STUDIES ON THE BIOSYNTHESIS OF COLLAGEN

II. The Conversion of ^{14}C-L-Proline to ^{14}C-Hydroxyproline by Fowl Osteoblasts in Tissue Culture

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INTRODUCTION

The formation of mature mammalian collagen fibre protein requires the incorporation in peptide linkage of, *inter alia*, 35 residues per cent of glycine, 12 residues per cent of proline, and 10.5 residues per cent of hydroxyproline. According to our present knowledge, in the animal kingdom hydroxyproline occurs only in the collagenous group of proteins and in elastin, which contains 1.5 residues per cent. Some of the characteristic mechanical, physical, and chemical properties of collagen fibres may be associated, at least in part, with its high content of hydroxyproline (*e.g.* Gustavson (2)).

In spite of the high hydroxyproline content of the collagen fibre protein, it seems that dietary hydroxyproline is not readily utilised by the intact, adult animal in the synthesis of this protein. Stetten (4) fed ^{15}N-labelled (L)-hydroxyproline to intact adult rats and found that the greater part of the ^{15}N-labelled material was recovered from the urine. Some of the ^{15}N administered was also recovered from the body protein, but the very low isotopic concentration of the isolated hydroxyproline indicated that in 3 days less than 0.1 per cent of the hydroxyproline of these rats had been derived from the dietary hydroxyproline. A higher concentration of ^{15}N was found in the glutamic acid, aspartic acid, and arginine of the body proteins and probably came directly from degradation products of the hydroxyproline. The body proline contained only traces of ^{15}N indicating that little, if any, of the proline of the body was derived from dietary hydroxyproline. In earlier experiments, Stetten and Schoenheimer (5) had shown that ^{15}N-labelled proline fed to intact, adult rats was readily incorporated into body protein, a significant part of the ^{15}N-labelling appearing in the hydroxyproline fraction of the carcass protein in 3 days. On the basis of this work, Stetten (4) wrote . . . "the hydroxyproline of proteins is not derived to any appreciable extent from dietary hydroxyproline, but rather from the oxidation of proline which is already bound, presumably in peptide linkage."

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If the mechanism proposed by Stetten for the formation of hydroxyproline is correct, it should constitute an important step in the sequence of reactions leading to the formation of the collagen fibre. Stetten's idea, with its far reaching implications for collagen research, seemed to us to demand evidence more direct than that obtained from feeding studies with the intact animal. In the present work we have therefore studied the ability of collagen-forming cells (fowl osteoblasts) grown in tissue culture, to form hydroxyproline from ^{14}C-(L)-proline.

Experimental Materials

Growth of Osteoblast Cultures.—Suspensions of osteoblasts, obtained from the frontal bones of fowl embryos, were grown on plates as described in the previous paper. ^{14}C-labelled amino acids in solution in the culture medium were added at the appropriate times.

Isotopes.—^{14}C-(L)-proline and ^{14}C-(L)-glutamic acid were uniformly labelled samples, prepared from algal protein, and obtained from the Radiochemical Centre, Amersham. The specific activities of each were 77 μc. per mg. The labelled proline contained no detectable ^{14}C-(L)-hydroxyproline or ^{14}C-(L)-glutamic acid when analysed by two-way paper chromatography.

L-Hydroxyproline (Roche).—was crystallised three times from aqueous ethanol.

Methods

Harvesting Cultures.—The cultures were harvested and washed with 5 per cent trichloroacetic acid solution and 3:1 alcohol-ether mixture as described in the previous paper. The efficiency of this method in removing free ^{14}C-labelled amino acids from the tissue was shown by control experiments in which 0.2 μc. ^{14}C-(L)-proline was added to a culture already grown at 38½°C. for 24 hours, and which was then incubated at 0°C. for a further 24 hours. It was assumed that under the conditions of incubation at 0°C., metabolic activity of the culture would be minimal and therefore little or no incorporation of ^{14}C-labelled amino acid into the culture should occur. It was found that a negligible amount of radioactivity (less than 20 counts per minute per mg. dry weight) was associated with the tissue after this treatment; thus it was demonstrated that non-specifically bound ^{14}C-(L)-proline was removed by the use of trichloroacetic acid and alcohol-ether.

