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# The Cornified Envelope of Terminally Differentiated Human Epidermal Keratinocytes Consists of Cross-Linked Protein

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## Summary

A small proportion of the protein of stratum corneum of human epidermal callus is insoluble even when boiled in solutions containing sodium dodecylsulfate and a reducing agent. This protein is present in the cornified envelope, a structure located beneath the plasma membrane. When cornified envelopes were dissolved by exhaustive proteolytic digestion and the products analyzed by chromatography, approximately 18% of the total lysine residues were found as the cross-linking dipeptide $\epsilon$-($\gamma$-glutamyl) lysine.

Labeled cornified envelope protein was synthesized by human epidermal keratinocytes allowed to differentiate terminally in culture. The extent of cross-linking, determined from the proportion of radioactive lysine in $\epsilon$-($\gamma$-glutamyl) lysine after exhaustive proteolysis, was similar to that in stratum corneum. The properties of the cornified envelopes (insolubility in detergent and reducing agents, and solubility following proteolytic digestion) are readily explained by a structure consisting of a cross-linked protein lattice.

## Introduction

The terminally differentiated squames of the mammalian epidermis have envelopes that are highly resistant to chemical treatment and contribute to the protective function of the integument (Matoltsy and Balsamo, 1955; Matoltsy, 1968). These envelopes are located beneath the plasma membrane of the cells in stratum corneum (for references see Raknerud, 1974) and develop in keratinocytes as they differentiate either in surface cultures (Sun and Green, 1976) or in suspension (Green, 1977). They can be obtained in purified form as a residue after extraction of the cells with alkali (Matoltsy and Matoltsy, 1966) or detergents and reducing agents (Sun and Green, 1976; Green, 1977). Since the envelopes are destroyed by proteolytic enzymes, their insolubility depends upon the integrity of the proteins (Sun and Green, 1976).

Such properties suggest the existence of a cross-linked structure (Matoltsy, 1976). A variety of lysine-derived covalent cross-links is known to occur in collagen (Bornstein and Piez, 1966; Tanzer, 1973) and elastin (Partridge, 1966), extracellular products of connective tissue cells. $\epsilon$-($\gamma$-glutamyl) lysine cross-links were first demonstrated in fibrin (Lorand et al., 1968; Matacic and Loewy, 1968; Pisano, Finlayson and Peyton, 1969), where they are formed through the action of transglutaminases (Folk and Chung, 1973). Similar cross-links have been found in epidermal appendages such as wool (Asquith et al., 1970), guinea pig hair and porcupine quill (Harding and Rogers, 1971). These cross-links presumably result from the activity of transglutaminases such as those present in guinea pig hair follicles (Harding and Rogers, 1972; Chung and Folk, 1972). The presence of transglutaminases in mammalian epidermis (Goldsmith et al., 1974; Goldsmith and Martin, 1975; Buxman and Wuepper, 1975) has suggested to some investigators that cross-links might be present in epidermal keratin; but since keratin of epidermal cells is rendered soluble by a combination of detergent and reducing agent and is not of very large molecular weight, it seemed improbable to us that $\epsilon$-($\gamma$-glutamyl) lysine cross-linking could be extensive in these proteins; the cornified envelope seemed a more probable site.

## Results

### Detection of $\epsilon$($\gamma$-Glutamyl) Lysine in Stratum Corneum Cells

Samples of stratum corneum of human plantar callus were homogenized and extracted with sodium dodecylsulfate under reducing conditions. The insoluble residue was exhaustively digested with a series of proteolytic enzymes, and the digests were examined for the presence of $\epsilon$-($\gamma$-glutamyl) lysine, whose isopeptide linkage is resistant to proteases (Pisano et al., 1969; Asquith et al., 1970; Harding and Rogers, 1971). Durrum amino acid analysis of the deproteinized and desalted digests revealed a peak of material eluting in the position of authentic $\epsilon$-($\gamma$-glutamyl) lysine. No such peak was observed in protease autodigests or in protease digests of $\alpha$-chymotrypsinogen.

