

Ascorbate Induction of Collagen Synthesis as a Means for Elucidating a Mechanism for Quantitative Control of Tissue-Specific Function

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Ascorbic acid displays the characteristics of an ideal inducer of tissue-specific function in primary avian tendon cells in culture. It is a highly specific, potent stimulator of collagen synthesis, it demonstrates slow reversible kinetics, and it has no effect on growth rate of the cultured cells. Kinetic analysis of ascorbate induction of collagen synthesis was used to determine the critical steps in this complex biosynthetic pathway. Full hydroxylation of the proline residues in collagen, although probably a necessary step for collagen induction, was in itself not sufficient for achieving either increased secretion or increased synthesis. On the other hand, an increase in secretion rate, which required both the presence of ascorbate and a high cell density, did correlate with the later stimulation in procollagen production. The process of procollagen secretion, therefore, meets the minimal requirements for the rate-limiting step. The fact that the cells maintained a large pool of intracellular procollagen despite changes in the rates of translation or secretion led us to postulate a possible feedback between the level of the internal procollagen pool and the rate of procollagen synthesis.

The quantitative expression of tissue-specific function is precisely regulated within the organism, but this regulation can be readily lost when cells are cultured (2, 6, 10, 35). In primary avian tendon (PAT) cells, the ability to maintain in ovo levels of collagen synthesis (30% of total protein synthesized [11]) is directly related to the cell culture environment (35, 36, 37). Of the many factors that influence cells in culture through poorly understood mechanisms, in this paper we have focused on ascorbic acid for several reasons. Ascorbic acid serves as a clear example of how a small molecule can influence the protein synthetic machinery and the level of post-translational modifications in eucaryotic cells. In PAT cells, ascorbic acid shifts the level of protein synthesis devoted to collagen from 5 to 10% to 20 to 30% (35) and greatly increases the hydroxylation of proline residues in collagen. This modulation becomes especially significant since ascorbate deprivation in vivo, i.e., scurvy, is characterized by the same quantitative and qualitative response as that observed in PAT cells in culture (3).

Additionally, although the general mechanism of ascorbate action appears to be straightfor-

ward, acting as a moderate reducing agent (25), little can be stated definitively about how it interacts with the cell. What complicates our understanding of the mechanism of ascorbate action is that its effects on cultured cells cannot be generalized. Some cell types respond to ascorbate by raising the level of collagen synthesis (35); in others, the presence of ascorbate does not change the makeup of the proteins synthesized (4, 29); some cell types have even been reported to reduce collagen synthesis when ascorbate is added (28). With regard to the proline hydroxylation step, most cell types will maximally hydroxylate in the presence of ascorbate (29); there is one report, however, that ascorbate is ineffective in increasing hydroxylation levels when the cells are at a low cell density (9); yet other cell types can fully hydroxylate their collagen molecules at high cell density whether or not ascorbate is present (14, 16, 29). Similarly, ascorbate appears to aid secretion of procollagen in some (7, 26, 34) but not all (4) cells. In those cells that are positively affected, ascorbate appears to act by changing the degree of hydroxylation and thereby the stability of the collagen triple helical structure (13, 20)—a hydroxylated helix is thought to be secreted more easily. However, large pools of both proline and procollagen within the cell make it difficult to interpret

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secretion rates solely on the basis of the rate of accumulation of radioactivity in the medium. Furthermore, an inhibitor of hydroxylation, α, α' -dipyridyl, which has been used in secretion studies, affects the specific activity of the synthesized proteins (23), further complicating the issue. Therefore, in each of the areas that ascorbate has been implicated in affecting collagen synthesis or processing, the data are confusing and the role of ascorbate remains unclear.

We feel that the noted lack of uniformity of ascorbate action can be traced to an inability to adequately define how the standard cell culture environment can affect the response of a cell. *In vivo*, but especially in culture, a cell is not a constant, but is a dependent variable affected in both reversible (35) and irreversible (37) ways by its milieu. A clearer and more reproducible analysis can be made of how ascorbate affects the synthesis and processing of the collagen molecule by using the PAT cell system, which has been developed for the maintenance of the original *in ovo* level of differentiated function by specifically controlling the cell culture environment.

The aim of this paper is to ask several questions. Is ascorbic acid a specific inducer of collagen synthesis? Is hydroxylation of proline the critical point—the rate-limiting step (33)—in collagen synthesis? Is the rate of collagen secretion directly related to the presence of ascorbate? And finally, how does ascorbate interact with the cell to affect the protein synthetic pattern?

(An abstract of part of this work has appeared previously [Fed. Proc. 37:1529, 1978].)

MATERIALS AND METHODS

Cell culture. PAT cells were isolated by a modification (36) of the Dehm and Prockop (11) procedure. Cells (8×10^6 cells in 25-cm² flasks; Falcon) were allowed to attach in 5 ml of F12 medium (17) for 40 min. The medium was then changed and the cells were grown in 10 ml of F12 with 0.15% fetal calf serum (GIBCO Laboratories; deactivated for 30 min at 56°C) with subsequent daily changes of medium. When ascorbic acid (J. T. Baker Chemical Co.) was used in the medium (50 $\mu\text{g}/\text{ml}$), it was added daily from 100 \times stock. The stock solution of ascorbic acid was freshly prepared every other day.

