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THE EFFECT OF DIPHENYLHYDANTOIN UPON THE BIOSYNTHESIS AND DEGRADATION OF COLLAGEN IN CAT PALATAL MUCOSA IN ORGAN CULTURE*

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Abstract—The effect of diphenylhydantoin (DPH) upon collagen metabolism in cat palatal mucosa was studied in organ culture using a grid technique. The incorporation of [^{3}H]proline into [^{3}H]hydroxyproline was taken as a measure for collagen synthesis. Using a 24 hr [^{3}H]proline pulse the collagen synthesis in the control explants was found to be constant from the third day until the end of the culture period at day 15. DPH, in the concentrations 5 and 20μg/ml, did not influence collagen or non-collagen protein synthesis. When collagen synthesis was studied using continuous labelling with [^{3}H]proline for 6 days, DPH increased the incorporation of radioactive collagen into the tissues. To study the collagen degradation cats were injected with [^{3}H]proline and six days later the palatal mucosa was excised and cultured in the absence and presence of DPH for 6 days. The release of radioactive hydroxyproline into the culture medium was taken as a measure for collagen degradation. DPH (20 μg/ml) resulted in about a 50 per cent decreased release of radioactive and non-radioactive hydroxyproline to the medium. To study the effect of DPH on the degradation of *in vitro* labelled collagen, mucosal explants were cultured in the presence of [^{3}H]proline for 24 hr, and post-cultured for another 24 hr period. The release of labelled and unlabelled hydroxyproline from the explants was then followed for 4 days. DPH was found to inhibit the release of radioactive hydroxyproline (from collagen 1–2 days old) only slightly, but caused a strong inhibition of the degradation of non-radioactive collagen. It is concluded that DPH is an inhibitor of collagen degradation and that the inhibition applies mainly to the degradation of older collagen.

Diphenylhydantoin (DPH) is a spasmolytic agent used in the treatment of epilepsy since 1938 [1]. Hyperplasia of the gingiva occurs as a frequent side effect when administered to humans [2] or animals [3–5]. This hyperplasia is characterized by a high content of collagen, as is healthy gingiva [2].

Administration of DPH to rats is shown to cause increased amounts of insoluble collagen and the appearance of a 'unique' insoluble non-collagenous protein in their skin [6]. DPH has also been shown to stimulate proliferation of fibroblasts *in vitro* [7–11], but opinions about its effect upon the collagen synthesis are divergent. Thus, Kasai and Hachimine [12] noticed that DPH stimulated collagen synthesis in different fibroblast cell-lines, whereas Houck *et al.* [13] and Hassell *et al.* [14] found no such effect. Blumenkrantz and Asboe-Hansen, when incubating chicken embryonic bone with DPH, found that the collagen synthesis was reduced [15]. Fibroblasts isolated from DPH-induced gingival hyperplasias had an increased protein and collagen synthesis compared to those from normal gingiva or gingiva from patients receiving DPH without contracting gingival hyperplasia [14]. These authors suggested that DPH either induces fibroblasts to increased synthetic activity or selects for fibroblasts with high synthetic capacity. However, not only the synthesis but also the degradation of collagen is of importance for the regulation of the collagen content in connective tissue. We have found only two reports dealing with the collagenolytic activity of gingival cells and tissues from DPH-treated patients. Fullmer [16] has mentioned that the collagenolytic activity in the medium after culturing hyperplastic gingiva-biopsies from DPH-treated patients was higher than from controls with normal gingiva. Rose and Robertson [17] registered reduced collagen lysis when fibroblasts isolated from DPH-induced gingival hyperplasias were cultured on collagen-coated cover slips compared to that produced by fibroblasts from inflamed gingiva. As the results of these two investigations are contradictory, the effect of DPH on collagen degradation remains unclear, especially as DPH was present in neither of the studies during the culture period. Consequently, the effect registered may only reflect persistent alterations in cellular activity or number of cells.

The aim of the present investigation is to study the synthesis and degradation of collagen in organ culture in the presence and absence of DPH.

MATERIAL AND METHODS

The animals used in the experiments were healthy mongrel cats of both sexes. They were fed a soft diet consisting of one part cereals, one part ground fish and one part cattle viscera and were given powdered milk *ad lib.*

Organ culture of cat palatal mucosa

Preparation of explants. The animals were anesthetised intraperitoneally (i.p.) with Mebumal (ACO, Läkemedel AB, Solna, Sweden) (30 mg/kg body weight) and exsanguinated by cardiac rupture. The palatal mucosa was rinsed thoroughly with sterile Ty-

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rode's solution. Using two scalpel blades mounted in parallel, 3 mm apart, 5–7 incisions were made in the hard palate at right angles to the midline, beginning just behind the papilla incisiva. An incision was also made along the tooth arch about 1 mm from the teeth, and the palatal mucosa, including the periosteum, was then dissected from the underlying palatal bone. The mucosal strips were laid upon a Teflon plate and cut into rectangular pieces by a punch equipped with 7 parallel razor blades mounted 1 mm apart. The pieces of mucous membrane had an epithelial surface area of 1 × 3 mm, while the thickness varied, depending upon which region of the palate the particular piece was obtained. Under a dissecting microscope, the tissue pieces were incised through the epithelium into the connective tissue perpendicular to the epithelial surface and it was checked that they were free of blood. The time lapse from the sacrifice of the animals to the onset of the culture was about 1 hr.

