ASCORBIC ACID DEFICIENCY IN CULTURED HUMAN FIBROBLASTS

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ABSTRACT

Fibroblasts grown in medium containing less than 1 μg of ascorbic acid per milliliter showed evidence of ascorbic acid deficiency when compared with cells grown in medium containing 50 μg of ascorbic acid per milliliter. This was manifested morphologically by dilated endoplasmic reticulum, a decrease in number, size, and intensity of staining of the mitochondria, by defective intercellular fibril formation, and by easy disaggregation of the cells from the intercellular matrix after treatment with pronase. When 50 μg per milliliter of ascorbic acid was incorporated into the medium, the altered morphology was corrected, banded fibrils were produced which were organized into bundles, and the cells were tightly bound in a matrix which was resistant to disaggregation with a variety of proteolytic enzymes. Collagen and sulfated glycosaminoglycan synthesis were less in the control than in the ascorbic acid supplemented cells. Similar morpholcgical and chemical changes have been reported in the connective tissue of scorbutic animals. The effects of low ascorbic acid concentration on fibroblasts in culture indicate that these cells require ascorbic acid to maintain connective tissue functions.

INTRODUCTION

Man is one of the few species that lack the liver microsomal enzyme L-gulono-oxidase and therefore, cannot synthesize ascorbic acid from glucose (1). Deficiency of this vitamin results in scurvy, a clinical syndrome characterized by tissue fragility, hemorrhage, impaired wound healing, and anemia (2). The molecular abnormalities in fibroblasts that produce these connective tissue lesions are only partially defined but are related to the metabolism of collagen and glycosaminoglycans (mucopolysaccharides) (3). Since cultured human fibroblasts synthesize both collagen and glycosaminoglycans, it seemed reasonable that they might require ascorbic acid for normal function, and several workers have already demonstrated a relation between hydroxyproline (collagen) synthesis and ascorbic acid concentration (4, 5). The purpose of this report is to present morphological and biochemical evidence that human fibroblasts grown in Eagle's minimal essential medium, with 10% fetal calf serum, showed lesions of ascorbic acid deficiency similar to those described in the connective tissue of scorbutic animals, and that these lesions were prevented when additional ascorbic acid was incorporated into the culture medium.

METHODS

Cell Cultures

These experiments were performed with cells derived from human skin, grown and maintained in

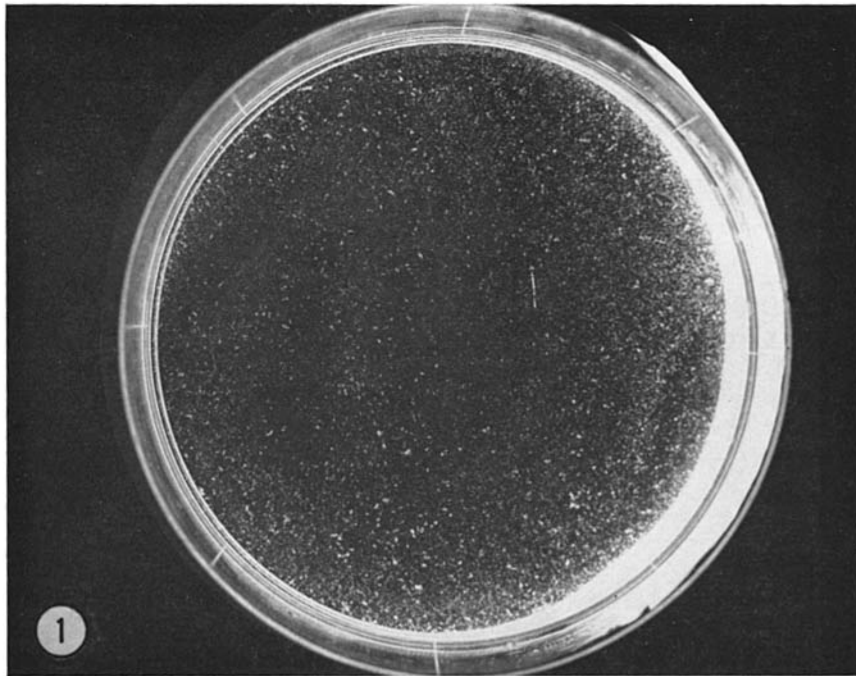


FIGURE 1 Control culture, 19 days after replication following a 10 min exposure to 0.025% pronase. The white specks represent aggregates of cells which can be easily disrupted into single cell suspension by gentle pipetting. See text.

Eagle's minimal essential medium (MEM), (6) supplemented with 10% fetal calf serum, 1 mmole pyruvate, 2 mmoles glutamine, 0.1 mmole serine, 10^4 units of penicillin and 500 μg of streptomycin per 100 ml of medium. All cultures were maintained and all experiments were performed in Falcon plastic tissue culture dishes (Falcon Plastic, Division of B-D Laboratories, Los Angeles, Calif.) (100 $\times$ 20 mm) in 10 ml of medium. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air.

Tissues

Skin specimens were obtained from a variety of sites: (*a*) foreskin, (*b*) tissues surgically removed from the back, thigh, and supernumerary finger, and (*c*) tissues removed from the forearm by biopsy with a high speed rotary drill which removed 2 mm of skin. The biopsy sites were never infiltrated with local anesthetic. The tissue was placed in a Petri dish on a sterile gauze sponge moistened with Earle's balanced salt solution, and extraneous fat and blood were removed. After the tissue was washed in four to five changes of unsupplemented MEM,[1] it was minced with a fine-bladed scissors in 3–4 ml of MEM,[1] and many small tissue fragments were transferred to each of three Petri dishes; 9 ml of medium was carefully added; displacement of the explants was avoided. Medium was replaced three times weekly.

