Site of Proline Hydroxylation during Collagen Synthesis in Mouse Fibroblasts*

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ABSTRACT: The established line of mouse fibroblasts, 3T6, was studied in saturated cultures to obtain information on the site of proline hydroxylation during collagen synthesis in these cells. Sucrose gradients prepared from cells labeled with [^{3}H]proline and a study of the distribution of radioactive proline and hydroxyproline in various cell fractions indicated that little or no hydroxylation takes place in the nascent collagen chains growing on the polysomes. Treatment of isolated cell fractions with the enzyme proline hydroxylase suggested that the intracellular pool of underhydroxylated collagenous material should be small. The results indicate that the proline hydroxylation takes place mainly with released completed collagen chains, and that hydroxylated collagen is rapidly secreted by the cells into the extracellular matrix.

Collagen contains two amino acids, hydroxyproline and hydroxylysine, found almost exclusively in this protein (Ramachandran and Gould, 1967–1968). For this reason, these amino acids can be used as natural labels in studies on the molecular aspects of collagen biosynthesis.

It has been known for many years that free hydroxyproline and hydroxylysine are not incorporated as such into the collagen molecule (Stetten, 1949; Green and Lowther, 1959; Sinex *et al.*, 1959). They are formed from proline and lysine, respectively, by enzymatic hydroxylation (Gould, 1968; Prockop and Kivirikko, 1968).

In recent years, the site of proline hydroxylation during collagen biosynthesis has been a subject for continued discussion. Several groups of investigators have claimed that they were able to isolate hydroxyprolyl- and hydroxylsyl-tRNA and concluded that proline and lysine are hydroxylated when present in the form of their respective aminoacyl-tRNAs (Coronado *et al.*, 1963; Manner and Gould, 1963; Jackson *et al.*, 1964; Urivetzky *et al.*, 1965). Such hydroxyprolyl-tRNA, however, was found not to serve as a source for collagen-hydroxyproline in cell-free systems, while prolyl-tRNA did (Urivetzky *et al.*, 1966). Also, other workers have obtained convincing evidence showing that the hydroxylation does not take place before the proline and lysine are present in a peptide-bound form (Peterkofsky and Udenfriend, 1963; Gottlieb *et al.*, 1966; Hutton and Udenfriend, 1966; Prockop *et al.*, 1966; Udenfriend, 1966).

The current disagreement therefore concerns the character of these peptide fractions. While some investigators claim that the hydroxylation of proline takes place when it is still present in nascent chains growing on the polysomes, other workers have presented evidence showing that this occurs mainly, if not exclusively, after release of the completed chains from the polysomes.

The hydroxylation of proline and lysine can be inhibited by the addition of α,α'-dipyridyl, a chelator for Fe^{2+} ions, which are an essential cofactor. Under these conditions collagen-like polypeptides which have a size similar to that of the α chains of collagen are released from the polysomes and accumulate intracellularly. These polypeptides become hydroxylated *in vivo* upon removal of the inhibitor and the addition of Fe^{2+}. When extracted from the cells they can be hydroxylated *in vitro* by means of partially purified preparations of the enzyme proline hydroxylase (protocollagen hydroxylase) (Hutton *et al.*, 1966, 1967; Juva and Prockop, 1966; Lukens, 1966; Chvapil *et al.*, 1967; Hausmann, 1967; Kivirikko and Prockop, 1967; Bhatnagar *et al.*, 1968; Goldberg and Green, 1968). This does not prove, however, that normal hydroxylation, in the absence of the inhibitor, occurs also with such a large free precursor molecule.

Results of sucrose gradient studies with chick embryo corium (Fernández-Madrid, 1967) and with mouse fibroblasts in tissue culture (Goldberg and Green, 1967) have been taken to indicate that the hydroxylation of proline takes place on growing nascent chains. On the other hand, Prockop and coworkers (Bhatnagar *et al.*, 1967a,b; Rosenbloom *et al.*, 1967) have concluded from kinetic studies of the incorporation of proline and its hydroxylation to hydroxyproline in whole embryonic chick bones *in vitro* that the hydroxylation occurs mainly, if not exclusively, with completed α chains released from the polysomes.

To obtain more information on this problem we designed experiments with 3T6 mouse fibroblasts in tissue culture and studied the distribution of radioactive proline and hydroxyproline in fractions of these cells during active collagen synthesis. Little hydroxyproline was found in the growing nascent chains present in these cells. Our findings indicate that the pool of nonhydroxylated collagen-like material in the cells must be small and that hydroxylated collagen is rapidly secreted into the extracellular matrix.

Materials and Methods

Fibroblasts. An established line of mouse fibroblasts, 3T6 cells, known for their capacity to rapidly synthesize collagen after reaching saturation density (Goldberg *et al.*, 1963), was obtained through the courtesy of Drs. Green

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The cells were grown in Roux flasks with Eagle's medium (Eagle, 1959), containing both serine and glycine at 40 mg/l. As soon as the cells became well attached to the glass wall and started to proliferate (after about 1 day), ascorbic acid was added at a concentration of 50 μg/ml. The medium was replaced after 3 days, when approximate saturation density had been reached and also 2 days later, a few hours before radioactive proline was added. To each flask 50 μCi of tritiated proline (generally labeled, 315 mCi/mmole, The Radiochemical Centre, Amersham, England) was added and the cells were incubated for an additional 15–17 hr. In some experiments the cells were incubated for only an additional 2 hr or pulse labeled with 100 μCi of tritiated proline for only 30 min (see Results).

Cell Extracts. At the end of the incubation period the radioactive medium was removed and the cells were washed twice with 10 ml of ice-cold PBS[1]/flask when still attached. The cells were then scraped off and washed once with 10 ml and twice with 5 ml of ice-cold PBS per amount of cells corresponding to the contents of one Roux flask. Each time the cells were collected by short low-speed centrifugation. The washed cells obtained in this way from 8 to 15 flasks were divided into 2 portions. Each portion was mixed with 1.5–2.25 ml of ice-cold TBS. The cells were broken by exposing them to ten strokes in a tight-fitting Dounce homogenizer. This procedure resulted in over 50% cell breakage and the cell nuclei remained intact. The ensuing homogenate was centrifuged in the cold at 15,000*g* for 10 min to separate the S_{15} sediment and supernatant. In several experiments an S_{15} supernatant was fractionated on a sucrose cushion according to the method of Bloemendal *et al.* (1967) to obtain information on the amount of microsome-bound ribosomes present in such supernatants. The results of these experiments indicated that at least 5% of the total RNA in the whole polysome fraction of such supernatants was present as microsome-bound RNA. In some of the experiments the S_{15} supernatant was centrifuged at 105,000*g* for 2 hr or at 137,000*g* for 75 min to prepare an S_{105} or S_{137} supernatant and a microsome pellet or a ribosome pellet (after prior treatment of the S_{15} supernatant with sodium deoxycholate for the ribosome pellet, see below). The microsome pellet prepared in this way consisted of a mixture of the free and microsome-bound ribosomes. The centrifugation at 137,000*g* was done to reduce the time required for preparing the various fractions.

