PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 178, 126–132 (1985)

RNA Metabolism in Cultures of Corneal Stromal Cells from Patients with Keratoconus (41993)

BEATRICE Y. J. T. YUE,*[,1] JOEL SUGAR,* AND KATHY BENVENISTE†

*Department of Ophthalmology, University of Illinois Eye and Ear Infirmary, Chicago, Illinois 60612 and †Department of Oral Biology, Northwestern University Dental School, Chicago, Illinois 60611

Abstract. Total cellular RNA was extracted from cultured keratoconus and normal human corneal stromal cells. The translational activity of these RNAs was examined in a cell-free translation system derived from reticulocyte lysate. Results indicated that keratoconus cells can be separated into two groups, as has been shown previously. Group I keratoconus cells contained the same amount of total RNA as normal cells. RNA activity and the rate of mRNA synthesis in this group of keratoconus cells were also normal. By these criteria it seems that the protein synthesizing system is functioning properly, and group I keratoconus cells should have a normal rate of protein synthesis. These results correlate well with previous findings. Group II keratoconus cells, in contrast, contained more RNA than normal cells. The translational efficiency of RNA was so markedly reduced that the elevation in RNA content did not compensate for the decrease in translational efficiency. It is likely that the reduced protein and collagen synthesis in this group of cells is related to the reduction in the RNA activity. An inhibitory component was present in the keratoconus RNA which affected synthesis of all proteins and suppressed translation of normal RNA.

Keratoconus is a slowly progressive and potentially blinding corneal disease whose pathogenesis is unknown (1). Histopathologic and structural studies (2–5) have demonstrated that, in the early stages of the disease, fragmentation of the epithelial basement membrane occurs together with disintegration of Bowman's membrane and fibrillation of the anterior stroma. In later stages, the central cornea is thinned, with destruction of Bowman's membrane and stromal scarring. In more advanced cases, the central portion of Descemet's membrane is ruptured with edematous clouding of the cornea.

Previous biochemical studies of keratoconus have suggested abnormalities in collagen crosslinking (6), in lysine hydroxylation (7), in hydroxylysine glycosylation (7), in collagen content (7, 8), and in collagenase activity (9, 10). However, data have been conflicting (11), and no clear mechanism of pathogenesis has been presented. Very recently, we have reexamined the biochemical properties of keratoconus corneas and have investigated the synthetic activities of keratoconus stromal cells in culture (12). We have demonstrated that keratoconus is a heterogeneous group of diseases. This heterogeneity may explain the contradictory data that exist in the literature.

We have demonstrated at least two subgroups of keratoconus corneas. Group I has a normal collagen content, while group II contains less collagen than normal corneas. Both groups have a reduced protein content. In tissue culture, group I keratoconus stromal cells seem to have a normal rate of protein and collagen synthesis. Conversely, group II cells show reduced levels of both protein and collagen synthesis.

Based on these data, we have hypothesized that the defect in group I corneas may not be related to protein synthesis. The defect in group II corneas, however, appears to be related to a reduction in total protein synthetic activities of corneal cells.

To determine whether the variation in synthetic activities seen in group II keratoconus cells is under transcriptional control, the present study was conducted. Total cellular RNA was extracted from both group I and II keratoconus cells and from normal

[1] To whom reprint requests and correspondence should be addressed: University of Illinois Eye and Ear Infirmary, 1855 W. Taylor St., Chicago, Ill. 60612.

0037-9727/85 $1.50

control cells and their translational activities were measured. This investigation provides information on the molecular-level defect in a pathologic condition.

Materials and Methods. *Cell cultures.* Corneal buttons from 15- to 67-year-old patients with typical clinical symptoms of keratoconus were obtained following transplantation. Normal human corneas from individuals 4 to 65 years old were obtained from the Illinois Eye Bank.

Descemet's membrane with the attached endothelium was stripped off from these corneas (13) and the superficial epithelial-stromal layer was removed. The mid- to posterior portion of stroma was then cultured by standard procedures (14) in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS, GIBCO Laboratories). After 4–5 weeks, when cultures were confluent, cells were trypsinized and subcultured.

