THE EFFECT OF ENVIRONMENTAL pH ON COLLAGEN SYNTHESIS BY CULTURED CELLS

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SUMMARY: The synthesis of collagen by cells in culture is markedly affected by environmental pH. In human cells the optimum pH is above pH 7.6, while a mouse fibroblast, L 929, demonstrated a more acid pH optimum. These optima coincide with those for general protein synthesis and growth. The pH optima for some specific steps in collagen synthesis and assembly have also been examined.

The marked fluctuations in medium pH that occur when cells are cultured in bicarbonate-buffered media are minimized by adding non-volatile organic buffers (1,2). Using such supplemented media, it was found that human diploid strains grow most rapidly and to highest density in the range pH 7.6 - 8.0, while a human cancer cell (HeLa), rabbit lens cells and mouse fibroblast (L 929) had more acid pH optima (2,3). Some functional activities of cultured cells are also pH-dependent. These include the synthesis of S100 protein by rat astrocytes[1] and of globulin by mouse myeloma cells[1], as well as reovirus synthesis[1], cell hybridization (4) and virus rescue after cellular fusion (5). Here we report the effect of environmental pH on collagen synthesis in cultured human and mouse cells.

MATERIALS AND METHODS

Cells were grown in closed T-15 flasks as previously described (1,6,7). The cells studied were the human diploid fibroblasts, KL, Renee and LaCol and the mouse line, L929. Strain LaCol was obtained from the American Type Culture Collection, Rockville, Md., and the other two human

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[1]Studies in progress.

strains were isolated in the laboratory of one of the authors (H.E.). Non-volatile buffers were combined as previously described (1,2) to obtain pH levels throughout the range pH 6.3 - 8.3. In some experiments with LaCol cells, it was necessary to halve the buffer concentration in order to avoid toxicity. In the dense cultures used to study collagen synthesis, medium pH changed by as much as 0.4 units in the course of the 24 hours of labeling even in the presence of nonvolatile buffers. The pH and protein values indicated in the figures and table are the geometric mean calculated from pH and protein measurements made at the beginning and end of the labeling period.

Cells were seeded in a minimal essential medium (8) containing 24 mM sodium bicarbonate as the buffering system, and supplemented with 5% each of fetal calf and calf serum. Forty-eight to seventy-two hours after seeding, the medium was replaced with fresh medium supplemented with 20 μg/ml of ascorbic acid and buffered to the desired pH with the nonvolatile buffers or with medium containing only bicarbonate buffer. Twenty-four hours later this medium was exchanged for 6 ml of identically buffered medium to which 20 μc each of (U-^{14}C)proline, (U-^{14}C)lysine and (U-^{14}C)glycine had been added. Two T-30 flasks were combined for each determination of isotope incorporation and collagen synthesis (9). Separate flasks without isotope were used for the determination of cell number and cell protein. In some cases, β-aminopropionitrile·HCl (50 μg/ml) was added to the medium to prevent the crosslinking of collagen and allow it to be extracted and characterized.

Cells were usually incubated with labeled media for 20 hours (LaCol) or 24 hours (the other cells). The medium was then decanted and dialyzed against 1% acetic acid until radioactivity in the dialysate approached background levels. The cell layer was detached with a rubber policeman and rinsed from the flask with a total of 10 ml of 1M NaCl, 0.05M Tris.HCl at pH 7.5. After stirring at 4° for at least 24 hours, soluble material was separated by centrifugation at 17,000 g for 15 min. Both the supernatant fluid and the pellet were resuspended in distilled water and dialyzed as

indicated above for the medium. Some samples prepared in this manner were hydrolyzed in 6N HCl at 105° for 48 hours prior to application on an amino acid analyzer equipped for continuous monitoring of the radioactivity in the column effluent (10).

To determine the chain composition of the collagen synthesized at different pH levels, confluent cultures were preincubated for 24 hours in media buffered at pH 7.0, 7.4 or 8.0. These media were replaced with fresh media to which the labeled amino acids as well as β-aminopropionitrile·HCl (50 μg.ml) and ascorbic acid (20 μg/ml) were added. Twenty hours later the media were decanted and the cell layers detached and extracted with 1M NaCl, 0.05M Tris-HCl (pH 7.4) at 4° for at least 24 hours. These suspensions were centrifuged at 17,000 g for 15 min and collagen (20 mg) prepared from the skins of lathyritic rats was added to each as carrier. Following dialysis against 0.05M acetate buffer adjusted to pH 4.8, the heat denatured preparations were chromatographed separately on carboxymethyl (CM)-cellulose at 45° (11). Radioactivity in the column effluent was measured in a commercially prepared solvent (Aquasol) by liquid scintillation counting.

