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Presence in Human Epidermal Cells of a Soluble Protein Precursor of the Cross-Linked Envelope: Activation of the Cross-Linking by Calcium Ions

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Summary

Late in the terminal differentiation of epidermis and cultured epidermal cells, a protein envelope located beneath the plasma membrane becomes cross-linked by cellular transglutaminase. The process of cross-linking can be initiated in cultured epidermal cells by agents affecting cell membrane permeability—nonionic detergents, high salt concentrations and ionophores. These agents initiate the cross-linking process by making calcium ions available to the transglutaminase. A soluble precursor of the cross-linked envelope has been identified in crude extracts of cultured epidermal cells by its ability to incorporate labeled amines through the action of transglutaminase. The protein has been purified to homogeneity by gel filtration and chromatography on columns of DEAE-cellulose and hydroxyapatite. Comprising an estimated 5–10% of the soluble cell protein, it has a molecular weight of about 92,000, is isoelectric at pH 4.5±0.3 and has an unusual amino acid composition (46% Glx residues). It is chemically and immunochemically unrelated to keratins. The following evidence confirms that the protein becomes incorporated into cross-linked envelopes: first, washed cross-linked envelopes bind antibody to the purified protein, as shown by indirect immunofluorescence; second, absorption of the antiserum with washed envelopes removes all detectable antibodies to the purified protein; and third, the protein cannot be extracted from keratinocytes after their envelopes have become cross-linked. Examination of sections of epidermis by immunofluorescence, using antiserum to the purified protein, reveals that in addition to the stratum corneum, the living cells of the outer half of the spinous layer react strongly. The envelope precursor is present in the cytoplasm, but becomes concentrated at the cell periphery, where it will be cross-linked later, when the cells have passed through the granular layer. The protein is also concentrated in a peripheral location in cultured epidermal cells.

Introduction

In terrestrial vertebrates, the protective function of the epidermis is largely performed by the superficial strata of terminally differentiated keratinocytes (stratum corneum). These dead cells are filled with disulfide-bonded keratins and have a chemically resistant envelope (Matoltsy and Balsamo, 1955) made of protein (Sun and Green, 1976) and stabilized by ϵ-(γ-glutamyl) lysine cross-links (Rice and Green, 1977). Once the keratinocytes leave the basal layer of the epidermis, they no longer multiply but instead undergo a process of maturation during which they prepare for envelope formation.

Recent improvements in the cultivation of human epidermal cells have permitted investigation of their differentiation under more controlled conditions than were available previously. In the presence of supporting lethally irradiated 3T3 cells, single epidermal keratinocytes grow into colonies of stratified squamous epithelium (Rheinwald and Green, 1975). The cells can be disaggregated and transferred repeatedly through many cell generations in the presence of epidermal growth factor (Rheinwald and Green, 1977). Cell growth is also stimulated by cholera toxin and other agents able to increase cellular levels of cAMP (Green, 1978). All cells in the stratified colonies contain abundant keratin filaments, but the cells in the superficial strata, like those of epidermis, enlarge and ultimately form flattened squames possessing cross-linked protein envelopes (Sun and Green, 1976; Rice and Green, 1977).

When surface cultures are growing rapidly, 5% or fewer of the cells have such envelopes, but in suspension culture a majority of cells develop them (Green, 1977). Formation of a cross-linked envelope can occur without protein synthesis and is even promoted by inhibitors of protein synthesis (Rice and Green, 1978). Since the cross-linking requires transglutaminase activity, a majority of the cells at any time must have sufficient transglutaminase and sufficient precursor protein to form envelopes. In this paper, we report the isolation of such a precursor and demonstrate that it participates in envelope assembly in living cells before the cross-linking takes place.

During terminal differentiation, the cross-linking process occurs at the time cells die, when the cell membrane is known to become permeable (Green, 1977). Various ways of artificially inducing permeability of the cell membrane of cultured epidermal cells to external calcium ion lead quickly to activation of the cross-linking process.

Results

Accelerated Formation of Cross-Linked Envelopes by Agents Affecting Cell Membrane Permeability

Nonionic Detergent

When cross-linked envelopes appear in cells incubated in suspension culture, the cells are usually permeable to trypan blue (Green, 1977). The process of permeabilization and cross-linking requires days under these conditions, and even when accelerated by cycloheximide it requires many hours (Rice and

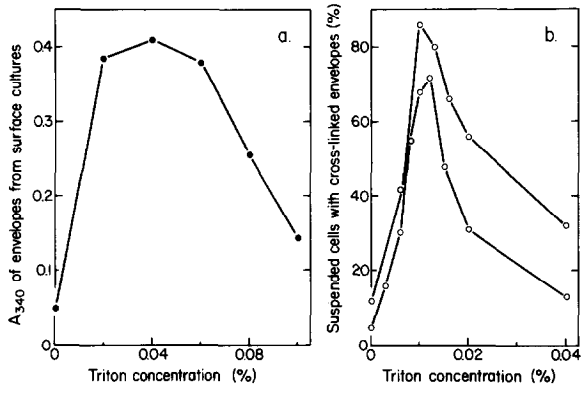

Figure 1. Envelope Formation as a Function of Triton X-100 Concentration

Confluent surface cultures (a) and trypsinized cell suspensions (b) were exposed to the indicated concentration of Triton for 4 hr and then extracted with 1% SDS and 20 mM dithiothreitol. The amount of cross-linked envelope formed was estimated either by the A_{340} of the envelopes after sonic disintegration (a) or by counts of the number of envelopes relative to the number of cells treated (b). The apparent difference in sensitivity between surface cultures and suspended cells may reflect the approximately 4 fold greater protein content of the former.

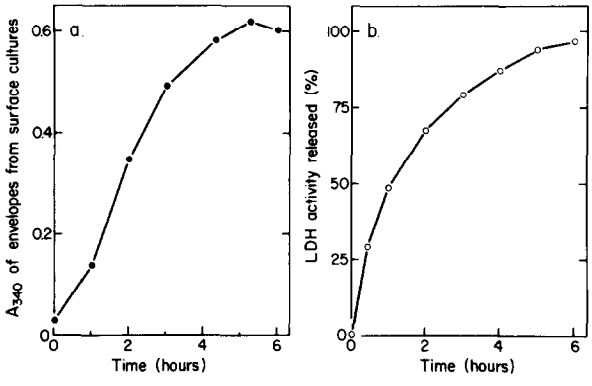

Figure 2. Kinetics of Envelope Cross-Linking and Enzyme Loss in Cells Treated With Triton

Confluent surface cultures of epidermal cells were washed with serum-free medium and a solution of 0.04% Triton X-100 in medium was added. (a) Kinetics of envelope formation determined by light attenuation (A^{340}). (b) Development of cell membrane permeability determined by release of lactate dehydrogenase into the medium.

Green, 1978). When confluent cultures of human epidermal keratinocytes were treated with low concentrations of Triton X-100 for 6 hr and then with sodium dodecylsulfate and dithiothreitol, the cellular contents dissolved, revealing a sheet of cross-linked envelopes covering the dish. The envelopes were quantitated by sonically disintegrating them and measuring the A^{340} due to light scattering by envelope fragments. As shown in Figure 1a, the amount of insoluble envelope material increased by 8–10 fold at the optimal concentration of Triton X-100 (0.04%). Nonidet P-40 was similarly effective over the same concentration range.

When trypsinized keratinocytes were treated in suspension with Triton X-100, they formed cross-linked

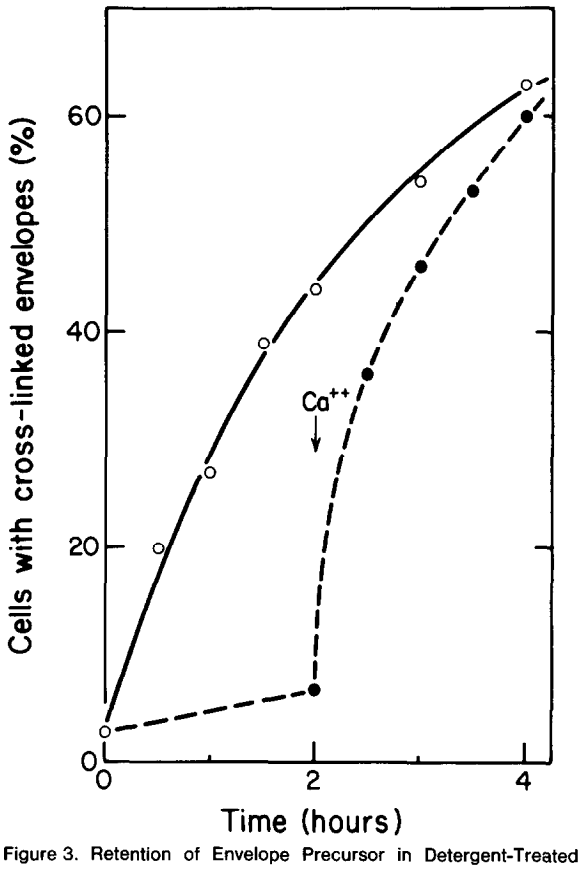

Figure 3. Retention of Envelope Precursor in Detergent-Treated Cells

Cell suspensions were treated with 0.01% Triton X-100. (○) Control, no other additions. (●) EGTA added to 10 mM; after 2 hr (arrow), $CaCl_2$ was added to 20 mM. The cells did not cross-link their envelopes in the absence of Ca^{++} but preserved their ability to do so when Ca^{++} was restored.

envelopes, and these were counted in a hemocytometer chamber. Envelopes formed optimally at a detergent concentration of 0.01% (Figure 1b), nearly the same concentration as was most effective on surface cultures, but higher concentrations reduced the effect more sharply in suspended cells than in surface cultures.