Collagen assays. Collagen synthesis was measured in two ways: (i) by collagenase digestion, using a modification (36) of the method of Peterkofsky and Diegelmann (31); and (ii) by the proline/hydroxyproline ratio method, using two-dimensional paper chromatography to separate the amino acids (37).

Polyacrylamide gel electrophoresis (PAGE). Samples were run on a Pharmacia slab gel apparatus (7.5% acrylamide and 0.112% bisacrylamide [Bio-Rad Laboratories], 0.08 M tris(hydroxymethyl)amino-

methane [Tris]-acetate, and 0.004 M ethylenediamine-tetraacetic acid [EDTA], pH 7.4; 0.3 by 14 cm). Gels were made in advance and refrigerated; before each run, they were pre-electrophoresed for 30 min at 100 V in running buffer (0.04 M Tris-acetate-0.002 M EDTA [pH 8.3]-0.1% sodium dodecyl sulfate [SDS]). Samples were applied to the gel and then electrophoresed for 16 h at 50 V. Gels were stained by the method of Scott et al. (39). The film used for autoradiography was Kodak X-ray film SB-5.

Sample preparation for PAGE. (i) Cells. Medium was removed and the cell layer was washed with F12 medium. Then 100 μl of lysis buffer (2.5% SDS, 5% β -mercaptoethanol, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co.], 0.08 M Tris-acetate, and 0.004 M EDTA, pH 7.4) was added. Cells were dispersed with a rubber policeman, and the solution was then heated to 55°C for 30 min.

(ii) Medium. Medium was concentrated 30-fold at 4°C by using a membrane vacuum ultrafilter (Millipore Corp.). The concentrate was then diluted 10-fold with buffer (0.08 M Tris-acetate-0.004 M EDTA, pH 7.4) and reconcentrated. Additions were made to the final concentrate to make it equivalent to the lysis buffer above. The sample from the medium was also heated at 55°C for 30 min.

Pulse-chase experiments. Seven-day-old PAT cultures were labeled with 10 μCi of [¹⁴C]proline/flask in 1.5 ml of F12 medium with 0.15% serum for 2 h. Cells were then washed three times with 10 ml of F12 medium with serum containing 100-fold excess (3×10^{-2} M) cold proline, and 1.5 ml of 100 \times proline medium was added to each flask. At various times (0, 10, 20, 30, and 45 min and 1.5, 2, 2.5, and 3 h), the cells were assayed for collagenase-sensitive radioactivity.

Specific activity of the radioactive proline. The method of Airhart et al. (1) was used. The ratio of a known specific activity [³H]dansyl chloride (Amersham Corp.), was compared with that of an unknown [¹⁴C]proline sample after reacting and separating the dansyl proline derivative by thin-layer chromatography.

RESULTS

Correlation between the level of proline hydroxylation and the level of collagen synthesis. PAT cells at low cell density (0.8×10^6 cells/25-cm² flask) synthesized a low percentage of collagen relative to total protein synthesis irrespective of the presence or absence of ascorbate. As the cells approached a confluent monolayer ($\approx 2 \times 10^6$ cells/flask), ascorbate caused the commitment to collagen synthesis to increase from 5 to 10% to 20 to 30% (35).

To understand the reason behind this restriction by cell density on the ability of ascorbate to stimulate collagen synthesis, we examined the possibility that ascorbate might assure a normal level of hydroxylation (47% of the total prolines in type I collagen) only when the cells were at high cell density. Such a restriction has been observed by Bruel et al. (9) for human lung

fibroblasts. To explore this hypothesis, we measured the percentage of collagen produced by PAT cells in two ways as they grew from low density to confluent monolayers. One method of determining the percentage of collagen synthesized, using collagenase to release incorporated counts in collagen, is independent of the level of hydroxylation; the other method, comparing incorporated counts in proline to that in hydroxyproline, is dependent on proline hydroxylation. The difference between the two measurements is thus directly related to and can be used for testing the degree of hydroxylation of the prolines in collagen. The results (Table 1) show that whenever ascorbate was present, the synthesized collagen was maximally hydroxylated ($99 \pm 16\%$). In scorbutic cultures, the level was only $22 \pm 8\%$ of maximal hydroxylation irrespective of cell density.

The degree of hydroxylation after ascorbate addition could further be demonstrated by means of a subtle shift to a slower mobility in the newly synthesized procollagen bands on SDS-PAGE (see autoradiogram of cellular procollagen in Fig. 2, lanes 9 and 10). A similar shift was also observed by Kao et al. (23) when inhibitors of hydroxylation were added to freshly isolated tendon cells. With PAT cells, this change in mobility of the procollagen bands was observed whether ascorbate was added to high- or low-density cultures. In PAT cells, therefore, ascorbate addition was equally effective at high or low cell density in producing collagen with maximal degree of proline hydroxylation, and the level of hydroxylation did not appear to relate directly to the level of collagen synthesis.

Induction of collagen: requirement for both high cell density and ascorbate. Ascorbate is capable of increasing proline hydroxylation at low cell density, yet is incapable of

stimulating collagen synthesis until the cells reach high cell density. This is not due to slow kinetics of ascorbate induction. PAT cells at low density can be subcultured and maintained in ascorbate-containing medium for over a week with no increase in collagen synthesis (37), and yet at any time during this period raising the cell density will stimulate collagen production (35, 37). To bring further insight into this effect of cell density, we developed a standard experimental protocol.

