SULFUR-CONTAINING PROTEINS AND EPIDERMAL KERATINIZATION

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INTRODUCTION

Sites of synthesis of sulfur-containing protein in keratinizing epithelium have been studied by radioautography after injection of sulfur^{35}-labeled cystine. Bern et al. (3) demonstrated concentration of silver grains immediately below the cornified layer, suggesting that much of sulfur is added at the final stages of keratinization as observed in hair. In contrast, Bélanger (4) reported that protein synthesis occurs mainly in the Malpighian cells, and Hoober and Bernstein (17) reached a similar conclusion. Radioautographs prepared with beta emission of sulfur^{35} lack resolution, and determination of precise localization of radioactivity with sulfur^{35} becomes very difficult because of its greater energy (167 Kev) compared to that of tritium (18 Kev) (24). In addition, light microscopy alone is not sufficient to study distribution and movement of newly synthesized protein,

especially in relation to the information available on epidermal keratinization at the ultrastructural level (5, 6, 23).

We have investigated incorporation of cystine-^{3}H into newborn rat epidermis by light and electron microscopic radioautography. This paper details the synthetic sites of cyst(e)ine-containing protein in the epidermis as well as the ultimate distribution of the radioactively labeled protein in cornified cells, relating it especially to plasma membrane changes occurring during the keratinization process.

MATERIALS AND METHODS

20 μc of cystine-^{3}H (uniformly labeled, spec. act. 3 mc/mmole, obtained from Schwarz BioResearch, Orangeburg, N.Y.) in 0.1 ml. saline were injected intradermally in the dorsal side of 12 newborn rats (4–5 days old, Sprague-Dawley strain). Biopsies were secured, at 1 and 6 hr after injection, from the injected sites, cut in small pieces, fixed in 3% glutaraldehyde, and postfixed in 2% osmium tetroxide, both fixatives being buffered with phosphate. Tissues were embedded in a mixture of Epon and Araldite. Three to five blocks randomly chosen from each animal were cut for both light (0.5 μ thick) and electron (approx. 800 A thick) microscopy on a Reichert ultramicrotome with a diamond knife. Thick sections were mounted on microslides and filmed with Kodak NTB-2 emulsion by a dipping method. After 3 wk of exposure, the films were developed and the tissues were stained with methylene blue. Thin sections placed on stainless steel grids coated with formvar and carbon were filmed with Ilford L4 emulsion by a modification of a loop method described by Caro and van Tubergen (8). The specimens were exposed for 7 wk, stained with uranyl acetate and lead citrate after photographic development, and examined with a Siemens Elmiskop 1A.

In a separate experiment, the possibility was examined that cystine-^{3}H was: (*1*) bonded to side groups by disulfide linkages; or (*2*) present in the tissue as a result of nonspecific binding of radioactivity by glutaraldehyde. Biopsies taken at 15, 30, and 60 min and at 6 hr after injection of cystine-^{3}H, were fixed in phosphate-buffered 3% glutaraldehyde solution at 4°C overnight. Tissues were then washed with phosphate-buffered 1% sucrose solution for 3 hr and were dehydrated in gradient ethanols. Specimens were divided into two samples and pulverized separately in a glass homogenizer. Sample No. 1 was suspended in 5% trichloracetic acid, homogenized for 5 min, and left at room temperature for 15 min. Samples were filtered through a paper, and the residues, washed twice with 5% trichloracetic acid, were hydrolyzed in 6 N hydrochloric acid at 100°C for 24 hr. The filtrate and washes were collected, and the radioactivity was counted by a Beckman liquid scintillation counter for comparison with the radioactivity found in the total specimen (sum of the filtrate, washes, and hydrolysate). Sample No. 2 was suspended in 2% thioglycollate solution (at pH 8) at 50°C for 2 hr with occasional homogenization and was filtered. The remaining residue was washed with thioglycollate solution and was hydrolyzed in 6 N hydrochloric acid for 24 hours. The radioactivity present in the thioglycollate solution and that present in the acid hydrolysate were counted.

