Age-Related Variations in Hydroxylation of Lysine and Proline in Collagen

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The effect of age on the extent of hydroxylation of lysine and proline both generally and at certain specific sites in collagens from bone, skin and tendon was examined in the chick from the 14-day embryo to the 18-month-old adult. For all collagens there was a marked fall in the overall extent of hydroxylation of lysine with increasing age in both α_1 and α_2 chains, this fall occurring mostly in a relatively short period immediately after hatching. Hydroxylation of lysine declined to a constant value which, as expected, differed appreciably for each collagen and was considered to be characteristic of the collagen according to its tissue of origin. Hydroxylation of lysine in the *N*-terminal, non-helical telopeptide region of both α_1 and α_2 chains, which is important with regard to cross-linking, was relatively high in embryonic collagens. There was, however, a rapid loss of hydroxylation at these sites in skin collagen, occurring both during development of the embryo and in the period immediately after hatching. In contrast some hydroxylation at these sites persisted in bone and tendon collagens and, as judged by examination of peptide α_1 -CB1, appeared to reach a constant value in time of about 33% in bone and about 15% in tendon collagen. The actual extent of hydroxylation of lysine in the *N*-terminal telopeptides and the size of the changes in these values with age appeared to be unrelated to the corresponding whole-chain values, and it is suggested therefore that hydroxylation of telopeptidyl lysine may be under separate enzymic control. The increased hydroxylation of lysine in the embryo was accompanied by only minimal changes in proline hydroxylation, which was very slightly increased in embryonic bone and tendon collagens. Increased hydroxylation of proline in the embryo was, however, readily observed in peptide α_1 -CB2 from the helical region of tendon collagen. This hydroxylation was close to the theoretical maximum, in contrast with that observed in post-embryonic tendon, where hydroxylation was incomplete, as in rat tendon (Bornstein, 1967), only four on average, of the six susceptible proline residues being hydroxylated.

The overall extent of hydroxylation of lysine in collagen to hydroxylysine is known to vary extensively from collagen to collagen both between different species and from tissue to tissue with a single species (e.g. Piez & Likins, 1960). The significance of this variation is as yet not clear, but may be related to the precise functioning of collagen within its particular tissue of origin.

In addition to the naturally occurring variations, differences are also known to arise under various pathological conditions, as for example with the increased hydroxylation in bone collagen in rickets (Toole *et al.*, 1972; Barnes *et al.*, 1973a), dietary calcium deficiency (Barnes *et al.*, 1973b) and osteogenesis imperfecta (Eastoe *et al.*, 1973) or the near absence of hydroxylation in skin collagen in some cases of the Ehlers-Danlos syndrome (Pinnell *et al.*, 1972).

Variation with respect to age may also occur: thus Miller *et al.* (1967) found in studies of bone

collagen from the young chick of 1 day to 10 weeks of age a decrease in the overall extent of hydroxylation with increasing age up to 7 weeks. In skin collagen from the same species, Barnes *et al.* (1971b) found a degree of hydroxylation in the embryo appreciably higher than that reported by Kang *et al.* (1969c) in the 3-week-old bird, again inferring age-related changes in hydroxylation. In the present study, a detailed examination of collagen lysine hydroxylation in relation to age has been undertaken in the chick to establish the course and extent of such changes. Bone, skin and tendon collagens have been examined from the 14-day embryo to the 18-month-old adult.

A study of the variation occurring at specific sites in the molecule may lead to a better understanding of the significance of variable hydroxylation of lysine in collagen. Thus variation in the extent of hydroxylation of the single lysine residue occurring in the *N*-terminal non-helical telopeptide of both α_1

