ISOLATION OF HYDROXYLYSYL-sRNA AND HYDROXYPROLYL-sRNA IN A CHICK EMBRYO SYSTEM.°

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Collagen contains two hydroxylated amino acids, hydroxyproline and hydroxylysine. It has been shown by several investigators (Stetten and Schoenheimer, 1944 and Sinex and Van Slyke, 1955) that proline and lysine, and not the hydroxylated derivatives, are the precursors of the protein bound amino acids. These findings presented two possibilities, 1) that the hydroxylation of the amino acids occurred previous to peptide formation with the amino acids in a bound or "activated" form or, 2) after they had entered the newly synthesized peptide.

Since collagen appears to be synthesized according to the accepted pattern for other proteins (Peterkofsky and Udenfriend, 1961) the first step in the process may be assumed to be the activation of the amino acids and their incorporation into soluble RNA. We have explored the first hypothesis stated above, and have been able to isolate hydroxyproline and hydroxylysine bound to sRNA using a partially purified enzyme system from chick embryo.

Some of these findings were reported by Coronado (1962).

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MATERIALS AND METHODS. Amino acid activating enzymes were prepared from the 105,000 x g supernatant fraction of a homogenate of 12 day chick embryos. The pH was adjusted to 5.2 and the precipitate resuspended in a buffer containing Tris (HCl) 0.01M, pH 7.5; Magnesium acetate 0.01M; KCl, 0.06M; and Glutathione, 0.01M.

Soluble RNA from chick embryo was prepared by the method of Rosenbaum and Brown (1961). Yeast sRNA was a commercial preparation of General Biochemicals Inc.

C^{14}-proline (10.8 mc/mmole) was obtained from Nuclear Chicago Corporation. C^{14}-lysine (140 mc/mmole) was purchased from Schwartz BioResearch Inc.

Isolation of aminoacyl-sRNA was performed by the method of Gierer and Schramm (1956). The bound amino acid was subsequently hydrolyzed off the sRNA by adjustment to pH 10 and incubation at 37° for 1 hour. The sRNA was reprecipitated with ethanol and the amino acids in the supernatant were desalted on a Dowex 50-H^+ column.

Proline and hydroxyproline were separated, after the addition of carrier C^{12} compounds (Mann Chemical Co.), in a paper chromatographic system of n-butanol: acetic acid: water (63:27:10). The respective Rf's were 0.41 and 0.27.

Lysine and hydroxylysine, also in the presence of commercial C^{12} carriers (Calbiochem), were separated on a Dowex-50-Na^+ column (Piez , 1954).

RESULTS. When C^{14}-proline was incubated with the system described in Table I, there appeared bound to sRNA a fraction which could be identified chromatographically as hydroxyproline. In these two experiments the hydroxyproline present was approximately 20% of the total counts recovered. This is consider-

ed significant because the two chromatographic bands were cleanly separated both in respect to ninhydrin color and C^{14}-counts, and the radioactivity recovered in the two bands accounted for 94% of the counts eluted from the whole chromato graphic paper.

TABLE I.

0.12 µmoles of C^{14}-proline (s.a.10.8 mc/mmole) were incubated with a system containing per ml.: 1.5 mg of chick embryo sRNA; 4 mg of pH 5 activating enzymes; cacodylate buffer pH 7.5, 75 µmoles; ATP 15 µmoles; magnesium acetate 7.5 µmoles and glutathione 10 µmoles, in a total volume of 10 ml. Incubation was carried out at 37° for 40 minutes. The reaction was stopped by addition of an equal volume of 90% phenol.

	c.p.m. Proline	c.p.m. Hydroxyproline	% Hydroxyproline
Experiment 1	552	140	20.2
Experiment 2	171	39	18.5
Control°	1944	4	0.2

° The control consisted of the chromatographic treatment of an aliquot of the supernatant remaining after the precipitation of the amino acyl-sRNA with ethanol.

In the control, where the free amino acids present in the incubation mixture were chromatographed, a small percentage of hydroxyproline was found. If significant, this amount could be a result of discharge from amino acyl sRNA or contamination of the commercial C^{14}-proline with a small amount of hydroxyproline (Lindsted and Prockop, 1961).