RESULTS

Light Microscopic Radioautography

At 1 hr after injection of cystine-^{3}H, radioactivity was demonstrated throughout the epidermis, but not over cornified cells (Fig. 1). Label was scattered diffusely in epidermal cells; most small keratohyalin granules were free of labeling, but label seemed to locate at the edge of larger karatohyalin granules in the upper granular cells.

Two specimens of different blocks were chosen from each animal, and grain counts were made in three layers of epidermis in order to determine the relation between synthesis of cystine-containing protein and cell differentiation. A Whipple eyepiece micrometer disc was used under 1280 magnification for nondiscriminate counting of grains appearing over the uppermost and lowermost and in-between areas of the epidermis. The number of silver grains observed outside of tissue sections in the same square measure, usually three to four, was considered to be background counts and was subtracted from the actual counts. The results summarized in Table I show that the density of grains in the granular layer was highest, 47.8 $\pm$ 3.3% of the total counted grains, indicating that protein synthesis continues in granular cells, probably at an increased rate.

At 6 hr after injection, a few horny cells located just above the granular layer were labeled (Fig. 2). The labeled cornified cells varied in size and shape, but none retained their nucleus. Some contained keratohyalin granules; others did not. Label in these cells no longer appeared diffuse but was localized at the cell periphery, whereas distribution of label in viable cells did not show specific localization.

Electron Microscopic Radioautography

Label appearing in granular cells at 1 and 6 hr after injection was situated diffusely over the nucleus, near tonofibrils, keratohyalin granules, the cell membrane, and other cytoplasmic organelles (Fig. 3). Low-power electron micrographs (× 3,000) were taken to cover the total field (200-mesh grid), and "mosaic maps" were constructed with four to five fields from different blocks of tissue of each animal. The number of silver grains associating with various cell organelles in the outer two granular cells was counted by the method