and α_2 chains of collagen has been observed. Hydroxylation at this site is relatively high in collagens from the chick embryo (Barnes *et al.*, 1971*a,b*). However, in skin collagen this hydroxylation is apparently only temporary, since no hydroxylation at the telopeptide site was observed in the 3-week-old chick (Kang *et al.*, 1969*a,b*). In contrast, hydroxylation at this site in bone collagen is still substantial at 3 weeks of age (Miller *et al.*, 1969; Lane & Miller, 1969). A similar situation appears to occur in the rat (Barnes *et al.*, 1971*a*) and, comparing skin and dentin collagens, in the cow (Volpin & Veis, 1973). Further evidence suggests a similar situation with respect to the C-terminal telopeptide (Stark *et al.*, 1971; Stoltz *et al.*, 1972, 1973; Becker & Timpl, 1972). The telopeptides at both N- and C-terminal ends of the collagen molecule are known to be involved in the formation of lysine-derived intermolecular cross-links (see Miller, 1971*a*; Kang, 1972; Dixit & Bensusan, 1973; Eyre & Glimcher, 1973; Miller *et al.*, 1973; Zimmerman *et al.*, 1973) and hydroxylation of lysine at these sites will determine the type of cross-links formed. Thus hydroxylation of this lysine appears correlatable with the occurrence of the cross-link dehydrohydroxylysino-hydroxy-norleucine derived from two molecules of hydroxy-lysine (see Bailey & Robins, 1972; Barnes, 1973). In the present study hydroxylation of lysine in the N-terminal telopeptides has been examined in relation to age to define the loss of hydroxylation at this site in skin collagen and to establish whether hydroxylation at this site in bone and tendon collagen, in contrast with skin collagen, remains substantial with increasing age.

Hydroxylation of collagen proline to hydroxyproline is generally regarded as showing little variation. Comparison of homologous peptides derived from CNBr cleavage has, however, revealed some distinct, albeit generally slight, differences between collagens (Butler, 1972; Volpin & Veis, 1973). In particular the six proline residues in the CNBr-derived peptide α_1 -CB2 that are theoretically susceptible to hydroxylation are hydroxylated in rat skin collagen to an overall extent of over 80%, compared with an overall extent of only 50% in rat-tail tendon collagen (Bornstein, 1967). In the present study, we have examined collagen proline hydroxylation in relation to age in the chick, to assess whether increased extents of hydroxylation occur in the embryo, as appears to be the case with lysine. We have, in particular, examined the peptide α_1 -CB2 to establish whether hydroxylation of proline in this peptide from tendon collagen is only partial as in the rat, and whether, if so, it is more complete in the embryo.

A preliminary account of some of this work has been given already (Barnes, 1972, 1973).

Materials and Methods

Materials

Radioactive amino acids. L-[4,5- ^3H]Lysine monohydrochloride and L-[G- ^3H]proline were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Dialysis. Visking 18/32 tubing was used.

Source of birds. Fertilized eggs were received from Winter Egg Farm, Thriplow, Cambs., U.K., and incubated in a Western incubator at 37°C until embryos reached the desired age. The 4-day-, 6-day- and 3-week-old chicks were reared from 1-day-old birds obtained from Sterling Poultry Services Ltd., Welwyn Garden City, Herts., U.K. Chicks were fed on the basic diet described by Barnes *et al.* (1973*b*). The 16-week- and 18-month-old birds were obtained from Winter Egg Farm. Birds were Rhode-Island Red crossed with Light Sussex breed.

Preparation of collagens

(a) **Skin collagen.** Skin collagen from chick embryos or very young chicks was labelled by incubation of the isolated tissue in a medium containing either [^3H]lysine or [^3H]proline. In various experiments, skin for incubation was removed from embryos (about 60 in number) of different specified ages (as given later in the Results and Discussion section), 1-day-old chicks (20 in number) and a single 4-day-old bird. Conditions of incubation and the procedure after incubation for the extraction and purification of the labelled collagen have been described previously (Barnes *et al.*, 1971*a,b*). Collagen α_1 and α_2 chains were separated by CM-cellulose chromatography as described below.

