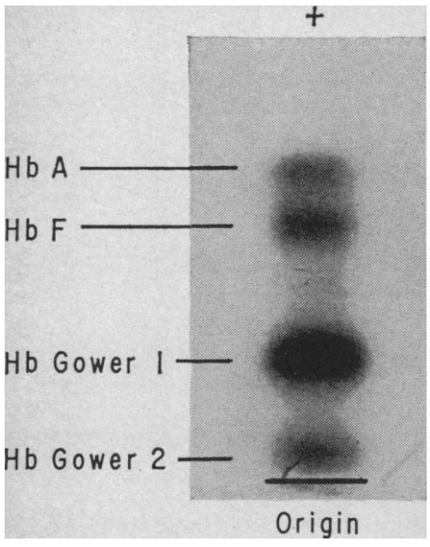

Fig. 1. Starch-gel electrophoresis of hemoglobins from 18.2-mm embryo in tris-citrate-borate buffer system, *p*H 8.6; stained with benzidene.

graphed (Fig. 1). The proportions of the various hemoglobins were estimated by transmission densitometry of the negatives; a Spinco model RB Analytrol was used.

In these three small embryos the combined proportion of the embryonic hemoglobins (Hb Gower 1 and Hb Gower 2) increased with decreasing embryonic size (Table 1). In the two smaller embryos Hb Gower 1 was the predominant fraction (*8*).

In each of the embryos the hemoglobin with the electrophoretic mobility of Hb A appeared to constitute at least 10 percent of the total hemoglobin. This concentration of Hb A is unexpectedly high for humans at this stage of development, in view of the finding that the proportion of Hb A is about 8 percent after a 35-week gestation period and in the range of 20 ± 10 percent at 40 weeks (*9*). Thus it might have been inferred that the concentration of Hb A should be much lower early in gestation.

Our data (Table 1) do not necessarily constitute evidence that the production of embryonic hemoglobins is initiated before that of Hb F and Hb A. The data do, however, indicate that there is either preferential production of Hb Gower 1 or preferential destruction of other hemoglobins in early embryonic life. This would signify either preferential production of polypeptide ϵ-chains, presumably the only ones in Hb Gower 1, or preferential destruction of other known (α, β, γ, δ) chains. We tend to favor the possibility of preferential production of ϵ-chains.

The significance of Hb Gower 1 early in human development may be that early embryos require a different type of hemoglobin for oxygen transport.

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Hydroxylation of Proline and the Intracellular Accumulation of a Polypeptide Precursor of Collagen

Abstract. *Autoradiographs of embryonic cartilage indicated that labeled protein accumulated intracellularly when the tissue was incubated with tritiated proline, and when the hydroxylation of proline was inhibited by anaerobic conditions or by a chelator for ferrous iron. The labeled protein apparently corresponds to protocollagen, the polypeptide precursor of collagen which serves as a substrate for the enzymatic synthesis of hydroxyproline.*

Although essentially all the hydroxyproline in vertebrate proteins is found in collagen, isotopic studies indicate that free hydroxyproline is not incorporated into collagen (*1*). These observations have recently been explained by evidence (*2*) suggesting that the hydroxyproline in collagen is synthesized by the hydroxylation of proline in a proline-rich polypeptide precursor of collagen. A proline-labeled polypeptide precursor of collagen was prepared by incubating embryonic cartilage with proline-C^{14} under anaerobic conditions (*3*). Because atmospheric oxygen was required for the hydroxylation (*4*), the synthesis of collagen hydroxyproline-C^{14} was inhibited to greater extent than the incorporation of proline-C^{14} into protein was inhibited. When protein fractions from the anaerobically labeled cartilage were subsequently incubated aerobically with a chick embryo homogenate, a net increase in protein-bound hydroxyproline-C^{14} was observed. The substrate for the hydroxylation was a large polypeptide for which the name "protocollagen" has been suggested (*3*). Since the enzymatic hydroxylation was inhibited by sodium (*3*), it occurred to us that the hydroxylation of protocollagen probably occurs intracellularly. We have found that when the hydroxylation is inhibited in embryonic cartilage, labeled protein which apparently corresponds to protocollagen accumulates within chondrocytes.