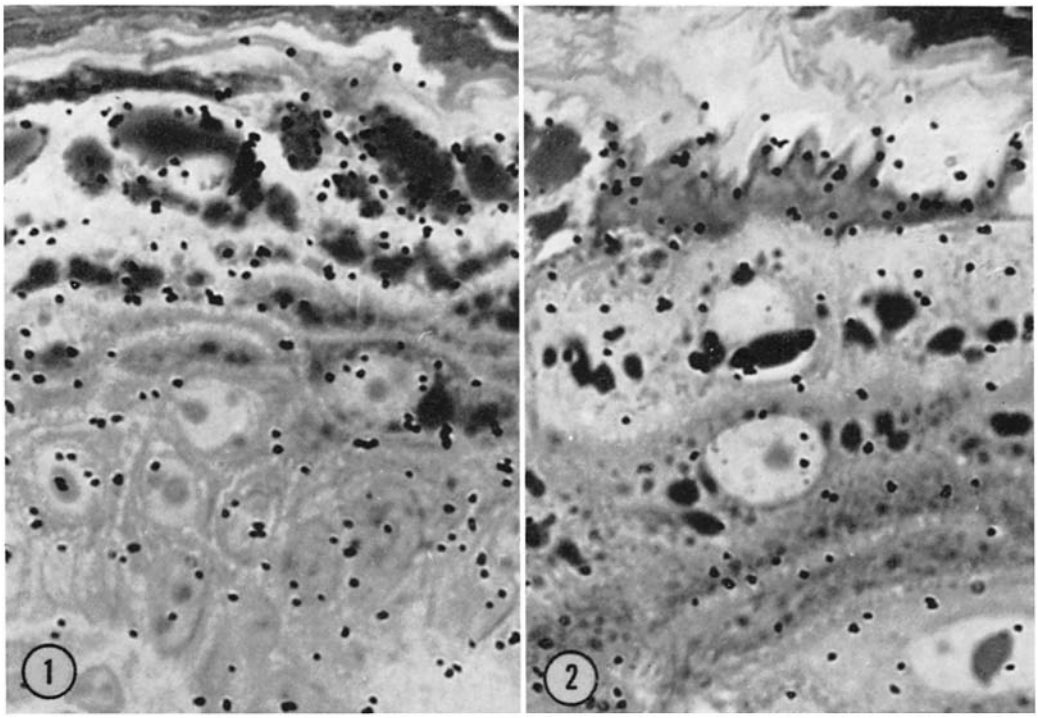

FIGURE 1 Sites of cystine-^{3}H incorporation in the newborn rat epidermis at 1 hr after injection. Radioactivity is concentrated over granular cells. × 3,000.

FIGURE 2 The labeled cell observed in the cornified layer at 6 hr after injection of cystine-^{3}H. Silver grains locate at the cell periphery. × 3,000.

TABLE I

Concentration of Silver Grains Observed in Basal, Spinous, and Granular Cells of Newborn Rats 1 Hr after Injection of Cystine-^{3}H

Animal No.	Total	Basal cell	Spinous cell	Granular cell
		%	%	%
1	1779	23.3	29.7	46.9
2	1139	26.7	29.1	44.0
3	575	26.0	27.4	46.4
4	1056	20.8	26.8	52.2
5	259	19.3	27.0	53.6
6	1391	25.3	28.2	46.4
Average		23.1 ± 2.7	27.6 ± 1.1	47.8 ± 3.3