In older chicks collagen was labelled *in vivo* by the intraperitoneal administration of [^3H]lysine as described previously (Barnes *et al.*, 1971*a*, 1973*a,b*). Each bird from a group of three 6-day-old and six 3-week-old chicks received, subcutaneously, 0.5 ml of a 20% (w/v) solution of 3-aminopropionitrile fumarate in 0.9% NaCl/100g body wt. (to inhibit the cross-linking of collagen α chains to β dimers), and about 2.5 h later, by intraperitoneal administration, 0.4 mCi of L-[4,5- ^3H]lysine monohydrochloride/100g body wt. A single 16-week-old bird received a total of 5 mCi of L-[4,5- ^3H]lysine intraperitoneally after subcutaneous injection of 5 ml of 20% (w/v) 3-aminopropionitrile fumarate. All birds were killed 4–5 h after administration of [^3H]lysine. Skins were combined within groups, and collagen was extracted with 1 M-NaCl, precipitated and redissolved before chromatography on CM-cellulose for separation of α chains as described previously (Barnes *et al.*, 1973*b*). Purified α chains were examined for lysine and hydroxylysine or proline and hydroxyproline radioactivity, as

appropriate, by procedures described below. In the 3-week-old group, proline and hydroxyproline were determined with ninhydrin as detailed below. In some instances, α chains were cleaved with CNBr for the isolation of peptides α_1 -CB1 and α_2 -CB1, corresponding respectively to the *N*-terminal telopeptide region of α_1 and α_2 chains (see below).

Unlabelled skin collagen was also separately extracted from one 16-week- and one 18-month-old bird with hot 5% (w/v) trichloroacetic acid. Proline and hydroxyproline were then determined (after hydrolysis) in the diffusible products obtained by collagenase digestion of the hot-trichloroacetic acid-extracted material. Details of this procedure have been given previously (Barnes *et al.*, 1970).

(b) *Bone collagen.* Frontal bones from chick embryos or 1-day-old chicks were incubated in the presence of [^3H]lysine or [^3H]proline and labelled α chains isolated and examined as described above for skin collagen labelled *in vitro*.

Bone collagen labelled *in vivo* with [^3H]lysine was extracted from the frontal bones dissected from the group of 6-day-old chicks used for the preparation of labelled skin collagen at this age. The procedure for extraction and purification of collagen and isolation and examination of α chains was the same as that used for embryonic frontal bones.

Tibiae and femora were removed from the group of 3-week-old chicks and the single 16-week-old bird that had been given [^3H]lysine. Labelled collagen was extracted during demineralization and purified as previously described (Barnes, 1973a,b). In the 3-week-old group, α chains after isolation on CM-cellulose were examined for lysine and hydroxylysine radioactivity and additionally for proline and hydroxyproline with ninhydrin. In the single 16-week-old bird incorporation of radioactivity was relatively low. The purified labelled collagen was therefore cleaved directly with CNBr as described below, by using the procedure of Bornstein & Piez (1966) without prior separation of α chains. After dialysis the low-molecular-weight peptide α_1 -CB1 from the *N*-terminal region of the α_1 chain was isolated from the diffusate by phosphocellulose chromatography (as described below) and the large collagen-derived peptides in the non-diffusible material, representing the bulk of the collagen molecule, were digested with collagenase as described previously (Barnes *et al.*, 1970) and the diffusible products so obtained examined for lysine and hydroxylysine radioactivity to derive an overall extent of hydroxylation.

Unlabelled bone collagen from 16-week- and 18-month-old birds was examined as follows. Tibiae and femora were removed from two birds of each age, combined within age groups and demineralized by exhaustive dialysis against 0.5M-acetic acid as previously described (Barnes *et al.*, 1971a). The de-

mineralized material was freeze-dried and the collagen was then solubilized by treatment of the freeze-dried product with CNBr under the conditions described by Miller *et al.* (1971) (see below). After removal of excess of CNBr the digest was dialysed and the diffusate examined on phosphocellulose. The non-diffusible material was digested with collagenase (Barnes *et al.*, 1970) and the diffusible products so obtained were analysed after hydrolysis for the extent of lysine and proline hydroxylation as described below. These experiments were performed in duplicate, by using separate sets of birds.