Extraction of total cellular RNA. Cultured corneal stromal cells were treated, as previously described (15), with 3 ml of 1% sodium dodecyl sulfate (SDS), 5 m*M* EDTA, 10 m*M* Tris, pH 7.5 (SET buffer) containing 65 μg proteinase K/ml per T-75 flask. Cells were lysed with a Dounce homogenizer and the mixture was incubated at 37°C for 1.5 hr. Triton-X (0.01 vol), 10% deoxycholic acid (DOC, 0.1 vol), and 4 *M* NaCl (0.1 vol) were added, and subsequently samples were shaken with 0.5 vol of phenol saturated with 0.5 *M* Tris–HCl, pH 7.5, followed by 0.5 vol of chloroform. The two phases were separated by centrifugation and the aqueous layer was removed and reextracted two more times. The RNA was precipitated by adding 2 vol of ethanol at −20°C. The precipitate was collected by centrifugation at 12,000*g* for 20 min, washed two times with 0.04 *M* NaCl in 66% ethanol to remove traces of phenol, and lyophilized. RNA was redissolved in water, and then was precipitated by the addition of 3 vol of 4 *M* sodium acetate, 5 m*M* EDTA, pH 7.0. The precipitate was washed twice with 3 *M* NaAc, 5 m*M* EDTA, and once with 0.04 *M* NaCl, 66% ethanol. After lyophilization and a further ethanol precipitation, the RNA was dissolved in water and stored in liquid nitrogen. The concentration was determined from absorbance at 260 nm, assuming that an absorbance of 20 is equivalent to 1 mg RNA/ml. DNA was determined as previously described (16) by a fluorometric method using 3,5-diaminobenzoic acid.

In some cases, confluent cultures of keratoconus and normal human corneal stromal cells were labeled for 2 hr with 8 μCi [5-^{3}H]uridine (New England Nuclear Corp., 25.2 Ci/mmole)/ml MEM containing 10% dialyzed FCS and 50 μg/ml of ascorbic acid. RNA was then extracted as described above.

Cell-free translation. RNAs derived from keratoconus and normal control cells were translated in a nuclease-treated rabbit reticulocyte lysate (Amersham, N-90) system. Each 15-μl translation mixture contained 12 μl of lysate, 5 μCi [2,3-^{3}H]proline (New England Nuclear Corp., 27.3 and 32.2 Ci/mmole), and RNA isolated from keratoconus or normal control cultures. Incubation was conducted at 28°C for 60 min. Translation was terminated by the addition of 1 *N* NaOH and H_2O_2 (20/0.8, v/v) to deacylate the transfer RNAs. After a further 15-min incubation at 37°C, the [^{3}H]proline incorporation was determined by precipitating 3-μl aliquots with 10% trichloroacetic acid (TCA) final concentration, and filtering onto 25-mm glass-fiber filters. The filters were washed with 5% TCA and 95% ethanol, and counted in toluene–Liquifluor in a Beckman scintillation counter.

For collagenase assays of cell-free translation products, a larger reaction (60 μl) was prepared. The RNA concentration was 60, 120, and 60 μg/ml for group I keratoconus, group II keratoconus, and normal control cells, respectively. Following translation, collagenase digestions were carried out at 400 units (Advanced Biofactures, Form III)/ml at 37°C for 3 min (17) and proteins were precipitated with TCA–tannic acid. Assays were performed in duplicate.

Gel electrophoresis of RNA. Radiolabeled RNA samples obtained from normal human, group I and group II keratoconus cells were electrophoresed through agarose gels to examine their molecular sizes. In each case, equal aliquots (containing approximately 60,000 cpm) of RNA were incubated for 1 hr either at 4 or 30°C. The samples were then denatured and electrophoresed on formamide denaturing 1.5% agarose gels (18). The gels were stained with ethidium bromide, destained, photographed, soaked in Enlighting

(New England Nuclear Corp.), and dried. Kodak XAR film was opposed to the gel at −20°C for 8 days and then developed.

Messenger RNA (mRNA) isolation. Confluent cultures of keratoconus and normal human corneal stromal cells were incubated with [5-^{3}H]uridine for 2 hr as described above. After labeling, the medium was removed, and the cells were washed, lysed, suspended in SET buffer, and incubated with proteinase K (65 μg/ml) at 37°C for 1.5 hr. Aliquots of the cell mixture were precipitated with 10% cold TCA, and the total radioactivity incorporated was determined.

To the remaining cell mixture, Triton-X (0.01 vol), 10% DOC (0.1 vol), and 4 *M* NaCl (0.1 vol) were added. This mixture was subsequently passed through an oligo(dT)-cellulose (T-2, Collaborative Research) column (0.6 ml) as described (19). Binding and wash buffer contained 0.4 *M* NaCl, 10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 0.5% SDS. The poly(A)-mRNA fraction was eluted with 1 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 0.1% SDS. The eluate was adjusted to 0.4 *M* NaCl, heated to 80°C for 5 min, rapidly chilled, and recycled through the column. The radioactivity associated with poly(A)-mRNAs was assayed by counting a 20-μl aliquot of the final eluate in a 3a70b cocktail (Research Products International Corp.).