To determine the purity of the isodipeptide peak and the amount of lysine involved in cross-linking, enzymatic digests were submitted to preliminary ion-exchange column chromatography. Figure 1a shows the elution positions of lysine and $\epsilon$-($\gamma$-glutamyl) lysine standards. $\alpha$-glutamyl lysine and $\alpha$-lysyl glutamic acid standards eluted much later than the isodipeptide. Material eluting in the position of authentic isodipeptide was collected and examined on the Durrum analyzer (Figure 2a). In addition to the dominant peak which matched $\epsilon$-($\gamma$-glutamyl) lysine in elution position, the material contained residual isoleucine, methionine and sometimes valine. The isodipeptide peak was also examined by thin-layer chromatography using methanol:pyridine: water (80:4:20) (Smith, 1969). A
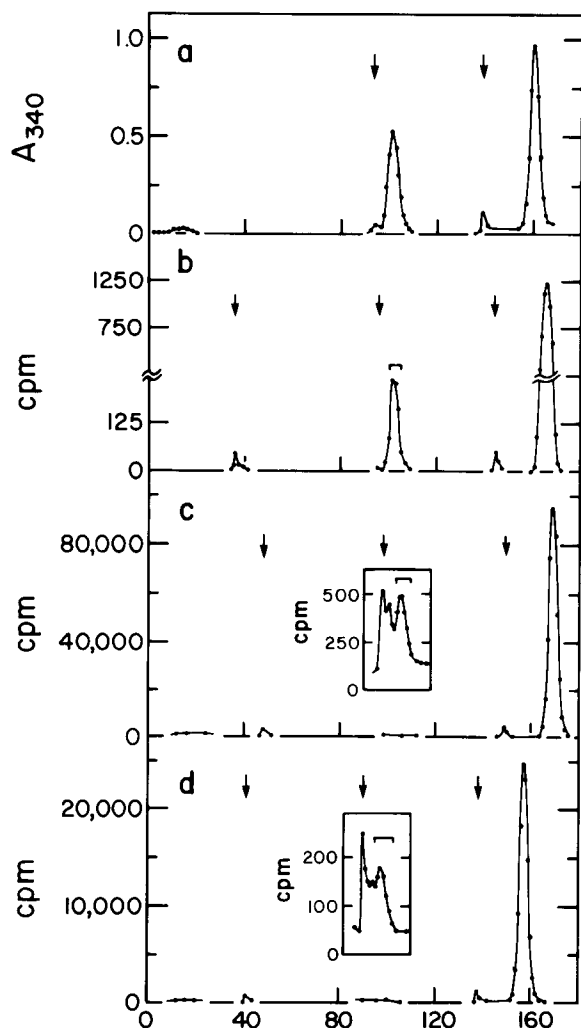
Figure 1. Ion-Exchange Column Chromatography of Isodipeptide Derived from Cornified Envelopes and Soluble Proteins

(a) Commercial standards $\epsilon$-($\gamma$-glutamyl) lysine (peak at fraction 92) and lysine (peak at fraction 161).

(b) Digest of $^3$H-L-lysine-labeled envelopes of cultured epidermal cells insoluble in sodium dodecylsulfate and dithiothreitol.

(c) Digest of $^3$H-L-lysine-labeled soluble proteins of cultured epidermal cells.

(d) Digest of soluble $^3$H-L-lysine-labeled dermal fibroblast protein.

In (c) and (d), the insets magnify the region of elution of $\epsilon$-($\gamma$-glutamyl) lysine. Fractions indicated by the bracket above the peaks were pooled for desalting and subsequent paper chromatography. Changes in pH at buffer discontinuities are indicated by arrows.

prominent ninhydrin-positive spot with the same mobility as $\epsilon$-($\gamma$-glutamyl) lysine was observed, as well as several faster migrating fainter spots in positions expected for the more hydrophobic amino acids revealed by the Durrum analyzer.

