

INTRACELLULAR ACCUMULATION OF PROTOCOLLAGEN AND EXTRUSION OF COLLAGEN BY EMBRYONIC CARTILAGE CELLS

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ABSTRACT

The synthesis of collagen can be interrupted, after the assembly of proline-rich and lysine-rich polypeptide chains called protocollagen, by incubating connective tissues anaerobically. Under these conditions the proline and lysine residues in protocollagen are not hydroxylated to hydroxyproline and hydroxylysine, and protocollagen molecules accumulate intracellularly. Chemical data and radioautographs at the level of the light and electron microscopes indicated that in tissues labeled with proline-3,4-³H under nitrogen, there appeared to be an accumulation of radioactivity over the ground cytoplasm. When the inhibition of protocollagen hydroxylase was reversed by exposing the tissue to oxygen, the accumulated protocollagen-³H was converted to collagen-³H and there was a rapid transfer of label from the ground cytoplasm to the extracellular matrix. There was no significant change in distribution of label over either the Golgi vacuoles or the cisternae of the endoplasmic reticulum. The failure to find a significant change in distribution of label over the Golgi vacuoles or the cisternae does not completely exclude the possibility that these two compartments are involved in the extrusion, but the data are consistent with the simpler notion that the completed collagen molecules pass directly from the ground cytoplasm to the extracellular matrix.

A number of efforts have been made for determination of how extracellular proteins synthesized on ribosomal complexes in cells are transferred to extracellular spaces. Extensive studies on pancreatic acinar cells indicated that digestive enzymes are synthesized on ribosomes of the endoplasmic reticulum of these cells and then pass through the cisternae of the reticulum to Golgi vacuoles before extrusion as zymogen granules (1, 2). Revel and Hay (3) suggested that collagen is synthesized and extruded by a similar mechanism. After they had injected radioactive proline into salamanders, they qualitatively observed that label first appeared over the endoplasmic reticulum of chondrocytes

and then over Golgi vacuoles. Ross and Benditt (4), however, found evidence for another pathway in similar studies in guinea pigs; they suggested that collagen synthesized on ribosomes may pass into the cisternae of the endoplasmic reticulum, and then into the extracellular matrix. Ross and Benditt (4) as well as Kajikawa et al. (5) suggested that extrusion not involving the Golgi vacuoles might occur by movement of collagen through the direct connections which are occasionally seen between the endoplasmic reticulum and plasma membrane of connective tissue cells. A third possible mechanism for extrusion was presented earlier in morphological studies which suggested a direct

passage of collagen from the ground cytoplasm through the plasma membrane. Wasserman (6)¹, obtained evidence for extrusion of collagen by "delamination" of the cell cortex during wound-healing in tendons, and Porter and Pappas (7) reported evidence for a similar process in cultured fibroblasts.

Recent studies (8-18) on the biosynthesis of collagen have provided a method of studying the extrusion of collagen molecules independently of their synthesis. The initial step in the synthesis of collagen is the assembly of polypeptides which are of the same size as the final protein molecule (9, 13, 14), but which are rich in proline and lysine and do not contain significant amounts of hydroxyproline or hydroxylysine. These unhydroxylated polypeptides have been called protocollagen (14). The enzyme which hydroxylates protocollagen has been isolated and characterized, and it has been shown to require α -ketoglutarate, ascorbic acid, oxygen, and ferrous iron (see references 15-17). When freshly isolated tissues are incubated either under anaerobic conditions (8, 9) or in the presence of an iron chelator (12), protocollagen molecules similar in size to the complete α -chains of collagen are synthesized and accumulate intracellularly (18). When inhibition of hydroxylation is reversed, appropriate proline and lysine residues in the accumulated protocollagen are hydroxylated and the hydroxylated polypeptides pass into the extracellular matrix (18).

In the experiments reported here, a combination of radioautographic and chemical procedures was used to examine the site at which protocollagen accumulates when hydroxylation of proline and lysine is inhibited in isolated cartilage. Also, the distribution of the labeled protein over various cellular compartments and the matrix was correlated with the conversion of protocollagen to collagen after inhibition of the hydroxylating enzyme had been reversed.

MATERIALS AND METHODS

In Vitro Incubations

Tibiae which consisted primarily of cartilage were removed from 10-day-old chick embryos under sterile conditions. All incubations were made in 2.7 ml of medium containing phosphate buffer, inorganic salts, and glucose (Krebs' medium A) (8, 9) in 2.5 × 10-cm tubes provided with rubber stoppers with two open-

ings to allow passage of gases through the tubes. The tubes were incubated in a metabolic shaker at 37°C, and in all cases the tibiae were preincubated for 20 min in the experimental medium with the appropriate gas phase. 8 μ c L-proline-3,4-³H (specific activity 5 mc/ μ mole; New England Nuclear Corp., Boston, Mass.) dissolved in 0.2 ml of medium was then injected through the rubber stopper. After incubation with the proline-³H for 1 hr, the radioactive proline was chased by injecting 180 μ g carrier L-proline in 0.1 ml of medium. Oxygen was introduced into the system either by exposing the samples to air or by passing 95% oxygen:5% carbon dioxide through the tubes. The use of air instead of 95% oxygen did not have any effect on the rate of hydroxylation of protocollagen. Each incubation tube contained two tibiae from the same embryo, and at the end of the incubation one tibia was taken for chemical assay and the contralateral tibia was taken for microscopy.

Chemical Assays

At the end of the incubation period, the medium was quickly removed from the incubation tubes, and the tubes containing the tibiae for chemical assay were placed in a bath of ethanol and dry ice. The tissues were homogenized in about 4 ml water in a Teflon and glass homogenizer with 10 strokes of the pestle at 3500 rpm, and the homogenates were dialyzed against running tap water for 18 hr. The dialyzed samples were hydrolyzed by adding an equal volume of concentrated HCl and autoclaving in sealed tubes for 15 hr at 120°C and 16-lb. pressure. The hydrolysates were evaporated to dryness *in vacuo*, and the residues were dissolved in distilled water. One aliquot of each sample was used to assay total ³H content in a liquid scintillation counter with the solvent system described previously (19), and the remainder of the sample was used to assay hydroxyproline-³H with a specific chemical assay procedure (20). Counting efficiencies for ³H in individual samples were estimated by the addition of internal standards of water-³H (United States National Bureau of Standards) or hexadecane-³H (Nuclear-Chicago Corporation, Des Plaines, Mich.), and appropriate corrections were made to convert observed counts per minute to disintegrations per minute (dpm). The liquid scintillation system employed had an efficiency of 10-12% for tritium, and the background was 20 cpm. All radioactive measurements were made with aliquots whose radioactivity level was at least 10 times background.