Culture method. The culture unit consisted of a culture chamber [18] in which a 6 cm disposable plastic petri-dish (A/S Nunc, Roskilde, Denmark) was placed. The petri-dish was equipped with a platform of titanium expanded metal (25 × 25 mm) supported by 3 mm legs. 3 strips (25 × 5 mm) of Selectron filter with a pore diameter of 0.45 μm (Schleicher and Schüll, Dassel, West Germany) were placed upon the platform. 4.5 ml of medium were added to the petri-dish, reaching a level to just wet the filter. The culture medium was equilibrated with a gas mixture (20% O_2, 75% N_2, 5% CO_2) for 30 min before it was pipetted into the petri-dishes. During the entire explantation procedure, the culture chambers were gassed with a flow of 100 ml/min. On each platform 6 mucous membrane pieces (2 pieces from the anterior, 2 from the middle and 2 from the posterior part of the palate) were placed on the filter paper with the epithelial surface perpendicular to the filter. After explantation, the culture chambers were placed in a 37° incubator and gassed for a further 15 min. The cultures were then gassed daily for 15 min.

Medium and supplements. The medium consisted of Eagle's [19] MEM (SBL, Stockholm, Sweden). As Fe^{2+} is a co-factor in collagen synthesis the medium was supplemented with Fe $(NO_3)_3 \cdot 9\ H_2O$ (0.1 μg/ml) and reducing agent (ascorbic acid 50 μg/ml). Benzylpenicillin (100 U/ml, AB Kabi, Stockholm, Sweden), streptomycin sulphate (50 μg/ml, Glaxo Laboratories Ltd., England) and mycostatin (50 U/ml, E. R. Squibb & Sons, Inc., USA) were also added. For the various experiments further additions to the medium were made: radioactive proline (L-[5-^{3}H]proline, 22 Ci/mole, cat. no. TRK 323, Amersham, Bucks, England, 5 μCi/ml), sodium 5,5-diphenyl hydantoinate (Sigma Chemical Co., St.Louis, MO, USA) from a solution prepared immediately before the experiment to give a final concentration of 5 μg/ml or 20 μg/ml in the medium. 0.01M-NaOH was used as solvent for DPH resulting in a concentration of NaOH in the culture medium of 5 × 10^{-5}M. The control medium contained the same amount of NaOH (5 × 10^{-5}M). These additions did not affect the pH of the medium. The medium was changed every third day, although freshly prepared aqueous solutions of ascorbic acid (50 μg/ml) were added daily to the media. The reason for choosing 5 and 20 μg DPH/ml medium was that the serum concentration of DPH in cats treated with 5mg DPH/kg body weight ranged between 10 and 20 μg/ml. This dose was found to give gingival hyperplasia in 6 of 8 animals within a 6 week period.

Experimental design

4 different experiments were designed:

1. To determine the kinetics of collagen synthesis in explants during a 15 day period.

2. To study the effect of DPH on the collagen synthesis with continuous labelling during a 6 day period in explants from cats of two age groups.

3 and 4. To investigate the effect of DPH on the degradation of *in vivo* and *in vitro* labelled collagen.

1. *Collagen and protein synthesis in cat palatal mucosa maintained in organ culture for up to 15 days.* Six 8-week-old cats originating from 3 litters (1♂, 1♀ + 1♂, 1♀ + 2♀) were used in the study. The material from each cat was distributed in 7 petri-dishes (6 pieces/dish), for the study of protein and collagen synthesis. The explants were harvested after 1, 3, 6, 9, 12 and 15 days, the medium being replaced with medium containing [^{3}H]proline for the final 24 hr prior to harvesting. In order to study the effect of DPH upon collagen and general protein synthesis one dish from each animal was exposed to DPH (20 μg/ml) and [^{3}H]proline for the 24 hr prior to the 6th day of harvesting.

