

RELATION OF PROTEIN SYNTHESIS  
AND TRANSGLUTAMINASE ACTIVITY TO FORMATION  
OF THE CROSS-LINKED ENVELOPE DURING  
TERMINAL DIFFERENTIATION OF THE CULTURED  
HUMAN EPIDERMAL KERATINOCYTE

ROBERT H. RICE and HOWARD GREEN

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

When serially cultivated human epidermal keratinocytes are placed in suspension culture they stop growing and form, beneath the plasma membrane, an insoluble envelope consisting of protein cross-linked by  $\epsilon$ -( $\gamma$ -glutamyl)lysine. The formation of envelopes in suspended cells is preceded by a sharp decline in the rate of protein synthesis, and most envelopes appear only after the average rate of protein synthesis has fallen to a very low level. If protein synthesis is reduced over 98% with cycloheximide or emetine at the time that surface-grown cells are placed in suspension culture, cross-linked envelopes form in most of the cells. This shows that the precursor of the envelope and the cross-linking enzyme are already in the cytoplasm in most cells of growing surface cultures. The process of envelope formation by suspension cultures is actually accelerated by the inhibitors of protein synthesis; an increased number of cells with cross-linked envelopes is observable within 4–6 h after the addition of cycloheximide. The inhibitor also induces a large fraction of the cells of surface cultures to form envelopes within a few days.

These findings suggest that arrest of protein synthesis leads to activation of the cross-linking process. Agents known to inhibit transglutaminase-mediated protein cross-linking—putrescine, iodoacetamide, and ethylene glycol-bis( $\beta$ -aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA)—also prevent envelope formation. Though the activity of the cross-linking transglutaminase depends on the presence of cellular  $\text{Ca}^{++}$ , we have not been able to activate the cross-linking process by high external  $\text{Ca}^{++}$  concentration or ionophores.

KEY WORDS transglutaminase · keratinocytes ·  
 $\epsilon$ -( $\gamma$ -glutamyl)lysine cross-linking · protein  
synthesis inhibition · cornified envelopes ·  
terminal differentiation

When human epidermal keratinocytes are culti-

vated together with lethally irradiated mouse 3T3 fibroblasts, they grow from single cells into colonies of stratified squamous epithelium (13). The keratinocytes have an increased culture lifetime and plating efficiency in successive subcultures

when grown in the presence of epidermal growth factor (14).

Maturation of the cells in culture resembles terminal differentiation in the epidermis in important respects. DNA synthesis and division are limited to cells of the basal layer. Cells of the upper layers increase in volume, accumulate disulfide-stabilized keratin filaments, and develop, beneath the plasma membrane, an insoluble "cornified" envelope consisting of cross-linked protein (20, 15, 19).

In large colonies growing in surface culture fewer than 10% of the cells have cross-linked envelopes, but when the cells are suspended in methylcellulose a majority of the cells develop envelopes (5). Though the mechanism by which suspension of the cells promotes the formation of cross-linked envelopes has not been fully elucidated, we described here studies implicating arrest of protein synthesis as an important factor and pointing to the existence of a detergent-soluble protein precursor of the envelope in growing cells.

## MATERIALS AND METHODS

### *Culture Conditions and Scoring of Cross-Linked Envelopes*

Serially cultivated human epidermal cells (strain N) were grown in the Dulbecco-Vogt modification of Eagle's medium containing 20% fetal calf serum and hydrocortisone at 0.4  $\mu\text{g}/\text{ml}$  (13). Mouse epidermal growth factor was added to 15 ng/ml beginning ~4 days after inoculation (14). After 20–50 generations of cultivation, large growing colonies were disaggregated with trypsin and EDTA. For preparation of suspension cultures, the cells were inoculated at  $2 \times 10^5/\text{ml}$  in medium supplemented with 1.1% methylcellulose (A4M, Dow Corning Corp., Midland, Mich.) and 20% fetal calf serum. After incubation for varying periods, the suspension was diluted sevenfold in isotonic buffer and the cells were recovered by centrifugation. The cells were resuspended in isotonic buffer (usually 0.4 ml) at a concentration of about  $10^6/\text{ml}$  and counted in a hemacytometer chamber. Sodium dodecyl sulfate (SDS) was then added to 1% and dithiothreitol to 10–20 mM; the suspension was heated at 90°C for 5 min and the envelopes were counted. Though the heating is not essential for scoring, it extracted the nonenvelope proteins more completely.