Gradients. For gradient studies the S_{15} supernatant was mixed rapidly with one-ninth its volume of a 25% sodium deoxycholate solution in water to reach a final concentration of 2.5%. Aliquots of 1 ml of the mixture were placed immediately on a linear 15–50% sucrose gradient in TBS. The gradients were centrifuged in a Spinco SW 25.1 rotor at 25,000 rpm for 3 hr. At the end of the run 100 drop fractions were collected through the bottom of the tubes, while absorbance at 260 mμ was monitored continuously. Gradient pellets were collected after first carefully rinsing the tubes above the pellets with distilled water to remove adhering liquid from the top of the gradients and then extracting the pellets with hot 5% trichloroacetic acid (see below).

Analysis. In the early experiments all gradients fractions, the gradient pellet, the S_{15} sediment and the remaining S_{15} supernatant were extracted with 5% trichloroacetic acid at 90° for 60 min to solubilize collagenous material. The resulting extracts were centrifuged at low speed to remove the precipitates, dialyzed exhaustively against tap water to remove free labeled proline, brought to 6 M HCl with concentrated HCl, hydrolyzed overnight at 120° in an oil bath, and analyzed for radioactivity. In later experiments only aliquots of the S_{15} sediment and of the S_{15} supernatant and further the whole gradient pellet were extracted with hot trichloroacetic acid. Other aliquots of the S_{15} sediment and the S_{15} supernatant and all the other subcellular fractions were dialyzed directly against cold TBS (from which the sucrose was omitted, but containing 100 mg of proline/l.) without prior hot trichloroacetic acid extraction, hydrolyzed, and analyzed. To the first 9 or 10 gradient fractions, 1.0 mg of bovine serum albumin was added before dialysis to reduce the loss of radioactive protein in the subsequent steps.

The hydrolyzed samples were rapidly evaporated to dryness in an oil bath under a stream of air. The residues were extracted by shaking with 4 ml of distilled water and centrifuged to remove precipitate. Aliquots of 0.5 ml of the extracts were mixed with 13 ml of an ice-cold mixture of toluene (600 ml), Triton X-100 (144 ml), and scintillator fluid (57 ml of a solution of 15 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene). These aliquots served to obtain a measurement of total radioactivity. The remainder of the extracts was subjected to a specific analysis for radioactive proline and hydroxyproline according to the procedures of Peterkofsky and Prockop (1962), as modified by Juva and Prockop (1966). A Nuclear-Chicago liquid scintillation spectrometer, type Mark I with external ^{133}Ba standard, was used for radioactivity measurements. The values were corrected for quenching. Counting efficiency for total radioactivity and that for the oxidation products of proline and hydroxyproline was about 30%. Not more than 0.1–0.2% proline radioactivity appeared as a contaminant in the toluene extract of the hydroxyproline oxidation product.

In this analysis the recovery of the oxidation product of radioactive proline in biological material was found to be impredictable and no simple way to determine this is available. This has also been observed by Prockop and coworkers in subsequent work (personal communication). For this reason, the radioactivity in this oxidation product was measured not for quantitative purposes, but only to check whether a normal oxidation of both proline and hydroxyproline had occurred during the oxidation step. Total radioactivity and specific hydroxyproline radioactivity were used for quantitative evaluation. The recovery of radioactive hydroxyproline was determined, as recommended by Prockop and coworkers, by analysis of the pyrole formed by oxidation of the nonradioactive hydroxyproline added to the tubes before oxidation. Generally, this recovery varied between 55 and 75% for tritiated hydroxyproline. The recovery of the radioactivity of generally labeled [^{14}C]proline and [^{14}C]hydroxyproline added to unlabeled gelatin samples processed in this way amounted to 90–97% for the total

[1] Abbreviations used are: PBS, phosphate-buffered saline; TBS, 0.05 M Tris (pH 7.4)–0.025 M KCl–0.015 M MgOAc–0.25 M sucrose.

radioactivity and to 75–90% for the hydroxyproline radioactivity, when due allowance was made for the loss of carbon in the decarboxylation step. Since it is difficult to predict the amount of tritium lost during the oxidation for analysis from generally labeled tritiated proline and from hydroxyproline formed from this proline metabolically, no corrections were made for this loss. Such corrections were considered unimportant, because only comparative values were required for the purpose of these investigations. From the values for total radioactivity and radioactive hydroxyproline the ratio of the latter to the former, the hydroxylation ratio, was calculated. For pure mouse collagen this ratio is 45% (Ramachandran and Gould, 1967–1968). Since it was impractical to take measurements of counting rates much longer than 20 min, values of less than 40 dpm of [^{3}H]hydroproline radioactivity (about 10 cpm) were considered to be not significantly higher than background which was 25–35 cpm.

Thin-layer chromatography of a hydrolysate of the S_{15} supernatant showed that more than 99% of the total radioactivity was present as proline and hydroxyproline even after 15–17-hr incubation of the cells with radioactive proline.

Proline Hydroxylase and Hydroxylation in Vitro of Cell Fractions. Fraction II of proline hydroxylase was prepared from 12-days-old chick embryos according to the procedure of Kivirikko and Prockop (1967). Enzyme activity was determined and hydroxylation *in vitro* of the cell fractions was carried out under the conditions as specified by these authors. Nonhydroxylated collagen served as a control substrate in all *in vitro* hydroxylation experiments. It was prepared according to the method of Nordwig *et al.* (1967).

Results

Characterization of Synthesized Polypeptides. In preliminary experiments the various cell fractions were extracted with hot 5% trichloroacetic acid in order to prove that the radioactive hydroxyproline formed by the cells was present in collagenous material. The dialyzed extracts contained radioactive proline and hydroxyproline in nondialyzable material. After treatment of such extracts with collagenase, partially purified according to the method of Keller and Mandl (1963), from 75 to 90% of the nondialyzable hydroxyproline radioactivity present in the dialyzed trichloroacetic acid extracts became dialyzable. Since no detectable protease activity was found in this purified collagenase preparation with bovine serum albumin and casein as a substrate, it can be concluded that the material containing nondialyzable hydroxyproline present in the trichloroacetic acid extracts was collagenous in nature.

Distribution of [^{3}H]Proline and [^{3}H]Hydroxyproline in Cell Fractions. In the early experiments it was noted that after 15–17-hr labeling with [^{3}H]proline a trichloroacetic acid extract of the S_{15} sediment showed a much higher radioactivity ratio (ratio of radioactive hydroxyproline to total radioactivity) than similar extracts of the other cell fractions. The pellet of sodium deoxycholate treated ribosomes had the lowest ratio (see Table I). The S_{15} sediment contains labeled extracellular collagen, while in the ribosome pellet labeled nascent collagen chains are present. The S_{105} supernatant contains completed collagen chains released from the polysomes and it showed an intermediate ratio. These findings suggested to us that the polysomes synthesizing collagen may well bear nascent collagen chains which are largely nonhydroxylated and that collagen is secreted rapidly soon after its proline has become hydroxylated.

TABLE I: Ratio of Hydroxyproline to Total Radioactivity (Radioactivity Ratio) in Various Subcellular Fractions of 3T6 Mouse Fibroblasts Labeled with [^{3}H]Proline for 17 hr.[a]

Experiment	Radioactivity Ratio × 100	
	1	2
S_{15} sediment	6.6	6.4
S_{15} supernatant	3.9	0.79
S_{105} supernatant	2.0	1.3
Ribosome pellet	<0.2	0.24

[a] The S_{15} supernatant was prepared and treated with sodium deoxycholate. All analyses were performed on hot trichloroacetic acid extracts. See Materials and Methods for further details concerning the preparation of the fractions and analysis.

