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Conversion of Proline to Collagen Hydroxyproline*

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ABSTRACT: Puromycin inhibits the incorporation of proline into the collagen proline and collagen hydroxyproline of carrageenan granuloma minces; however, the formation of soluble hydroxyproline-containing peptides continues, suggesting that hydroxylation of proline takes place prior to the release of the collagen polypeptide from the ribosome. Chlortetracycline inhibits the incorporation of proline into collagen and the hydroxylation of proline to about the same extent, suggesting that hydroxylation takes place on the ribosome. Evidence was obtained indicating that hydroxyproline can occur at the carboxyl-terminal position of the growing peptide chain attached to soluble ribonucleic acid (s-RNA) on the ribosome. Free hydroxyprolyl s-RNA could not be demonstrated, while, under the same experimental conditions, prolyl s-RNA was invariably obtained. When labeled proline was incubated with a granuloma mince from an ascorbic acid deficient guinea pig, the collagen isolated from the microsome fraction contained somewhat less labeled proline as compared to the control, while the incorporation into hydroxyproline was markedly reduced; this suggests formation of a proline-rich polypeptide. A cell-free system was obtained which incorporates proline into a peptide form which does not contain hydroxyproline. The available data support the conclusion that ribosome-bound peptidyl-prolyl s-RNA is a substrate for hydroxylation.

Collagen differs from many other proteins both in its amino acid composition and in the manner of its biosynthesis. Thus, collagen contains certain amino acids (4-hydroxy-L-proline, 3-hydroxy-L-proline, and 5-hydroxy-L-lysine) that are not usually found in other proteins; these are not derived from the corresponding free amino acids, but there is evidence that proline and lysine are hydroxylated during the biosynthesis of collagen (Stetten and Schoenheimer, 1944; Stetten, 1949; Sinex and Van Slyke, 1955). Stetten (1949) suggested that collagen hydroxyproline might arise by hydroxylation of peptide-bound proline. Subsequent studies led to consideration of the possibility that proline is hydroxylated at an intermediate step prior to its incorporation into the peptide chain (Robertson *et al.*, 1959; Green and Lowther, 1959; Stone and Meister, 1962). More recently, experimental support for the belief that proline is hydroxylated in peptide linkage has come from experiments with a cell-free chick embryo system capable of incorporating proline into peptide-bound proline and hydroxyproline (Peterkofsky and Udenfriend, 1963, 1965; Juva and Prockop, 1964; Prockop and Juva, 1965). On the other hand, work in several laboratories has provided evidence for the existence of hydroxyprolyl soluble ribonucleic acid (s-RNA) (Manner and Gould, 1962, 1963; Coronado *et al.*, 1963; Jackson *et al.*, 1964; Urivetzky *et al.*, 1965).

Earlier work in this laboratory showed that the incorporation of tritiated proline into collagen hydroxyproline by minces of carrageenan granuloma is directly related to the release of tritiated water from tritiated proline, and therefore that the formation of tritiated water can be used as a valid measure of hydroxyproline formation in this system (Stone and Meister, 1962). Subsequent studies indicated that puromycin inhibits the formation of collagen hydroxyproline markedly, but that hydroxylation (as determined by the formation of tritiated water and tritiated hydroxyproline) continues (Meister *et al.*, 1964). The experiments reported here support this conclusion; thus, evidence is presented that,

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although the formation of collagen hydroxyproline is markedly reduced in the presence of puromycin, peptides containing hydroxyproline are formed and accumulate to a greater extent (in relation to the total amount of hydroxyproline formed) in the presence of puromycin than in its absence. Evidence has also been obtained for ribosome-bound peptidyl s-RNA in which some of the carboxyl-terminal amino acid residues (*i.e.*, the residues esterified to s-RNA) are hydroxyproline.

Experimental Section

Materials. DL-[^{3}H]Proline (44 c/mole), prepared by the method of Wilzbach (1957), was obtained from the Comissariat à l'Energie Atomique, Centre d'Etudes Nucleaires de Saclay, Gif-sur-Yvette, France; L-[3,4-^{3}H]proline (5000 c/mole), prepared by catalytic reduction of 3,4-dehydro-L-proline with tritium gas,[1] and L-[^{14}C] proline (185 c/mole), stated to be uniformly labeled, were obtained from New England Nuclear Corp., Boston, Mass. L-Proline, hydroxy-L-proline, and L-ascorbic acid were purchased from Mann Research Laboratories, Inc., New York, N. Y.; ascorbic acid deficient diet and puromycin dihydrochloride were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; lithium borohydride was purchased from Metal Hydrides, Inc., Beverly, Mass.; carrageenan was a gift from Marine Colloids, Inc., Springfield, N. J. Adenosine triphosphate[2] (ATP) was obtained from Sigma Chemical Co., St. Louis, Mo.; disodium creatine phosphate was purchased from Calbiochem, Los Angeles, Calif.; yeast s-RNA was obtained from General Biochemicals, Chagrin Falls, Ohio; trypsin (twice crystallized) was a product of Worthington Biochemicals Corp., Freehold, N. J.; ribonuclease (five times crystallized) was obtained from Pentex, Inc., Kankakee, Ill.; tannic acid was purchased from Fisher Scientific Co., Fair Lawn, N. J.

Preparation of Hydroxy-L-prolinol and L-prolinol. Hydroxy-L-prolinol and L-prolinol were synthesized by reduction of the corresponding imino acid methyl esters with lithium borohydride. The methyl esters were prepared by dissolving 10 g of the imino acid in 150 ml of absolute methanol. The solution was saturated with dry hydrogen chloride at 0° and refluxed for 1 hr (Greenstein and Winitz, 1961). Hydroxy-L-proline methyl ester was isolated in 70% yield as the hydrochloride (mp 162–165°). L-Proline methyl ester hydrochloride could be obtained only as an oil. The imino acid methyl ester hydrochloride (2.8 mmoles) was refluxed with lithium borohydride (13.8 mmoles) in 15 ml of tetrahydrofuran for 2 hr; conversion of ester to alcohol was 99% complete as determined by the chromotropic acid procedure (*vide infra*). The reaction mixture was acidified by adding 6 ml of 6 N HCl. The tetrahydrofuran was removed by evaporation on a steam bath, and the imino alcohol hydrochloride was extracted from the residue with 30 ml of boiling 2-propanol. Ethyl acetate (300 ml) was then added and the precipitated imino alcohol hydrochloride was collected by filtration. The product was crystallized from 2-propanol–ethyl acetate.

Anal. Calcd for $C_5H_{11}NO_2 \cdot HCl$: C, 39.1; H, 7.9; N, 9.2; Cl, 23.1. Found: C, 39.2; H, 7.5; N, 8.8; Cl, 23.4 (hydroxyprolinol hydrochloride, mp 127–128°). Calcd for $C_5H_{11}NO \cdot HCl$: C, 43.6; H, 8.8; N, 10.2. Found: C, 43.7; H, 8.4; N, 10.5 (prolinol hydrochloride, mp 57–58°; reported 57–58°, Blicke and Lu, 1955).