PAT cells were grown for 4 days without ascorbate; ascorbate was added on day 5 (time zero) and with each subsequent medium change. Cultures were monitored during the following 48 h, the time required for maximal induction. Figure 1 shows the SDS-PAGE pattern of cellular proteins and the proteins from concentrated medium of cells that had been pulsed for 3 h with [^{14}C]proline at time zero (day 5) and at 45 h later (day 7). The Coomassie blue-stained gels of the cellular components (Fig. 1A) showed a complex pattern expected whenever whole cell lysates are applied to a gel. At this level of resolution, the protein banding patterns were extremely similar and independent of the duration of the ascorbic acid treatment (the greater intensity on day 7 was due to a doubling in cell number). A considerable simplification of these patterns was achieved by comparing the corresponding autoradiograms. Since the cells were pulsed with proline and proline occurs approximately four times more frequently in procollagen than in the average protein, the proline-labeled procollagen bands were especially prominent. The protein pattern in the medium was also simplified because the serum proteins, being unlabeled, did not interfere.

The autoradiograms revealed the $\text{pro}\alpha_1$ and $\text{pro}\alpha_2$ bands within the cell (Fig. 1B). These were identified by mobility, high specific activity of [^{14}C]proline in the band, collagenase susceptibility (27), and hydroxyproline content (data not shown). The medium showed four intense bands in the procollagen region. Two migrated with the same mobility as the $\text{pro}\alpha_1$ and $\text{pro}\alpha_2$ bands inside the cell, and two had the mobility of the cleavage products resulting from the action of procollagen peptidases (24). Three much less intense bands were observed in the α -chain region of the gel.

The autoradiograms and the Coomassie blue-stained gel showed that most bands increase as a function of cell number as expected. The exception was the procollagen bands in the medium. Thus, the increase in the percentage of procollagen synthesis with cell density in the presence of ascorbate could be accounted for, almost entirely, by an increase in the absolute

TABLE 1. Lack of effect of cell density on proline hydroxylation^a

Culture	Hydroxylation (%)	
	Low cell density	High cell density
+ Ascorbate	112 ± 17	90 ± 5
- Ascorbate	24 ± 12	20 ± 3

^a In fully hydroxylated type I collagen, 47% of the prolines are hydroxylated. In the table, 100% represents fully hydroxylated collagen. Measurements were made on cells grown from low density (1.6×10^4 cells/cm²) to confluent cultures (7.2×10^4 cells/cm²). No cell density effects on hydroxylation were noted. To simplify the presentation of the data, we have combined all data obtained with cells at less than 0.8×10^6 cells/flask as low cell density and above that point as high cell density.

