main components of the extracellular matrix of PDL, the sulphated GAG metabolize at the highest rate: 5–6 times faster than collagen and twice as fast as the structural non-collagenous proteins.

As this half-life refers to all GAG, we are studying different pools of proteoglycans in the PDL with different half-lives. We have some preliminary indications that those proteoglycans that are more difficult to extract and are more firmly bound to collagen fibres metabolize faster than proteoglycans belonging to the ground substance. Compared with GAG from other tissues of the rat, this half-life of PDL GAG is extremely short. Other tissues of rat have reported half-lives of 3–14 days (Kofod, Bozzini and Tocci, 1971; Margolis and Margolis, 1977), whereas, in other species, *in-vitro* studies have yielded

Table 3. Half-lives of PDL components from rat incisors with impeded and unimpeded eruption

	Unimpeded	Impeded
Insoluble collagen	10.5 days ( $-0.043 < B < -0.015$ )	8.9 days ( $-0.070 < B < -0.002$ )
Structural non-collagenous proteins	3.6 days ( $-0.105 < B < -0.063$ )	3.4 days ( $-0.130 < B < -0.049$ )
Sulphated GAG	1.6 days ( $-0.009 < B < -0.006$ )	1.7 days ( $-0.009 < B < -0.006$ )

The 95 per cent confidence interval of the regression coefficient B of each linear regression curve is given in parentheses.

much longer half-lives (bovine cornea: 10–30 days; human cartilage: 200–300 days; Kennedy, 1979). The reason why the components of the PDL should have such short half-lives is unknown. A rapid turnover rate would permit the process of intensive remodelling needed for the continuous eruption. However, increased remodelling due to unimpeded eruption does not result in alterations of the metabolic activity of the PDL components. Beertsen and Everts (1977) and Rippin (1976, 1978) found, by autoradiography, an increased turnover in unimpeded teeth, whereas our biochemical results indicate a comparable turnover rate for collagen and non-collagenous proteins. This difference may be, at least partly, ascribed to the higher amount of structural non-collagenous proteins that we found in the unimpeded state combined with the high turnover rate of this fraction. Biochemically, but not autoradiographically, collagen and non-collagenous proteins can be fully distinguished.

Sodek (1978) has shown that the half-life of collagen and the process of eruption are not correlated; the half-life of collagen in the rat molar PDL (not erupting) is even shorter than that in the incisor PDL. When rat molars are made unopposed, the hypofunctional PDL collagen possesses a 2-fold higher specific radioactivity than collagen of control molar PDL, permitting an increased remodelling of this collagen (Kanoza *et al.*, 1980). However, when the results of Kanoza *et al.* (specific radioactivities of hydroxyproline: their Tables 1 and 2) are logarithmically plotted against time, no significant differences in slopes of regression lines are found, indicating that half-lives of collagen are almost identical. Thus the turnover rate of collagen would be the same for normal and hypofunctional PDL collagen. The higher specific radioactivity for hypofunctional PDL collagen may be ascribed to a decreased amount of collagen, a lower degree of hydroxylation or a higher supply of radioactive precursor caused by a denser vascularization.

Slootweg (1976) observed in guinea-pig incisor PDL that increase in eruption rate did not result in shorter half-lives for collagen. Our evidence shows that the turnover rate of sulphated GAG of PDL is not altered when the eruption rate is increased. In our opinion the metabolism of the PDL components is already at maximal rate and capacity permitting a rapid remodelling. When the opposing force is diminished by trimming the incisors out of occlusion, the high, but not altered metabolism of the components would permit the more rapid eruption rate, unhindered by the antagonists. Shore *et al.* (1982) concluded from an ultrastructural study of PDL of rat incisors that the amount of ground substance within the extracellular matrix was decreased in the unimpeded state. We could not confirm this biochemically: the amount of GAG was the same in both situations. This agrees with the result of Beertsen and Everts (1977) who did not find any ultrastructural differences. Our results neither support nor disprove the proposed role of proteoglycans in the PDL in the process of tooth eruption (Pearson, 1982). The concentration of proteoglycans (0.3 per cent/mg dry weight), and as consequence their hydrodynamic volume, is high enough to give the hydrostatic pres-

sure necessary for eruption (Moxham and Berkovitz, 1982). However, the proteoglycans cannot be considered as the sole factor as changes in eruption rates are not related to changes in proteoglycan concentration. In our view, a multifactorial and synergistic system, composed of fibroblasts, collagen fibres, proteoglycans and glycoproteins is responsible for the process of eruption and the high rate of turnover permits this process. On the other hand, this same high turnover may facilitate the repair of micro-traumata, frequently occurring in the PDL as result of the strong forces during physiological stress (Kanoza *et al.*, 1980).

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