2. *The effect of DPH on the content of newly synthesized collagen in palatal mucosa maintained for a 6 day period in organ culture.* One series of experiments was performed upon 2 male and 5 female 4-week-old cats and another series upon 4 female 8-month-old cats. The explants from each cat were distributed between 6 dishes. The medium contained [^{3}H]proline from the beginning of the experiment. Two of the dishes contained 5 μg/ml DPH, two 20 μg/ml and two were controls. All explants were harvested after 6 days culture and the media from each dish were pooled separately and retained.

3. *Degradation of* in vivo *labelled collagen.* Two litters, each consisting of 4 nine-week-old kittens, were used (1 male, 3 females + 4 females). The litters were put into cages and received gelatin capsules daily, half of the animals empty capsules and the other half receiving capsules containing sodium 5,5-diphenyl hydantoinate, 5 mg/kg body weight. After 6 days, radioactive proline dissolved in saline was injected intraperitoneally (0.75 or 1 μCi/g body weight). The DPH administration was sustained for a further 6 days after which the animals were sacrificed. Dissection and explantation of the palatal mucosa was done as described. The material from each of the eight animals was divided into an experimental and a control group with four petri-dishes in each and six explants in each dish (64 dishes in all). The explants in the experimental group were cultured in medium supplemented with DPH (20 μg/ml) and in the control group in medium with addition of the vehicle (NaOH). The culture period was six days with a change of medium after three days.

4. *Degradation of* in vitro *labelled collagen.* Explants from the palatal mucosa of 2 female 7-week-old kittens from the same litter were distributed to 12 petri-dishes. The explants were cultured for 12 hr after which the medium was changed to one containing L-[5-^{3}H]proline

(20 μCi/ml, 22 Ci/mole) and the culture was continued for 24 hr. The medium was then changed to non-radioactive one and culture was continued for a further 24 hr with a change after 12 hr. Finally, the material from each animal was divided into three groups, one of which was cultured in medium with only vehicle, one in medium containing 5 μg DPH/ml and one in medium containing 20 μg DPH/ml. The culture period was 4 days.

Analytical methods

Weight determination. Prior to culture the wet weights of the explants were determined. Filter paper strips (Munktell's No 3, Grycksbo Pappersbruk AB, Grycksbo, Sweden) of a standardized humidity were obtained by dipping the strips in Tyrode's solution and then pressing them between 2 spring-loaded Teflon rollers. The mucous membrane pieces, kept in Tyrode's solution, were placed on the pressed filter paper strips to absorb the excess solution, and were then immediately transferred to sterile pre-weighed stoppered tubes to determine the wet weight. The weighing procedure prolonged the explantation by 10–15 min. The harvested explants were dried under vacuum in the presence of phosphorous pentoxide for 20 hr at 70° and stored at −20°.

Homogenization and dialysis. The dried mucous membrane pieces were placed in small glass tubes together with distilled water and then frozen in a mixture of dry ice and ethanol. The frozen material was homogenized with a siliconized dental drill having a diameter somewhat smaller than that of the glass tubes. The procedure was repeated until an even homogenate was obtained. In the 15-day study only the homogenates were analyzed. These were dialyzed extensively against running tap water before determination of total radioactivity and radioactive hydroxyproline. In the 6-day study both medium and explants were examined. After the explants had been harvested the medium from each dish was pooled separately, centrifuged at 10,000 *g* for 20 min, and the supernatants were stored at −20°. The homogenized explants and the media were dialyzed separately against 25 vol. of distilled water at 4° for 24 hr and further 25 vol. for another 24 hr.

In the degradation studies the homogenized explants were extensively dialyzed against tap water. The media were centrifuged and stored at −20°.

Determination of incorporated radioactivity and radioactive hydroxyproline. All the media and the tissue homogenates were evaporated to dryness in a rotary evaporator and hydrolyzed in 6M-HCl in sealed glass ampules under nitrogen for 20 hr at 120°. The hydrolyzates were filtered through a Selectron filter, 0.22 μm evaporated to dryness under vacuum and dissolved in 4 ml distilled water (8 ml for the diffusible fraction of the medium). For measurement of the radioactivity, samples usually 0.2 ml were mixed with Instagel (Packard Instrument Company Inc., Downers Grove, Il, USA) before scintillation counting. Each sample was corrected for quenching by the use of the external standard ratio method and quenched standards containing L-[5-^{3}H]proline. This measured radioactivity incorporation of [^{3}H]proline into the non-diffusible tissue proteins was used as an estimate of total protein synthesis. Radioactive hydroxyproline was analyzed according to Juva and Prockop [20] and non-radioactive proline according to Kivirikko *et al.* [21]. Contamination of hydroxyproline radioactivity by radioactive proline was corrected for with the aid of L-[5-^{3}H]-proline standards analyzed in parallel with the samples. The values of radioactive pyrrole were corrected for each sample by means of an external standard ratio and quenched standards containing [^{3}H]toluene. The radioactivity in the samples was measured in a Beckman LS-250 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). The coefficient of variation for determination of radioactive hydroxyproline varied according upon the analyzed fraction. It was lowest for the non-dialyzable fraction of the explants (1.7%) and highest for the dialyzable fraction of the medium (5.1%). To calculate the amount of radioactivity associated with collagen the values for hydroxyproline radioactivity were multipled by 2.3 [22]. The coefficient of variation for determination of non-radioactive hydroxyproline was 3.8 per cent.