The imino alcohols were determined by a modification of the method of Frisell *et al.* (1954). The imino alcohols were oxidized by treatment with sodium periodate at pH 2 for 2 hr at 37° to yield 0.85 mole of formaldehyde/mole of imino alcohol. Under these conditions, no formaldehyde was produced from the corresponding imino acids. The absorbancies obtained were proportional to imino alcohol concentration over the range 0.15–1.3 μmoles/ml.

Methods

Preparation of Granuloma Minces. Male American short-hair guinea pigs, weighing 400–600 g each, were injected subcutaneously with 5 ml of a 1% solution of carrageenan containing penicillin (250 μg/ml) and streptomycin (500 μg/ml) in 0.9% NaCl as described by Robertson and Schwartz (1953). The guinea pigs were maintained on an adequate diet plus an oral dose of 50 mg of L-ascorbic acid in water every other day. After 7–10 days the animals were decapitated; the granuloma (20–30 g) was excised and immediately placed in 100 ml of ice-cold Medium M [NaCl (0.122 M), KCl (0.003 M), $MgSO_4$ (0.0012 M), $CaCl_2$ (0.0013 M), KH_2PO_4 (0.0004 M), D-glucose (0.01 M), and $NaHCO_3$ (0.025 M), pH 7.5]. The tissue was minced finely with a pair of scissors just prior to incubation. In some experiments the minces were incubated with Medium A (Keller and Zamecnik, 1956), which contains sucrose (0.35 M), $MgCl_2$ (0.004 M), KCl (0.025 M), K_2HPO_4 (0.020 M), and $KHCO_3$ (0.035 M); the pH was adjusted to 7.0 at 25° by addition of 2 N HCl. Although the release of tritiated water from tritiated proline was the same with both media, the large amount of sucrose in Medium A made lyophilization and subsequent homogenization difficult; for this reason Medium M is preferred; $MgSO_4$ and $CaCl_2$ were omitted from Medium M in the studies with chlortetracycline since this compound is insoluble in the presence of these salts.

In some experiments the animals were given an ascorbic acid deficient diet for 2 weeks before injection with carrageenan, and then maintained on the same ration during the period of granuloma formation.

Isolation of Collagen Proline, Collagen Hydroxyproline, and Tritiated Water. Minces of carrageenan granuloma

[1] Studies carried out in our laboratory on two batches of this material indicated that these had a different distribution of tritium in the proline molecule; however, the ratio of the tritiated water formed to the tritiated hydroxyproline formed in minces of carrageenan granuloma is constant for a given batch of tritiated proline (Meister *et al.*, 1964).

[2] Abbreviation used: ATP, adenosine triphosphate.

were incubated with labeled proline in rubber-stoppered Erlenmeyer flasks at 37° under an atmosphere of 95% O_2-5% CO_2; the flasks were shaken (100–120 excursions/min). After incubation, the reaction mixture was lyophilized and the tritiated water was collected in a coldfinger trap cooled by a mixture of Dry Ice and acetone. The radioactivity of the water was determined by liquid scintillation counting on 1-ml aliquots mixed with 15 ml of Bray's solution (Bray, 1960). The dry residue was homogenized in 10 ml of cold 5% trichloroacetic acid in a Virtis 45 homogenizer for 30 sec at 0.75 maximum speed, and the homogenate was centrifuged at 1000*g* for 2 min. The supernatant fraction was decanted and extracted four times with an equal volume of ether; hydroxyproline was isolated from the aqueous phase before and after treatment with 6 N HCl at 110° as described below for collagen. The insoluble fraction was washed 10 times with 4 ml of cold 5% trichloroacetic acid and twice with 4 ml of ether. Collagen was extracted by mixing the insoluble residue with 3 ml of 5% trichloroacetic acid at 90° for 75 min (Fitch *et al.*, 1955). The extract was cooled to 0° and the collagen was precipitated by adding an equal volume of cold 5% tannic acid (Meister *et al.*, 1964). The collagen tannate was collected by centrifugation at 1000*g* for 2 min and washed once with 2 ml of cold 5% tannic acid. It was then dissolved in 1 ml of 2 N NH_4OH at 60°, and aliquots of the protein solution were plated on stainless steel planchets. The [^{14}C] radioactivity of the collagen tannate was determined in a Nuclear-Chicago gas flow counter.

In some experiments collagen tannate (80–100 mg) was hydrolyzed by treatment with 3 ml of 6 N HCl for 16 hr at 110°. The hydrolysate was decolorized by adding a mixture of Dowex 1–charcoal (2:1) (Prockop and Udenfriend, 1960); after deamination of the amino acids with HNO_2 (Hamilton and Ortiz, 1950), the mixture was desalted on a column of Dowex 50W (H^+; 200–400 mesh; 1.2 × 12 cm) at 25° using 2 N NH_4OH to elute the imino acids; the eluate was concentrated by evaporation under reduced pressure at 37°. The pH was adjusted to 1–2 and the proline and hydroxyproline (20–30 μmoles of each) were separated on a column of Dowex 50W-X8 (H^+; 200–400 mesh; 1 × 30 cm) at 25° using 1.5 N HCl as the eluent. Hydroxyproline and proline were eluted from the column in the 55–60-ml and the 110–120-ml fractions, respectively. Hydroxyproline was determined by the methods of Neuman and Logan (1950) and of Peterkofsky and Prockop (1962). Proline was determined as described by Troll and Lindsley (1955) and by Piez *et al.* (1956). The peak fractions of each imino acid were pooled and concentrated to dryness by evaporation at 37° under reduced pressure; each residue was dissolved in 2 ml of water and the radioactivity ([^{14}C] or [^{3}H]) of an aliquot was determined in 15 ml of Bray's solution (Bray, 1960) using a Nuclear-Chicago liquid scintillation counter.

Preparation of Subcellular Fractions of Carrageenan Granuloma. After incubation with labeled proline, the mince was collected by centrifugation and washed twice with 10 ml of 0.25 M sucrose at 4°; it was then homogenized with 2 volumes of cold 0.25 M sucrose in a Virtis 45 homogenizer at 0.75 maximum speed for 15 sec. The homogenate was centrifuged at 1000*g* for 10 min at 4°. The sediment, which contains cell debris and insoluble collagen, was washed twice with 10 ml of 0.25 M sucrose. In some experiments collagen was extracted from the 1000*g* pellet with hot trichloroacetic acid as described above. The 1000*g* supernatant solution was centrifuged at 10,000*g* for 10 min and, after removal of the sediment, the microsome fraction was collected by centrifugation at 100,000*g* for 75 min in a Spinco Model L ultracentrifuge. The microsome fraction was washed 10 times with 4 ml of 95% ethanol, twice with 4 ml of absolute ether, and then dried to a fine powder at 50°. In certain experiments collagen was extracted from the microsome fraction with hot trichloroacetic acid and then precipitated with tannic acid as described above.

Isolation of Iminoacyl Soluble RNA. In the studies with cell-free preparations, the soluble fraction that is precipitable at pH 5 was obtained as described by Keller and Zamecnik (1956). The concentration of protein was determined by the method of Kalckar (1947). The iminoacyl s-RNA fraction was isolated as described by Moldave (1963) and by Rosenbaum and Brown (1961). The isolation of iminoacyl s-RNA from the microsome fraction was carried out by adding sodium dodecyl sulfate (final concentration, 1%) to an aqueous microsome suspension containing carrier yeast s-RNA (25–50 mg) and stirring for 10 min at 26°. An equal volume of water-saturated phenol was added and the mixture was stirred vigorously for 1 hr at 4° (Kurland, 1960). The iminoacyl s-RNA was isolated (Moldave, 1963) and either incubated at pH 10 for 1 hr or treated with $LiBH_4$ as described below.