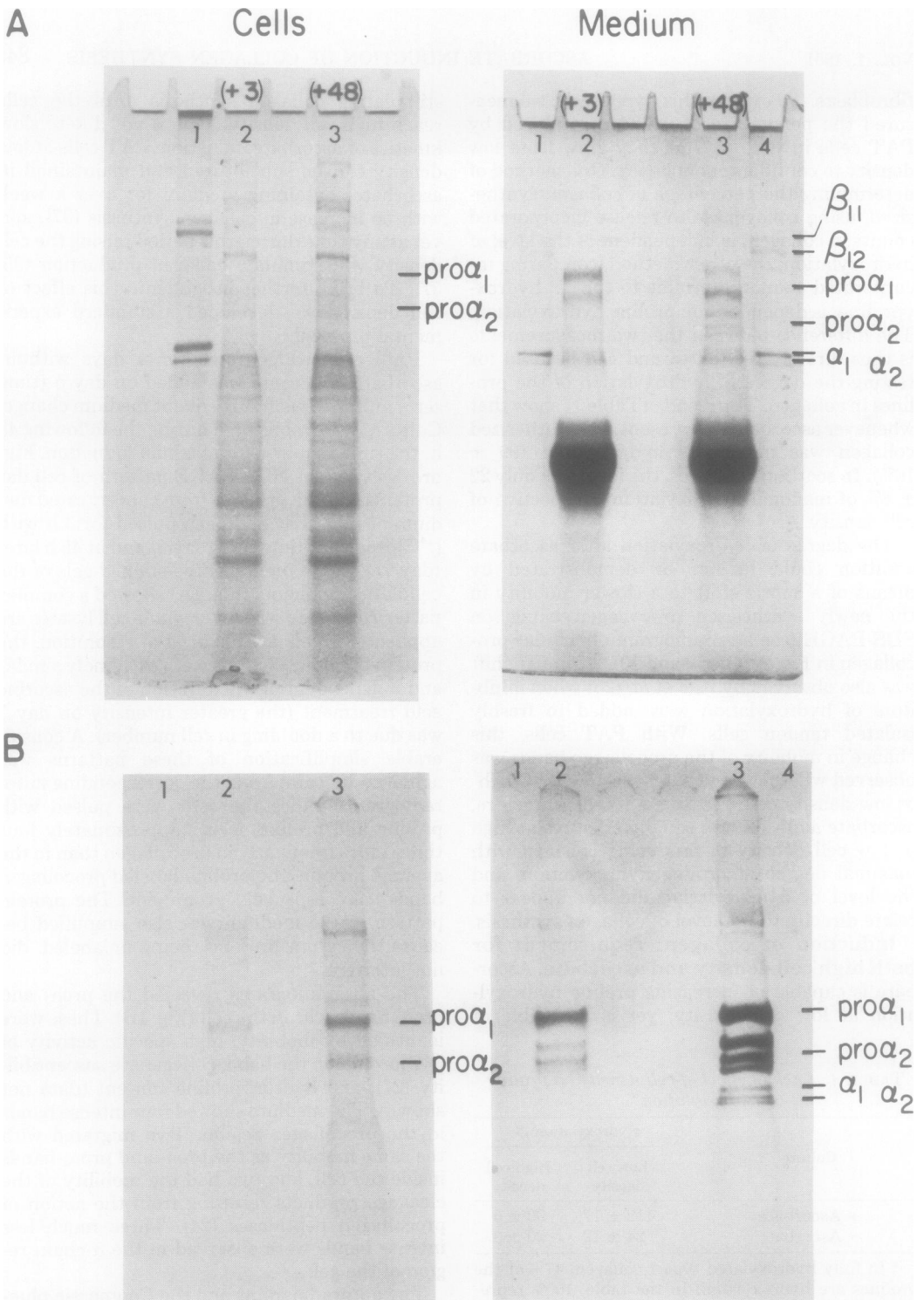


FIG. 1. Effect of cell density on the protein synthetic pattern of PAT cells in the presence of ascorbate. Cells were grown for 4 days without ascorbate. At day 5, time zero, cells were given ascorbate (50 $\mu\text{g}/\text{ml}$). Cells at time zero and again 45 h later were pulsed with 5 μCi of [^{14}C]proline for 3 h in 1.5 ml of F12 plus 0.15% serum and ascorbate. At the end of the pulse, the medium was concentrated and applied to a slab gel as described (see text); the cell layer was solubilized and also applied to the gel (see text). (A) Coomassie blue-stained gel of both cell-associated proteins (left) and medium-associated proteins (right) 3 h (lane 2) or 48 h (lane 3) after receiving ascorbate. Lanes 1 and 4 were loaded with a calf skin collagen (Calbiochem) as a standard. (B) Autoradiogram of the above gels.

synthesis of procollagen which accumulated in the medium.

The qualitative observations of Fig. 1 could be put into a quantitative form by measuring the total collagenase-sensitive and -insensitive counts (Table 2). Similarly, with this method, the major change in protein synthesis as the cells approached confluency was an increase in procollagen synthesis, and this was mostly secreted into the medium at the end of a 3-h pulse.

The interpretation of the data presented in Table 2 depends on the assumption that cell density and the presence of ascorbate do not alter the specific activity of the proline pool. Observations by Bruel et al. (9) have shown that the specific activity of the proline pool can change with cell density. Their studies, however, were very different from ours in that they used Dulbecco modified Eagle medium and not F12. The significant difference between the two media is that the former contains no proline, whereas the latter contains 3×10^{-4} M proline. In Dulbecco modified Eagle medium, the level of cellular production of proline critically affects the specific activity. In F12 medium, the high external concentration of proline and its uptake by the cells would be expected to turn off or overwhelm cellular production of proline; i.e., it is expected that the specific activity of the internal proline pool would equilibrate with the large external pool and be relatively independent of cell culture conditions.

This assumption was tested in several ways. One was to actually measure the specific activity of the internal and external proline pools at the end of the assay period (3 h) to ascertain equilibration. By using the elegant technique of reacting a known specific activity [^3H]dansyl chloride molecule with an unknown specific activity [^{14}C]proline molecule (1), one can obtain the proline specific activity by measuring the ratio

of $^{14}\text{C}/^3\text{H}$ in the dansyl proline derivative. In all cases tested (high density, low density, minus ascorbate, plus ascorbate), the pools had indeed equilibrated by 3 h (data not shown).

The invariance of the specific activity of the internal proline pool was tested further by observing the rate of synthesis of noncollagen proteins. If one follows the kinetics of incorporation of [^3H]proline into noncollagen proteins in fully induced and scorbutic cultures, and if specific activities are identical under both conditions, then the rate of incorporation should be identical. This was indeed the case (see Fig. 4).

Another test for whether the internal and external proline pools did indeed equilibrate was to compare the calculated rate of collagen synthesis using a radioactive label with the rate measured by an independent method. By using the known specific activity of [^3H]proline in the medium and knowing the rate of incorporation into collagen, one can calculate the rate of procollagen production to be $0.5 \mu\text{g}/\text{h}$ per 10^6 cells. Alternatively, due to the high levels of collagen production in PAT cells, the level of procollagen synthesis during the same 3-h period could be ascertained by comparing the level of staining of secreted procollagen by Coomassie blue on SDS-PAGE with that of standards (chick calvaria collagen). By this method, the cell synthesized approximately $0.6 \mu\text{g}/\text{h}$ per 10^6 cells (the values obtained by these two methods compare favorably with the value obtained by Kao et al. [22] for freshly isolated tendon cells, $0.63 \mu\text{g}/\text{h}$ per 10^6 cells). Again, the agreement between these two methods indicates equilibration of the internal and external proline pools. It can be concluded that in PAT cells in F12 medium, the internal and external proline pools do equilibrate within 1 h (see Fig. 4) and that this is independent of cell density or the addition of ascorbate.

