# Effects of Ascorbic Acid on Collagen Synthesis in Nonmitotic Human Diploid Fibroblasts (37647)

ROBERT T. DELL'ORCO, AND JAMES H. NASH
(Introduced by R. F. Kampschmidt)
*Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73401*

Our laboratory recently reported a simplified method for the maintenance of human diploid fibroblasts in a nonmitotic state for extended periods of time (1). The present study concerns itself with one aspect of the metabolism of these nondividing cells.

There are numerous reports in the literature describing the biosynthesis of collagen and the role of ascorbic acid in human diploid cell strains (2–6), as well as in mammalian cell lines (*e.g.*, 7, 8). It was our purpose to determine if nonmitotic human diploid cell populations maintained by our method had the ability to synthesize collagen to the same extent as that reported (2, 5, 6).

*Materials and Methods.* The cells used for these experiments were derived from human foreskin material and designated CF-1. These cells characteristically enter Phase III after 35 ± 5 population doublings *in vitro* (1). Only intermediate passage level or Phase II cells were used in these experiments. Cells were routinely transferred at weekly intervals at a 1:4 split ratio with McCoy's Medium 7a (9) supplemented with 10% fetal bovine serum but no antibiotics. Routine tests for mycoplasma contamination were negative.

For the induction of the nonmitotic state, confluent cultures were incubated with medium supplemented with 0.5% serum and 25 $\mu$g/ml of both penicillin and streptomycin. These cultures were fed twice weekly. The medium used for the low serum cultivation was McCoy's 7a with no free hydroxyproline added. This medium contained either the normal amount of ascorbic acid, 0.5 $\mu$g/ml, or ascorbic acid was added to a final concentration of 50 $\mu$g/ml. The nonmitotic nature of cells cultivated in this manner has been determined previously by autoradiography with $^3$H-thymidine and by the calculation of the mitotic index, which was 0.1% after 7 days and 0 after 14 days of cultivation with medium containing 0.5% serum (1).

In these experiments, hydroxyproline content was used as an indicator of collagen content (4). Replicate cultures were harvested at the time of addition of low serum medium to establish a base level of hydroxyproline in the cell layer. In addition to this, a sample of fresh medium was taken to determine the amount of hydroxyproline contributed by the serum. After 7, 14, and 21 days of incubation in the presence of low serum medium, replicate cultures from both ascorbic acid treatments were harvested. After washing the cell layer three times with phosphate buffered saline, the cells were harvested by the addition of 10 ml of 0.1 *M* citric acid (10). An aliquot of the citric acid suspension was taken for nuclei counts with crystal violet and for protein determination by the Lowry method (11). The remainder of the citric acid suspension was extracted with cold 10% trichloroacetic acid (TCA). The precipitate was washed with ethanol and ether followed by hydrolysis in 0.3 *N* KOH for 18 hr at 37° and reprecipitation with 10% TCA. The precipitate was then dissolved in 6 *N* HCl and hydrolyzed with refluxing for 18 hr. The hydrolysate was taken to dryness and redissolved in distilled water. Hydroxyproline was determined on this hydrolysate by the method of Bergman and Loxley (12). The culture medium from each flask was taken at the time of feeding and at the time of harvest and extracted for hydroxyproline determination in a manner similar to that described

TABLE I. Effect of Ascorbic Acid on the Net Synthesis of Collagen by Nonmitotic Cells.

| Ascorbic acid | Incubation period (days) | μg Hydroxyproline/$10^6$ cells[a] |
|---|---|---|
| 0.5 μg/ml | 0– 7 | 0.63 ± 0.05 |
| | 7–14 | 0.31 ± 0.08 |
| | 14–21 | 1.27 ± 0.27 |
| 50.0 μg/ml | 0– 7 | 1.73 ± 0.49 |
| | 7–14 | 1.32 ± 0.69 |
| | 14–21 | 2.69 ± 0.28 |

[a] Values are mean ± SEM from five experiments using three different cell populations and represent the net increase in precipitable hydroxyproline during the incubation period (final minus initial) for both cell layer and incubation medium.

above.

*Results.* Table I shows the results of the net synthesis of collagen as determined by hydroxyproline for each 7-day incubation period. These values include the precipitable hydroxyproline contained in both the cell layer and the incubation medium. Both levels of ascorbic acid showed a net increase in hydroxyproline during each of the incubation periods. However, the increase was from 2- to 4-fold greater in the samples incubated with the higher ascorbic acid concentration. The average net increase for the entire 21-day experimental period was 5.74 μg/$10^6$ cells for the higher ascorbic acid level and 2.21 μg/$10^6$ cells for the lower level. These results, with respect to both the biosynthetic capability as well as the sensitivity to ascorbic acid, are comparable to those reported in the literature in which human diploid fibroblasts were employed (3, 4, 6).

*Discussion.* The primary purpose for the development of a system for the cultivation of nonmitotic diploid cell populations was for its use in the study of senescence *in vitro* (1). The usefulness of such a system depends upon its characterization with respect to biochemical, physiological, and morphological parameters. The parameter investigated in the present study, *i.e.*, synthesis of collagen, compares well with published reports that have employed other human diploid cell systems (2–6). The CF-1 cells, while having lost temporarily their ability to divide due to cultivation with medium containing 0.5% serum, have retained the ability to synthesize hydroxyproline (collagen). This biosynthetic capability is maintained for an extended time and is sensitive to ascorbic acid concentration as shown in Table I. These results offer a preliminary indication that cells maintained in this manner are metabolically functional and are subject to at least one control that is present in rapidly dividing populations. The extension of these studies may offer a true *in vitro* model system for nondividing tissues *in vivo*.

*Summary.* Human diploid fibroblasts maintained in a nonmitotic state by reducing the serum concentration of the incubation medium to 0.5% were shown to be metabolically functional with respect to collagen synthesis. Collagen synthesis was maintained for an extended time and responded to elevated ascorbic acid levels in a manner similar to that reported for other human diploid cell populations.

1. Dell'Orco, R. T., Mertens, J. G., and Kruse, P. F., Jr., Exp. Cell Res. 77, 356 (1973).

2. Macek, M., Hurych, J., and Chvapil, M., Cytologia 32, 426 (1967).

3. Schafer, I. A., Silverman, L., Sullivan, J. C., and Robertson, W. G., J. Cell Biol. 34, 83 (1967).

4. Green, H., and Goldberg, B., Proc. Soc. Exp. Biol. Med. 117, 258 (1964).

5. Manner, G., Exp. Cell. Res. 65, 49 (1971).

6. Houck, J. C., Sharma, V. K., and Hayflick, L., Proc. Soc. Exp. Biol. Med. 137, 331 (1971).

7. Levene, C. L., and Bates, C. J., J. Cell Sci. 7, 671 (1970).

8. Green, H., and Goldberg, B., Nature (London) 204, 347 (1964).

9. Kruse, P. F., Jr., Whittle, W., and Miedema, E., J. Cell Biol. 42, 113 (1969).

10. Paul, J., "Cell and Tissue Culture," 3rd Edition, p. 327. Williams and Wilkins, Baltimore, Md. (1965).

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

12. Bergman, M., and Loxley, R., Anal. Chem. 35, 1961 (1963).

Received April 26, 1973. P.S.E.B.M., 1973, Vol. 144.