Synthesis and Degradation of Collagen in the Developing Corium of the Chick Embryo

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1. Indices of collagen synthesis and degradation were developed in a chick corium system *in vitro*. 2. These indices were determined by quantitatively measuring non-diffusible and diffusible hydroxy[^{14}C]proline in the tissues after a standard period of incubation. 3. Under these incubation conditions collagen metabolism of the corium appears to be stable for at least 180 min. 4. The indices of collagen synthesis and degradation seem to reflect the conditions of collagen metabolism *in vivo*. 5. A rapid collagen turnover occurs in the corium of the 13–14-day embryo owing to an accelerated collagen degradation at that period. 6. Epidermal elements may influence the synthesis as well as the degradation of collagen.

A number of reports indicate a rapid metabolism of connective tissue in the chick embryo during and immediately after the mid-period of development. Coleman, Herrmann & Bess (1962) observed a sevenfold increase in the uptake of [^{14}C]glycine into collagen of the chick embryo cornea *in vitro* during the 9–16-day period of development. Peterkofsky & Udenfriend (1963) noted a high activity of a mixed-function oxidase that converts a specific peptidyl proline residue into collagen hydroxyproline in the 8–9-day chick embryo. Kivirikko (1963) found a rapid increase in collagen of the chick skin during the 12–14-day period with a peak concentration of diffusible hydroxyproline at 13 days. Woessner, Bashey & Boucek (1967) also observed an abrupt increase in collagen concentration in the chick skin at 12 days of development and a high catheptic activity at 13–14 days that Woessner (1968) associates with rapid collagen degradation at that time. Mottet & Hall (1966) found hydroxyproline in the whole fertilized avian egg at 5 days with more than an eightfold increase during the 9–16-day period. Our Laboratories are interested in using the chick corium to study collagen metabolism and to develop an expression for relative contributions of synthesis and degradation at selected periods during the development of the corium.

To measure the relative contributions of collagen synthesis and degradation, the corium from 9–14-day embryos was incubated in the presence of [^{14}C]proline, and the hydroxy[^{14}C]proline content of the tissue fractions was determined. After a

standard incubation time, the amount of hydroxy-[^{14}C]proline in the non-diffusible fraction was used as an index of collagen synthesis, and that in the diffusate expressed collagen degradation (Hurych & Chvapil, 1965).

METHODS

Fertilized eggs of the Kimber strain White Leghorn chicken (Watkins Laboratories, Miami, Fla., U.S.A.) were placed in the incubator between 10.30 and 11.00 a.m., so that the 24 hr. period for each day of embryo development began at 10.30 a.m. In most experiments the embryos were obtained at 10.30 a.m. The skin was collected in chilled Krebs–Ringer solution (Umbreit, Burris & Stauffer, 1951), the collection requiring approx. 1 hr. The embryo was decapitated as soon as it was removed from the egg because of previous evidence that tryptophan metabolism in the chick embryo is influenced by exposure to light (Boucek, Boucek, Hlavackova & Dietrich, 1967), and acute changes in circulating concentrations of tryptophan or its derivatives could affect protein synthesis in general (Wunner, Bell & Munro, 1966; Sidransky, Bongiorno, Sarma & Verney, 1967).

Preparation of the tissues. The fragments of skin were divided so that there was approx. 500 mg. of tissue in each beaker. To each beaker was added 3 ml. of the following incubation medium: amino acid mixture (Eagle's no. 1 mixture; Grand Island Biological Co., Grand Island, N.Y., U.S.A.), 2 ml.; glucose, 5.5 m-moles; sulphate, 70 μ moles; pyridoxine, 0.113 m-mole; nicotinic acid, 32.4 μ moles; ascorbic acid, 10 m-moles; Krebs–Ringer solution, 100 ml.; final pH 7.4 (adjusted with NaOH). The mixture was equilibrated at 37° for 10 min. in a metabolic shaker, after which 2 μ C of L-[U- ^{14}C]proline (197 mc/m-mole) (New England Nuclear Corp., Boston, Mass., U.S.A.) was added. The reaction was stopped by

adding 3 ml. of ice-cold incubation medium, and the sample was transferred to a 40 ml. polyethylene tube and centrifuged at 320g for 2 min. in an International refrigerated centrifuge (International Equipment Co., Needham Heights, Mass., U.S.A.). The pellet was washed five times with 2.5 ml. of cold Krebs-Ringer solution in the refrigerated centrifuge at 2° and then homogenized in 2 ml. of 0.15M-NaCl in a Potter-Elvehjem homogenizer.