(c) *Tendon collagen.* Labelled α chains from 20-day-old embryos and 1-day-old chicks were prepared after incubation of leg tendons in the presence of the appropriate radioisotope, exactly as described above for skin and frontal-bone collagens.

Leg tendons from the group of 3-week-old chicks injected with [^3H]lysine were extracted with hot trichloroacetic acid and the extracted material was digested with collagenase as described above in connexion with the extraction of unlabelled skin collagen. The diffusible products were examined after hydrolysis for the extent of hydroxylation of lysine and proline as described below.

Tendons from 16-week- and 18-month-old birds were examined as follows. The tendons from two birds of each age (the same birds as used for the examination of bone collagen, as described above) were combined within each age group. Tendons were freed of adhering tissue, severed into small pieces, crushed in a stainless-steel mortar after freezing in liquid N_2 and the crushed material was freeze-dried. Collagen was solubilized by CNBr treatment as described by Miller *et al.* (1971) and the cleaved products were then examined exactly as described above for the bones removed from these birds.

Separation of collagen α chains

Collagen α chains were obtained by chromatography of denatured collagen samples on CM-cellulose as described by Piez *et al.* (1963) and Bornstein & Piez (1966). The elution of α_1 and α_2 chains was detected by counting the radioactivity in the eluate, as previously described (Barnes *et al.*, 1971a). Fractions of the eluate corresponding to either α_1 or α_2 peaks were combined, dialysed against 0.15M-acetic acid and freeze-dried.

Isolation of peptides α_1 -CB1, α_1 -CB2 and α_2 -CB1 from CNBr-cleaved collagens

Purified collagen α -chains were cleaved with CNBr under the conditions described by Bornstein & Piez (1966). Excess of CNBr was removed by freeze-drying and the digest was then dialysed against

water. The diffusate was chromatographed on phosphocellulose as previously described (Barnes *et al.*, 1971a), the procedure of Bornstein & Piez (1966) being used to permit isolation of peptide α_1 -CB1 or α_2 -CB1 corresponding to the *N*-terminal telopeptide of the α_1 or α_2 chain, and peptide α_1 -CB2 from the helical region of the α_1 chain adjacent to the *N*-terminal telopeptide. Elution of peptide α_1 -CB1 and α_2 -CB1 derived from [3 H]lysine-labelled collagen and peptide α_2 -CB2 from [3 H]proline-labelled collagen was detected by counting the radioactivity in the eluate (Barnes *et al.*, 1971a).

In some instances, as detailed above, collagen was cleaved with CNBr without prior extraction from the tissue, by using the procedure of Miller *et al.* (1971). Excess of CNBr, as before, was removed by freeze-drying and the diffusible products were desalted on a column (3.5 cm $\times$ 65 cm) of Bio-gel P2 and then chromatographed on phosphocellulose for isolation of peptides α_1 -CB1 and α_1 -CB2. Their elution positions were ascertained by measurement of the absorption at 230 nm of fractions of the eluate (since collagen in these instances was not isotopically labelled).

Hydrolysis

Samples of either purified α chains, of the collagen-derived peptides α_1 -CB1, α_1 -CB2 and α_2 -CB1, or of the diffusible material obtained from collagenase digests of the non-diffusible fraction from tissue cleaved directly with CNBr were hydrolysed by refluxing under N_2 in 80 ml of 6 M-HCl for 24 h. HCl was removed from hydrolysates by rotary evaporation *in vacuo*.

Determination of hydroxylation of collagen lysine and proline. In chick embryos and young chicks, where collagen synthesis is rapid, synthesis was allowed to proceed either *in vitro* or *in vivo* in the presence of [4,5- 3 H]lysine or [*G*- 3 H]proline. The extent of hydroxylation in α chains or the peptides α_1 -CB1, α_1 -CB2 and α_2 -CB1 was then determined, after hydrolysis, by measuring the distribution of radioactivity between lysine and hydroxylysine or proline and hydroxyproline as required. Lysine was separated from hydroxylysine and proline from hydroxyproline and the radioactivity in each was determined by procedures described previously (Barnes *et al.*, 1970, 1971a). Radioactivity values were adjusted to an identical counting efficiency (of 40%). Hydroxylysine values were corrected for a 25% loss of radioactivity occurring when [4,5- 3 H]lysine in collagen is converted into hydroxylysine (Popenoe *et al.*, 1965).