Amino Acid Analyses.—The tissues were hydrolysed in 6 N HCl as described in the previous paper. Proline and hydroxyproline were determined in aliquots of the hydrolysate by the method of Chinard (1) and Neuman and Logan (3) respectively.

Chromatography.—The hydrolysed tissue was analysed by two-way paper chromatography as described previously. The efficacy of this method for separating proline and hydroxyproline was tested by chromatographing ^{14}C-(L)-proline together with carrier proline and hydroxyproline. A slight trailing of activity could be detected between the proline and hydroxyproline spots when 2,000 counts per minute of ^{14}C-(L)-proline were used, but the contamination of the hydroxyproline spot itself by the radioactive proline was less than 0.2 per cent.

The amino acid spots, after being visualised with ninhydrin, were cut out and eluted with 3 × 1 ml. portions of water made alkaline with ammonia. The eluates were transferred to standard (2.2 cm. diameter) aluminum counting discs and evaporated to dryness on a hot plate with a stream of air.

Radioactivity Measurements.—The radioactive samples were counted with a General Electric Company geiger counter, type EHM 2^s, contained in a square lead castle. The window of this counter was of mica, having a thickness of 1.7 mg. cm.$^{-2}$. A quenching probe unit (E. K.

Cole type NK 558) was used in conjunction with the counting tube, in order to reduce errors due to spurious pulses. In view of the low counting rates encountered throughout this work, no corrections were necessary for pulses occurring during the quenching period of the probe unit. The pulses from the probe unit were counted with a scaler (E. K. Cole type N530 C) which also supplied the potential necessary to polarise the counting tube.

The samples were placed in the castle as close as possible to the counting tube so that about 25 per cent of the beta particles emitted by the source were intercepted by the counter window. Although some of the beta particles from carbon-14 were absorbed in the air space between the source and the counter, and also in the counter window, the proportion so absorbed was constant. The samples consisted of small and constant weights of solid material, which was spread uniformly over the counting trays. It was assumed that to the degree of

TABLE I

Radioactivity of the Amino Acids from 5 Day Osteoblast Cultures Grown for 3 Days in the Presence of ^{14}C-(L)-*Proline.*

Amino acid	C.P.M. per mg. dry weight of tissue
Proline	1,171
Hydroxyproline	237
Glutamic acid	38
Aspartic acid	16
Cystine	0
Glycine	12
Serine	6
Threonine	0
Alanine	18
Valine	17
Tyrosine	23
Leucine	0
Phenylalanine	0
Arginine	12
Lysine	0
Histidine	0

accuracy achieved, errors due to source absorption, source scattering, and back scattering, were constant, and thus no corrections were made to the observed counting rates.

RESULTS

Distribution of Radioactivity in Cultures Grown in the Presence of ^{14}C-(L)-*Proline:*

Table I gives the radioactivity of the component amino acids in the hydrolysates of a typical collagen-forming osteoblast culture grown for 2 days in normal nutrient and for a further 3 days in the presence of 0.2 μc. ^{14}C-(L)-proline. Radioactivity is associated chiefly with the proline and hydroxyproline fractions, although small activities are also shown by glutamic acid, aspartic acid, and some other fractions. As will be seen later, more glutamic

acid is formed by some cultures than the amount recorded in Table I; the activity of the aspartic acid fraction appears always to be approximately half that of glutamic acid.