Next pulse-labeling experiments were done to obtain more detailed information on the distribution of nondialyzable radioactivity in different cell fractions of 3T6 cells. For this reason the fractions were dialyzed as such without prior extraction with hot trichloroacetic acid and then analyzed (see Materials and Methods).

Table II shows the results of two such experiments, it is clear that already after 30 min, the bulk of radioactive hydroxyproline present in nondialyzable peptides (collagen) was found in the S_{15} sediment. The microsomes (*i.e.*, the free polysomes and microsomes combined) and the buffer in which they were washed before analysis (TBS_1 and TBS_2) contained comparatively large amounts of proline radioactivity at that time. The S_{15} sediment had by far the highest radioactivity ratio. On the basis of its composition, pure mouse collagen should show a radioactivity ratio of 45% (Ramachandran and Gould, 1967–1968). Using this figure, the per cent of [^{3}H]proline present as collagen in the various cell fractions was calculated from the radioactivity ratios, under the assumption that all the collagenous material was hydroxylated. The values indicate (see Table II) that the collagen content is by far the highest in the S_{15} sediment. Since over 50% cell breakage was obtained and extracellular collagen is known to occur abundantly in saturated cultures of 3T6 cells (Goldberg *et al.*, 1963), the collagen in the S_{15} sediment represented mainly extracellular collagen. The results suggest therefore that collagen is rapidly secreted as soon as it is hydroxylated.

Table III shows an experiment in which microsomes were compared with sodium deoxycholate treated polysomes and ribosomes. An aliquot of the S_{15} supernatant was fractionated as such, another aliquot was first treated with sodium deoxycholate. The washed microsomes and their wash fluids contained a larger amount of total and hydroxyproline radioactivity than the sodium deoxycholate treated polysomes and ribosomes and their wash fluids. The hydroxylation ratio and the collagen content of the sodium deoxy-

TABLE II: Analysis of Total Hydroxyproline Radioactivity in Various Cell Fractions of 3T6 Mouse Fibroblasts Pulse Labeled with [^{3}H]Proline.[a]

	Experiment 1						Experiment 2					
	Total ^{3}H		[^{3}H]Hydroxyproline				Total ^{3}H		[^{3}H]Hydroxyproline			
Fraction	dpm	% of Total[b]	dpm	% of Total[b]	rr × 100[c]	Collagen %[d]	dpm	% of Total[b]	dpm	% of Total[b]	rr × 100[c]	Collagen %[d]
Microsomes	148,500	10.6	960	1.4	0.6	1.3	50,190	4.4	502	1.3	1.0	2.2
TBS_2	34,000	2.4	40	0.1	0.1	0.2	66,130	5.8	<40	<0.1	<0.1	<0.2
TBS_1	49,300	3.5	650	1.0	1.3	2.9	25,630	2.2	372	1.0	1.5	3.3
S_{137} supernatant	311,500	22.2	4,420	6.5	1.4	3.1	206,200	18.0	3,427	8.8	1.7	3.8
S_{15} sediment	861,000	61.3	61,400	91.0	7.1	15.8	796,800	69.6	34,480	88.9	4.3	9.6
Whole cells[e]	1,404,000	100	67,470	100	4.8	10.7	1,145,000	100	38,820	100	3.4	8.4

[a] Pulse labeling was for 30 min with 100 μCi of [^{3}H]proline/Roux flask. In two different experiments the labeled cells were washed, broken, and homogenized as described in Materials and Methods. No sodium deoxycholate was added. The homogenate was centrifuged at 15,000*g* for 10 min to obtain the S_{15} sediment and S_{15} supernatant. The latter was centrifuged at 137,000*g* for 75 min. The microsomal pellet was washed twice with TBS (TBS_1 and TBS_2). The microsomal pellet is a mixture of free and membrane-bound polysomes and ribosomes. There was no extraction with hot trichloroacetic acid. For further details on the analysis, see Materials and Methods. [b] Per cent of total ^{3}H or of [^{3}H]hydroxyproline present in all the fractions combined which occurs in each separate fraction. [c] rr = hydroxylation ratio = [^{3}H]hydroxyproline: total ^{3}H. [d] These values were calculated from the radioactivity ratio found and this ratio for pure mouse collagen: 45% (see text). The values are given as per cent of [^{3}H]proline present as collagen in nondialyzable peptides. [e] Values for all fractions combined.

TABLE III: Analysis of Total and Hydroxyproline Radioactivity in Various Cell Fractions of Pulse-Labeled 3T6 Cells. Comparison between the Polysome and Ribosome Fraction and the Microsomes.[a]

	I[c]						II[c]					
	Total ^{3}H		[^{3}H]Hydroxyproline				Total ^{3}H		[^{3}H]Hydroxyproline			
Fraction	dpm	% of Total[b]	dpm	% of Total[b]	rr × 100[e]	Collagen %[e]	dpm	% of Total[b]	dpm	% of Total[b]	rr × 100[e]	Collagen %[e]
Pellet[c]	13,460	1.5	126	0.3	0.9	2.0	32,980	3.5	748	1.8	2.3	5.1
TBS_2	2,300	0.3	40	0.1	0.1	0.2	14,800	1.6	190	0.5	1.3	2.9
TBS_1	6,600	0.7	81	0.2	1.2	2.7	45,100	4.9	825	2.0	1.8	4.0
S_{137} supernatant	270,000	29.5	4,810	11.8	1.8	4.0	209,000	22.6	3,270	8.0	1.6	3.6
S_{15} sediment[d]	624,000	68.0	35,770	87.7	5.7	12.6	624,000	67.4	35,770	87.7	5.7	12.6
Whole cells[e]	916,400	100	40,830	100	4.6	9.8	925,900	100	40,790	100	4.4	9.8

[a] The cells were pulse labeled for 30 min with 100 μCi of [^{3}H]proline/flask, washed, broken, and homogenized as described in Materials and Methods. The S_{15} supernatant was divided into two equal parts. To one part sodium deoxycholate was added to a final concentration of 2.5%. Both samples of the S_{15} supernatant were then centrifuged at 137,000*g* for 75 min. Both the microsome pellet and the total polysome and ribosome pellet were washed twice with TBS (TBS_1 and TBS_2). There was no extraction with hot Cl_3CCOOH. For further details, see Materials and Methods. [b] See Table II, footnote *b*. [c] In series I the pellet consisted of washed polysomes and ribosomes, in series II of washed microsomes (see Table II, footnote *a*). [d] All fractions obtained from the sodium deoxycholate treated and untreated S_{15} supernatants were analyzed separately. The analytical values for the S_{15} sediment were common to both of these supernatants and their fractions. [e] See Table II, footnotes *c–e*.

cholate treated polysomes and ribosomes was lower than that of the microsomes.