### *Protein Synthesis by Suspended Cells*

After varying periods, the methocel-stabilized suspen-

sion was diluted 15-fold in medium and the cells were recovered by centrifugation. Approximately  $10^6$  cells were resuspended in 1.5 ml of medium containing 15  $\mu\text{Ci}$  of [ $^3\text{H}$ ]glycine and incubated for 1 h in a 35-mm bacteriological petri dish. The cells were then centrifuged, and resuspended in 1 ml of 0.05 N NaOH for 20 min at room temperature. Acid-precipitable material was recovered on glass fiber filters after dilution with 2 ml of 15% TCA made 1 mM in glycine. In experiments in which methylcellulose stabilization was not used, aliquots of freshly trypsinized cells were incubated in bacteriological petri dishes in 1.5 ml of medium. At intervals, 0.5 ml of medium containing 15  $\mu\text{Ci}$  of [ $^3\text{H}$ ]glycine was added to some dishes. After 1 h of additional incubation, the labeled cells were recovered and their acid-precipitable radioactivity was measured.

### *Determination of $\epsilon$ -( $\gamma$ -Glutamyl)Lysine Cross-Linking in Insoluble Envelopes*

Cells were labeled in surface culture with L-[ $^3\text{H}$ ]lysine, trypsinized, and placed in suspension culture in the presence or absence of cycloheximide, 100  $\mu\text{g}/\text{ml}$ . After 11 days,  $2 \times 10^6$  cells were centrifuged, rinsed in isotonic phosphate buffer (pH 7.2), and heated at 90°C for 5 min in a neutral lysis buffer containing 1% SDS and 20 mM dithiothreitol. The insoluble residue was recovered by centrifugation, rinsed and gently stirred in fresh lysis buffer for 2 days at 37°C. The insoluble residue was recovered, rinsed in water, and rinsed twice in 80% ethanol and once in 0.2 M *N*-ethylmorpholine-HCl buffer, pH 8.2. The final residue (~1 mg of protein) was digested at 37°C for 1 day in 0.3 ml of the *N*-ethylmorpholine buffer containing 1 mg of pronase. To the digest were then added 0.1 mg of leucine aminopeptidase and  $\text{MgCl}_2$  to 5 mM for a 2nd day of incubation. After adding 140  $\mu\text{g}$  of carboxypeptidase A and 100  $\mu\text{g}$  of carboxypeptidase B, digestion was continued for a 3rd day. The final digest (0.5 ml) was dried under vacuum and extracted with 0.5 ml of cold 10% TCA. Aliquots of the extract were adjusted to 20% in pyridine and spotted directly on Whatman no. 1 filter paper. A descending chromatogram was developed (17) using as solvent 88% phenol:ethanol:28% ammonia:water (68:20:1.5:10.5). The dried chromatogram was cut into 2-cm squares and counted by liquid scintillation. The locations of the lysine and  $\epsilon$ -( $\gamma$ -glutamyl)lysine spots were determined with ninhydrin using nonlabeled standards run in parallel. Control experiments indicated that the extent of digestion (routinely 98% of the radioactivity became acid soluble) and the percentage of label migrating as isodipeptide were not altered by longer digestion times. Ion-exchange column chromatography of the extract before paper chromatography (15) was unnecessary in the present work due to the large proportion of the lysines cross-linked.

## RESULTS AND DISCUSSION

### *Reciprocal Relation between the Rate of Protein Synthesis and Formation of the Cross-Linked Envelope*

Serially cultivated human epidermal keratinocytes were trypsinized and the disaggregated cells were placed in suspension culture. At intervals, aliquots were withdrawn to determine the number of cells possessing cross-linked envelopes by the criterion of insolubility in 1% SDS and 10 mM dithiothreitol; other aliquots were used to determine the rate at which cells were able to incorporate tritiated glycine into protein.

As seen in Fig. 1, the suspended cells showed an increase in the rate of amino acid incorporation, somewhat variable in magnitude, during the first few hours, and then a rapid decrease. After a period of about a day, when protein synthesis had declined to a low level, the number of cross-linked envelopes began to increase and generally reached maximum values of 40–70% of the total cell number within the next 2 days. The same pattern of decreasing protein synthesis followed by the appearance of cellular envelopes was observed whether the cells were suspended in medium stabilized with methylcellulose (Fig. 1a) or were simply inoculated into bacteriological petri dishes, in which the cells settle to the bottom but do not readily attach (Fig. 1b). In all subsequent experiments, suspension cultures were stabilized with methylcellulose.