Tibiae which consisted primarily of cartilage were removed from 10-day-old chick embryos under sterile conditions. The tibiae were incubated with

L-proline-C^{14} or proline-3,4-H^3 (Table 1). After the incubation, the tibiae were homogenized in distilled water at 5000 rev/min in a tightly fitting Teflon and glass homogenizer. The homogenates were dialyzed with 100 mg of carrier L-proline and dialyzed against running tap water overnight; the dialyzed material was hydrolyzed by heating with an equal volume of concentrated HCl at 120°C and 16 pounds pressure (2 atm absolute pressure) for 15 hours. The hydrolyzates were evaporated to dryness at reduced pressure, and the residues were dissolved in 4.0 ml of water; 0.2-ml and 3.5-ml samples were taken for assay of total C^{14} (*5*) and of hydroxyproline C^{14} (*6*), respectively.

When the tibiae were incubated under anaerobic conditions for 1 hour, in some experiments the total incorporation of labeled proline was reduced to about one-half that of control values (Table 1), but essentially no difference from control values was observed in other experiments. The synthesis of labeled hydroxyproline was consistently inhibited to a greater extent than the incorporation of proline was. The fraction of nondialyzable C^{14} in the tissues which was accounted for by hydroxyproline-C^{14} decreased from 17.0 to 2.4 percent. When the tibiae were incubated with α,α'-dipyridyl, a chelator for the ferrous iron required by the enzymatic system (*3*), the incorporation of labeled proline was only partially inhibited; but the synthesis of labeled hydroxyproline was almost completely inhibited. The fraction of the nondialyzable C^{14} which was accounted for by hydroxyproline-C^{14} decreased from 17.0 percent to less than 0.1 percent. Unlabeled proline was added as a carrier (chase experiment) after the labeling in order to determine whether the labeled protein that accumulated under anaerobic conditions was subsequently hydroxylated when the tissue was exposed to air. Matched samples were incubated anaerobically for 1 hour, and a hundredfold excess of carrier L-proline was added before the incubation was continued for a 2nd hour. One sample was exposed to oxygen after the carrier was added, and the other was kept under nitrogen. Addition of the carrier inhibited further incorporation of labeled proline during the 2nd hour of incubation (compare samples E and F to samples A and B in Table 1), but there was a maximum degree of hydroxylation of the labeled