Both ascorbate and high cell density were

TABLE 2. Effect of cell density and ascorbate on the level of collagen synthesis^a

Culture	Collagen synthesis, pc/nc (cpm/cell $\times 10^{-5}$)				Ratio, 48 h/3 h	
	3 h		48 h			
	Cells	Medium	Cells	Medium	Cells	Medium
+ Ascorbate	63/160	24/34	86/158	145/31	1.4/1.0	5.6/0.9
- Ascorbate	100/208	19/51	68/175	15/30	0.7/0.8	0.8/0.6
Ratio, + Ascorbate/- Ascorbate	0.63/0.8	1.4/0.7	1.3/0.90	9.7/1.0		

^a PAT cells were grown for 4 days without ascorbate. On day 5, half of the flasks received ascorbate ($50 \mu\text{g}/\text{ml}$). Cells were pulsed with [^{14}C]proline ($5 \mu\text{Ci}$) in 1.5 ml of F12 plus 0.15% serum plus ascorbate for 3 h at zero time (time of ascorbate addition) and 45 h later. Collagen counts were determined by the collagenase assay. To compensate for the procollagen (pc) extensions, which act to first approximation like noncollagen (nc) proteins in this assay, 10% of the collagen counts were added to the procollagen column and subtracted from the noncollagen total. This assumes that the procollagen extensions are about half the length of the collagen molecules and that they have a proline composition similar to that of an average protein (12, 15, 18).

critical for high levels of procollagen synthesis (Table 2). Without ascorbate, as the cells approached a confluent monolayer, the level of procollagen synthesis in fact dropped slightly on a per-cell basis along with most other proteins. This was further confirmed by pulsing cells with [14 C]proline and comparing the electrophoretic profile of the proteins in the cell and the medium in a manner similar to that described in Fig. 1. In this case, control confluent cultures (day 7) were compared with those fully induced with ascorbate (Fig. 2). The conclusions, however, were the same: the major difference between fully induced and uninduced cells was a 10-fold greater accumulation of procollagen in the medium whenever the variable was cell density or ascorbate.

Kinetics of synthesis and secretion. In comparing fully induced PAT cells with their scorbutic controls, there was a large difference in the procollagen levels in the medium but little difference inside the cell. This raised the possibility that the rate of secretion may play a role in the induction process. Although ascorbate has been known to influence the rate of secretion (23, 26, 30), rates of secretion and synthesis have not previously been shown to be directly coupled (30). To explore this, we analyzed the kinetics of synthesis and secretion.

Cells were pulsed with [14 C]proline for up to 4 h. At various time points, flasks were analyzed for cell-associated and medium-associated counts in collagen and noncollagen proteins. We

chose three conditions: fully induced (48 h with ascorbate), noninduced, and induced only for the duration of the experiment (Fig. 3). The data for total collagen synthesis (the sum of cell-associated and medium-associated counts; Fig. 3A) showed what was previously implied, that ascorbate stimulated the rate of procollagen synthesis almost threefold over the uninduced cells after 48 h. However, if ascorbate was present only during the pulse, the apparent synthetic rate was unaffected (35). In contrast to collagen production, the rate of noncollagen protein synthesis was almost identical between induced and uninduced cells (Fig. 4). These results (Fig. 3A and Fig. 4) again confirm the previous observations (Fig. 1 and 2, Table 2) that the effect of ascorbate was due to an increase in the rate of procollagen synthesis specifically.

In contrast to the long period required to stimulate the rate of procollagen synthesis, ascorbate acted rapidly on the rate of procollagen secretion (Fig. 3B). The addition of ascorbate to scorbutic cultures caused a drop in the internal pool of procollagen due to an increased flux to the medium. The smaller procollagen pool caused a rapid equilibration of the radioactively labeled procollagen as noted by the fact that the rate of procollagen synthesis equaled the rate at which it was secreted. The data are consistent with ascorbate addition causing a rapid increase in procollagen secretion in scorbutic cultures.