Similar experiments were carried out to study the hydroxilation of lysine. In these experiments the separation of the amino acids was achieved on a Dowex 50-X8-Na^+ column. The results are shown in Figure 1 A.

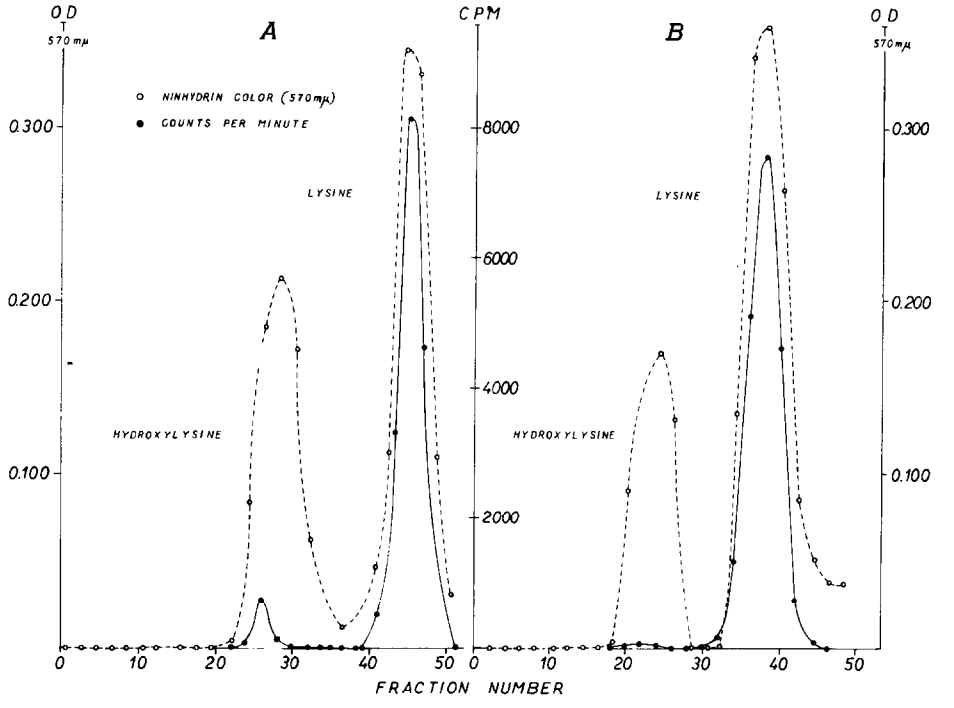


Figure 1. 16.4 mμmoles of C^{14}-lysine (s.a. 140 mc/mmole) were incubated in a system that contained per ml: 1 mg of chick embryo sRNA in curve A and 1 mg of yeast sRNA curve B; 3.6 mg of pH 5 enzyme, buffer TRIS-HCl pH 7.5 25 μmoles, magnesium acetate 5 μmoles; ATP 2.4 μmoles; glutathione 2 μmoles; in a total volume of 10 ml. The incubation was carried out at 37° for 30 minutes and the amino acids bound to the sRNA were isolated as described in Methods. The desalted amino acids in the presence of 1 mg each of carrier L-lysine and DL + allo-ʃ-hydroxylysine were chromatographed in a Dowex 50-X8 Na^+ column (30 x 0.7 cm). Two ml fractions were collected and aliquots of 0.2 ml were withdrawn for ninhydrin determination (Moore and Stein, 1948). The remainder of the fraction was counted in a gasflow windowless counter.

The radioactivity present in the hydroxylysine peak (980 c.p.m.) was approximately 5% of the total counts recovered from the column (18311 c.p.m.). The displacement of the radioactivity peak towards the earlier part of the ninhydrin curve may be explained by the fact that the carrier hydroxylysine added contained a large amount of allohydroxylysine. As shown by Piez (1954) in a similar column, hydroxylysine runs slightly faster than its isomer.

When sRNA from yeast was substituted in the system, it showed lysine acceptor activity comparable to that of the chick embryo sRNA but much smaller hydroxylysine incorporation, as shown in figure 1 B. One could then assume that the formation of the hydroxylsyl-sRNA requires a specific sRNA present in the chick embryo preparation but not in yeast. The small amount of hydroxylysine in curve B might be explained by the presence of sRNA in the pH 5 chick embryo enzyme fraction (Schweet,1962). When a control incubation was carried out in the presence of pancreatic ribonuclease (25 micrograms per ml) no detectable counts were found in the ethanol precipitable material.