To study the influence of epidermal structures on collagen metabolism, the corium was collected, and the epidermal layer, including the feather buds, was removed from half of the tissues by teasing the epidermis away from the dermis with small curved ophthalmic forceps. The dissection was carried out in an iced Petri dish.

The incubation medium was selected to provide excess of ascorbic acid (Jeffrey & Martin, 1966) for collagen synthesis and the minimum tissue-culture requirements of amino acids for fibroblasts (Eagle, 1959). Inorganic sulphate was included, since earlier work with the chick corium preparation indicated its importance in the synthesis of glycosaminoglycans (Noble & Boucek, 1965). The pH of the incubation medium was adjusted to 7.4, although the metabolism of collagen did not seem to be affected over the pH range 6.8–7.4.

The homogenate was transferred into Visking bags and dialysed overnight at 4° against 20 ml. of water followed by two changes at 2–3 hr. intervals. On completion, the diffusate and the non-diffusible material were dried and hydrolysed with 6N-HCl at 110° for 16 hr. In some experiments the dialysed homogenate was centrifuged at 40000g for 20 min. in the Spinco model L ultracentrifuge (rotor no. 40) and the hydroxy[¹⁴C]proline determined in the supernatant and pellet fractions.

Radioactive hydroxyproline was measured in the incubation medium in some experiments, and the tissue pellet washes were combined with the incubation medium. The incubation medium was dialysed against large volumes of water over a 3-day period, and the diffusate and non-diffusible fractions were evaporated to dryness, hydrolysed and then analysed.

After hydrolysis a small amount of activated charcoal was added to each tube and the contents were filtered through Whatman no. 40 paper into evaporating dishes. The HCl was evaporated off and the residue was dissolved in 0.2 ml. of 70% (v/v) ethanol and applied to Whatman no. 1 paper for separation of the amino acids by the technique of Pasiëka & Morgan (1956). The proline and hydroxyproline areas were eluted; the eluates were dried down and transferred to aluminium planchets for counting in a gas-flow counter with a thin-window Geiger-Müller tube (model D47; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). The area between the amino acid spots was tested for radioactivity and found to have approximately background values. Corrections were made for background radioactivity. The thickness of the sample was such that the count did not require a correction for self-absorption. After the radioactivity determination, the sample was eluted from the planchet and the hydroxyproline determined by a modification of the Stegemann (1958) procedure (Woessner, 1961).

Radioactivity expressions. For comparing collagen synthesis in embryos of different ages, the amount of hydroxy[¹⁴C]proline was related to the embryo in the following manner. The sum of the hydroxyproline value of

all the samples in one experiment was divided by the number of embryos used in that experiment to obtain the amount of hydroxyproline/embryo tissue. By using this value, the hydroxyproline value for each incubation beaker was converted into its equivalent in number of embryo skins. The hydroxy[¹⁴C]proline was then expressed as counts/min./embryo skin. The specific radioactivity of hydroxyproline was also determined, in counts/min./μg. of hydroxyproline.

Tissue acid phosphatase reaction. The 14-day embryos were mounted *en bloc* in a gelatin-polyvinylpyrrolidone mixture and frozen with an acetone-solid CO₂ slurry. The block was mounted in a cryostat and 10 μ sections were made for histochemical determination of the acid phosphatase reactions according to the technique of Burstone (1962).

RESULTS

Characteristics of the system in vitro. Omission of ascorbic acid decreases hydroxy[¹⁴C]proline synthesis by 25% (Table 1), and therefore ascorbic acid was included in the incubation medium as a routine. A decrease in ambient oxygen concentration decreases hydroxy[¹⁴C]proline synthesis by more than 25%. Incubation at 4° results in a 75% decrease in hydroxy[¹⁴C]proline synthesis. Puromycin decreases the synthesis by more than 70%.

Tissue hydroxy[¹⁴C]proline is found in three fractions: diffusible, salt-soluble non-diffusible (0.15M-sodium chloride-extractable) and salt-insoluble non-diffusible. The specific radioactivity of the hydroxyproline in each fraction increases during the 180 min. incubation, the increase in the non-diffusible fraction being greater than that in the diffusible fraction (Table 2). The specific radioactivity of hydroxyproline is highest in the diffusate and in the salt-soluble non-diffusible fractions. Non-diffusible hydroxy[¹⁴C]proline appears in the tissue within 10 min. and increases over 180 min. (Fig. 1). A considerable amount of diffusible hydroxy[¹⁴C]proline appears in the incubation medium and this is discussed in more detail below.