Reduction with Lithium Borohydride. The samples to be reduced were dried by washing with ethanol and ether or by lyophilization and then placed in a 100-ml round-bottom flask. Carrier proline and hydroxyproline (2–3 mg of each) were added to the samples (100–200 mg), and 3 ml of 0.15 M $LiBH_4$ in tetrahydrofuran was added (Golub *et al.*, 1965). The mixture was shaken at 25° for 1 hr, after which 2 ml of H_2O and 0.2 ml of 6 N HCl were added to destroy the excess $LiBH_4$. After removal of the tetrahydrofuran by evaporation on a steam bath, carrier imino alcohols (2–3 mg of each) and an equal volume of concentrated HCl were added, and the mixture was heated at 110° for 16 hr. The hydrolysate was decolorized, treated with nitrous acid, and desalted as described above except that 7.5 N NH_4OH was used to elute prolinol from the desalting column. Ammonia was removed from the eluate by evaporation under reduced pressure at 10–15°; the eluate was then made acid to Congo Red paper by adding 6 N HCl and was then concentrated under reduced pressure at 37°. The imino acids and imino alcohols were separated as described in Figure 1. The fractions containing each compound were pooled and counted as described above.

Preparation of a Cell-Free Proline-Incorporating System. Granuloma mince (10 g) was weighed into the

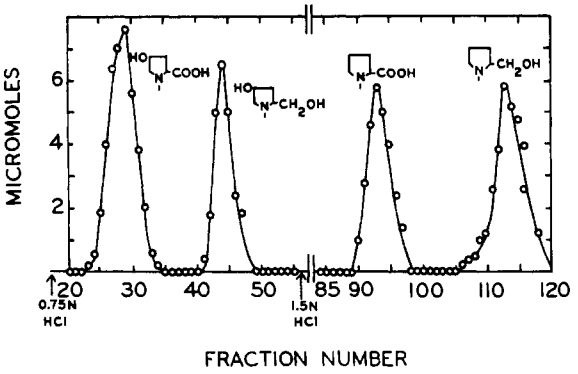

FIGURE 1: Separation of imino acids and imino alcohols. A solution (2–3 ml) containing L-proline, hydroxy-L-proline, and the corresponding imino alcohols (20–30 μmoles of each) at pH 1–2 was applied to the top of a column of Dowex 50W (H^+; 200–400 mesh; 1 × 38 cm) at 25°. The compounds were eluted with 0.75 N HCl. The first 100 ml of the eluate was discarded; then 2-ml fractions of the eluate were collected. After 55 fractions had been collected, the eluent was changed to 1.5 N HCl. The imino alcohols were determined as described in the text; ordinate = micromoles per fraction.

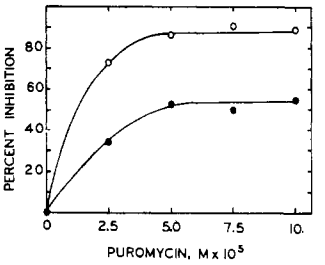

FIGURE 2: Effect of puromycin on the incorporation of [^{14}C]proline into collagen and on the release of tritiated water from [^{3}H]proline. Each vessel contained 1 g of granuloma mince, 2.5 ml of Medium A, DL-[^{3}H]proline (134 mμmoles; 1.3 × 10^6 cpm), L [^{14}C]proline (6.5 mμmoles; 1.3 × 10^6 cpm), and puromycin dihydrochloride in the concentrations indicated in a final volume of 4.0 ml; incubated at 37° for 1 hr. After incubation, the reaction mixtures were lyophilized and the tritiated water was collected and counted. The collagen was isolated from the dry residue as described in the text. Open circles—inhibition of incorporation of [^{14}C]proline into collagen; closed circles—inhibition of formation of tritiated water.

5–30-ml glass cup of a Virtis 45 homogenizer and 10 ml of ice-cold Medium M was added. The cup was flushed with nitrogen for 3 min and the mixture was homogenized at half-maximum speed for 10 sec at 4°. The homogenate was then centrifuged at 1000*g* for 10 min. The supernatant fraction was filtered through a single layer of cheesecloth and the filtrate was used in the experiments described here. The reaction was stopped by adding 0.33 volume of cold 20% trichloroacetic acid. The precipitated protein was washed 10 times with 4 ml of 95% ethanol, twice with 4 ml of ether, and then hydrolyzed with 2 ml of 6 N HCl at 110° for 16 hr. The hydrolyzate was assayed for radioactive proline and hydroxyproline by the method of Peterkofsky and Prockop (1962).

Results

Effect of Puromycin on the Incorporation of [^{14}C]-*Proline and* [^{3}H]*Proline into Collagen and on the Formation of Tritiated Water.* In the experiments described in Figure 2 granuloma minces were incubated with both [^{14}C]proline and [^{3}H]proline in the presence of various concentrations of puromycin. The incorporation of [^{14}C]proline into collagen was inhibited by about 90% with puromycin concentrations of 5 × 10^{-5} M or greater. However, under these conditions the formation of tritiated water was inhibited by only about 50%. Previous studies have shown that the formation of tritiated water is a valid measure of the conversion of proline to hydroxyproline in this system (Stone and Meister, 1962); the data therefore indicate that hydroxylation of proline can occur when collagen formation is markedly decreased.

In an experiment similar to those described in Figure 2, the isolated collagen was hydrolyzed with 6 N HCl and the free proline and hydroxyproline thus liberated were isolated. The specific radioactivity of the collagen hydroxyproline was 460 cpm/μmole in the absence of puromycin and 68 cpm/μmole in its presence (85% inhibition). The specific radioactivity of the collagen proline was 1180 cpm/μmole and 200 cpm/μmole in the absence and presence, respectively, of puromycin (83% inhibition). These data indicate that puromycin inhibits the incorporation of proline into both collagen proline and collagen hydroxyproline and that the incorporation into both imino acids is inhibited to about the same extent.