Radioautography

Tibiae contralateral to those used for the chemical assays were cut into two pieces and fixed at 0°C in 2% glutaraldehyde in a 0.1 M sodium cacodylate

¹ Wassermann, F. Personal communication. W

buffer, pH 7.2, for 4–18 hr. The tissues were briefly rinsed in buffer containing 0.22 M sucrose and were postfixed for 1 hr in 1% OsO₄ in Dalton's buffer, pH 7.4. The tissues were dehydrated in a graded ethanol series at 0°C and embedded in ethanol:Araldite (1:1) at room temperature on a rotating platform for 18 hr, and for 24 hr each in 100% Araldite without catalyst and in Araldite with catalyst. The tissues were then placed in gelatin capsules and incubated at 45°C in Araldite with catalyst for 24 hr and at 60°C for 2 days. Embedded material was sectioned on a Porter-Blum microtome with a diamond knife at 0.3 μ for light microscopy, and gold to silver sections were cut for electron microscopy.

To prepare the radioautographs for light microscopy (21), we transferred the 0.3-μ sections with a stainless steel loop to gelatin-coated slides. The slides were coated with Ilford L-4 nuclear research emulsion (Ilford Ltd., Ilford, Sussex, England) which was diluted 1:7 with distilled water in order to obtain a layer of emulsion of about a single grain thickness (22). The coated slides were exposed at 4°C for 5 days or 2 wk. The radioautographs were developed at 19°–20°C for 5 min, and they were examined unstained with phase-contrast optics.

To prepare the radioautographs for electron microscopy, we transferred the thin sections to parlodion-coated glass slides by means of a stainless steel loop. The sections were stained with Reynolds' lead citrate (23) or with 4% uranyl acetate in 70% ethanol, and they were coated with carbon (24). The sections were then coated with the 1:7 diluted Ilford L-4 emulsion and photoincubated at 4°C for 1–3 months. The radioautographs were developed by the technique of Lettré and Paweletz (25) by using 1-phenyl-3-pyrazolidone (K & K Laboratories Inc., Plainview, N.Y.) as the developing agent. The sections were removed from the glass slides in absolute alcohol, they were floated onto copper grids, and then examined with an RCA model EM 3 microscope. In

most instances the sections examined for any given sample were obtained from a single block.

RESULTS

Conditions for the Synthesis of Protocollagen and Collagen

Tibiae from 10-day-old embryos synthesize collagen at a rapid rate *in vitro*. The tibiae contain chondrocytes in four different stages of differentiation, and most of the total synthesis of proteins is accounted for by the postmitotic cells in the "flattened zone region" (26). Because the flattened zone cells are the most active in incorporating radioactive amino acids and sulfate-³⁵S (18), most of the observations here were based on changes in these cells.

A pulse-label and chase type of procedure was used to trace the synthesis of complete protocollagen molecules, the hydroxylation of protocollagen to collagen, and the extrusion of collagen. Fig. 1 summarizes the general format of these experiments. Tibiae were incubated under either oxygen or nitrogen with tritiated proline for 1 hr, and carrier proline was then added to chase the radioactive proline during a further 1 hr incubation. After the chase period, tibiae which had been incubated under nitrogen were exposed to oxygen for varying periods of time in order to trace the hydroxylation of protocollagen to collagen as indicated by the appearance of labeled hydroxyproline (Fig. 1). As indicated, most of the incorporation of radioactivity occurred during the initial 1 hr incubation with radioactive proline, and there was no further incorporation of proline after the chase period. During incubation under nitrogen, little

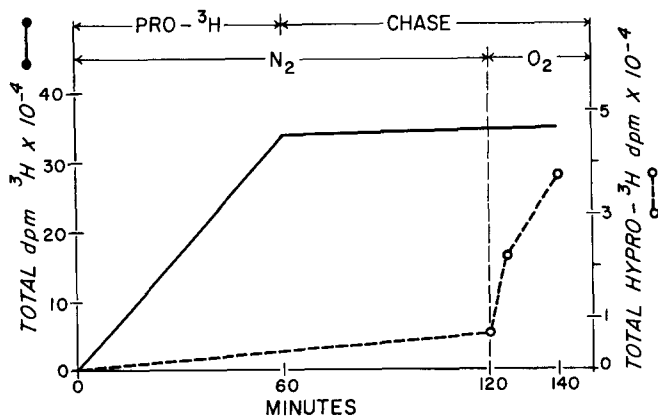


FIGURE 1 Schematic representation for pulse-label and chase experiments. As described in text, tibiae were incubated with proline-³H for 1 hr, and then carrier proline was added to chase the radioactive proline. The data on which this representation is based are presented in Tables I and II and elsewhere (18, 30).

TABLE I
Incorporation of Proline-³H and Synthesis of Hydroxyproline-³H in
Cartilage under Aerobic and Anaerobic Conditions

Sample	N ₂	O ₂	Total ³ H	Hydroxy- proline ³ H	Hydroxyproline- ³ H	
					Total ³ H	× 100
			dpm × 10 ⁻⁵	dpm × 10 ⁻⁴	%	
A, control		0-120	5.9	9.6	16.4	
B, inhibited	0-120		3.9	0.69	1.8	
C, 5 min reversal	0-120	120-125	4.1	2.2	5.4	
D, 15 min reversal	0-120	120-135	2.9	3.1	10.7	
E, 20 min reversal	0-120	120-140	3.4	3.7	10.9	

The conditions for the experiment are described in the text and in Fig. 1. Exposure to nitrogen or oxygen is indicated in minutes from the beginning of the experiment.

TABLE II
Incorporation of Proline-³H and Synthesis of Hydroxyproline-³H
in Cartilage under Aerobic and Anaerobic Conditions

Sample	N ₂	O ₂	N ₂	Total ³ H	Hydroxyproline- ³ H	Hydroxyproline- ³ H	
						Total ³ H	× 100
				dpm × 10 ⁻⁵	dpm × 10 ⁻⁴	%	
F		0-240		3.7	6.6	17.8	
G			0-240	2.3	0.36	1.6	
H	0-60	60-120		3.4	5.0	14.7	
I	0-60	60-240		4.1	5.7	13.9	
J		0-60	60-120	5.2	7.0	13.5	
K		0-60	60-240	4.7	6.7	14.3	

The conditions for the experiment are described in the text and in Fig. 1. Exposure to nitrogen or oxygen is indicated in minutes from the beginning of the experiment.

hydroxyproline-³H was synthesized. After exposure of the tibiae to oxygen, the proline-³H incorporated into protocollagen during the labeling period was hydroxylated to hydroxyproline-³H.

