CONVERSION OF PROLINE-C^{14} TO PEPTIDE-BOUND HYDROXYPROLINE-C^{14} IN A CELL-FREE SYSTEM FROM CHICK EMBRYO

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Previous studies on carrageenin tumor slices (Lowther et al., 1961) and on intact chick embryos (Prockop et al., 1961) demonstrated the conversion of proline to hydroxyproline and suggested that the microsomes are the initial site of collagen biosynthesis. This paper presents similar results obtained in a cell-free system. In the presence of fortified microsomes from chick embryo homogenates, proline-C^{14} is converted to radioactive hydroxyproline which is found in microsomal protein. The hydroxyproline containing protein, like collagen (Fitch et al., 1955), is soluble in hot TCA[1/]. Some of the properties and requirements of this system are described.

EXPERIMENTAL

Preparation of enzyme extract. All operations were performed at 0 - 4°. Nine- or ten-day-old chick embryos were excised, quickly blotted, dropped into 0.25 M sucrose, washed through cheesecloth with the same medium and finally homogenized for 10 - 15 seconds in a Lourdes homogenizer. In early experiments, Medium A of Littlefield and Keller (1957) was used for homogenization, but later 0.25 M sucrose was used. The homogenate was centrifuged at 15,000 x g for 10 minutes and the microsome-rich supernatant was used as the enzyme source.

1/ Abbreviations used: TCA, trichloroacetic acid; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)amino methane; CP, creatine phosphate.

Measurement of the incorporation of proline-C^{14} into TCA precipitable material

Incubation at 37° was carried out in glass centrifuge tubes in a metabolic shaker. The following components were present in 1.0 ml. (in μmoles): L-proline-C^{14} 2/, 1.0 (5 x 10^5 dpm); Tris.HCl, pH 7.6, 50; KCl, 20; $MgCl_2$, 3.0; K_2ATP, 1.0; sodium creatine phosphate, 20; enzyme protein, 10 - 20 mg. The reaction was stopped after 20 minutes by adding an equal volume of cold 10 per cent TCA. The precipitate was collected by centrifugation, washed four times with cold 5 per cent TCA, once with ethanol:ether (1:4), once with absolute ether and was finally dried by warming. The dry powder was dissolved in 2 ml. Hyamine base by warming for 5 - 10 minutes at 50° (Vaughan et al., 1957) and the solution was prepared for liquid scintillation counting with 13 ml. toluene and 2 ml. of phosphor (Prockop et al., 1961).

Measurement of proline-C^{14} incorporation and conversion to peptide-bound hydroxyproline in protein extractable with hot TCA

The components of the system were used in the same concentrations as in the previous assay, but the volume was increased to 10 or 12 ml. Incubations were carried out in rubber stoppered 50 ml. erlenmeyer flasks for $2\frac{1}{2}$ hours, after which time the flasks were chilled to 0° and the reaction mixtures centrifuged at 105,000 x g for 90 minutes. The soluble fractions were poured off, the tubes drained and wiped, and the microsomes suspended in cold distilled water. Both fractions were precipitated with one-third of a volume of cold 20 per cent TCA and the precipitates collected by centrifugation. The microsomal precipitates were washed 3 times and the soluble fractions 4 times with cold 5 per cent TCA. The washed precipitates were resuspended in 5 per cent TCA (microsomal, 5 ml.; soluble fraction, 10 ml.) and extracted for 75 minutes at 90° with occasional stirring. This method consistently removed approximately 85 per cent of the hydroxyproline

2/ Uniformly labelled L-proline-C^{14}, 10 μc (22 x 10^6 dpm)/μmole, was obtained from Nuclear-Chicago Corp. and diluted with non-radioactive L-proline to 5 x 10^5 dpm/μmole.

from the precipitates. The precipitates were centrifuged and washed with 5 ml. of cold 5 per cent TCA. The extracts and washes were combined, dialyzed against distilled water and the retentates were hydrolyzed in 6 N HCl, for 2½ hours at 130°, under pressure. The hydrolysates were decolorized (Prockop and Udenfriend, 1960), filtered, the HCl removed in vacuo and the residues taken up in distilled water. The solutions were then analyzed for proline and hydroxyproline radioactivity (Peterkofsky and Prockop, 1961). In some instances, quantitative assays for proline (Troll and Lindsley, 1955) hydroxyproline (Prockop and Udenfriend, 1960) and α-amino acids (Moore and Stein, 1948) were performed.

RESULTS AND DISCUSSION

As stated above, approximately 85 per cent of the hydroxyproline in the cold TCA precipitable material was extracted with hot TCA in a non-dialyzable form. Collagen also exhibits these properties. Eastoe (1961) has reported the isolation of a collagen-like protein from the microsomes of granuloma tissue.

The incorporation of proline-C^{14} into cold TCA precipitable material accounted for 0.37 per cent of the added radioactivity. DL-Leucine-1-C^{14} and L-glutamic acid-U-C^{14} were incorporated to the extent of 0.43 per cent and 0.19 per cent, respectively, under comparable conditions. The requirements for the incorporation of proline-C^{14} and its conversion to peptide-bound hydroxyproline in the microsomal, hot TCA extract are presented in Table I. The components were used at concentrations determined as optimal for the incorporation of proline-C^{14} into cold TCA precipitable material.

