The Effect of Oxygen Partial Pressure on Protein Synthesis and Collagen Hydroxylation by Mature Periodontal Tissues Maintained in Organ Cultures

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(Received 7 August 1978)

Mature periodontal tissues from adult-mouse first mandibular molars were cultured in a continuous-flow organ-culture system which allowed the regulation of both ascorbic acid concentration and pO_2 (oxygen partial pressure). Protein synthesis was measured by analysing the incorporation of [3H]proline into collagenous and non-collagenous proteins during the last 24h of a 2-day culture. At low pO_2 [16.0kPa (approx. 120mmHg)] approx. 60% of protein-incorporated [3H]proline was found in collagenous proteins. However, it was evident that this collagen was considerably underhydroxylated. At high pO_2 [56.0kPa (approx. 420mmHg)], both the amount of collagen deposited in the tissues and the degree of hydroxylation were increased considerably. In contrast, no significant effect on non-collagenous protein was observed. Tissues cultured at low pO_2 for the first 48h were unable to respond to a subsequent increase in pO_2 during the last 24h. Analysis of pepsin-solubilized collagen α -chains labelled with [^{14}C]glycine demonstrated the synthesis of both type-I and type-III collagens by explants cultured for 48h at high pO_2 . Type-III collagen comprised 20–30% of the radioactivity in α -chains in both the periodontal ligament and the tissues of the alveolar process. The pattern of protein synthesis in the alveolar tissues at high pO_2 was similar to that observed in these tissues *in vivo*. However, in the cultured periodontal ligament the proportions of non-collagenous proteins and type-III collagens were increased in comparison with the tissue *in vivo*.

The periodontium, the organ that supports the teeth, comprises four connective tissues: the cementum, which is attached to the root of the tooth; the alveolar bone, which is continuous with the bone of the jaw; the periodontal ligament, which is a cellular and vascular fibrous connective tissue that extends between the cementum and the alveolar bone; and the lamina propria of the gingiva that is protected by overlying epithelium. The collagen of functioning periodontal tissues of adult rat molars has been shown to exhibit a high rate of turnover compared with collagen of other connective tissues (Sodek, 1976, 1977; Sodek *et al.*, 1977). Although it is known that the periodontium is actively remodelled during orthodontic therapy (Crumley, 1964; Koumas & Matthews, 1969; Baumrind & Buck, 1970; Diaz, 1978), the mechanisms whereby the synthesis and resorption of bone and periodontal-ligament collagen are regulated during turnover and remodelling are unknown.

Goldhaber (1966) and co-workers (Stern *et al.*, 1966) have suggested that there is a relationship between pO_2 and bone remodelling *in vitro* and, on

Abbreviations used: pO_2 and pCO_2 , partial pressures of oxygen and carbon dioxide respectively in the culture system.

the basis of results drawn from studies *in vivo*, have further suggested a relationship between pO_2 and remodelling of alveolar bone during orthodontic tooth movement (Khouw & Goldhaber, 1970). Other regulators of bone remodelling have also been proposed, including piezo-electric stimuli (Zengo *et al.*, 1974) and pressure and tension (Baumrind, 1969).

It is unlikely that a direct role of pO_2 or any other regulator in collagen metabolism can be established by experiments *in vivo*, because of the difficulty of controlling the numerous variables that are present. A system *in vitro* in the form of an organ culture therefore provides a promising experimental model for elucidation of the exact role of such potential regulators.

As the periodontium of teeth of limited eruption in small animals is not fully functional until young adulthood, the culture of foetal or neonatal tissues is not appropriate. Organ culture of a single molar and its associated periodontium from adult mouse in a modified New circulator has been described (Turnbull & Melcher, 1974; Melcher & Turnbull, 1976). However, it became evident in preliminary experiments that pO_2 and other parameters, including ascorbic acid (Feng *et al.*, 1977), could not be controlled readily by using this culture system. A con-

tinuous-flow culture system has been modified from Feng *et al.* (1977) to facilitate the manipulation of culture conditions (Yen & Melcher, 1978). The present paper describes the use of this continuous-flow culture system to examine in organ culture, in the continual presence of ascorbic acid, the effect of pO_2 on collagen synthesis and hydroxylation and on synthesis of non-collagenous protein by mouse periodontal tissues.