Chemical data on the tibiae used for radioautography in two separate experiments are presented in Tables I and II. In samples incubated under nitrogen for 120-240 min, the synthesis of hydroxyproline-³H was less than one-tenth the value in samples incubated under oxygen, even though the total incorporation of ³H was about 65% of the control values (sample A versus sample B in Table I, and sample F versus sample G in Table II). When tissues labeled and chased under nitrogen were exposed to oxygen, the ratio of the hydroxyproline-³H to total-³H increased toward control values (samples C, D, and E in Table I, and samples H and I in Table II). As noted previously

(27), the maximal value for the ratio of radioactive hydroxyproline to total radioactivity incorporated was 17-25% when proline-¹⁴C was used, but somewhat lower values were obtained with L-proline-3,4-³H. The difference is probably explained by the varying degrees of randomization of label during the chemical synthesis of different preparations of the L-proline-3,4-³H, which make it difficult to make the appropriate corrections in converting counts per minute of pyrrole-³H observed in the chemical assay to disintegrations per minute of hydroxyproline-³H (20, 28).

Light Microscopic Studies of Distribution of Label in the Tissues

One tibia from a chick embryo was assayed chemically (Tables I and II), and the contralateral

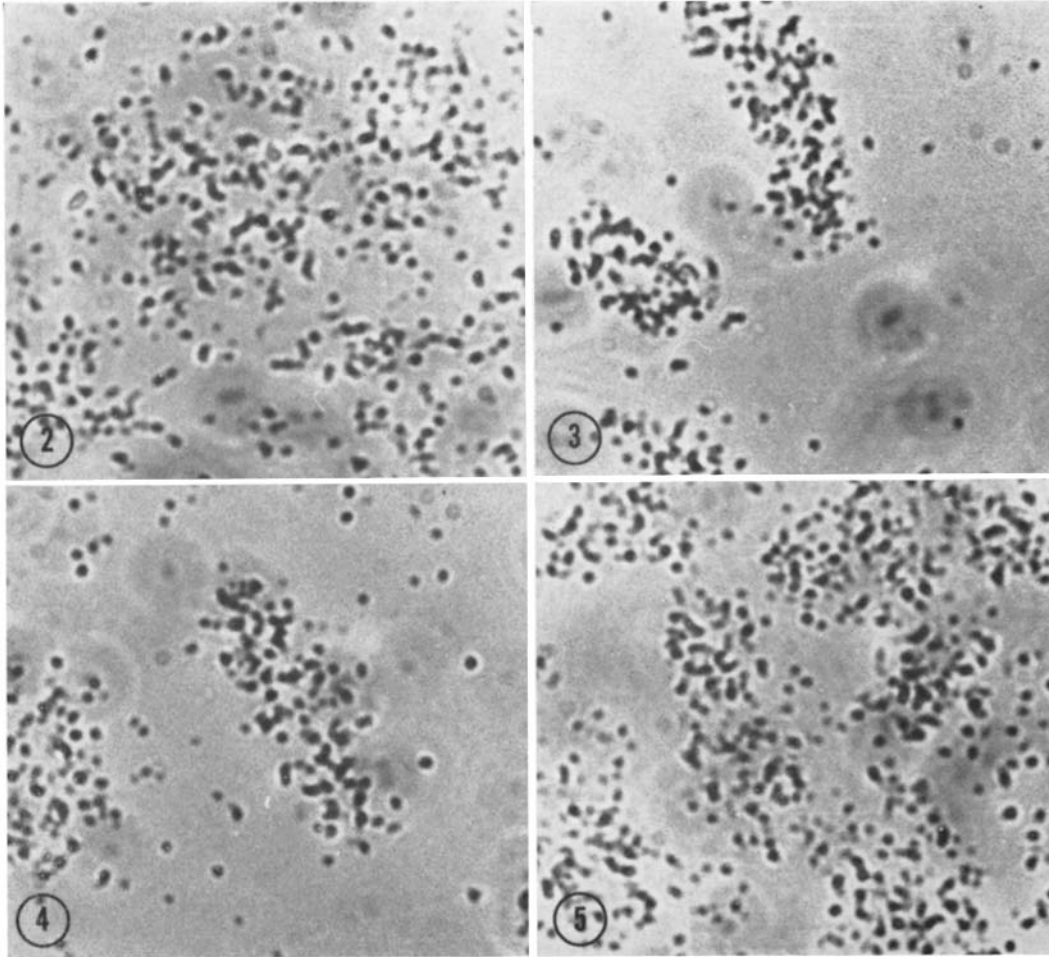


FIGURE 2 Light microscopic radioautographs of embryonic cartilage. Tibiae labeled and chased under oxygen (see sample A in Table I). This indicates a large fraction of the radioactivity has been incorporated into the extracellular matrix. $\times 2,000$.

FIGURE 3 Tibiae labeled and chased under nitrogen (sample B in Table I). A small amount of the label is over the extracellular matrix, but most of the label is over the cellular spaces. $\times 2,000$.

FIGURE 4 Tibiae labeled and chased under nitrogen, and then exposed to oxygen for 5 min (sample C in Table I). The amount of extracellular label appears to be slightly greater than in sample not exposed to air (Fig. 3). $\times 2,000$.

FIGURE 5 Tibiae labeled and chased under nitrogen, and then exposed to oxygen for 20 min (sample E in Table I). Amount of extracellular label greater than Fig. 4, but somewhat less than control sample (Fig. 2). $\times 2,000$.

tibia was fixed for light and electron microscopy. Even though the samples were fixed directly in glutaraldehyde, free proline- ^3H should not have appeared in the radioautographs (3), since all the samples were chased with carrier proline.