RESULTS

1. *Collagen and protein synthesis in cat palatal mucosa maintained in organ culture for up to* 15 *days.* The incorporation into non-dialyzable protein decreased continuously up to day 12, when it attained its lowest value. The incorporation decreased linearly between days 1 and 9 (Fig. 1). No significant difference was found in the incorporation of [^{3}H]proline during day 6 between explants cultured in control medium and explants cultured in the presence of 20 μg DPH/ml.

Incorporation of [^{3}H]proline into [^{3}H]hydroxyproline of non-dialyzable protein decreased between the first and the third day. Hereafter the synthesis was unaltered throughout the rest of the culture period (Fig. 1). There was no significant difference between explants

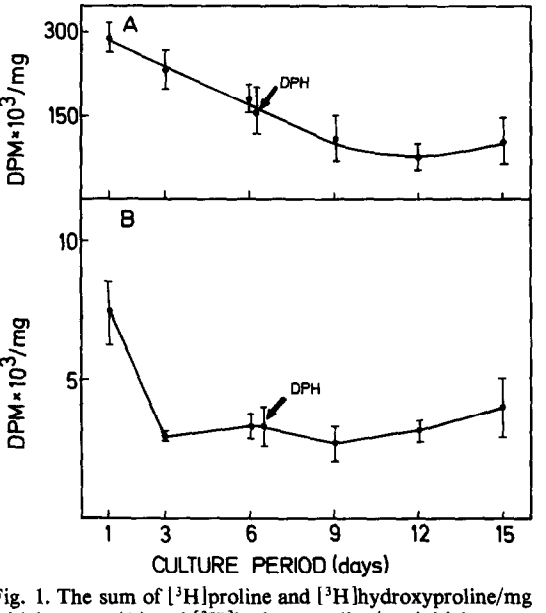

Fig. 1. The sum of [^{3}H]proline and [^{3}H]hydroxyproline/mg initial wet wt (A) and [^{3}H]hydroxyproline/mg initial wet wt (B) in palatal mucosa from 8-week-old cats cultured for 15 days and pulse labelled with [^{3}H]proline for 24 hr periods. The results are expressed as means ± S.E.M. ($n = 6$). During day 6 parallel experiments were performed in which the explants were cultured in the presence of 20 μg DPH/ml.

cultured in the presence or absence of DPH (20 μg/ml) during day 6. A calculation of the amount of radioactivity associated with collagen molecules showed that the collagen protein radioactivity accounted for 5.85 ± 0.80 per cent of the radioactive amino acids during the first day. The highest value was 9.05 ± 0.66 per cent and was obtained on day 12.

2. *The effect of DPH on the content of newly synthesized collagen of palatal mucosa maintained for a 6 day period in organ culture.* The synthesis of [^{3}H]hydroxyproline was 3 times higher in tissues from 4-week-old than in tissues from 8-month-old animals (Table 1). Of the total amount of [^{3}H]hydroxyproline formed, about twice as much was deposited in the younger animals than in the older ones (Table 2). The total amount of [^{3}H]hydroxyproline in the explants was 7 times higher in the younger than in the older animals (Table 1). The total synthesis of [^{3}H]hydroxyproline was not significantly affected by the addition of DPH (5 or 20 μg/ml) to the medium (Table 1). The effect was the same for explants from both young and older animals. The amounts of non-dialyzable radioactive hydroxyproline in the explants of 8-month-old animals was raised significantly in the presence of 5 μg/ml DPH ($P<0.05$). For explants from 4-week-old animals there was a significant increase with 20 μg/ml DPH ($P<0.05$). Of the total amount of synthesized [^{3}H]hydroxyproline, 18.1 per cent was deposited and retained during culture of tissues from 8-month-old animals and this increased to 22.5 per cent when the culture was done in the presence of 20 μg/ml DPH ($P < 0.0025$) (Table 2). A similar increase was also observed after culturing the tissues from the younger animals.