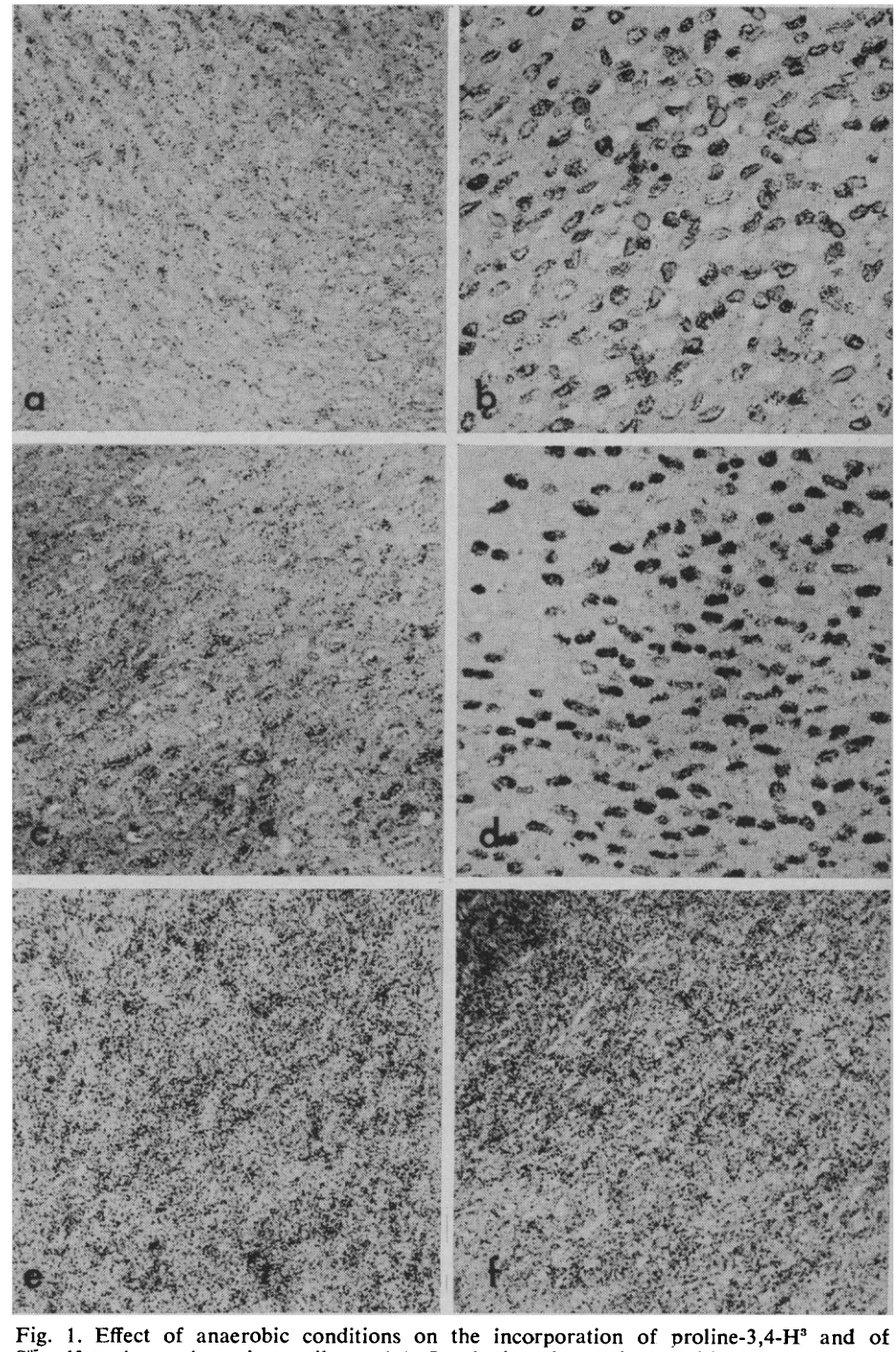

Fig. 1. Effect of anaerobic conditions on the incorporation of proline-3,4-H^3 and of S^{35}-sulfate by embryonic cartilage. (*a*) Incubation for 1 hour with 20 μc of proline-3,4-H^3 (4000 μc/μmole) in an atmosphere of 95 percent O_2 and 5 percent CO_2. (*b*) Incubation similar to (*a*) except the atmosphere was 95 percent N_2 and 5 percent CO_2. (*c*) Incubation for 1 hour with 20 μc of proline-3,4-H^3 in an atmosphere of 95 percent N_2 and 5 percent CO_2. Carrier L-proline (360 μg) was then added, and the gas mixture was changed to 95 percent O_2 and 5 percent CO_2 for a 2nd hour of incubation. (*d*) Same as (*c*) except that incubation for the 2nd hour was continued under 95 percent N_2 and 5 percent CO_2. (*e*) Incubation for 1 hour with 5 μc of sodium sulfate-S^{35}, 17 μc/μmole (New England Nuclear), in an atmosphere of 95 percent O_2 and 5 percent CO_2. (*f*) Incubation similar to (*e*) except that the atmosphere was 95 percent N_2 and 5 percent CO_2 ($\times$ 500). Conditions for the incubations are described in Table 1. After incubation, the tissues were fixed in 2 percent glutaraldehyde and 0.1*M* sodium phosphate, *p*H 7.5, for 1 hour, and they were dehydrated with graded ethanol solutions. The tissues were embedded in paraplast, and 5-μ sections were mounted on glass slides and covered with NTB3 liquid nuclear track emulsion (Kodak). After incubation at 4°C in the dark for 48 hours, the autoradiographs were developed with D-19 developer at 10°C for 5 minutes. The sections were stained with Alcian blue–hematoxylin before the emulsion was applied.

Table 1. Effect of anaerobic conditions and of α,α'-dipyridyl on the incorporation of labeled proline and the synthesis of hydroxyproline by embryonic cartilage. Four tibiae from two embryos were cross-paired for each set of control and test samples. The tibiae were incubated in 2.5 ml of a simple medium containing glucose, inorganic salts, and bicarbonate-phosphate buffer (*3*). Aerobic conditions were provided by flushing the incubation tubes with a mixture of 95 percent O_2 and 5 percent CO_2. Sample D contained α,α'-dipyridyl, $1 \times 10^{-3}M$. After the samples were equilibrated with the appropriate gas mixture at 37°C for 30 minutes, 5 μc of L-proline-C^{14}, 180 μc/μmole (New England Nuclear), in 0.5 ml of medium was injected through rubber stoppers used to seal the tubes. With samples E and F, 360 μg of carrier l proline was injected after 1 hour, and sample E was flushed with 95 percent O_2 and 5 percent CO_2. The number of counts per minute observed were converted to disintegrations per minute (dpm) by reference to a benzoic acid–C^{14} standard obtained from the National Bureau of Standards.