In tibiae incubated for 2 hr under oxygen in pulse-label and chase experiments, much of the label was incorporated into the extracellular matrix (Fig. 2). Some radioactivity remained over the cellular nuclei, and some was randomly dis-

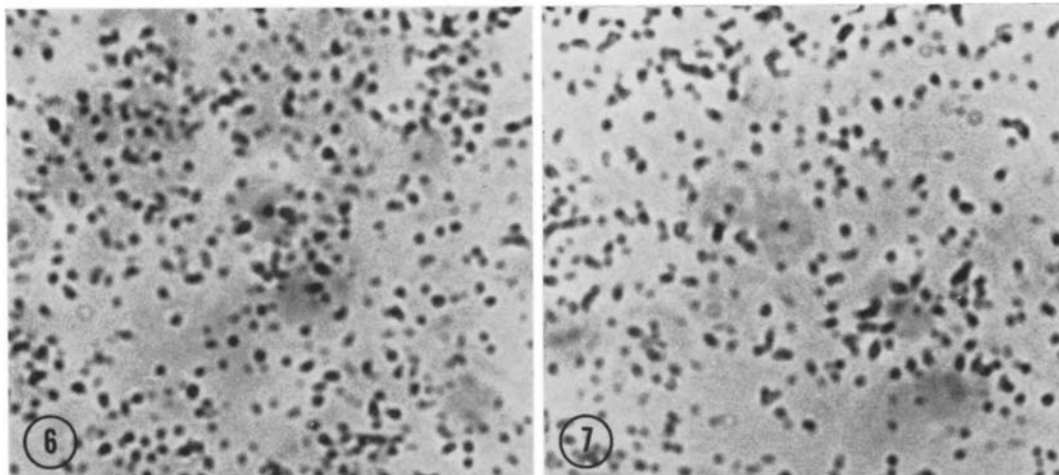


FIGURE 6 Tibiae labeled and chased under nitrogen and then exposed to oxygen for 60 min (sample H in Table II). Amount of extracellular label is about the same as in control (Fig. 2). $\times 2,000$.

FIGURE 7 Tibiae labeled under oxygen for 60 min and then chased under nitrogen for 60 min (sample J in Table II). Amount of extracellular label is about the same as in control (Fig. 2); this indicates that the absence of oxygen does not in itself prevent the extrusion of fully hydroxylated collagen molecules. $\times 2,000$.

tributed over the cytoplasm. The residual intracellular label remained there even with chase periods of up to 3 hr (not shown). When tibiae were labeled with tritiated proline under nitrogen and the label was chased with carrier proline under nitrogen, most of the label remained intracellular (Fig. 3). A small amount of label appeared over the extracellular matrix, and this label probably represents proline- ^3H which was incorporated into polypeptides associated with mucopolysaccharides, since the synthesis and extrusion of mucopolysaccharides is not significantly inhibited by incubation of this tissue under nitrogen (30).

The transfer of accumulated protein from the cells to the extracellular matrix was followed in experiments in which the tissue was labeled and chased under nitrogen and then exposed to oxygen for 5, 20, and 60 min (Figs. 4–6). After exposure to oxygen, the amount of extracellular label increased rapidly over the first 20 min (Figs. 4 and 5). A small additional increase in extracellular label appeared to occur after exposure to oxygen for 60 min (compare Figs. 5 and 6), but this was not apparent in statistical evaluation of the data obtained by electron micrographs (see below).

Control experiments indicated that incubation under nitrogen did not in itself prevent the extru-

sion of fully hydroxylated collagen molecules. In tibiae labeled with proline- ^3H under oxygen, large amounts of the label were still intracellular after 1 hr (not shown). In tibiae labeled with proline- ^3H under oxygen for 1 hr and chased for 1 hr with carrier proline under nitrogen the radioautographs (Fig. 7) were indistinguishable from those obtained when tibiae were labeled and chased under oxygen (Fig. 2); this indicates that the labeled collagen synthesized under oxygen was transferred to the extracellular matrix during the incubation under nitrogen. As expected, the values for the incorporation of proline- ^3H and the synthesis of hydroxyproline- ^3H under these conditions were close to the values in control samples (compare samples F, J, and K in Table II).

Electron Microscopic Studies on the Distribution of Label

Sections for electron microscopy were prepared from the same blocks used for light microscopy. For quantitation of the changing distribution of label in the sections, all electron micrographs were taken at a magnification of 5,300, and the plates were enlarged five times to give 7×10 -inch prints. The grains over the various subcellular compartments and the matrix were counted, and the

