COLLAGEN SYNTHESIS BY ISOLATED BONE CELLS*

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Recent development of suitable techniques for isolating viable cells from well differentiated bone now permits direct investigation of bone cell metabolism (Flanagan and Nichols, 1963; Peck, Birge, and Fedak, 1964). Since bone formation includes the synthesis of collagen rich organic matrix, a function that is retained by whole bone fragments in vitro (Deiss, Holmes,and Johnston, 1962; Flanagan and Nichols, 1962) and cells derived from bone anlage (Fitton-Jackson and Smith, 1957; Smith and Fitton-Jackson, 1957), the characteristics of collagen synthesis by isolated bone cells were examined. Freshly dispersed cells were found to synthesize collagen briefly while incubated in suspension. Cells cultured for longer periods of time on a flat surface formed collagen after several days, and the rate of collagen synthesis increased as growth and multiplication produced high cell densities and rates of cell proliferation waned. Cells were initially resistant to the collagen synthesis promoting action of ascorbic acid, but became sensitive after

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several days in primary culture.

METHODS

Bone cells, obtained by digestion of fetal and newborn rat calvaria with crude collagenase as previously described (Peck, Birge, and Fedak, 1964), were suspended in appropriate medium after extensive washing. Cell densities were estimated by direct counting in a standard hemocytometer.

Collagen synthesis by freshly isolated cells was estimated by measuring labeled hydroxyproline in protein after incubation of cells in suspension with medium containing L-proline-U-C^{14}. Since collagen hydroxyproline is derived almost totally from free proline (Stetten, 1939), and since the ratio of proline to hydroxyproline in mammalian collagen is approximately one (Harrington and Von Hippel, 1961), synthesis of non-collagen protein was estimated from the amount of proline incorporated in excess of this ratio. After incubation, cells and medium were dispersed for one minute at 0°C with a Branson ultrasonic sonifier; the protein was then recovered by filtration through columns containing Sephadex G-25 (1 X 45 cm.) and hydrolyzed in sealed tubes at 110°C for 18 hours in 6 N HCl. Hydrolysates were dried *in vacuo* and radioactivity of individual amino acids in the hydrolysates was estimated following separation by descending chromatography on Whatman 3 MM paper using a phenol:water:sodium cyanide 75:25:02 w/w solvent system. Standard amino acids were used for reference in chromatography. Areas containing labeled amino acids were cut from the papers and assayed for radioactivity by liquid scintillation spectrometry. Overall recovery of labeled standards was consistently 60%. Virtually all of the radioactivity was located in areas corresponding to proline, hydroxyproline and glutamic acid. The identity of hydroxyproline was further substantiated by a solvent partition method (Peterkofsky and Prockop, 1962) and by

two-dimensional chromatography using two different solvent system combinations.

Collagen synthesis by cells dispersed on a flat surface and cultured for a longer period of time was studied with similar techniques. Cells were grown on the bottom of 25 ml. Erlenmeyer flasks at 37°C in minimal essential medium (Eagle, Microbiological Associates) containing Pencillin and Streptomycin 100 units each/ml. and 20% calf serum, or 1% bovine serum albumin. Radioactive proline was added 12 hours before termination of the culture. At termination, the medium was decanted, the cell layer washed briefly with neutral salt solution, and then dispersed by sonification. The resultant suspension was divided into two fractions. DNA was precipitated from one fraction with 5% cold perchloric acid (PCA), extracted with hot PCA and estimated colorimetrically (Ceriotti, 1952). The concentration of DNA was used as a measure of the number of cells (since direct cell counting requires enzymatic redispersion of cells from the flat surface). The other fraction was centrifuged and the insoluble residue washed briefly before hydrolysis. Radioactivity of the individual amino acids in each hydrolysate was estimated after separation by paper chromatography.