Apart from the hydrophobic amino acids included in the peak fractions, the degree of contamination of the isodipeptide peak by other small pep-
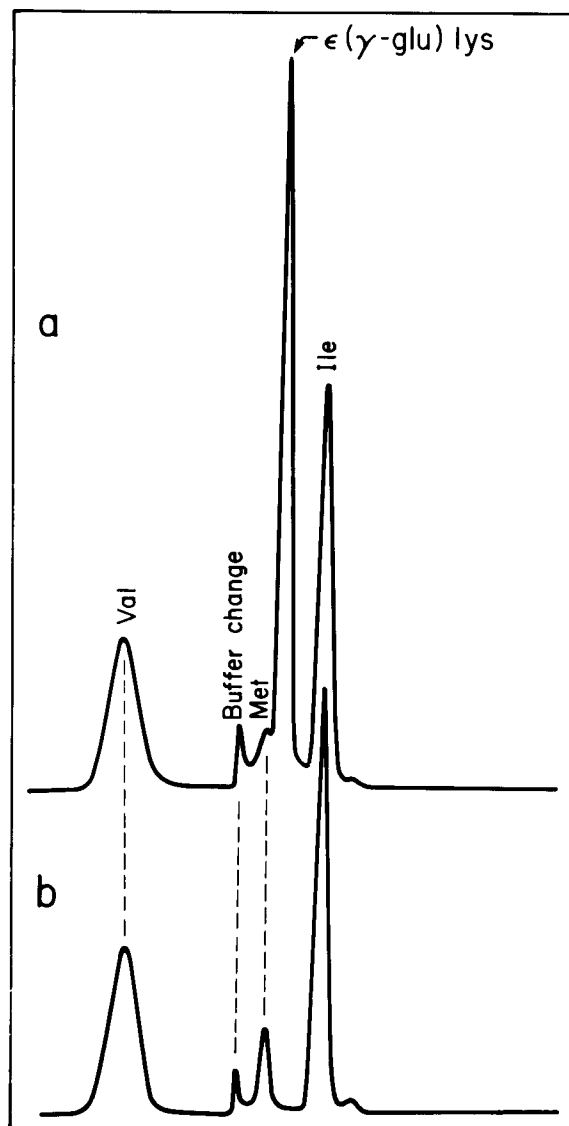


Figure 2. Amino Acid Analysis of a Sample of the Isodipeptide Region Isolated from an Enzymatic Digest of Stratum Corneum by Ion-Exchange Column Chromatography

(a) before and (b) after hydrolysis for 24 hr at 108°C in 6 N HCl.

tides was estimated from the amounts of free amino acids released by acid hydrolysis of the peak fraction. The isodipeptide peak completely disappeared (Figure 2b). Lysine and glutamic acid accounted for only about one third of the products. The major contaminants were proline and glycine. Asquith et al. (1970) observed the presence of contaminating peptides containing neutral amino acids after a similar purification of $\epsilon$-($\gamma$-glutamyl) lysine from wool.

In an effort to reduce this contamination, the isodipeptide fraction was treated with prolidase and reisolated by ion-exchange chromatography.

Amino acid analysis of an acid hydrolysate of the isodipeptide fraction showed that the purity of the isodipeptide had increased to about 50%. The main contaminant remaining was glycine, and there were smaller amounts of several other amino acids, but no aspartic acid. The disappearance of the isodipeptide peak was accompanied by the appearance of equimolar amounts of lysine and glutamic acid. From the amount of lysine released by acid hydrolysis, the proportion of envelope lysine of stratum corneum present in the form of isodipeptide was estimated at 18% (Table 1).

## Detection of Labeled Isodipeptide Synthesized by Keratinocytes in Cell Culture

When cultured human epidermal keratinocytes are placed in suspension stabilized with methyl cellulose methocel, the cells stop growing and most develop cornified envelopes (Green, 1977). Cells were allowed to differentiate terminally in suspension in the presence of $^3$H-L-lysine. Extraction of the matured cells with sodium dodecylsulfate under reducing conditions dissolved 95% of the trichloroacetic acid-precipitable radioactivity, leaving the insoluble envelopes free of most of the cellular contents. Enzymatic digests of these envelopes were examined by chromatography (Figures 1 and 3). In a representative experiment shown in Figure 1b, 19% of the lysine label eluted in the position of the isodipeptide. Nearly all the remaining label was found in the elution position of lysine.

In contrast to the cornified envelopes, keratinocyte protein soluble in detergent and reducing agent (including the water-soluble protein and the keratin) contained only a small amount of label eluting in the position of isodipeptide after enzymatic digestion and column chromatography (Figure 1c). The peak material was obviously heterogeneous, and its radioactivity was 0.5–1% of that in lysine. Human diploid fibroblasts, the protein of which is essentially all soluble in detergent, also

gave rise in the region of the isodipeptide to a small peak that contained 0.5–1% of the total radioactive lysine (Figure 1d). The radioactivity profile was identical when the fibroblasts were labeled while growing in surface cultures.