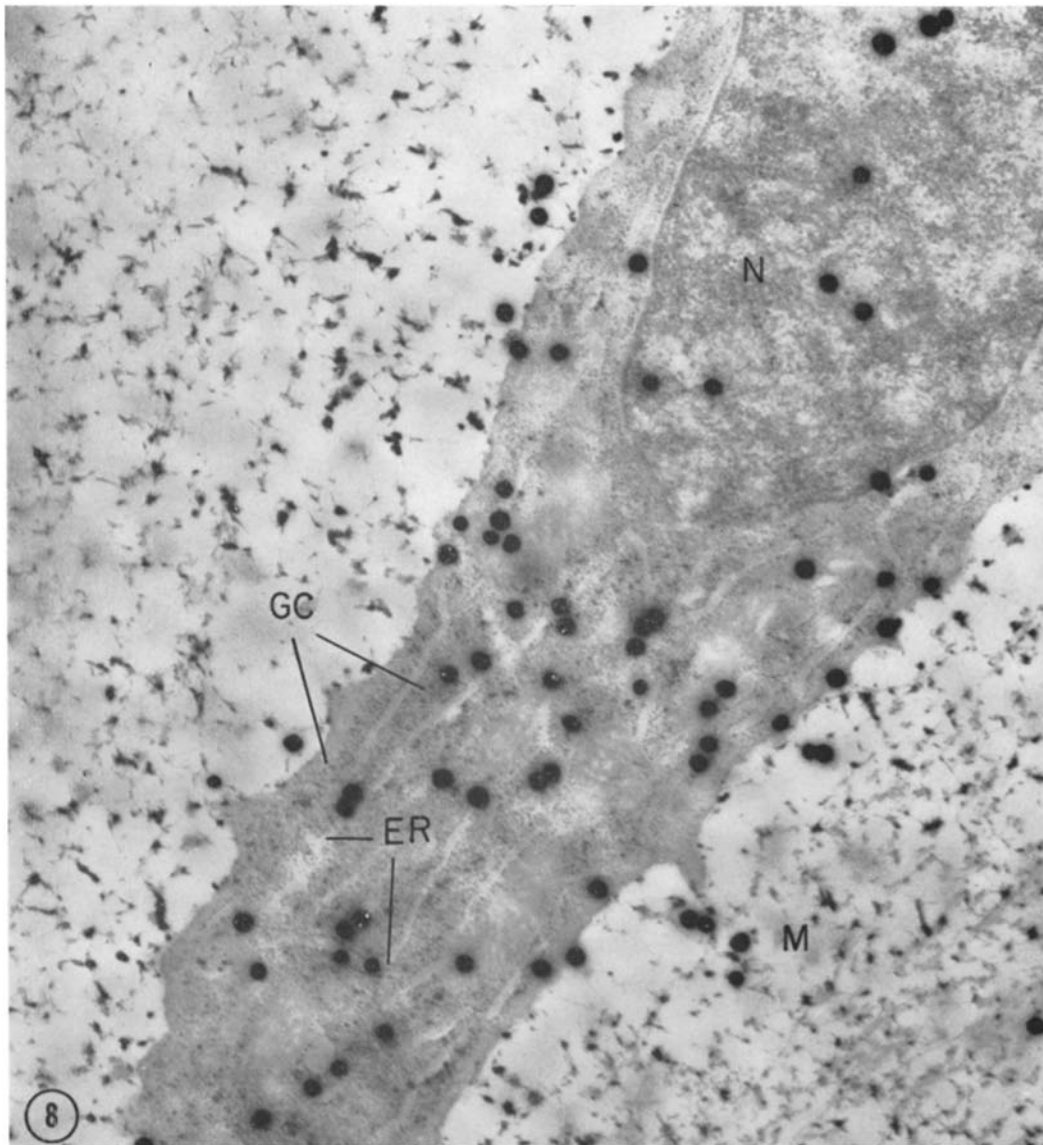


FIGURE 8 Electron microscopic radioautograph of embryonic cartilage. Tibiae labeled and chased under nitrogen (sample B in Table I). Section from same block as Fig. 3. Subcellular compartments: *N*, nucleus; *ER*, cisternae of the endoplasmic reticulum; and *GC*, ground cytoplasm; *M*, matrix. $\times 21,000$.

number of grains over each cellular compartment or matrix was expressed as per cent of total grains for each experimental sample. The subcellular compartments were defined as indicated in Figs. 8 and 9. In the instances where grains were near the boundaries between compartments, the centers of the grains were determined, and the grains were arbitrarily assigned to the compartment in which

the centers lay. Intracellular grains which were not over nuclear, vacuolar, or cisternal compartments were assigned to the ground cytoplasm. The values for the ground cytoplasm also included the small number of grains occasionally seen over mitochondria. As a rule of thumb, the number of photographs examined for a given sample was determined by the number required to give a value

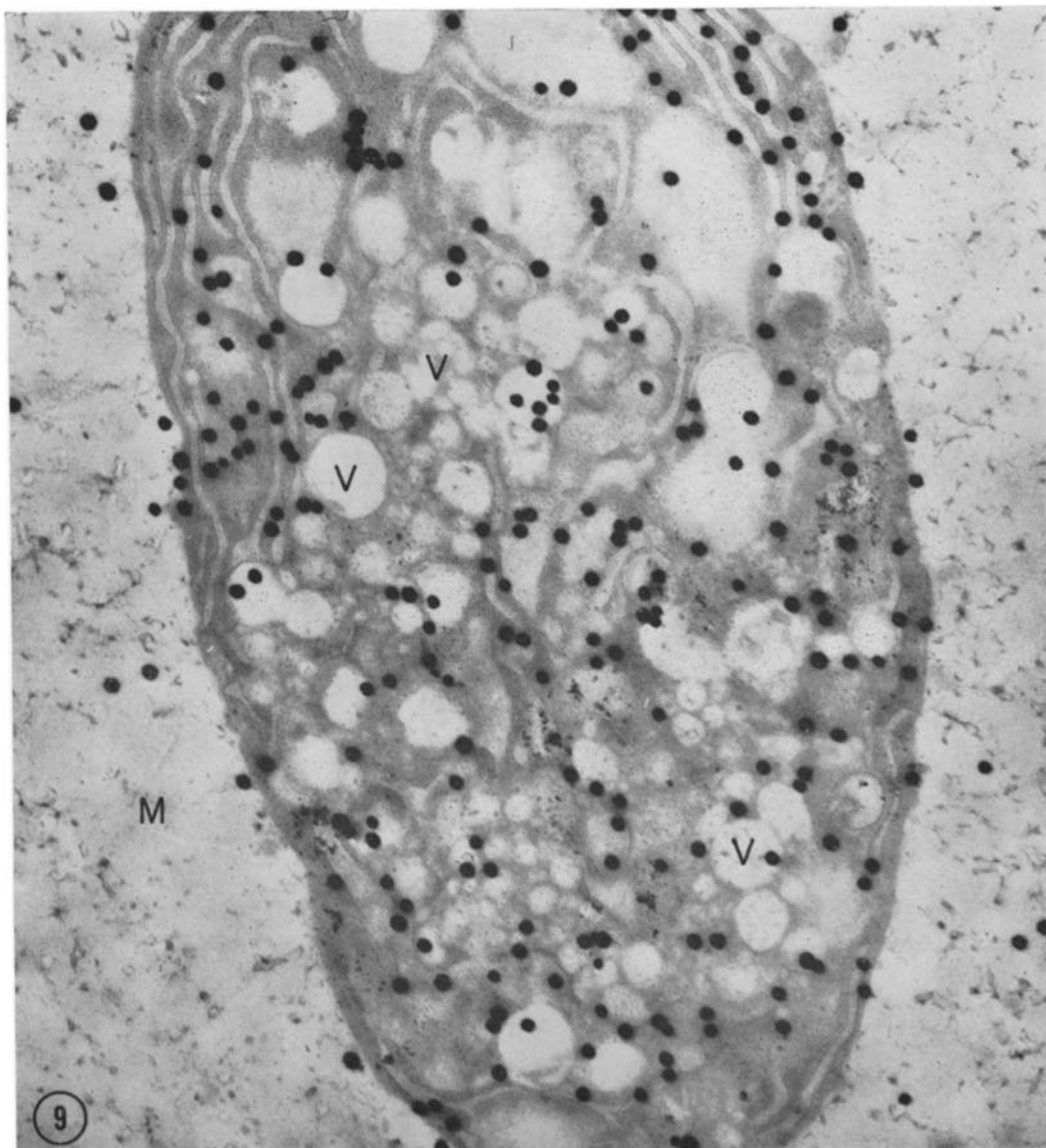


FIGURE 9 Electron microscopic radioautograph of embryonic cartilage. Tibiae labeled and chased under nitrogen, and then exposed to oxygen for 5 min (see sample C in Table I). Section from same block as Fig. 4. Subcellular compartments indicated as in Fig. 8; *V*, vacuoles; *M*, matrix. $\times 21,000$.

for over-all per cent grains per compartment (100 times total grains in compartment in all photographs for the sample divided by total grains counted for the sample) which corresponded closely to the value obtained by taking the mean of the per cent of grains for that compartment in the individual photographs.