RESULTS AND DISCUSSION

The rate of appearance of labeled hydroxyproline in protein was most rapid during the first two hours of incubation and slowed considerably thereafter (Fig. 1). The rate of proline incorporation was more rapid than the rate of hydroxyproline formation and remained constant for at least six hours. When labeled proline was added to cells that had been preincubated in suspension for 12 hours and the incubation continued for three more hours, hydroxyproline was not incorporated into protein although proline continued to be incorporated, indicating cessation of collagen synthesis. Protein

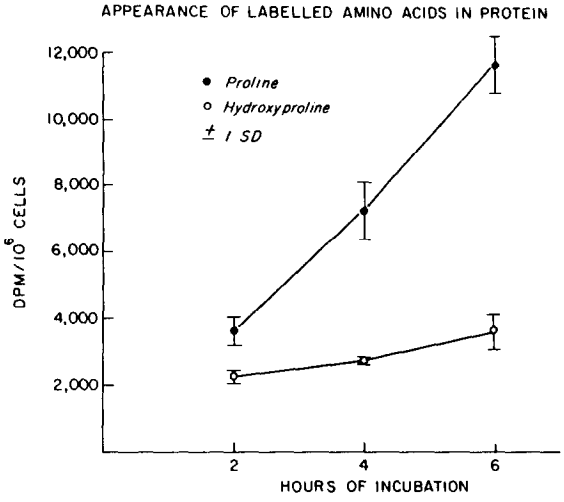

Fig. 1. Rates of appearance of proline and hydroxyproline radioactivity in protein. Each point represents the mean of three determinations. Freshly harvested cells were incubated in Krebs' Ringer bicarbonate buffer, pH 7.4, gas phase 95% O_2, 5% CO_2, containing L-proline-U-C^{14}, dialyzed bovine serum albumin 0.5%, glucose 0.011 M. and penicillin-streptomycin 100 units/ml. Proline incorporation was linear for six hours; the appearance of hydroxyproline increased most rapidly during the first two hours.

and hydroxyproline formation were directly proportional to the initial cell density (Table I) for cells incubated in Krebs' Ringer bicarbonate buffer. Ratios of hydroxyproline to proline radioactivity indicate that collagen comprises approximately 20% of the accumulated protein after incubation for six hours in Krebs' Ringer bicarbonate buffer. These findings indicate that collagen formation is an evanescent property of freshly isolated bone cells, but formation of non-collagen proline containing proteins continues. Furthermore, the character of the incubation medium may modify the types of proteins that are produced. In contrast to these results, Nichols, Flanagan, and Woods (1965) have found that cells isolated mechanically from the bones of young pigs do not synthesize collagen when incubated in suspension suggesting that the method of isolation may be critical in determining the preservation of this function, or that only cells derived from extremely rapidly growing tissues will continue to form collagen after isolation.

TABLE I

No. Cells/ml.	Incubation Medium	Radioactivity in Protein		Hydroxyproline / Proline Radioactivity Ratio
		Hydroxyproline	Proline	
8×10^6	KRB*	34,600	157,000	0.22
8×10^6	KRB*	37,000	172,600	0.21
8×10^6	KRB	18,600	84,000	0.22
8×10^6	KRB	18,500	108,000	0.17
4×10^6	KRB	9,800	45,300	0.22
4×10^6	KRB	9,300	59,500	0.16
4×10^6	MEM	5,200	13,200	0.40
4×10^6	MEM	5,000	14,400	0.34

* Initial ^{14}C proline radioactivity 0.5 microcuries/ml. In all other flasks radioactivity was 0.25 microcuries/ml.

Collagen synthesis by freshly isolated bone cells incubated in suspension. Relationship between the appearance of amino acid radioactivity in protein, the initial cell density and the type of incubation medium. Freshly isolated cells were incubated for six hours at 37°C in 25 ml. polyethylene bottles. Incubation medium was either Krebs' Ringer bicarbonate buffer (KRB) or minimal essential medium (MEM - Eagle), that contained glucose, 0.011 M, ^{14}C proline (12 microcuries/micromole, New England Nuclear Corporation), and dialyzed bovine serum albumin, 0.5%. The rate of appearance of radioactive proline and hydroxyproline in protein is directly proportional to the initial cell density in Krebs' Ringer bicarbonate buffer. Incubation in minimal essential medium produced a disproportionately large decrease in proline incorporation resulting in a significantly ($p < .01$) higher mean ratio of hydroxyproline to proline radioactivity (0.37) compared to incubation in Krebs' Ringer bicarbonate buffer (0.20).