The kinetics of cross-linking induced by Triton X-100 were examined in surface cultures. Within 1 hr after the addition of the detergent, the amount of cross-linked envelope was much increased, and reached a maximum in 4–5 hr (Figure 2a). Although not obvious in the microscope at low power, probably because of envelope formation, the detergent produced considerable damage in the keratinocyte membranes. This was evident from the release of cellular protein into the medium. When the activity of lactate dehydrogenase (molecular weight 140,000) was assayed at intervals, it was found that half the enzyme was lost from the cells in an hour and essentially all was lost within 5 hr (Figure 2b). Transglutaminase diffused out of the cells at a similar or perhaps slightly slower rate. The cross-linking enzyme therefore

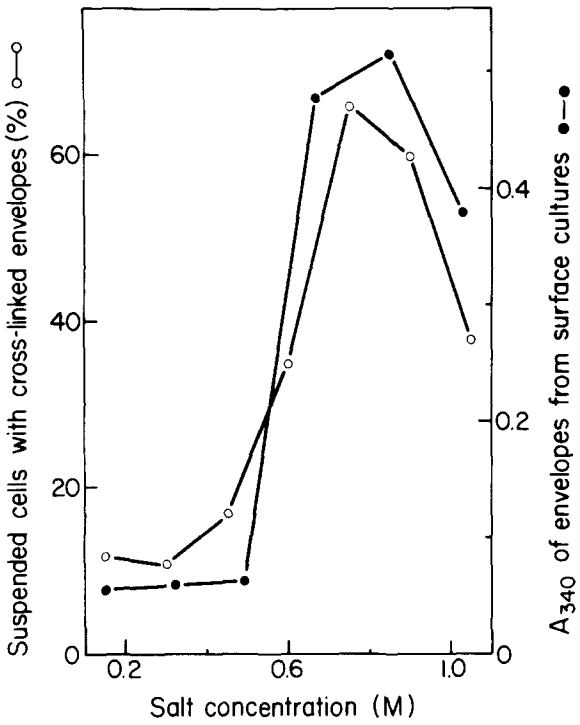

Figure 4. Effect of Salt Concentration on Envelope Formation by Epidermal Cells in Suspension (3.5 Hr Incubation) or in Confluent Surface Culture (6 Hr Incubation)

Sodium chloride was added to serum-free medium to give the final concentrations shown on the abscissa. The maximum number of envelopes formed at a sodium concentration of 0.7–0.9 M.

seems to be lost from the cells more readily than the envelope precursor.

Triton-activated cross-linking had the same enzymatic requirements as those shown earlier for transglutaminase in other cross-linking processes (Folk and Chung, 1973) and for spontaneous envelope cross-linking by keratinocytes (Rice and Green, 1978). The process was completely prevented by 20 mM cystamine, a potent transglutaminase inhibitor known to form a mixed disulfide with a sulfhydryl group at the active site of the enzyme (Siefring et al., 1978). Chelation of calcium ion with EDTA or EGTA also prevented cross-linking in cell suspensions treated with 0.01% Triton for 2 hr; when cross-linking was prevented by 2 mM EGTA and an excess of Ca^{++} was then added, envelopes formed to the same extent as in control suspensions whose cross-linking had never been blocked (Figure 3), and the rate of envelope formation was slightly faster. Similarly, when Triton-treated cell suspensions were chilled to 0°C to prevent envelope formation and then rewarmed to 37°C, there was no decrease in final yield of envelopes. These experiments show that in a low detergent concentration the envelope precursor was effectively retained by the cells over the period during which the cross-linking was prevented.

High Salt Concentration and Ionophores

High concentrations of sodium chloride have recently been shown to make cell membranes permeable to a variety of small molecules (Castellot, Miller and Pardee, 1978). Serum-free medium adjusted with NaCl to a total of about 0.75 M in Na^+ was found to be effective in promoting envelope formation (Figure 4). Suspended keratinocytes quickly became shriveled due to loss of water, but they formed envelopes to approximately the same extent as in Triton X-100. Envelope formation began generally after a lag of approximately 1 hr, presumably because of the time required for cells to become permeable (Castellot et al., 1978). Attempts to demonstrate envelope formation in hypotonic medium were unsuccessful.

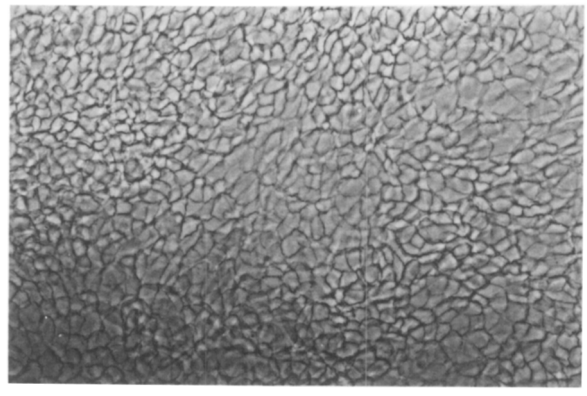

Figure 5. Sheets of Cross-Linked Envelopes Produced by a Calcium Ionophore

Confluent cultures were incubated for 6 hr in serum-free medium containing 100 μg of X537A per ml. Sodium dodecylsulfate was then added to 2% and dithiothreitol to 20 mM. The contents of the cells are dissolved and the cross-linked envelopes are clearly visible. The appearance of Triton-treated cells was identical.

The ionophores X537A and A23187 are known to make membranes permeable to Ca^{++} (Pressman, 1976). In the presence of Ca^{++}, A23187 has been shown by Lorand et al. (1976) to be effective in activating transglutaminase of intact erythrocytes. At the same concentrations neither A23187 nor X537A was effective in initiating cross-linking of the keratinocyte envelope (Rice and Green, 1978), but at concentrations considerably higher than those used earlier they are quite effective.

The ionophore X537A promotes flux of mono- and divalent cations across membranes (Pressman, 1976). A confluent epidermal culture treated with this ionophore produced cross-linked envelopes similar to those produced by Triton (Figure 5). The optimum ionophore concentration in surface cultures was ~160 μM and in suspension cultures ~40 μM (Figure 6a); in both cases there were over 50 nmole of ionophore per mg of cell protein. In other experiments, the calcium ionophore A23187 promoted envelope formation as did X537A, but was so insoluble that the effective concentration was uncertain.

The ionophore X537A induced envelope formation in suspended cells more quickly than Triton X-100 or

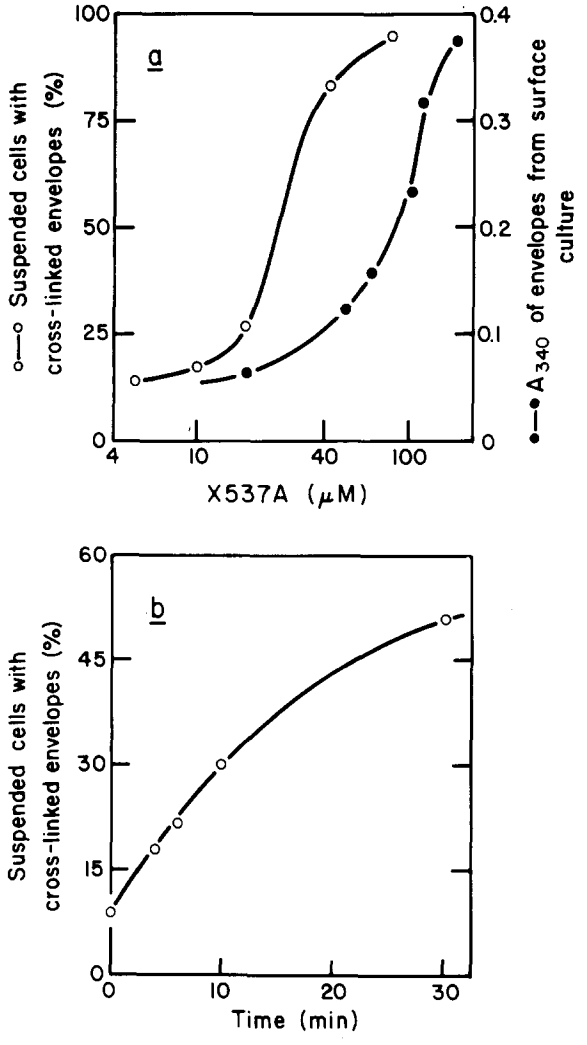

Figure 6. Effect of Ionophore Concentration on Cross-Linked Envelope Formation

(a) Keratinocytes in suspension were treated with X537A for 3.5 hr. Confluent surface cultures were treated for 6.5 hr. Note the very high concentrations of ionophore necessary to attain maximum envelope formation. The apparent difference in sensitivity between suspended cells and surface cultures may reflect the approximately 4 fold greater protein content of the latter.

(b) Kinetics of cross-linked envelope formation in suspended keratinocytes following addition of X537A to 50 μg/ml.

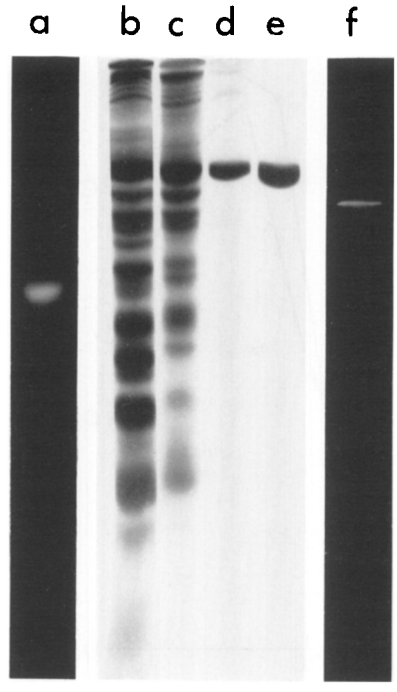

Figure 7. Envelope Precursor Protein at Different Stages of Purification

Electrophoresis in acrylamide gels was carried out in 0.1% sodium dodecylsulfate (Weber and Osborn, 1969).

