*PROTEIN SYNTHESIS RELATED TO EPIDERMAL DIFFERENTIATION**

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DNA is synthesized in the epidermis of the newborn rat only in the cells of the basal or germinative layer,[1-3] while RNA is produced in the spinous and granular layers as well.[1, 4, 5] Radioautographic studies have shown that although amino acids are incorporated into the cells of all three layers[6-8], there is a difference in the initial localization of certain amino acids.[6] Whereas tritiated leucine, phenylalanine, and methionine are first incorporated in the cells of the basal and lower spinous layers, tritiated glycine first appears more concentrated in the cells of the upper spinous and granular layers, and tritiated histidine first localizes in the cells of the upper layer. Methionine-S^{35} and cystine-S^{35} seem to be first incorporated in the inner layers.[7, 8]

This difference in the initial incorporation of amino acids and the histochemically demonstrated[9] higher concentration of histidine in the granular relative to the other layers suggest that as epidermal cells differentiate during their migration from the basal layer toward the cornified layer, the synthesis of new and unusual proteins is initiated. In support of this hypothesis, fractions of epidermal protein have been isolated which are soluble in 8 *M* urea and dilute $HClO_4$, have high concentrations of glycine and histidine but little or no methionine, leucine, phenylalanine, or cyst(e)ine, and account for a major portion of the tritiated glycine and histidine initially incorporated in the epidermis under conditions of the previous radioautographic experiments.

Materials and Methods.—Glycine-α-H^3 (1 mc/ml H_2O, 200 mc/mmole), D,L-phenylalanine-ring-H^3 (0.5 mc/ml H_2O, 20 mc/mmole), and L-histidine-H^3 (0.25 mc/ml 25% ethanol, 1.1 c/mmole), respectively, were injected intraperitoneally (0.01 ml/animal) to 4–5-day-old rats of the Sprague-Dawley or CFN strain. One to 1.5 hr later, the skin was excised, cooled, and soaked for 15 min at 0° in 0.24 *M* NH_4Cl, pH 9.5, to separate the epidermis from the dermis.[10] The epidermis was immediately frozen in liquid N_2 and pulverized with a mortar and pestle. The powdered tissue was incubated for 1 hr at room temperature in freshly prepared 8 *M* urea–0.2 *M* Tris acetate, pH 8.5 (2 ml/epidermis), followed by homogenization in a Ten Broeck tissue grinder. The suspension was centrifuged at 40,000 × *g* for 10 min at 15°, the residue was again homogenized in $^1/_2$ of the original volume of urea-Tris, and again centrifuged as above. The RNA in the urea extract was degraded at pH 7 by stirring with RNase (5-times recrystallized, protease-free, Sigma Chemical Company, 0.5 mg enzyme/ml) at room temperature for 90 min.[11] After the pH was adjusted to 10, the extract was dialyzed for 36 hr at 2–4° once against 20 vol of 0.1 *M* NH_4OH and then twice against 20 vol of 0.01 *M* NH_4OH. The dialyzed solution was lyophilized and the residue was kept overnight under high vacuum in the presence of anhydrous $CaCl_2$. The dry powder (in batches of 500 mg) was stirred with 0.11 *N* $HClO_4$ (1 ml/10 mg) at room temperature for 30 min. The mixture was then filtered through glass wool and the filtrate was neutralized to pH 4.5–5.5 with 2 *M* Na_2CO_3 and cooled to 0°. The resulting gummy precipitate was collected by centrifugation at 3,000 × *g* for 5 min at 0° and then extracted with 0.02 *M* Na_2CO_3 (1 ml/10 mg original dried powder) at room temperature for several hours. The suspension was centrifuged at 6,000 × *g* for 10 min at 15° and the protein in the supernatant solution was called the "0.1 *N* $HClO_4$-soluble" fraction.

When the residue remaining after extraction with 0.11 N $HClO_4$ was further treated with 0.55 N $HClO_4$ (1 ml/10 mg original dried powder) at 80° for 30 min, additional protein was solubilized. The hot mixture was filtered through glass wool, and the filtrate was neutralized to pH 7 with KOH and cooled to 0°. The precipitated $KClO_4$ was removed by centrifugation and the supernatant solution was called the "0.55 N $HClO_4$-soluble" fraction.