Rate of Conversion of Proline to Hydroxyproline:

In two serial experiments summarised in Table II, cultures were grown in the presence of ^{14}C-(L)-proline from zero time, and were harvested at intervals

TABLE II

Age of culture		Dry weight	Radioactivity						
			Proline		Hydroxyproline			Glutamic acid	
On ^{14}C-(L)-proline addition	On harvesting		C.P.M. in whole culture	Specific activity C.P.M./mg.	C.P.M. in whole culture	Specific activity C.P.M./mg.	Ratio of hydroxyproline: proline activity	C.P.M. in whole culture	Ratio of glutamic acid: proline activity
hrs.	*hrs.*	*mg.*							
Series A									
0	15	1.64	683	5.89×10^3	58	2.46×10^3	0.085	17.7	0.026
0	22	0.812	971	17.11×10^3	78	4.65×10^3	0.080	13.5	0.014
0	45	1.19	1,097	12.66×10^3	113	4.87×10^3	0.103	26.6	0.024
0	69	0.98	805	12.32×10^3	134	7.88×10^3	0.166	19.4	0.024
Series B									
0	16	0.745	1,290	21.9×10^3	92	5.31×10^3	0.072	87	0.067
0	24	0.931	1,765	27.8×10^3	130	8.12×10^3	0.074	89	0.050
0	45	0.823	1,515	23.3×10^3	188	17.0×10^3	0.124	70	0.046
0	72	0.850	1,305	20.5×10^3	237	17.2×10^3	0.181	135	0.103
Series C									
48	72	1.51	1,412	18.8×10^3	159	10.22×10^3	0.113	54	0.033
48	96	1.33	850	9.15×10^3	206	8.95×10^3	0.242	20	0.024
48	120	1.25	1,425	16.2×10^3	296	5.92×10^3	0.208	47	0.034
48 and 144	168	0.81	1,237	22.1×10^3	138	10.62×10^3	0.112	142	0.115

of up to 72 hours. Maximum weight and proline and hydroxyproline content were reached in 24 to 48 hours (the corresponding curves for series A are given in Text-fig. 6 of the previous paper), but the activity of the protein-bound hydroxyproline continued to increase after this period. The rate of formation of protein-bound radioactive hydroxyproline, expressed as increase in counts per minute per hour, plotted against the mean age of the cultures between successive times of harvesting, is seen in Fig. 1. The rate of formation falls exponentially with increasing age, while the rate of incorporation of proline falls off rapidly, and there is some loss of radioactivity in the 70 hour culture.

The rate of incorporation of ^{14}C-(L)-proline and the formation of protein-

bound hydroxyproline in successive 24 hour periods was studied in cultures of different ages, and the results are summarised in Table III and Fig. 2. The rates of incorporation of proline and the formation of hydroxyproline are closely parallel and both decline precipitately in cultures grown for longer periods.