Relation of secretion rate to rate of col-

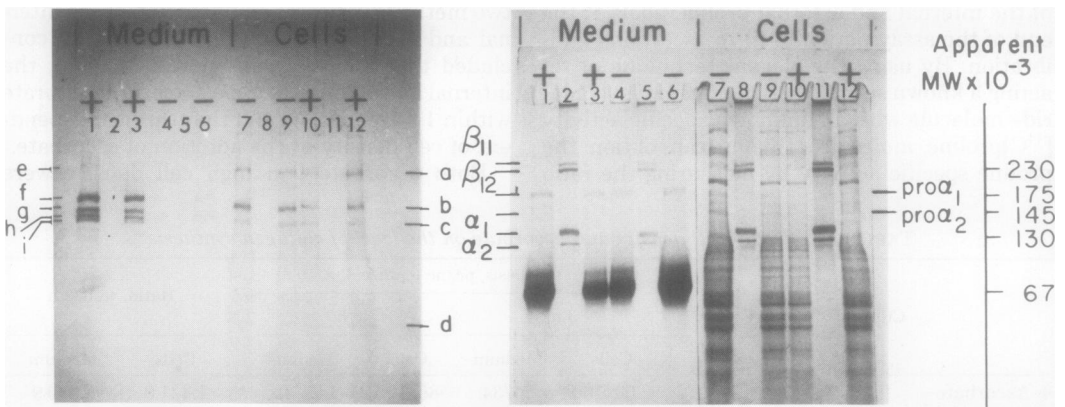


FIG. 2. Protein synthetic pattern of PAT cells 48 h after induction by ascorbate. Induced (+) and uninduced (-) cells were pulsed with 5 μ Ci of [14 C]proline for 3 h. The solubilized cell layer (lanes 7 to 12) and concentrated medium (lanes 1 to 6) were applied to gels. Two concentrations of each were used to resolve both weak and intense bands. (Right) Coomassie blue-stained gels of cell layer and medium. Calf skin collagen standards separated the higher concentration of samples (in lanes 1, 6, 7, and 12) from the lower concentration of samples (lanes 3, 4, 9, and 10). (Left) Autoradiograms of the above gels. The intense bands have been alphabetically labeled. The pro α_1 bands are b, f, and g; the pro α_2 bands are c, h, and i. Several bands (a, e, and d) are intensely labeled but are not affected by ascorbate addition. Bands a and e have been identified as fibronectin (27).

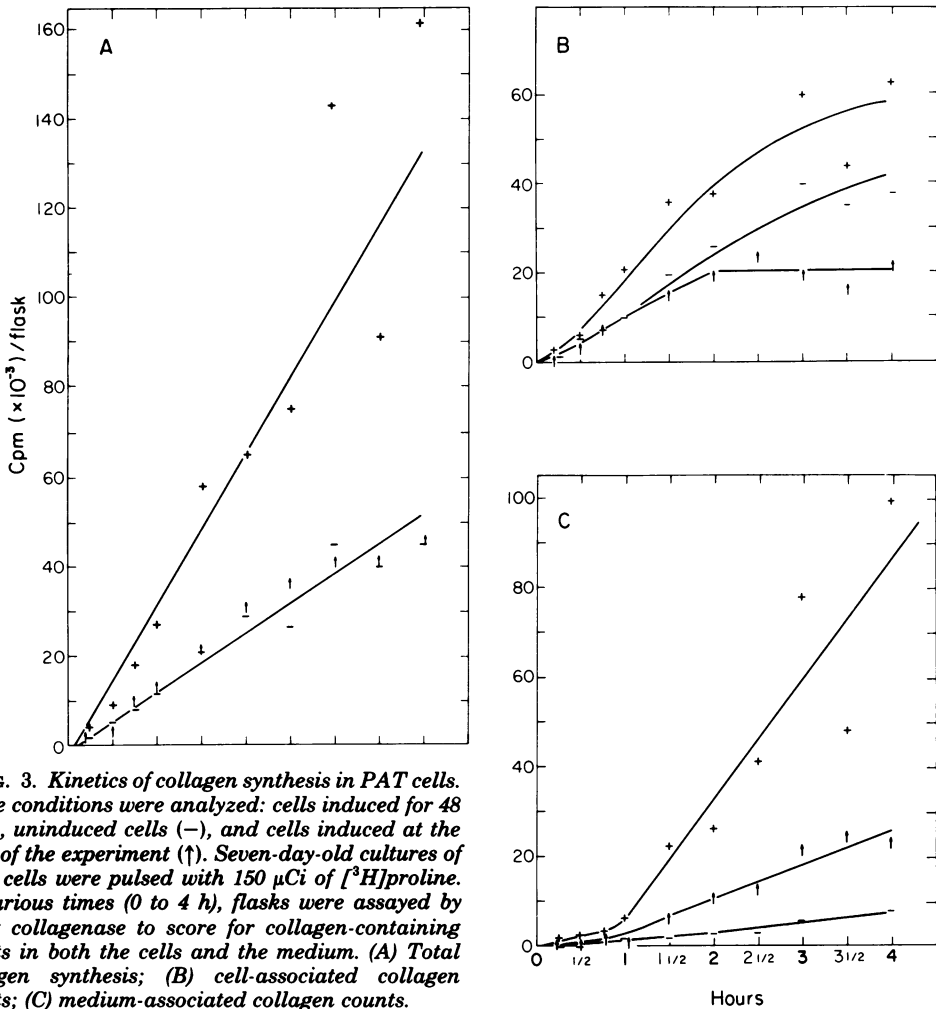


FIG. 3. Kinetics of collagen synthesis in PAT cells. Three conditions were analyzed: cells induced for 48 h (+), uninduced cells (-), and cells induced at the start of the experiment (↑). Seven-day-old cultures of PAT cells were pulsed with 150 μ Ci of [3 H]proline. At various times (0 to 4 h), flasks were assayed by using collagenase to score for collagen-containing counts in both the cells and the medium. (A) Total collagen synthesis; (B) cell-associated collagen counts; (C) medium-associated collagen counts.

lagen synthesis. The above-mentioned results led us to suspect that high rates of procollagen secretion may lead to high levels of procollagen synthesis. In other words, in the kinetics of ascorbate induction of collagen synthesis, stimulation of secretion precedes and appears to trigger a higher level of synthesis. Assuming this is correct, then rates of secretion should be dependent on cell density to the same extent as the rate of collagen synthesis. This same argument was used to rule out the proline hydroxylation step as the limiting factor in increased collagen synthesis (Table 1). The data necessary for deciphering whether cell density is a cofactor in the ascorbate stimulation of secretion have already been shown (Table 2 and Fig. 3). The pertinent information is presented in Fig. 5. The data are derived from experiments where cells received a short exposure to ascorbate and the total level of collagen synthesis did not change;

therefore, the percentage of collagen counts in the medium directly reflected secretion rates. We found that ascorbate enhancement of secretion did depend on high cell density.

