COLLAGEN PROLINE HYDROXYLATION IN CHICK EMBRYOS

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In the study of collagen biosynthesis, a vexing question has been the chemical bonding of proline at the time of its hydroxylation. Hydroxyproline is known to arise from proline only after the proline has ceased to be free [1], i.e., after it has become incorporated into peptide linkage or during the activation that precedes incorporation. Both views have had experimental support. Data published by workers from several laboratories are consistent with hydroxylation after incorporation [2]. On the other hand, isolation of hydroxyprolyl-sRNA has been reported from several laboratories [3], raising the possibility that hydroxyproline may *per se* be incorporated into peptides *via* hydroxyprolyl-sRNA. Manning and Meister [4] obtained hydroxyprolinol following reduction and hydrolysis of carrageenan granulomas, which implies that at least some of the hydroxylated proline may still be attached in ester linkage. Urivetzky et al. [5] have reported enzymatic hydroxylation, by a crude chick-embryo fraction, of purified sRNA charged with [^{14}C]proline, but failed to obtain incorporation of the hydroxylated material with a chick-embryo ribosomal preparation [6].

Because of its importance, we have re-investigated this question through isolation of labelled aminoacyl hydroxamates from nine-day-old chick embryos which had been injected with [^{14}C]proline. At this developmental stage, intensive collagen synthesis occurs and the specific activity of collagen-hydroxyproline is particularly high if labelled proline is given [7]. Previous isolations of hydroxyprolyl-sRNA frequently used nine-day-old embryos as starting material. However, it is shown here that no authentic labelled hydroxyprolylhydroxymate can be isolated under conditions where significant radioactivity is associated with the prolylhydroxamate fraction [8].

Reference aminoacylhydroxamates were prepared by standard procedures from the free amino acids via the methyl esters [9]. Both prolylhydroxamate (PH) and hydroxyprolylhydroxamate (HPH) form in high yield from the methyl esters [10]. Each gives a single wine-red spot with $FeCl_3$ spray after descending paper chromatography in the solvent mixture developed by Wieland and Fritz (*sec*-butanol/formic acid/water; 75:15:10 [11]). Relative mobilities of hydroxamate spots are here expressed as R_{PH}, i.e., in reference to the mobility of freshly prepared PH. Thus, PH has an R_{PH} of 1.00 in this solvent system, HPH one of 0.64 (column 2, table 1). We were unable to crystallize PH, but HPH crystallizes readily as the monohydrate (m.p. 119–123°; elementary analysis: found C = 36.53% (36.60% calculated); H = 7.32% (7.31% calculated); N = 17.00% (17.08% calculated). When either of the synthetic hydroxamates is hydrolyzed in dilute acid for 15 min at 100°, the theoretical amount of free amino acid is regenerated [12,13].

Table 1
Chromatographic mobility and radioactivity of aminoacyl hydroxamates

Hydroxamate	R_{PH}		Activity in region of synthetic hydroxamate (cpm)
	before acid treatment	after acid treatment	
PH	1.00	0.65	2370
"fast" HPG	0.64	0.46	850
"slow" HPH	0.47	0.46	0

Column 2: Movement, relative to PH, of PH and HPH in *sec*-butanol/formic acid/water before exposure to pH 2 (= "acid treatment").
Column 3: Movement after acid treatment.
Column 4: cpm of ^{14}C-labelled chick-embryo aminoacyl-hydroxamates eluted from designated regions of chromatogram. A background count of 40 cpm/inch has been subtracted from all values given. "Slow" HPH: HPH of altered mobility formed after exposure to pH 2. See text for abbreviations.