The effect of puromycin on the distribution of labeled hydroxyproline in granuloma minces was examined as described in Table I. In expt 1 granuloma mince was incubated with tritiated proline in the presence and absence of puromycin. After lyophilization of the vessel contents and determination of the tritiated water formed, the dry residue was homogenized with cold trichloroacetic acid and separated by centrifugation into soluble and insoluble fractions. The total hydroxyproline of the insoluble fraction was isolated after hydrolysis with 6 N HCl and its radioactivity determined; labeled free hydroxyproline was isolated from an unhydrolyzed aliquot of the soluble fraction; total bound hydroxyproline was isolated after acid hydrolysis of the soluble fraction. In this experiment (and also in expt 2) negligible amounts of free hydroxyproline were found in the soluble fraction. The values given in Table I for the hydroxyproline present in the soluble fraction were

TABLE I: Effect of Puromycin on the Distribution of Labeled Hydroxyproline in Granuloma Minces.[a]

Expt No.	Fraction	Hydroxyproline			Soluble Hydroxyproline/ Total Hydroxyproline × 100 (%)	
		without Puromycin (cpm)	with Puromycin (cpm)	Inhibition (%)	without Puromycin	with Puromycin
1	Insoluble	41,500	11,600	72	..	..
	Soluble[b]	12,500	8,340	33	23	42
2	Microsome	9,250	1,750	81	..	..
	Soluble[b]	5,780	3,760	35	38	68

[a] Expt 1: Granuloma mince (2 g) was incubated with L-[3,4-^{3}H]proline (2.0 mμmoles; 2.2×10^6 cpm) and 5 ml of Medium M in a final volume of 8 ml at 37° for 3.5 hr. Puromycin (final concentration 2.5×10^{-5} M) was added initially as indicated. After incubation, the reaction mixture was lyophilized and the tritiated water was determined. In this experiment the formation of tritiated water was reduced by 44% in the presence of puromycin. The lyophilized residue was homogenized in cold 5% trichloroacetic acid and separated by centrifugation at 1000*g* into a soluble and an insoluble fraction. The hydroxyproline of the insoluble fraction and that of the cold trichloroacetic acid soluble fraction (before and after acid hydrolysis) were determined as described in the text. Expt 2: Granuloma mince (6 g) was incubated with L-[^{14}C]proline (55 mμmoles; 1.1×10^7 cpm) and 12 ml of Medium M at 37° for 1.5 hr. Puromycin (final concentration. 5.0×10^{-5} M) was added initially as indicated. The hydroxyproline of the microsome fraction and the free and bound hydroxyproline present in the cold trichloroacetic acid soluble fraction were determined as described in the text. [b] Corrected by subtraction of the values for free hydroxyproline (less than 5% of the total).

corrected by subtracting the values for free hydroxyproline (less than 5% of the total hydroxyproline found). When an aliquot of the unhydrolyzed soluble fraction was chromatographed on Dowex 50W, a broad distribution of radioactive peaks was found (usually at least 7 distinguishable components) and there was little labeled free hydroxyproline; after hydrolysis in 6 N HCl at 110°, more than 90% of the total radioactivity was recovered as free hydroxyproline. It thus appears that most of the hydroxyproline present in the soluble fractions is bound hydroxyproline. In the presence of puromycin, the incorporation into hydroxyproline of the insoluble and soluble fractions was inhibited by 72 and 33%, respectively. It is of significance that the percentage of hydroxyproline found in the soluble fraction in the presence of puromycin is about twice that of the control. Experiment 2 (Table I) describes a similar experiment carried out with [^{14}C]-proline. After incubation, the granuloma mince was homogenized with sucrose and the microsome fraction was isolated as described under Methods. The 100,000*g* supernatant fraction was treated with trichloroacetic acid and the trichloroacetic acid soluble fraction was worked up as in expt 1. In expt 2, the formation of microsomal hydroxyproline was markedly reduced in the presence of puromycin, while the formation of bound hydroxyproline in the soluble fraction was not as greatly inhibited. As in expt 1, a much larger percentage of bound hydroxyproline was found in the cold trichloroacetic acid soluble fraction in the presence of puromycin than in its absence. These experiments are consistent with the belief that appreciable formation of peptide-bound hydroxyproline continues while there is marked inhibition of insoluble collagen formation by puromycin.

Isolation of Prolinol and Hydroxyprolinol from the Microsome Fraction of Granuloma after Treatment with Lithium Borohydride. The experiments described above suggest that the ribosome-bound peptide precursor of insoluble collagen (*i.e.*, peptidyl s-RNA) contains hydroxyproline. Previous studies have shown that treatment of aminoacyl s-RNA and peptidyl s-RNA compounds with lithium borohydride leads to the formation of the corresponding amino alcohol derivatives (Golub *et al.*, 1965; Soda *et al.*, 1965). Thus, Soda *et al.* (1965) found that reduction of polylysyl s-RNA by lithium borohydride gave polylysyllysinol. Application of this procedure to the collagen-forming system of carrageenan granuloma was attempted as follows. Granuloma minces were incubated with labeled proline and then homogenized in sucrose. The microsome fraction was isolated and treated with lithium borohydride as described under Methods. The formation of labeled prolinol and hydroxyprolinol was determined before and after hydrolysis with 6 N HCl at 110°. No free hydroxyprolinol was found before acid hydrolysis. Some free prolinol was found before acid hydrolysis; this represented about 3% of the total prolinol found after acid hydrolysis. The results of several representative experiments in which the isolated microsome fraction was treated with lithium borohydride and then hydrolyzed with acid are summarized in Table II. In the control experiments an equivalent quantity of the microsome fraction was incubated at pH 12 for 3 hr at 37°. After incubation, the solution was adjusted to pH 5 by addition of 1.5 N HCl and then

TABLE II: Treatment of the Microsome Fraction of Granuloma with Lithium Borohydride.

Expt No.	Fraction[a]	Hydroxy-prolinol (cpm)	Hydroxyproline (cpm)	Prolinol (cpm)	Proline (cpm)
1	Microsome	400	11,700	1,700	64,500
	Microsome control[b]	50	11,000	230	58,500
2	Microsome	380	13,900	1,490	56,000
	Microsome control[b]	110	12,000	170	46,800
3	Microsome	216	9,250	1,440	55,000
	Microsome control[b]	60	8,600	338	57,000
4	Microsome	400	16,000	2,850	206,000
	Microsome control[b]	110	17,000	410	200,000
5	Microsome	...	...	1,970	115,000
	Microsome control[b]	...	...	220	115,000
6	Microsome	...	...	3,050	105,000
	Microsome control[b]	...	...	180	105,000

[a] Minced granuloma (48 g, expt 1; 64 g, expt 2; 24 g, expt 3; 30 g, expt 4; 28 g, expt 5) was incubated with L-[^{14}C]-proline (520 mμmoles, 1.1 $\times$ 10^8 cpm, expt 1; 710 mμmoles, 1.5 $\times$ 10^8 cpm, expt 2; 260 mμmoles, 5.5 $\times$ 10^7 cpm, expt 3; 620 mμmoles, 1.3 $\times$ 10^8 cpm, expt 4; 325 mμmoles, 6.7 $\times$ 10^7 cpm, expt 5) in Medium M (96 ml, expt 1; 64 ml, expt 2; 48 ml, expt 3; 45 ml, expt 4; 45 ml, expt 5) for 1–2 hr at 37°. In expt 6, minced granuloma (30 g) from guinea pigs on ascorbic acid deficient diet was incubated with L-[^{14}C]proline (325 mμmoles, 6.7 $\times$ 10^7 cpm) in 45 ml of Medium M for 1 hr. The microsome fractions were isolated by centrifugation and treated with lithium borohydride as described under Methods. [b] Control: the microsome fraction was isolated by centrifugation after incubation with labeled proline; it was then incubated at pH 12 for 3 hr at 37° prior to treatment with lithium borohydride.