Protein was measured by the biuret method.[12] Amino acid analyses[13] were carried out on samples of protein hydrolyzed in 6 M HCl at 108–110° *in vacuo* for 24 hr with norleucine added as an internal standard.[14] Radioactivity was measured in a liquid scintillation counter using a scintillation solution[15] containing Cab-O-Sil (Cabot Corporation) (40 gm/liter) and corrected for quenching.

Results.—Formation of $HClO_4$-soluble polypeptides: Initially, the epidermis from newborn rats which had received glycine-H^3 was fractionated into cold $HClO_4$-soluble, lipid, nucleic acid, and protein fractions.[16] The protein fraction contained 80 per cent of the nonvolatile H^3 in the epidermis, while about 12 per cent of the H^3 was found in the nucleic acid fraction. When a dialyzed urea extract of the epidermis (containing 98% of the nondialyzable H^3 originally present in the epidermis) was heated in 0.55 N $HClO_4$ at 80° to fragment the nucleic acids into soluble form,[17] over 50 per cent of the H^3 and about 35 per cent of the protein were found in the supernatant solution after the residue was removed by centrifugation. Chromatography of this hot acid-soluble fraction on Sephadex G-50 (Pharmacia Fine Chemicals, Inc.) resulted in the elution of protein and H^3 together whether glycine-H^3 or histidine-H^3 had been administered *in vivo* (Fig. 1). The nucleic acid fragments

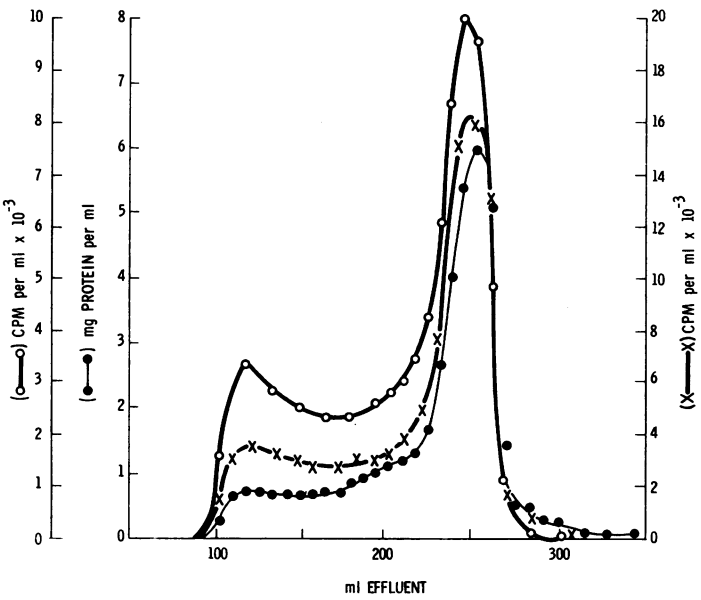

FIG. 1.—Elution diagram of the hot $HClO_4$-soluble fraction chromatographed on a column (2.2 × 77 cm) of Sephadex G-50 in 0.1 N acetic acid. To 100 ml of the dialyzed extract of epidermis were added 5 ml of 70% $HClO_4$. The mixture was incubated at 80° for 30 min, cooled to 20°, and centrifuged at 20,000 × g for 5 min. The soluble fraction was neutralized with $KHCO_3$, the precipitated $KClO_4$ removed by centrifugation, and the supernatant solution lyophilized. The residue was dissolved in 0.1 N acetic acid and applied to the column. *Key:* ●, protein; ×, radioactivity from glycine-H^3; and ○, radioactivity from histidine-H^3.