3. *Degradation of* in vivo *labelled collagen.* The weight of the DPH-treated animals remained unchanged during the experimental period while the untreated animals gained in weight. Palatal mucosa from animals which had received DPH did not contain significantly more radioactive hydroxyproline than mucosa from control animals, 348 ± 142 d.p.m./mg and 263 ± 161 d.p.m./mg, respectively. When explants

Table 1. [^{3}H]Hydroxyproline in explants and medium after culturing cat palatal mucosa for 6 days with and without DPH

		[^{3}H]Hydroxyproline d.p.m. 10^3/mg wet wt*			
	DPH μg/ml	8-month-old animals $n = 4$	P	4-wk-old animals $n = 7$	P
Explant					
Non-dialyzable	0	0.98 ± 0.29		7.47 ± 1.28	
	5	1.41 ± 0.36	< 0.05	9.72 ± 0.75	
	20	1.29 ± 0.23		9.87 ± 1.18	< 0.05
Dialyzable	0	0.15 ± 0.09		0.22 ± 0.06	
	5	0.08 ± 0.03		0.20 ± 0.08	< 0.05
	20	0.09 ± 0.04		0.18 ± 0.07	
Medium					
Non-dialyzable	0	1.39 ± 0.27		1.79 ± 0.22	
	5	1.30 ± 0.31		1.62 ± 0.17	
	20	1.19 ± 0.27	< 0.01	1.57 ± 0.22	
Dialyzable	0	3.90 ± 0.80		10.68 ± 1.46	
	5	3.80 ± 0.67		11.05 ± 1.90	
	20	4.17 ± 0.95		11.72 ± 1.08	
Total synthesis	0	6.42 ± 0.83		20.16 ± 2.48	
	5	6.59 ± 0.73		21.59 ± 2.76	
	20	6.47 ± 1.13		23.34 ± 1.94	

Values are means ± S.E.M. of explants and media of 2 dishes from each animal. Control groups and experimental groups are compared by Student's paired t test. P = significance for the t values. n = number of animals, * = initial wet wt of explants.

Table 2. The effect of DPH upon the per cent incorporation of non-dialyzable [^{3}H]hydroxyproline in explants cultured for 6 days

	Per cent incorporated [^{3}H]hydroxyproline			
DPH concn μg/ml	8-month-old animals $n = 4$	P	4-wk-old animals $n = 7$	P
0	18.11 ± 7.64		37.05 ± 3.69	
5	23.46 ± 7.93	< 0.025	45.02 ± 3.04	n.s.
20	22.50 ± 7.38	< 0.0025	42.29 ± 2.51	< 0.05

The data are based upon the experiments summarized in Table 3. The values (means ± S.E.M.) represent non-dialyzable [^{3}H]hydroxyproline in the explants expressed as per cent of total [^{3}H]hydroxyproline in explant and medium; n.s. = not significant.

Table 3. Per cent released [^{3}H]hydroxyproline and non-radioactive hydroxyproline to the culture medium after six days culture of pieces of palatal mucosa pulse-labelled *in vivo* with [^{3}H]proline. The culture was performed in the presence of DPH or in control medium with a change of medium after three days

Medium	Per cent released [^{3}H]hydroxyproline to the medium*											
	Untreated animals				Mean ± S.D.	P	DPH treated animals				Mean ± S.D.	P
	1	2	3	4			5	6	7	8		
Control	17.4	42.5	40.6	60.8	40.3 ± 17.8		64.5	30.9	47.3	76.7	54.9 ± 20.0	
DPH (20 μg/ml)	11.2	18.9	20.5	23.1	18.4 ± 5.12		40.5	7.7	23.6	64.4	34.1 ± 24.3	
Per cent decrease	35.6	55.5	49.5	62.0	50.7 ± 11.3	<0.05	37.2	75.1	50.1	16.0	44.6 ± 24.7	<0.01
Medium	Per cent released hydroxyproline to the medium											
	Untreated animals				Mean ± S.D.	P	DPH treated animals				Mean ± S.D.	P
	1	2	3	4			5	6	7	8		
Control	13.7	37.9	39.7	59.4	37.7 ± 18.7		52.2	19.6	28.3	79.2	44.8 ± 26.7	
DPH (20 μg/ml)	8.2	15.9	19.3	20.5	16.0 ± 5.5		27.5	5.3	20.5	61.8	28.8 ± 23.9	
Per cent decrease	40.2	58.1	51.4	65.5	53.8 ± 10.7	<0.05	47.3	73.0	46.5	22.0	47.2 ± 20.8	<0.01

* Mean of analyses from four petri dishes with six explants in each. The per cent release of [^{3}H]hydroxyproline and non-radioactive hydroxyproline in experiment and control groups are compared with Student's paired *t* test. P = significance. $^+$ = Initial wet weight of explants.