Comparison of the per cent of total grains over

each compartment in five samples (Table III) did not indicate any marked change in the distribution of grains over the nuclei, vacuoles, or cisternae of the endoplasmic reticulum with the different experimental treatments. There were, however, complementary changes in ground cytoplasm and the matrix. In tibiae labeled and chased under nitrogen, 56% of the total grains was over the

TABLE III
Distribution of Grains over Cellular Compartments and Matrix

Sample	No. of photographs	Grains counted	Total Grains					
			Nuclei	Vacuoles	ER	Cytoplasm	Combined ER and cytoplasm	Matrix
			%	%	%	%	%	%
B, N ₂ inhibited	9	1,157	8.6	3.5	21	56	77	11
C, 5 min reversal	16	1,859	8.0	4.0	24	47	71	17
E, 20 min reversal	9	2,684	13	3.1	26	33	59	25
H, labeled in N ₂ , excluded in O ₂ (60 min reversal)	12	439	15	6.4	25	30	55	23
J, labeled in O ₂ , excluded in N ₂	10	468	15	2.7	20	31	51	33

Electron micrographs were prepared from samples from same experiments as presented in Tables I and II. Grains were counted on a total of 56 micrographs as described in text.

ground cytoplasm. When tibiae labeled and chased under nitrogen were exposed to oxygen, the concentration of grains over the ground cytoplasm decreased to 33% in 20 min. The combined values for grains over the cisternae of the endoplasmic reticulum and ground cytoplasm showed a similar change from 77 to 59%. In the same samples the concentration of grains over the matrix increased from 11 to 25%. The value for the distribution of grains in the sample exposed to oxygen for 20 min in the first experiment (sample E) was similar to that observed in samples exposed to oxygen for 60 min in the second experiment (samples H and J), even though the latter values are probably less reliable because a smaller number of grains were counted. The apparent differences in the per cent of total grains over the vacuoles between samples H and J, and between either of these samples and samples B, C, and E, were not significant when evaluated by the statistical tests described below.

Since the area occupied by each compartment varied among different photographs (compare Figs. 8-10), the technique of Loud (31) was used to estimate the per cent of total area in each print occupied by the various compartments. A total of 34 prints from samples B, C, and E were examined, and the mean values for per cent of the total area occupied by each compartment were compared. In the three samples the mean values for per cent of total area were 7.8, 7.1, and 6.9% for nuclei; 2.4, 4.4, and 4.5% for vacuoles; 13, 13, and 17% for cisternae of the endoplasmic reticu-

lum; 19, 21, 21% for the ground cytoplasm; and 58, 55, and 50% for the matrix. In 10 micrographs the area accounted for by each compartment was also measured directly with a planimeter. No significant difference was found in the values obtained by these direct measurements and the estimates obtained by the Loud technique.

The relatively narrow range of mean values for per cent of the total area occupied by individual compartments in samples B, C, and E suggested that the changes observed in the per cent of total grains over the ground cytoplasm and matrix (Table III) were not explained by sampling errors. In order to obtain a statistical evaluation of the results, however, we undertook a more detailed analysis of the data for these three samples. In order to correct for variations in area occupied by a given compartment in different photographs, we obtained the values for per cent of grains per compartment (y) and per cent area per compartment (x) for each photograph. The values for y versus x for a given compartment in all the photographs were then plotted, and appropriate statistical tests were used to establish that the regressions of y on x for that compartment in each of the three samples were parallel (32).² The mean values for per cent

² Standard text indicated in reference 32 was used for the statistical tests. The test for parallelism of the regression of y on x was performed as indicated on page 137 of the text; the analysis of covariance was performed as described on page 384.