To prepare *in vivo* labelled material, 5 μC of [U-^{14}C] proline (189 μC/μmole; New England Nuclear Corp.) in 0.9% sterile NaCl was injected into a chorioallantoic vein of a nine-day-old chick embryo. After a period of incubation following injection, the embryo was removed from the egg, quickly rinsed, and homogenized in 1 ml of ice-cold neutral salt-free hydroxylamine solution (approximately 2.5 M) in a Dounce homogenizer. Cold carrier PH and HPH were added at this time. To isolate aminoacylhydroxamates from the homogenate, a modification of the method of Elliott and Coleman [14] was used. Proteins were precipitated with perchloric acid and perchlorate was removed as the insoluble potassium salt. The neutral solution was taken to dryness on a flash evaporator and the residue was dissolved in 5 ml of M/100 ammonium acetate buffer (pH 5.0). The aminoacylhydroxamates were absorbed on a column of Biorex 70 resin and eluted with 5N methylamine. The eluted aminoacylhydroxamates, which had by these preliminaries been freed from both free amino acids and other acylhydroxamates, were again taken to dryness on the flash evaporator, dissolved in a small amount of water and streaked on Whatman No. 3MM paper. They were separated by descending chromatography as before. Regions corresponding to synthetic PH and HPH were eluted and their radioactivity determined in a windowless gas-flow counter. The remaining portions of the chromatogram were divided into horizontal strips which were eluted and counted. With the purification procedures as outlined, counts above background were confined to the two regions mentioned except for some activity near the lower margin of the chromatogram. Radioactivity in this spot was assigned to a small residual amount of the injected proline on the basis of chromatographic behavior and coincidence of activity with a deep-blue spot which formed upon reaction with isatin.

Counts in the various fractions from a 40-min chick-embryo incubation are shown in the fourth column of table 1. A substantial number of counts is found in the region of synthetic HPH, designated as "fast" HPH in the table.

To confirm identification of the putative HPH, the labelled hydroxamate isolated by chromatography as HPH was hydrolyzed in dilute acid at 100°. The regenerated free amino acids were separated by descending paper chromatography in phenol/cresol and radioactivity determined along the length of the chromatogram by eluting and counting successive horizontal strips. The majority of counts was recovered not as hydroxyproline but as free proline; the remainder was distributed in regions tentatively attributed to glutamic acid and alanine. No radioactivity at all was recovered from the hydroxyproline region. When the experiment was repeated with a 50-min incubation, about half of the counts associated with the region of synthetic HPH were recovered as free proline; again, no labelled hydroxyproline was obtained.

To obviate the possibility that a small amount of authentic labelled HPH might have been lost during chromatography on Biorex 70, a control experiment was run which bypassed the entire purification procedure. Hydroxamates were prepared from chick embryo following a 30-min incubation with [^{14}C] proline. Proteins were coagulated by treatment with ice-cold methanol and removed by centrifugation. The supernatant was reduced in volume on the flash evaporator and an aliquot subjected to descending chromatography. Of 3000 counts which were isolated from the region of authentic HPH, none appeared as hydroxyproline in chromatographic separation of the free amino acids, while 46% were recovered as proline.

We concluded from these results that the counts in the region of "fast" HPH could not be due to authentic HPH. Further experimentation revealed that PH and HPH share with several other aminoacylhydroxamates a rather curious property. During short-term exposure to pH 2 at room temperature (not to be confused with dilute acid hydrolysis at 100°) they are converted into compounds that still react with $FeCl_3$, hence retain a hydroxamate structure, but which run as discrete spots with reduced mobility in the chromatographic system of Wieland and Fritz [11]. They are designated "slow" or pseudohydroxamates. The effect of acid treatment on PH and HPH is shown in columns 2 and 3 of table 1. Synthetic or "fast" PH is converted from R_{PH} 1.00 to R_{PH} 0.65, while synthetic ("fast") HPH shifts from 0.64 to 0.46. "Slow" HPH, R_{PH} 0.47, is not affected by further acid treatment. An observation relevant to this report is that on 15-min hydrolysis in boiling dilute acid, the "slow" hydroxamates, unlike the "fast", do not yield the free amino acids quantitatively. This fact no doubt accounts for the less than quantitative recovery of labelled proline after hydrolysis of the radioactive material isolated from the "fast" HPH region. The chemical nature of the slow compound is under investigation.