Experimental

Dissection and culture system

Explants were dissected from 8–9-week-old female white mice (C.M.R.L. strain; Connaught Laboratories, Toronto, Ont., Canada). The mice were killed by cervical dislocation and the two mandibles removed. Gem razor blades (Phillip Morris Inc., Harry Reid and Co., Toronto, Ont., Canada) were used to remove muscle and soft tissue, including gingiva, from the surface of each mandible, and to excise an explant that consisted of the first molar plus the supporting structures and surrounding bone (Turnbull & Melcher, 1974). The explants were cultured in a continuous-flow culture system designed on the principle described by Feng *et al.* (1977) and modified to allow the regulation of pO_2 (Yen & Melcher, 1978). In brief, the system consists of a reservoir from which the medium is pumped by using a peristaltic pump (Minipuls II; Gilson, Villiers-le-Bel, France) through a glass outlet tube and polyvinyl tubing (Tygon 6419-1; Cole-Palmer, Chicago, IL, U.S.A.) into an oxygenator flask housed in an incubator at 37°C. The medium passes through a 6m length of gas-permeable silastic tubing (Silastic Tubing, 602-235; Dow Corning, Midland, MI, U.S.A.) contained in the sealed oxygenator flask before entering a small glass tube of 4mm internal diameter, 6mm external diameter and 20mm in length, which serves as the first of three culture chambers. The next two culture chambers, which are identical, are connected directly in series by using short lengths (10mm) of silastic tubing. The medium flows through the culture chambers, then through a short length of silastic tubing, and leaves the flask to pass through polyvinyl tubing to a collecting flask. A gas source enters and exits from the oxygenator flask through two stainless-steel tubes of external diameter 1.0mm and internal diameter 0.8mm, which are 90mm in length. The tubing and plugs for the flasks were sterilized by irradiation from a ^{137}Cs source, and the flasks were autoclaved before use. Two explants together with a small amount of medium were placed in each of the culture chambers. The chambers were connected to the tubing and the system was assembled and filled with medium. Medium was pumped at a rate of 10ml/h.

Medium, gas mixture and radioisotopes

The medium used was Waymouth's 752/1 (Waymouth, 1959) (Gibco, Burlington, Ont., Canada) supplemented with 10% heat-inactivated foetal bovine serum (Flow Laboratories, Rockland, MD, U.S.A.), 300 μg of ascorbic acid (Fisher Scientific Co., Fairlawn, NJ, U.S.A.)/ml, and an antibiotic-antimycotic mixture (1.5ml/100ml of medium) (Gibco) comprising penicillin (6mg/ml), fungizone (25 μg /ml) and streptomycin (10mg/ml). Dissection was performed in medium containing 3ml of antibiotics/100ml of medium. Fresh ascorbic acid-containing medium was prepared each 24h and added to the reservoir to maintain a relatively constant concentration of ascorbic acid (Feng *et al.*, 1977).

The oxygenator was flushed continuously with O_2/CO_2 (19:1) or air/ CO_2 (19:1). The flow of gas was adjusted to provide a pO_2 in the medium of either 13.3–16.0kPa (100–120mmHg) or 53.4–56.0kPa (400–420mmHg). The pO_2 , pCO_2 and the pH of the medium were measured in 100 μl samples that were withdrawn by inserting the needle of a Hamilton 100 μl syringe (Hamilton Co., Reno, NV, U.S.A.) into polyvinyl collars located at the inlet and outlet of the oxygenator flask. The samples were then analysed in a Radiometer BMS 2 pHM72 Mk2 Blood Micro System, Digital Acid-Base Analyser for pO_2 , pCO_2 and pH (Radiometer, Copenhagen, Denmark). The pCO_2 was 4.0–5.3kPa (30–40mmHg) and the pH 7.4.

By using the assay method described by Feng *et al.* (1977), ascorbic acid concentrations were determined from 1.0ml samples of medium withdrawn in the same manner as those taken for gas analysis. From these measurements the value for ascorbic acid concentration in the culture tubes was estimated to be approx. 95 μg /ml of medium.

Two culture systems were used in each experiment, one for high pO_2 and the other for low pO_2 . After an initial culture period of 24h, 5 μCi of [3H]proline (NET-323 L-[2,3- 3H]proline; New England Nuclear, Boston, MA, U.S.A.; sp. radioactivity 27.8 μCi /mmol)/ml was added to the medium during the last 24h of culture. At each pO_2 range 18 explants were cultured. In addition, 12 explants were cultured for 48h at the low- pO_2 range, followed by another 24h at the high- pO_2 range. Simultaneously 12 explants were cultured for 72h at low pO_2 as controls to determine whether the effect of low pO_2 was reversible with subsequent administration of high pO_2 .

