Hydroxylysine in the N-Terminal Telopeptides of Skin Collagen from Chick Embryo and Newborn Rat

By M. J. BARNES, B. J. CONSTABLE, L. F. MORTON and E. KODICEK

*Dunn Nutritional Laboratory, Medical Research Council
and University of Cambridge, Milton Road, Cambridge CB4 1XJ, U.K.*

(Received 4 October 1971)

The extent of hydroxylation of the single lysine residue occurring in both the α_1 - and α_2 -CB1 peptides obtained by cyanogen bromide cleavage of the α_1 - and α_2 -chains of collagen appears to vary according to the nature of the tissue from which the collagen is derived. Thus in the collagen of skin isolated from young rat and chick (Bornstein & Piez, 1966; Kang, Bornstein & Piez, 1967; Kang, Piez & Gross, 1969a; Kang, Igarashi & Gross, 1969b), as in all skin collagens so far examined (Bornstein & Nesse, 1970; Click & Bornstein, 1970; Fietzek, Münch, Breitzkreutz & Kühn, 1970; Epstein, Scott, Miller & Piez, 1971; Rauterberg & Kühn, 1971; Volpin & Veis, 1971), no hydroxylation of this residue has been found, whereas in the bone collagen from these same two species about half or more of the lysine in question is converted into hydroxylysine (Miller, Lane & Piez, 1969; Lane & Miller, 1969; Barnes, Constable, Morton & Kodicek, 1971). Barnes *et al.* (1971) have also observed hydroxylation of the corresponding lysine in the collagen of membranous bone and tendon isolated from chick embryos.

This hydroxylation may be of significance in the formation of collagen cross-links. The intermolecular cross-link, syndesine, is formed by condensation between one residue each of lysine and hydroxylysine after oxidation to the corresponding aldehyde, allysine and hydroxyallysine. Syndesine is a major cross-link in collagen of bone (from 1-day-old chicks; Bailey, Fowler & Peach, 1969) and tendon (from chick embryo, calf, young rat and young human; Bailey, Peach & Fowler, 1970a; Bailey & Shimokomaki, 1971), but is present only in trace amounts, if at all, in skin collagens (Bailey, Peach & Fowler, 1970b; Bailey & Shimokomaki, 1971). Further, although the precise location of syndesine within the collagen molecule is not known, it has been shown that the non-helical telopeptides of collagen participate in the formation of intermolecular cross-links (see Bornstein, Kang & Piez, 1966; Bailey *et al.* 1970a). The α_1 - and α_2 -CB1 peptides referred to above correspond to these telopeptides occurring at the N-terminal end of the collagen molecule (Bornstein *et al.* 1966), and thus

it is possible that the hydroxylysine occurring in this region of bone and tendon collagen is involved in the formation of syndesine known to occur in these collagens.

To investigate the factors controlling this hydroxylation we decided to isolate radioactively labelled chick-embryo skin collagen as a substrate for studies of the hydroxylation *in vitro*, since it seemed likely, in the light of the facts outlined above, that the N-terminal telopeptide lysine in this collagen would not occur hydroxylated. However, as reported below, it was, in fact, found that about half of the lysine in both α_1 - and α_2 -CB1 peptides from chick-embryo skin collagen was converted into hydroxylysine. In corroboration of this finding, some hydroxylation of the corresponding lysine was also found in skin collagen of the newborn rat.

Small pieces of skin dissected from 14-day-old chick embryos (12 per incubation) and 1-day-old rats (two per incubation) were incubated for 4-5 h in a medium (10 ml and 5 ml respectively) containing 0.25 mCi of L-[4,5- 3 H]lysine in the same way as described by Barnes *et al.* (1971). After incubation the procedure for the extraction of labelled collagen with 0.5 M-acetic acid, its precipitation and the isolation of α_1 - and α_2 -chains from denatured collagen solutions by chromatography on CM-cellulose was as described previously (Barnes *et al.* 1971). The separation of α_1 - and α_2 -chains from chick-embryo skin collagen is shown in Fig. 1(a). Fractions were pooled within each peak, dialysed and freeze-dried. The radioactivity in β_{11} - and β_{12} -components (formed by cross-linking between two α -chains) was only slight (3-aminopropionitrile was included in the incubation medium; see Barnes *et al.* 1971), and no attempt was made therefore to collect α_1 -fractions free of the β_{11} -component.