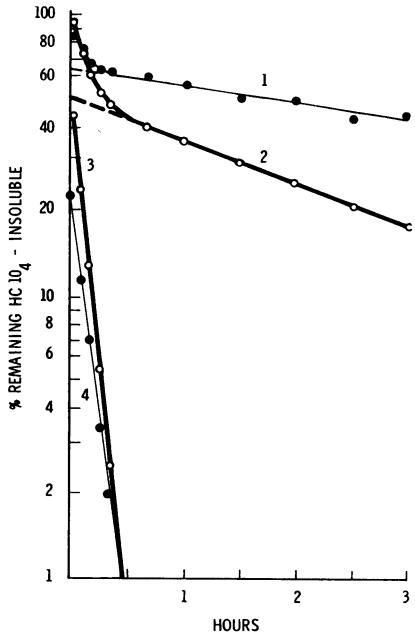

FIG. 2.—Effect of 0.55 *N* $HClO_4$ at 80° on epidermal protein with glycine-H^3 as the tracer as a function of time. Semi-logarithmic plot of the percentage of protein and radioactivity remaining insoluble in $HClO_4$. Two-ml portions of the dialyzed urea extract (in 0.1 *N* acetic acid) were heated with 0.10 ml of 70% $HClO_4$ in sealed tubes at 80° ± 2°. For each time point, the soluble and insoluble fractions of one portion were separated by filtration and analyzed for tritium and for protein. Curves *1* and *2* were experimentally determined. Curves *3* and *4* are plots of the difference values between the extrapolated and the experimental curves during the first 0.5 hr. *Key:* ●, protein; and ○, radioactivity.

eluted after the protein, were not labeled. Amino acid analysis of the eluted protein revealed 85–90 per cent of the H^3 to be in glycine and the remainder in serine when glycine-H^3 had been used and greater than 99 per cent in histidine when histidine-H^3 was the tracer. Figure 2 shows that after initial precipitation, the protein and H^3 were progressively solubilized during the treatment with hot $HClO_4$. These biphasic rate curves indicate that 22 per cent of the protein and about 43 per cent of the H^3 from glycine-H^3 was solubilized at the more rapid rate in 30 min.

Similar $HClO_4$-soluble protein was *not* obtained by heating the urea-extracted protein in 1 *M* HCOOH at 80° for 30 min,[18] by procedures to reduce disulfide bonds,[19] or by treating the extract in 8 *M* urea with RNase at pH 7.[11] No ester groups were detected in the urea-extracted protein[20] nor was carbohydrate found in the hot acid-soluble fraction.

Table 1 presents the distribution of protein and H^3 in the fractions, soluble and insoluble in hot acid, after administration, *in vivo*, of glycine-H^3, histidine-H^3, and phenylalanine-H^3 in separate experiments. Only 10 per cent of the H^3 in the urea extract became soluble in hot $HClO_4$ when phenylalanine was the tracer, as com-

TABLE 1

DISTRIBUTION OF RADIOACTIVITY FROM TRITIATED AMINO ACIDS IN EPIDERMAL PROTEIN

Fraction	Glycine-H^3		Histidine-H^3		Phenylalanine-H^3	
	Mg protein	Cpm	Mg protein	Cpm	Mg protein	Cpm
Dialyzed extract	100	255,000	100	80,000	100	45,200
$HClO_4$-soluble	31	127,000	29	38,300	32	4,700
$HClO_4$-insoluble	64	107,000	60	32,900	68	41,000

The epidermal proteins labeled (*in vivo*) with the various tritiated amino acids were obtained by extraction with 8 *M* urea at pH 8.5 and were dialyzed against 0.1 *N* acetic acid. Portions of the extracts were heated with 0.05 vol of 70% $HClO_4$ at 80° for 30 min, then cooled, and filtered. The soluble and insoluble fractions were analyzed for tritium and for protein.

pared with 50 and 48 per cent in the other two experiments. In a similar experiment with leucine-H^3, 6 per cent of the H^3 became soluble.[21]

Further purification and analysis of epidermal protein soluble in $HClO_4$: Further study of the $HClO_4$-soluble protein led to the introduction of the drying procedures and the extraction of the dried material with 0.11 *N* $HClO_4$ at room temperature (as described under *Materials and Methods*) prior to using 0.55 *N* $HClO_4$ at 80°. The distribution of protein in the various fractions is shown in Table 2.

When the protein solubilized in 0.02 *M* Na_2CO_3 was chromatographed on a column (2 × 42 cm) of Sephadex G-50 in 0.01 *N* NH_4OH at room temperature, all the protein was excluded from the gel particles. This material, however, was retarded when chromatographed with 0.1 *N* acetic acid on Sephadex G-100 (Fig. 3). The elution pattern of H^3 was similar whether histidine-H^3 or glycine-H^3 was the tracer.