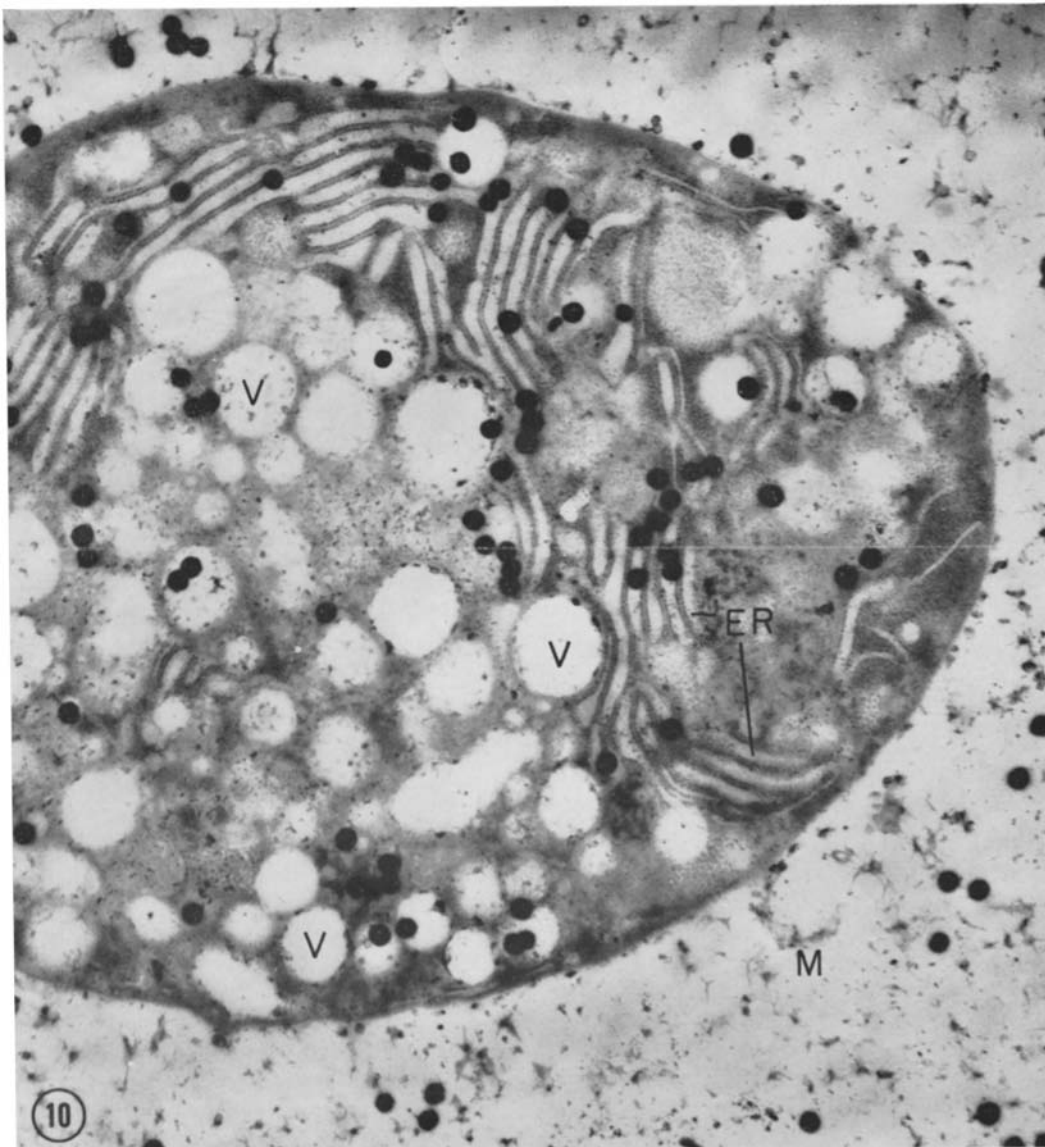


FIGURE 10 Electron microscopic radioautograph of embryonic cartilage. Tibiae labeled and chased under nitrogen, and then exposed to oxygen for 20 min (sample E in Table I). Section from same block as Fig. 5. Subcellular compartments as in Figs. 8 and 9; *V*, vacuoles; *M*, matrix; *ER*, cisternae of endoplasmic reticulum. $\times 21,000$.

of grains per compartment (Table III) were corrected, for variations in area occupied by a given compartment in the three samples, with the formula,

$$\bar{y}_{ia} = \bar{y}_i - b(\bar{x}_i - \bar{x})$$

where \bar{y}_{ia} is the mean per cent of total grains per compartment adjusted for area; \bar{y}_i is the mean value for per cent of grains in a compartment for the *i*th sample; *b* is the slope of the linear regression of *y* versus *x*; \bar{x}_i is the mean value for area of a compartment in the *i*th sample; and \bar{x} is the common

TABLE IV
Distribution of Grains over Cellular Compartments and Matrix Corrected for Mean Area Accounted for by Each Compartment in the Electron Micrographs

Sample	Mean per cent total grains adjusted for mean area					
	Nuclei	Vacuoles	ER	Cytoplasm	Combined ER and cytoplasm	Matrix
B, N ₂ inhibited	19 ± 2.1	4.2 ± 0.95	21 ± 2.4	54 ± 3.7	(75 ± 3.8)	12 ± 2.9
C, 5 min reversal	15 ± 1.9	3.7 ± 0.67	25 ± 1.8	43 ± 2.7	(69 ± 2.8)	20 ± 2.2
E, 20 min reversal	14 ± 2.7	3.4 ± 0.98	25 ± 2.6	33 ± 3.7	(58 ± 2.8)	29 ± 2.9
p value	N.S.*	N.S.*	N.S.*	<0.005	<0.05	<0.001

* Nonsignificant with p values >0.1.

Values shown are mean values and standard errors. Statistical corrections for mean area per compartment were made as described in text. 15 of the 34 photographs examined for the three samples did not contain any nuclear compartment (see Fig. 10). Accordingly, the n value for the nuclear compartment was less than that for the other compartments, and the sum of mean per cent grains per compartments as shown here is greater than 100% for each sample.

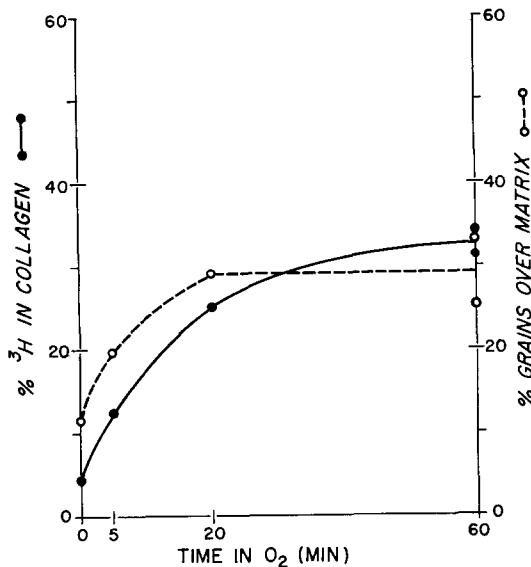


FIGURE 11 Increase in grains over matrix and increase in collagen-³H when tibiae labeled and chased under nitrogen were exposed to oxygen. Values for per cent grains over matrix after 0, 5, and 20 min in oxygen are the mean values adjusted for area as given in Table IV. The values for per cent grains over matrix after 60 min in oxygen are the uncorrected mean per cent grains over matrix in 12 photographs examined for sample H and in 10 photographs examined for sample J. Values for per cent of total ³H in collagen (C_i) in the same samples were calculated from values for ratios of hydroxyproline-³H to total ³H presented in Tables I and II according to the equation,

$$\% \text{ } ^3\text{H in } C_i = \frac{\text{hypro-}^3\text{H}}{\text{total } ^3\text{H}} \times 100 \times \frac{1.00}{0.43}$$

mean area for the compartment in all the photographs for the three samples.

The standard errors for the mean per cent of total grains adjusted for mean area were calculated with the formula,

$$SE \text{ of } \bar{y}_{ia} = s_E \sqrt{\frac{1}{n_i} + \frac{(\bar{x}_i - \bar{x})^2}{E_{xx}}}$$

where s_E is the mean square error obtained from the analysis of covariance; n_i is the number of photographs in the i th sample; \bar{x}_i and \bar{x} are defined as above; and E_{xx} is the denominator of the slope term b (32).² Analysis of covariance (32) indicated that there was no significant difference in the adjusted values for mean per cent of total grains over nuclei, vacuoles, or cisternae in the three samples (Table IV). There was, however, a highly significant difference among the adjusted values for grains over the ground cytoplasm and among the adjusted values for the matrix. The difference among the values for the combined compartments of cisternae and ground cytoplasm was significant at the level of $p < 0.05$.

An independent statistical evaluation of the results was also carried out by calculating the ratio of per cent of total grains to per cent of total area per compartment for individual photographs (ratio of $y:x$), and the values for this ratio were examined with the Kruskal-Wallis one-way analysis of variance (33). The p values obtained with this nonparametric analysis were similar to those shown in Table IV.