Seven 8–9-week-old female white mice were given intraperitoneal injections of [3H]proline (20 μCi /g body wt.) to compare the protein-synthetic activity of the periodontal organ explants *in vitro* with that of the same tissues *in vivo*. The mice were killed 24h after injection and the first mandibular molars together with their supporting tissues were excised as described above. The periodontal ligament and alveolar tissues

were then assayed for newly synthesized collagen and non-collagenous protein by using the same procedure as that described for the explants labelled *in vitro*.

Assay for collagen and non-collagenous-protein synthesis

The tissues from each pair of explants that had been cultured in the same culture chamber were pooled. The teeth were extracted from the alveolar processes. Soft connective tissue remaining on the periodontal surface of the alveolar bone was removed, and the alveolar tissues were then assayed separately from the periodontal ligament attached to the extracted teeth. Each pair of teeth and alveolar process were then washed briefly in radioisotope-free medium, freeze-thawed twice and dialysed once against 500 ml of fresh medium for 6 h at 4°C. Dialysis was continued against two 500 ml changes of 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-CaCl₂ (assay solution). Each pair of dialysed specimens was placed in 400 µl plastic centrifuge tubes which contained 200 µl of the assay solution, 25 µl of bovine serum albumin as a carrier protein (10 mg/ml in assay solution) and 25 µl of bacterial collagenase (1 mg/ml in assay solution, also containing 0.25 mM-N-ethylmaleimide) purified as described by Peterkofsky & Diegelman (1971) from a commercial preparation (Collagenase, *Clostridium histolyticum* CLSP; Worthington Biochemical Corp., Freehold, NJ, U.S.A.), and then incubated for 1 h at 37°C. The digestion specifically removed the collagen of the periodontal ligament from the teeth and the newly formed unmineralized

on a Beckman 121 M instrument. A second portion was separated in the same system, but the effluent was collected in fractions in scintillation vials to determine the radioactivity associated with proline and hydroxyproline. Radioactivity was measured in a Nuclear-Chicago Isocap 300 liquid-scintillation counter (Searle Instrumentation, Toronto, Ont., Canada) after the addition of 7 ml of Aquasol scintillation fluid (New England Nuclear). The counting efficiency for ³H in these analyses was consistently around 45%.

The non-collagenous protein precipitate was heated in 0.5 ml of 7% trichloroacetic acid at 80°C for 30 min to remove any remaining collagenous proteins. After centrifugation for 5 min at 10000g, the precipitate was dissolved in 90% (v/v) formic acid and the radioactivity measured. The counting efficiency in these analyses was consistently around 55%. As bovine serum albumin was present in the precipitate the specific radioactivity of the non-collagenous protein was expressed as d.p.m. of trichloroacetic acid-precipitated protein/nmol of proline analysed in the collagen fraction.

Specific radioactivities were determined for collagenous hydroxyproline and proline, and for non-collagenous protein proline in periodontal tissue samples radiolabelled in experiments *in vivo* and *in vitro*. Also, the ratios of collagenous proline specific radioactivity to hydroxyproline specific radioactivity were used to assess the efficiency of collagen hydroxylation. The percentage of [³H]proline incorporated into collagen relative to that incorporated into total protein was calculated as follows:

$$\frac{\% \text{ of } [^3\text{H}] \text{ proline in collagen}}{\text{collagen (proline + hydroxyproline) d.p.m.} + \text{non-collagenous protein (proline) d.p.m.}} = \frac{\text{collagen (proline + hydroxyproline) d.p.m.}}{\text{collagen (proline + hydroxyproline) d.p.m.} + \text{non-collagenous protein (proline) d.p.m.}}$$

connective tissue from the bone of the alveolar process. After centrifugation for 5 min at 10000g in an Eppendorf Microfuge (Brinkman Instruments, Toronto, Ont., Canada) the non-collagenous proteins, which had not been digested, were precipitated from the supernatant by the addition of trichloroacetic acid to a final concentration of 7% (v/v).