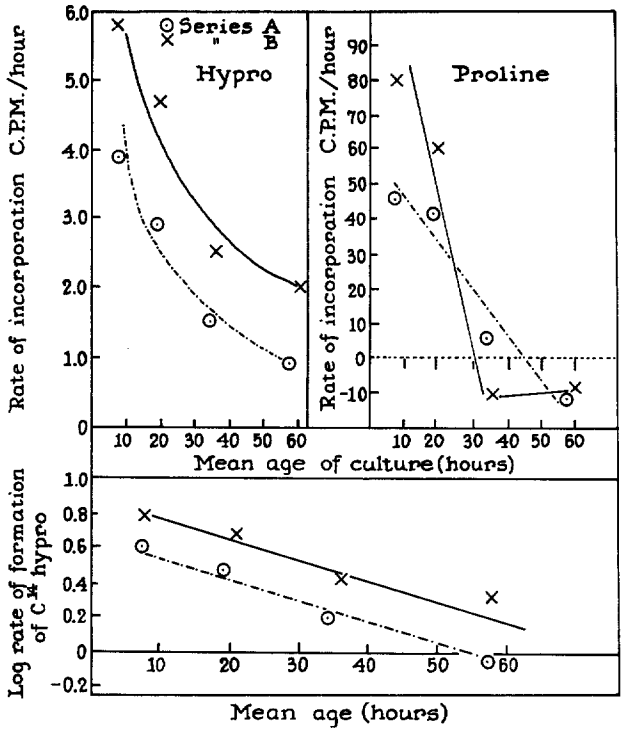

FIG. 1. (top) Graphs of the rate of increase in radioactivity, counts per minute per hour (C.P.M./hour), of hydroxyproline and proline fractions of whole cultures plotted against mean age of the cultures between successive times of harvesting. (bottom) Graph of the logarithmic rate of increase in radioactivity (C.P.M./hour) of hydroxyproline of cultures plotted against mean age. ^{14}C-(L)-proline was added at zero time; in series A each plate received 0.12 μc., and in series B each plate received 0.2 μc. The points given in series A represent single cultures; those given in series B, pairs of cultures. The growth curves for A series are shown in Text-fig. 6 of the previous paper.

Formation of ^{14}C-Hydroxyproline by Older Cultures:

In the serial experiment C (Table II) ^{14}C-(L)-proline was added to the cultures which had been grown for 48 hours. There was no increase in weight after 72 hours, but both the content of hydroxyproline and its radioactivity increased after this period (see Text-fig. 2 of previous paper). This increase was paralleled by an increase in radioactivity in the hydroxyproline fraction. The cultures grown for 7 days showed signs of morphological degeneration, and

formed appreciably more glutamic acid than the younger cultures. In another experiment, cultures were grown for 4 days in the normal nutrient and for a further 2 days in the presence of fresh medium containing 0.2 μc. ^{14}C-(L)-proline per 0.5 ml. Under these conditions the amount of labelled amino acid incorporated was small, but appreciable conversion to hydroxyproline was obtained (Table IV). In two further experiments there was a very small conversion to hydroxyproline between the 4th and 6th days of growth.

Experiments which show conversions of ^{14}C-(L)-proline to protein-bound ^{14}C-hydroxyproline by cultures to which the isotope was added after different times of growth are also summarised in Table IV. This gives the mean ratios of the radioactivities of hydroxyproline to proline and of glutamic acid to

TABLE III

Effect of Age of Culture on Incorporation of ^{14}C-(L)-Proline and Formation of Hydroxyproline

Age of culture		Dry weight	Radioactivity						
			Proline		Hydroxyproline			Glutamic Acid	
On ^{14}C-(L)-proline addition	On harvesting		C.P.M. in whole culture	Specific activity C.P.M./mg.	C.P.M. in whole culture	Specific activity C.P.M./mg.	Ratio C.P.M. hydroxyproline: proline	C.P.M. in whole culture	Ratio C.P.M. glutamic acid: proline
hrs.	*hrs.*	*mg.*							
4	24	0.67	933	22.6×10^3	155	12.7×10^3	0.166	22	0.024
24	48	1.31	1,240	11.93×10^3	201	7.65×10^3	0.162	28	0.023
48	72	0.94	281	4.46×10^3	15	0.93×10^3	0.053	20	0.071
72	96	0.79	123	2.00×10^3	9.3	0.60×10^3	0.076	26	0.212

proline in 26 sets of cultures. The ratio hydroxyproline/proline decreased while the ratio glutamic acid/proline increased in the older cultures.

In a few experiments in which the tissue cultures grew rather poorly and in which the percentage collagen content as determined by hydroxyproline assay was low, the cultures formed less protein-bound ^{14}C-hydroxyproline than actively growing cultures. For example, two cultures grown for 65 hours in the presence of 0.5 μc. ^{14}C-(L)-proline had a mean dry weight of 0.62 mg. and the collagen content of 5.6 per cent as compared with approximately 15 per cent for an active culture. The ratios of the radioactivities of the hydroxyproline/proline and glutamic acid/proline fractions were 0.052 and 0.056 respectively.