Despite the transient nature of collagen synthesis by freshly isolated cells, cells that were maintained in culture on a flat surface regained the ability to produce collagen after several days, and thereafter the rate of collagen synthesis increased as the cell density rose and cell proliferation slowed (Fig. 2). These results suggest that the initial loss of collagen formation was related to factors other than death of competent cells, possibly

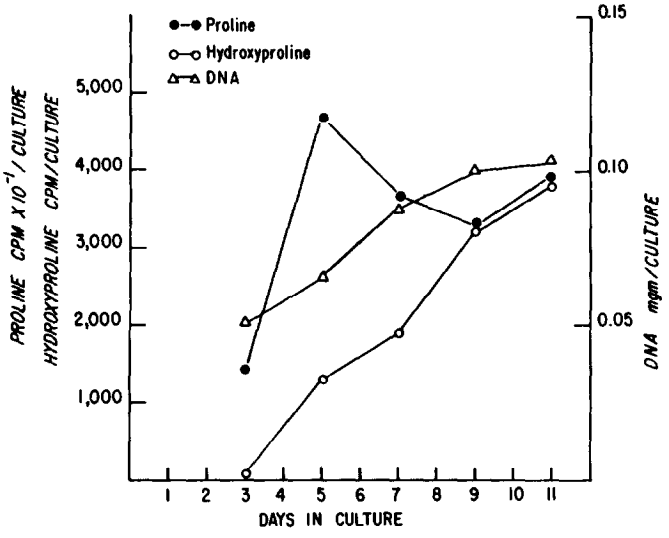

Fig. 2. Relationship between cell proliferation and appearance of labeled amino acids in protein during culture of bone cells on flat surfaces. Monolayers formed after culture for 8-10 days. L-proline-U-C^{14} was added to each culture 12 hours before termination, hence data indicate radioactivity appearing in protein during the preceding 12 hours. The rate of hydroxyproline formation increased dramatically as the cell population began to increase, and continued to rise after cell proliferation slowed. This was not accompanied by similar changes in proline incorporation which showed no consistent trend after the fifth day of culture

to an increase in the rate of cell proliferation. Recent studies have indicated that in vitro collagen synthesis by other connective tissue cell types is reduced during periods of rapid cell proliferation (Green and Goldberg, 1964; Prockop, Pettengill, and Holtzer, 1964). In addition, rapid cessation of collagen synthesis by freshly isolated cells could be explained by a progressive decrease in the influence of a substance such as ascorbic acid, an agent that is known to stimulate collagen formation in vitro (Shimizu, McCann, and Keech, 1965) and that could be carried by the cells through the process of isolation. However, addition of ascorbic acid did not enhance collagen formation by the freshly isolated cells. Seven to nine day-old cultures of bone cells did respond to the addition of ascorbic acid with a dramatic

increase in collagen synthesis (Table II) suggesting that ascorbic acid deficiency had developed during this period. Since bone cells in culture became sensitive to ascorbic acid *in vitro*, it is possible that

TABLE II

Ascorbic Acid γ/ml.	Hydroxyproline Radioactivity DPM/γ DNA ± S.D.	Proline Radioactivity DPM/γ DNA ± S.D.
0	60 ± 12	845 ± 290
50	384 ± 136*	1390 ± 280
100	302 ± 52*	1100 ± 200

*Values differ significantly from controls ($p < .01$).

Stimulation by ascorbic acid of collagen synthesis by bone cells grown in primary culture on a flat surface. Cells were maintained for 8-10 days in minimal essential medium (Eagle), 1% bovine serum albumin, glutamine and penicillin-streptomycin. Ascorbic acid was added to the culture 24 hours before termination and L-proline-U-C^{14} 12 hours before termination. Collagen synthesis was monitored by the appearance of labeled hydroxyproline in protein.

they will develop sensitivity to a variety of humoral agents, thus providing a suitable model for further study of the regulation of bone metabolism.

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