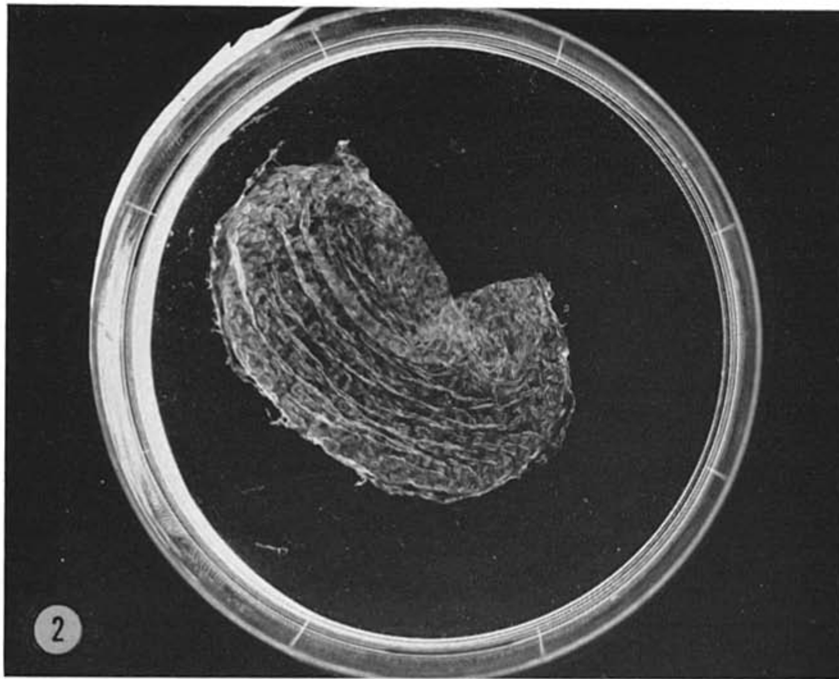
In 3–7 days, the initial outgrowth of cells appeared and within 3–4 weeks sufficient cells were available for subculture. To subculture these fibroblasts, the medium was removed and the cells were rinsed with a balanced salt solution free of calcium and magnesium.[2] Pronase obtained from Calbiochem (Los Angeles, California) was made up to a concentration of 0.025% in the same salt solution and was added to the dishes which were then incubated at 37°C for 7–10 min. Complete disaggregation of the cells occurred. The cell suspension was removed and centrifuged for 5 min at 1000 rpm, and the supernatant solution was discarded. Cells were resuspended in medium and new Petri dishes inoculated. Serial propagation was performed by subculture approximately every 2 wk.

Experimental Procedures

Experiments were performed on cells which had been cultured 6–62 wk. Pronase treatment, as de-

[1] MEM used for washing the tissue was prepared to include 1 mmole pyruvate, 0.1 mmole serine, without added glutamine, serum, or antibiotics.

[2] Pomerat, C. M. 1963. Unpublished data.



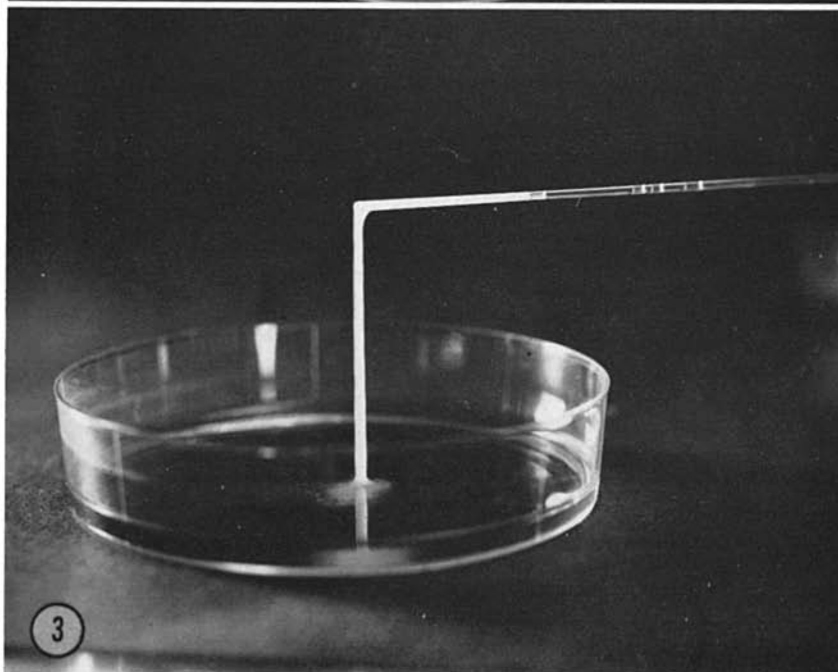


FIGURE 2 Ascorbic acid-supplemented culture, 50 μg/ml for 14 days, 19 days after replication following a 10 min exposure to 0.025% pronase. The membrane persists after 24 hr exposure to a variety of proteolytic enzymes. See text.

FIGURE 3 The membrane demonstrated in Fig. 2 has been lifted from the Petri dish and can be again spread out to its original form. Note the mucoid and stringy consistency of the membrane. See text.

tailed above, was used to prepare replicate dishes containing 2.5×10^5 cells per plate in 10 ml of medium. The medium was replaced three times weekly. 5 days after replication, ascorbic acid 50 μg/ml was added to one-half the plates while the other one-half served as controls and received no added ascorbic acid. Fetal calf serum contributed 0.01 -0.61 μg of ascorbic acid to each milliliter of medium in all cultures (7). The experiments were generally terminated 14 days after the addition of ascorbic acid, and the cells were processed for chemical or morphological studies.

Chemical Methods

When hydroxyproline determinations were to be performed, the cellular layer was rinsed with distilled water, then suspended in water, and the cells were disrupted by sonication. Nondialyzable hydroxyproline was determined after dialysis against running tap water for 20 hr at 5°C with a flow rate of 40 liters of water per hour. Medium was processed in the same manner except that the sonication step was omitted. Hydroxyproline was measured by the method of Prockop and Udenfriend (8). The results are expressed as micromoles of hydroxyproline per milligram of cell nitrogen. Nitrogen determinations were performed by the micro-Kjeldahl method (9).

When glycosaminoglycan (GAG) determinations were performed, the medium was removed and the cellular layer rinsed with Earle's BSS at 37°C. The rinse was discarded and the cells were removed by scraping in 2 ml distilled water. Pools of cells were then shell frozen, lyophilized, and weighed. Glycosaminoglycans were isolated from lyophilized cells following the method of Kofoed and Robertson, (10) and total sulfated GAG's assayed by a modification of the method of Whitehouse and Bostrom (11). Uronic acid was measured by the method of Bitter and Muir (12). The mixed GAG's were then fractionated by Svejcar's modification (13) of the procedures originally described by Antonopoulos and Gardell (14). The results are expressed as micrograms of glycosaminoglycans per milligram dry weight of cells.