The data indicate that the conversion to peptide-bound hydroxyproline has a greater dependency on an energy source than does proline incorporation. This suggests that a reaction leading to the hydroxylation of proline may also involve the ATP generating system. In a similar experiment the same pattern of results was obtained and, in addition, it was shown that with a boiled extract incorporation and hydroxylation were 7 per cent of that

obtained with the complete system. These results are comparable to values obtained for zero time controls.

TABLE 1

Requirements for the Incorporation of Proline-C^{14} and Its Conversion to Peptide-Bound Hydroxyproline-C^{14} in Microsomal, Hot TCA Extractable Material

	cpm above background *	
	Proline	Hydroxyproline
Complete system	1110	117
Minus ATP	1110	123
" CP	580	6
" ATP, CP	448	6
" Mg++	186	4

Two flasks with 10 ml. of reaction mixture were used for each experimental condition. These were then combined for analysis. The protein concentration was 17 mg./ml.

* Radioactivity was corrected only for the recoveries of the carrier imino acids using isotope dilution principles (Peterkofsky and Prockop, 1961).

The incorporation of proline and its conversion to peptide-bound hydroxyproline in microsomal, hot TCA extracts were followed over a period of 3 hours (Figure 1). While proline incorporation began almost immediately and was maximal at 20 minutes, radioactivity in hydroxyproline was not detected until about 20 minutes had elapsed. The lag period may represent the time necessary for the hydroxylation of proline to occur. This suggests the possibility of studying the hydroxylation process independent of protein formation.

Although the hot TCA extracts of the soluble fraction and microsomes contained comparable amounts of radioactive proline, only the microsomal extracts contained radioactive hydroxyproline. This finding is further evidence that collagen biosynthesis takes place in the microsomes.

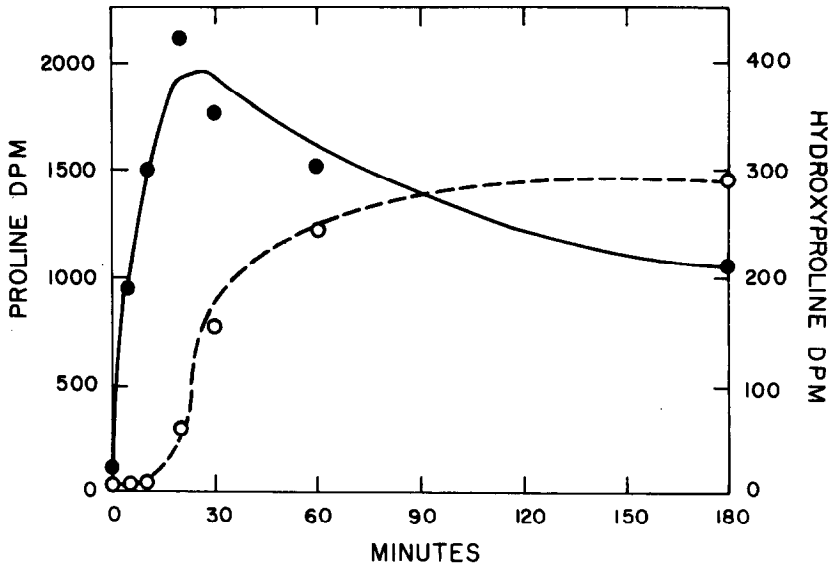

Figure 1. Time course of incorporation of radioactive proline and its conversion to peptide-bound hydroxyproline in microsomal, hot TCA extractable material.

● - ● proline, o - o hydroxyproline

The results represent the average of two independent experiments. Medium A, which contains KCl and $MgCl_2$ in addition to 0.25 M sucrose, was used as the homogenization medium. Incubation conditions, in a 12 ml. volume, were as described in the text.

In order to determine whether the radioactive imino acids were present in peptide material, hydrolyses of a dialyzed hot TCA extract from a large-scale preparation were carried out for various time periods as described above. Total α-amino acids and radioactive proline and hydroxyproline were found to be released at comparable rates. At 15 minutes approximately 30 - 40 per cent of each component had been released; by $2\frac{1}{2}$ hours the release of all components was maximal. These results indicate that the radioactive imino acids are incorporated into peptide bonds.

The method used for determining radioactivity in hydroxyproline is fairly specific and involves the conversion of the imino acid to pyrrole, which is extracted into toluene (Prockop et al., 1961; Peterkofsky and Prockop, 1961). However, the identity of the radioactive hydroxyproline was established even further. A hydrolysate of a dialyzed, microsomal, hot TCA extract was treated

with nitrous acid to destroy α-amino acids (Hamilton and Ortiz, 1950) and then desalted on a Dowex - 50 (H+) column. The resulting solution was chromatographed on a 25 cm Dowex - 50 (H+) column, using 1 N HCl as the eluting agent. Two well-separated radioactive peaks were obtained; the first reacted positively in the hydroxyproline assay and the second appeared to be proline. The first fraction was concentrated in vacuo and chromatographed in two solvent systems, along with appropriate markers. Strips cut from the area of the unknown compound were scanned in a Vanguard paper strip counter. When these strips were sprayed with isatin (Smith and Taylor, 1953), a blue-green spot characteristic of hydroxyproline was apparent in the area where radioactivity was detected. Tracings of the scannings and color reactions are shown in Figure 2. There seems little doubt that proline-C^{14} was converted to hydroxyproline-C^{14} by the enzyme system.