Comparison of the chemical data (Tables I and

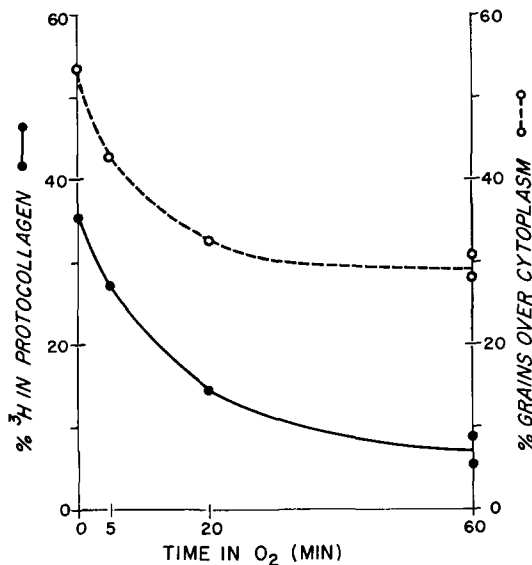


FIGURE 12 Decrease in grains over ground cytoplasm and decrease in protocollagen-³H when tibiae labeled and chased under nitrogen were exposed to oxygen. Values for per cent grains over ground cytoplasm after 0, 5, and 20 min in oxygen are the mean values adjusted for area as given in Table IV. The values for per cent grains over cytoplasm after 60 min in oxygen are the uncorrected mean per cent grains over matrix in 12 photographs examined for sample H and in 10 photographs examined for sample J. Values for per cent total ³H in protocollagen were calculated from the equation,

$$\% \text{ } ^3\text{H in } P = C_f - C_t$$

where P is protocollagen; C_f is the final mean value (39.8%) for per cent ³H in collagen when the tibiae were incubated under oxygen for 2 and 4 hr (samples A and F in Tables I and II); and C_t is the per cent of total ³H in collagen after exposure to oxygen for time t (calculated as described in Fig. 11).

II) and the distribution of grains (Table IV) in the same samples indicated a close correlation between the two sets of data. Since the ratio of hydroxyproline to total hydroxyproline and proline in collagen is about 0.43, the per cent of total ³H in collagen can be calculated (14) from the values for per cent of hydroxyproline-³H given in Tables I and II. Also, the per cent of ³H in protocollagen can be calculated as the difference between the final value for per cent of ³H in collagen after incubation of the tissue in oxygen and the value for per cent of ³H in collagen in any given sample. When tissues were exposed to oxygen, the

increase in per cent total grains over matrix closely paralleled the increase in per cent total ³H in collagen (Fig. 11). In the same samples, the values for per cent of total ³H in protocollagen closely paralleled the decrease in per cent of total grains over the ground cytoplasm (Fig. 12).

DISCUSSION

The ability to inhibit the hydroxylation of proline and lysine in a relatively specific manner provides a unique tool for radioautographic studies on the synthesis of collagen by connective tissue cells. When protocollagen hydroxylase is inhibited in embryonic cartilage, protein synthesis continues at 40–90% of the control rate, and sulfated chondromucoproteins are synthesized and extruded into the matrix at a normal rate (8, 9, 18, 30). Under these conditions the radioactive proline incorporated into protocollagen can be identified in radioautographs by the fact that it will remain intracellular until inhibition of the hydroxylase is reversed and the accumulated protocollagen is hydroxylated to collagen. Cartilaginous tibiae from chick embryos have several advantages for the type of study undertaken here, since the isolated tissue synthesizes collagen at a rapid rate; there is no significant conversion of radioactive proline to other amino acids; and one-half to two-thirds of the radioactive proline incorporated is accounted for by protocollagen and collagen (14, 27).

The results obtained here indicate that when protocollagen hydroxylase is inhibited, the major compartment in which protocollagen molecules accumulate is the ground cytoplasm. They also suggest that after protocollagen molecules which have accumulated during inhibition of the enzyme are hydroxylated, the completed collagen molecules pass rapidly from the ground cytoplasm to the extracellular matrix. When tissues which were pulse labeled and chased under nitrogen were exposed to oxygen, the mean per cent of total grains over the ground cytoplasm decreased from 54 to 33%, and the per cent of total grains over the matrix increased from 12 to 29% (Table IV). Chemical assays on the same samples indicated that the shift in radioactivity from the ground cytoplasm to the matrix closely paralleled the conversion of protocollagen-³H to collagen-³H in the tissue, and that there was no significant lag between the hydroxylation of protocollagen-³H and the transfer of radioactivity to the matrix

(Figs. 11 and 12). There was no significant change in concentration of grains over the Golgi vacuoles or over the cisternae of the endoplasmic reticulum under the conditions of these experiments.

As is generally recognized, any evaluation of the data from electron microscope radioautographs must consider the question of whether the resolution of the technique is sufficient to permit accurate localization of the radioactivity to specific subcellular compartments. The new procedure (25) used to develop the radioautographs made it possible to assign grains near the boundaries between compartments in a systematic manner. Of even greater importance, however, was the fact that because of the special chemical and morphological features of the experimental system, the accuracy of the data could be evaluated statistically. For example, if adequate resolution had not been obtained for the distribution of radioactivity between the cisternae and the ground cytoplasm, a significant decrease of label over both of these compartments would have been expected with the conversion of protocollagen-³H to collagen-³H and the transfer of grains from cells to the matrix. Statistical analyses of the data, however, indicated that there was no significant change in the distribution of grains over the cisternae, and they suggested therefore that a valid assignment of grains between the cisternae and the ground cytoplasm had been made. It should be noted that, although expressing the data in terms of "mean per cent of total grains adjusted for mean area" (Table IV) involves a more complex presentation than is usually employed, this type of presentation makes it possible to apply common statistical tests in evaluating the data.

The failure to find a significant change in the distribution of label over the Golgi vacuoles or the cisternae does not completely exclude the possibility that these compartments are involved in the extrusion of collagen, since after the conversion of protocollagen to collagen, the labeled collagen may pass through one of these compartments so rapidly

that no change in the concentration of radioactivity is detectable. The data, however, are consistent with the simpler notion that the completed collagen molecules pass directly from the ground cytoplasm to the extracellular matrix. This conclusion differs from that suggested by Revel and Hay (3) and from that suggested by Ross and Benditt (4). The reasons for the discrepancies are not apparent, but it is clear that the ability to correlate chemical and radioautographic data provides important advantages in the system used here.

Recent biochemical studies (34-36) suggest that the pathway for the synthesis of collagen is essentially the same in tissues in which protocollagen hydroxylase is not inhibited as in tissues in which it is inhibited, and that most of the hydroxylation of proline and lysine does not normally occur until after complete protocollagen polypeptides are released from ribosomes. Also, it has been shown that protocollagen hydroxylase is a soluble enzyme (16) presumably present in the ground cytoplasm. The biochemical data suggest therefore that the accumulation of protocollagen in the ground cytoplasm demonstrated here reflects the normal pathway for the synthesis and extrusion of collagen.

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