The precipitate was washed with 300 µl of 7% trichloroacetic acid and centrifuged at 10000g for 5 min, and the supernatants containing the digested collagenous proteins were pooled. Internal standards consisting of 25 µl of [¹⁴C]glycine (0.5 µCi/ml) and norleucine (0.4 mM) in water were added and the mixture was extracted three times with approximately equal volumes of diethyl ether to remove the trichloroacetic acid. The collagenous supernatant was freeze-dried and hydrolysed in 6M-HCl at 110°C for 24 h under vacuum. After drying, the samples were dissolved in 0.2M-sodium citrate buffer, pH 2.2, and a portion was taken for automatic amino acid analysis

Analysis of the nature of collagens synthesized

Periodontal organ explants were continuously labelled for 24 h as described above, except that [¹⁴C]glycine (L-[1-¹⁴C]glycine, CFA.30; The Radiochemical Centre, Amersham, Bucks., U.K.) was used instead of [³H]proline. Similarly, a single intraperitoneal injection of 0.45 mCi of [¹⁴C]glycine was given to 8–9-week-old mice which were killed 24 h after injection for a comparison *in vivo*. After brief washing of the extracted teeth and alveolar bone samples, they were digested for 24 h at 15°C in 100 µl of a 1.0 mg/ml solution of pepsin (2× crystallized pepsin, P-7012; Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.5M-acetic acid. The solubilized labelled proteins were separated by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis in the system developed by Laemmli (1970), but into which the interrupted electrophoretic step described by Sykes *et al.* (1976) to separate type-I from type-III collagen α-chains had been introduced. The radiolabelled

bands were detected by fluorography (Bonner & Laskey, 1974) with Kodak SB-5 X-ray film (Eastman Kodak Co., Rochester, NY, U.S.A.). Quantification of the radiolabelled collagen α -chains was obtained by scanning individual tracks at 550nm on a Gilford spectrophotometer (model 240; Gilford Instruments, Oberlin, OH, U.S.A.) equipped with a film-scanning attachment. Corrections for non-linearity were made from standard curves of collagen α -chains loaded over a range of different concentrations and exposed for various times (H. F. Limeback & J. Sodek, unpublished work).

Results

Analysis of the tissue residues remaining after digestion with the highly purified bacterial collagenase revealed that approx. $55.7 \pm 7.8\%$ of the radioactivity had been removed from the extracted teeth and $45.5 \pm 5.1\%$ from the alveolar bone. Although prolonged digestions released further radioactivity, the digestion was limited to 1 h to minimize the possibility of pulp tissue being digested. Amino acid analysis of the collagenase-digested proteins gave a composition close to that expected for collagen, whereas the non-digested protein contained minimal ($<5\%$) amounts

of hydroxyproline. Analyses of the hot-trichloroacetic acid extracts of protein precipitated after the collagenase digests revealed that only a small percentage (less than 1%) of the radioactivity in the precipitate was solubilized.

The effect of pO_2 on the synthesis and deposition of collagen and non-collagenous proteins in the periodontal ligament is shown in Table 1. At low pO_2 the collagen synthesized was highly underhydroxylated, as indicated by the collagen hydroxyproline/collagen proline specific-radioactivity ratio of 1:1.63. Nevertheless, the percentage incorporation of [3H]proline into collagenous proteins was still appreciable. At higher pO_2 both the collagen proline and hydroxyproline specific radioactivities were increased, whereas the collagen proline/hydroxyproline specific-radioactivity ratios had decreased. The specific radioactivity of the non-collagenous protein, however, was apparently unaffected by the pO_2 of the culture.

Similar results were obtained from analyses of the tissues of the alveolar process (Table 2). The specific radioactivities of the imino acids at the low pO_2 were slightly higher than in periodontal ligament, but the percentage of label incorporated into collagen was on average slightly lower. Considerable underhydroxylation was apparent from the ratio of the collagen

Table 1. *Collagen and non-collagenous-protein synthesis in periodontal ligament in organ culture at different oxygen partial pressures*

Specific radioactivities of imino acids of collagenous proline and hydroxyproline from periodontal ligament of explants labelled for 24 h with [3H]proline were determined as described in the text. Collagenous proteins were separated from non-collagenous proteins by collagenase digestion. All values are expressed as means $\pm 95\%$ confidence limits, for nine pairs of explants. Mean values for each particular pO_2 range refer to nine pairs of explants pooled from three separate experiments, each consisting of three pairs of explants. [3H]Proline incorporation is the percentage of [3H]proline incorporated into collagen relative to total [3H]proline incorporation into total protein.