In samples not labelled with the appropriate radioisotope the extent of hydroxylation of lysine and/or proline was determined by hydrolysis, followed by determination of lysine, hydroxylysine, proline

and hydroxyproline on a Technicon amino acid autoanalyser.

Results and Discussion

The overall extent of lysine hydroxylation in bone, skin and tendon collagens from the chick and the changes in these values with age are shown in Table 1. In all instances the extent of hydroxylation in collagens synthesized in the embryo and 1-day-old chick was substantially higher in both α_1 and α_2 chains than the corresponding value recorded in post-embryonic collagens isolated from birds 3 weeks or more of age. The decrease in hydroxylation from the elevated embryonic values occurred mostly in the period immediately after hatching, particularly in the skin where (at least as indicated by examination of the α_1 chain) a decrease to a relatively constant value appears to occur within the space of 4 days or less after hatching. In bone and tendon collagens the degree of hydroxylation at 3 weeks of age was still slightly higher than observed in the 16-week-old bird. There appeared, however, to be no change in hydroxylation between 16 weeks and 18 months of age, suggesting that a constant value was reached somewhere between 3 and 16 weeks.

As expected, the actual value of hydroxylation found, once age changes had ceased and hydroxylation had become relatively constant, differed from one collagen to another. Collagen from tendon exhibited the highest degree of hydroxylation, and that from bone was least hydroxylated (although in the embryo bone collagen exhibited a higher extent than skin). These values, shown in Table 1, are similar to those reported for the corresponding collagens from human, bovine and turkey tissues (Piez & Likins, 1960). The particularly high extent of hydroxylation in rat bone collagen, in comparison with other bone collagens (Piez & Likins, 1960; Barnes *et al.*, 1973a; Stoltz *et al.*, 1973), and the low extent, relative to other tendon collagens, in rat tail-tendon collagen (Piez *et al.*, 1963) suggest that the rat may be anomalous in this respect.

As pointed out above the degree of hydroxylation of lysine in collagen from embryos or young birds was determined by radioisotopic methods and therefore represents the value in collagen synthesized at the time of investigation, i.e. the time of administration of the radioisotope. In older birds, particularly those of 18 months of age, the incorporation of radioactivity was generally too low to permit the use of radioisotopic methods, and colorimetric analysis with ninhydrin was used. Further, since in these birds the solubility of collagen in acetic acid was negligible, collagen was treated *in situ* with CNBr and the degree of hydroxylation was determined in the collagen-derived products solubilized by this means. The degree of hydroxylation in these

Table 1. *Extent of lysine hydroxylation in relation to age in various collagens of the domestic fowl*

Hydroxylation percentages, except those denoted below, were derived from the distribution of radioactivity between lysine and hydroxylysine in collagen synthesized in the presence of L-[4,5-³H]lysine, as described in the text. ³H-labelled collagen α chains were separated by CM-cellulose chromatography. Hydroxylation percentages in labelled collagens were either calculated from the individual values for isolated α chains, by using a chain composition of $(\alpha_1)_2\alpha_2$, or in those instances where α chains were not isolated, measured directly after extraction of labelled collagen and digestion with collagenase. The 'bone' data refer to frontal bones of the embryo and 1–6-day-old chicks and to tibiae and femora of the 3-week-old to 18-month-old birds. The number of birds used in each age group is given in the text.