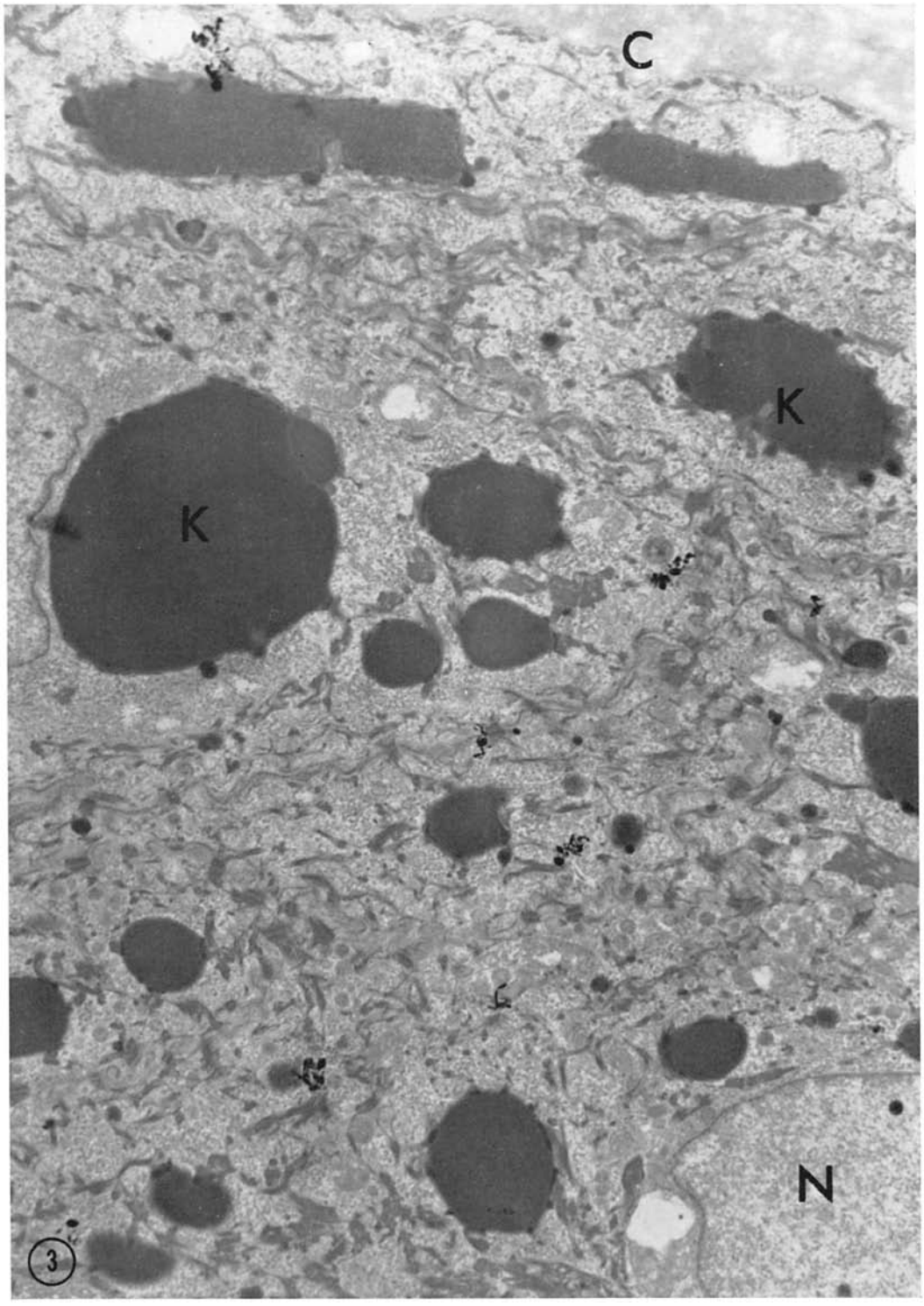

FIGURE 3 Distribution of silver grains in granular cells at 1 hr after injection of cystine-^{3}H. Label is not associated with any specific cellular structures. *C*, cornified cell; *N*, nucleus; *K*, keratohyalin granules. × 8,250.

described by Ross and Benditt (25) (the mean geometric error was calculated to be 0.24 μ). Since the field containing the most granular cells was selected automatically from each block, the amount or distribution of grains did not influence our choice of fields for counting. Background grains were negligible, none or one per unit area, as has been described (9, 25); they were not taken into consideration for the grain counts. Table II indicates the counts made at 1 and 6 hr after injection of cystine-^{3}H. At both time intervals, more than 24% of grains appeared over: (*1*) tonofibrils; (*2*) ribosomes; and (*3*) keratohyalin granules. About 14% of grains were associated with the plasma membrane and a small percentage of grains was found over the nucleus, mitochondria, vesicles, and membrane-coating granules. Label associating with keratohyalin granules located usually at the edge of the granules; label seldom appeared in the center of granules. A concentration of radioactivity over any specific structure was not observed, even at 6 hr after injection.

Horny cells labeled 6 hr after injection were the "transitional cells" described by Brody (5). In these cells, label was located in areas where the thickened cell membrane becomes visible (Fig. 4). In 30 fields obtained from 30 blocks made from five animals, we found 25 of these labeled cells with a total of 203 silver grains, of which 162 grains (78%) were over the cell membrane, and others (21%) were inside the cell.

Radioactivity Present in Glutaraldehyde-Fixed Tissue as Nonprotein Cystine-^{3}H

The amounts of radioactivity appearing in 5% trichloracetic acid and in 2% thioglycollate solution as compared with the total radioactivity present in tissue specimens are summarized in Table III. The radioactivity soluble in 5% trichloracetic acid was about 1% of the total counts in tissue at 15, 30, and 60 min after injection of cystine-^{3}H. Relatively higher per cents of radioactivity, 2.6–3.0% and 5.6–7.1%, were solubilized in 2% thioglycollate solution at 50°C from the tissue obtained after injection of cystine-^{3}H at 1 and 6 hr, respectively.