Results. Total cellular RNA was extracted from both group I and group II keratoconus cells and from normal control cells to examine the RNA metabolism in corneal cultures. The grouping of keratoconus stromal cells was based on the synthesis data shown in a previous report (12). The RNA content (expressed as μg/μg of DNA) in group I keratoconus cells was similar to that found in normal control cells (Table I). Group II keratoconus cells, however, contained a significantly ($P < 0.001$) higher amount of RNA. The concentration of these RNAs was determined from absorbance at 260 nm. The ratio of absorbance at 260 nm to that at 280 nm varied from 1.85 to 2.0, indicating a minimal contamination with proteins. The DNA content in the RNA preparations ranged from 0.2 to 1.0%. It was not significantly different among the group I and group II keratoconus and normal samples.

TABLE I. RNA CONTENT IN KERATOCONUS AND NORMAL HUMAN CULTURES

Sample	Age of donor (years)	RNA content (μg/μg DNA)
Keratoconus		
Group I	28	2.0
	30	3.2
	33	2.6
		2.7
	33[a]	3.7
		2.3
		2.8 ± 0.6[b]
Group II	15	5.0
	17	4.2
	51[c]	4.4
		4.5 ± 0.4[d]
Normal	4	3.0
		3.0
		3.1
	5	2.5
	53	3.1
	55	2.0
	56	1.8
	58	2.5
	65	3.0
		2.5
		2.7 ± 0.5

Note. The data are presented as means ± SD. Each line represents an individual experiment.

[a] This cornea was analyzed biochemically and had a normal collagen content.

[b] Compared to control, N.S.

[c] This cornea was analyzed biochemically and had a reduced collagen content.

[d] Compared to control, $P < 0.001$.

The ability of RNAs extracted from corneal cells to direct protein synthesis was assessed using a cell-free translation system derived from rabbit reticulocyte lysate. The RNA used was prepared individually from different cell lines. The extent of ^{3}H incorporation was linearly proportional to the RNA added throughout the concentrations tested (up to approximately 60 μg/ml in the case of group I keratoconus and normal cells, and 180 μg/ml in the case of group II keratoconus cells). RNA from group I keratoconus cells and normal control cells showed comparable activity in terms of total protein synthesis (Fig. 1). In contrast, the translational efficiency of RNA from group II keratoconus cells was

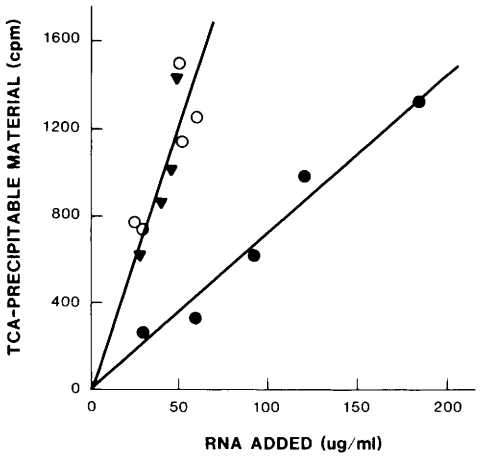

FIG. 1. Translation of RNA isolated from keratoconus and normal control cells. RNA was translated in a nuclease-treated rabbit reticulocyte lysate system. The translation mixture (15 μl) contained 12 μl of lysate, 5 μCi of [^{3}H]proline, and RNA isolated from keratoconus or normal control cultures. Incubation was conducted at 28°C for 60 min. Translation was terminated by the addition of 1 *N* NaOH and H_2O_2 (20/0.8, v/v) followed by a 15-min incubation at 37°C. The radioactivity incorporated into proteins in 3 μl aliquots was measured after TCA precipitation. RNA was from (▼) group I keratoconus cultures, (●) group II keratoconus cultures, and (○) normal control cultures.

markedly reduced (Fig. 1) and did not always show a linear response to an increase in RNA concentration. However, linearity is difficult to access when the incorporation is as low as it was with this RNA.

The radiolabeled collagenous protein synthesized in the cell-free system was quantitated by digesting the translation products with purified bacterial collagenase. Table II shows that the percentage of collagenase-labile proteins present in the products translated from group I and group II keratoconus RNAs was comparable to that found in normals.