The values for radioactive hydroxyproline in explants and medium from untreated animals ranged between 102 and 433 d.p.m./mg initial wet weight and for DPH-treated animals between 196 and 460 d.p.m./mg initial wet weight. The values for hydroxyproline in explants and medium from untreated animals ranged between 8.81 and 13.39 μg/mg initial wet wt and for DPH-treated animals between 8.36 and 13.39 μg/mg initial wet wt.

from the pulse-labelled control animals were cultured in control medium, 40.3 per cent of the radioactive and 37.7 per cent of the non-radioactive hydroxyproline were released to the medium. Explants from the DPH-treated animals released 54.9 per cent of the radioactive and 44.8 per cent of the non-radioactive hydroxyproline when cultured in control medium (Table 3). The differences in the degradation of labelled and non-labelled collagen between the DPH-treated and the untreated animals was not significant. It is important to note that in all 8 animals, the proportions of labelled and non-labelled collagen released into the medium were virtually the same. When culture was performed in the presence of DPH there was a decreased release of both radioactive and non-radioactive hydroxyproline to the medium whether the animals had been treated with DPH or not. For radioactive hydroxyproline the decrease was 50.7 ± 11.3 per cent (P<0.05) in tissues from control animals and 44.6 ± 24.7 per cent (P<0.01) in tissues from DPH-treated animals. As regards non-radioactive hydroxyproline, the reduction was 53.8 ± 10.7 per cent (P<0.05) in tissues from control animals and 47.2 ± 20.8 per cent (P<0.01) for DPH-treated animals (Table 3).

4. *Degradation of* in vitro *labelled collagen.* Table 4 shows the results obtained when pulse labelling was performed *in vitro*. After culture in control medium the release of radioactive hydroxyproline was about twice as high as the release of non-radioactive hydroxyproline. DPH caused a decreased release of both radioac-

Table 4. Per cent release of [^{3}H]hydroxyproline and non-radioactive hydroxyproline to the culture medium*

	Per cent released [^{3}H]hydroxyproline		Per cent released non-radioactive hydroxyproline	
	Animal		Animal	
Medium	I	II	I	II
Control	51	59.2	25.3	34.3
DPH 5 μg/ml	44.6	49.4	8.9	9.7
DPH 20 μg/ml	41.3	47.3	5.5	11.2

* The pieces of palatal mucosa were pulse-labelled *in vitro* for 24 hr with [^{3}H]proline and 'postcultured' for 24 hr. During the following four days the culture was carried out in DPH-containing and control medium.

Each value represents the mean of duplicate or triplicate dishes with 6 explants in each. The values for radioactive hydroxyproline in explants and medium ranged between 7000 and 11800 d.p.m./mg initial wet wt and for hydroxyproline between 6.75 and 9.34 μg/mg initial wet wt.

tive as well as non-radioactive hydroxyproline from the tissues, the reduction being considerably more pronounced in the latter case.

DISCUSSION

Diphenylhydantoin in the concentration 20 μg/ml, did not influence the synthesis of collagen in the study with pulse labelling during 24 hours. Neither did it influence the collagen synthesis in the study using continuous labelling during 6 days when tested in the concentrations 5 and 20 μg/ml. 20 μg/ml is within the human therapeutic serum concentration range [23, 24] and also within the serum concentration range registered in the cats used in this study receiving a dose of DPH equivalent to the human.

In the first study DPH was found to have no effect upon total protein synthesis. These results agree with those of Houck *et al.* [13] and Hassell *et al.* [14] who found no direct effect of DPH on the synthesis of collagen in cultured fibroblasts. DPH has, however, in one report been shown to increase the collagen content in cells and media after culturing cells from different fibroblast lines for one week or longer [12]. In this latter study inhibition of degradation of collagen which was deposited in the cell layer may have influenced the registered amount of collagen. The collagen in the medium was estimated after ethanol precipitation and the degree of degradation of the collagen may therefore have influenced the estimation of the amount of collagen in the medium. In an *in vitro* study using chicken embryo tibiae DPH inhibited the formation of hydroxyproline and hydroxylysine in the concentration 137 μg/ml or higher but in the concentration 44 μg/ml the drug had no such effect [15]. Even the latter concentration is higher than the human therapeutic serum concentration and it would therefore seem likely that DPH, at least when studied in clinical concentrations, has no direct effect on collagen synthesis *in vitro*.

When the tissues were incubated with [^{3}H]proline for 6 days in the presence of DPH the amount of non-dialyzable [^{3}H]hydroxyproline in the explants was increased, the effect being similar for both age groups. Since the collagen synthesis did not seem to be increased, the accumulation of newly synthesized collagen in the tissue could be due to a reduced degradation or increased deposition of collagen (i.e. increased fibril formation). When the tissues were incubated with [^{3}H]proline for 24 hr there was, however, no difference in the amount of [^{3}H]hydroxyproline between tissues incubated in the presence and in the absence of DPH. This seemingly contradictory result is probably due to the shorter labelling time, the degradation products not having enough time to completely diffuse from the explants.