Since the radioactive hydroxyproline found in the pulse-labeled cultures represent mainly extracellular hydroxylated collagen present in the S_{15} sediment (see above), the results indicate that from 8 to 10% of the [^{3}H]proline incorporated under these conditions was present as collagen in these cultures. The calculated collagen content for fractions other than the S_{15} sediment may be lower than the actual content, because of the presence of nonhydroxylated collagenous material in these fractions. The actual values could also be low in these fractions because of the presence of newly synthesized noncollagenous proteins (free of hydroxyproline) and which are not secreted by the cells. Thus, although the results shown in Tables II and III suggest that proline hydroxylation occurs at a late state of collagen synthesis they do not give information on the exact site of the hydroxylation, *i.e.*, whether it occurs on the nascent chains or after release.

Sucrose Gradients. In order to determine whether significant hydroxylation of proline takes place in the nascent collagen chains growing on the polysomes sucrose gradients were prepared from labeled cells. The conditions chosen minimized both the amount of material pelleted during centrifugation of the gradients and the association between free collagen and collagenous nascent chains (see for this Discussion). Labeling of the cells was for 17 hr to ensure homogeneous labeling of the intracellular pools.

All together more than 50 gradients were prepared. In all gradients total radioactivity (largely [^{3}H]proline) occurred throughout the gradients, while [^{3}H]hydroxyproline was present mainly in the top layer. The gradient pellet contained only a small amount of [^{3}H]hydroxyproline. In the bottom region of the gradients, corresponding to the heavier polysomes, radioactive hydroxyproline was found in slightly larger amounts than in the region of the lighter polysomes. The radioactivity ratio was always significantly higher in the bottom region than in that of the lighter polysomes and was above the background ratio of 0.1–0.2% to be expected from the analytical methods used (see Materials and Methods). The size and position of these "peaks" of [^{3}H]hydroxyproline were not very reproducible, as is shown clearly by the results of two experiments in which duplicate sucrose gradients were run simultaneously with one and the same S_{15} supernatant (Figures 1 and 2). In the first experiment the S_{15} sediment, the S_{15} supernatant and the two gradient pellets showed a radioactivity ratio ([^{3}H]hydroxyproline: total ^{3}H) of 1.76, 0.76, 0.47 (pellet of gradient 1), and 3.3 (pellet of gradient 2, probably contaminated with a small particle of the S_{15} sediment). In the second experiment these figures were 2.53, 0.24, 0.49, and 0.10. The addition of polyvinyl sulfate at 2 μg/ml or of bentonite at 1 mg/ml to the buffer during breakage and fractionation and to the sucrose gradients to diminish the possible effect of RNase did not affect the distribution of ultraviolet-absorbing material and of radioactivity. The results of our gradient studies indicate that the bulk of the hydroxylated collagen was present as free collagen in the top layer of the gradients, while only very small amounts of hydroxylated collagenous material occurred on the polysomes and in the gradient pellets.

Since it has been claimed that radioactive hydroxyproline can be found in the nascent chains in sucrose gradients (Fernández-Madrid, 1967; Goldberg and Green, 1967)

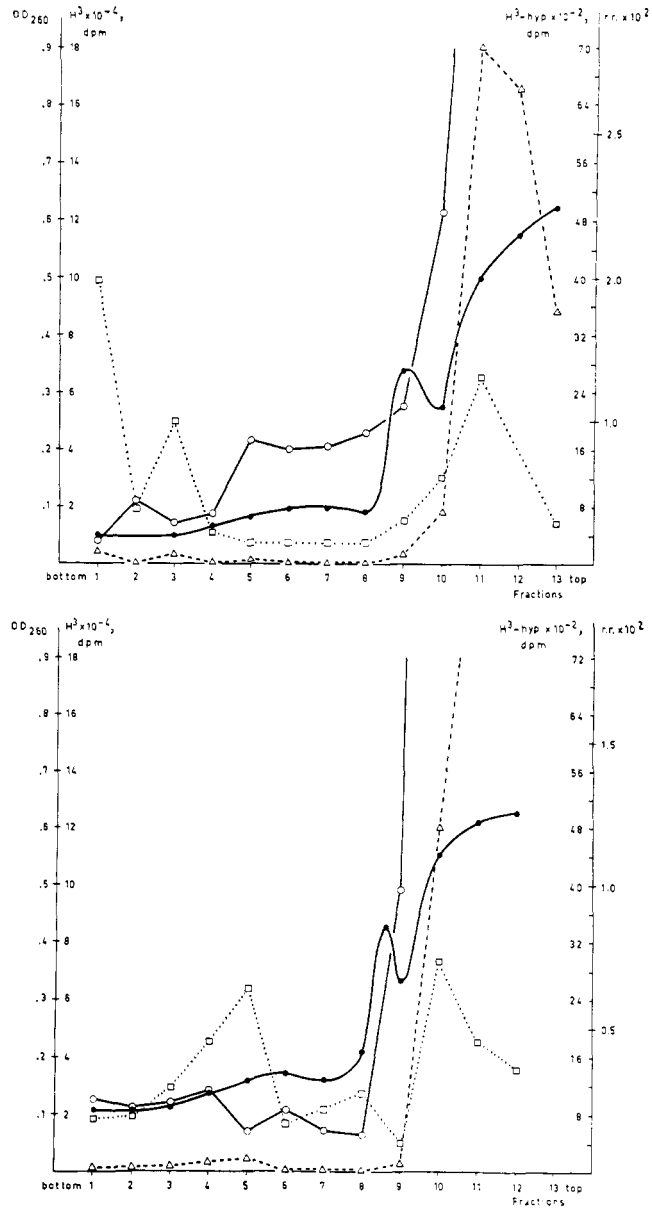

FIGURE 1: Duplicate sucrose gradients (15–50% in TBS) of one single S_{15} supernatant treated with sodium deoxycholate and prepared from 3T6 mouse fibroblasts labeled with [^{3}H]proline for 17 hr. Centrifugation: 3 hr at 25,000 rpm. Fractions: 100 drops. For further experimental details, see text. (●——●) OD_{260}, (○——○) total nondialyzable radioactivity, (Δ- - - -Δ) nondialyzable [^{3}H]hydroxyproline, and (□·····□) rr = radioactivity ratio = [^{3}H]hydroxyproline: total ^{3}H. The values for total radioactivity (^{3}H × 10^{-4}) in the gradient top fractions of both gradients and for hydroxyproline radioactivity ([^{3}H]hydroxyproline × 10^{-2}) of the bottom gradient were too high to be shown in the figure. These values were:

Fraction	Top Gradient ^{3}H × 10^{-4} (dpm)	Bottom Gradient ^{3}H × 10^{-4} (dpm)	Bottom Gradient [^{3}H]-Hydroxyproline × 10^{-2} (dpm)
10	See figure	65.8	See figure
11	53.8	214.0	97.3
12	192.2	266.3	93.0
13	204.9		