To determine the amounts of labeled isodipeptide more precisely, the peak fractions obtained by column chromatography were examined by paper chromatography. In the alkaline phenolic solvent system used, lysine and the isodipeptide were well separated, as shown in Figure 3 (top). About 90% of the labeled peak obtained from keratinocyte envelope preparations had the mobility of authentic isodipeptide (Figure 3a). After acid hydrolysis, all the radioactivity migrated in the position of lysine (Figure 3b). Isodipeptide fractions from cell envelope preparations were also examined by thin-layer chromatography on cellulose using methanol:pyridine:water (80:4:20) (Smith, 1969). The results were similar to those obtained by paper chromatography.

Paper chromatography of the isodipeptide fraction obtained from the soluble protein of the epidermal cells (Figure 3c) and the fibroblasts (Figure 3d) showed that 50% and 35%, respectively, of the counts had the mobility of the $\epsilon$-($\gamma$-glutamyl) lysine standard. As in the case of envelope protein, virtually all the $^3$H label migrated in the position of lysine after acid hydrolysis.

The extent of lysine participation in cross-linking was calculated from the relative amounts of free and isodipeptide lysine liberated in digests of radioactive cell protein (Table 1), using paper chromatography to correct for contaminating peptides eluting within the isodipeptide peak. In the cornified envelopes, about 17% of the total lysine was present as isodipeptide. For proteins soluble in sodium dodecylsulfate and dithiothreitol, the fraction was 0.3%, whether those proteins were derived from keratinocytes or human diploid fibroblasts. In cells of the mouse fibroblast line 3T3

Table 1. Abundance of $\epsilon$-($\gamma$-Glutamyl) Lysine Cross-Links

| Digest | Isodipeptide Lysine[b] | Total Lysine[b] | Proportion of Lysine Cross-Linked |
|---|---|---|---|
| Insoluble[a] protein of stratum corneum | 30 n moles[c] | 168 n moles[e] | 18% |
| Insoluble[a] protein of cultured keratinocyte envelopes | 890 cpm[d] | 5,320 cpm | 17% |
| Soluble[a] protein of cultured keratinocytes | 1370 cpm[d] | 412,000 cpm | 0.3% |
| Soluble[a] protein of dermal fibroblasts | 340 cpm[d] | 106,000 cpm | 0.3% |

[a] In solution containing 1% sodium dodecylsulfate and 20 mM dithiothreitol.
[b] Determined after initial ion-exchange column chromatography by either amino acid analysis (stratum corneum) or scintillation counting ($^3$H-labeled cultured keratinocytes) of lysine and isodipeptide peaks.
[c] Determined as lysine after acid hydrolysis.
[d] Corrected for non-isodipeptide radioactivity detected by paper chromatography (see Figures 3a, 3c and 3d).
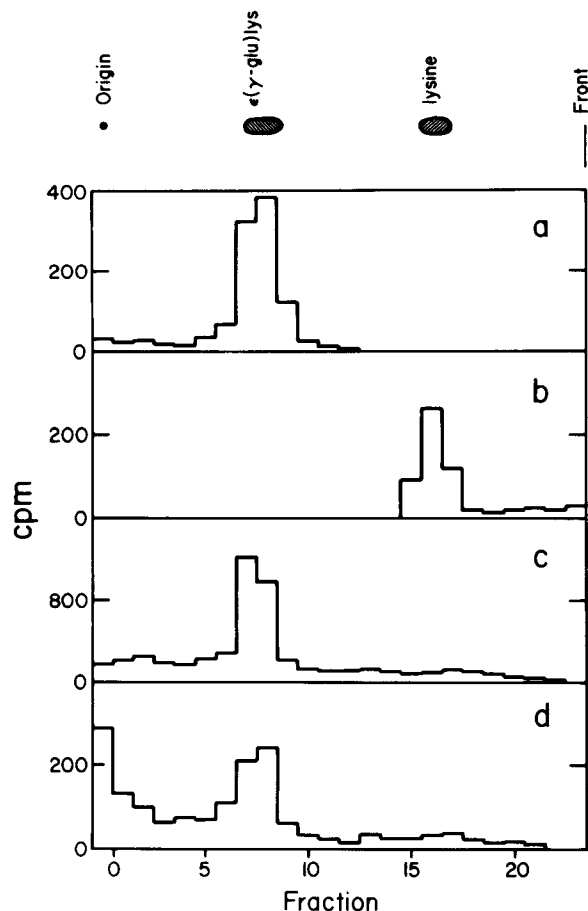[e] Corrected for lysine arising from enzyme autodigestion.