We surmised that the $FeCl_3$-positive spot from chick-embryo extracts running in the region of "fast" HPH is in reality a mixture of "fast" HPH (added as cold carrier) and "slow" PH. The latter might possibly be formed from "fast" PH during exposure of the homogenate to perchloric acid in the isolation procedure. If such a partial acid conversion could be observed with PH, one would expect HPH to undergo a similar conversion from the "fast" into the "slow" form under the same conditions. Re-examination of the chromatograms of hydroxamates obtained from chick-embryos led to the conclusion that such a conversion had in fact taken place for HPH also, since these chromatograms showed a $FeCl_3$-positive spot at the R_{PH} expected of acid-treated HPH. This spot, which was doubtlessly formed from added carrier HPH, had earlier been eluted and counted, but radioactivity associated with it had proved negligible ("slow" HPH, table 1).

In previously reported work [15] in which chick-embryo tissues (from embryos ranging in age from 7 to 12 days) had been exposed to radioactive proline for periods of up to 1 hour, isolated collagen-hydroxyproline contained radioactivity of several thousand counts per minute per embryo. Proline label was often reported as greater, but the proline/hydroxyproline specific activity ratio never exceeded 11 to 1. A comparable ratio for the hydroxamates should have given a minimum of 220 counts for hydroxyproline in the 40-min incubation.

Procedures that detect derivatives of proline and hydroxyproline based on hydroxamate formation thus failed to point to the presence of aminoacyl hydroxyprolyl compounds prior to their entry into peptide linkage at a time of intensive *de novo* synthesis of collagen hydroxyproline. These results raise further doubts concerning the involvement in collagen synthesis in chick embryos of hydroxyprolyl-sRNA, the presence of which in very small amounts has been reported, and strengthens the conclusion that the latter is unrelated to collagen amino acid assembly [16].

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References

[1] M.R.Stetten and R.Schoenheimer, J. Biol. Chem. 153 (1944) 113;
M.R.Stetten, J. Biol. Chem. 181 (1949) 31.
[2] For a thoroughgoing review, see S.Udenfriend, Science 152 (1966) 1335.
[3] G.Manner and B.S.Gould, Fed. Proc. 21 (1962) 169;
G.Manner and B.S.Gould, Biochim. Biophys. Acta 72 (1963) 243;
A.Coronado, E.Mardones and J.E.Allende, Biochem. Biophys. Res. Commun. 13 (1963) 75;
D.S.Jackson, D.Watkins and A.Winkler, Biochim. Biophys. Acta 87 (1964) 152.
[4] J.M.Manning and A.Meister, Biochemistry 5 (1966) 1154.
[5] M.Urivetzky, J.M.Frei and E.Meilman, Arch. Biochem. Biophys. 109 (1965) 480.
[6] M.Urivetzky, J.M.Frei and E.Meilman, Arch. Biochem. Biophys. 117 (1966) 224.
[7] B.Peterkofsky and S.Udenfriend, J. Biol. Chem. 238 (1963) 3966.
[8] K.Moldave, in: Methods in Enzymology, vol. 6, eds. S.P.Colowick and N.O.Kaplan (1963) p. 757.
[9] B.F.Erlanger, H.Sachs and E.Brand, J. Am. Chem. Soc. 79 (1954) 1806;
K.G.Cunningham, G.T.Newbold, F.S.Spring and J.Stark, J. Chem. Soc. (London) 1949, 2091.
[10] R.S.Schweet, Biochim. Biophys. Acta 18 (1955) 566.
[11] Th.Wieland and H.Fritz, Chem. Ber. 86 (1953) 1186.

[12] F.D.Chinard, J. Biol. Chem. 199 (1962) 91.
[13] F.Woessner, Arch. Biochem. Biophys. 93 (1961) 440.
[14] W.H.Elliott and G.Coleman, Biochim. Biophys. Acta 57 (1962) 236.
[15] L.N.Lukens, J. Biol. Chem. 240 (1965) 1661;
K.Juva and D.J.Prockop, Biochim. Biophys. Acta 91 (1964) 174.
[16] B.S.Gould, Int. Rev. Conn. Tissue Res., vol. 4 (Academic Press, London, 1968) p. 55;
Treatise on Collagen, vol. II, part A, ed. B.S.Gould (Academic Press, 1968) p. 171.