The Influence of Hydroxyproline in the Medium on the Conversion of ^{14}C-(L)-Proline to ^{14}C-Hydroxyproline:

If hydroxyproline is not directly utilised by the cell for the formation of collagen the addition of relatively high concentrations of free, non-radioactive

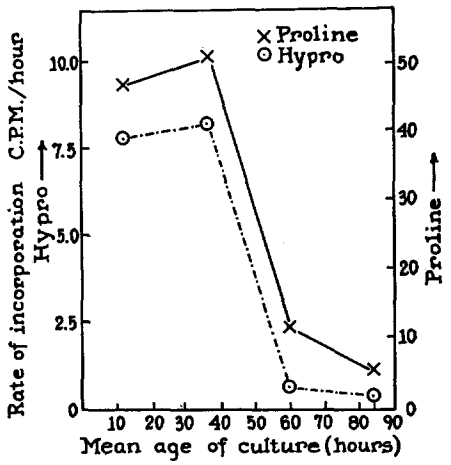

FIG. 2. Graphs of the rate of increase in radioactivity (C.P.M./hour) of hydroxyproline and proline fractions of cultures plotted as a function of age of cultures between successive times of harvesting. ^{14}C-(L)-proline (0.2 μc. per culture) was added to pairs of cultures 24 hours before harvesting them. The growth curves for these cultures are given in Text-fig. 4 of the previous paper.

TABLE IV

Age of culture on addition of ^{14}C-(L)-proline	Mean ratio C.P.M. $\frac{\text{Hydroxyproline}}{\text{Proline}}$ for time of contact:		Mean ratio C.P.M. $\frac{\text{Glutamic acid}}{\text{Proline}}$ for time of contact:	
	0 to 48 hrs.	48 hrs.	0 to 48 hrs.	48 hrs.
hrs.				
0 to 24	0.108 (4)	0.178 (6)	0.035	0.064
25 to 48	0.078 (9)	—	0.05	—
49 to 72	0.061 (3)	0.058 (1)	0.102	0.040
72	0.014 (2)	—	0.101	—
72	0.075 (1)	—	0.02	

Numbers in brackets indicate number of sets of cultures used.

TABLE V

Effect of Added Hydroxyproline on Conversion of ^{14}C-(L-)Proline to Hydroxyproline

Amount of hydroxyproline added μg.	Radioactivity		
	Proline	Hydroxyproline	
	Specific activity C.P.M./mg.	Specific activity C.P.M./mg.	Ratio C.P.M. hydroxyproline: proline
0	29.3×10^3	16.2×10^3	0.107
25	20.92×10^3	18.2×10^3	0.109
100	22.55×10^3	12.02×10^3	0.096
250	33.80×10^3	19.76×10^3	0.122

hydroxyproline would not be expected to influence the rate of conversion of added ^{14}C-(L)-proline to hydroxyproline. If, on the other hand, free hydroxyproline is directly incorporated by the cell, the presence of a high concentration would be expected to reduce the amount of proline converted to hydroxyproline.

In the present experiments each culture plate contained 0.5 ml. of nutrient medium, the total free and bound hydroxyproline and proline content of which was 10 μg. and 410 μg. respectively. Table V summarises a serial experiment in which pairs of cultures were grown for 66 hours in the presence of 0.5 μc. ^{14}C-(L)-proline and amounts of non-radioactive hydroxyproline of 0 to 250 μg. Since the ratio of the activities of hydroxyproline to proline fractions was not lowered by these additions, it is concluded that, under these conditions, proline is utilised preferentially to free hydroxyproline for the formation of protein-bound hydroxyproline.

The Incorporation of ^{14}C-(L)-Glutamic Acid by Osteoblast Cultures:

Cultures grown in the presence of ^{14}C-(L)-glutamic acid for 48 hours readily incorporated this amino acid, but negligible radioactivity was found in the hydroxyproline fraction derived from the tissue.

DISCUSSION

Fowl osteoblasts grown in tissue culture in nutrient medium containing ^{14}C-(L)-proline incorporated this amino acid in such a way that it was not removed by exhaustive washing with trichloroacetic acid; thus it is assumed that the labelled proline has been incorporated in peptide linkage into the proteins of the cell. It was considered inadvisable to attempt to isolate collagen protein from the other proteins of the culture, prior to counting and hydroxyproline assay, since the use of extraction methods might well have led to the loss of a collagen precursor of peptide nature.