lyophilized; the dry residue was then treated with lithium borohydride. As indicated in Table II, the amounts of hydroxyprolinol and prolinol found after lithium borohydride treatment and hydrolysis of the isolated microsome fractions were about 3.5–17 times higher than those of the corresponding alkali-treated microsome controls. The formation of some prolinol and hydroxyprolinol in the controls might possibly result from incomplete hydrolysis of peptidyl s-RNA under the conditions employed.[3] Some reduction of the free carboxyl-terminal imino acid residues and some reductive cleavage of prolyl- and hydroxyprolyl-peptide bonds may also occur. Thus, it has recently been shown that treatment of peptides containing internal proline residues with lithium aluminum hydride yields an aldehyde and N-terminal proline peptides (Ruttenberg *et al.*, 1964). Wilcheck *et al.* (1965) have found that sodium in liquid ammonia cleaves peptides containing acyl proline linkages as well as polyproline in a similar manner. Chibnall and Rees (1958) found that lithium borohydride cleaves some peptide bonds in β-lactoglobulin and other proteins. It seems probable that some peptide bond cleavage occurs even under the mild conditions used here, and that such cleavage followed by reduction of the resulting imino aldehydes could account for the imino alcohols found in the control studies. However, the data indicate that such reactions occur to a relatively small extent under the conditions employed. When the insoluble collagen fraction and the pellet obtained by centrifugation at 1000*g* were isolated from minces (previously incubated with labeled proline) and then lyophilized, treated with lithium borohydride, and hydrolyzed with HCl, very small amounts of radioactivity were found in prolinol and hydroxyprolinol (about 0.4% of the corresponding imino acid radioactivity). Similarly low values were obtained after these fractions were incubated for 3 hr at pH 12 prior to treatment with lithium borohydride. As stated below, treatment with lithium borohydride of the bound hydroxyproline and proline of the soluble fraction of granuloma homogenates did not yield significant amounts of the corresponding imino alcohols.

A number of attempts were made to isolate peptidyl s-RNA from the microsome fraction. In these experiments, the granuloma mince was incubated with labeled proline and the microsome fraction was isolated as described under Methods. The microsome fraction was treated with sodium dodecyl sulfate and then extracted with phenol (Kurland, 1960). In such extractions only relatively small amounts of peptidyl s-RNA entered the aqueous phase; most of the labeled proline is apparently present in the phenol phase as large peptidyl s-RNA complexes which exhibit solubility more similar to proteins than to nucleic acids. Nevertheless, the findings indicate that a significant amount of peptidyl s-RNA was isolated free of microsomal protein. Thus, in a typical experiment, 24 g of granuloma mince was

[3] Slapikoff (1964) has found that peptidyl soluble RNA complexes from rat liver ribosomes exhibit varying degrees of stability in alkaline solution; they are, however, considerably more stable than aminoacyl soluble RNA derivatives.

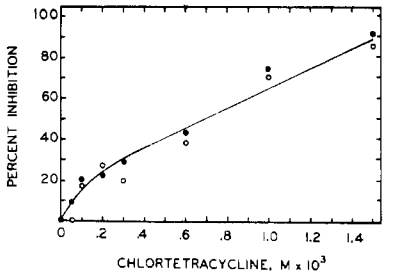

FIGURE 3: Effect of chlortetracycline on the incorporation of L-[^{14}C]proline into collagen and on the release of tritiated water from L-[3,4-^{3}H]proline. Each vessel contained 2 g of granuloma mince, 4.7 ml of Medium M (minus Ca^{2+} and Mg^{2+}), and the indicated concentrations of chlortetracycline hydrochloride. After preincubation for 15 min at 37°, L-[3,4-^{3}H]proline (8.5 mμmoles; 9.3×10^{6} cpm) and L-[^{14}C]proline (44 mμmoles; 8.8×10^{6} cpm) were added; final volume, 8.0 ml. Incubated for 75 min. Open circles—inhibition of incorporation of [^{14}C]proline into collagen; closed circles—inhibition of formation of tritiated water.

incubated with 48 ml of Medium M containing L-[^{14}C]-proline (1 μmole; 2×10^{8} cpm) for 60 min at 37°. After homogenization and isolation of the microsome fraction, 25 mg of yeast s-RNA was added as carrier; sodium dodecyl sulfate (final concentration 1%) was added, and the mixture was stirred for 10 min at 26°. An equal volume of water-saturated phenol was added and the mixture was extracted at 4° (Kurland, 1960). The peptidyl s-RNA fraction was isolated from the aqueous layer as described under Methods; this material was treated with lithium borohydride and then hydrolyzed with 6 N HCl at 110°. The radioactivity of the hydroxyprolinol was 26 cpm and that of hydroxyproline was 452 cpm (counting error 1–2%). A control in which the isolated material was incubated at pH 12 for 3 hr at 37° prior to treatment with lithium borohydride was also carried out; no [^{14}C]hydroxyprolinol was found.

It is possible to make a rough estimate of the average chain length of the peptides attached to s-RNA if the following assumptions are made: (1) The labeled proline and hydroxyproline residues are equally distributed throughout the growing peptide chains. (2) Proline and hydroxyproline occur in the carboxyl-terminal position of the growing peptide chain attached to s-RNA at a frequency proportional to the occurrence of these imino acids in the peptides being synthesized. (3) The hydroxyprolinol and prolinol formed (corrected for the control values) are valid measures of the corresponding carboxyl-terminal imino acid residues attached to s-RNA on the microsome. Thus, in expt 1, about 3.0% of the hydroxyproline and about 2.3% of the proline was converted to the corresponding imino alcohol suggesting average chain length values in the range of 35–45. Similar calculations from the data obtained in the other experiments given in Table II give average chain length values of from 38 to 84. It is clear that small errors in these determinations would have a considerable effect on the values obtained for the average chain length, so that such estimates can be accepted only as an indication of the order of magnitude.

When an experiment was carried out with the microsome fraction obtained from an experiment such as that described in Table II, but in which puromycin (5×10^{-5} M) was added, the incorporation of [^{14}C]proline into the microsome fraction was inhibited by about 75%; when the microsome fraction was treated with lithium borohydride, small but significant quantities of hydroxyprolinol and prolinol were obtained. The corrected values for conversion of proline and hydroxyproline to the corresponding imino alcohols were 2.5 and 2.0%, respectively, indicating that the average chain length of the ribosome-bound peptides in the presence of puromycin was of the same order of magnitude as in the experiments described in Table II. When the supernatant fraction obtained as described in Table I in the presence of puromycin was lyophilized and treated with lithium borohydride, no evidence for imino alcohol formation was obtained. This finding indicates that significant amounts of imino acid esters do not accumulate in the soluble fraction in the presence of puromycin.

The Effect of Chlortetracycline on the Incorporation of Proline into Collagen. Figure 3 describes the effect of chlortetracycline on the incorporation of [^{14}C]proline into collagen and on the formation of tritiated water from [^{3}H]proline. At each concentration of chlortetracycline studied, the hydroxylation of proline (as determined by the formation of tritiated water) and the incorporation of [^{14}C]proline into collagen were inhibited to approximately the same extent. These findings are in contrast to those described above on the effects of puromycin.