Source of collagen ...		Hydroxylation (%)								
		Skin			Bone			Tendon		
		Chain		Collagen	Chain		Collagen	Chain		Collagen
		α_1	α_2		α_1	α_2		$(\alpha_1)_2\alpha_2$	α_1	
Age		α_1	α_2	$(\alpha_1)_2\alpha_2$	α_1	α_2	$(\alpha_1)_2\alpha_2$	α_1	α_2	$(\alpha_1)_2\alpha_2$
Embryo	14 day	32	50	38	31	50	37			
	17 day	30	45	35	36	52	41			
	20 day	31	41	34	35	52	41			
Chick	1 day	24	39	29	35	45	38	45	64	51
	4 day	12								
	6 day	14			17	25	20			
	3 week	13	27	18	22	29	24			39
	16 week	17	31	22			12			36*
							15*			
							17*			
18 month							12*			33*
							16*			
							14			
							14			35
							14			34

* These values, obtained in duplicate as explained in the text, were measured in unlabelled collagen. Collagen was cleaved with CNBr without prior extraction from the tissue and the soluble products were then digested, after dialysis, with collagenase. The diffusible peptides obtained by collagenase treatment were analysed, after hydrolysis, with ninhydrin for lysine and hydroxylysine.

Table 2. *Extent of lysine hydroxylation in relation to age in the non-helical peptides α_1 -CB1 and α_2 -CB1 from the N-terminal regions of various collagens from the domestic fowl*

Values for the embryo and 1-day-old chick and that of 23% in the 16-week-old group were obtained by measurement of the distribution of radioactivity between lysine and hydroxylysine in peptides α_1 -CB1 and α_2 -CB1 isolated from [³H]lysine-labelled collagen by phosphocellulose chromatography as described in the text. Values for the 3-week-old chick are taken from the data of Kang *et al.* (1969a,b), Lane & Miller (1969) and Miller *et al.* (1969). Values for the 16-week-old and 18-month-old bird were, with the one exception noted above, derived in duplicate from unlabelled collagen. Collagen was cleaved with CNBr without prior extraction. Peptide α_1 -CB1 was isolated by phosphocellulose chromatography, and then analysed, after hydrolysis, for lysine and hydroxylysine with ninhydrin. The source of bone collagen was as described in Table 1. The number of birds used in each age group is given in the text.

Source of collagen		Hydroxylation (%)					
		Skin		Bone		Tendon	
		α_1 -CB1	α_2 -CB1	α_1 -CB1	α_2 -CB1	α_1 -CB1	α_2 -CB1
Age							
Embryo	14 day	48	62	35	—		
	17 day	26	32	49	66		
Chick	1 day	8	—	41	—	31	43
	3 week	0	0	50	50		
	16 week			23		21	—
				35		11	
			30	29			
	18 month			33		15	—
				36	35	13	

cases therefore relates to the total collagen present in the tissue rather than that just synthesized. Because of this we cannot exclude the possibility that although the degree of hydroxylation in the tissues becomes constant, some collagen in which hydroxylation was further decreased may be synthesized but not retained by the tissue. Because of the constancy of the degree of hydroxylation in the tissue, it follows that replacement of collagen lost during the normal turnover process must be by collagen of the same degree of hydroxylation.

Hydroxylation of lysine in the *N*-terminal non-helical telopeptides was determined by isolation of the peptides α_1 -CB1 and α_2 -CB1. Extents of hydroxylation are shown in Table 2. There was a rapid loss of hydroxylation at this site in skin collagen, occurring during growth of the embryo and in the period immediately after hatching. This loss correlates with the substitution of dehydrohydroxylysino-hydroxynorleucine, derived from two residues of hydroxylysine, as a cross-link in embryonic skin collagen by dehydrohydroxylysino-norleucine, derived from one residue each of lysine and hydroxylysine in post-embryonic skin (Bailey & Robins, 1972). In bone and tendon collagen hydroxylation at this site, at least as measured by examination of peptide α_1 -CB1, although showing some decrease, nevertheless remained appreciable with increasing age, particularly in bone where approximately one-third of the lysine was still hydroxylated in the 18-month-old adult. The data suggest that a constant extent of hydroxylation at this site is achieved, since there was little if any difference between 16 weeks and 18 months of age. The initial decline in bone collagen is in accord with the data of Mechanic *et al.* (1971), who observed, when comparing bovine foetal and adult bone collagens, that there was some decrease with age in the preponderance of dehydrohydroxylysino-hydroxynorleucine over dehydrohydroxylysino-norleucine as an intermolecular cross-link.