Collagen degradation. The question can be raised whether the difference in the level of collagen synthesis after addition of ascorbate is due to an increase in the rate of synthesis or a decrease in the rate of degradation. To test for degradation, we pulsed cells for 2 h with radioactive proline and chased with excess cold proline for 3 h (see Materials and Methods). At intermediate time points during the chase, we analyzed for total counts of collagen in the cell layer and in the medium. If significant degradation was present, then a drop should have been observed in total counts during the chase. We observed for 10 time points a tight distribution around the mean for cells fully induced and those grown without the vitamin (day 7): plus

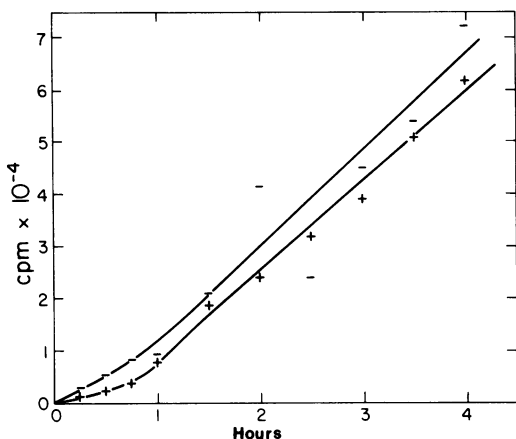


FIG. 4. Kinetics of noncollagen protein synthesis. Two conditions were analyzed: cells induced for 48 h (+) and uninduced cells (-). Experimental details are described in legend of Fig. 3. The data were corrected for the fact that collagenase recognizes the procollagen as mostly a noncollagen protein (see footnote a, Table 2).

ascorbate, 755 ± 103 cpm; minus ascorbate, 350 ± 68 cpm. There was no evidence for increased degradation of already formed collagen in the minus-ascorbate cells. Although we cannot rule out low levels of degradation by this method (<20%), the 300% increase in the rate of procollagen synthesis after full induction by ascorbate was not due to a decrease in the rate of degradation of procollagen.

DISCUSSION

In this report, we have improved our understanding of how ascorbate stimulates the synthesis of collagen in PAT cells. Previously we had shown that the percentage of total protein synthesis devoted to collagen changed by three-fold on addition of ascorbate (35). We now show that the change in the percentage value is due specifically to the increase in the rate of collagen synthesis. Previously we had shown that PAT cells after a week in culture synthesized approximately the same percentage of collagen as they did at the time of isolation. We now show that this is also true on an absolute scale: $0.6 \mu\text{g}/\text{h}$ per 10^6 cells (22). Previously we had shown that ascorbate induction of a high percentage of collagen synthesis was a slow process. We now confirm the slow kinetics, but, more important, we demonstrate that this fact can be used to advantage to separate the events which precede, and appear to be required for, an increased synthetic rate, namely, a stimulation in the rate of procollagen secretion. In addition, we present evidence that the ascorbate effect is due to in-

creased rates of synthesis rather than decreased rates of procollagen degradation. PAT cells are, therefore, an easily manipulated and controllable culture system, and ascorbate is close to an ideal inducer; the combination can be used to reveal the underlying molecular mechanisms of the disease scurvy and also to further our understanding of the regulation of tissue-specific function in eucaryotic cells.

Compared with other fibroblasts (4, 9, 29), the ascorbate effect in PAT cells is much more dramatic and reproducible. We believe that this is due to a careful selection of the cell culture conditions. For instance, high serum (>0.5%) acts as a complex negative modulator and obscures the ascorbate effect (35, 37). Subculturing cells by repeatedly growing them to confluency will result in a reduction of both the rate of collagen synthesis and the ability of ascorbate to act as an inducer (37). Due to the slow kinetics of induction, the practice of adding ascorbate only for the duration of a short-term experiment is ineffective in stimulating collagen production (Fig. 3). One can conclude that the type of response that a cell in culture will exhibit after

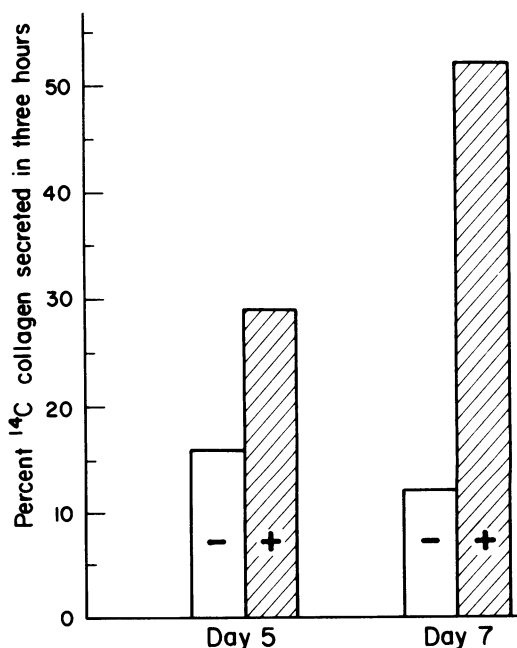


FIG. 5. Effect of cell density on the ability of ascorbate to increase collagen secretion. Scurvitic cells with and without ascorbate were pulsed with radioactive proline. The percentage of the total collagen counts that was secreted into the medium is plotted for both day 5 (4.2×10^6 cells/flask) and day 7 (1.5×10^6 cells/flask).