DISCUSSION. The finding of hydroxylysyl-sRNA and hydroxyprolyl-sRNA in a chick embryo system substantiates the hypothesis of Stone and Meister (1962) that the hydroxylation process occurs prior to the synthesis of the collagen peptide chain.

The question now arises whether the hydroxylation occurs before or after the incorporation of the amino acids into s-RNA.

The results given above do not allow one to discriminate between these two possibilities.

If one arbitrarily disregards a pathway for the hydroxylation of lysine previous to the formation of the activating enzyme-AMP-amino acid complex and assumes a specific sequence in the collagen molecule, the three hypotheses below could be considered.

In numbers one and two the hydoxylation would take place at the level of the activating enzyme.

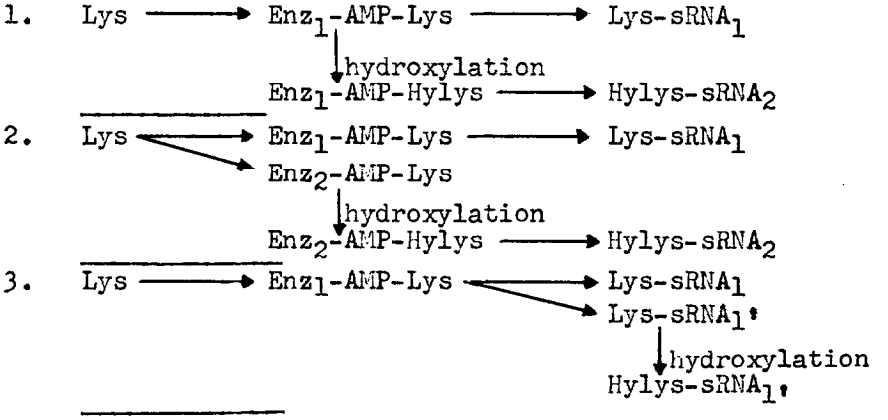
Possibility one, having one aminoacyl-sRNA synthetase for both amino acids would require that the enzyme change its specificity for transfer RNA after the hydroxylation.

Possibility two postulates two different activating enzymes for lysine, one of which allows the hydroxylation to take place

and can only transfer hydroxylysine to its specific sRNA. An analogous case has been described by Bergmann et al. (1961) for the E. coli isoleucyl-sRNA synthetase which can also activate valine but only transfer isoleucine to sRNA.

Possibility three would have the hydroxylation occurring on one of two different lysyl-sRNA's. In this case the same $sRNA_1'$ would bind two different amino acids. Since the specificity of incorporation of amino acids into proteins seems to depend only on the sRNA (Chapeville et al.1962), this would mean that the reading of the collagen code would be ambiguous. However, this might be obviated by the reaction being highly displaced toward the formation of the hydroxylysyl-$sRNA_1'$.

When this manuscript was in preparation we learned of a recent publication by Manner and Gould (1963) in which they report the formation of hydroxylprolyl-sRNA in an in vitro chick embryo system.



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ERRATA

Biochem. Biophys. Res. Commun. 12, 247 (1963) in the communication "On the Biosynthesis of Ubichromenol" by V.C. Joshi, J. Jayaraman, and T. Ramasarma:

Page 248, the legend for figure 1 should read:

"Column chromatography of the unsaponifiable lipids of liver and kidney of rats treated with mevalonic acid 2-C^{14}.

'The fractions eluted with 5% and 10% ether from the first column were rechromatographed on columns of alumina (Merck, chromatography grade, deactivated by adding 0.4 ml. water/10g.) using successively the following eluents: light petroleum (100ml.), 5% ether - light petroleum (50 ml.), 10% ether - light petroleum (50 ml.), and 20% ether - light petroleum (50 ml.). 5 ml. fractions were collected. Radioactivity was measured in each fraction and coenzyme Q and ubichromenol were estimated spectrophotometrically in appropriate fractions."