Table 1. *Hydroxy[¹⁴C]proline formation by the chick corium*

Each value represents the mean of the results of at least two incubations.

	Hydroxy[¹⁴ C]proline formation (counts/min./corium)		Difference (%)
	Control	Experimental	
Without ascorbic acid	335	245	–27
Under N ₂	364	268	–26
At 4°	364	77	–79
Puromycin	335	96	–71

Table 2. *Hydroxyproline in chick corium fractions incubated for 180 min.*

Each value represents the mean $\pm$ s.d. of the results of the number of determinations given in parenthesis.

Corium	Time ...	Specific radioactivity of hydroxyproline (counts/min./ μ g.)		
		60 min.	90 min.	180 min.
9-day embryo (7 μ g. of hydroxyproline/corium)				
Diffusible		326 $\pm$ 41 (4)	376 $\pm$ 94 (4)	371 $\pm$ 146 (4)
Non-diffusible				
Salt-soluble		98 $\pm$ 58 (4)	192 $\pm$ 41 (3)	238 $\pm$ 141 (4)
Salt-insoluble		13 $\pm$ 5 (4)	16 $\pm$ 6 (4)	19 $\pm$ 8 (4)
13-day embryo (35 μ g. of hydroxyproline/corium)				
Diffusible		150 $\pm$ 11 (4)	195 $\pm$ 44 (4)	237 $\pm$ 56 (6)
Non-diffusible				
Salt-soluble		113 $\pm$ 20 (4)	179 $\pm$ 43 (4)	204 $\pm$ 51 (6)
Salt-insoluble		2.3 $\pm$ 0.5 (4)	3.7 $\pm$ 0.7 (4)	6.8 $\pm$ 1.2 (6)

Fig. 1. Kinetics of hydroxy[¹⁴C]proline accumulation in the non-diffusible fraction of the chick corium at 9–10 days of development. Each point is the average of two tissue samples.

Influence of embryo age on collagen metabolism. Although the embryos were removed between 10.30 and 11.30 a.m., some of the 10-day embryos were less well developed and were classified as late 9-day embryos.

An oscillation in the index for collagen synthesis (non-diffusible hydroxy[¹⁴C]proline/90 min./corium) occurs, with high values seen in tissues containing approx. 8 μ g. of hydroxyproline/corium (9–10-day embryo), 25–30 μ g. of hydroxyproline/corium (11–12-day embryo) and more than 30 μ g. of hydroxyproline/corium (13–14-day embryo) (Fig. 2). This oscillation in the index of collagen synthesis occurs with a series of eggs incubated on the same day (indicated by asterisks in Fig. 2) as well as with pooled data.

Fig. 2. Indices for collagen synthesis (●) and collagen degradation (○) plotted against the hydroxyproline (μ g.) content of the skin of the 9–15-day chick embryo. The asterisks identify serial determinations on a single batch of embryonated eggs. Mean $\pm$ s.d. of at least six experiments is given for each point.

The index of collagen degradation (diffusible hydroxy[¹⁴C]proline/corium/90 min.) is relatively low in tissues, with a hydroxyproline concentration of 7–20 μ g./corium (9–11-day embryo) (Fig. 2) and

increases at 13–14-days. The diffusible hydroxyproline makes up less than 5% of the total-tissue value until day 12 (Fig. 3), after which the percentage increases to more than 30% during the 13–14-day period. Thereafter the value decreases.

The distribution of hydroxy[^{14}C]proline between the tissue and the incubation medium is different at 9 days from that at 14 days (Table 3). With the 9–10-day tissues more than 50% of the total hydroxy[^{14}C]proline remains in the tissue after 90 min. of incubation, whereas at 13–14-days less than 15% is in the tissue; the remainder is in the incubation medium. (The percentage of hydroxy[^{14}C]proline in the non-diffusible fraction of the incubation medium is similar at the two periods of tissue development.)

Fig. 3. Amount of diffusible hydroxyproline in the corium as a function of the hydroxyproline ($\mu\text{g.}$) content of the skin and the embryo age. Mean $\pm$ s.d. of at least five experiments is given for each point.