RESULTS

Previous studies have shown that human cell strains KL and Renee grow best at a more alkaline pH than do L929 cells. The experiments reported here were designed to establish how collagen synthesis varied in cells grown and labeled at various pH levels. The formation of nondialyzable ^{14}C-hydroxyproline from ^{14}C-proline was used as a measure of collagen synthesis, and the incorporation of ^{14}C-proline into nondialyzable macromolecules was used to estimate total protein synthesis (12). The protein content of the cell layer was also measured. In the human strains reported here 3-10% of the incorporated ^{14}C-proline was hydroxylated in different experiments. The total amount of ^{14}C-hydroxyproline formed was dependent on the pH at which the cells had been grown (Fig. 1). In general, ^{14}C-hydroxyproline formation

paralleled the incorporation of ^{14}C-proline into nondialyzable material (presumably reflecting total protein synthesis (12)(Table 1). The results of Figure 1 are modified quantitatively but not qualitatively if expressed as ^{14}C-hydroxyproline formation per unit protein in the culture flask rather than per culture flask.

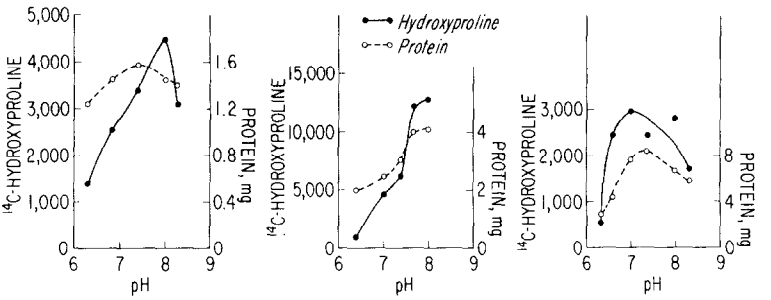

Fig. 1. ^{14}C-Hydroxyproline formed by cells grown at different pH levels. The protein levels in the cell layer are also given. Data on the left were obtained from human strain KL; those in the center from human strain Renee, and those on the right from the mouse line L929.

Similar measurements made on the incorporation of ^{14}C-lysine into macromolecules and the appearance of ^{14}C-hydroxylysine are shown in part in Table 1. These and other results (not shown) demonstrated that variation of pH had an identical effect on the incorporation of ^{14}C-lysine and ^{14}C-proline incorporation. ^{14}C-Hydroxylysine formation was proportional to the incorporation of ^{14}C-lysine into macromolecules.

Since the mouse cell line L929 shows a higher rate of growth at lower pH values than human cell strains, we carried out studies similar to those outlined above on the mouse line. The pH optimum for ^{14}C-hydroxyproline formation (Fig. 1) and ^{14}C-hydroxylysine formation (not shown) in these cells were lower than those observed with the human cell strains. Again the formation of the amino acids specific for collagen paralleled the incorporation of ^{14}C-proline and ^{14}C-lysine into macromolecules, which was in turn proportional to cell protein (not shown).

Human skin fibroblasts cultured in bicarbonate-buffered media synthesize

TABLE 1

INCORPORATION OF LABELED AMINO ACIDS INTO MACROMOLECULES IN HUMAN CELLS WITH BUFFERED BICARBONATE OR WITH A NONVOLATILE BUFFER AT pH 7.9

	^{14}C cpm	
Cells grown in HCO_3^- buffer*	Cell layer	Medium
Hydroxyproline	2,380	791
Proline	63,700	37,759
Hydroxylysine	7,646	1,146
Lysine	44,490	13,112
Cells grown in nonvolatile buffer, average pH 7.7†		
Hydroxyproline	7,114	5,354
Proline	149,638	98,046
Hydroxylysine	11,784	2,232
Lysine	85,241	21,910

* pH varying widely during period of incubation.