Cell free translation was subsequently performed with a mixture of 30 μg/ml each of RNAs from group II keratoconus cells and from normal cells. Parallel experiments were also performed with the same concentration of RNAs from each cell type. As shown in Fig. 2, the extent of ^{3}H incorporation was not additive with the mixed RNA preparation. In fact, incorporation was less than that observed with the normal RNA alone. It appears that the RNA from group II keratoconus cells inhibited protein synthesis, suggesting the possible presence of an inhibitory component in these RNAs. In two additional experiments, similar levels of inhibition were observed (data not shown).

In order to eliminate the possibility that the observed inhibition and the poor translational efficiency were due to ribonuclease contamination in the RNA preparations from group II keratoconus cells, RNAs from both normal and keratoconus cells were examined by gel electrophoresis. Samples were incubated for 1 hr at either 4 or 30°C and then electrophoresed on denaturing agarose gels. Figure 3 shows the fluorogram of these RNAs. Incubation at the temperature used for translations did not alter the size or distribution of RNAs in any of the samples. This result indicates that none of the RNA preparations contain significant levels of ribonuclease.

TABLE II. COLLAGENASE DIGESTION OF THE *in Vitro* TRANSLATION PRODUCTS

Sample	Total TCA-precipitable products (cpm × 10^{-3})	Percentage collagen synthesis[a]
Experiment 1		
Keratoconus Group II	6.0	1.9
Normal	4.9	2.1
Experiment 2		
Keratoconus Group I	4.7	4.7
	5.7	6.2
Normal	6.0	5.6

Note. The cell-free translation was carried out as described in Fig. 1 except that a larger reaction (60 μl) was prepared. The RNA concentrations were 60, 60, and 120 μg/ml for normal, group I, and group II keratoconus cells, respectively. Following incubation, four aliquots of the reaction mixture were transferred to glass test tubes for collagenase assays. Calcium chloride, *N*-ethylmaleimide, Tris buffer, and collagenase (400 units/ml) were added to two samples. No enzyme, but an equal volume of the buffer was added to the other two samples. Tubes were incubated at 37°C for 3 min and proteins were precipitated with TCA–tannic acid. Each value is the average from duplicate experiments. Different lots of reticulocyte lysate were used for Experiments 1 and 2.

[a] Collagen synthesis is expressed as a percentage of total protein synthesis after correcting for the 5.4-fold enrichment of proline in collagenous proteins.

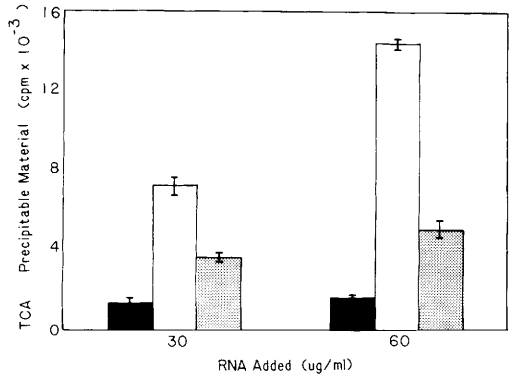

FIG. 2. Translation of RNA mixed from group II keratoconus and normal control cells. The experimental conditions for cell-free translation were the same as described in Fig. 1. Radioactivity in 15-μl translation mixtures is presented. The bars represent the range of the values from duplicate experiments. RNA was from (■) group II keratoconus cells, (□) normal control cells, and (□) a mixture of either 30 or 60 μg/ml of each cell type.

The percentage of [^{3}H]uridine-labeled poly(A)-mRNAs relative to total RNA was determined after pulse-labeling corneal cells for 2 hr. The values in both group I and group II keratoconus cultures were similar to those found in control cultures (Table III). Since mRNA is a short-lived species, and the relatively short incubation period used preferentially labeled mRNA, our data suggested that the rate of mRNA synthesis by both groups of keratoconus cells was normal.

Discussion. The present study confirms our previous finding that keratoconus is a heterogeneous group of diseases. In addition to the cell synthetic activities shown previously (12), cellular RNA content and RNA template activity may also be criteria for segregation of keratoconus corneas into two subgroups. Medical histories in terms of duration of disease, size and shape of the cone, degree of scarring, and the presence of other systemic diseases were examined. No difference in these clinical factors, however, could be identified between the two groups of keratoconus corneas.