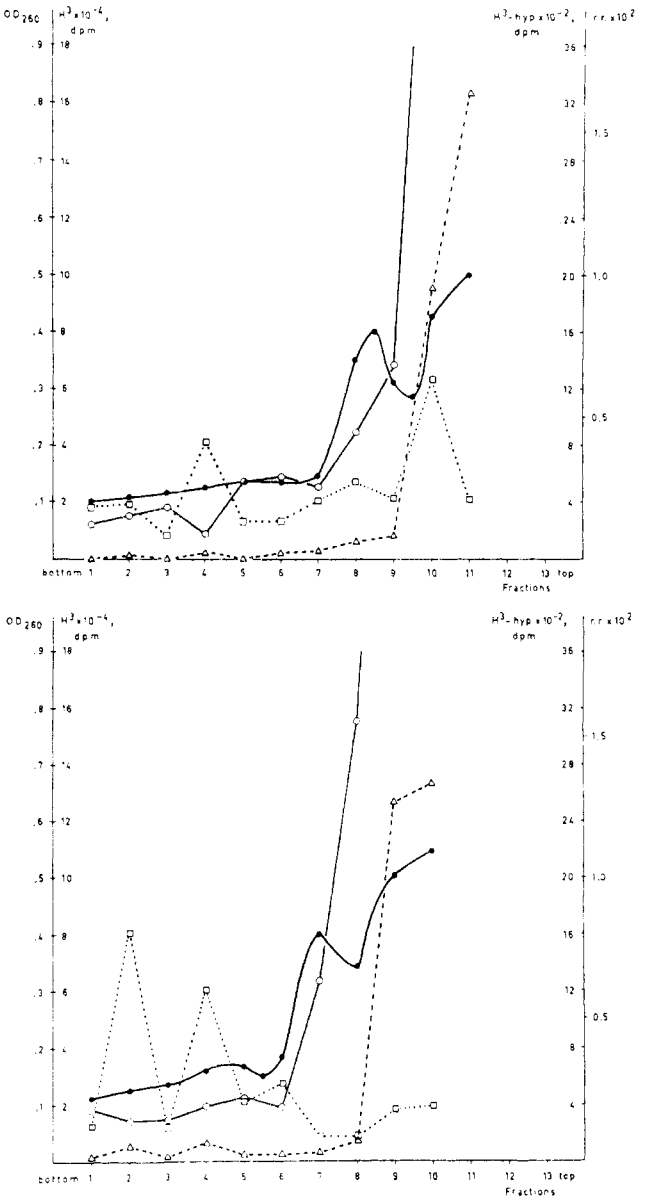

FIGURE 2: Similar duplicate sucrose gradients as shown in Figure 1. For further experimental details, see text. The radioactivity values too high to be shown in the figure were

Fraction	Top Gradient $^3H \times 10^{-4}$ (dpm)	Bottom Gradient $^3H \times 10^{-4}$ (dpm)
9	See figure	136.8
10	30.4	142.0
11	152.0	

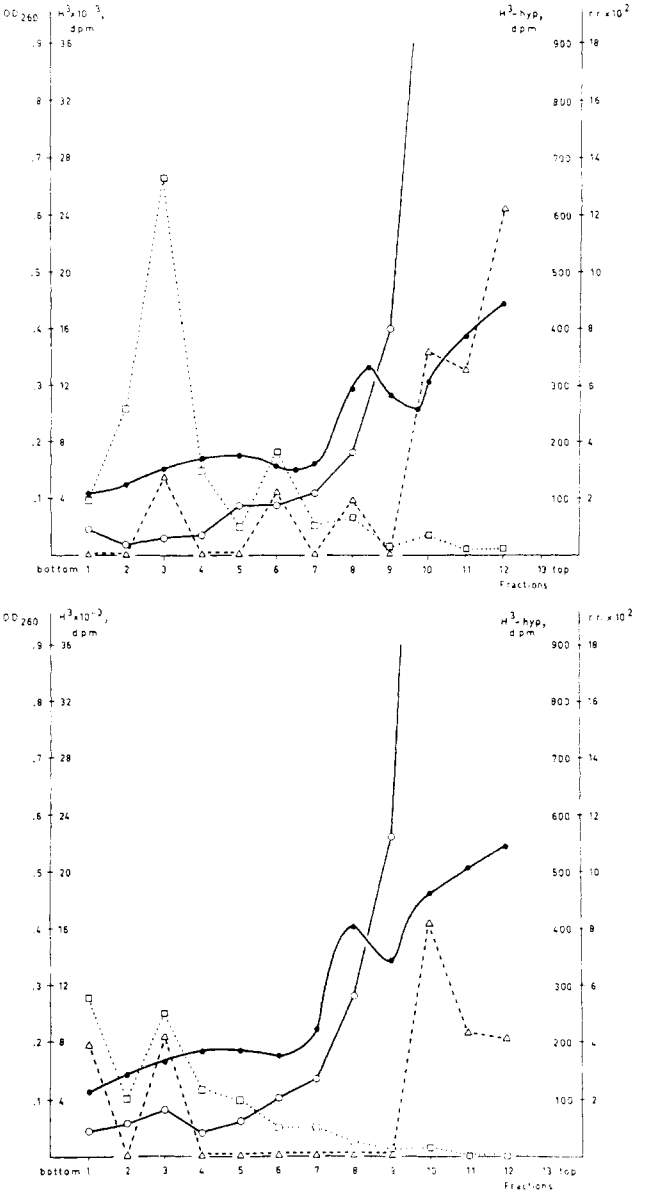

FIGURE 3: Similar duplicate sucrose gradients as shown in Figures 1 and 2. In this experiment unlabeled cells were broken in prelabeled S_{105} supernatant. For further experimental details, see text. The radioactivity values too high to be shown in the figure were

Fraction	Top Gradient $^3H \times 10^{-3}$ (dpm)	Bottom Gradient $^3H \times 10^{-3}$ (dpm)
10	52.5	76.1
11	253.0	254.0
12	574.0	426.9

and that collagen is synthesized on very heavy polysomes (Kretsinger *et al.*, 1964; Manner *et al.*, 1967), we considered it necessary to prove that the small amount of [^{3}H]hydroxyproline occurring in the bottom regions of our gradients was not present in growing nascent collagen chains.

To this end unlabeled 3T6 cells were broken in a prelabeled S_{105} supernatant obtained from labeled cells. The [^{3}H]hydroxyproline in such an S_{105} supernatant is present in completed collagen chains released from the polysomes. Duplicate sucrose gradients were prepared from the ensuing S_{15} supernatant. The results (see Figure 3) show that the collagen was sedimenting with the unlabeled polysomes and tended to accumulate in the bottom region of the gradients (note in particular the pattern of the radioactivity ratio). We may conclude therefore that the small amounts of [^{3}H]hydroxyproline present in the bottom region of the gradients prepared from labeled cells are likely to be due rather to the association of completed and hydroxylated collagen released from the polysomes with the largely nonhydroxylated nascent

collagen chains growing on the polysomes than to the presence of very heavy polysomes on which collagen is synthesized and hydroxylated.

Pool Size of Nonhydroxylated Collagen. Having shown that the hydroxylation of proline in 3T6 cells takes place in the ultimate stages of collagen synthesis and that it occurs hardly at all in the nascent chains growing on the polysomes we tried to obtain direct evidence for the presence of nonhydroxylated collagenous material in the microsome-bound polysomes or in other cell fractions. In five experiments attempts were made to demonstrate such material in the microsomes.

In the first experiment the microsomes, washed twice with TBS, were dialyzed against 0.05 M Tris (pH 7.5) and 1 mM EDTA or against this buffer and 0.1 mM MgOAc. The supernatant obtained after centrifugation of the dialyzed microsomes was divided into two parts. One part was subsequently incubated at pH 10 and 37° for 60 min to hydrolyze released peptidyl-tRNA.