Table 2 shows the distribution of H^3 in the various fractions when glycine-H^3 or histidine-H^3 had been used as the tracer. The "0.11 *N* $HClO_4$-soluble" fraction contained 42 per cent of the H^3 originally present in the epidermis from histidine-H^3 but only 20 per cent from glycine-H^3. Glycine-H^3 contributed 17 per cent of the H^3, and histidine-H^3 14 per cent, to the "0.55 *N* $HClO_4$-soluble" fraction. The specific activity, in cpm/mg protein and in cpm/μmole of labeled amino acid, of the material purified from the 0.11 *N* $HClO_4$-extract was greater than that of the protein soluble in 0.55 *N* $HClO_4$ or that of the $HClO_4$-insoluble final residue.

Table 3 shows the content of amino acids in the various fractions. Serine, glutamic acid, glycine, histidine, arginine, alanine, aspartic acid, and threonine accounted for 89 per cent of the amino acid residues in the "0.11 *N* $HClO_4$-soluble" fraction. The level of histidine was over three times greater than it was in the residue. Glycine contributed 1/3 of the residues in the "0.55 *N* $HClO_4$-soluble" fraction, with serine and glutamic acid together accounting for an additional 1/3 of the residues. Hydroxyproline was not detected in either $HClO_4$-soluble fraction. The level of proline was less than four residues per 100 residues in the combined $HClO_4$-soluble fractions. Only small amounts of leucine and phenylalanine were present, while

TABLE 2

RECOVERIES OF PROTEIN AND H^3 DURING ISOLATION OF $HClO_4$-SOLUBLE PROTEIN

Fraction*	Protein (%)	Precursor: Glycine-H^3 Cpm (%)	Cpm/mg protein	Cpm/μmole glycine	Precursor: Histidine-H^3 Cpm (%)	Cpm/mg protein	Cpm/μmole histidine
Urea-extract	100†	100‡	1,080	760	100§	560	2,900
Dialyzed extract	100	73	—	—	96	—	—
0.11 *N* $HClO_4$-extract	28	20	1,030	825	42	740	1,800
pH 4.5-insoluble	12	17	2,700	2,100	40	1,680	4,100
pH 4.5-soluble	16	3	270	—	2	64	160
Na_2CO_3-extract of pH 4.5 insoluble	6	—	—	—	38	3,010	—
Sephadex G-100 eluate ml:							
37–44	0.9	0.9	1,700	—	5	3,000	9,100
57–68	0.6	2.6	3,500	—	10	7,200	14,300
77–110	4	9.5	3,400	2,740	16	2,200	4,000
0.55 *N* $HClO_4$-extract	10	17	2,200	690	14	700	2,800
Final residue	63	36	—	—	37	310	2,800

* Fractions obtained as described in the text.
† Amount of protein isolated given a value of 100%.
‡ Represents 98% of the H^3 originally in epidermis. The specific activity of glycine calculated assuming 85% of the H^3 to be in glycine.
§ Represents 97% of the H^3 originally in epidermis.