Electron Microscopy

The cells examined by electron microscopy had been serially cultured for 30 wk. Representative cultures were fixed for 1 hr by addition of 4% cold glutaraldehyde in 0.33 M sucrose buffered with 0.05 M phosphate (pH 7.4). They were then washed twice for ½ hr with phosphate-buffered sucrose. This was followed by fixation in 1% OsO_4 in phosphate-buffered sucrose for 20 min. The specimens were then dehydrated with graded ethanols and embedded in Epon 812 (15). A number of areas were selected and sectioned on a Huxley ultramicrotome. Sections showing silver interference colors were stained in 50% ethanol saturated with uranyl acetate and in lead citrate, (16) and were examined in a Hitachi HU-11A microscope.

RESULTS

Gross Morphology

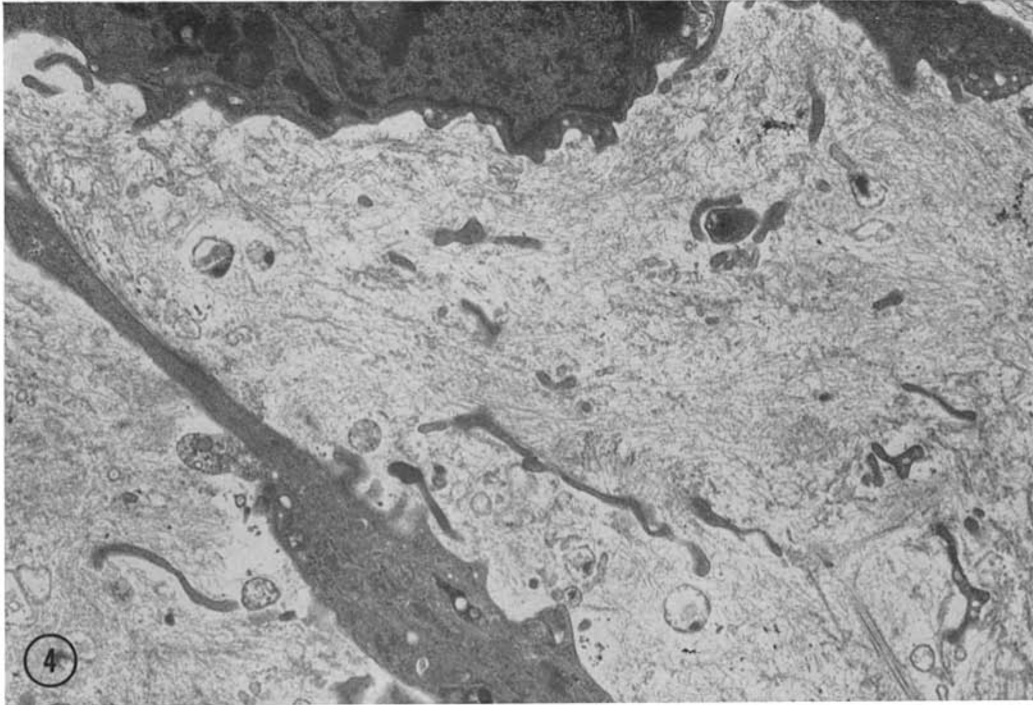
Consistent differences were observed between the appearance of control cultures and the appearance of those supplemented with ascorbic acid when exposed to proteolytic enzymes. The white, opaque specks shown in Fig. 1 represent small aggregates of cells observed in the control culture after 10 min of exposure to 0.025% pronase. The aggregates were easily dispersed into a single cell suspension by gentle pipetting.

In contrast a "membrane structure" was observed in ascorbic acid-supplemented cultures (Fig. 2). The cells were tightly bound together in a membrane that could be lifted from the culture dish (Fig. 3) and then spread out again in its original form. The membrane could not be disrupted even after 24 hr exposure to pronase, trypsin, papain, or hyaluronidase alone or in combination with EDTA. Tetraphenylboron sodium, a compound which chelates potassium, had no effect. A collagenase, containing some protease and peptidase, partially dissolved the membrane after 18 hr, but large segments still remained intact and in no way showed the complete disaggregation of cells seen in control cultures after pronase treatment. In eight separate human fibroblast lines, the membrane was observed regularly in 19-day-old cultures which had been supplemented with ascorbic acid for 14 days, but the membrane was never observed in the unsupplemented control cultures.

Electron Microscopy

Several notable differences were observed when control cultures and ascorbic acid-supplemented cultures were examined in the electron microscope at 19 days.

(*a*) The intercellular spaces in the control culture were filled predominantly with a wavy tangle of very fine fibrils, along with occasional thicker and straighter fibrils (Fig. 4). The spaces between the cells from ascorbic acid-supplemented cultures contained predominantly fibrils which were straighter, thicker, and longer, and which were often arranged in parallel to form bundles (Fig.