### *The Effect of Arresting Protein Synthesis on Formation of Cross-Linked Envelopes*

Cycloheximide, which suppresses protein synthesis within seconds (21), was used to determine whether the protein synthesis initially observed upon suspension of the cells was necessary for their subsequent formation of cross-linked envelopes. At concentrations of 1, 10, and 100  $\mu\text{g}/\text{ml}$ , the drug inhibited protein synthesis in keratinocyte surface cultures by 92, 96, and 99%, respectively, and thus is effective at the concentrations employed with other cell types (18). In numerous experiments, epidermal keratinocytes treated in suspension culture with cycloheximide at 100  $\mu\text{g}/\text{ml}$  were able to form cross-linked envelopes almost to the same final extent as control suspensions lacking inhibitor (Table I). Preparing the cell suspension by disaggregating the cells with

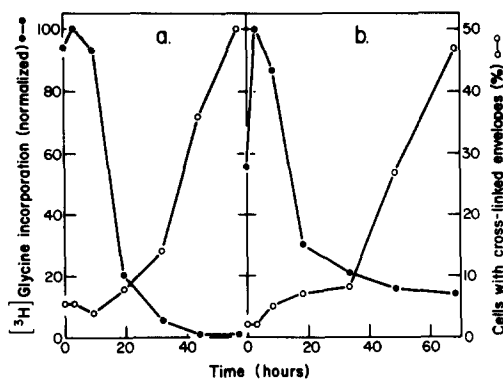


FIGURE 1 Arrest of protein synthesis and appearance of cross-linked envelopes in suspended human epidermal keratinocytes. (a) Cells stably suspended with methylcellulose; (b) cells in bacteriological petri dishes. The maximal values for protein synthesis, represented as 100, corresponded approximately to 5,000 cpm. At time zero, the cross-linked envelopes are those that had developed previously in the growing surface cultures.

TABLE I  
Effect of Inhibitors of Protein Synthesis on Cross-Linked Envelope Formation by Suspended Keratinocytes

Exp	Cells with cross-linked envelopes		
	Control	Cycloheximide, 100 $\mu\text{g}/\text{ml}$	Emetine, 100 $\mu\text{g}/\text{ml}$
		% of Total	
1	54	46	38
2	54	65	52
3	96	92	—
4	40	38	—

Cells incubated in methocel-stabilized suspension for 3–11 days. Values did not increase after the 4th day.

trypsin and EDTA in the presence of cycloheximide did not alter this result. Emetine (6) at 100  $\mu\text{g}/\text{ml}$  inhibited over 99% of protein synthesis in keratinocyte surface cultures, and similarly did not prevent envelope assembly by the suspended cells. Although in some experiments the proportion of cells forming envelopes in the absence of protein synthesis was slightly lower than in control suspension, it may be concluded that most if not all of the cells in growing cultures contain sufficient amounts of envelope precursor and cross-linking activity to assemble envelopes even if all protein synthesis is arrested at the time that the cells are placed in suspension culture.

Shorter-term experiments showed that the ini-

tial rate at which the cells acquired cross-linked envelopes was markedly accelerated by the presence of cycloheximide at 100  $\mu\text{g/ml}$  (Fig. 2). In a total of five experiments, an average period of 60–90 h was required for 50% of the cells to develop cross-linked envelopes in the absence of cycloheximide, whereas in the presence of cycloheximide the time was reduced to 20–35 h. Inhibition of protein synthesis by emetine (100  $\mu\text{g/ml}$ ) produced similar though sometimes not so marked acceleration of envelope formation. These results indicate that continuing protein synthesis delays the onset of envelope formation.

In surface cultures, in which normally fewer than 10% of the cells possess cross-linked envelopes (20), the keratinocytes were induced to make envelopes with much higher frequency by inhibitors of protein synthesis. In several experiments, inclusion of 100  $\mu\text{g/ml}$  of cycloheximide or emetine in the medium of surface cultures resulted in envelope formation by 30–70% of the cells in as little as 2 days. Hence, cell detachment itself appears not to be required for initiation of