ascorbate addition relates directly to its previous history (including the tissue of origin) and on its current culture environment. Considering this, the wide variations in the response to ascorbate reported for different cell types are, indeed, expected.

Although fibroblast cell lines are useful for many studies, they appear to make poor models for understanding the regulation of differentiated function (32). After many generations in culture, cell lines may appear stable, but this is, perhaps, a consequence of their becoming more and more insensitive to their environment with time (2, 6). In many cases, the percentage of collagen synthesized by cell lines is very low (<5%) and is independent of the cell culture environment, in complete contrast to PAT cells. Even so, fibroblast cell lines were exploited when first developed for their ability to retain differentiated function in culture (16). Although retention of differentiated function was used as evidence for the "normal" nature of these cells, their low level of collagen synthesis and their insensitivity to the environment indeed reflect abnormal (in fact, tumorigenic) characteristics (2, 8). Whether insensitivity to the environment will prove to be a more accurate measure of transformation (2, 38) than the multitude of currently used parameters remains to be tested. In any case, the need for a responsive system such as we have described is critical if we are going to understand the range of controls used by the cell to precisely regulate the level of differentiated function.

One area of control that needs to be better understood is procollagen secretion, because in PAT cells changes in this parameter correlate well with increased synthesis. But we must immediately interject a qualifying remark. With any kinetic analysis, all steps beyond the rate-limiting step are more or less a function of that step. An increase in secretion may reflect only an increased rate in a preceding but as yet unassayable step. Notwithstanding this limitation, two observations in our results stand out as unexpected: one is the large internal pool of procollagen, also observed by others (23); the second is the correlation between increased procollagen secretion and high cell density.

There is clearly a need to better understand how cell density influences the collagen pathway and to elucidate the exact requirements for rapid secretion of high levels of procollagen (22). At the present time, our knowledge of the cell density effect is based on indirect observations. For instance, the effect does not appear to be due to a long-lived soluble factor because conditioned medium has no effect when compared with fresh medium on the level of collagen synthesis (R. I.

Schwarz and M. J. Bissell, unpublished data). Cell contact does not seem to be critical because the effects are observable at densities well below confluency. Similarly, the cell density effect occurs before the cells show a decline in generation time (37), thus implying that the rate of cell growth is not the critical factor. Even though common theories for cell density effects are not readily applicable to this situation, we continue to explore other possibilities in order to better understand the mechanism by which cell density can alter the ability of PAT cells to secrete procollagen.

Although secretion does appear to be an important regulatory step in the ascorbate induction process, collagen degradation (5) does not seem to play a major role. In pulse-chase experiments described in this paper, the collagen synthesized during the pulse was stable throughout the chase. Even in scorbutic cultures where the collagen produced was underhydroxylated and thus not triple helical, a conformation which is more susceptible to protease attack *in vitro* and which is retained inside the cell longer before being secreted, the collagen synthesized was equally stable. It could be argued that the degradation of collagen in the absence of ascorbate is a rapid event and occurred before the molecule could be assayed. If this were the case, ascorbate addition to scorbutic cultures should cause a rapid stabilization and thus an immediate increase in the apparent rate of synthesis. However, our results (Fig. 3) show no change in the rate of procollagen synthesis for at least 4 h after the addition of ascorbate to scorbutic cultures. Therefore, collagen degradation rates do not appear to be a significant factor in the ascorbate induction of PAT cells.

The apparent complexity of ascorbate action in PAT cells can be reduced to a rather simple model. We postulate that the critical point regulating the level of synthesis of procollagen is the maintenance of a large pool of procollagen within the cell. Adding ascorbate to cultures at high cell density causes an increased secretion rate and a reduction in the procollagen pool within the cell. The decreased pool size, we postulate, triggers an increase in synthesis which we observe by 24 h. With an increase in synthesis, the pool returns to approximately the original size. According to this hypothesis, we would predict that any set of conditions that leads to a reduction in the pool size would lead to higher rates of synthesis and vice versa. This is the case in ascorbate addition at high cell density as we have discussed above. In contrast, adding agents like α, α' -dipyridyl, which lower secretion rates, would be predicted to lower the rate of procollagen synthesis in order to retain the pool size.

Observations concerning the effect of α, α' -dipyridyl by Kao et al. (23), performed for a different reason, are in complete agreement with this hypothesis.

The underlying implication of this model is that there is a feedback between the pool size of procollagen and the rate at which procollagen messenger ribonucleic acid is transcribed or translated. Clearly, more experiments need to be done to establish the operative control steps or to invalidate the hypothesis.

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