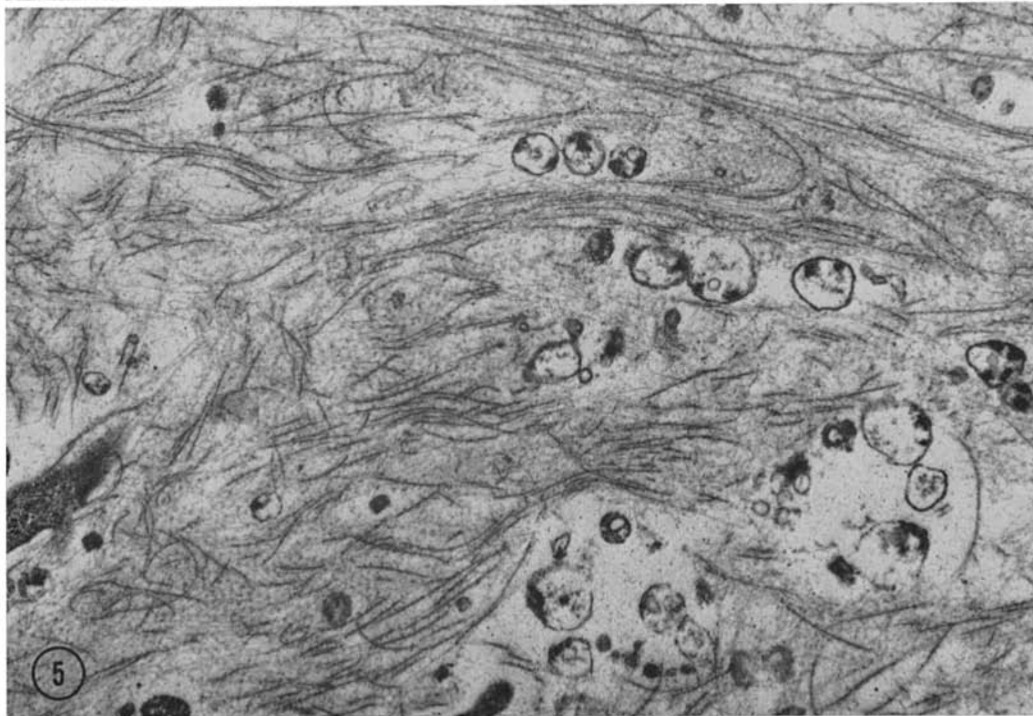
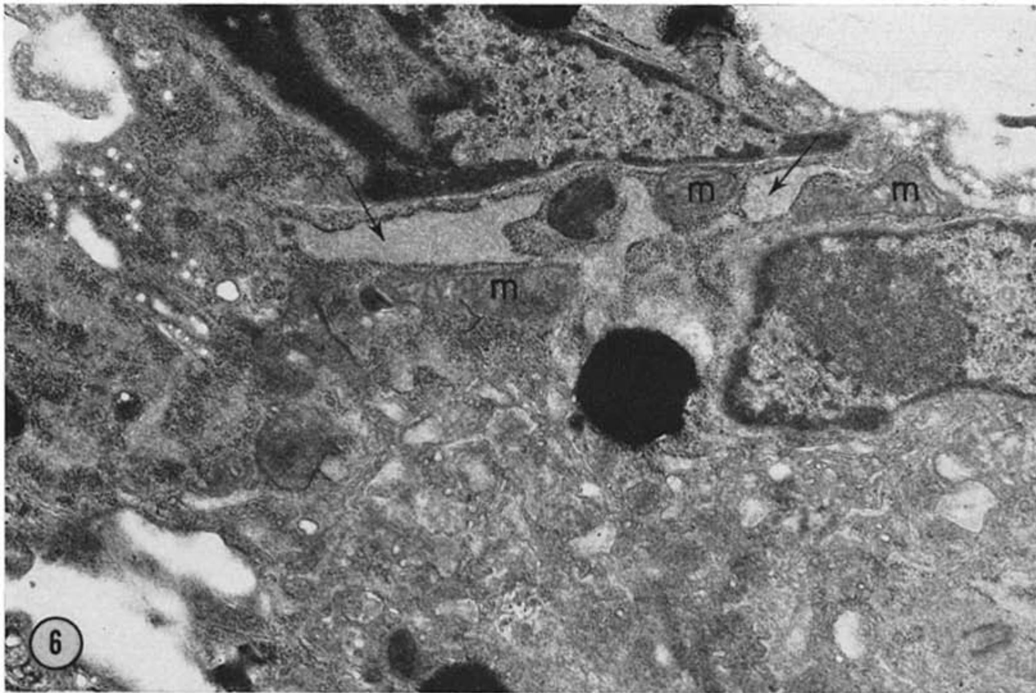


FIGURE 4 Control tissue culture, 19 days. Numerous fine unbanded fibrils, 60–70 A in width, fill the spaces between the cells. × 15,000.

FIGURE 5 Ascorbic acid-supplemented culture, 19 days. Straighter and longer fibrils, 120–140 A in width, are often arranged in parallel to form bundles. A number of fibrils display beading at intervals of approximately 640 A. × 15,000.



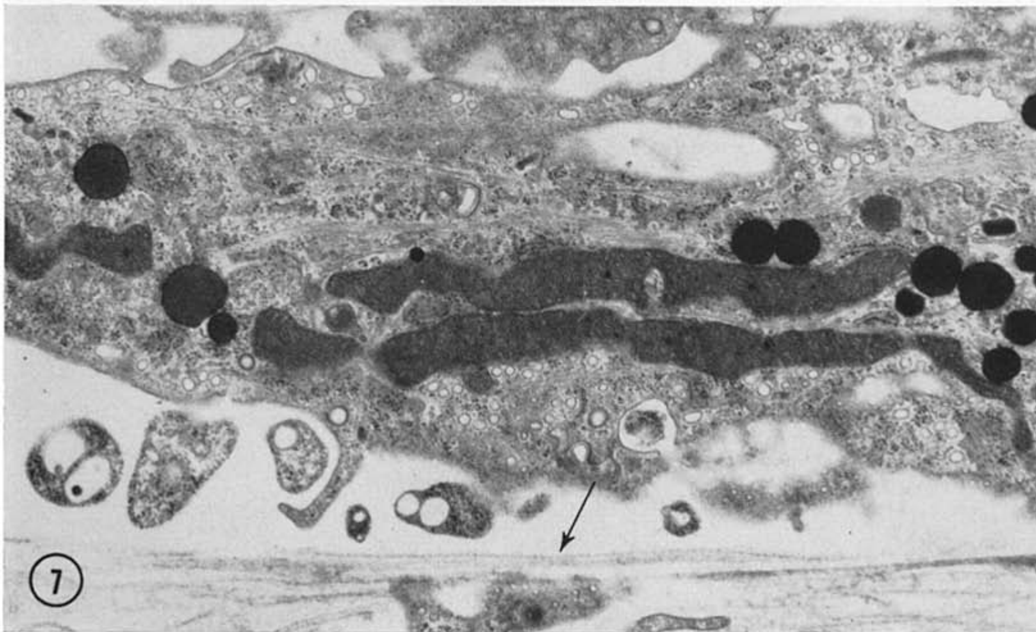


FIGURE 6 Control culture, 19 days. Dilated endoplasmic reticulum (arrow) is filled with a finely granular, moderately electron-opaque material. A few small mitochondria with electron-lucent matrices are seen (*m*). × 15,000.

FIGURE 7 Ascorbic acid-supplemented culture, 19 days. Mitochondria are much larger and show denser matrices than those in Fig. 6. See Fig. 10 for comparison of endoplasmic reticulum. Periodic banding of 640 A is seen in a fiber (arrows). × 15,000.