Purified α -chains were cleaved with cyanogen bromide by the procedure of Bornstein & Piez (1966). After the removal of cyanogen bromide the cleaved products were dialysed, and the difusate, containing the low-molecular-weight peptides, including peptide CB1, was chromatographed on phosphocellulose (Bornstein & Piez, 1966) to

permit the isolation of the CB1 peptide. As illustrated in Figs. 1(b) and 1(c) the eluate contained, in the case of both α_1 - and α_2 -chains, only one major radioactive peak as expected (see Barnes

et al. 1971), corresponding to the peptides α_1 - and α_2 -CB1 respectively. Analysis of each peak after hydrolysis, as described by Barnes *et al.* (1971), gave, for chick-embryo skin collagen, the following

Fig. 1. (a) CM-cellulose chromatography of denatured chick-embryo skin collagen at 40°C. The column (1.8 cm $\times$ 18 cm) was eluted with a 0–0.1M-NaCl gradient in 60 mM-sodium acetate buffer, pH 4.8, over a volume of 800 ml. Fractions of volume 10 ml were collected and the radioactivity in a 0.2 ml portion of each was counted at 33% efficiency in 10 ml of Kennedy's (1969) solution by using a Beckman model LS-250 liquid-scintillation counter. Approx. 7×10^6 c.p.m. was applied to the column. (b) Phosphocellulose chromatography of the low-molecular-weight peptides obtained by CNBr cleavage of the α_1 -chains of chick-embryo skin collagen. The column (1.8 cm $\times$ 18 cm) was eluted at 40°C with a 0–0.3M-NaCl gradient in 1 mM-sodium acetate buffer, pH 3.8, over a volume of 820 ml. Fractions of volume 10 ml were collected and the radioactivity in a 0.5 ml portion of each was counted (at 28% efficiency) as described above. Approx. 120 000 c.p.m. (at 33% efficiency) was applied to the column. (c) Phosphocellulose chromatography of the low-molecular-weight peptides obtained by CNBr cleavage of the α_2 -chains of chick-embryo skin collagen. The column was eluted as described above. The radioactivity in a 1.0 ml portion of each fraction of the eluate was counted (at 24% efficiency) as above. Approx. 24 000 c.p.m. (at 33% efficiency) was applied to the column.

results in which hydroxylysine radioactivity (after correction for the loss occurring during hydroxylation; Barnes *et al.* 1971) is expressed as a percentage of the total (lysine plus hydroxylysine) as determined for two separate incubations: peptide α_1 -CB1: 52 and 44; peptide α_2 -CB1: 58 and 66. (Occasionally the α_1 -CB1 peak showed a partial resolution into two, but analysis of fractions taken to represent mostly either one or the other of the two components showed no significant difference in the distribution of radioactivity between lysine and hydroxylysine.) These results, indicative of appreciable hydroxylation of the lysine in these peptides from skin collagen synthesized in the 14-day-old embryo, are in contrast with the lack of similar hydroxylation in the 3-week-old chick (Kang *et al.* 1969*a,b*). Likewise the α_1 -CB1 peptide isolated from the α_1 -chains of collagen synthesized in the skin of newborn rats was found to be partially hydroxylated (to the extent of approx. 15%), although in the same collagen isolated from the 6-week-old rat this hydroxylation does not occur (Barnes *et al.* 1971). These findings imply that the state of lysine hydroxylation in the *N*-terminal telopeptide region of collagen is affected by the age as well as the nature of the tissue from which the collagen is derived.

The relatively high degree of hydroxylation in this region of skin collagen from the embryo or newborn appears to be reflected in the general extent of lysine hydroxylation in the rest of the molecule. Thus examination of the dialysis residue from chick-embryo skin collagen α_1 - or α_2 -chains, obtained after dialysis following cyanogen bromide treatment and containing the high-molecular-weight peptides from the helical region (peptides α_1 -CB3, α_1 -CB6, α_1 -CB7 and α_1 -CB8 from the α_1 -chains or peptides α_2 -CB3, α_2 -CB4 and α_2 -CB5 from the α_2 -chains), indicated an overall extent of lysine hydroxylation in these peptides (as synthesized by the 14-day-old embryo) of 31% and 50% respectively. This is considerably higher than the corresponding values (10% and 29% as calculated from the data of Kang *et al.* 1969*a,b*) in skin collagen from the 3-week-old chick. Likewise the extent of hydroxylation of 19% we found in the corresponding high-molecular-weight peptides from the α_1 -chains of newborn rat skin collagen is approx. 50% higher than the corresponding value in skin collagen from rats a few weeks old (12.5% from the data of Butler, Piez & Bornstein, 1967).

It is concluded that a marked fall occurs with age in lysine hydroxylation in skin collagen in the embryo and newly born, leading to a complete loss of hydroxylation of the telopeptide lysine. A comparable change in the extent of proline hydroxylation does not appear to occur (Hurtych & Chvapil, 1965). In view of the possible correlation between

the occurrence of hydroxylysine in the *N*-terminal telopeptide region of collagen and the occurrence of syndesine as a cross-link, it seems pertinent to consider whether syndesine occurs in skin collagen from the embryo and the newborn before hydroxylation of the telopeptide lysine ceases.