Cultures	Hydroxyproline (d.p.m./nmol)	Proline (d.p.m./nmol)	Proline:hydroxyproline (d.p.m./nmol:d.p.m./nmol)	Non-collagenous protein (d.p.m./nmol)	[3H]Proline incorporation (%)
Low pO_2 (16.0 kPa)	73 $\pm$ 30	109 $\pm$ 34	1.63 $\pm$ 0.50	64 $\pm$ 20	63 $\pm$ 6
High pO_2 (56.0 kPa)	218 $\pm$ 33	253 $\pm$ 50	1.26 $\pm$ 0.22	71 $\pm$ 24	82 $\pm$ 4

Table 2. *Collagen and non-collagenous-protein synthesis in alveolar process in organ culture at different oxygen partial pressures*

Specific radioactivities of imino acids of collagenous proline and hydroxyproline from alveolar process of explants labelled for 24 h with [3H]proline were determined as described in the text. Collagenous proteins were separated from non-collagenous proteins by collagenase digestion. All values are expressed as means $\pm 95\%$ confidence limits, for nine pairs of explants. Mean values for each particular pO_2 range consist of nine pairs of explants pooled from three separate experiments, each consisting of three pairs of explants. [3H]Proline incorporation is the percentage of [3H]proline incorporated into collagen relative to total [3H]proline incorporation into total protein.

Cultures	Hydroxyproline (d.p.m./nmol)	Proline (d.p.m./nmol)	Proline:hydroxyproline (d.p.m./nmol:d.p.m./nmol)	Non-collagenous protein (d.p.m./nmol)	[3H]Proline incorporation (%)
Low pO_2 (16.0 kPa)	82 $\pm$ 24	141 $\pm$ 35	1.78 $\pm$ 0.28	76 $\pm$ 28	58 $\pm$ 8
High pO_2 (56.0 kPa)	233 $\pm$ 40	310 $\pm$ 69	1.33 $\pm$ 0.12	119 $\pm$ 30	77 $\pm$ 3

imino acid specific radioactivities. At the higher pO_2 the specific radioactivities were again slightly higher than the ligament values, as was the average percentage incorporation of label into collagenous proteins. The collagen imino acid radioactivities increased with the increase in pO_2 , as found in the tissues of the periodontal ligament, but in contrast the non-collagenous-protein proline specific radioactivity in the tissue of the alveolar process did not increase significantly. Increased hydroxylation was apparent from the decreased collagen proline/hydroxyproline specific-radioactivity ratios.

The results of experiments in which tissues were cultured at low pO_2 for 48 h and then at high pO_2 for the final 24 h are shown in Tables 3 and 4. The specific radioactivities of the collagen imino acids and the non-collagenous proline in both tissues of the experimental explants were essentially unchanged from values obtained from tissues cultured continuously at low pO_2 . In addition, there was no apparent difference in the degree of hydroxylation as indicated from the ratios of collagen imino acid specific radioactivities.

The values for collagen synthesis and hydroxyl-

Table 3. *Recovery of collagen and non-collagenous-protein synthesis in periodontal ligament in organ culture at high oxygen partial pressures after an initial period at low oxygen partial pressures*

Specific radioactivities of imino acids of collagenous proline and hydroxyproline from periodontal ligament of explants labelled for 24 h with [3H]proline were determined as described in the text. Collagenous proteins were separated from non-collagenous proteins by collagenase digestion. All values are expressed as means $\pm 95\%$ confidence limits, for 12 pairs of explants. Mean values for each particular pO_2 range consist of 12 pairs of explants pooled from four separate experiments, each consisting of three pairs of explants. [3H]Proline incorporation is the percentage of [3H]proline incorporated into collagen relative to total [3H]proline incorporation into total protein.