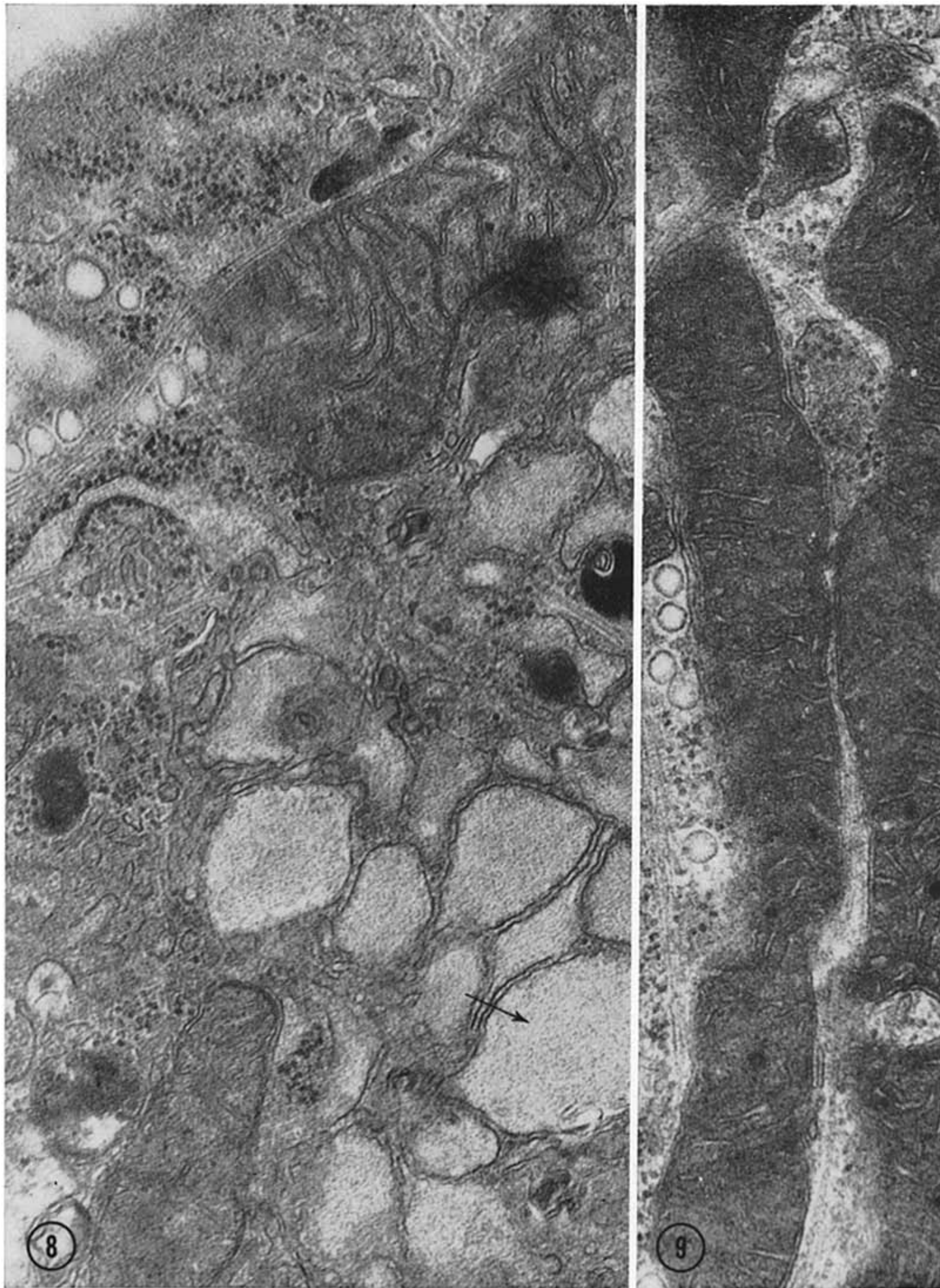


FIGURE 8 Control culture, 19 days. One of the largest mitochondria is seen in the upper half. Note matrix density. Dilated endoplasmic reticulum is prominent (arrow). × 45,000.

FIGURE 9 Ascorbic acid-supplemented culture, 19 days. This demonstrates the large mitochondria generally observed in supplemented cultures. The increased stain density was a consistent finding. × 45,000.

5). Many fine fibrils were also present. Periodic banding was not observed in control cultures, but several fibrils in the supplemented cultures showed fine beading at intervals of 640–720 A (Fig. 5, Fig. 7). More commonly, fibrils showed periodicities of approximately 120 A (Fig. 11).

(*b*) The endoplasmic reticulum of control cells was often markedly dilated, and contained abundant amounts of a finely granular, moderately electron-opaque material (Figs. 6 and 8), whereas the endoplasmic reticulum in cells from supplemented cultures showed parallel, closely apposed membranes (Fig. 10). The endoplasmic reticulum of cells from both cultures was predominantly rough surfaced.

(*c*) The mitochondria in control cells were small and few and showed an electron-lucent matrix, (Figs. 6 and 8) while those in the supplemented cells were much larger and more numerous and had a dense matrix (Figs. 7 and 9).

(*d*) Smudgy densities, which often displayed a definite periodicity, were seen between the fibers of the supplemented cultures (Fig. 11). The nature of these structures was not defined.

Densely staining lipid droplets were evident in cells of both control and supplemented cultures. This was not interpreted as evidence of cell injury, since vesiculated endoplasmic reticulum or myelin figures were absent.

Chemical Analyses

Hydroxyproline has been commonly used as an index of collagen concentration because it comprises 8–10% of the amino acid residues and is uniquely present in this protein, except for a small amount in elastin (17). Both total and nondialyzable hydroxyproline were assayed in these experiments; the latter represents hydroxyproline incorporated into compounds with molecular weights greater than 10,000. Table I presents pooled data on the concentration of cell-bound hydroxyproline in two cell lines. Cell bound is defined as hydroxyproline contained both in the cell and in the intercellular matrix. It includes hydroxyproline associated with the cellular layer after rinsing with distilled water. The ascorbic acid-supplemented cells showed higher concentrations of total and nondialyzable hydroxyproline than the control cultures after 7 and 14 days.

Since increased hydroxyproline might reflect either increased collagen synthesis in the supplemented cultures or diffusion of collagen from the control cells into the medium, the net synthesis of hydroxyproline was measured. This is defined as the quantity of hydroxyproline in the medium and cells minus the quantity of hydroxyproline contributed by the fetal calf serum in the medium. These data are given in Table II and show that the supplemented cells synthesized more nondialyzable hydroxyproline than the control cells. The dialyzable hydroxyproline was about the same in both groups. In all cultures most of the nondialyzable hydroxyproline synthesized by the cells diffused into the medium, but in the ascorbic acid-supplemented cells a larger proportion of hydroxyproline remained associated with the cellular layer. Fetal calf serum, exhaustively dialyzed to remove ascorbic acid, was used in one experiment. The cultures grown in this medium showed a distribution for hydroxyproline similar to that found in control cultures grown in regular medium. It was concluded that ascorbic acid-supplemented cultures showed an increase in total collagen synthesis and that an increased proportion of this protein was retained in the cellular layer.