(a) Precursor protein labeled with dansylcadaverine by transglutaminase in crude extracts of cultivated epidermal cells. The single fluorescent band visible under ultraviolet illumination in this 6% gel migrated in 8% gels as shown in (e) for the 92K protein.

(b–e) Polyacrylamide concentration 8%; proteins stained with Coomassie blue. (b) Crude extract of cultured epidermal cells. (c) After gel filtration on Bio-Gel A-1.5 M. (d) After chromatography on DEAE cellulose. Several faint contaminating bands are present near the top of the gel. (e) After chromatography on hydroxyapatite. The purified protein had the same mobility as rabbit muscle phosphorylase (92K).

(f) Isoelectric focusing of the purified protein. Location of the band near the acidic end of the gel is shown by light scattering of the trichloracetic acid-precipitated protein, using visible illumination at an oblique angle.

hypertonic medium. Increases in the number of cross-linked envelopes were detectable in less than 5 min; the number was half-maximal in about 20 min (Figure 6b). This ionophore appeared to be the mildest of the three agents, since the cell shape was unaffected and loss of cellular lactate dehydrogenase was very slow (≤1% per hour in surface culture). The efficacy of the ionophores in cross-linking depended upon their ability to bring external Ca^{++} into the cells; when suspended cells were exposed to these agents in medium lacking Ca^{++}, there was no activation of cross-linking over a 4 hr period.

In a brief survey of other agents, the channel-forming ionophore Gramicidin S promoted envelope formation well at 100 μg per ml and partially at 10 μg per ml, while valinomycin, which transports K^+ (Pressman, 1976), had no effect at 100 μg per ml. Lysolecithin, used according to the method of Miller, Castellot and Pardee (1978), also promoted envelope formation in surface cultures.

Isolation of an Envelope Precursor

Detection of a Protein Substrate of Transglutaminase

Assays of transglutaminase exploit the ability of the enzyme to incorporate labeled aliphatic amines into proteins having available glutamine residues (Folk and Chung, 1973). Since crude extracts of epidermis contain transglutaminase (Goldsmith et al., 1974; Buxman

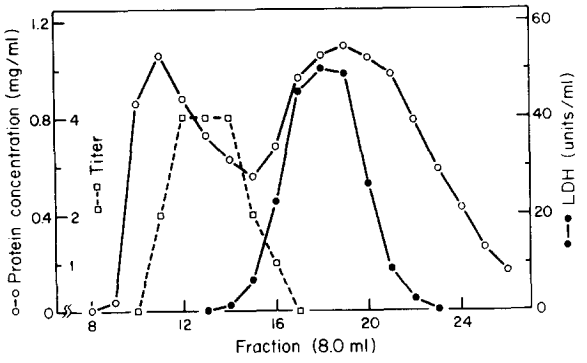

Figure 8. Fractionation of Crude Extract of Cultivated Epidermal Cells on Biogel A-1.5 M

The location of the envelope precursor protein was determined by precipitin formation using a specific antiserum. The precursor protein (□) eluted near the void volume (Fraction 9). Most cell protein, including lactate dehydrogenase, eluted much later. In other stages of purification, fractions containing the precursor protein were often identified by the presence of the 92K protein demonstrated by gel electrophoresis.

and Wuepper, 1976; Ogawa and Goldsmith, 1976), a search was made for a cellular protein labeled by radioactive or fluorescent amines. Figure 7a shows the result of an experiment in which a keratinocyte extract was incubated with dansylcadaverine and the products were subjected to electrophoresis in a polyacrylamide gel containing sodium dodecylsulfate (Lorand et al., 1972). Under ultraviolet light, a single fluorescent band was prominent at the same location as a protein of 92,000 daltons identified by Coomassie blue staining. When the gels were more heavily loaded, faint fluorescent bands were visible at positions of about 45,000 and 20,000 daltons, but Coomassie blue staining did not reveal definite protein bands at these locations. In cells treated with Triton it was possible to label some of the unpolymerized precursor protein without preparing a cell extract; within 30 min, added dansyl cadaverine labeled enough of the 92K protein for the product to be identified by gel electrophoresis.

A substrate of transglutaminase in bovine epidermis was reported previously to have a molecular weight of 150,000 (Buxman, Buehner and Wuepper, 1976), but we observed no fluorescent protein with this molecular weight in extracts of cultured human keratinocytes. No fluorescent bands were visible when the incubation was carried out in the presence of 20 mM iodoacetamide or 20 mM EGTA, known inhibitors of transglutaminase (Folk and Chung, 1973). A fluorescent protein of 92,000 daltons was also formed in extracts of serially cultivated rabbit epidermal cells, but not in extracts of diploid human or rabbit dermal fibroblasts (even though both possess considerable transglutaminase activity) or in extracts of 3T3 cells. The 92K protein of epidermal cells could also be labeled enzymatically with ^{3}H-putrescine or ^{14}C-aminoethanol,

Table 1. Purification of Envelope Precursor Protein

Stage of Purification	Purity (%)[a]	Yield (%)[a]
Crude Extract	6.4	100
Bio Gel A-1.5 M	27	88
DE-52	84	42
Hydroxyapatite	100	18

[a] Assayed immunochemically, average of three experiments.

known amine donors (Folk and Chung, 1973).

Gel Filtration of the Transglutaminase Substrate-Protein and Estimation of its Native Molecular Weight

Figure 8 gives the elution profile of the proteins of a crude isotonic keratinocyte extract after filtration through Bio Gel A-1.5 M. Most cellular proteins, including lactate dehydrogenase (140,000 daltons), were considerably retarded, whereas the envelope precursor protein eluted near the void volume, suggesting a molecular weight of over 500,000. Gel filtration led to 4 fold purification of the precursor, with only small losses (Table 1 and Figures 7b and 7c). Gel filtration also permitted clean separation of the precursor protein from transglutaminase activity, the latter eluting in the region of lactate dehydrogenase. When transglutaminase was removed in this way, the precursor could be labeled with dansyl cadaverine by adding the enzyme in the form of a crude extract of dermal fibroblasts, showing that the fibroblast transglutaminase also recognizes the precursor protein as a preferred substrate.

The discrepancy between the size of the precursor molecule estimated from gel electrophoresis in SDS and that estimated from gel filtration under nondenaturing conditions could be due to an aggregated native state of the protein or to a highly elongated shape. The gel filtration properties of the precursor protein were not changed either by inclusion of 1% Triton X-100 in the sample and column buffer or by its prior chromatographic purification on DEAE cellulose and hydroxyapatite columns (described below). This showed that the molecule was not dissociated by the nonionic detergent or by purification.

Information about the shape of the molecule was obtained by comparing its sedimentation and gel filtration properties (Siegel and Monty, 1966), as shown previously for the tau protein of microtubules (Cleveland, Hwo and Kirschner, 1977). Such an analysis led to the conclusion that the envelope precursor protein is highly elongated. In gel filtration in columns of BioGel A-5 M (Figure 9a), the precursor protein eluted between E. coli β-galactosidase (465K) and bovine thyroglobulin (660K), permitting estimation of its diffusion coefficient ($D_{20,w}$) as 2.8×10^{7} cm^2 per sec. In 5–20% linear sucrose gradients (Martin and Ames,

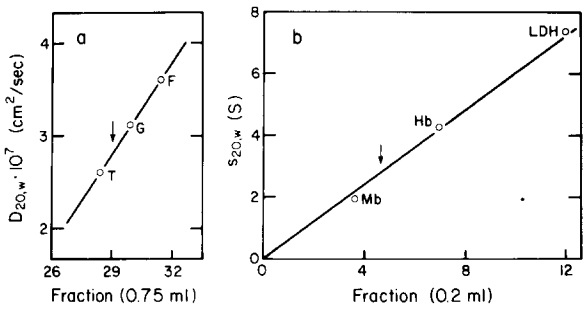

Figure 9. Estimates of Diffusion and Sedimentation Coefficients of Envelope Precursor Protein

Epidermal cell extracts were centrifuged at 10,000 × g for 10 min and the supernatants were applied to (a) BioGel A-5 M gel filtration or (b) 5–20% linear sucrose gradients (Martin and Ames, 1961) in isotonic phosphate buffer. Values for diffusion and sedimentation coefficients of standards are as given by Smith (1970).

(a) The locations of bovine thyroglobulin (T), E. coli β-galactosidase (G) and horse spleen ferritin (F) are shown. Envelope protein (arrow) was located with specific antiserum.

(b) 0.2 ml of epidermal cell extract containing 0.8 mg of either bovine hemoglobin (Hb) or sperm whale myoglobin (Mb) was layered on 5 ml gradients and centrifuged for 20 hr at 3°C in an SW 50.1 rotor at 38,000 rpm. The tubes were punctured and 4 drop (0.2 ml) fractions were collected. The fractions were numbered from the top. The peak of cellular lactate dehydrogenase (LDH) activity (Fraction 12) was used to normalize gradients run in parallel.