In the second experiment a washed microsome pellet was extracted with 0.5 M acetic acid. Another such pellet was extracted first with 0.15 M NaCl and then with 0.5 M acetic acid. This procedure will yield extracted collagenous material.

In a third experiment a washed microsome pellet was incubated with puromycine under the conditions of Ibuki and Moldave (1968) to release the nascent chains. A supernatant was prepared by centrifugation. The pellet was extracted with 0.5 M acetic acid to dissolve collagen.

In a fourth and fifth experiment a microsomal pellet was extracted with 7 M urea in a boiling-water bath for 20 min to completely denature ribosomal material.

Although in all of the extracts, supernatants, and wash fluids peptide-bound radioactive proline was present in measurable amounts, the hydroxylation ratio was below background. No measurable increase in this ratio was obtained upon exposure to partially purified proline hydroxylase under conditions where the standard proline hydroxylase substrate was readily hydroxylated by the enzyme. The hydroxylation ratio of the residual pellets after extraction was also below background. The results of these experiments indicated that certainly not more than at most 5% of the nondialyzable radioactive proline present in the combined microsome and ribosome pellet were present as underhydroxylated collagenous material.

These experiments were done with material obtained from 15 Roux flasks. Calculations based on the level of total radioactivity present in our extracts showed that it would require at least 5 or 6 times as many or from 80 to 100 Roux flasks per experiment to enable a possibly significant measurement of an increase in the hydroxylation ratio of microsome extracts upon exposure to proline hydroxylase. It seemed impractical to do such a large-scale experiment which still might have yielded results of only doubtful significance. It appeared more useful to try and demonstrate the presence of underhydroxylated released collagen in the S_{15} sediment or the S_{137} supernatant, since it seemed reasonable to assume that some of such material might well excape from the microsomal vesicles during cell breakage. Therefore, in a final hydroxylation experiment the S_{15} sediment was extracted twice with 0.14 M NaCl. The combined extracts and the S_{137} supernatant were dialyzed exhaustively against tap water and the resulting precipitates were extracted with 0.14 M NaCl. Thus, the preparation of proline hydroxylase substrate from chick embryo minces in which proline hydroxylase is inhibited with α,α'-dipyridyl, according to the procedure of Nordwig *et al.* (1967), was followed closely with both the S_{15} sediment and the S_{137} supernatant of uninhibited 3T6 cells. The resulting extracts were treated with proline hydroxylase.

As shown in Table IV there was a rise in the hydroxylation ratio upon purification of the collagenous material. No increase in hydroxylation was found upon exposure of the fractions to proline hydroxylase. The fraction of greatest interest is sup_2S_{137}, since the highest proportion of collagenous material is expected to occur in this fraction, of all S_{137} supernatant fractions. This is apparent from its hydroxylation ratio. On the basis of the hydroxylation ratio of 4.3% in this fraction about 10% of the total radioactivity should have been present in hydroxylated collagenous material. The sensitivity of our analysis (about 40 dpm of [^{3}H]hydroxyproline) would have allowed us to measure an increase of not less than about 1.6% in this ratio upon exposure to proline hydroxylase. This means that we could have detected not less than about 3.5% of the total radioactivity as being present in underhydroxylated collagenous material in this fraction. The absence of a significant increase in the hydroxylation ratio of this fraction upon exposure to proline hydroxylase indicates therefore that not more than about 3.5% of the total radioactivity in this fraction consisted of underhydroxylated collagen. For the whole S_{137} supernatant it must have been considerably smaller than that.

Discussion

The following conclusions can be drawn from our studies.

Electron microscopy and chemical analysis (Goldberg *et al.*, 1963; Green and Goldberg, 1964) have shown that the collagen synthesized by 3T6 cells is secreted by these cells and accumulates in a fibrous form in the extracellular matrix present between them. Part of the secreted collagen leaks out of the matrix into the growth medium. We found that about 90% peptide-bound radioactive hydroxyproline was present in the S_{15} sediment after pulse labeling with [^{3}H]proline for 30 min. As some of the synthesized and secreted collagen will have escaped into the growth medium by that time and part of it will have been lost from the extracellular matrix during washing of the cells, this value for the S_{15} sediment is a minimum value. We calculated that 8–10% [^{3}H]proline incorporated into nondialyzable peptides was present in collagen in whole pulse-labeled cells (including intracellular and extracellular material). For the same reasons this is also a minimum value.

Since the S_{15} sediment represents largely extracellular collagen the results of our pulse-label experiments indicate that collagen is rapidly secreted by the 3T6 cells as soon as it is hydroxylated. Prockop and coworkers (Juva *et al.*, 1966) have found by means of autoradiography that upon release of inhibition of the hydroxylation in embryonic chick bones the intracellularly accumulated nonhydroxylated collagenous material is rapidly hydroxylated and secreted by the cells. This suggests that hydroxylated collagen is secreted rapidly also by the cells in chick bones in the absence of inhibition. Our results (Table III) suggest further that the hydroxylation of proline in 3T6 cells may take place in the microsomes,

TABLE IV: Hydroxylation *in Vivo* and *in Vitro* of Collagenous Fractions Prepared from the S_{15} Sediment and the S_{137} Supernatant from Pulse-Labeled 3T6 Cells According to the Procedure of Nordwig *et al.* (1967) for the Preparation of Proline Hydroxylase Substrate.[a]

Fractions	Hydroxylation *in Vitro*[b]	Total ^{3}H (dpm)	[^{3}H]Hydroxyproline (dpm)	rr × 100[c]
Whole S_{137}	−(2)	18,420	306	1.7
Sup_1 S_{137}[d]	−(2)	28,830	80	0.28
	+(2)	15,730	63	0.40
Sup_2 S_{137}[e]	−(2)	3,884	167	4.3
	+(2)	2,580	86	3.3
P–S_{137}[f]	−(2)	27,220	549	2.0
Sup_1 S_{15} sediment[g]	−(2)	52,890	271	0.51
	+(2)	11,940	48	0.40
Sup_2 S_{15} sediment[h]	−(2)	4,936	173	3.5
	+(2)	6,634	178	2.7
P_1–S_{15} sediment[i]				6.5
P_2–S_{15} sediment[j]	−(2)	31,330	1171	3.6
Hydroxylase substrate	−(2)	18,750	<40	<0.2
	+(2)	11,550	854	7.3

[a] The cells present in 14 Roux flasks were pulse labeled for 30 min with 100 μCi of [^{3}H]proline/flask. The labeled cells were washed, broken, homogenized, and fractionated as described in Tables II and III. An aliquot of the S_{137} supernatant was dialyzed separately and analyzed together with the precipitate which had formed in the bag during dialysis (whole S_{137}). The remainder of the S_{137} supernatant and the S_{15} sediment were fractionated as described below. Aliquots of all the extracts were analyzed as such and after exposure to proline hydroxylase. [b] − without hydroxylase, + with added hydroxylase. The number of parentheses gives the number of tubes used in each analysis or hydroxylation *in vitro*. Each value given is an average. When no number is given, only one sample was analyzed. (See Materials and Methods for details on the hydroxylation procedure and analysis.) [c] rr = hydroxylation ratio = [^{3}H]hydroxyproline:total ^{3}H. [d] Supernatant after dialysis against tap water and centrifugation of whole S_{137} supernatant. [e] 0.14 M NaCl extract from the pellet obtained after dialysis *ad d*. [f] Extracted pellet *ad e*. [g] Supernatant after dialysis and centrifugation of a 0.14 M NaCl extract. [h] 0.14 M NaCl extract from the pellet *ad g*. [i] Extracted pellet *ad h*. [j] Extracted pellet *ad i*.

as has been found to be the case in other systems (for a review, see Gould, 1968).