Miller, Martin, Piez & Powers (1967) have reported a fall with age in the overall extent of lysine hydroxylation, unaccompanied by any change in the extent of proline hydroxylation, in bone collagen in the young chick. Nevertheless, compatible with the possible derivation of syndesine from hydroxylysine in the *N*-terminal telopeptide region of bone collagen, we have observed, in the 6-week-old rat, that the degree of hydroxylation of the telopeptide lysine is still high when hydroxylation of the corresponding lysine in skin collagen has already ceased (Barnes *et al.* 1971). A similar situation appears to exist in the 3-week-old chick (Kang *et al.* 1969*a,b*; Miller *et al.* 1969; Lane & Miller, 1969). It is not yet known whether hydroxylation of this lysine in bone collagen eventually declines with age. Bailey & Shimokomaki (1971) have reported a decrease with age in syndesine in tendon collagen, but this is assumed to reflect the maturation of syndesine to some as-yet-unidentified stabilized form of the cross-link.

Trelstad, Kang & Gross (1971) have reported the presence of a second collagen in chick-embryo skin that is not present in skin from 3-week-old chicks, of chain composition $[\alpha_1(\text{III})]_3$, in addition to the usual collagen of chain composition $[\alpha_1(\text{I})]_2\alpha_2$. In our studies CM-cellulose chromatography of chick-embryo skin collagen gave an α_1/α_2 ratio of about 3–4:1 instead of the expected 2:1 ratio (see Fig. 1*a*), which may indicate the presence of both types of α_1 -chain. This may offer a possible explanation for the occasional partial resolution of the α_1 -CB1 peak into two. However, since the loss in hydroxylation of the telopeptide lysine in skin collagen with age was observed, not only in peptide α_1 -CB1, but also in peptide α_2 -CB1 (which can only be derived from collagen $[\alpha_1(\text{I})]_2\alpha_2$), it is not considered that this loss can be attributed to the disappearance from the skin of the collagen $[\alpha_1(\text{III})]_3$.

- Bailey, A. J., Fowler, L. J. & Peach, C. M. (1969). *Biochem. biophys. Res. Commun.* **35**, 663.
- Bailey, A. J., Peach, C. M. & Fowler, L. J. (1970*a*). *Biochem. J.* **117**, 821.
- Bailey, A. J., Peach, C. M. & Fowler, L. J. (1970*b*). In *Chemistry and Molecular Biology of the Intercellular Matrix*, vol. 1, p. 385. Ed. by Balazs, E. A. London and New York: Academic Press.
- Bailey, A. J. & Shimokomaki, M. S. (1971). *FEBS Lett.* **16**, 86.
- Barnes, M. J., Constable, B. J., Morton, L. F. & Kodicek, E. (1971). *Biochem. J.* **125**, 433.

- Bornstein, P., Kang, A. H. & Piez, K. A. (1966). *Biochemistry, Easton*, **5**, 3803.
- Bornstein, P. & Nesse, R. (1970). *Archs Biochem. Biophys.* **138**, 443.
- Bornstein, P. & Piez, K. A. (1966). *Biochemistry, Easton*, **5**, 3460.
- Butler, W. T., Piez, K. A. & Bornstein, P. (1967). *Biochemistry, Easton*, **6**, 3771.
- Click, E. M. & Bornstein, P. (1970). *Biochemistry, Easton*, **9**, 4699.
- Epstein, E. H., Scott, R. D., Miller, E. J. & Piez, K. A. (1971). *J. biol. Chem.* **246**, 1718.
- Fietzek, P. P., Münch, M., Breitreutz, D. & Kühn, K. (1970). *FEBS Lett.* **9**, 229.
- Hurych, J. & Chvapil, M. (1965). *Biochim. biophys. Acta*, **107**, 91.
- Kang, A. H., Bornstein, P. & Piez, K. A. (1967). *Biochemistry, Easton*, **6**, 788.
- Kang, A. H., Igarashi, S. & Gross, J. (1969*b*). *Biochemistry, Easton*, **8**, 3200.
- Kang, A. H., Piez, K. A. & Gross, J. (1969*a*). *Biochemistry, Easton*, **8**, 1501.
- Kennedy, J. F. (1969). *Experientia*, **25**, 1120.
- Lane, J. M. & Miller, E. J. (1969). *Biochemistry, Easton*, **8**, 2134.
- Miller, E. J., Lane, J. M. & Piez, K. A. (1969). *Biochemistry, Easton*, **8**, 30.
- Miller, E. J., Martin, G. R., Piez, K. A. & Powers, M. J. (1967). *J. biol. Chem.* **242**, 5481.
- Rauterberg, J. & Kühn, K. (1971). *Eur. J. Biochem.* **19**, 398.
- Trelstad, R. L., Kang, A. H. & Gross, J. (1971). *Fedn Proc. Fedn Am. Socs exp. Biol.* **30**, 1196 (Abstr.).
- Volpin, D. & Veis, A. (1971). *Biochemistry, Easton*, **10**, 1715.