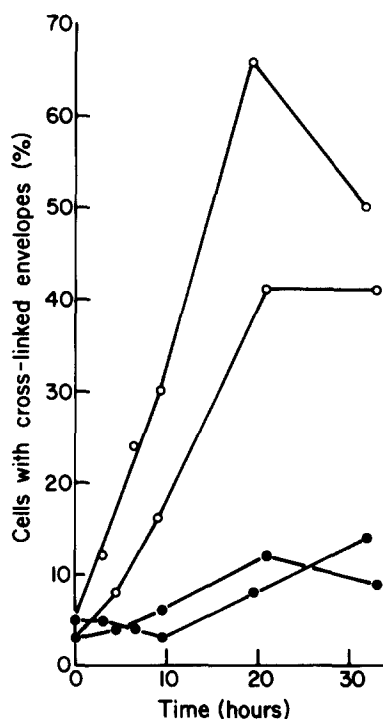


FIGURE 2 Effect of cycloheximide on initial rate of cross-linked envelope formation by suspended human epidermal keratinocytes. Two independent experiments. Open circles, cycloheximide, 100  $\mu\text{g/ml}$ ; closed circles, controls. The number of envelopes was increased by the drug within 4–6 h.

envelope assembly, but may have this effect because protein synthesis is arrested. In growing surface cultures, the cells containing envelopes may be those in which protein synthesis has already ceased as part of the process of terminal differentiation.

The visual scoring of cross-linked envelopes by phase or Nomarski microscopy does not reveal the amounts of protein incorporated into the envelopes or the extent of their cross-linking. Therefore, envelopes formed in the presence and absence of protein synthesis were characterized by these sensitive criteria for proper assembly. Cells were labeled with L- $^3\text{H}$ lysine while growing in surface culture, and inoculated into methylcellulose-stabilized suspension in the presence or absence of cycloheximide at 100  $\mu\text{g/ml}$ . The resulting envelopes were isolated as a residue after extraction of the detergent-soluble cellular protein under reducing conditions. As indicated in Table II, the cells incubated in the presence of cycloheximide and the controls had approximately the same proportion of  $^3\text{H}$ lysine-labeled cellular protein in the form of insoluble envelopes (8–12%).

The envelopes were then exhaustively digested with proteolytic enzymes, and the relative amounts of radioactive lysine and  $\epsilon$ -( $\gamma$ -glutamyl)lysine were measured after their separation by paper chromatography. In the envelope protein, the proportion of total lysines present as the cross-linking isodipeptide  $\epsilon$ -( $\gamma$ -glutamyl)lysine was only marginally lower for cells incubated in the presence of cycloheximide. All values were between 14 and 19%, indicating that the envelopes that formed in the absence of protein synthesis were essentially normal.

Previous work has shown that terminal differentiation of keratinocytes in suspension culture includes not only envelope formation but also accumulation of disulfide-stabilized keratin filaments and loss of cell nuclei (5). These processes were not inhibited and may actually have been slightly enhanced by the presence of cycloheximide or emetine.

To accelerate envelope formation, cycloheximide was required at a concentration sufficient to block protein synthesis by over 98%. A concentration of 3  $\mu\text{g/ml}$ , which is sufficient to inhibit protein synthesis by 90–95%, did not induce envelope formation in surface culture or accelerate the appearance of envelopes in suspension culture.

The effectiveness of inhibitors of protein syn-

TABLE II  
Extent of Envelope Cross-Linking in the Absence of Protein Synthesis

Trial	Sample	Proportion of cellular protein in envelopes*	Proportion of envelope [ <sup>3</sup> H]lysine cross-linked
		%	
1	100 μg/ml Cycloheximide	10	14
	Control (no cycloheximide)	8	17
2	100 μg/ml Cycloheximide	10	15
	Control (no cycloheximide)	12	18

\* Calculated from ratio of [<sup>3</sup>H]lysine in envelopes to total cellular acid-precipitable [<sup>3</sup>H]lysine.

thesis in promoting envelope formation in surface culture does not seem to be simply due to inhibition of cell growth. Sodium azide (10 mM), which interferes with ATP generation, arrested growth of surface cultures but did not promote envelope formation. In suspension cultures of nongrowing cells, neither sodium azide, colchicine (10 μg/ml), nor cytochalasin B (10 μg/ml) had any significant effect on initiation of envelope formation.