TABLE II

Number of Grains Appearing over Different Cellular Structures of Newborn Rat Granular Cells at 1 Hr and 6 Hr after Injection of Cystine-^{3}H

Time after injection:	1 hr		6 hr	
No. of animals:	6		6	
Total fields counted:	30		20	
Cell organelles	No. of grains	%	No. of grains	%
Tonofibrils	123	26.8	88	29.1
Ribosomes	112	24.4	75	24.8
Keratohyalin granules	137	29.9	74	24.5
Plasma membrane	64	13.9	44	14.5
Nucleus	12	2.6	15	4.9
Mitochondria	5	1.9	1	0.3
Vesicle	3	6.5	4	1.3
Membrane-coating granules	2	0.4	1	0.3
Total	458		302	

DISCUSSION

Observations by both light and electron microscopic radioautography were made to investigate incorporation of cystine-^{3}H into the epidermis. The possibility that injected cystine-^{3}H may be present in the tissue in a form other than that of a peptide bonding was examined by counting the radioactivity solubilized in 5% trichloracetic acid and 2% thioglycollate solutions. The amount of radioactivity as nonprotein cystine-^{3}H, soluble in 5% trichloracetic acid, was about 1% of the total radioactivity found in the tissue at 15, 30, and 60 min after injection of cystine-^{3}H. Methods of tissue preparation for radioautography were, therefore, considered to exclude administered free amino acid. Thioglycollate solution was used to reduce —S—S— groups present in the tissue, and to solubilize cystine-^{3}H which was attached to side groups by formation of disulfide linkages. Since amorphous protein can be extracted from glutaraldehyde-fixed epidermis at alkaline pH (14), the solubilized radioactivity may be due, at least partially, to some contamination by alkaline-soluble polypeptide originally linked to other peptide chains by disulfide linkages. Nevertheless, the amount of contamination was insignificant for the final radioautographic observations. As interpreted by Bern et al. (3), all silver grains observed in radioautography were considered to demonstrate sites of cystine-^{3}H incorporation into protein, probably as cysteine-^{3}H by reduction. Both sulfhydryl and disulfide groups were demonstrated at all levels of the epidermis by chemical and histochemical studies (2, 26, 27).

Reduced silver grains were found throughout the epidermis, except in cornified cells, at 1 hr after injection of cystine-^{3}H. Synthesis of cyst(e)ine-containing protein seems to be a continuous process in the living epidermis, and not limited to basal and spinous cells (4, 17) or immediately below the keratin layer (3). The number of grains appearing over basal, spinous, and granular cells was counted for comparing the synthetic rate of this/these protein(s) in cells at different stages of differentiation (the cornification process). Since the cells are not of the same size, grains were counted in the set area measured by the micrometer disc, rather than in the unit number of cells of each layer. The results indicated that granular cells incorporate about twice as much radioactivity as either the basal or spinous cells. Distribution of grains was least dense in basal cells, 23.1 ± 2.7% of the total grains counted, and slightly more in spinous cells, 27.6 ± 1.1. According to our previous classification, cystine-^{3}H belongs with those amino acids, namely histidine-^{3}H, serine-^{3}H, and arginine-^{3}H, which primarily concentrate over granular cells (12), and protein synthesized in granular cells was considered to result in formation of specific cellular structures to complete epidermal cornification. The specific cellular organelles of granular cells involved in synthesis of cyst(e)ine-containing protein was studied by means of electron microscopic radioautography at 1 and 6 hr after injection of cystine-^{3}H. This approach has provided information on synthesis and migration of proteins in the epidermal cell (13) and other organs (7, 9, 25, 28). The technical details have been worked out (8), and the technique has been discussed in depth with respect to its background, efficiency, and resolution (9, 25). Silver grains associated with cellular organelles were always counted in low-power electron micrographs taken on the field containing the most granular cells, in order to choose samples randomly. Three to five different blocks were processed from each animal so as to obtain sufficient numbers of grains. A higher percentage (24–29%) of label appeared over ribosomes, tonofibrils, and keratohyalin granules and about 14% was over the plasma membrane, whereas very little label was found over the nucleus, mitochondria, vesicles, and membrane-coating granules. Distribution of label in granular cells seemed to change between 1 and 6 hr after injection, but the significance of these changes remains uncertain since statistical analysis to determine the chance of labeling specific structures (using volume of the cellular organelles as correction factors (9)) was not employed.