Incorporation of Proline into the Microsome Fraction of Scorbutic Granuloma. Granuloma minces from ascorbic acid deficient guinea pigs and from normal controls were incubated with [^{14}C]proline; after incubation the minced granuloma was homogenized with sucrose and the microsome fraction was isolated as described under Methods. The collagen was obtained from the microsome fraction as the tannate and hydrolyzed with 6 N HCl; the radioactivity of the imino acids isolated from these hydrolysates was determined (Table III). The incorporation of proline into collagen proline was reduced by 35% as compared to the control, while the incorporation of proline into collagen hydroxyproline was reduced by 86%. The data therefore show that proline is incorporated into the microsome fraction in a form which, like collagen, is soluble in hot trichloroacetic acid and is precipitated by tannic acid. This material evidently contains relatively little radioactive hydroxyproline. The ratio of radioactivity incorporated into hydroxyproline to that incorporated into proline for the control is not far from the value of 0.8, which is typical of collagen of mammalian origin. On the other hand, this ratio is markedly reduced in experiments on scorbutic granuloma, suggesting forma-

TABLE III: Incorporation of Proline into the Microsomal Collagen of Normal and Scorbutic Granuloma.[a]

Tissue	Radioactivity (cpm) Proline	Hydroxyproline	Ratio Hydroxyproline:Proline
Normal	13,900	9,560	0.69
Scorbutic	9,100	1,350	0.15
% Reduction	35	86	

[a] Minced granuloma (7 g) from normal and scorbutic guinea pigs was incubated with 17 ml of Medium M containing L-[^{14}C]proline (55 mμmole; 1.1 × 10^7 cpm) for 2 hr at 37°. The microsome fractions were isolated and the collagen was isolated as the tannate; the collagen was hydrolyzed with 6 N HCl and the imino acids were isolated and counted as described in the text.

TABLE IV: Evidence for Formation of Iminoacyl Soluble RNA.[a]

Expt No.	Hydroxyproline[b] (cpm)	Proline[b] (cpm)
1	0	1100
2	0	1200
3	0	1100

[a] Expt 1: Granuloma mince (40 g) was incubated in 60 ml of medium M containing L-[^{14}C]proline (220 mμmoles; 4.4 × 10^7 cpm) for 35 min at 26°. The iminoacyl s-RNA was isolated as described in the text. Expt 2: Same as expt 1, except that puromycin dihydrochloride (7.4 × 10^{-5} M) was added to the incubation mixture. Expt 3: Granuloma mince (20 g) was incubated in 30 ml of 0.25 M sucrose containing 10^{-3} M chlortetracycline hydrochloride for 15 min; then L-[^{14}C]proline (850 mμmoles; 1.7 × 10^8 cpm) was added and the mixture was incubated for 90 min at 24°. The iminoacyl s-RNA was isolated as described in the text. [b] The isolated iminoacyl s-RNA was incubated at pH 10 for 1 hr at 37°; the radioactivity of the imino acids released was determined as described under Methods.

tion of a collagen-like peptide containing less than normal amounts of hydroxyproline.

When the microsome fraction of scorbutic granulomas was isolated (after incubation of the minced granuloma with [^{14}C]proline), and then treated with lithium borohydride as described above, significant quantities of prolinol were found. Thus, in expt 6 (Table II) considerably more radioactive prolinol was obtained than in the alkali-treated control; the percentage of radioactive proline converted to prolinol was of the same order of magnitude as observed in the experiments carried out with granulomas from nondeficient guinea pigs.

Attempts to Demonstrate Formation of Hydroxyprolyl s-RNA. In the course of these studies a number of attempts were made to detect hydroxyprolyl s-RNA. In the studies described above on the treatment of the microsome fraction with lithium borohydride, some free prolinol but no free hydroxyprolinol was found prior to acid hydrolysis. The data given in Table IV summarize additional efforts to demonstrate the formation of hydroxyprolyl s-RNA. In expt 1 and 2, granuloma minces were incubated with labeled proline and were then homogenized with an equal volume of phenol; the s-RNA was isolated as described by Rosenbaum and Brown (1961). After incubation of the isolated s-RNA fraction at pH 10 for 60 min, the proline and hydroxyproline released from s-RNA were determined and taken as a measure of the presence of the corresponding iminoacyl s-RNA derivative prior to hydrolysis. As indicated in Table IV, no evidence was obtained for hydroxyprolyl s-RNA, while substantial amounts of proline (presumably formed from prolyl s-RNA) were found. In expt 3, the minced granuloma was incubated in the presence of chlortetracycline. Subsequently, the mince was homogenized and centrifuged at 1000*g*. The supernatant solution was treated with an equal volume of phenol and the s-RNA was isolated as described by Moldave (1963). Evidence for the formation of prolyl s-RNA but not hydroxyprolyl s-RNA was obtained. In this experiment, a sample of the isolated s-RNA fraction was treated with lithium borohydride; no hydroxyprolinol was found, but the amount of prolinol formed was close to that found for free proline after hydrolysis at pH 10. In another experiment the isolated s-RNA fraction was treated with lithium borohydride and subsequently hydrolyzed with 6 N HCl; this treatment would be expected to yield hydroxyprolinol if an intermediate such as *N*-formylhydroxyprolyl s-RNA was present. No radioactive hydroxyprolinol was detected in this experiment.

Another approach is described in Table V; in these experiments, labeled proline, ATP, and s-RNA, and a soluble pH 5 precipitable fraction of granuloma (see Methods), were incubated; the s-RNA fraction was isolated after incubation. The s-RNA fraction was incubated at pH 10 for 60 min and the radioactivity of the proline and hydroxyproline released was determined as described by Peterkofsky and Prockop (1962). Again, although significant amounts of proline were found, no hydroxyproline was detected. Coronado *et al.* (1963) have reported that a similar system from chick embryo catalyzed the formation of hydroxyprolyl s-RNA from proline. We have attempted to repeat these experiments as described by Coronado *et al.* (1963) with chick embryo preparations. Apparently positive results were obtained in 6 of 10 experiments carried out; however, the formation of hydroxyproline was much less than reported and it was too small to permit adequate char-

TABLE V: Isolation of Free Iminoacyl s-RNA from a Cell-Free Granuloma System.[a]

Expt No.	Hydroxyproline[b] (cpm)	Proline[b] (cpm)
1	0	25,000
2	0	17,000
3	0	1,000

[a] The reaction mixtures contained L-[^{14}C]proline (270 mμmoles; 5.5×10^7 cpm), potassium cacodylate buffer (pH 7.5; 550 μmoles), ATP (110 μmoles), magnesium acetate (60 μmoles), reduced glutathione (75 μmoles), added granuloma soluble RNA (8.0 mg), and granuloma soluble pH 5 precipitable enzyme fraction (35 mg) in a final volume of 7.5 ml. Incubated at 37° for 1–2 hr. The iminoacyl s-RNA fraction was then isolated as described under Methods. [b] The isolated iminoacyl s-RNA was incubated at pH 10 for 1 hr at 37°; the radioactivity of the imino acids released was determined as described under Methods.

TABLE VI: Incorporation of Proline by a Cell-Free Granuloma Preparation.