Figure 3. Paper Chromatography of Isodipeptide Region Isolated from Enzymatic Digests of $^3$H-L-Lysine-Labeled Protein by Ion-Exchange Column Chromatography

The positions of ε-(γ-glutamyl) lysine and lysine markers are shown above the radioactivity profiles.
(a) Cultured epidermal cell envelopes.
(b) Cultured epidermal cell envelopes, isolated peptides hydrolyzed for 24 hr at 108°C in 6 N HCl.
(c) Soluble epidermal cell protein.
(d) Soluble fibroblast protein.

labeled while growing in surface culture, only 0.02% of the lysine label appeared in the isodipeptide fraction after column and paper chromatography.

**Discussion**

These experiments show that a large fraction of the lysine residues contained in the cornified envelopes of human stratum corneum and cultured human keratinocytes participate in cross-links. By three chromatographic criteria, the cross-linked lysine had the properties of the isodipeptide ε-(γ-glutamyl) lysine. The chromatographically purified isodipeptide was completely cleaved by acid conditions normally used for peptide bond hydrolysis.

This distinguishes ε-(γ-glutamyl) lysine cross-links from some lysine-derived cross-links in connective tissue (Tanzer, 1973), as well as lysinoalanine observed in many food proteins (Sternberg, Kim and Schwende, 1975).

It seems appropriate to view the cornified envelope as a cross-linked structure, whose stability would depend upon the molecular weight of the constituent protein(s). The envelope protein is 8% lysine (based on amino acid analyses of cultured keratinocyte envelopes). If, for example, the envelopes were made from 30,000 dalton monomers, each monomer would have nearly four lysines and an equal number of glutamines participating in ε-(γ-glutamyl) lysine cross-links. Even if some of the isopeptide bonds were intramolecular, this amount of cross-linking seems more than enough to account for the insolubility of the envelope.

The proportion of envelope lysine residues participating in cross-links was 18% for stratum corneum and approximately the same for cultured keratinocytes. Since nonenvelope proteins may not have been fully extracted by the detergent and reducing agent, these values may be slight underestimates, but they are rather similar to the values reported for guinea pig hair medullary proteins (Harding and Rogers, 1971). In contrast, the degree of cross-linking attributed to the keratin proteins in wool is much lower (Asquith et al., 1970). In our protein fractions soluble in detergent and reducing agents, the amount of cross-linking was very small, and not significantly different in cultured keratinocytes and cultured fibroblasts, even though about one third of the proteins of the cultured keratinocytes consists of keratins (T.-T. Sun and H. Green, manuscript in preparation). This suggests that cytoplasmic keratin filaments are not specifically involved in this type of cross-linking but rely exclusively on disulfide bonds for their detergent insolubility (Baden, Lee and Kubilus, 1976; T.-T. Sun and H. Green, manuscript in preparation). Proteins soluble in detergent and dithiothreitol may contain partially cross-linked envelope precursor protein; on the other hand, the amount of soluble cross-links was similar (0.3%) in human diploid fibroblasts, which do not develop detergent-resistant envelopes. Isopeptide cross-linking has also been reported in cell membrane fractions from mouse L cells to the extent of 0.3% of the lysines (Birck-bichler et al., 1973) and has been postulated to occur in surface proteins of human fibroblasts (Keski-Oja, Mosher and Vaheri, 1976). It is possible that a low level of cell protein cross-linking might be the accidental result of possession of transglutaminase activity, the products being neither useful nor harmful to the cells. It should be pointed out, however, that since 95% of the cell protein of the

keratinocytes was soluble in the detergent and reducing agent, the total amount of soluble cellular isodipeptide was quite appreciable, even in comparison with that in the cross-linked envelope.

The detailed mechanism of envelope assembly, including characterization of the proteins subject to cross-linking, remains to be elucidated. Since transglutaminase activity is present in extracts of our cultured keratinocytes, as it is in epidermis in vivo (Goldsmith et al., 1974), this enzyme is very probably responsible for the formation of the isopeptide bonds.