Part of the radioactivity was found to appear in the hydroxyproline fraction of the tissue. Since no hydroxyproline was present in the added proline, it is concluded that it was formed by the cell either by direct hydroxylation of the proline or by its partial or complete degradation followed by resynthesis of the labelled fragments. The low radioactivities observed in most of the other amino acids would appear to exclude the occurrence of extensive degradation of the proline into small carbon fragments, for it is improbable that these would be preferentially resynthesised into hydroxyproline.

The formation of protein-bound ^{14}C-hydroxyproline was always accompanied by the formation of glutamic acid and a smaller amount of aspartic acid. The proportions of ^{14}C-hydroxyproline and glutamic acid produced varied in different series of cultures, but there was a tendency for more glutamic acid to be formed as the cultures aged. The oxidative ring cleavage of

proline by tissues *in vivo* and *in vitro* to give glutamic acid is a well established fact (*e.g.* Weil-Malherbe and Krebs (6)) and was to be expected in the osteoblast cultures. The possibility that glutamic acid is an intermediate in the formation of hydroxyproline from proline was excluded by the observation that ^{14}C-(L)-glutamic acid, although readily incorporated by the cultures, formed negligible amounts of radioactive hydroxyproline. The present experiments thus point to hydroxyproline arising from direct oxidation of the proline in the osteoblasts.

The failure of relatively high concentrations of free, non-isotopic hydroxyproline to influence the conversion of ^{14}C-(L)-proline to hydroxyproline is in agreement with Stetten's finding (4) that in the whole animal free hydroxyproline is not utilised to any appreciable extent in the formation of collagen protein. Whether this apparent inability of collagen-forming cells to incorporate efficiently free hydroxyproline in peptide linkage is merely associated with the impermeability of the cell to the compound, or with the steric effect of the hydroxyl group in preventing peptide bond formation is not at present clear. In this connection, however, it is interesting that Wiseman (7) has shown that both proline and hydroxyproline are actively absorbed in the intestine.

In the preceding paper it was recorded that in actively growing osteoblasts in tissue culture, the dry weight and hydroxyproline content of the tissue reached their maxima within the first 48 hours of cultivation. Studies of the rate of formation of hydroxyproline from ^{14}C-(L)-proline here described, show that the maximum rate of this process also occurs during the early part of the growth period, the half-period of the reaction, calculated from the curves given in Fig. 1 *a*, being 22 hours. The experiments summarised in Table II, (A) and (B), and in Fig. 1 show, however, that protein-bound ^{14}C-hydroxyproline continues to be formed at a low rate after the tissue has reached its maximum dry and hydroxyproline content. A possible explanation of these apparently contradictory findings is that during the early period of cellular growth and rapid formation of protein-bound hydroxyproline, proline peptides from the nutrient medium are used in preference to free proline for the formation of collagen precursor. Later, when an equilibrium between tissue breakdown and resynthesis has been established, and the rate of synthesis of collagen precursor is considerably reduced, the isotopically labelled proline may compete more favourably with the proline peptides of the medium for incorporation into the collagen precursor, so that the turnover of the protein is associated with a net increase in radioactivity.

It is interesting that the most rapid rate of formation of protein-bound ^{14}C-(L)-hydroxyproline occurs prior to the formation of collagen fibrils, which can be distinguished by morphological methods (as described in the previous paper) and, when collagen fibrils are apparent, this rate has declined.

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SUMMARY

1. Fowl osteoblasts grown in bulk tissue cultures in the presence of ^{14}C-(L)-proline incorporated this amino acid into peptide linkage. A significant amount of the incorporated radioactivity was found in the hydroxyproline, glutamic acid, and aspartic acid fractions of the cultures.

2. The rate of formation of protein-bound ^{14}C-hydroxyproline from ^{14}C-(L)-proline was maximal in cultures grown for 15 hours and fell exponentially with the increasing age of the cultures.

3. ^{14}C-(L)-glutamic acid was incorporated by the osteoblast cultures, but no significant amount was converted to hydroxyproline.

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