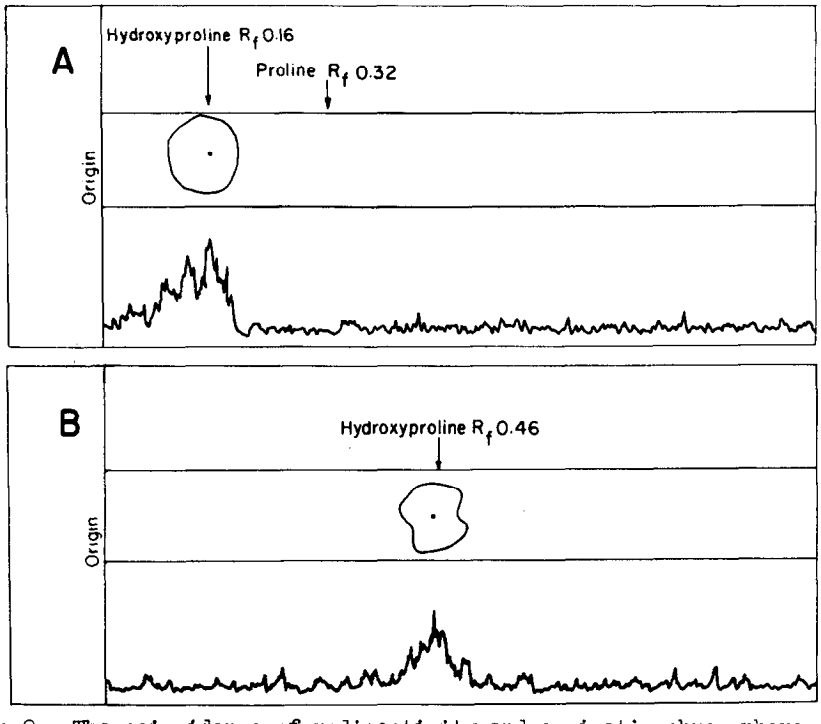

Figure 2. The coincidence of radioactivity and an isatin chromophore on paper chromatograms.

The material used was purified from hydrolysates of microsomal, hot TCA extractable material as described in the text. Developed with: A. n-butanol:water:formic acid (10:2:1); B. 77 per cent ethanol in water (v/v).

The L-proline-C^{14} used in these experiments was contaminated with 0.01 per cent of hydroxyproline-C^{14} (Lindstedt et al., 1961). In order to show that the hydroxyproline radioactivity measured in these experiments was not derived from this contaminating source, tracer DL-hydroxyproline-C^{14} 3/ was incubated for 3 hours, in the presence of non-radioactive L-proline, in the 1.0 ml. assay system. No incorporation of free hydroxyproline was detected. It is of interest that in this cell-free system, as in the intact animal (Stetten, 1949), proline is a far better precursor of peptide-bound hydroxyproline than is hydroxyproline itself.

The hydroxyproline containing, hot TCA extractable, protein in chick embryo microsomes has not been further characterized. However, it should now be possible to prepare sufficient enzymatically formed material to determine whether the C^{14} labelled protein is indeed collagen or a precursor of it.

REFERENCES

Eastoe, J. E., Biochem. J., 79, 648 (1961).
Fitch, S. M., Harkness, M.L.R. and Harkness, R. D., Nature, 176, 163 (1955).
Hamilton, P. B. and Ortiz, P. J., J. Biol. Chem., 187, 733 (1950).
Lindstedt, S. and Prockop, D. J., J. Biol. Chem., 236, 1399 (1961).
Littlefield, J. W. and Keller, E. B., J. Biol. Chem., 224, 13 (1957).
Lowther, D. A., Green, N. M. and Chapman, J. A., J. Biophysic. and Biochem. Cytol., 10, 373 (1961).
Moore, S. and Stein, W. H., J. Biol. Chem., 176, 367 (1948).
Peterkofsky, B. and Prockop, D. J., in preparation, 1961.
Prockop, D. J., Peterkofsky, B. and Udenfriend, S., in preparation, 1961.
Prockop, D. J. and Udenfriend, S., Anal. Biochem., 1, 228 (1960).
Prockop, D. J., Udenfriend, S. and Lindstedt, S., J. Biol. Chem., 236, 1395 (1961).
Smith, M.J.H. and Taylor, K. W., Biochem. J., 55, xxx (1953).
Stetten, M. R., J. Biol. Chem., 181, 31 (1949).
Troll, W. and Lindsley, J., J. Biol. Chem., 215, 655 (1955).
Vaughan, M., Steinberg, D. and Logan, J., Science, 126, 446 (1957).

3/ A gift from Dr. C. Mitoma.