Influence of epidermal structures on collagen metabolism. Feathers appear and major histological changes occur in the corium during the 9–14-day period (Wessells, 1965). To test for an effect of the epidermal elements on the metabolism of collagen in the corium, the distribution of hydroxy[^{14}C]proline in incubation systems of the intact corium was compared with that of the dermis. As shown in Table 4, the non-diffusible hydroxy[^{14}C]proline content of the intact corium (150 ± 14 counts/min./corium) is significantly lower than that of the dermis (539 ± 176 counts/min./corium), and the intact corium has a lower percentage of the total hydroxy[^{14}C]proline than the dermis. The total hydroxy[^{14}C]proline synthesized by the dermis is somewhat greater than that by the intact corium. Removal of the epidermal structures decreases the amount of diffusible unlabelled hydroxyproline of the tissue by more than 50% (Table 5). This difference in the amount of diffusible hydroxyproline was observed with embryos from eggs that started incubating on the same day or with separate batches of 13–14-day embryos.

These results indicate that the epidermis is active in the process of collagen breakdown. An intense stain for acid phosphatase activity indicates lysosome-like cellular components concentrated in the epi-peridermal area and in the feather-bud area, and these may be the site of proteolytic enzymes related to the collagen-breakdown process.

DISCUSSION

The formation of hydroxyproline in the incubation system with the chick embryo corium has features analogous to a number of previously described systems for studying collagen metabolism *in vitro*, i.e. slices of the carrageenin granuloma (Green & Lowther, 1959), isolated rabbit skin (Nimni, De Guia & Bavetta, 1967), chick embryo corium (Hurych & Chvapil, 1965) and minces of the skin from a growing rat (Tsurufuji & Ogata, 1965).

Table 3. Percentage distributions of hydroxy[^{14}C]proline in the incubation system as the corium develops

Each value for tissue represents the mean of the results of six incubations; each value for medium represents the analysis of pooled medium from six incubations.

Expt. no.	Embryo age (days)	Distribution of hydroxy[^{14}C]proline (%)			
		Tissue		Incubation medium	
		Non-diffusible	Diffusible	Non-diffusible	Diffusible
1	9–10	43	20	6	31
2	9–10	49	6	6	39
3	13–14	6	6	2	86
4	13–14	8	4	6	82

Table 4. *Influence of the epidermal structures on hydroxy[¹⁴C]proline synthesis and distribution in the 14-day embryo corium*

Each value represents the mean $\pm$ S.D. of the results of six 90 min. incubations with tissue from 16 embryos. The tissue washes were combined with the incubation medium. The *P* values indicate significance of difference between mean values (Student's *t* test).

	Tissue hydroxy[¹⁴ C]proline		Incubation-medium hydroxy[¹⁴ C]proline		Total hydroxy[¹⁴ C]proline (counts/min.)
	(counts/min./corium)	(% of total)	(counts/min./corium)	(% of total)	
Matched embryos					
Intact corium	150 $\pm$ 14	15	868 $\pm$ 275	85	1018 $\pm$ 276
Dermis	539 $\pm$ 176	37	903 $\pm$ 189	63	1442 $\pm$ 333
<i>P</i> (vertical) ...	< 0.001		< 0.9 > 0.8		< 0.05 > 0.02

Table 5. *Influence of epidermal elements on the diffusible unlabelled hydroxyproline in tissues from the 13-14-day embryo*

Each value represents the mean $\pm$ S.D. of the results of the number of determinations given in parenthesis.

	Tissue hydroxyproline (% of total)	Difference (%)
Same group of embryos		
Intact corium	32 $\pm$ 6 (6)	
Dermis	10 $\pm$ 1 (6)	-69
Separate group of embryos		
Intact corium	11 $\pm$ 5 (9)	
Dermis	5 $\pm$ 2 (9)	-52

In these systems hydroxy[¹⁴C]proline appears in the 0.15M-sodium chloride-soluble fraction, and some then passes into a salt-insoluble form.