1961), the envelope protein was found to have a sedimentation coefficient of about 2.8S, between bovine hemoglobin (4.3S) and sperm whale myoglobin (2.0S), as shown in Figure 9b. Substituting these values in the Svedberg equation $S=M(1-\bar{\nu}\rho)D/RT$ gives a molecular weight estimate of 83K, which is in reasonable agreement with the value of 92K obtained by electrophoresis. In this estimate, the partial specific volume ($\bar{\nu}$) was calculated as 0.720 from the amino acid composition of the purified protein (Table 2), according to the method of Cohn and Edsall (1943). It seems most probable from this analysis that the envelope precursor is a monomeric protein of highly asymmetric shape. Its low diffusion coefficient, as compared to globular proteins of similar molecular weight, corresponds to a frictional coefficient of about 2.5 and an axial ratio greater than 30:1.

Purification of the Precursor by Chromatography on DEAE Cellulose

Preliminary experiments indicated that at neutral pH the precursor protein was not bound to negatively charged resins (carboxymethylcellulose, phosphocellulose or sulfopropyl-Sephadex), but was retained by positively charged resins (QAE-Sephadex, DEAE- or ECTEOLA-cellulose). To locate the protein more con-

Table 2. Amino Acid Composition of Envelope Precursor and of Envelopes After Cross-Linking

	Envelope Precursor[a]	Cross-Linked Envelopes			
		X537A[b]	Triton[c]	NaCl[c]	Methocel[d]
Asx	2.8	8.6	9.3	9.2	9.1
Thr	1.6	4.8	5.1	4.9	4.8
Ser	1.6	6.5	9.0	6.7	7.2
Glx	45.8	16.6	13.3	14.9	15.9
Pro	5.7	8.0	7.6	7.5	7.8
Gly	6.7	6.8	7.6	7.7	9.2
Ala	1.5	5.5	6.9	6.9	7.0
Val	3.7	4.8	4.3	5.4	5.1
Met	0.9	2.5	2.5	2.4	1.5
Ile	0.4	4.0	3.5	3.8	3.5
Leu	14.6	8.6	9.0	8.6	7.9
Tyr	0.8	3.5	3.4	2.9	1.8
Phe	0.6	3.4	3.4	3.3	2.9
His	4.7	2.2	1.8	2.0	1.9
Lys	7.4	7.8	7.7	8.1	7.6
Trp	0.2	2.9	1.2	1.1	e
Arg	0.7	3.5	4.4	4.6	4.3
$CySO_3$	0.3	N.D.[e]	N.D.[e]	N.D.[e]	2.5
	100.0[f]	100.0[f]	100.0	100.0	100.0

[a] Mean of three analyses.
[b] Confluent surface culture, one analysis.
[c] Cell suspension, one analysis.
[d] Cell suspension stabilized with methyl cellulose (Rice and Green, 1978), mean of three analyses.
[e] N.D. = not determined.
[f] Less than 2% carbohydrate by weight in anthrone test according to Spiro (1966).

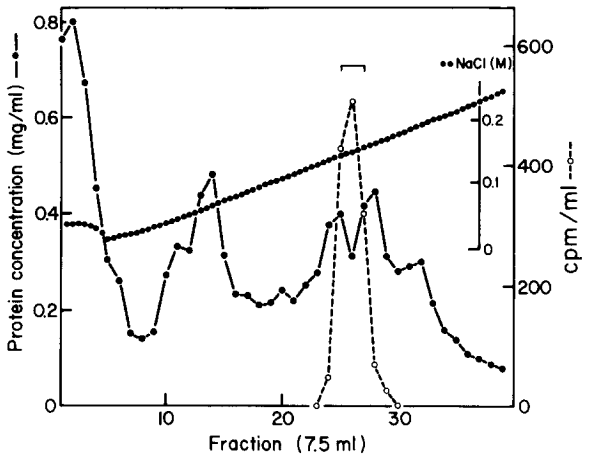

Figure 10. Fractionation of Crude Extract of Cultivated Epidermal Cells on DEAE Cellulose

A crude cell extract was applied to the column together with a marker of previously purified precursor protein labeled with ^{14}C-2-aminoethanol. The labeled precursor (○) eluted sharply at about 0.15 M NaCl. Bracket shows fractions pooled for further purification.

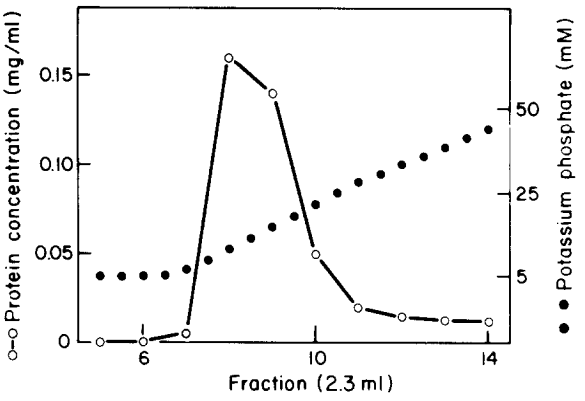

Figure 11. Chromatography of Envelope Precursor Protein on Hydroxyapatite

Following gel filtration on BioGel A-1.5 M and DEAE cellulose chromatography, the envelope precursor was applied to a column of hydroxyapatite. Most of the protein eluted in a single peak at a potassium phosphate concentration of about 10 mM. This protein appeared homogeneous in SDS gel electrophoresis (Figure 1e).

veniently during chromatography, it was labeled enzymatically in crude keratinocyte extracts with ^{14}C-aminoethanol. As in the case of dansyl cadaverine, the 92K protein was preferentially labeled, but aminoethanol has the advantage over putrescine or dansyl cadaverine that it avoids alteration in net charge or hydrophilic character of the protein. Triton X-100 and unreacted aminoethanol were removed by chromatography on DEAE-cellulose, the labeled protein eluting as a single peak of radioactivity at about 0.15 M NaCl in a linear salt gradient. This marker was then added to a larger quantity of keratinocyte extract for preparative purification.

Figure 10 shows the profile of keratinocyte proteins eluted from DEAE-cellulose with a linear salt gradient. The peak of radioactivity indicates the location of the envelope precursor protein. Electrophoresis in SDS gels confirmed that only fractions containing the label also contained the 92K protein. The chromatography resulted in purification of about 8 fold, but a contaminating protein with electrophoretic mobility of rabbit muscle actin remained even after subsequent purification. Since it was removed by gel filtration on Biogel A-1.5 M, this step was henceforth performed before DEAE-cellulose chromatography. Under these conditions, approximately half the starting precursor protein was recovered from the ion exchange column in nearly homogenous form (Table 1, Figure 7d).

Final Purification of the Precursor by Chromatography on Hydroxyapatite

Chromatography on columns of hydroxyapatite readily removed the remaining contaminating proteins (Figure 11). The overall purification was about 15 fold and the yield about 18% (Table 1). The protein appeared homogeneous by gel electrophoresis (Figure 7e), and its mobility in the presence of SDS was not significantly affected by omission of reducing agent. The protein was also homogeneous upon isoelectric focusing in polyacrylamide gels (Figure 7f), and had an isoelectric point of 4.5±0.3, consistent with its affinity for positively charged ion-exchange resins at neutral pH. Unpurified precursor protein labeled in crude extracts with dansyl cadaverine had approximately the same isoelectric point.

Immunological Identification of the 92K Protein as Envelope Precursor

To follow the envelope precursor protein during envelope assembly and cross-linking, we prepared a rabbit antiserum to the purified protein. When tested by immunodiffusion, the antiserum formed a precipitin band with the protein, whether purified or in a crude cell extract (Figure 12). As little as 0.2 μg of the pure protein gave a visible band. When an immune precipitate made in solution from a crude cell extract was centrifuged, washed in isotonic saline and submitted to electrophoresis in the presence of SDS, the 92K protein was virtually the only cell protein present. No immune precipitate was produced by crude extracts of human dermal fibroblasts or 3T3 cells (Figure 12) or by isolated keratins of stratum corneum, using sodium dodecylsulfate to keep the keratins in solution (Sun and Green, 1978). When crude epidermal cell extract was passed through BioGel A-1.5 M or chromatographed on DEAE-cellulose, there was only one peak of immunoreactive antigen, and this peak was coincident with that of the protein labeled with ^{14}C-aminoethanol by transglutaminase.

To examine the immunoreactivity of envelopes, cross-linking was initiated in confluent keratinocyte cultures with Triton X-100 or X537A. After the process was complete, the envelopes were washed with guanidinium chloride, sectioned and treated with the antiserum. As seen by indirect immunofluorescence, the

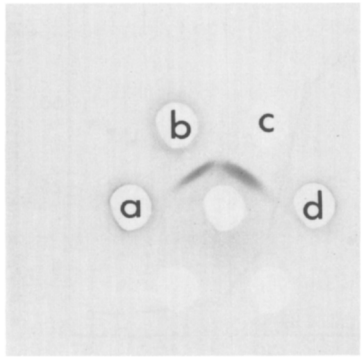

Figure 12. Immunoprecipitation of Envelope Precursor Protein

10 μL of antiserum were placed in the center well of a diffusion chamber and allowed to react with 10 μg of purified envelope precursor (c) and 100 μg of crude keratinocyte extract (b). A common precipitin band resulted. No precipitin band was produced by 100 μg of cell protein extracted from human dermal fibroblasts (a) or 3T3 cells (d). All cell extracts were prepared by homogenization in isotonic buffer containing 10 mM EDTA and clarified by centrifugation at 10,000 × g.

envelopes were stained well by the antiserum (Figure 13a).