† The initial medium pH of 7.9 fell to pH 7.5 after 20 hours in culture.

a collagen identical to the major protein component of human skin, with the chain composition $(\alpha 1)_2\alpha 2$ (9). To establish the nature of the collagen synthesized by cells grown at different pH, the human diploid strain La Col was grown in medium buffered with the nonvolatile buffers at pH 7.0, 7.4 or 8.0 or with bicarbonate alone, and the collagen extracted and chromatographed on CM-cellulose as described in Methods. Three major radioactive peaks were observed in all samples (Fig. 2) corresponding to the elution positions of proα1, α1 and α2. Proα2 is not well resolved from α2 on CM-cellulose (13) and may account for a portion of the material eluting in the region of α2. The ratio of α1 type chains to α2 type chains was 2:1, with no consistent qualitative differences between the collagen formed in the bicarbonate or nonvolatile buffers. More of the ^{14}C-amino acids were incorporated into

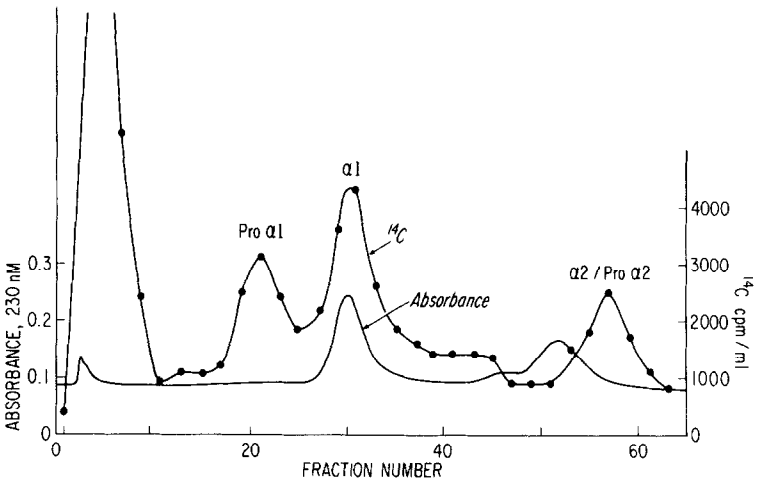

Fig. 2. CM-cellulose elution pattern of the salt soluble radioactive protein extracted from the cell layer of the human strain La Col maintained in non-volatile buffer at average pH 8 (initial pH 8.2, final 7.8). Pattern of absorbance of denatured rat skin collagen was used to mark recoveries and positions of α1 and α2 chains.

collagen at 7.4 and 8.0 than at 7.0 or in bicarbonate buffer. Similar observations were made with the human strain Renee.

Previous studies have shown that 50 to 60% of the nondialyzable ^{14}C-hydroxyproline synthesized by cells grown and labeled in bicarbonate buffer is present in the medium (12,9). In this study no change was noted in the distribution of nondialyzable ^{14}C-hydroxyproline formed by human cells (Renee) grown and labeled in the presence of nonvolatile buffers over the pH range shown in Fig. 1.

Finally, we have measured the proportion of nondialyzable ^{14}C-hydroxyproline that could be extracted with 1M NaCl, 0.05M Tris-HCl, pH 7.4 from the cell layer. With the exception of the material synthesized at the very lowest pH level, the extractability of cell layer associated ^{14}C-hydroxyproline decreased with increasing pH (Fig. 3).

DISCUSSION

Many quite different types of cells in culture synthesize collagen, as judged by the formation of peptide-bound hydroxyproline or the occurrence of extracellular structures with the characteristic collagen fibril band

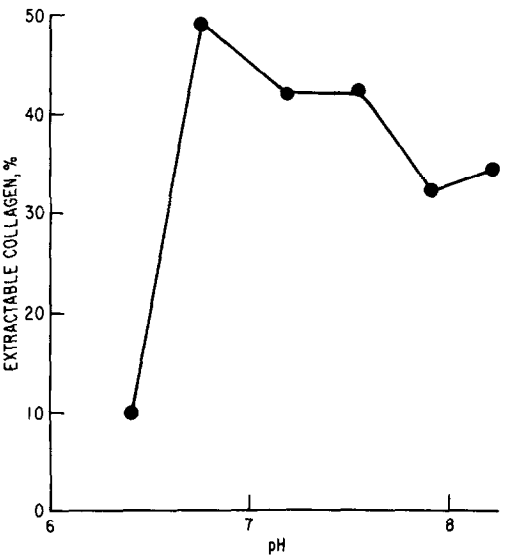

Fig. 3. The extractability in 1M NaCl, 0.05M Tris.HCl of collagen in cell layers of the human strain Renee maintained at varying pH.