It may be important to note that label associating with keratohyalin granules usually located at the edge of the granules, even at 6 hr after injection, unlike the localization of radioactivity at the center portion of keratohyalin granules seen 6 hr after injection of histidine-^{3}H (13). Those observations agree with the histochemical findings of Matoltsy and Matoltsy (19), and Barrnett and Sognnaes (2) that both sulfhydryl and disulfide groups were absent in keratohyalin granules studied by the Barrnett-Seligman method. We conclude that cyst(e)ine-containing protein locates in granular cells, but does not contribute directly to formation of keratohyalin granules, as histidine-containing protein evidently does (13).

TABLE III

Rates of Solubilized Radioactivity in Trichloracetic Acid and Thioglycollate as Compared to the Total Radioactivity Present in Glutaraldehyde-Fixed Tissue

Intervals	Trichloracetic acid soluble	Thioglycollate soluble
	%	%
15 min	1–2	
30 min	1	
60 min	0.9	2.6–3.0
6 hr		5.6–7.1

Labeled transitional cells appeared in the cornified layer 6 hr after injection of cystine-^{3}H and were considered to be cells that had just moved from the granular layer. Most label was located at the edge of these cells, suggesting that a cyst(e)ine-containing protein moved from the sites of synthesis to the periphery of the cells, most probably to the plasma membrane. Since radioactivity did not concentrate over any specific cellular sites in granular cells at 6 hr after injection, addition of cyst(e)ine-containing protein to the plasma membrane must take place in granular cells during the final stages of differentiation. Morphologically, epidermal cells undergo a recognizable differentiation as they move into the cornified layer. The cells contain filaments and amorphous substances (5, 23), and the plasma membrane becomes thickened and resistant to trypsin digestion or keratolytic agents (11, 19, 20). Farbman observed that dense