The ability of the washed envelopes to absorb the antibodies to the precursor protein was then examined. After the antiserum was absorbed with 0.42 mg of envelope protein per 0.2 ml, it was no longer able to form a precipitin band against a crude keratinocyte extract (Figure 14). In contrast, absorption of the antiserum with the same amount of washed red cell ghosts (prepared by lysis of human red cells in water) led to no detectable loss in titer. Absorption of an antiserum to purified keratins with washed envelopes did not reduce its reactivity against the keratins by immunodiffusion in solution containing SDS. The envelope precursor thus seems to bear no immunological relation to the keratins.

An experiment was performed to examine the effect of the cross-linking process on the amount of extractable envelope precursor. The ionophore X537A was added to 160 μM to confluent cultures of epidermal cells. After incubation for 7 hr, the cells were homogenized, the extract was centrifuged at 10,000 × g for 10 min and the supernatant was tested by immunodiffusion against the antiserum. The cells contained virtually no extractable protein able to give an immune precipitate (Figure 15b). Inclusion of 20 mM EDTA during the incubation of the cells with the ionophore prevented the disappearance of the extractable protein (Figure 15c), showing that the disappearance of this protein depended on the cross-linking process.

Additional experiments established that the precursor protein could be cross-linked in solution. The purified protein was treated with ^{14}C–formaldehyde so as to label only a small fraction of the lysine residues (Rice and Means, 1971). The labeled protein was incubated with crude keratinocyte extract and Ca^{++}

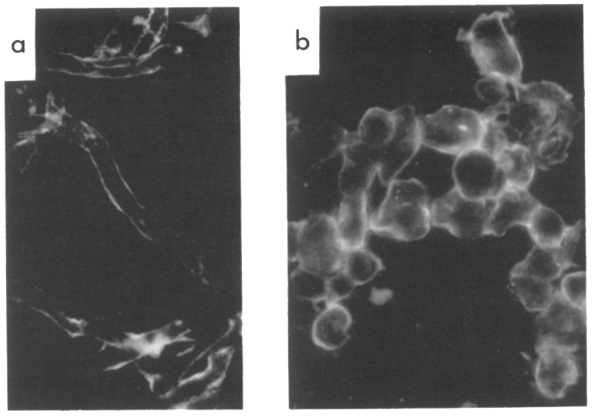

Figure 13. Staining of Envelopes by Indirect Immunofluorescence Using Antiserum to the Precursor Protein

(a) Cross-linked envelopes were formed in confluent surface cultures using 160 μ M X537A. The envelopes were then extracted with 6 M guanidinium hydrochloride, prepared as a pellet, sectioned and tested with the antiserum. The fluorescent envelopes show the flattened contours of cells growing on a surface.

(b) Expanding colonies were trypsin-disaggregated, and the cells were formalin-fixed without extraction. The fluorescent borders show the spherical shape adopted by the trypsinized cells. In most of these cells, the envelopes have not yet been enzymatically cross-linked.

at 5 mM for 1 hr and then submitted to electrophoresis. Most of the radioactivity remained at the very top of the gel. The polymerization of the labeled protein was prevented when 10 mM EDTA was included in the incubation.

Keratinocytes cultivated from human corneal epithelium (Sun and Green, 1977) and tracheal epithelium have been observed to form envelopes in suspension culture. Like epidermal cells, these cell types possess a protein that reacts with the antiserum to epidermal cell envelope precursor, and that can be labeled enzymatically with dansyl cadaverine to give a single prominent fluorescent protein band in SDS gel electrophoresis.

Localization of Envelope Precursor in Living Cells

The rapidity with which Triton X-100 promotes envelope cross-linking in cells, even though the plasma membrane is degraded and cytoplasmic macromolecules are lost from the cells, made it seem improbable that the assembly of the envelope could take place after the addition of the detergent. For this reason, the location of the envelope precursor in living cells was investigated by immunofluorescence, using antiserum to the precursor protein.

Frozen sections were prepared from human skin and stained with antiserum. There are two significant aspects (Figure 16). First, the protein was located not only in stratum corneum, which possesses cross-linked envelopes, but also in the living cell layer, usually down to about the superficial half of the spinous cell layer; though there was some variation in the position of the line of demarcation, the deepest

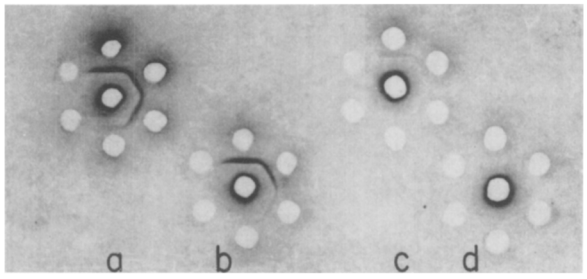

Figure 14. Absorption of Antibody to Envelope Precursor by Whole Washed Cross-Linked Envelopes

0.2 ml of antiserum was absorbed with washed envelopes containing 0 (a), 0.12 mg (b), 0.42 mg (c) and 1.2 mg (d) of protein. 2 fold serial dilutions of the antiserum were placed in the outer wells beginning at 12 o'clock (no dilution) and proceeding clockwise. In the inner well of each set keratinocyte extract containing 10 μg of protein was added. Note that absorption with 0.12 mg of envelope protein diminished the reactivity of the serum toward the soluble keratinocyte extract (b) and 0.42 mg removed nearly all reactivity (c).

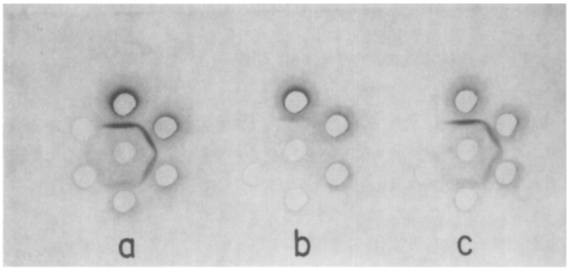

Figure 15. Loss of Immunoreactive Soluble Envelope Precursor from Cells during Formation of Cross-Linked Envelopes

Extracts were prepared from (a) suspended cells incubated for 7 hr in serum-free medium; (b) cells incubated with X537A in medium for 7 hr; and (c) cells incubated for 7 hr with the ionophore in the presence of 20 mM EDTA. Antiserum to the purified precursor was placed in the center wells, and 2 fold serial dilutions of the keratinocyte extract were placed in the outer wells, beginning at 12 o'clock (no dilution) and proceeding clockwise. Note that during the cross-linking process nearly all soluble immunoreactive precursor disappeared from the cells (b).

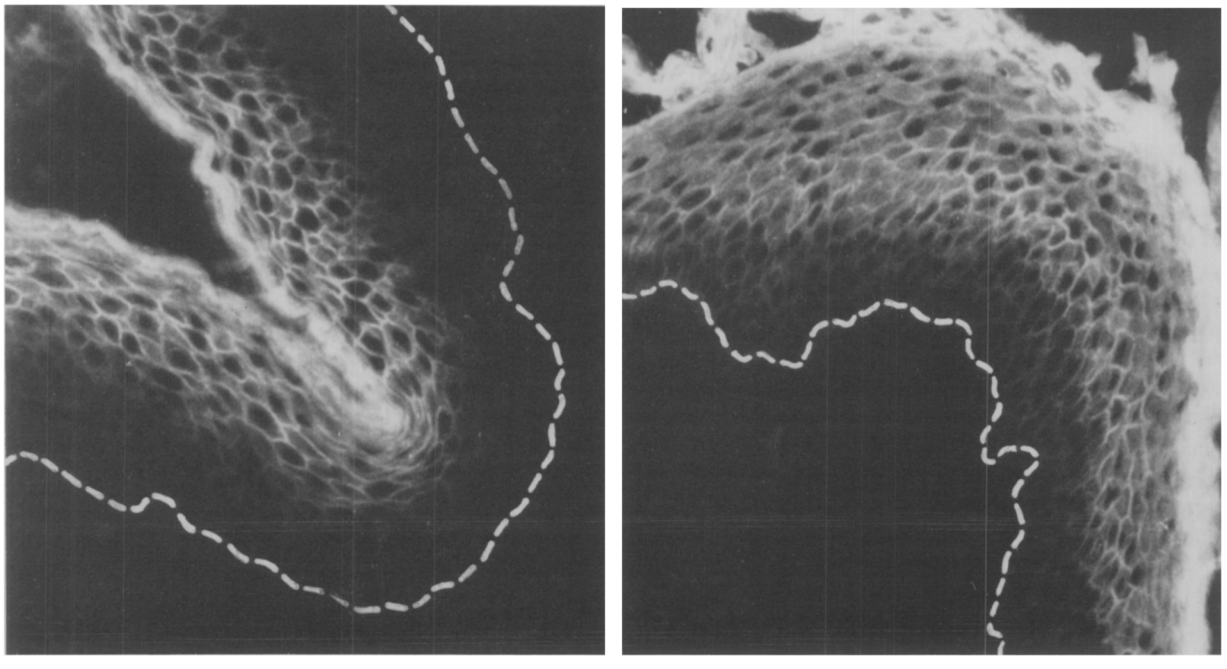

Figure 16. Localization of Envelope Precursor in Sections of Human Skin

Frozen sections (5 μ thickness) of newborn human foreskin were examined for the presence of envelope precursor protein by indirect immunofluorescence. The antigen is present in cells of stratum corneum and in the outer layers of cells in the living part of the epithelium, usually down to about the middle; examples of variability are shown. The deepest layers rarely show fluorescence, and the dermis never does. The envelope precursor is seen in the interior of the cell cytoplasm, but becomes concentrated peripherally long before the cross-linking takes place at the site of stratum corneum formation.

part of the spinous layer and the basal cells rarely contained significant fluorescence. Second, the precursor protein was found in the cytoplasm, but had a distinct tendency to concentrate in the periphery of the cells (Figure 16). From the appearance of the sections alone it could not be stated whether this peripheral concentration was intracellular or extracellular, but in view of the known intracellular site of the envelope after subsequent cross-linking (Hashimoto, 1969; Green, 1977), it may be assumed that the peripherally located envelope precursor is also on the cytoplasmic side of the cell membrane. The cytoplasmic staining visible in many cells is patchy, and probably signifies the location of precursor protein that has not yet moved to the cell periphery. Earlier investigations had already suggested that the epidermal transglutaminase is not localized to membranes (Buxman and Wuepper, 1975).