Our gradient studies have shown that little radioactive hydroxyproline is found in the nascent collagen chains growing on the polysomes. It appears therefore that proline hydroxylation occurs in the ultimate stages of collagen synthesis, *i.e.*, mainly after release of the completed chain from the polysomes. This is in keeping with the results of Prockop and coworkers (Bhatnagar *et al.*, 1967a,b; Rosenbloom *et al.*, 1967) who studied the kinetics of proline hydroxylation in whole embryonic chick bones *in vitro*.

The intracellular pool of nonhydroxylated collagenous material was found to be small. In pulse-labeled cells this pool must have been smaller than 5% of the proline incorporated into the nondialyzable peptides in the microsomes and free polysomes. This value of 5% represents a maximum value. The actual pool size may even be smaller than that. Nonhydroxylated collagen represented less than 3.5% of the nondialyzable labeled peptides present in partially purified collagen prepared from the S_{137} supernatant of pulse-labeled cells. For the whole S_{137} supernatant the pool of such material should have been far smaller than that. A sizeable amount of nonhydroxylated material can be formed in 3T6 cells, however, when the hydroxylation of proline is inhibited with α,α′-dipyridyl and such material can be extracted from the cells and be hydroxylated *in vitro* with proline hydroxylase (Goldberg and Green, 1968).

Our observations on the site of proline hydroxylation are at variance with those of Goldberg and Green (1967) who used a similar system of 3T6 mouse fibroblasts. These investigators employed purified bacterial collagenase (Worthington) to demonstrate collagenous material in nondialyzable polypeptides present in the polysome fractions and which became cold trichloroacetic acid extractable after digestion with the enzyme. In some experiments we have found that a preparation of this enzyme digests tryptophan-containing nascent chains present in these cells to a considerable extent, although such chains are not collagenous, since collagen is free of tryptophan. It is known that even the purified Worthington collagenase contains detectable amounts of proteases and peptidases (Strauch and Graszmann, 1966). Therefore it seems dangerous to use susceptibility to this enzyme as a measure for the content of collagenous material in nascent chains. The experiments in which labeled hydroxyproline

occurring in the polysomes was determined (Goldberg and Green, 1967; Table I) showed that after labeling for 70 min the cold trichloroacetic acid soluble material obtained after treatment with collagenase from heavy polysomes was 30% hydroxylated. The light and medium polysomes yielded material with 15% hydroxylation. After 15-min labeling the hydroxylation was 15% for the whole polysome region and after 3-min labeling it was 5%. This decreasing degree of hydroxylation with decreasing labeling time is unexpected if the hydroxylation of proline should occur in the nascent chains, since the translation time for an α chain of collagen has been estimated to be of the order of 2 min only (Rosenbloom *et al.*, 1967). It seems possible that the extracted material may well have represented fully hydroxylated completed collagen released from the polysomes which had become associated with nonhydroxylated chains growing on the polysomes. Such material is digested by collagenase and is then cold trichloroacetic acid extractable. The problem of association and aggregation in the study of collagen synthesis in subcellular fractions has also been pointed out by Malt and Speakman (1965), Speakman (1968), and Prockop and coworkers (Rosenbloom *et al.*, 1967; Kivirikko and Prockop, 1967). Our gradient studies have shown that such association is difficult to avoid, but it was not disturbing under our experimental conditions. Under the conditions of Goldberg and Green it may become quite a problem. They added 50–100 μCi of labeled proline to 50-mm petri dishes in only about 1 ml of medium. This large concentration of labeled proline in contact with far fewer cells than in our case (50 or 100 μCi of [^{3}H]proline in 50 ml/Roux flask) and the isolation of material digested with collagenase will greatly exaggerate the effect of said association because of the presence of extremely highly labeled released collagen susceptible to collagenase. We suggest that the decreasing degree of proline hydroxylation with decreasing labeling times, as found by Goldberg and Green, may well be due to such association. With shorter labeling times smaller amounts of less highly labeled hydroxylated collagen has been released from the polysomes and that should reduce both the effect of the association and the degree of hydroxylation found in the nascent chains under their conditions.

Other reports in the literature have dealt with the site of proline hydroxylation and the size of the polysomes synthesizing collagen. The groups of Rich and Gould (Kretsinger *et al.*, 1964; Manner *et al.*, 1967) used homogenates of pulse-labeled whole chicken embryos injected with radioactive proline. In their buffer system at low ionic strength they found hardly any radioactive hydroxyproline throughout the gradient (15–30% sucrose). Only the pellet and the top layer showed significant amounts of hydroxyproline radioactivity. They observed rapidly sedimenting large collagen-containing (collagenase susceptible, radioactive hydroxyproline containing) polysome complexes which were collected in the gradient pellet in about 1 hr. They concluded that these findings indicated the presence of very large polysomes containing mRNA coding for polypeptides having a length corresponding at least to that of the single α chains. These polysomes were thought to be aggregated due to aggregation of the nascent chains. Fernández-Madrid (1967) also used this low ionic strength buffer in sucrose gradient studies of chick embryo corium pulse labeled *in vitro* with radioactive proline. He found hydroxyproline radioactivity throughout the 15–60% sucrose gradients used by him, but particularly in the heaviest fractions. Nonradioactive hydroxyproline was similarly distributed. The material in the heaviest fractions of these 15–60% sucrose gradients is probably comparable with that in the pelletable material present in the 15–30% sucrose gradients of Rich, Gould, and coworkers.

The buffer used in these studies was of low ionic strength and contained 0.01 M Tris (pH 7.4), 0.01 M KCl, and 0.0015 M $MgCl_2$. In this buffer triple-chained tropocollagen is insoluble. The buffer of Goldberg and Green (1967) was also used in our studies. It consisted of 0.05 M Tris (pH 7.4), 0.025 M KCl, 0.015 M $MgCl_2$, and 0.25 M sucrose. We have found that because of its higher ionic strength it was adequate in keeping in solution small amounts of acid-soluble collagen from rattail tendons for at least 4 hr, the minimum time required for preparing, running and fractionating our gradients. In this system relatively little radioactive hydroxyproline was found in the gradient pellets. The aggregates found by Rich, Gould, and coworkers and the results of Fernández-Madrid have little physiological meaning, because the radioactive and unlabeled hydroxyproline found in pellets and gradient fractions in this buffer system may have consisted of aggregates of completed α chains released from the ribosomes and triple-chained collagen molecules with the nascent chains on the polysomes. In preliminary experiments with chicken embryo homogenates we obtained results similar to those of Rich, Gould, and coworkers when using their buffer.