The different patterns of hydroxylation, both generally and at the telopeptide site, suggest strict control of lysine hydroxylation. In particular, the changes in the telopeptide, which are important in relation to cross-linking, may be under separate enzymic control. Degrees of hydroxylation at this site appear to be unrelated to the overall state of hydroxylation. Thus despite the relatively high overall degree of hydroxylation in tendon, hydroxylation in the telopeptide is very much less than in bone collagen. The complete loss of hydroxylation in the telopeptides of skin collagen is accompanied by a decrease in the overall hydroxylation which is less extensive than that occurring in bone collagen. The lysine residue in the telopeptides, in contrast with other lysine residues in collagen, is situated in a non-helical region of the molecule, located within a sequence of amino acids distinct from that occurring

around lysine residues within the helical region, where glycine occurs at every third residue (see Kang *et al.*, 1967; Rauterberg *et al.*, 1972; Hulmes *et al.*, 1973). These facts suggest that a lysyl hydroxylase, specific for the telopeptide lysine residue, may control hydroxylation at the telopeptidyl site. In support of this, the enzyme lysyl oxidase, which oxidizes collagen telopeptidyl lysine to allysine, does not appear to oxidize lysine residues within the helical structure of the molecule (Siegel & Martin, 1970).

The fall in hydroxylation with age presumably reflects changes either in the concentration or activity of lysyl hydroxylase(s) and/or cofactors. Anttinen *et al.* (1973) have reported a fall with age in lysyl hydroxylase activity in human skin. All the collagens studied in the present work have a chain composition at least in post-embryonic tissues, represented as $(\alpha_1 [\text{Type I}])_2\alpha_2$. Evidence has been presented, however, for the occurrence in embryonic skin of an additional collagen of chain composition $(\alpha_1 [\text{Type III}])_3$ (Miller *et al.*, 1971; Trelstad *et al.*, 1971). It seems unlikely to us, however, that the fall in hydroxylation can be interpreted simply in terms of the replacement of this embryonic collagen by one of composition $(\alpha_1 [\text{I}])_2\alpha_2$, since we noted comparable changes in hydroxylation in α_2 as well as α_1 chains. The presence of appreciable amounts of a collagen of chain composition $(\alpha_1 [\text{III}])_3$ would increase the $\alpha_1 : \alpha_2$ radioactivity ratio to a value appreciably higher than 2.1–2.2, the theoretical value anticipated for collagen of chain composition $(\alpha_1 [\text{I}])_2\alpha_2$. We observed values for this ratio sometimes as high as 3.5 (see also Barnes *et al.*, 1971*b*; 1973*b*) which could imply the presence of up to about one-third of collagen of chain composition $(\alpha_1 [\text{III}])_3$. Nevertheless, in many instances, particularly in the embryo, the ratio was close to the theoretical value for collagen of composition $(\alpha_1 [\text{I}])_2\alpha_2$ which is indicative of the presence of little if any collagen of structure $(\alpha_1 [\text{III}])_3$. Further, we observed no continuous decline in this ratio from a relatively high value to one of around 2 which would be anticipated if collagen of structure $(\alpha_1 [\text{III}])_3$ was being increasingly replaced by collagen of composition $(\alpha_1 [\text{I}])_2\alpha_2$ either during embryonic growth or in the period immediately after hatching, when lysine hydroxylation mostly declines. The slightly elevated ratios that we sometimes observed in both embryonic and post-embryonic tissues we do not consider therefore necessarily to indicate the presence of collagen $(\alpha_1 [\text{III}])_3$.