Newly synthesized collagen is soluble in dilute, buffered salt solution at pH 7.4, but as collagen matures, interchain cross-links are introduced and dilute acid solutions are required to solubilize the protein. With further maturation, the degree of interchain cross-linking increases so that little or no collagen can be extracted by mild extraction procedures (18). The cellular layers of ascorbic acid-supplemented and control cultures were repeatedly extracted at 5°C with large volumes of 0.14 M sodium chloride in phosphate buffer pH 7.4, then by 0.14 M citrate buffer at pH 3.4 and finally 2.0 M acetic acid. The cellular material remaining after these extractions was dried *in vacuo*, weighed, and assayed for its hydroxyproline content. The concentration of mature collagen was three to four times as high in the ascorbic acid-supplemented cultures as in the control cultures and in both it accounted for about 75% of the total hydroxyproline. This indicated that ascorbic acid not only increased the amount of collagen but that this was highly cross-linked mature collagen.

The GAG composition of scorbutic repair tissues has been found different than that of normal tissues (3). The composition of cellular glycosaminoglycans was examined in order to test the possibility of a similar effect of ascorbic acid on fibroblasts grown in culture. Table III summarizes data from six cell lines. Although the total concen-

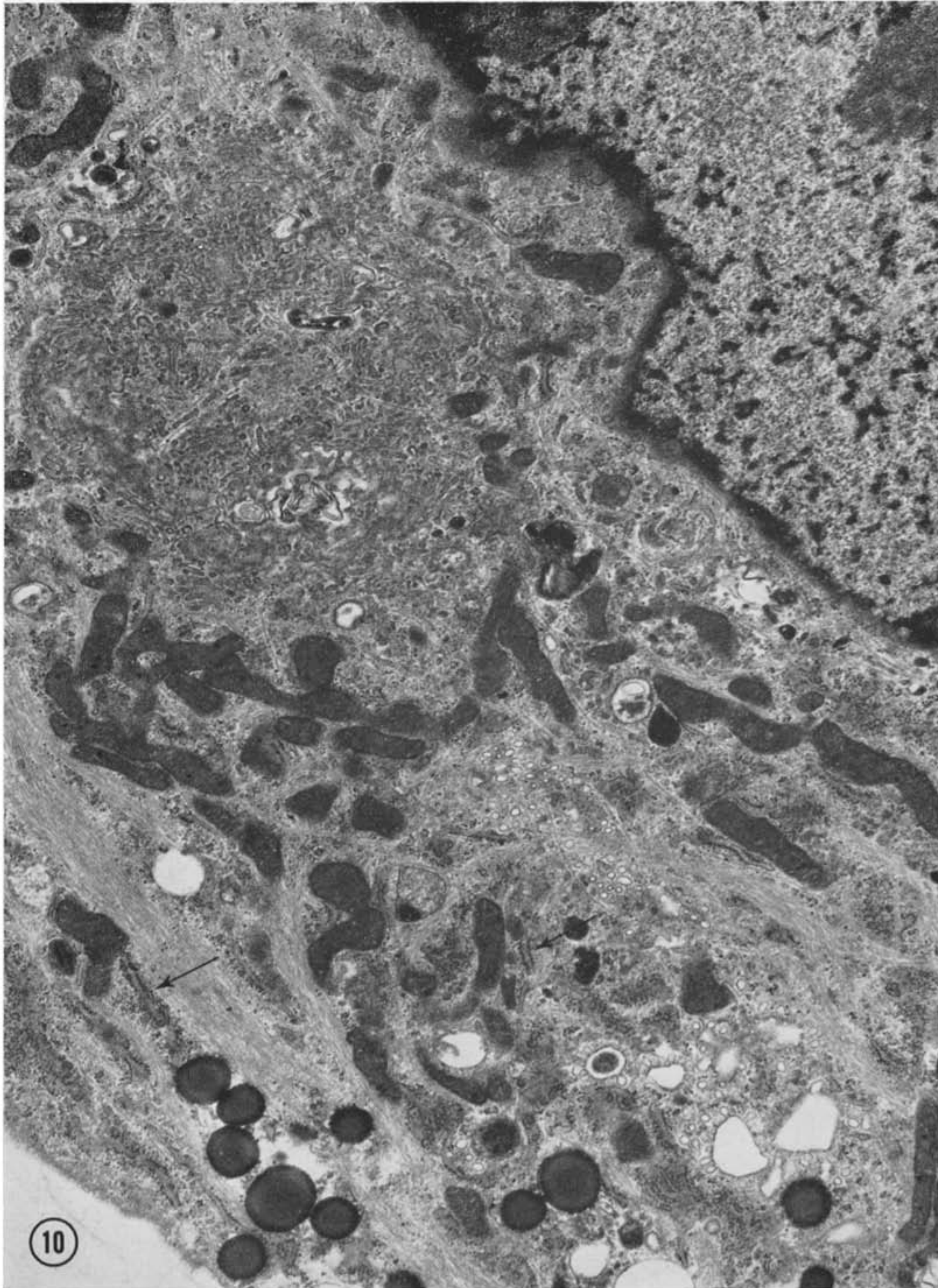


FIGURE 10 Ascorbic acid-supplemented culture, 19 days. This illustrates the increased number of mitochondria generally seen. Compare the tightly apposed endoplasmic reticulum (arrows) with that seen in Fig. 6. × 15,000.