Cultures of keratinocytes were trypsinized and frozen sections were made of a centrifuged cell pellet. When these were examined by immunofluorescence, most of the cells showed peripheral concentration of the stain (Figure 13b), even though the envelopes are already cross-linked in only 5–10% of the cells of such cultures.

Amino Acid Composition of the Precursor Protein

Precursor protein isolated by the three-stage procedure described above was analyzed for its amino acid composition (Table 2). The protein shows a striking preponderance of glutamic acid/glutamine residues (46% Glx). The proportion of each is unknown; but even a relatively small proportion of glutamine would be sufficient to provide the degree of cross-linking found in the envelopes, where 15–20% of the lysine residues participate in the formation of ϵ-(γ-glutamyl) lysine bonds (Rice and Green, 1977). The low content of aromatic residues is consistent with the low extinction coefficient for absorption of ultraviolet light (A^{280}) noted during purification.

The amino acid composition of the precursor protein was compared with that of cross-linked envelopes prepared in all the ways described above. The cross-linking was promoted by incubating cells in methocel-stabilized suspension culture for 4 days, and by exposure to Triton X-100, to X537A or to high salt concentrations, in each case for 6 hr. The envelopes were then extracted with sodium dodecylsulfate and dithiothreitol several times, hydrolyzed and analyzed. Table 2 shows that all methods gave amino acid compositions in excellent agreement with each other, but in poor agreement with that of the purified envelope precursor. The most striking difference is in the content of Glx residues, comprising 46% of the precursor and 13–17% of the envelope. Distinct differences are also apparent in the residues of lowest frequency in the precursor (Ser, Ala, Ile, Tyr, Phe, Trp, Arg, $CySO_3$), as these are at least 4 fold more abundant in the cross-linked envelopes. No citrulline, ornithine or hydroxyamino acids were observed. Little if any carbohydrate was detected either in the precursor or in envelopes.

Discussion

Transglutaminase is a widely distributed enzyme (Chung, 1972; Folk and Chung, 1973). Until recently, the most clearly identified sites of action have been extracellular; the two best-studied examples are the cross-linking of fibrin during blood clotting (Lorand, 1972; Doolittle, 1973) and the remarkable cross-linking process that forms the copulation plug of rodents (Notides and Williams-Ashman, 1967; Williams-Ashman et al., 1972). The isodipeptide ϵ-(γ-glutamyl) lysine has been detected in many proteins (Folk and Finlayson, 1977; Matacic and Loewy, 1979), some of which are cross-linked by intracellular transglutaminase. It has recently been found that when Ca^{++} is introduced into erythrocyte ghosts, spectrin becomes aggregated with other membrane proteins (Carraway, Triplett and Anderson, 1975). This takes place by transglutaminase-catalyzed polymerization, a process that can be induced in intact erythrocytes by Ca^{++} ionophores. (Lorand et al., 1976). Although the amount of cross-linked product must be small compared with that of the epidermal cell, the degree of cross-linking—10% of the lysines participating (Siefring et al., 1978)—is not much lower.

Transglutaminase has been implicated in cross-linking processes in the epidermis and epidermal appendages by many studies (Asquith et al., 1970; Harding and Rogers, 1971, 1972a; Chung and Folk, 1972; Goldsmith et al., 1974; Buxman and Wuepper, 1975, 1976; Buxman et al., 1976). Attention has often been directed to keratins as possible cross-linked proteins, but at least in epidermal cells, the keratins are not specifically involved (Rice and Green, 1977), and it would seem that the role of transglutaminase is to cross-link the cell envelope (Sugawara, 1977; Rice and Green, 1977), thus forming the structure referred to earlier by electron microscopists as the marginal band (Hashimoto, 1970; Raknerud, 1974). In hair, medullary and internal root sheath proteins are cross-linked by transglutaminase (Harding and Rogers, 1971, 1972a, 1972b), and a protein fraction containing a putative precursor of these cross-linked hair proteins has been isolated (Rogers, Harding and Llewellyn-Smith, 1977). The possible relation of this fraction, which has a high Glx and Leu content, to the epidermal envelope precursor remains to be explored.

In the erythrocyte, it seems quite clear that it is the entry of Ca^{++} that initiates the activity of the transglutaminase in polymerizing membrane constituents (Lorand et al., 1976). In our earlier experiments on keratinocytes, we were unable to initiate cross-linking with commonly used ionophore concentrations, and sug-

gested that the cross-linking might be initiated by movement of the envelope precursor to the cell membrane. This possibility must now be rejected, since the envelope precursor is located adjacent to the cell membrane before the cross-linking begins and the cross-linking process can be quickly activated by agents that remove the cell permeability barrier to Ca^{++}, including high concentrations of ionophores. It may be concluded that, as in the erythrocyte, the activation of cross-linking is due to the appearance of free Ca^{++} in the cytoplasm.

Though cultured epidermal cells spontaneously become permeable during their terminal differentiation in culture, it is not clear that the Ca^{++} necessary for transglutaminase activity can only originate outside the cells. In contrast to erythrocytes, the keratinocytes must, like other cell types (Carafoli and Lehninger, 1971), have large amounts of sequestered calcium in organelles such as the mitochondria. Since terminal digestion in the keratinocyte affects both organelles and cell membrane, the Ca^{++} necessary for transglutaminase might originate from either internal or external sources. In detergent- or ionophore-assisted cross-linking, the rapidity of the process depends on external Ca^{++}, but in the relatively slow process that occurs spontaneously in suspended cells, internal stores can release Ca^{++} in amounts adequate to support envelope cross-linking. For this reason, cells suspended even in calcium-free medium can ultimately cross-link their envelopes (Rice and Green, 1978); to prevent this slow cross-linking, it is necessary to add a chelator that can prevent the internally generated Ca^{++} from being effective.

When the cross-linking process was initiated in cultured cells by four different means, the amino acid compositions of the envelopes were in remarkably good agreement (Table 2), but the composition of the envelopes differed significantly from that of the precursor. The amino acid composition of envelopes formed in suspension cultures is also not in very close agreement with that of envelopes formed in vivo (Matoltsy and Matoltsy, 1966; our unpublished observations). A number of explanations are possible for these discrepancies:

—The envelopes, though sonically disintegrated and washed under denaturing and reducing conditions, may still be contaminated with other cellular constituents. Contamination with keratins could not explain such features as the higher proline content, since the keratins are low in proline (Fuchs and Green, 1978) and no contamination by keratins was detected immunochemically.

—Some part of the envelope precursor may be excised before or after the cross-linking. The purified protein was frequently found to be degraded by storage at 4°C, and this may indicate high susceptibility to proleoytic cleavage. We do not know, for example, whether the glutamic/glutamine residues are clustered and, if so, whether they could easily be eliminated.

—The precursor described is quite possibly not the only protein participating in envelope formation. For example, the precursor was identified and followed by its ability to act as an amine acceptor, but any other protein acting as an amine donor would not have been recognized. The extracellular transglutaminase substrates cited previously are known to be able to form homopolymers (Notides and Williams-Ashman, 1967; Williams-Ashman et al., 1972; Lorand, 1972); on the other hand, spectrin is not the only protein subject to cross-linking in erythrocytes (Carraway et al., 1975; Lorand et al., 1976). The amount of envelope precursor present in the epidermal cells seems adequate to account for the entire protein content of the cross-linked envelope, but we wish to leave open the possibility that other proteins may contribute to it and perhaps even play a role in the localization of the precursor we have isolated.

Experimental Procedures

Cell Culture

Serially cultivated human epidermal cells (strains N and I) were used after 30–60 generations of culture life. The cells were grown in the Dulbecco-Vogt modification of Eagle's medium supplemented with 20% fetal calf serum and 0.4 μg/ml hydrocortisone (Rheinwald and Green, 1975). Mouse epidermal growth factor (Cohen and Taylor, 1974) was added to 10 ng/ml beginning ~4 days after inoculation. For large scale preparations of envelope subunit protein, cultures were expanded in the presence of cholera toxin at 10^{-10} M (Green, 1978).

Promotion of Envelope Cross-Linking

For preparation of suspension cultures, 3T3 feeder cells were removed from surface cultures by vigorous pipetting with EDTA (Sun and Green, 1976), and the adherent epidermal cells were disaggregated by treatment with trypsin and EDTA and recovered by low speed centrifugation in medium containing serum. They were washed in similar medium supplemented with 0.5 mg/ml soybean trypsin inhibitor (Sigma) and resuspended at 8×10^5 per ml in a small volume (usually 0.5 ml) of serum-free medium containing nonionic detergent, NaCl or ionophore at the appropriate concentration. After gentle dispersion, the number of cells were counted and the sample was incubated at 37°C. At intervals, aliquots were treated with 2% sodium dodecylsulfate and 20 mM dithiothreitol (Green, 1977), and cross-linked envelopes were scored under Nomarski optics. In early experiments, the cells were suspended in methylcellulose to prevent clumping, but the short incubation periods made stabilization of the medium unnecessary.