Sample	Incubation conditions	Time (hr)	Total C^{14} (10^3 dpm)	Hypro-C^{14} (10^3 dpm)	Hypro-C^{14}: total C^{14} (%)
A control	O_2	0–1	129	21.9	17.0
B test	N_2	0–1	58	1.4	2.4
C control	O_2	0–1	129	22.0	17.0
D test	O_2; α,α'-dipyridyl	0–1	117	< 0.1	< 0.1
E control	N_2	0–1			
	O_2	1–2	73	15.2	20.9
F test	N_2	0–2	77	2.1	2.8

proline in the sample exposed to air. Similar results (Table 1) were also obtained with L-proline-3,4-H^3 (New England Nuclear).

Autoradiographs of tibiae incubated with tritiated proline were prepared in order to determine the site at which the labeled precursor of collagen accumulated when the hydroxylation was inhibited. Cells in four stages of differentiation are normally observed in tibiae from 10-day-old chick embryos (*7*) as follows: (i) small, round cells in the articular condyles; (ii) flattened cells in the adjacent region; (iii) enlarging cells in the next region; and (iv) terminally hypertrophied chondrocytes in the middiaphysis. Cells in each of these regions have been shown to differ metabolically and functionally (*8*). Inhibition of the hydroxylation was accompanied by a marked effect on the cellular and extracellular distribution of isotope. When the tibiae were incubated aerobically for 1 hour or longer, the tritium was evenly distributed between the cellular spaces and the matrix (Fig. 1*a*). When the tibiae were incubated anaerobically, most of the tritium was retained over the cells (Fig. 1*b*). Autoradiographs of tibiae incubated with tritiated proline and α,α'-dipyridyl were similar to those obtained from tibiae incubated anaerobically with tritiated proline. The relatively small amount of label seen over the matrix in the inhibited samples may represent tritiated proline in proteins other than collagen (*9*). With tibiae in which the hydroxylation was inhibited, the density of labeling in different regions was similar to that in controls, and the regions containing the enlarging cells and the terminally hypertrophied cells exhibited the highest grain densities.

In other experiments, a chase procedure similar to that described in Table 1 was employed. Tibiae were incubated with tritiated proline under nitrogen for 1 hour, and then the tritiated proline was chased with carrier proline prior to a 2nd hour of incubation. When the incubation for the 2nd hour was conducted aerobically, tritium appeared predominantly over the matrix of the tissue (Fig. 1*c*). When the incubation for the 2nd hour was conducted anaerobically, most of the tritium remained over the cellular spaces (Fig. 1*d*).

Incubation under nitrogen or with α,α'-dipyridyl did not affect the distribution of sulfate-S^{35} incorporated by the tissue (Fig 1, *e* and *f*).

Recent reports (*2*) have indicated that labeled proline is incorporated into polypeptides when the synthesis of hydroxyproline is inhibited. Our results are the first demonstrations that polypeptide precursors of collagen accumulate intracellularly and that the accumulated precursors are subsequently converted to extracellular collagen when the inhibition of the hydroxylating reaction is released. Collagen contains approximately equal amounts of proline and hydroxyproline, and, since up to 20 percent of the labeled proline incorporated by inhibited tissue was subsequently converted to labeled hydroxyproline, at least one-third of the initially incorporated proline must have entered collagen precursors. Most of the labeled proline was retained intracellularly, and therefore the results indicate that, although major components of the polypeptide structure of collagen can be formed without the synthesis of hydroxyproline, the hydroxylation of specific proline residues is essential to incorporate the intracellular polypeptides into the matrix of connective tissue. We have not completely excluded the possibility that the two conditions used to inhibit the hydroxylation also inhibited other reactions essential to the secretion or extrusion of large molecules such as collagen. This possibility seems unlikely, and our observations indicate that the incorporation of sulfate-S^{35} into matrix was not affected. The capacity of chondrocytes to accumulate "protocollagen" appears to constitute a relatively unique mechanism whereby the overall rate of collagen synthesis is not appreciably affected by intermittent inhibition of the hydroxylating reaction necessary to complete the structure of the protein.

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