Collagen formation in the chick corium is characterized by increasing specific radioactivities of hydroxyproline in the salt-soluble, salt-insoluble and diffusible fractions over a 3 hr. period (Table 2). The specific radioactivity for hydroxyproline is the highest in the salt-soluble and in the diffusible fractions, and in tissues from the 13-day embryo the specific radioactivities of these two fractions are similar (Table 2). This similarity indicates that a considerable amount of salt-soluble collagen is degraded before its incorporation into an insoluble form in the chick corium, as suggested by Hürtych & Chvapil (1965). Nimni *et al.* (1967) arrived at a similar conclusion from studies on collagen metabolism in rabbit skin *in vitro*.

Woessner *et al.* (1967) have reported on the accumulation of collagen in the chick embryo corium during 9-17 days of development. The most rapid collagen accumulation occurs between 12 and 16 days with the inflexion point occurring at approx. 14 days. Kivirikko (1963) found a

four- to five-fold increase in collagen of the chick skin between 12 and 14 days of development. Indices for collagen synthesis are high in the corium of the 13- and 14-day chick embryos studied *in vitro* (Fig. 2), suggesting that the index was reflecting conditions *in vivo*.

The oscillation in the index for collagen synthesis suggests a periodic acceleration of collagen synthesis during the 9-14-day period (Fig. 2). Tanzer & Gross (1964) observed variations in the incorporation of labelled glycine and hydroxyproline in chick embryo collagen and suggested discontinuous synthesis of collagen to permit organization of collagen fibrils. The fluctuations reported by these authors were damped by flooding the system with large amounts of proline. The availability of intracellular free amino acids, such as proline, was shown by Goodwin (1963) and Finerman, Downing & Rosenberg (1967) to affect proline incorporation and hydroxyproline formation in animal cells. It is possible that the intracellular amino acid pool size varies during the 9-14-day period in the chick embryo corium, and these variations would contribute to the oscillations in collagen synthesis.

Conveniently, a diffusible form of hydroxy[¹⁴C]proline exists in the tissue in spite of repeated tissue washings (five or six times). The diffusible hydroxy[¹⁴C]proline may be within intracellular organelles, such as lysosomes, or trapped in the extracellular matrix. Collagen degradation in the system *in vitro* seemed to correlate with the conditions *in vivo*, since a high index of collagen degradation occurs in the corium from the 13-14-day embryo, a period when concentrations of diffusible hydroxyproline of the whole egg (Mottet & Hall, 1966) as well as that of the skins (Kivirikko, 1963; Woessner, 1968) are elevated.

The tissue diffusible hydroxyproline passes into the incubation medium. The amount of diffusible hydroxyproline in the incubation medium increases as the tissue develops. Whereas at 9-10 days less

than 50% of the total hydroxy[^{14}C]proline is found in the incubation medium, at 13–14 days more than 75% of the total is found in the incubation medium. Between these two periods the epidermal elements develop and the epidermal region may be a major site for collagen degradation.

The increase in the amount of diffusible hydroxy[^{14}C]proline in the 13–14-day period is accompanied by a sharp increase in the amount of diffusible non-radioactive hydroxyproline (Table 5 and Fig. 3). The presence of large amounts of diffusible non-radioactive hydroxyproline suggests that the accelerated degradation at 13–14 days includes previously formed collagen, or older collagen, as well as the newly formed collagen (salt-soluble fraction) that contains hydroxy[^{14}C]proline. Kivirikko (1963) and Woessner (1968) have reported high concentrations of free hydroxyproline in the chick corium at that time of development and these may be a part of the remodelling process of the skin to accommodate the growth in size of the embryo.

Histochemical studies of the acid phosphatase reaction locate an intense activity in the epidermal elements and may indicate the site of concentrated proteolytic enzymes. These enzymes may participate in the process of collagen degradation. Proteolytic enzymes arising from the epidermal elements have been reported by Eisen & Gross (1965) to influence collagen degradation in the resorption of connective tissue occurring during amphibian metamorphosis. Grillo & Gross (1967) proposed that proteolytic enzymes from the epithelial cells in wound tissue appear to digest the underlying collagen of the dermis so as to facilitate migration of epithelial cells.

Collagen synthesis may also be influenced by the epidermal elements. This is suggested by the acceleration of hydroxy[^{14}C]proline synthesis after the removal of the epidermal structures. Although the possibility exists that cell stimulation attends the blunt resection of the epidermal layer, the high concentration of diffusible hydroxyproline of the intact corium (Table 2) may in itself decrease protein synthesis in a manner analogous to that reported by Cleland (1967) and Finerman *et al.* (1967) in plants and animal tissues.

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