Group I keratoconus cells contained the same amount of RNA as normal cells. The RNA activity in terms of directing total protein synthesis, and the rate of mRNA synthesis were also normal. This suggests that the protein synthesizing system in group I keratoconus cells functions properly, and correlates with the normal level of protein synthesis observed in culture (12).

In this group of corneas, a reduced level of noncollagenous proteins was noted (12). The accumulation of macromolecules in a cell and in the extracellular matrix is a net sum of synthetic and degradative processes. The synthetic processes depend, in part, on the levels of mRNA and the efficiency of translation by the protein synthetic apparatus of the cell. Since the mRNA synthesis, the RNA level, and the RNA translational efficiency were all normal in group I keratoconus cells, the reduction in noncollagenous protein may result from an alteration in the protein degradative processes (12). This hypothesis, however, awaits to be tested.

Group II keratoconus cells appear to have a different defect. They contained almost twofold more total RNA than control cells (Table I), but the translational efficiency of the RNA was reduced approximately threefold (Fig. 1). Thus the elevation in RNA

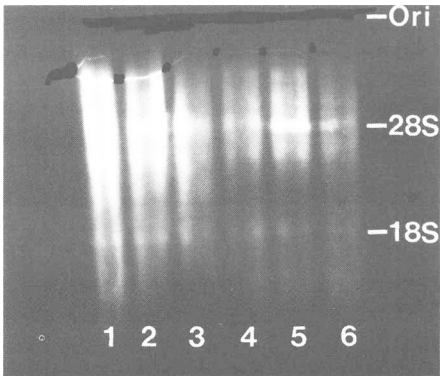

FIG. 3. Fluorogram of RNAs isolated from keratoconus and normal control cells. RNAs were incubated for 1 hr at either 4° or 30°C, denatured and electrophoresed on formamide denaturing 1.5% agarose gels (18). Lane 1, normal RNA, 4°C; Lane 2, normal RNA, 30°C; Lane 3, group I keratoconus RNA, 4°C; Lane 4, group I keratoconus RNA, 30°C; Lane 5, group II keratoconus RNA, 4°C; Lane 5, group II keratoconus RNA, 30°C. All lanes contained approximately equal quantities of radioactivity. Positions of origins (Ori) and 18 S and 28 S ribosomal RNAs are indicated.

TABLE III. PERCENTAGE OF Poly(A)-mRNA RELATIVE TO TOTAL RNA SYNTHESIZED BY KERATOCONUS AND NORMAL CONTROL CELLS

Cell	Age of donor (years)	Number of subcultures	% Poly(A)-mRNA
Keratoconus			
Group I	28	2	10.7
		4	9.8
	30	1	13.1
		3	11.0
		3	13.0
			11.5 ± 1.5[a]
Group II	15	1	10.3
	51	4	6.6
		4	7.9
	67	4	12.0
		5	9.3
			9.2 ± 2.1[a]
Normal	5	3	10.6
		4	10.1
	12	3	11.4
		3	11.5
	49	2	11.8
	64	3	8.0
		4	8.8
		5	8.0
		5	8.4
			9.8 ± 1.5

Note. Confluent cultures of corneal stromal cells were labeled with 8 μCi/ml of [5-^{3}H]uridine for 2 hr. After labeling, cells were separated from the medium and were then suspended in 10 m*M* Tris, pH 7.5, 5 m*M* EDTA, 1% SDS and incubated with proteinase K (65 μg/ml) at 37°C for 1.5 hr. Total radioactivity incorporated was determined by precipitating aliquots of the cell mixture with 10% cold TCA and filtering onto glass-fiber filters. The poly(A)-mRNA fraction was isolated by oligo(dT)-cellulose column chromatography. Percent poly(A)-mRNA relative to total RNA was determined. Each line represents an independent experiment. In each group, no significant differences were associated either with age of donors or number of passages.

[a] Compared to normal, N.S.

content does not compensate for the decrease in template activity, and the reduced protein and collagen synthesis observed in this group of cells is possibly related to the reduction in RNA activity. A decrease in translation efficiency might be caused by a decline in mRNA. However, this explanation is unlikely. When keratoconus RNA is combined with the normal RNA in a translation assay, there is a suppression of synthesis from the normal template (Fig. 2). These observations appear unrelated to a ribonuclease activity, since no change in size or distribution of any RNA samples (Fig. 3) was observed by incubating RNAs at the temperature used for cell-free translations (30°C). The data argue that keratoconus RNA contains an inhibitory factor that affects the protein synthesizing mechanism. All proteins appear to be equally affected, since we found that the percentage of collagenous material in products synthesized from the RNA obtained from group II keratoconus cells was close to that obtained from normal control cells (Table II). The presence of an inhibitory factor rather than a defect in the mRNA per se is supported by the normal level of mRNA synthesis (Table III) observed in these cells. At present, the identity of the inhibitor is unknown.