Reaction Mixtures[a]	Incorp of [^{14}C]Proline[b] (cpm)
Complete system	2,200
Complete system (zero time)	120
Complete system − creatine phosphate	125
Complete system − Mg^{2+}	120
Complete system + ascorbic acid	2,250
Complete system + poly UC (1:1)	2,880
Complete system + poly UC (2:1)	3,570
Complete system + trypsin	155
Complete system + ribonuclease	125
Complete system (control)[c]	100
Complete system (incubated under N_2)	2,640

[a] The complete system contained Tris–HCl buffer (pH 7.4; 300 μmoles), ATP (12 μmoles), creatine phosphate (240 μmoles), $MgCl_2$ (90 μmoles), KCl (120 μmoles), L-[^{14}C]proline (17 mμmoles; 3.3×10^6 cpm), and cell-free preparation (110 mg of protein) in a final volume of 6 ml. Poly UC (1:1) (2.5 mg), poly UC (2:1) (1.4 mg), ascorbic acid (30 μmoles), crystalline trypsin (2 mg), and crystalline ribonuclease (1.6 mg) were added as indicated. The reaction was incubated at 37° under air for 1 hr; then trichloroacetic acid was added as described under Methods. [b] The radioactivity of the imino acids was determined as described by Peterkofsky and Prockop (1962). There was no detectable radioactivity in the hydroxyproline fraction in any of the experiments. [c] The enzyme preparation was heated at 100° for 5 min prior to the experiment.

acterization of this imino acid. Nevertheless, in all of these experiments substantial amounts of prolyl s-RNA were formed. When [^{14}C]prolyl s-RNA was incubated with a cell-free system from chick embryo as described by Peterkofsky and Udenfriend (1963), no [^{14}C]hydroxyprolyl s-RNA was found.

In our experience, the determination of hydroxyproline and proline by the method of Peterkofsky and Prockop (1962) gives valid values when relatively large amounts of radioactive hydroxyproline are present in the sample. However, we have occasionally observed an artifact with samples containing much more radioactive proline than hydroxyproline. In this procedure proline is oxidized and decarboxylated to Δ^1-pyrroline, and hydroxyproline is oxidized to pyrrole-2-carboxylic acid. Δ^1-Pyrroline is extracted into toluene and the radioactivity of this extract is taken as a measure of [^{14}C]proline. The aqueous layer is then heated to convert pyrrole-2-carboxylic acid to pyrrole, which is extracted into toluene and counted. We have found that with some samples a small amount of the proline radioactivity appears in the final toluene extract containing pyrrole. This might occur if a small amount of Δ^1-pyrroline-2-carboxylate remains after oxidation of proline; it would subsequently be decarboxylated on heating and therefore be extracted along with the pyrrole formed from hydroxyproline. Thus, incomplete decarboxylation of this type in the course of the assay might lead to the erroneous conclusion that hydroxyproline is present in the sample.

Incorporation of Proline by a Cell-Free Granuloma Preparation. The experiments described in Table VI were carried out with the cell-free preparation described under Methods. When this preparation was incubated with [^{14}C]proline, ATP, Mg^{2+}, and creatine phosphate, there was significant incorporation of proline into the cold trichloroacetic acid insoluble fraction. However, there was no detectable conversion of proline to hydroxyproline. The incorporation of proline was linear with time and stopped after 30–60 min under these conditions. Addition of ascorbic acid, fumarate, catalase, Fe^{2+}, or a mixture of the 20 common protein amino acids did not stimulate the incorporation of proline or the conversion of proline to hydroxyproline. Incorporation was markedly decreased when creatine phosphate or Mg^{2+} was omitted and when either trypsin or ribonuclease was added. Addition of the synthetic polyribonucleotide, poly U–C (2:1),[4] produced moderate stimulation of incorporation into proline. The cell-free preparation was obtained by homogenization of the granuloma in an atmosphere of nitrogen; when homogenization was done in air, only about 30% as much incorporation of proline was observed. Apparently the ability of the preparation to

[4] The polynucleotides, poly U–C (1:1) and poly U–C (2:1), were generously supplied by Dr. Severo Ochoa and Dr. Albert Wahba of New York University School of Medicine.

incorporate proline is reduced by reactions related to the presence of oxygen. It is of interest that when the incubation was carried out under nitrogen, incorporation was increased to about 20%. The presence of 2-mercaptoethanol (0.05 M) during homogenization led to cell-free preparations that were often twice as active as those prepared without addition of this mercaptan.

When the trichloroacetic acid insoluble material was extracted at 90° with 5% trichloroacetic acid as described under Methods, 75% of the radioactivity was solubilized; however, 80% of this material was dialyzable.[5] About 33% of the total [^{14}C] incorporated was released as free proline when the cold trichloroacetic acid insoluble material was suspended in water (adjusted to pH 10 with KOH) and incubated for 1 hr at 37°. An equivalent amount of [^{14}C] was found as free prolinol when the cold trichloroacetic acid insoluble material was treated with lithium borohydride suggesting that about one-third of the observed incorporation represents formation of prolyl s-RNA. Some of the product resembles collagen in that it is soluble in hot trichloroacetic acid; however, the bulk of the solubilized material is dialyzable and is not precipitated with tannic acid. The data, therefore, suggest that prolyl s-RNA and also peptides containing proline (but not hydroxyproline) are formed in the cell-free system.

A similar cell-free system was prepared from granuloma from ascorbic acid deficient guinea pigs. There was significant incorporation of proline into the cold trichloroacetic acid insoluble fraction; however, there was no detectable conversion of proline to hydroxyproline. Addition of ascorbic acid did not stimulate incorporation into proline or the conversion of proline to hydroxyproline.

Discussion

The question as to whether collagen hydroxyproline arises by hydroxylation of peptide-bound proline or whether proline is hydroxylated at a step prior to its incorporation into peptide linkage has been difficult to answer because of the complexity and lability of collagen-forming systems and the limitations of the available experimental procedures. A number of reports have appeared describing the identification of hydroxyprolyl s-RNA; if this compound is formed and if it is a true intermediate in collagen formation, then it would appear that hydroxylation takes place at or prior to the stage of iminoacyl s-RNA. Assuming this to be true, it would seem that the amino acid sequence of collagen is determined by m-RNA in the manner now generally accepted for the synthesis of other proteins. On the other hand, if proline is hydroxylated in peptide linkage it seems probable that there is a mechanism for the specific hydroxylation of certain prolyl residues of the newly formed collagen peptide chain.

The literature contains apparently conflicting information, and the problem cannot yet be unequivocally resolved. Nevertheless, the present findings seem to support certain conclusions. The observation that the formation of hydroxyproline continues in the presence of concentrations of puromycin that inhibit virtually all incorporation of proline into collagen proline and hydroxyproline suggests that hydroxylation of proline takes place prior to release of the collagen polypeptide from the ribosome.[6] The data indicate that in the presence of puromycin the percentage of hydroxyproline in the acid-soluble fraction is about twice that found in the absence of puromycin (Table II); almost all of the soluble hydroxyproline is present in bound forms rather than as the free imino acid. This result is consistent with the observations made on other protein synthesis systems which show that puromycin promotes the premature release of nascent peptide chains from the ribosome (see *e.g.*, Morris and Schweet, 1961; Allen and Zamecnik, 1962). The present findings are in accord with other data which indicate that puromycin inhibits incorporation of proline into collagen (Lukens, 1965; Juva and Prockop, 1964; Manner and Gould, 1963; Peterkofsky and Udenfriend, 1963). Manner and Gould (1963) found that the formation of free hydroxyproline in chick embryos was only moderately inhibited by puromycin; however, significant formation of free hydroxyproline was not observed in the presence or absence of puromycin under the conditions employed here with the granuloma system. Lukens (1965) also investigated the effect of puromycin in chick embryos; although radioactivity was associated with the hydroxyproline of the acid-soluble fraction in these experiments, its significance was considered to be questionable because of the presence of radioactive impurities.