For studies on envelope formation in surface cultures, cells grown to confluence in 50 mm dishes were rinsed 3 times in serum-free medium and given 1.5 ml of similar medium containing nonionic detergent, NaCl or ionophore. After incubation at 37°C, the medium was gently decanted and discarded, desquamation being negligible. The cultures were treated with sodium dodecylsulfate and dithiothreitol in 2 ml of neutral buffer, sonicated lightly in test tubes to reduce the viscosity of the released DNA and centrifuged for 10 min at 1000 × g. After discarding the clear supernatants, the pellets (consisting of broken envelopes) were rinsed once in 0.1% sodium dodecylsulfate and finally resuspended in the same detergent for quantitation by light attenuation at A^{340}.

Gel Filtration

Confluent cultures of epidermal cells were rinsed 3 times in serum-

free medium and disrupted by homogenization (Dounce) in an ice bath in isotonic phosphate buffer containing EDTA (10 mM). In preliminary experiments, extraction was not much affected by substituting cystamine (20 mM) for EDTA in the isotonic phosphate buffer. The extract was clarified by centrifugation for 10 min at 10,000 × g and the pellet was re-extracted. Routinely, 30 cultures in 100 mm dishes were harvested to yield a final pooled extract of about 15 ml. The extract was reclarified and applied to a 2.5 x 47 cm column of BioGel A-1.5 M (BioRad), 100–200 mesh, maintained in isotonic phosphate buffer (pH 7.3) containing 0.02% sodium azide.

Analytical gel filtration for estimation of diffusion coefficient was done with a 0.9 x 59 cm column of BioGel A-5 M, 100–200 mesh. The sample for each run contained blue dextran, tritiated leucine and either a crude keratinocyte extract (prepared as above without re-extraction of the pellet) or commercially purified marker protein. Column runs were compared using peaks of blue dextran (fraction 17) and ^{3}H–Leu (fraction 47) for normalization. The peak of envelope precursor protein was located by titer with specific antiserum. Horse spleen ferritin (Boehringer-Mannheim) was located by A^{340}; E. coli β-galactosidase (Boehringer-Mannheim) by activity using the substrate 0-nitrophenyl-β-D-galactopyranoside; and bovine thyroglobulin (Sigma) colorimetrically after reaction of its amino groups with trinitrobenzenesulfonic acid (Habeeb, 1966) using 0.2 M sodium borate buffer (pH 9.2) in place of sodium bicarbonate buffer.

DEAE Cellulose Chromatography

Fractions from gel filtration were dialyzed against 0.05 M Tris buffer (pH 7.5), pooled, clarified by centrifugation at 10,000 × g for 10 min and applied to a 1.4 x 25 cm column of DE-52 (Whatman) equilibrated with buffer containing azide. Protein was eluted with a 440 ml linear gradient of NaCl (0–0.4 M) in the buffer. Fractions containing the envelope precursor protein were usually located by gel electrophoresis.

Hydroxyapatite Chromatography

Fractions containing envelope precursor protein eluted from DE-52 columns were pooled and dialyzed overnight against 2 L of 0.005 M potassium phosphate buffer (pH 6.8). At lower pH, the protein tended to precipitate. After application of the sample to a 1 x 10 cm column of hydroxyapatite HTP (BioRad), protein was eluted with a 120 ml linear gradient of the same buffer (0.005–0.3 M).

Transglutaminase Assay and Labeling of Envelope Precursor Protein in Crude Extracts

For assay of transglutaminase activity, samples of 0.5 ml contained 1 mg of dimethyl casein (Means and Feeney, 1968), 50 μmole of Tris–HCl (pH 8), 5 μmole of $CaCl_2$, 0.5 μmole of EDTA, 1 μmole of DTT, 1 μCi of ^{3}H–putrescine (50 μCi/μmole) and cell extract (10–100 μg of protein). The samples were incubated for 1 hr at 35°C. The reaction was stopped by addition of 2.5 ml of 12% TCA containing putrescine (10 mM), the samples were chilled for 20 min on ice, and the precipitated casein was recovered by centrifugation at low speed. The casein was resuspended in 3 ml of cold 5% TCA containing 1 mM putrescine, deposited onto glass fiber filters, washed with cold 5% TCA, rinsed with 95% ethanol and counted by liquid scintillation.

Six confluent 50 mm cultures were rinsed 3 times in serum-free medium, harvested by scraping, sonically disintegrated in 3 ml of 0.05 M Tris–Cl buffer (pH 8.3) containing 1 mM EDTA and centrifuged for 10 min at 10,000 × g. The supernatant, containing approximately 3 mg of protein per ml, was adjusted to 1% in Triton X-100, 1 mg/ml in dithiothreitol, 6 mM in $CaCl_2$ and 5 μCi/ml in ^{14}C–2–aminoethanol (3.9 μCi/μmole; New England Nuclear). After incubation at 37°C for 1 hr, the mixture was clarified by centrifugation at 10,000 × g for 10 min and diluted with water to the conductivity of column buffer [0.05 M Tris–Cl (pH 7.5) containing 1 mM EDTA]. The labeled envelope precursor protein was freed of Triton X-100 and excess ^{14}C–2–aminoethanol by preliminary chromatography on a 1 x 12 cm column of DE-52, eluting with a 100 ml linear gradient of NaCl (0–0.4 M) in column buffer. For use as a marker in gel electrophoresis, single confluent epidermal cultures were sonicated in 1–2 ml of buffer and incubated in the presence of either dansyl cadaverine (0.5 mg/ml) or ^{14}C–2–aminoethanol.

Isoelectric Focusing

Gels 8 cm in length were 5% in acrylamide and 2% in ampholine (LKB, pH range 3.5–10). Samples containing 30 μg of protein were applied to either acidic or basic side of the gels and focused 5–6 hr at 120 V. Bands were made visible by fixation in 15% trichloracetic acid (Wrigley, 1968). Isoelectric points were estimated from the pH profiles in unfixed gels by eluting 2 mm slices, each in 1 ml of water.

Antiserum to Envelope Precursor Protein

Rabbits were injected with 0.2 mg of purified protein dissolved in isotonic phosphate buffer and emulsified in Freund's complete adjuvant. Booster injections of 0.2 mg in complete adjuvant were given after 4 and 6 weeks. The rabbits were bled of 50 ml every week for 4–6 weeks after the final injections. No more than a slight decrease in titer was apparent during this interval. Preimmune sera and antisera were stored frozen in aliquots of several milliliters until needed. Antigens or antisera in isotonic buffer containing .02% NaN_3 were titered by 2 fold dilutions in Ouchterlony double diffusion gels of 1% agarose (BioRad). Preimmune sera did not form visible precipitin lines when tested against purified envelope precursor protein or crude keratinocyte extracts. Antisera were absorbed with test substances by gentle magnetic stirring for 4.5–16 hr at 37°C.

Binding of antisera to epidermal cells or tissues was monitored by indirect immunofluorescence. Newborn human foreskin was frozen in isopentane chilled in liquid nitrogen, mounted in Tissue Tek (Fisher Scientific) and sectioned in a cryostat. 5 μ sections were air-dried on glass slides for 0.5–1 hr, incubated at 37°C for 45–60 min with antiserum diluted 1:20, rinsed thoroughly in isotonic buffer and mounted in gelvatol (Rodriguez and Deinhardt, 1960) under glass coverslips. Fixation of skin samples in formaldehyde before or after sectioning greatly reduced the peripheral staining of cells in the stratum corneum and upper spinous layers but had less effect on cytoplasmic staining. Cultured cells were trypsinized, fixed for 30 min in 3.7% formaldehyde in isotonic buffer at room temperature, rinsed in buffer and centrifuged; the pellet was frozen in chilled isopentane and sectioned as tissue. Sections stained with preimmune sera in parallel with those treated with antisera gave only very dim and diffuse fluorescence.

Cross-linked envelopes of cultured cells were purified for treatment with antisera by multiple extractions of other cellular material with 6 M guanidine HCl made 0.2 M in Tris HCl (pH 8.3) and 20 mM in dithiothreitol. The envelopes were rinsed several times in isotonic phosphate buffer prior to use.

Miscellaneous Procedures

Lactate dehydrogenase activity was measured spectrophotometrically (Decker, 1977) in 0.1 M sodium phosphate buffer (pH 7.2) made 4 mM in sodium pyruvate and 0.22 mM in NADH. Protein concentration was estimated by the method of Lowry et al. (1951). Salt concentration was determined by conductivity. The protein content of envelopes was measured by reaction with ninhydrin after digestion with sulfuric acid (Schiffman, 1966). Amino acid analysis was performed on a Durrum D 500 analyzer; values for tryptophan were obtained after hydrolysis in 4 N methanesulfonic acid (Simpson, Neuberger and Liu, 1976); cysteine was measured as cysteic acid after performic acid oxidation (Hirs, 1967) and hydrolysis in redistilled 6 N HCl. The ionophores X537A and A23187 were gifts from Hoffman-LaRoche and Eli Lilly and Company, respectively.

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References

Asquith, R. S., Otterburn, M. S. Buchanan, J. H., Cole, M., Fletcher, J. C. and Gardner, K. L. (1970). The identification of εN-(γ-glutamyl)-L-lysine cross-links in native wool keratins. Biochim. Biophys. Acta *207*, 342–343.

Buxman, M. M. and Wuepper, K. D. (1975). Keratin cross-linking and epidermal transglutaminase. J. Invest. Dermatol. *65*, 107–112.