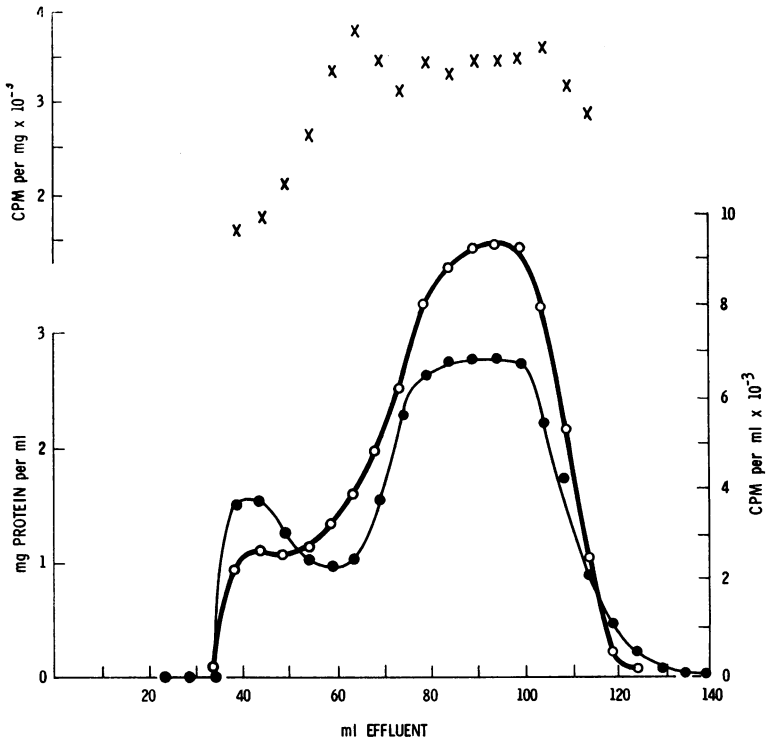

FIG. 3.—Chromatography of the "pH 4.5-insoluble" fraction on Sephadex G-100. Glycine-H^3 was the tracer. The pH 4.5-insoluble fraction was extracted with 0.02 *M* Na_2CO_3, the extract passed through a column (2 × 42 cm) of Sephadex G-50 in 0.01 *N* NH_4OH, and the eluate lyophilized. The residue was dissolved in 10 ml of 0.1 *N* acetic acid and fractionated on a column (2 × 41 cm) of Sephadex G-100 using 0.1 *N* acetic acid. *Key:* ●, protein; ○, radioactivity; and ×, specific activity.

TABLE 3

PARTIAL COMPOSITIONS OF SEVERAL FRACTIONS OBTAINED DURING PURIFICATION OF $HClO_4$-SOLUBLE PROTEINS

	Residues per 100 Residues†					
		0.1 *N* $HClO_4$-Soluble				
Amino acid*	Urea extract	Extract	pH 4.5 Insoluble	Sephadex G-100 eluate‡	0.55 *N* $HClO_4$-soluble	Final residue
Serine	11.2	14.4	14.4	15.6	19.2	9.2
Glutamic acid	14.8	19.2	19.8	20.6	11.6	14.8
Glycine	15.8	14.6	15.2	13.6	33.8	15.0
Arginine	6.8	10.6	11.4	11.6	6.9	5.7
Alanine	7.6	11.4	12.0	10.8	5.9	6.4
Histidine	2.5	5.8	5.7	6.4	3.2	1.6
Threonine	4.8	6.0	4.2	6.0	3.8	4.8
Aspartic acid	8.3	5.7	5.4	5.3	3.9	11.0
Lysine	4.6	2.7	1.7	1.6	2.1	5.2
Leucine	6.2	1.4	1.2	1.0	1.0	7.7
Isoleucine	3.1	1.9	1.8	1.8	1.0	3.7
Phenylalanine	2.6	0.7	0.5	0.4	1.7	3.3
Tyrosine	2.7	1.0	1.2	0.7	2.7	3.2
Valine	3.9	1.9	1.2	1.2	1.7	5.1

* Contents of cystine (and cysteic acid) and methionine in hydrolysates were determined by chromatography and checked independently with ammonium chloropallidite.[22] Only traces of these amino acids were found.

† Data are expressed as the number of residues of each amino acid per 100 residues of the total amino acids recovered in the analyses.

‡ Eluate fraction: 80–103 ml from Table 2.

cystine and methionine were essentially undetectable in either $HClO_4$-soluble fraction.

Discussion.—The epidermis of the newborn rat represents a differentiating biological system in which cells arising in the single germinative layer either stay in this basal layer and retain mitotic capability or migrate toward the surface of the skin through the spinous layer (several cells in thickness) and the granular layer (cells filled with "granules") to the keratinized layer. The migrating cell normally does not divide. In passing from the granular layer into the keratinized layer, the cell loses its nucleus, becomes flattened, and attains the appearance in the electron microscope of being filled with microfibrils[23] which are assumed to contain "keratin-type" protein. Keratinogenesis is presently considered to involve sequential processes occuring during cellular migration and differentiation culminating in the combination of a fibrous protein with an amorphous fraction to form an insoluble complex.[23–26] The fibrous protein is thought to arise in the inner layers while the second component is supposed to be synthesized with the addition of cysteine residues, in the "keratogenic" zone just under the cornified layer.[23–27] Radioautographic data[7, 8, 28] on the localization of cystine-S^{35}, appear to be at variance with this concept. It is entirely possible that the synthesis of protein represented by the initial localization of histidine-H^3 and glycine-H^3 in the cells of the granular and upper spinous layers is related to a specific step in epidermal keratinization.