Cultures	Hydroxyproline (d.p.m./nmol)	Proline (d.p.m./nmol)	Proline:hydroxyproline (d.p.m./nmol:d.p.m./nmol)	Non-collagenous protein (d.p.m./nmol)	[3H]Proline incorporation (%)
Low pO_2 then high pO_2	93 $\pm$ 21	121 $\pm$ 22	1.35 $\pm$ 0.13	89 $\pm$ 21	67 $\pm$ 4
Low pO_2	68 $\pm$ 24	124 $\pm$ 33	2.11 $\pm$ 0.65	73 $\pm$ 18	64 $\pm$ 9

Table 4. *Recovery of collagen and non-collagenous-protein synthesis in alveolar process in organ culture at high oxygen partial pressures after an initial period at low oxygen partial pressures*

Specific radioactivities of imino acids of collagenous proline and hydroxyproline from alveolar bone of explants labelled for 24 h with [3H]proline were determined as described in the text. Collagenous proteins were separated from non-collagenous proteins by collagenase digestion. All values are expressed as means $\pm 95\%$ confidence limits, for 12 pairs of explants. Mean values for each particular pO_2 range consist of 12 pairs of explants pooled from four separate experiments, each consisting of three pairs of explants. [3H]Proline incorporation is the percentage of [3H]proline incorporated into collagen relative to total [3H]proline incorporation into total protein.

Cultures	Hydroxyproline (d.p.m./nmol)	Proline (d.p.m./nmol)	Proline:hydroxyproline (d.p.m./nmol:d.p.m./nmol)	Non-collagenous protein (d.p.m./nmol)	[3H]Proline incorporation (%)
Low pO_2 then high pO_2	200 $\pm$ 48	257 $\pm$ 79	1.66 $\pm$ 0.24	131 $\pm$ 44	72 $\pm$ 7
Low pO_2	130 $\pm$ 38	222 $\pm$ 63	1.73 $\pm$ 0.15	119 $\pm$ 45	71 $\pm$ 7

Table 5. *Collagen and non-collagenous-protein synthesis in periodontal ligament and alveolar process in vivo*

Specific radioactivities of imino acids of collagenous proline and hydroxyproline from periodontal ligament and alveolar bone of explants labelled for 24 h with [3H]proline were determined as described in the text. Collagenous proteins were separated from non-collagenous proteins by collagenase digestion. All values are expressed as means $\pm 95\%$ confidence limits for seven pairs of explants. Mean values for each type of tissue consist of seven pairs of explants from seven animals. [3H]Proline incorporation is the percentage of [3H]proline incorporated into collagen relative to total [3H]proline incorporation into total protein.

Tissue	Hydroxyproline (d.p.m./nmol)	Proline (d.p.m./nmol)	Proline:hydroxyproline (d.p.m./nmol:d.p.m./nmol)	Non-collagenous protein (d.p.m./nmol)	[3H]Proline incorporation (%)
Periodontal ligament	116 $\pm$ 36	127 $\pm$ 50	1.10 $\pm$ 0.09	19 $\pm$ 5	89 $\pm$ 5
Alveolar tissue	233 $\pm$ 77	238 $\pm$ 72	1.04 $\pm$ 0.09	71 $\pm$ 35	84 $\pm$ 8

ation, and for non-collagenous protein synthesis for the periodontal ligament and alveolar process cultured continuously at high pO_2 , were compared with values obtained from their counterparts *in vivo* (Table 5). The specific radioactivities of the collagen imino acids of the cultured periodontal ligament were approximately twice as much as *in vivo*, whereas those of the non-collagenous protein were approximately three times higher. For the alveolar tissues *in vitro*, values were much closer to their counterparts *in vivo*. In both periodontal ligament and alveolar tissues the ratios of collagen imino acid specific radioactivities *in vitro* were slightly higher than those *in vivo*, but the percentage incorporation of label into collagenous protein was similar to the results *in vivo*.

The nature of the collagens synthesized by the periodontal tissues cultured at high pO_2 was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and fluorography of the collagen

α -chains produced by pepsin digestion. Representative densitometric scans of the fluorogram tracks of tissues labelled *in vivo* and at high pO_2 *in vitro* are shown in Fig. 1. In each case three major peaks, corresponding to type-III collagen α -chains and type-I collagen α_1 - and α_2 -chains, were resolved. Minor radiolabelled bands occurred in positions expected for β - and γ -chains. In addition, minor bands were also observed between the α_1 (III) and α_1 (I) bands. These bands, which were more prominent in alveolar-process samples, may correspond to αA and αB collagens (Burgeson *et al.*, 1976). The proportion of type-III collagen was $25 \pm 5\%$ for both ligament and alveolar tissues *in vitro*. A similar value was obtained for the alveolar tissues *in vivo*. However, in the periodontal ligament *in vivo* the type-III collagen comprised $14 \pm 3\%$ of the total α -chains. The ratio between the radioactivity associated with type-I collagen α_1 - and α_2 -chains was close to 2:1 both *in vitro* and *in vivo*.