## Experimental Procedures

### Materials
Methyl cellulose A4M was donated by the Dow Chemical Co. (Midland, Michigan). $^3$H-L-lysine was purchased from New England Nuclear (Boston, Massachusetts); AG 50X8 from BioRad Laboratories (Richmond, California); chymotrypsin, pronase, leucine aminopeptidase, carboxypeptidases A and B from Sigma Chemical Co. (St. Louis, Missouri); trypsin from Worthington Biochemical Co. (Freehold, New Jersey); prolidase from Miles Laboratories, Inc. (Elkhart, Ind.); α-glutamyl-lysine, α-lysylglutamic acid and ε-(γ-glutamyl) lysine from Vega-Fox Biochemicals (Tucson, Arizona); constant boiling HCl and N-ethyl-morpholine, sequanal grade, from Pierce Biochemical Co. (Rockford, Illinois). Plantar callus was obtained from a healthy male subject.

### Labeling of Cells In Methocel Suspension
Serially cultured newborn human diploid epidermal keratinocytes (strain N) were grown according to Rheinwald and Green (1975). 20–30 generations after their isolation, cells were harvested by disaggregation with trypsin and EDTA of log-phase cultures grown in the presence of 15–30 ng/ml of mouse epidermal growth factor (Savage and Cohen, 1972), prepared by Dr. T.-T. Sun of this laboratory. The cells were resuspended at 3-4 × 10$^5$/ml in fortified Eagle's medium whose lysine concentration was reduced to 37 μg/ml and supplemented with 1.1% methyl cellulose, 20% fetal calf serum and 5 μCi/ml $^3$H-L-lysine. The cells were incubated for 11 days at 37°C. Diploid fibroblasts were subcultured twice to eliminate epidermal cells in fortified Eagle's medium supplemented with 10% bovine serum. The cells were recovered from the methocel by centrifugation after 15 fold dilution of the suspension with isotonic phosphate buffer and washed once with the buffer.

### Preparation of Callus and Epidermal Cells for Enzymatic Digestion
Human callus (100 mg) was vigorously homogenized by hand in a ground glass Tenbroek tissue grinder in water or aqueous buffer to produce a fine suspension containing 20 mg protein per ml. Approximately 95% of the original protein was recovered in the pellet upon centrifugation at 2000 × g for 10 min. This insoluble material was resuspended in 5 ml of 2% sodium dodecylsulfate made 25 mM in dithiothreitol and 10 mM in sodium phosphate buffer (pH 7.2) and heated for 5 min in a 90°C water bath. After centrifugation at 10,000 × g for 10 min, the pellet was resuspended in 2.5 ml of the same solution, reheated for 5 min at 90°C and recovered by centrifugation at 10,000 g for 10 min. To remove the detergent, the insoluble residue, approximately 5% of the starting protein, was rinsed in water, twice in 80% ethanol and twice in 5% (v/v) formic acid.

Cells recovered from methocel suspension were resuspended at 3-4 × 10$^6$/ml in 1% sodium dodecylsulfate made 25 mM in dithiothreitol and 10 mM in sodium phosphate buffer (pH 7.2) and heated for 5 min at 90°C. Insoluble material was recovered by centrifugation at 1000 g for 10 min. It was rinsed in 0.1% sodium dodecylsulfate, water, twice in 80% ethanol and finally in 0.2 M N-ethylmorpholine HCl buffer (pH 8.2). The protein soluble in detergent and dithiothreitol was precipitated with 10% trichloroacetic acid and rinsed 3 times with 5% trichloroacetic acid, twice with 80% ethanol and once with 0.2 M N-ethylmorpholine HCl buffer (pH 8.2).

### Enzymatic Digestions
Protein from keratinocytes or fibroblasts (up to 1 mg per sample) was suspended in a final volume of 0.5 ml of N-ethylmorpholine HCl buffer (pH 8.2) and digested by sequential treatment at 37°C with a series of proteases for the times indicated: 10 μg of trypsin (1 day), 50 μg of chymotrypsin (1 day), 250 μg of pronase (3 days and repeated for a second period of 3 days), 50 μg of leucine aminopeptidase (2 days) and 80 μg of carboxypeptidase A together with 50 μg of carboxypeptidase B (2 days). Samples were adjusted to 5 mM in MgCl$_2$ upon addition of leucine aminopeptidase. Inclusion of prolidase (Davis and Smith, 1957) in digests of labeled epidermal cells did not significantly affect the amount of label appearing in the isodipeptide fraction.