This investigation tested the hypothesis that this increased labeling by histidine-H^3 and glycine-H^3 of the granular and upper spinous layers as compared with the inner layers of the epidermis in the newborn rat resulted from the initiation of synthesis of different proteins as the cells moved through the outer layers. Such proteins might contain relatively high levels of glycine and histidine and little leucine, methionine, and phenylalanine. Since the initial incorporation of leucine, methionine, and phenylalanine into this region of the epidermis was low, the isolation of proteins low in these three amino acids and high in glycine and histidine would support this hypothesis.

Polypeptide fractions which are soluble in $HClO_4$ have been found to have this predicted unusual composition. Furthermore, glycine-H^3 and histidine-H^3 were mainly incorporated into the protein(s) soluble in 0.11 *N* $HClO_4$ at room temperature and 0.55 *N* $HClO_4$ at 80°. Very little of the incorporated tritiated leucine and phenylalanine appeared in the $HClO_4$-soluble polypeptides. The synthesis in the granular layer of this unusual protein(s) could explain the radioautographically observed different incorporation of amino acids. Further work on the localization of these fractions, *in situ*, will be necessary to determine whether this proposed explanation is the correct answer to the question. Nevertheless, the fact that the specific activities of the glycine and histidine residues in the protein soluble in 0.11 *N* $HClO_4$ and retarded on Sephadex G-100 were severalfold greater than those in the original extract indicates that there is some type of difference in the synthesis of this unusual protein fraction relative to that of the bulk of the urea-soluble, epidermal protein.

The solubilization of protein by treatment with $HClO_4$ possibly means that the solubilized material resulted from the fragmentation of protein in the urea extract. Although intuitively one might expect fragmentation of a protein exposed to 0.55 *N* $HClO_4$ at 80°, it was surprising to observe the solubilization of protein in 0.11 *N*

$HClO_4$ at room temperature in 30 min. Drying the protein prior to the treatment with $HClO_4$ must have had an influence, since merely adjusting a dialyzed urea extract to a concentration of 0.11 *N* $HClO_4$ and incubating the preparation at room temperature did not yield a similar quantity of soluble protein. Peracetic acid and performic acid have been used to fragment wool protein but this appears to occur through oxidation of disulfide linkages.[29] Solubilization of epidermal protein by treatment with $HClO_4$ does not appear to be a random fragmentation since the soluble material differs from the $HClO_4$-insoluble residue with respect to amino acid composition and specific activity when labeled amino acids are involved. Even the two $HClO_4$-soluble fractions differ in these parameters. Although the soluble fractions have not been purified to homogeneity, there is no indication that further purification will yield a broad spectrum of degraded products.

Available histochemical evidence supports the postulated localization of the 0.11 *N* $HClO_4$-soluble protein in the granular layer. The basophilic aggregates seen with the light microscope in the layer just under the keratinized layer of the epidermis are stained intensely with general strains for protein[30] and reagents which detect histidine,[9] but do not appear to contain tyrosine,[9, 30] tryptophan,[9, 31] or cysteine residues.[9, 30, 32, 33] Furthermore, the initial incorporation of histidine-H^3 is seen in the cells of the granular layer by radioautography.[6] Unequivocal localization of the "0.11 *N* $HClO_4$-soluble" material, however, must await future investigation.

A variety of proteins have been isolated from mammalian epidermis (cf. refs. 34, 35), but none appear to be similar to those reported in this paper.

Summary.—Two protein fractions have been obtained by extraction with 8 *M* urea which could explain the unusual initial incorporation of histidine-H^3 and glycine-H^3 in the cells of the granular and upper spinous layers of the epidermis of the newborn rat. One fraction of the urea extract is soluble in 0.11 *N* $HClO_4$ at room temperature and the other dissolves in 0.55 *N* $HClO_4$ at 80°. Together, these fractions account for 50–60 per cent of the tritiated glycine or histidine but only 6–10 per cent of the phenylalanine, leucine, or methionine incorporated into epidermal protein when these labeled amino acids were administered intraperitoneally. These $HClO_4$-soluble fractions contained about 16 per cent of the urea-extractable protein and were unusually low in amino acids having nonpolar side chains.

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