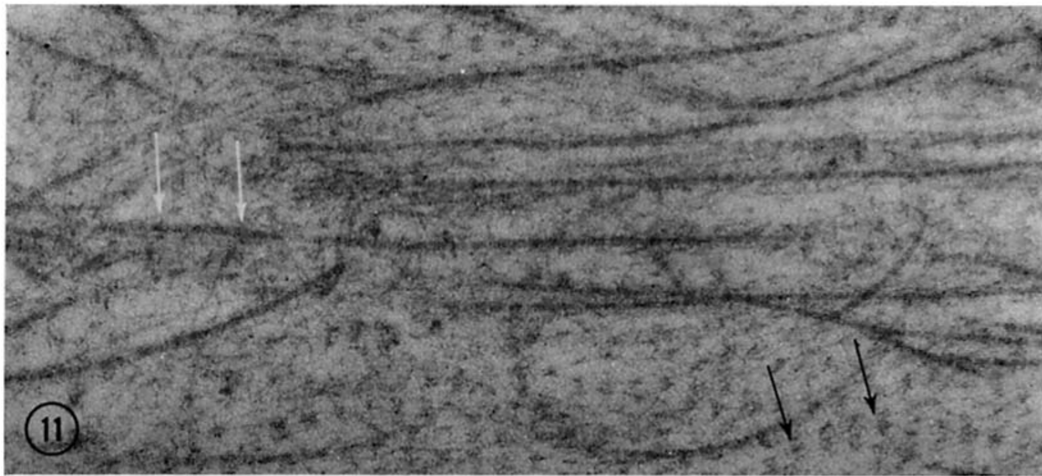


FIGURE 11 Ascorbic acid-supplemented culture, 19 days. The fibers show a fine periodicity of 120–140 A (white arrows). Between the fibers are smudgy periodic densities (arrows). × 45,000.

TABLE I

Cellular Hydroxyproline Concentration

Time in culture	No. of exper	Control			Ascorbic acid		
		μM Hydroxyproline/mg nitrogen			μM Hydroxyproline/mg nitrogen		
		Total OHPro	Nondialyzable OHPro		Total OHPro	Nondialyzable OHPro	
days				%*			%*
5	6	0.05 (9) ± 0.01	0.02 (6) ± 0.01	37	‡		
12	3	0.04 (5) ± 0.01	0.03 (5) ± 0.01	57	0.13 (5) ± 0.02	0.11 (5) ± 0.03	84
19	6	0.07 (9) ± 0.003	0.04 (8) ± 0.01	58	0.31 (9) ± 0.09	0.26 (8) ± 0.09	84

Mean values ± SEM.
Figures in parentheses indicate number of cell pools studied.
Represents pooled data from two cell lines #64-5 and #65-8.
* Per cent of total hydroxyproline that was nondialyzable.
‡ Ascorbic acid added on day 5.

tration of GAG was not changed by ascorbic acid, the sulfated GAG were increased in five of six cell lines. Fractionation of this mixture further defined these changes in GAG composition (Table IV). Fraction 1 is composed of a mixture of compounds whose identities are not completely defined. Glycoproteins, keratan sulfate, depolymerized hyaluronic acid, depolymerized or incompletely sulfated GAG may be eluted in this fraction. Dermatan sulfate is eluted in fraction 6. The tentative identities of compounds in fractions 2–5 are given in Table IV. These data must be interpreted with caution until the compounds eluted in these fractions are verified, since the extent of polymerization, degree of sulfation, and other factors affecting net charge can alter the elution patterns. The total concentration of GAG (fractions 1–6) and total sulfated GAG (fractions 3–6) compare well with values obtained by different methods (Table III). It is clear that ascorbic acid increases the concentration of sulfated GAG, particularly dermatan sulfate, and that a reciprocal decrease in compounds eluted in fraction 1 accounts for the similarity in the total concentrations of GAG in ascorbic acid-supplemented and control cells.

DISCUSSION

Four lines of evidence in these experiments indicate that human fibroblasts grown in medium not supplemented with ascorbic acid showed lesions simi-

TABLE II

Net Synthesis of Hydroxyproline

Time in culture	No. of exper	Control				Ascorbic acid			
		μM hydroxyproline/mg nitrogen				μM hydroxyproline/mg nitrogen			
		Dialyzable	Nondialyzable			Dialyzable	Nondialyzable		
			Total	Cell bound			Total	Cell bound	
days					%*				%*
5	3 (5)	0.57	0.18			‡			
12	3 (5)	0.63	0.78	0.03	4	0.70	1.28	0.10	8
19	3 (5)	1.23	0.97	0.04	4	1.08	2.00	0.34	17

Data from two cell lines #64-5 and #65-8.
Figures in parentheses indicate number of cell pools analyzed.
Net synthesis: hydroxyproline of the cell layer + the medium − hydroxyproline contributed to medium by fetal calf serum.
Hydroxyproline in fetal calf serum: dialyzable 0.02–0.03 μM/ml; nondialyzable 0.01–0.02 μM/ml.
* Per cent of nondialyzable hydroxyproline that was cell bound.
‡ Medium supplemented with 50 μg/ml ascorbic acid beginning on day 5.

TABLE III

Cellular Glycosaminoglycans

Normal cell line	Total GAG*		Total sulfated GAG	
	Control	Vit C	Control	Vit C
64-5	3.15	2.04	0.84	0.78
65-6	16.20	15.30	2.16	3.60
65-8	19.95	14.70	4.09	12.20
65-9	8.67	19.17	1.62	2.28
66-2	3.78	4.86	2.30	3.47
66-3	13.20	12.18	2.66	4.43
avg	10.83	11.38	2.28	4.49

* These values were obtained by multiplying the uronic acid concentration of the isolated GAG by 3. The differences in the total sulfated GAG were tested by the Wilcoxon matched pairs signed ranks test (two sided). T = 1, P = 0.06. Table shows μg/mg dry weight of cells.

lar to those described for repair tissue in scorbutic animals.

(*a*) The ultrastructure of fibroblasts grown in unsupplemented medium showed a dilated endoplasmic reticulum, and tangled masses of fine, unbanded, intercellular fibrils, a morphology similar to that reported in healing wounds of scorbutic guinea pigs (19). By contrast, in cultures supplemented with ascorbic acid, the fibroblasts displayed an endoplasmic reticulum composed of tightly apposed membranes and banded intercellular fibrils organized into bundles, a morphology similar to that described in the healing wounds of normal guinea pigs (19). These effects of ascorbic acid on fibril formation in tissue culture have been observed previously by Shimizu et al. (4). Their electron micrographs of unsupplemented cells also showed evident dilatation of the endoplasmic reticulum, but the change was not commented upon in the text.