The significance of the changes in lysine hydroxylation is not known. However, the elevated extent of hydroxylation in embryonic collagens, reflected in extensive hydroxylation in the telopeptides, means that dehydrohydroxylysino-hydroxynorleucine is the major reducible cross-link in these collagens. The

Table 3. *Extent of proline hydroxylation in relation to age in various collagens of the domestic fowl*

Values for the embryo were derived from the distribution of radioactivity between proline and hydroxyproline in α chains from collagen synthesized in the presence of [G - 3H]proline. Collagen values were calculated from the data for individual chains. Values for other groups were derived from the measurement with ninhydrin of proline and hydroxyproline either in individual collagen α chains or the whole molecule. Collagen was solubilized as described in the text and either separated into α chains by CM-cellulose chromatography or treated with collagenase and the diffusible products so obtained were analysed for proline and hydroxyproline. The source of bone collagen was as described in Table 1. The number of birds used in each age group is given in the text.

Source of collagen		Hydroxylation (%)								
		Skin			Bone			Tendon		
		Chain		Collagen	Chain		Collagen	Chain		Collagen
		α_1	α_2	$(\alpha_1)_2\alpha_2$	α_1	α_2	$(\alpha_1)_2\alpha_2$	α_1	α_2	$(\alpha_1)_2\alpha_2$
Embryo	14 day	46	47	46	53	52	53			
	20 day				52	51	52	51	51	51
Chick	3 week	47	49	48	48	46	47			45
	16 week			48			49*			47*
	18 month			48			48*			46*

* Values represent the mean from duplicate experiments.

Table 4. *Extent of proline hydroxylation in relation to age in the peptide α_1 -CB2 from bone and tendon collagens of the domestic fowl*

The value for the 20-day embryo was obtained from the measurement of proline and hydroxyproline radioactivity in the peptide isolated from [3H]proline-labelled collagen. The value for the 3-week-old chick is taken from the data of Miller *et al.* (1969). Values for the 16-week-old and 18-month-old bird were obtained from duplicate experiments in which collagen was cleaved with CNBr without prior extraction, as described in the text. The peptide in question was isolated by phosphocellulose chromatography and then analysed after hydrolysis for proline and hydroxyproline with ninhydrin. The source of bone collagen was as described in Table 1. The number of birds used in each age group is given in the text.

Age		Hydroxylation (%)	
		Bone	Tendon
20-day embryo			45
3-week chick		48	
16-week bird		49	34
		49	36
18-month bird		46	35
		42	37

derived from the helical portion of α_1 chains close to the *N*-terminus of the chain. As in the rat (Bornstein, 1967), it was found that hydroxylation of the susceptible proline residues in this peptide in tendon collagen was only partial (Table 4). However, in the embryo, hydroxylation was substantially increased and much nearer the theoretical maximum of 50% for this peptide. The reason for the appreciable underhydroxylation in this region from tendon collagen, in contrast with other collagens, is unknown. Interestingly, it occurs in a collagen that exhibits a relatively high overall degree of lysine hydroxylation.

In this study, only collagens of composition $(\alpha_1[I])_2\alpha_2$ have been examined. It is therefore not possible to state whether other collagens, such as that of cartilage of composition $(\alpha_1[II])_3$ or the collagen mentioned above of composition $(\alpha_1[III])_3$ show similar age changes. Cartilage collagen generally exhibits a much higher degree of lysine hydroxylation in comparison with bone, skin and tendon collagens (Trelstad *et al.*, 1970; Miller, 1971*b*; Strawich & Nimni, 1971; Miller & Lunde, 1973).

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presence of this cross-link may conceivably be an essential feature of embryonic collagen.

The general extent of proline hydroxylation in relation to age is shown in Table 3. The elevated extents of hydroxylation of lysine in the embryo are reflected, but only minimally, in proline hydroxylation, which in bone and tendon collagen is very slightly increased in the embryo, by about 10%. Increased hydroxylation of proline in the embryo was more easily demonstrable in the peptide α_1 -CB2

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