There was a striking difference in the synthesis pattern for total protein and collagen. During the first 9 days the protein synthesis decreased linearly as indicated by the decreasing content of non-diffusable [^{3}H]proline. The collagen synthesis on the other hand was reduced after the first day but showed a steady state synthesis from day 3 to the end of the experiment. There may be many reasons why the synthesis patterns of total protein and collagen differ but the fact that the explants consist of two tissue components, connective tissue and epithelium, must provide at least a part of the explanation. In an autoradiopraphic study with [^{3}H]proline labelling, Bergenholtz (unpublished) has noticed a considerable uptake of radioactivity in the epithelium of palatal mucosa maintained for 6 days in organ culture. This indicates that a large part of the non-collagenous protein was synthesized in the epithelium. The reduction in thickness of the epithelium shown by Bergenholtz [25] to occur after 6 days culture may thus partly explain the successive reduction in protein synthesis during culture of the explants. The reduction in collagen synthesis after the first day is probably due to a reduction in the cell population responsible for collagen production and the fact that collagen synthesis was unchanged from the third day can be interpreted as an adjustment of the remaining cells to the culture conditions.

The total synthesis of [^{3}H]hydroxyproline was 3 times higher in explants from 4-week-old cats than in those from 8-month-old ones. The amount of non-dialyzable [^{3}H]hydroxyproline in the explants was about 7 times higher for the younger than for the older animals. This difference could be due to a higher incorporation of newly synthesized collagen in tissues from the younger animals or to lower degradation. It has, however, been shown from *in vivo* studies that collagen degradation is faster in younger animals than in older [26] and that there is an age-dependent reduction in the turnover of collagen in different organs of the rat [27]. Little is known about the effect of age upon fibril formation and collagen maturation in oral soft tissues. Schneir *et al.* [28] have shown that collagen in palatal mucosa matures more rapidly in older than in younger rats. This contrasts to the condition in rat skin, where conversion of soluble to insoluble collagen decreases with age [29]. There may, however, be other explanations of our findings such as species differences or differences in adaptability between cells from young and old animals to the *in vitro* conditions.

DPH, in a concentration of 20 μg/ml, inhibited degradation of collagen in cultured cat oral mucosa. The concentration in the gingiva of humans has been shown to be, on average, lower than that of serum [30]. It would also seem likely that a part of the drug is bound to proteins in the gingiva, as has been shown to be the case in serum [31]. Therefore, all DPH molecules may not be available to the cells. Our findings, that even a concentration of 5 μg/ml DPH reduced the collagen degradation to the same degree as did 20 μg/ml DPH, may therefore be relevant when considering the side-effects of the drug on the gingiva. Thus it appears that the effect of DPH on collagen degradation in organ culture, is not dose-related within the tested concentrations.

That DPH inhibits collagen degradation in soft tissues is a new observation. DPH has been shown to inhibit PTH and 25-hydroxycholecalciferol-induced ^{45}Ca mobilisation from cultured bone [32–34] and in a recent study also to inhibit degradation of bone collagen during PTH-stimulated bone resorption [35]. The inhibition of both soft tissue and bone collagen degradation indicates that DPH influences collagenolytic enzymes, for instance reduces their synthesis, their release from the cells or has a direct inhibiting effect upon enzyme activity.

The finding in study 3 that DPH inhibited the degradation of both *in vivo* labelled and unlabelled collagen to the same degree, indicated that DPH, when exerting its inhibitory effects, did not discriminate between 6-day-old collagen and collagen of older age. The finding in study 4, however, that DPH only slightly inhibited the degradation of the 1–2-day-old labelled collagen, indicated that DPH did not significantly interfere with the degradation of newly synthesized collagen. This observation together with a previous one [36] that gold salts, when added to bone cultures, preferentially influenced the degradation of a collagen pool with a half-life longer than 3 days, suggests that fundamental differences may exist in the degradative process between newly synthesized and older collagen. This may be partly due to the maturation of collagen. The soluble newly synthesized collagen is easily degraded so that only a part of the synthesized collagen can be incorporated into the insoluble fraction [37, 38]. In rat, only one-third of the degradation products of collagen recovered in the urine are considered to be derived from insoluble collagen [39]. From the present study it would also appear that in organ culture, newly synthesized collagen is rapidly degraded. The fact that DPH had little influence on degradation of newly synthesized collagen also explains the observations in the first study in which continuous labelling was used. In this case the tissue content of radioactive hydroxyproline, which represents recently synthesized collagen, was only slightly increased by DPH. The pattern of collagen degradation observed in organ culture, and the selective effect of DPH on mature collagen, may be considered to be important factors when trying to elucidate the mechanisms by which DPH gives rise to gingival hyperplasia.