Fig. 1. Densitometric tracings of fluorographic band patterns of collagen α -chains produced by pepsin digestion and separated by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis

Collagenous proteins of periodontal ligament or alveolar process tissue labelled *in vivo* or *in vitro* under conditions of high pO_2 and in the presence of ascorbic acid were digested with pepsin. The digests were freeze-dried and separated on a 7.5% (w/v) polyacrylamide slab gel. The type-III α -chains were separated from the type-I α -chains by initially running the electrophoresis under non-reduced conditions. After 1 h the electrophoresis was stopped and the samples were treated with 20% (v/v) mercaptoethanol placed in the sample wells. The electrophoresis was then resumed. The slab gel was impregnated with 2,5-diphenyloxazole, vacuum-dried, then exposed to X-ray film, which was developed and scanned at 550 nm. The resulting densitometric scans are shown. Electrophoresis was carried out from negative to positive electrode. Peaks representing the β -chains and α -chains are identified. (a) Periodontal ligament *in vivo*; (b) periodontal ligament *in vitro*; (c) alveolar-process tissue *in vitro*; (d) alveolar-process tissue *in vivo*.

Discussion

The incorporation of [^3H]proline into collagenous and non-collagenous proteins of periodontal tissues in organ culture has been used to measure the synthesis and deposition of these proteins in the periodontal ligament and tissues of the alveolar process *in vitro* at two different ranges of $p\text{O}_2$. It is important to point out that the values given are for $p\text{O}_2$ in the medium, and do not represent the $p\text{O}_2$ in the tissues, the values of which have not been measured, but which we believe to be much lower than in the medium. The results demonstrate that an adequate oxygen supply is essential to achieve normal hydroxylation of the collagen. In addition, it is evident from the collagen imino acid specific radioactivities that the accumulation of newly synthesized collagen within the tissues is dramatically increased at elevated $p\text{O}_2$. Since the nature of the continuous-flow culture system currently precludes the analysis of intact or degraded collagen released from the explant, the increased collagen imino acid values cannot be unequivocally attributed to increased collagen synthesis. However, the apparent lack of effect of oxygen on non-collagenous protein suggests that increased collagen synthesis is probable.

The requirement of atmospheric oxygen for the hydroxylation of collagenous peptidylproline (Fujimoto & Tamiya, 1962; Prockop *et al.*, 1963) predicts the production of underhydroxylated collagen in environments having a relatively low $p\text{O}_2$. This has also been demonstrated in our studies by the higher ratio between collagenous proline and hydroxyproline specific radioactivities in both alveolar bone tissue and periodontal ligament from explants cultured at the lower $p\text{O}_2$ as compared with that at the higher $p\text{O}_2$. Our results are in agreement with previous studies demonstrating marked inhibition of hydroxyproline formation in relatively anaerobic culture conditions (Juva *et al.*, 1966; Hutton *et al.*, 1967; Uitto & Prockop, 1974), but are contrary to the findings of Levene & Bates (1976), who demonstrated that relatively anoxic conditions in short-term 3T6 fibroblast cultures stimulated prolyl hydroxylase activity, which was accompanied by increased hydroxylation of cell-layer collagen. The discrepancy may be explained by the fact that the periodontal-organ explant was not ascorbate-deficient, as were the cell cultures of Levene & Bates (1976).

The inability of the explants to 'recover' from 48 h of low $p\text{O}_2$ when cultured subsequently at high $p\text{O}_2$ may be due to decreased cell viability within the explant resulting from the low $p\text{O}_2$. However, the selective effect of $p\text{O}_2$ on collagen synthesis and deposition suggests that increased $p\text{O}_2$ does not simply amplify cellular activity. It is conceivable that the $p\text{O}_2$ may determine the survival of certain cell types within the tissue. In contrast with these findings, we