#### Role of Transglutaminase in Envelope Formation

Transglutaminase has been detected in cells of vertebrate epidermis (4) and has been purified from human stratum corneum (9) and cow snout (2). During terminal differentiation the epidermal cell envelope becomes cross-linked by ε-(γ-glutamyl)lysine (15, 19), suggesting participation of the transglutaminase. The enzyme is readily detectable in growing keratinocyte cultures, and must be present in sufficient amount to cross-link the envelope after cycloheximide is added. Evidence consistent with the importance of this enzyme was obtained by altering conditions known to affect its cross-linking activity in vitro. Initial observations that iodoacetamide (10 mM) completely suppressed envelope formation in methylcellulose suspension while, in the absence of serum, dithiothreitol (10 mM) enhanced it, are explainable in terms of the active site sulfhydryl groups of transglutaminases (3). More specific inhibition was obtained with putrescine and ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-te-

traacetate (EGTA).

Aliphatic amines inhibit transglutaminase cross-linking of natural substrates such as fibrin (7, 10). Putrescine (1, 4-diaminobutane), which serves as an acceptor amine in transglutaminase assays, inhibited the formation of cross-linked envelopes over 50% at 10 mM and completely at 100 mM. This experiment was performed in methylcellulose suspension in the absence of serum to avoid possible conversion of amine to aldehyde by serum oxidases.

The formation of cross-linked envelopes also depended upon calcium, as expected for a transglutaminase-catalyzed reaction (3). It was found that the addition of EGTA (Table III) or EDTA to a suspension culture of keratinocytes in excess of available Ca<sup>++</sup> prevented nearly completely the formation of cross-linked envelopes. Addition of Mg<sup>++</sup> in excess of free EGTA, which preferentially binds Ca<sup>++</sup> (16), did not permit significant envelope formation (Table III). Similarly, EGTA prevented the appearance of envelopes in cells suspended in the presence of cycloheximide.

The possibility that the activity of the enzyme is regulated by calcium ions was then considered. It has been suggested that this is the case for certain functions of transglutaminase in the erythrocyte (8). Two types of experiments were performed to affect envelope formation through the action of Ca<sup>++</sup>. One was to change the calcium concentration of the medium. The use of calcium-free medium did not prevent envelope formation (Table III), indicating that calcium retained by the cells was sufficient for enzyme activity. This

TABLE III  
Effect of Ca<sup>++</sup> Concentration on the Formation of Cross-Linked Envelopes by Suspended Keratinocytes

Addition	Proportion of cells with cross-linked envelopes*
	%
None	59
2 mM CaCl <sub>2</sub>	57
2 mM CaCl <sub>2</sub> + 3 mM EGTA	6
4.8 mM CaCl <sub>2</sub> + 3 mM EGTA	82
2 mM CaCl <sub>2</sub> + 3 mM EGTA + 2 mM MgCl <sub>2</sub>	10

The methylcellulose suspensions were prepared using calcium-free medium supplemented with 20% fetal calf serum depleted of free calcium by overnight dialysis against 10 vol of calcium-free medium.

\* Scored after 5 days.

was also demonstrated by allowing envelopes to form in calcium-free medium without serum supplementation. Increasing the calcium concentration of the medium to high levels (2 mM) had little or no effect on the proportion of cells developing cross-linked envelopes.

The second method was to add the ionophores A23187 (12) and X537A (11) to the medium (which contains  $\text{Ca}^{++}$  at 1.8 mM). Although the cells in surface culture were affected, judging by their fusiform morphology and inhibited growth at 1–20  $\mu\text{g}/\text{ml}$ , treatment with 0.1–20  $\mu\text{g}/\text{ml}$  of the ionophores had no significant effect on envelope formation even after 6 days. Moreover, addition of the ionophores (1–20  $\mu\text{g}/\text{ml}$ ) to suspended cell cultures did not alter the initial rate of envelope formation.

These observations therefore failed to provide any evidence that the onset of cross-linking was  $\text{Ca}^{++}$  activated. Evidently, there exists a sufficient quantity of intracellular calcium (perhaps mitochondrial) that becomes available, during the terminal organelle destruction, in sufficient amount to support the activity of the enzyme. The addition of EGTA or EDTA is effective in stopping the cross-linking by binding not extracellular but intracellular  $\text{Ca}^{++}$ , to which the chelator presumably gains access when the keratinocyte membrane becomes permeable during the process of terminal differentiation (5).

The activation of the cross-linking process need not proceed through an effect on the enzyme. For example, the precursor of the cross-linked envelope may be a potentially membrane-seeking protein, and the arrest of protein synthesis might lead to changes in membrane composition permitting the precursor protein to localize there. The action of the transglutaminase at this site would be favored by suitable alignment of the precursor molecules. Such a mechanism is consistent with evidence suggesting that the transglutaminase is itself not concentrated in the membrane (1).