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REFERENCES

1. H. H. Merrit and T. J. Putnam, *J. Am. med. Ass.* **11**, 1068 (1938).
2. E. Aas, *Acta odont. scand.* **21**, suppl. 34 (1963).
3. J. D. King and A. P. Gimson, *Br. dent. J.* **83**, 148 (1947).
4. J. Ishikawa and I. Glickman, *J. Periodont.* **32**, 149 (1961).
5. P. H. Staple, M. J. Reed and P. A. Mashimoto, *J. Periodont.* **48**, 325 (1977).
6. J. C. Houck, R. A. Jacob and G. D. Maengwyn-Davies, *J. clin. Invest.* **39**, 1758 (1960).
7. W. G. Shafer, *Proc. Soc. exp. Biol. Med.* **104**, 198 (1960).
8. W. G. Shafer, *Proc. Soc. exp. Biol. Med.* **106**, 205 (1961).
9. W. G. Shafer, *Proc. Soc. exp. Biol. Med.* **108**, 694 (1961).
10. W. J. Nease, *J. Periodont.* **36**, 22 (1965).
11. S. Kasai and T. Yoshizumi, *Bull. Tokyo dent. Coll.* **12**, 233 (1971).
12. S. Kasai and K. Hachimine, *Bull. Tokyo dent. Coll.* **15**. 53 (1974).
13. J. C. Houck, R. F. Cheng and M. D. Waters, *Proc. Soc. exp. Biol. Med.* **139**, 969 (1972).
14. T. M. Hassell, R. C. Page, A. S. Narayanan and C. G. Cooper, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2909 (1976).
15. N. Blumenkrantz and G. Asboe-Hansen, *Acta Neurol. scand.* **50**, 302 (1974).
16. H. M. Fullmer, *J. dent. Res.* **50**, 288 (1971).
17. G. G. Rose and P. B. Robertson, *J. dent. Res.* **56**, 416 (1977).
18. O. A. Trowell, *Expl. Cell Res.* **6**, 246 (1954).
19. H. Eagle, *Science* **130**, 432 (1959).
20. K. Juva and D. J. Prockop, *Analyt. Biochem.* **15**, 77 (1966).
21. K. I. Kivirikko, O. Laitinen and D. J. Prockop, *Analyt. Biochem.* **19**, 249 (1967).
22. K. Piez, E. Eigner and M. Lewis, *Biochemistry* **2**, 58 (1963).
23. F. Buchtal, O. Svensmark and P. J. Schiller, *Archs Neurol.* **2**, 624 (1960).
24. E. H. Reynolds, D. Chadwick and A. W. Galbraith, *Lancet* May 1, 923 (1976).
25. A. Bergenholtz, *Acta odont. scand.* **27**, suppl. 54 (1969).
26. S. Lindstedt and D. J. Prockop, *J. biol. Chem.* **236**, 1399 (1961).
27. K. T. Kao, D. M. Hilker and T. H. McGovack, *Proc. Soc. exp. Biol. Med.* **106**, 335 (1961).
28. M. Schneir, D. Furuto and K. Berger, *J. periodont. Res.* **11**, 235 (1976).
29. E. Heikkinen and E. Kulonen, *Biochim. biophys. Acta* **160**. 464 (1968).
30. G. J. Conard, H. Jeffay, L. Boshes and A. D. Steinberg, *J. dent. Res.* 53, 1323 (1974).
31. F. Bochner, W. Hooper, J. Tyrer and M. Eadie, *Proc. Aust. Assoc. Neurol.* **9**, 171 (1973).
32. M. Harris, M. V. Jenkins and M. R. Wills. *Br. J. Pharmac.* **50**, 405 (1974).
33. M. V. Jenkins, M. Harris and M. R. Wills, *Calcif. Tissue Res.* **16**, 163 (1974).
34. M. J. Somerman, W. Y. W. Au and B. R. Rifkin, *J. dent. Res.* **56**, B196 (1977).
35. T. J. Hahn, C. R. Scharp, C. A. Richardson, L. Halstead, A. J. Kahn and S. L. Teitelbaum, *J. clin. Invest.* **62**, 406 (1978).
36. C. Onkelinx, R. Srivastava and N. Lefebvre, *J. dent. Res.* **55**, B80 (1976).
37. K. I. Kivirikko, *Int. Rev. Connect. Tissue Res.* **5**, 93 (1970).
38. R. S. Bienkowski, M. J. Cowan, J. A. McDonald and R. G. Crystal, *J. biol. Chem.* **253**, 4356 (1978).
39. O. Laitinen, *Acta Endocr.* (Suppl.) (Kbh). **120**, 50 (1967).