Mitochondria in ascorbic acid-deficient fibroblasts were fewer and smaller, and their matrix stained less intensely than in the supplemented cells. Altered mitochondrial structure has not been reported previously in cells from ascorbic acid-deficient tissue cultures or in the fibroblasts of scorbutic animals. However, mitochondria contain the enzymes of electron transport and oxidative phosphorylation and, in scurvy, the urinary excretion of citric acid cycle intermediates is increased, while the activities of cytochrome and succinic oxidase, lactic, malic, and succinic dehydrogenases are decreased (20, 21, 22). The morphological changes of the mitochondria appear consistent with the altered metabolism of Krebs cycle intermediates reported in animal studies.

(*b*) The increased hydroxyproline concentration of the ascorbic acid-supplemented cultures confirms reports of other investigators (4) (5). In vivo, ascorbic acid appears rate limiting for the hydroxylation of proline to hydroxyproline in rapidly synthesizing collagen systems; i.e. during wound repair (3); however, growing animals are able to synthesize some collagen even though scorbutic

TABLE IV

Glycosaminoglycans

Normal cell line	Fractions 1–6		Fraction 1		Fraction 2		Fraction 3–6		Fraction 6	
	Control	Vit C	Control	Vit C	Control	Vit C	Control	Vit C	Control	Vit C
64-5	2.9	1.7	2.1	0.7	0.3	0.1	0.5	0.9	0.0	0.1
65-6	20.8	16.9	13.5	6.0	1.7	3.9	5.6	7.0	1.3	1.9
65-8	19.2	18.4	12.0	2.3	1.7	2.7	5.5	13.4	0.9	3.6
65-9	4.5	7.2	1.5	2.0	1.0	2.0	2.0	3.2	0.3	1.0
66-2	2.6	4.1	1.0	0.6	0.5	0.8	1.1	2.7	0.1	0.7
66-3	8.8	10.0	5.1	2.8	1.7	3.1	2.0	4.1	0.2	1.3
avg	9.80	9.72	5.87	2.40	1.15	2.10	2.78	5.22	0.47	1.43

Wilcoxon matched pairs signed ranks test (two sided), difference between vitamin C and control.
Fraction 1 P = 0.09, fraction 2 P = 0.06, fractions 3–6 P = 0.03, fraction 6 P = 0.03.
Table shows μg/mg dry weight of cells.
Nominal characterization of fractions: fraction 1, keratan sulfate, glycoproteins, low molecular weight hyaluronate, etc; fraction 2, hyaluronic acid; fraction 3, heparan sulfate; fraction 4, chondroitin 4-sulfate: fraction 5, chondroitin 6-sulfate; fraction 6, dermatan sulfate.

(23). Our studies indicate a similar pattern in culture. Cells grown in dialyzed serum, which contained little ascorbic acid, synthesized some collagen. Most of the collagen synthesized by cells grown in regular medium diffused into the medium, but in the ascorbic acid-supplemented cells a greater proportion of collagen remained bound to the cellular layer. Much of this cell-bound collagen was highly cross-linked, as evidenced by its solubility characteristics. These chemical data cannot delineate changes in the molecular structure of the collagen synthesized by the cells. The data do allow us to conclude that in the absence of ascorbic acid there is decreased synthesis of both soluble and highly cross-linked collagen, but that this is not the sole defect.

(*c*) Ascorbic acid altered the glycosaminoglycan (GAG) composition of the cells. In the few reports of GAG synthesis by cultured cells, the medium rather than the cellular layer was analyzed and, under these circumstances, hyaluronic acid was the principal GAG detected (24–26). When the effect of ascorbic acid on GAG synthesis was studied, analyses of the medium showed no differences in GAG composition with added ascorbic acid (4). Similar conclusions would have been made in these experiments if only the medium had been studied. Hyaluronic acid was the principal GAG detected in the medium and its concentration was not changed by the addition of ascorbic acid. However, when the GAG composition of the cellular layer was analyzed, the concentration of sulfated GAG, particularly dermatan sulfate, was increased in the ascorbic acid-supplemented cultures.

The decrease in sulfated GAG concentration in the presence of an unchanged total GAG concentration in unsupplemented cells parallels the changes described in scurvy. Hyaluronic acid accumulates in the scorbutic carrageenan granuloma in guinea pigs (27); the sulfated GAG concentration of healing wounds in scorbutic animals is less than normal, (28) and a lower dermatan sulfate concentration has been observed in the aorta of scorbutic guinea pigs (29).

(*d*) The formation of the membrane structure in the ascorbic acid-supplemented cultures was a reproducible observation. This structure was composed of cells embedded in a matrix of collagen, glycosaminoglycans, and other compounds whose identities are not yet established. Similar observations of membrane formation have been reported when human fibroblasts were grown in medium 1066 which contains 50 μg of ascorbic acid per milliliter (30). The toughness of the connective tissue matrix produced by the cells grown in the presence of ascorbic acid may be contrasted with the ease with which ascorbic acid-deficient cells were disrupted from their matrix. This pattern is strikingly similar to that described for the weakened tensile strength of healing wounds in ascorbic acid-deficient animals (31).

All of our experiments focused on the function of ascorbic acid in relation to the connective tissue

components produced by fibroblasts. We have concluded from these data that human fibroblasts grown in medium containing less than 1 μg of ascorbic acid per milliliter showed the morphological and chemical lesions of ascorbic acid deficiency. The minimal concentration of ascorbic acid necessary to reverse these lesions was not determined, but cells grown in medium containing ascorbic acid in concentrations of 50 μg per milliliter did not show the lesions found in the ascorbic acid-deficient cultures.

Although these data are limited to human fibroblasts, the possibility should be considered that fibroblasts derived from species that can synthesize ascorbic acid in vivo may require ascorbic acid in tissue cultures. Ascorbic acid is synthesized in vivo by liver and/or kidney microsomes, a source of supply not available to fibroblasts in culture. Therefore, cultured fibroblasts regardless of origin may require ascorbic acid to maintain normal function. Supporting this view is the observation of greatly dilated endoplasmic reticulum in mouse fibroblasts cultured in unsupplemented medium (32) and the later report of increased collagen synthesis in these same cell lines when ascorbic acid was added (33). Since tissue cultures of fibroblasts are now finding increasing use in studies of connective tissue metabolism, it is important to consider the ascorbic acid content of each experimental system in order to control the effect of this variable on cell metabolism.

Supported by grants from the National Foundation, Hartford Foundation and Nos. HD 00560, AM 05223, HE 06716 (the latter two to Dr. David Glick) from the United States Public Health Service.

Received for publication 1 December 1966.

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