These investigations were aided by grants from the National Cancer Institute.

Received for publication 18 July 1977, and in revised form 18 November 1977.

## REFERENCES

1. BUXMAN, M. M., and K. D. WUEPPER. 1975. Keratin cross-linking and epidermal transglutaminase. *J. Invest. Dermatol.* **65**:107–112.
2. BUXMAN, M. M., and K. D. WUEPPER. 1976. Isolation, purification, and characterization of bovine epidermal transglutaminase. *Biochim. Biophys. Acta.* **452**:356–369.
3. FOLK, J. E., and S. I. CHUNG. 1973. Molecular and catalytic properties of transglutaminases. *Adv. Enzymol.* **38**:109–191.
4. GOLDSMITH, L. A., H. P. BADEN, S. I. ROTH, R. COLMAN, L. LEE, and B. FLEMING. 1974. Vertebral epidermal transaminases. *Biochim. Biophys. Acta.* **351**:113–125.
5. GREEN, H. 1977. Terminal differentiation of cultured human epidermal cells. *Cell.* **11**:405–415.
6. GROLLMAN, A. P. 1968. Inhibitors of protein biosynthesis. V. Effects of emetine on protein and nucleic acid biosynthesis in HeLa cells. *J. Biol. Chem.* **243**:4089–4094.
7. LORAND, L., N. G. RULE, H. H. ONG, R. FURLANETTO, A. JACOBSEN, J. DOWNEY, N. ONER, and J. BRUNER-LORAND. 1968. Amine specificity in transpeptidation. Inhibition of fibrin cross-linking. *Biochemistry.* **7**:1214–1223.
8. LORAND, L., L. B. WEISSMAN, D. L. EPEL, and J. BRUNER-LORAND. 1976. Role of the intrinsic transglutaminase in the  $\text{Ca}^{++}$ -mediated cross-linking of erythrocyte proteins. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4479–4481.
9. OGAWA, H., and L. A. GOLDSMITH. 1976. Human epidermal transglutaminase. I. Preparation and properties. *J. Biol. Chem.* **251**:7281–7288.
10. PISANO, J. J., J. S. FINLAYSON, and M. P. PEYTON. 1969. Chemical and enzymatic detection of protein cross-links. Measurement of  $\epsilon(\gamma\text{-glutamyl})\text{lysine}$  in fibrin polymerized by factor XIII. *Biochemistry.* **8**:871–876.
11. PRESSMAN, B. C., and N. T. DEGUZMAN. 1975. Biological applications of ionophores: Theory and practice. *Ann. N. Y. Acad. Sci.* **264**:373–386.
12. REED, P. W., and H. A. LARDY. 1972. A23187: A divalent cation ionophore. *J. Biol. Chem.* **247**:6970–6977.
13. RHEINWALD, J. G., and H. GREEN. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell.* **6**:331–344.
14. RHEINWALD, J. G., and H. GREEN. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.)*. **265**:421–424.
15. RICE, R. H., and H. GREEN. 1977. The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell.* **11**:417–422.
16. SCHMID, R. W., and C. N. REILLEY. 1957. New complexon for titration of calcium in the presence of magnesium. *Anal. Chem.* **29**:264–268.
17. SMITH, I. 1969. Chromatographic and Electrophoretic Techniques. Vol. 1. John Wiley and Sons, Inc., New York. 104.

18. STANNERS, C. P., and H. BECKER. 1971. Control of macromolecular synthesis in proliferating and resting syrian hamster cells in monolayer culture. I. Ribosome Function. *J. Cell. Physiol.* **77**:31-42.
19. SUGAWARA, K. 1977. Intermolecular cross-links in epidermal differentiation. In *Biochemistry of cutaneous epidermal differentiation*. M. Seiji and I. A. Bernstein, editors. University of Tokyo Press, Tokyo. 387-397.
20. SUN, T-T., and H. GREEN. 1976. Differentiation of the epidermal keratinocyte in cell culture. Formation of the cornified envelope. *Cell.* **9**:511-521.
21. WARNER, J. R., M. GIRARD, H. LATHAM, and J. E. DARNELL. 1966. Ribosome formation in HeLa cells in the absence of protein synthesis. *J. Mol. Biol.* **19**:373-382.