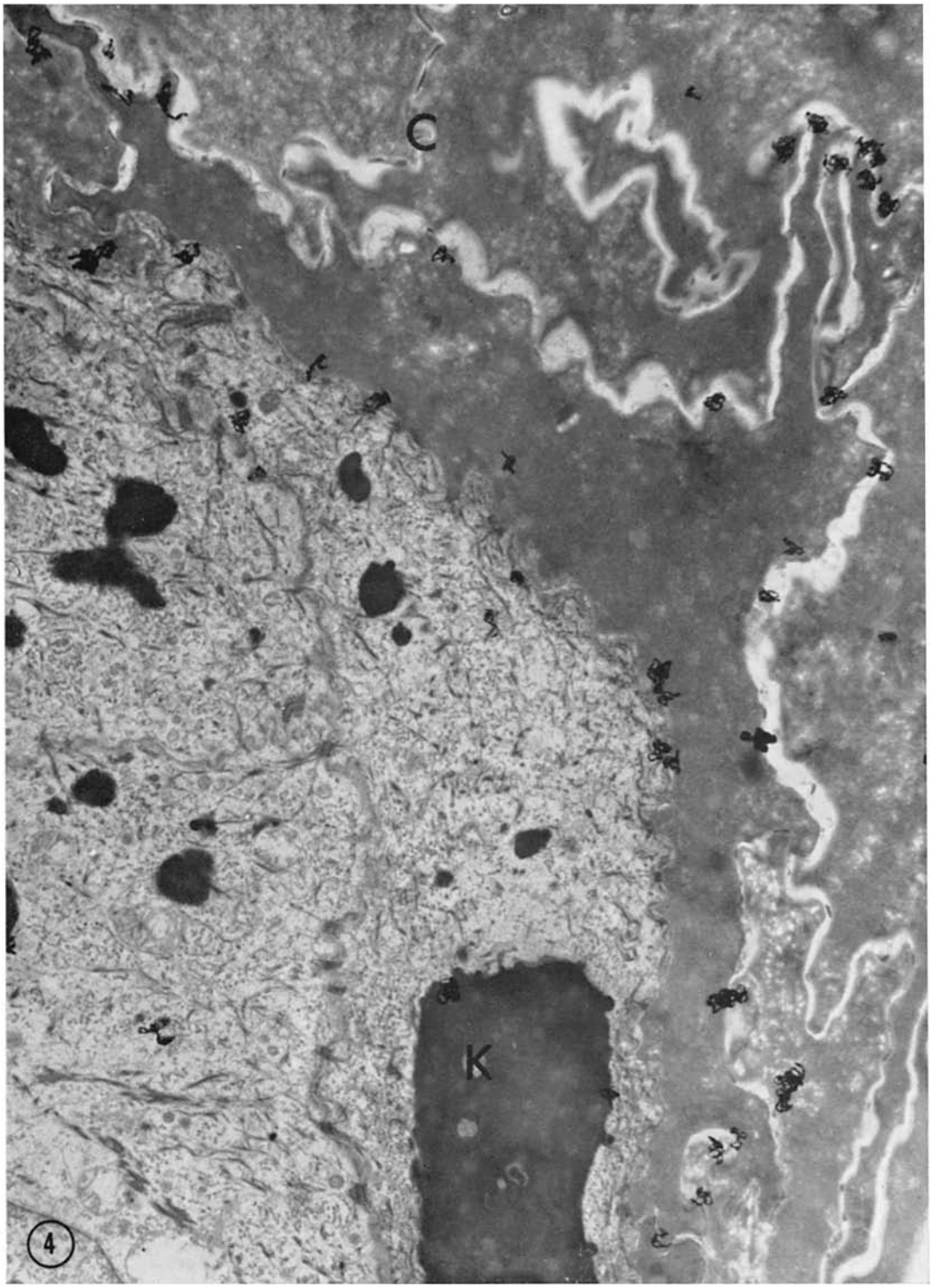

FIGURE 4 Most of the label appears near the plasma membrane in cornified cells at 6 hr after injection of cystine-^{3}H. *C*, cornified cell; *K*, keratohyalin granules. × 8,250.

material is deposited at the periphery of granular cells and that this material becomes indistinguishable from the inner layer of the plasma membrane in cornified cells (11). The cyst(e)ine-containing protein that has moved to the cell periphery may correspond to the dense deposit, as cyst(e)ine is known to be strongly osmiophilic (1). It is possible that added protein formed disulfide bridges to make the cornified cell membrane rigid and chemically resistant. The results also coincide with a chemical study of Matoltsy reporting a high cystine content in the membrane protein isolated from human horny cells (20).

In hair, large amounts of sulfur are known to be added to the fibers of cortical cells during cornification (4, 10, 15, 16, 21). This occurs primarily in the keratogenous zone and is readily demonstrated by electron microscopic radioautography (22). Whether or not this "sulfur enrichment" occurs in the epidermis or other keratinizing epithelium has been widely debated (3, 17, 27). The present study demonstrates that the incorporation of cyst(e)ine into protein in granular cells is twice that seen in basal cells, but no specific association with any specific cellular structure occurs until the cells move into the cornified layer. At this time, radioactivity associates with a thickened plasma membrane and not especially with the fibers. An important difference between keratinization in the epidermis and that in hair, then, appears to be the cellular structures formed as a result of synthesis of sulfur-containing protein.

SUMMARY

Incorporation of cystine-^{3}H into protein(s) of the newborn rat epidermis was studied by means of light and electron microscopic radioautography. Synthesis of cyst(e)ine-containing protein was a continuous process throughout the basal cells to granular cells. However, in granular cells a unique movement of cyst(e)ine-containing protein seemed to occur. This protein appeared at the plasma membrane as the membrane became thickened in cornified cells. This protein may contribute to the stability and chemical resistance of the cornified cell membrane.

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