pattern when examined in the electron microscope (12). Steps in the formation of collagen and its deposition as fibers include the synthesis of precursor polypeptide chains, the hydroxylation of certain prolyl and lysyl residues, macromolecular assembly of the proα chains into a triple helical procollagen molecule, conversion of procollagen to collagen, precipitation of collagen into fibers and crosslinking (14, 15). It is not known if the control for collagen synthesis is specific to this protein, and if so, whether any one of the steps in its synthesis is limiting. Since environmental pH affects the synthesis of other proteins, we have examined its effects on collagen synthesis as well.

As judged by the formation of ^{14}C-hydroxyproline, as well as labeled polypeptide chains, the synthesis of collagenous proteins varied markedly with the pH at which cells are grown. Hydroxylysine formation paralleled that of hydroxyproline, which might have been expected since the two occur together in the same polypeptides. In general, hydroxyproline formation was related to the amount of cell protein, but the differences in hydroxyproline formation at varying pH usually exceeded the differences in cell protein.

Even if the synthesis of peptide-bound hydroxyproline were affected to exactly the same extent as that of other cell proteins, we would expect relatively greater accumulation of the peptide-bound hydroxyproline due to differences in turnover, since cultured fibroblasts are not known to produce enzymes capable of degrading native, triple helical, collagenous proteins.

Preliminary characterization of the collagenous protein associated with the cell layer demonstrated that the ratio of α1 to α2 species was independent of pH and buffer type, in the range pH 7 to 8. Alpha 1 and α2 type chains were recovered in the ratio of 2:1 which suggests that the cells synthesized a collagen molecule with the chain composition $(\alpha 1)_2\alpha 2$.

In contrast to skin collagen which is not-soluble under physiological conditions, a considerable portion of the peptide bound hydroxyproline synthesized by cultured cells is found in the medium. Recent studies have shown that most of this hydroxyproline is present in procollagen, a macromolecular precursor of collagen (9, 16). Procollagen is efficiently converted to collagen *in vivo* (unpublished observation) and by tissue explants *in vitro* (17), but for unknown reasons cells in culture convert only half or less of the procollagen, and it accumulates in the medium. As judged by the proportion of newly synthesized hydroxyproline that appeared in the medium, environmental pH in the range pH 7.0 - 8.0 did not affect the conversion of procollagen to collagen. This is in accord with the observation of Lapiere *et al.* (18) that procollagen peptidase, an enzyme tentatively identified as mediating this conversion, is not affected by fluctuations in pH over this same range.

The crosslinking of the collagen associated with the cell layer did however change with pH. These cross-links are formed from lysine- and hydroxylysine-derived aldehydes. Aldehyde formation is catalyzed by lysyl oxidase which has a pronounced optimum for activity at pH 7.8 (19). We examined the extractability in neutral salt of collagen from cell layers grown and labeled at varying pH. Uncrosslinked collagen dissolves, while

crosslinked collagen does not (20). As the pH was decreased from pH 8.0 to 6.8, the collagen formed was increasingly extractable. This may reflect changes in lysyl oxidase activity. A marked, abrupt and unexplained decrease in the extractability of collagen was observed at the very lowest pH level studied.

Previous studies have shown that the formation of peptide-bound hydroxyproline increases as cells in culture approach saturation density (12). The possibility that the more acidic conditions prevailing in dense cultures increased the rate of collagen synthesis has been investigated (21). It was found that lactate ion, not decreased pH, stimulated the formation of peptide bound hydroxyproline by cells in culture particularly at low cell density (21). Subsequently it was shown that prolyl hydroxylase is limiting during the early log phase of growth (22) and that lactate stimulates the conversion of a precursor form to active enzyme (23). We are probably dealing with a different effect of pH on collagen synthesis. Changes in collagen synthesis at various pH levels were found both by measuring the synthesis of polypeptide chains by human cells as well as peptide bound hydroxyproline. Since the optimal pH for collagen synthesis coincides with that for cell growth in cells with different pH optima, it is likely that the changes in collagen in contrast to certain other activities of cultured cells (4,5) reflects effects on general protein synthesis.

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