Digestions of insoluble callus protein (1–2 mg) were performed using double the amounts of enzyme, and included treatment with 30 μg of pepsin in 5% formic acid (1 day) with subsequent lyophilization prior to addition of the other proteases. All digestion samples contained a crystal of thymol to retard bacterial growth. The completed digests were deproteinized by addition of trichloroacetic acid to 12%. The insoluble protein was centrifuged and extracted twice with 10% trichloroacetic acid. The pooled supernatants (1.5–2.5 ml) were diluted with 4 vol of water, and aliquots were applied directly to ion-exchange columns.

Digestion of keratinocyte and fibroblast samples appeared essentially complete, generally <2% of the radioactivity in the labeled samples being recovered in the washed trichloroacetic acid precipitates. The extent of digestion of insoluble callus protein was not accurately quantitated due to the large amount of added enzyme protein, but it was noted that any one of pepsin, trypsin, chymotrypsin or pronase added at 1:50 enzyme:callus protein weight ratio was able to solubilize over half the protein sample at 37°C during overnight digestion.

### Column Chromatography
Sample volumes up to 11 ml were applied to a 0.9 × 40 cm column of AG50 × 8 resin (200–400 mesh) maintained at 38°C. Fractions of 1.1 ml were collected at a flow rate of 30 ml/hr. Acidic and neutral amino acids were eluted with 0.2 M sodium citrate buffers of pH 3.42 (90 ml) and pH 4.25 (65 ml), prepared as described by Moore and Stein (1951) without additions of thiodiglycol or benzyl alcohol. A pH 7.3 buffer of 0.1 M sodium phosphate in 1 M NaCl was used for elution of lysine. Histidine, glutamyl-lysine and lysyl-glutamic acid eluted with the buffer breakthrough, while ammonia and arginine were not eluted in the fractions collected. The column was calibrated with amino acid standards (typically 1 μmole each of several applied at once) detected by A$^{340}$ after reaction of 0.2 ml of each eluate fraction with 0.8 ml of 0.2 M sodium borate buffer (pH 9.2) containing 0.15 mg trinitrobenzenesulfonic acid (Satake et al., 1960). Radioactivity was determined by liquid scintillation counting of 0.2 ml aliquots of each fraction after dilution with 0.8 ml of water and addition of 8 ml of Scinti-Verse (Fisher Chemical). Changes in pH at breakthroughs of the second and third buffers gave rise to small peaks of trinitrobenzenesulfonic acid-positive or radioactive material and served as reference points in the elution profiles. Commercial standards of ε-(γ-glutamyl) lysine and lysine eluted with peaks 7 tubes after the pH 4.25 breakthrough (between methionine and isoleucine) and 21 tubes after the pH 7.3 breakthrough, respectively. Application of labeled samples in dilute trichloroacetic acid also led to a pH discontinuity in the vicinity of tube 40 which resulted in a small peak of radioactivity at that point in the elution profile. The resin was

cleaned with 0.2 N NaOH and reequilibrated with the pH 3.42 buffer between runs.

Pooled peak fractions of material eluted in the position of ε-(γ-glutamyl) lysine or lysine were desalted by dilution with 2 vol of 0.1 N HCl and application to 0.6 × 5 cm columns of AG 50 × 8 (sodium form). The columns were rinsed with water, and samples were eluted with 1 M pyridine to recover ε-(γ-glutamyl) lysine or 2 M pyridine made 1 N in acetic acid to recover lysine and dried in vacuo over NaOH pellets and $P_2O_5$. Aliquots of these samples were submitted to amino acid analysis on a Durrum D500 analyzer (callus) or to paper or thin-layer chromatography (epidermal cells or fibroblasts).

## Paper Chromatography

Samples were dissolved in 1 M pyridine, spotted on Whatman #1 chromatograpy paper and separated in phenol (88%):-ethanol:$NH_3$(28%):$H_2O$ (68:20:1.5:10.5) (Smith, 1969) by allowing the front to migrate 46 cm beyond the origin. Positions of lysine and ε-(γ-glutamyl) lysine markers were determined by spraying with ninhydrin. Radioactivity profiles were obtained by scintillation counting 2 cm squares of chromatography paper.

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