have been able to show that the tissues in the explant can recover from the effects of a 24 h period of avitaminosis C (J. Sodek, J. Feng, E. H. K. Yen & A. H. Melcher, unpublished work). The restricted availability of oxygen in the low- $p\text{O}_2$ cultures may also have allowed prolyl hydroxylation to become the rate-controlling step in collagen synthesis (Prockop *et al.*, 1976). Since prolyl hydroxylation has been shown to be inefficient in the absence of co-substrates such as O_2 (Barnes & Kodicek, 1972), a decreased rate of collagen synthesis might be expected. Stimulation of collagen synthesis by oxygen has been demonstrated *in vitro* by using tibia explants cultured at various $p\text{O}_2$ ranges (Shaw & Bassett, 1967), and also *in vivo* (Chvapil *et al.*, 1968; Niinikoski & Kivisaari, 1973). Alternatively, lower hydroxyproline and collagenous-proline specific radioactivities in low- $p\text{O}_2$ cultures could be explained by the possible release of procollagen extracellularly (Ramaley *et al.*, 1973) and the subsequent degradation of the unstable underhydroxylated collagen (Switzer & Summer, 1973; Schiltz *et al.*, 1977), which then would not be detected if lost during dialysis to remove free [^3H]proline.

Interestingly, both alveolar tissue and periodontal ligament from explants cultured at low $p\text{O}_2$ do not demonstrate dramatic decreases in non-collagenous-protein specific radioactivity during the first 48 h of culture, as could be expected from the work of Glinos *et al.* (1973) and Uitto & Prockop (1974), who used even shorter culture periods than those used here.

Analysis of the nature of the collagens synthesized by mouse periodontal tissues *in vitro* has demonstrated that both types-I and -III collagens are produced. The proportion of type-III collagen comprising the separated α -chains was found to be similar for alveolar tissues and periodontal ligament *in vitro* and alveolar tissues *in vivo*. However, the proportion of type-III collagen in periodontal ligament *in vivo* was considerably lower, and corresponded to the proportions found in rat periodontal ligament (J. Sodek & H. F. Limeback, unpublished work) and in bovine incisor periodontal ligament *in vivo* (Butler *et al.*, 1975). These results indicate that type-III collagen synthesis is increased relative to that of type-I collagen when periodontal ligament, but not alveolar-process tissue, is maintained in organ culture.

The synthesis of type-III collagen in bone tissues has not previously been reported, although its presence in the fibrous tissue of haematopoietic marrow spaces has been demonstrated by using immunohistochemical methods (Reddi *et al.*, 1977). The significance of the occurrence and the rapid metabolism of type-III collagen in the tissues of the alveolar process is unknown, but it is possible that it is located in the endosteal spaces and not in the bone tissues.

Although the actual specific radioactivities obtained

from experiments *in vivo* cannot be compared directly with the results *in vitro*, they can be used to demonstrate that the pattern of protein and collagen synthesis by periodontal tissues *in vitro* cultured at high pO_2 is comparable with the pattern *in vivo* (Table 5). The percentage incorporation of label into collagenous proteins by the ligament and alveolar process *in vitro* is only slightly lower than *in vivo*. In addition, analysis of the nature of the collagen synthesized has shown that both type-I and -III collagens are produced by these tissues *in vitro* and *in vivo*. There are some differences, however. Firstly, it is apparent that complete hydroxylation has not been obtained *in vitro*. This is probably due to an oxygen-gradient effect resulting from the size of the explant (Yen & Melcher, 1978). Secondly, the hydroxyproline specific radioactivities indicate that collagen synthesis in the periodontal ligament is increased relative to that in the alveolar tissues *in vitro* as compared with the situation *in vivo*. This difference may be due to an actual increase in collagen synthesis in the periodontal ligament *in vitro*, a decrease in the alveolar tissues, or a net loss of ligament collagen during culture, which has been shown morphologically to occur (Melcher & Turnbull, 1976). Thirdly, the periodontal ligament synthesized proportionately more type-III collagen *in vitro*. Whether the differences in the rate and type of collagen synthesis can be related to the fact that the tooth is non-functional *in vitro* remains to be determined.

The organ-culture system used in these experiments is unique in that mature, contiguous hard and soft connective tissues from adult animals being cultured *in vitro* in a relatively stable concentration of ascorbic acid and adequate pO_2 have been shown to synthesize proteins in a manner similar to corresponding tissues *in vivo*. Previous organ-culture techniques have utilized embryonic material to study collagen metabolism *in vitro*. Because periodontal tissues are fully functional only in the adult animal, this culture system provides a model *in vitro* suitable for analysing the effect of various regulators involved in the physiology and pathology of the periodontal tissues.

We thank Mr. J. Feng and Mr. H. F. Limeback for their assistance in this project.

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