Manning and Meister (1966) have presented evidence suggesting that some hydroxyproline may be present at the carboxyl end of the growing nascent collagen chains. They showed the presence of this amino acid in ester linkage when reducing the microsomal fraction of carrageenin granuloma minces with lithium borohydride. It was not proved unequivocally however, that this hydroxyproline was bound in ester linkage to the CCA end of tRNA. Moreover, it seems unlikely that proline hydroxylase should have access to proline residues still bound in ester linkage to the CCA end of tRNA, while it is known that at least the first 30 residues from the COOH-terminal end of a nascent chain growing on the ribosomes are protected from proteolytic action (Malkin and Rich, 1967).

Recently, Bekhor and Bavetta (1967) obtained small hydroxyproline-containing peptides (with mol wt <10,000) which were isolated from embryonic rabbit skin synthesizing collagen *in vitro*. This suggested to them that proline hydroxylation occurs on the growing nascent chains. These investigators did not show, however, that these peptides were nascent chains originally bound to the ribosomes. In view of the rather elaborate and drastic methods of isolation used these peptides may rather represent breakdown products of completed and released chains. In any case, whole collagen chains were not subjected to this procedure as a control to show that breakdown does not occur under the conditions of the peptide isolation.

After evaluating our own results and the relevant literature we suggest that the following sequence of events takes place during collagen biosynthesis. Nonhydroxylated or largely nonhydroxylated α chains of collagen are synthesized on polysomes bound to the endoplasmatic reticulum. After completion and release from the polysomes they remain

associated with the microsomal membranes in which the enzymes which hydroxylate proline and lysine and other modifying enzymes seem to be localized. Specific proline and lysine residues are hydroxylated on the completed chains. Further modifications of the molecule, such as the linking of one galactose and one glucose residue to the hydroxyl group of hydroxylysine (Rosenbloom *et al.*, 1968; Bosmann and Eylar, 1968) and aldehyde formation from lysine residues, which is needed for the subsequent extracellular formation of certain cross-links (Kang *et al.*, 1967), will also take place after release. The collagen molecules are rapidly secreted by the cell into the extracellular matrix during or after which some of said secondary modifications take place.

It appears that none of the studies published so far provide reliable information on the size of the polysomes on which collagen synthesis takes place (Speakman, 1968). The estimations of Rich, Gould, and coworkers are unreliable due to the association and aggregation occurring in their experiments. Those of Goldberg and Green (1967) are equally unreliable due to the use by these investigators of impure collagenase and the application of the equations of Martin and Ames (1961) in their determination of the s values and particle weights of large polysomal aggregates in sucrose gradients as concentrated as 15–60%. Work in this laboratory is in progress to obtain more reliable information on this problem.

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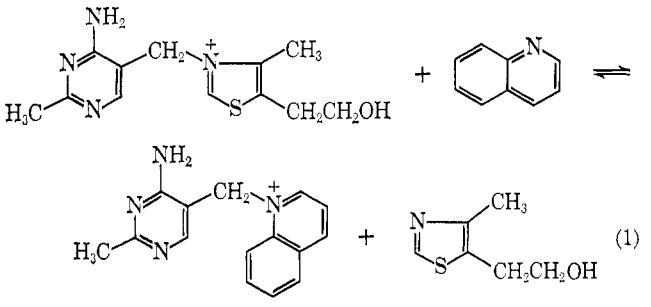
Kinetic Evidence for a (4-Amino-2-methyl-5-pyrimidinyl)methyl-Enzyme Intermediate in the Thiaminase I Reaction*

Gustav E. Lienhard

ABSTRACT: Thiaminase I (thiamine:base (4-amino-2-methyl-5-pyrimidinyl)methyl (PM) transferase, EC 2.5.1.2) catalyzes the transfer of the PM group from thiamine and other similar PM-N^+ compounds to aromatic nitrogenous bases and thiols. A kinetic study of this reaction has been made with the bacterial enzyme.

Rates were measured spectrophotometrically or by the incorporation of radioactive label. The following findings suggest that the reaction proceeds by way of a PM-enzyme intermediate. (a) The values of V_{max} for the reaction of 1-PM-quinolinium with pyridine, benzenethiol, and *p*-nitrobenzenethiol are nearly identical. (b) The values of V_{max} for the reaction of quinoline with thiamine, pyrithiamine, and 1-PM-3-chloropyridinium are identical. (c) The observed value of V_{max} for the reaction of 1-PM-pyridinium with quinoline can be calculated from the value of V_{max} for the reaction of 1-PM-pyridinium with *p*-nitrobenzenethiol and the value of V_{max} in b, if the kinetic scheme is assumed to be the Ping-Pong type. (d) The second-order rate constants for the reaction aniline with five PM-N^+ compounds are identical. The following new compounds have been prepared and characterized: 1-PM-quinolinium chloride hydrochloride, 1-PM-pyridinium chloride hydrochloride, 1-PM-3-chloropyridinium chloride hydrochloride, 1-PM-3-methylimidazolium chloride hydrochloride, *S*-PM-benzenethiol, *S*-PM-*p*-nitrobenzenethiol, and [2-^{3}H]quinoline.

Thiaminase I is an enzyme that catalyzes the transfer of the PM group[1] from thiamine and certain PM-N^+ analogs of thiamine to aromatic nitrogenous bases and to thiols (Fujita, 1954; Murata, 1965). Equation 1 shows this reaction with



(1)

thiamine and quinoline. The substrate specificities of thiaminase I's from various sources have been examined in some detail (Fujita, 1954; Murata, 1965), but almost nothing is known about the mechanism of this enzymatic aralkyltransfer reaction. This paper describes a kinetic study of the thiaminase I reaction, the goal of which was to determine whether or not the reaction proceeds by way of a PM-enzyme intermediate. The bacterial enzyme (Ebata and Murata, 1961; Wittliff and Airth, 1968) was used.

Methods

Kinetics. The rates of the reactions catalyzed by thiaminase I were followed spectrophotometrically with a Gilford Model 240 recording spectrophotometer, the cell compartment of which was thermostatted at 25.0 ± 0.1°. Reaction mixtures that contained the buffer and the substrates were prepared in 3.0-ml, 1-cm, Teflon-stoppered cuvets and were temperature equilibrated in a water bath at 25.0° for 15 or more min. Reaction was initiated by the addition of an aliquot from a stock solution of thiaminase I maintained at 25.0°. The recording of the rate was started between 15 and 25 sec after the addition of enzyme and was continued for several minutes, against a blank identical with the reaction mixture except for the absence of enzyme. The total volume of each reaction mixture was 3.0 ml. The buffer present in all the reaction mixtures was 0.1 M sodium phosphate (pH 6.45). The activity of thiaminase I is maximal in this buffer (Wittliff and Airth, 1968). The amount of enzyme present in each reaction mixture was such that no more than 10% of either substrate was consumed during the first minute of reaction. In every case the recording of absorbance against time was linear during the ini-

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[1] The abbreviation PM is used for the (4-amino-2-methyl-5-pyrimidinyl)methyl group. The names, PM-aniline, PM-3-chloropyridinium, PM-pyridinium, PM-*N*-methylimidazolium, and PM-quinolinium refer to the compounds in which the PM group is bonded to the nitrogen of aniline, 3-chloropyridine, pyridine, *N*-methylimidazole, and quinoline, respectively. The names PM-benzenethiol and PM-*p*-nitrobenzenethiol refer to the compounds in which the PM group is bonded to the sulfur of benzenethiol and *p*-nitrobenzenethiol.