The results of our experiments with chlortetracycline are in marked contrast to those with puromycin. Thus, chlortetracycline inhibited both the incorporation of proline into collagen and the hydroxylation of proline. Recent work indicates that chlortetracycline (and tetracycline) inhibits interaction between aminoacyl s-RNA and ribosomes, thus preventing formation of peptide bonds (Hierowski, 1965; Suarez and Nathans, 1965). The present findings, therefore, suggest that the hydroxylation reaction takes place on the ribosome. It is of interest that little tritiated water was formed from tritiated proline in the presence of chlortetracycline, a finding in agreement with previous data (Stone, 1962) indicating that there is little, if any, metabolism of proline unrelated to collagen formation in carrageenan granuloma.

It is now generally believed that the synthesis of proteins begins with attachment of the amino-terminal amino acid to the ribosome in the form of its aminoacyl

[5] Inflated diameter ($^{22}/_{32}$) Visking tubing (soaked in water at 4° for 3 hr prior to use) was employed.

[6] Although it is conceivable that hydroxylation occurs after the release of the peptide from the ribosome, the finding of hydroxyprolinol after the microsome fraction was treated with lithium borohydride indicates that hydroxylation takes place while the growing peptide chain is still attached to s-RNA on the ribosome. In addition, the finding that microsomal collagen contains about the same amount of labeled proline and hydroxyproline indicates that hydroxylation takes place before completion of the polypeptide chain and its release from the ribosome.

s-RNA. After subsequent attachment of the next aminoacyl s-RNA to the ribosome, its amino nitrogen atom attacks the activated carboxyl carbon atom of the amino-terminal amino acid releasing the amino-terminal s-RNA moiety; additional amino acid residues are added to the peptide chain by repetition of this step. Support for this mechanism, according to which peptidyl s-RNA is an intermediate in protein synthesis, has come from a number of approaches such as the studies cited above in which puromycin was employed, and also other experiments (see, *e.g.*, Takanami, 1962; Gilbert, 1963; Bretscher, 1963; Slapikoff *et al.*, 1963). Additional evidence has come from the studies of Soda *et al.* (1965) who treated polylysyl s-RNA (formed in a polyadenylic acid stimulated ribosome system from *Escherichia coli*) with lithium borohydride and obtained evidence for the formation of polylysyllysinol. As stated above the present observations are consistent with the view that the formation of hydroxyprolinol and prolinol under the conditions employed represents a measure of the corresponding carboxyl-terminal imino acids bound by ester linkage to s-RNA.

Earlier work in this laboratory (Stone and Meister, 1962) showed that the incorporation of proline into the collagen of ascorbic acid deficient granuloma was moderately reduced, while incorporation of proline into collagen hydroxyproline was markedly inhibited. This observation has been elucidated further here; thus, we found substantial incorporation of proline into the microsome fraction of scorbutic granuloma but relatively little incorporation into hydroxyproline. The protein associated with the scorbutic microsome fraction appears to resemble collagen in that it is soluble in hot trichloroacetic acid, and precipitable by tannic acid. It is not clear whether the peptides synthesized in the ascorbic acid deficient system are collagen analogs, *i.e.*, peptides in which hydroxyproline residues are replaced by proline residues, or whether the protein formed is a true precursor of collagen which fails to be hydroxylated in the absence of ascorbic acid. However, in either case the present finding suggests that the collagen isolated from scorbutic granuloma in previous studies (Stone and Meister, 1962; Meister *et al.*, 1964) may have contained a small amount of highly labeled proline-rich peptide, even though the ratio of total proline to total hydroxyproline was essentially normal.

In studies carried out under a variety of experimental conditions we have been unable to obtain definite evidence for hydroxyprolyl s-RNA, although prolyl s-RNA was invariably found. These negative findings contrast with those of several other groups, especially Urivetzky *et al.* (1965), whose findings on the conversion of prolyl s-RNA to hydroxyprolyl s-RNA in a chick embryo system appear to be convincing. We cannot exclude the possibility that we simply failed to achieve the experimental conditions necessary for isolation of hydroxyprolyl s-RNA; however, should this be true, it would appear that the properties of hydroxyprolyl s-RNA are considerably different from those of prolyl s-RNA and indeed a variety of other aminoacyl s-RNA compounds.

Although we feel that the available data are insufficient to unequivocally establish the site of hydroxylation, our present findings taken as a whole suggest that hydroxylation of proline requires transfer to the ribosome, and that hydroxylation occurs prior to release of the collagen peptide from its attachment to s-RNA on the ribosome. It appears that if a proline-containing peptide is the substrate for hydroxylation, it need not be very large since our data indicate that relatively small peptides containing hydroxyproline are synthesized in the presence of puromycin. Other studies and the present work show that when either oxygen or ascorbic acid is limiting, proline-rich polypeptides are synthesized; the studies of Gottlieb *et al.* (1965) suggest that such polypeptides can be hydroxylated. It has also been reported that a preparation from chick embryo can hydroxylate a synthetic polypeptide $(\text{pro-gly-pro})_n$ of mol wt 15,000 (Juva and Prockop, 1965). However, this type of hydroxylation may not represent an intermediate stage in the normal formation of collagen. The present results indicate that in the course of normal collagen synthesis the growing peptide chain can have hydroxyproline in the carboxyl-terminal position. It would seem to be of significance that treatment of the microsome fraction with lithium borohydride did not lead to formation of free hydroxyprolinol, although some free prolinol was detected. This suggests that ribosome-bound prolyl s-RNA is not a substrate for hydroxylation. Our results, therefore, favor the conclusion that peptidyl–prolyl s-RNA (in which the peptidyl moiety contains one or more amino acid residues) is a substrate for hydroxylation. The frequent occurrence of the -gly-pro-hypro– sequence in collagen (Schrohenloher *et al.*, 1959) is at least consistent with the possibility that the terminal proline residue of an X-gly-pro-pro-s-RNA sequence can be hydroxylated. Indeed the data support the conclusion that some of the carboxyl-terminal proline residues attached to s-RNA are hydroxylated under the experimental conditions used in this study. However the findings do not exclude the possibility that proline residues located elsewhere in the growing peptide chain can also be hydroxylated. If a peptide bound to s-RNA on the ribosome is the substrate for hydroxylation, it seems possible that the RNA template recognizes prolyl s-RNA both for proline residues that remain as such in completed collagen peptide chains as well as for proline residues that are to be converted to hydroxyproline residues. The positions of the hydroxyproline residues of collagen would then be established by the action of the hydroxylase system, the specificity of which might be determined by the amino acid sequence of a particular segment of the growing peptide chain. On the other hand, the possibility must also be considered that the s-RNA moiety itself functions to influence the specificity of the hydroxylase. There may be two types of prolyl s-RNA, only one of which participates in the action of the hydroxylase on the carboxyl-terminal proline residue attached to s-RNA. In conclusion, the present findings indicate that a ribosome-bound s-RNA-linked peptide is the substrate for hydroxylation; the factors that de-

termine the specificity of the hydroxylase require further study.

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