The mechanism that produces a higher than normal RNA content in group II keratoconus cells (Table I) is also not clear, nor is it known whether the excess RNA is poly(A)$^+$ or poly(A)$^-$. Further studies are required to identify the inhibitor and to determine whether a translational control factor exists in group II keratoconus cells. Determination of collagenase and other protease activities in keratoconus cell cultures is also underway in our laboratory. A full understanding of the molecular mechanism involved in keratoconus must wait completion of these studies. In any event, it seems that the changes in some cases of keratoconus are related to changes in RNA metabolism.

We thank Ms. Judy Elvart for her technical assistance. This work was supported by Grants EY 03890 and PHS EY 1792 from the National Eye Institute.

1. Nirankari VS, Karesh J, Bastion F, Lakhanpal V, Billings E. Recurrence of keratoconus in donor cornea 22 years after successful keratoplasty. Brit J Ophthalmol **67**:23–28, 1981.
2. Chi HH, Katzin HM, Teng CC. Histopathology of keratoconus. Amer J Ophthalmol **42**:847–860, 1956.
3. Teng CC. Electron microscopic study of the pathology of keratoconus. Part I. Amer J Ophthalmol **55**:18–47, 1963.
4. Pataa CL, Joyon L, Roucher E. Ultrastructure of keratoconus. Arch Ophtalmol (Paris) **30**:403–417, 1970.

5. Bron AJ, Tripathi RC, Harding JJ, Crabbe MJC. Stromal loss in keratoconus. Trans Ophthalmol Soc UK **98**:393–396, 1978.
6. Cannon D, Foster CS. Collagen crosslinking in keratoconus. Invest Ophthalmol Visual Sci **17**:63–65, 1978.
7. Robert L, Schillinger G, Moczar M, Junqua S, Moczar E. Biochemical studies of keratoconus. Arch Ophtalmol (Paris) **30**:590–608, 1970.
8. Buddecke E, Wollensak J. Saure Mucopolysaccharide und Glykoproteine der Menschlichen Cornea in Abhangigkeit vom Lebensalter und bei Keratoconus. Albrecht von Graefes Arch Klin Exp Ophthalmol **71**:105–120, 1966.
9. Kao WWY, Vergnes J-P, Ebert J, Sundar-Raj CV, Brown SI. Increased collagenase and gelatinase activities in keratoconus. Biochem Biophys Res Commun **107**:929–936, 1982.
10. Rehany U, Lehav M, Shoshan S. Collagenolytic activity in keratoconus. Ann Ophthalmol **14**:751–754, 1982.
11. Andreassen TT, Simonsen AH, Oxlund H. Biomechanical properties of keratoconus and normal corneas. Exp Eye Res. **31**:435–441, 1980.
12. Yue BYJT, Sugar J, Benveniste K. Heterogeneity in keratoconus: Possible biochemical basis. Proc Soc Exp Biol Med **175**:336–341.
13. Stocker FW, Eiring A, Georgiade R, Georgiade N. A tissue culture technique for growth of corneal epithelial, stromal and endothelial tissues separately. Amer J Ophthalmol **46**:294–298, 1958.
14. Yue BYJT, Baum JL, Silbert JE. The synthesis of glycosaminoglycans by cultures of rabbit corneal endothelial and stromal cells. Biochem J **158**:567–573, 1976.
15. Rowe DW, Moen RC, Davidson JM, Byers PH, Bornstein P, Palmiter RD. Correlation of procollagen mRNA levels in normal and transformed chick embryo fibroblasts with different rates of procollagen synthesis. Biochemistry **17**:1581–1590, 1978.
16. Fiszer-Szafarz B, Szafarz D, Guevara de Murillo A. A general, fast and sensitive micromethod for DNA determination: Application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts and yeast cells. Anal Biochem **110**:165–170, 1981.
17. Peterkofsky B, Diegelmann B. Use of mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry **10**:988–994, 1971.
18. Maniatis T, Fritsch EF, Sambrook J. Electrophoresis of RNA through Gels Containing Formaldehyde in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, pp202–203, 1982.
19. Aviv H, Leder P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA **69**:1408–1412, 1972.

Received March 26, 1984. P.S.E.B.M. 1985, Vol. 178.
Accepted September 27, 1984.