Buxman, M. M. and Wuepper, K. D. (1976). Isolation, purification and characterization of bovine epidermal transglutaminase. Biochim. Biophys. Acta *452*, 356–369.

Buxman, M. M., Buehner, G. E. and Wuepper, K. D. (1976). Isolation of substrates of epidermal transglutaminase from bovine epidermis. Biochem. Biophys. Res. Commun. *73*, 470–478.

Carafoli, E. and Lehninger, A. L. (1971). A survey of the interaction of calcium ions with mitochondria from different tissues and species. Biochem. J. *122*, 681–690.

Carraway, K. L., Triplett, R. B. and Anderson, D. R. (1975). Calcium-promoted aggregation of erythrocyte membrane proteins. Biochim. Biophys. Acta *379*, 571–581.

Castellot, J. J., Miller, M. R. and Pardee, A. B. (1978). Animal cells reversibly permeable to small molecules. Proc. Nat. Acad. Sci. USA *75*, 351–355.

Chung, S. I. (1972). Comparative studies on tissue transglutaminase and factor XIII. Ann. NY Acad. Sci. *202*, 240–255.

Chung, S. I. and Folk, J. E. (1972). Transglutaminase from hair follicle of guinea pig. Proc. Nat. Acad. Sci. USA *69*, 303–307.

Cleveland, D. W., Hwo, S-Y. and Kirschner, M. W. (1977). Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J. Mol. Biol. *116*, 207–225.

Cohen, S. and Taylor, J. M. (1974). Epidermal growth factor: chemical and biological characterization. In Recent Progress in Hormone Research, *30*, R. O. Greep, ed. (New York: Academic Press), pp. 553–550.

Cohn, E. J. and Edsall, J. T. (1943). Proteins, Amino Acids and Peptides (Rheinhold: New York), pp. 370–375.

Decker, L. A. (1977). Worthington Enzyme Manual (Freehold, N.J.: Worthington Biochemical Co.), pp. 19–22.

Doolittle, R. F. (1973). Structural aspects of the fibrinogen to fibrin conversion. Adv. Protein Chem. *27*, 1–109.

Folk, J. E. and Chung, S. I. (1973). Molecular and catalytic properties of transglutaminases. Adv. Enzymol. *38*, 109–191.

Folk, J. E. and Finlayson, J. S. (1977). The ε-(γ-glutamyl) lysine cross-link and the catalytic role of transglutaminases. Adv. Protein Chem. *31*, 1–133.

Fuchs, E. and Green, H. (1978). The expression of keratin genes in epidermis and cultured epidermal cells. Cell *15*, 887–897.

Goldsmith, L. A., Baden, H. P., Roth, S. I., Colman, R., Lee, L. and Fleming, B. (1974). Vertebral epidermal transamidases. Biochim. Biophys. Acta *351*, 113–125.

Green, H. (1977). Terminal differentiation of cultured human epidermal cells. Cell *11*, 405–416.

Green, H. (1978). Cyclic AMP in relation to proliferation of the epidermal cell: a new view. Cell *15*, 801–811.

Habeeb, A. F. S. A. (1966). Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. *14*, 328–336.

Harding, H. W. J. and Rogers, G. E. (1971). ε-(γ-glutamyl) lysine cross-linkage in citrulline-containing protein fractions from hair. Biochemistry *10*, 624–630.

Harding, H. W. J. and Rogers, G. E. (1972a). Formation of the ε-(γ-glutamyl) lysine cross-link in hair proteins. Investigation of transamidases in hair follicles. Biochemistry *11*, 2858–2863.

Harding, H. W. J. and Rogers, G. E. (1972b). The occurrence of the ε-(γ-glutamyl) lysine cross-link in the medulla of hair and quill. Biochim. Biophys. Acta *257*, 37–39.

Hashimoto, K. (1969). Cellular envelopes of keratinized cells of the human epidermis. Arch. Klin. Exp. Derm. *235*, 374–385.

Hashimoto, K. (1970). Infolded plasma membranes of keratinized cells. Arch. Klin. Exp. Derm. *238*, 53–60.

Hirs, C. H. W. (1967). Determination of cystine as cysteic acid. In Methods in Enzymology, *11* (New York: Academic Press) pp. 59–62.

Lorand, L. (1972). Fibrinoligase: the fibrin-stabilizing factor system of blood plasma. Ann. NY Acad. Sci. *202*, 6–30.

Lorand, L., Chenoweth, D. and Gray, A. (1972). Titration of the acceptor cross-linking sites in fibrin. Ann. NY Acad. Sci. *202*, 155–171.

Lorand, L., Weissman, L. B., Epel, D. A. and Bruner-Lorand, J. (1976). Role of the intrinsic transglutaminase in the Ca^{2+}-mediated cross-linking of erythrocyte proteins. Proc. Nat. Acad. Sci. USA *73*, 4479–4481.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. *193*, 265–275.

Martin, R. G. and Ames, B. N. (1961). A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. *236*, 1372–1379.

Matacic, S. S. and Loewy, A. (1979). Presence of the ε-(γ-glutamyl) lysine cross-link in cellular proteins. Biochim. Biophys. Acta *576*, 263–268.

Matoltsy, A. G. and Balsamo, C. A. (1955). A study of the components of cornified epithelium of human skin. J. Biophys. Biochem. Cytol. *1*, 339–360.

Matoltsy, A. G. and Matoltsy, M. N. (1966). The membrane protein of horny cells. J. Invest. Dermatol. *46*, 127–129.

Means, G. E. and Feeney, R. E. (1968). Reductive alkylation of amino groups in proteins. Biochemistry *7*, 2192–2201.

Miller, M. R., Castellot, J. J. and Pardee, A. B. (1978). A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. Biochemistry *17*, 1073–1080.

Notides, A. C. and Williams-Ashman, H. G. (1967). The basic protein responsible for the clotting of guinea pig semen. Proc. Nat. Acad. Sci. USA *58*, 1991–1995.

Ogawa, H. and Goldsmith, L. A. (1976). Human epidermal transglutaminase. Preparation and properties. J. Biol. Chem. *251*, 7281–7288.

Pressman, B. C. (1976). Biological application of ionophores. Ann. Rev. Biochem. *45*, 501–530.

Raknerud, N. (1974). The ultrastructure of the interfollicular epidermis of the hairless (hr/hr) mouse. II. Plasma membrane modifications during keratinization. Virchows Arch. B. Cell Path. *17*, 113–135.

Rheinwald, J. G. and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6*, 331–343.

Rheinwald, J. G. and Green, H. (1977). Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature *265*, 421–424.

Rice, R. H. and Means, G. E. (1971). Radioactive labelling of proteins in vitro. J. Biol. Chem. *246*, 831–832.

Rice, R. H. and Green, H. (1977). The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell *11*, 417–422.

Rice, R. H. and Green, H. (1978). Relationship of protein synthesis and transglutaminase activity to formation of the cross-linked envelope during terminal differentiation of the cultured human epidermal

keratinocyte. J. Cell Biol. *76*, 705–711.

Rodriguez, J. and Dienhardt, F. (1960). Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology *12*, 316–317.

Rogers, G. E., Harding, H. W. J. and Llewelly-Smith, I. J. (1977). The origin of citrulline-containing proteins in the hair follicle and the chemical nature of trichohyalin, an intracellular precursor. Biochim. Biophys. Acta *495*, 159–175.

Shiffman, G. (1966). Immunological methods for characterizing polysaccharides. Methods in Enzymology, *8* (New York: Academic Press), pp. 79–85.

Siefring, G. E., Jr., Apostol, A. B., Velasco, P. T. and Lorand, L. (1978). Enzymatic basis for the Ca^{2+}-induced cross-linking of membrane proteins in intact human erythrocytes. Biochemistry *17*, 2598–2604.

Siegel, L. M. and Monty, K. J. (1966). Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite hydroxylamine reductases. Biochim. Biophys. Acta *112*, 346–362.

Simpson, R. J., Neuberger, M. R. and Liu, T. Y. (1976). Complete amino acid analysis of proteins from a single hydrolysate. J. Biol. Chem. *251*, 1936–1940.

Smith, M. A. (1970). Peptides and Proteins. In Handbook of Biochemistry, H. A. Sober, ed. (Cleveland: Chemical Rubber Co.), pp. C10–C21.

Spiro, R. G. (1966). Analysis of sugars found in glycoproteins. In Methods in Enzymology, *8* (New York: Academic Press), pp. 3–52.

Sugawara, K. (1977). Intermolecular cross-links in epidermal differentiation. In Biochemistry of Cutaneous Epidermal Differentiation, M. Seiji and I. A. Bernstein, eds. (Tokyo: University of Tokyo Press), pp. 387–397.

Sun, T-T. and Green, H. (1976). Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. Cell *9*, 511–521.

Sun, T-T. and Green, H. (1977). Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states. Nature *269*, 489–493.

Sun, T-T. and Green, H. (1978). Keratin filaments of cultured human epidermal cells. J. Biol. Chem. *253*, 2053–2060.

Weber, K. and Osborn, M. (1969). The reliability of molecular weight estimates for proteins in polyacrylamide gel electrophoresis. J. Biol. Chem. *244*, 4406–4412.

Williams-Ashman, H. G., Notides, A. C., Pabalan, S. S. and Lorand, L. (1972). Transamidase reactions involved in the enzymic coagulation of semen: isolation of γ-glutamyl-ϵ-lysine dipeptide from clotted secretion protein of guinea pig seminal vesicles. Proc. Nat. Acad. Sci. USA *69*, 2322–2325.

Wrigley, C. W. (1968). Analytical fractionation of plant and animal proteins by gel electrofocusing. J. Chromatography *36*, 362–365.