SULPHUR IN EPIDERMAL KERATOHYALIN GRANULES: A QUANTITATIVE ASSAY BY X-RAY MICROANALYSIS

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SUMMARY

The elemental composition of different types of keratohyalin granules from the epidermis of newborn and adult rats was studied by means of an EMMA-4 analytical electron microscope, equipped with an energy-dispersive X-ray spectrometer. An absolute quantitation of the sulphur concentration in keratohyalin granules was performed.

The results demonstrate that epidermal keratohyalin granules are chemically heterogeneous. A type of keratohyalin granule present in the nuclei and cytoplasm of epidermal cells from both newborn and adult rats—termed single granules—is rich in sulphur, having a content of 2.5–3.6%. Other types of keratohyalin granules, which differ in newborn and adult rats, contain a sulphur-poor component; they often have a sulphur-rich component as well. The sulphur-poor keratohyalin contains 0.6–0.9% sulphur.

It is suggested that the sulphur-rich keratohyalin granules are the source of the peripheral envelope protein of cornified cells.

INTRODUCTION

Keratohyalin granules are synthesized in the living cells of keratinizing epithelia. The granules are generally thought to contribute to the keratin content of the dead, cornified cells. A distinguishing feature of the chemical composition of keratin is the occurrence of the sulphur-containing diamino acid cystine (see Fraser, McRae & Rogers, 1972).

In a preceding paper we have reported that sulphur is present in high concentration in a particular type of keratohyalin in rat mucosa, using X-ray microanalysis at the electron-microscope level (Jessen, Peters & Hall, 1974).

There are a number of recent papers dealing with the composition of keratohyalin granules from epidermis either by biochemical methods (Matoltsy & Matoltsy, 1970, 1972; Ugel, 1971; Ugel & Idler, 1972; Guss & Ugel, 1972; Elias, Montague & Ugel, 1972; Tezuka & Freedberg, 1972, 1974; Sibrack, Gray & Bernstein, 1974) or biochemical methods (Matoltsy & Matoltsy, 1970, 1972; Ugel, 1971; Ugel & Idler, 1972; Guss & Ugel, 1972; Elias, Montague & Ugel, 1972; Tezuka & Freedberg, 1972, 1974; Sibrack, Gray & Bernstein, 1974) or

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by electron-microscopic autoradiography (Fukuyama & Epstein, 1967, 1969). The results of these studies are divergent and appear irreconcilable as to one important point, namely the possible presence of sulphur-containing amino acids in keratohyalin granules. Most authors seem to agree that sulphur-containing amino acids are not important components of keratohyalin. Matoltsy & Matoltsy (1970, 1972), however, found a significant content of cystine.

Newborn rat epidermis contains numerous keratohyalin granules. It has therefore been the tissue of choice for most biochemical studies of keratohyalin. In order to elucidate the discrepancies between the biochemical findings, we have performed X-ray microanalysis on newborn as well as adult rat epidermis. In this paper we will demonstrate that keratohyalin from rat epidermis varies in elemental compositions. It will be shown by quantitative X-ray microanalysis, that there is a sulphur-rich as well as a sulphur-poor type of keratohyalin.

METHODS

Epidermis from the back of newborn rats (2–3 days old) and from the back and tail of adult rats (weighing approximately 60 g) was used.

Newborn rat epidermis was fixed by immersion in 3 % glutaraldehyde in 0.15 M cacodylate buffer at pH 7.4 for 2 h. Adult rat epidermis was fixed by perfusion via the ascending aorta with 200 ml of fixative containing 3 % glutaraldehyde and 2 % Dextran in 0.15 M cacodylate buffer at pH 7.4 within a period of 30 min. Tissue blocks were dehydrated in graded ethanol and embedded in Araldite. Some tissue blocks were postfixed in 1 % osmium tetroxide in 0.15 M cacodylate buffer at pH 7.4 for 2 h before embedding.

Unstained sections of glutaraldehyde-fixed epidermis were analysed in an EMMA-4 analytical electron microscope by the method described by Jessen et al. (1974) in examining lingual and oesophageal epithelium.

Fig. 1. Micrograph showing cells from the granular layer of newborn rat epidermis. Several types of keratohyalin granules are present. Dense keratohyalin granules (dg) are seen in all levels of the granular layer. Single keratohyalin granules (sg) and composite keratohyalin granules (eg) are seen in the upper part of the granular layer. Cornified cells (cc) are seen in the left part of the micrograph. Glutaraldehyde/osmium-fixed, stained section. × 4900.

Fig. 2. Intranuclear single keratohyalin granule (sg) from newborn rat epidermis after glutaraldehyde fixation. n, nucleus. Stained section. × 24,500.

Fig. 3. Composite keratohyalin granule (eg) from newborn rat epidermis after glutaraldehyde fixation. The granule contains an electron-lucent 'single granule component' and a dense component. Stained section. × 28,500.

Fig. 4. Micrograph showing cell from the uppermost part of the granular layer from newborn rat epidermis. Single keratohyalin granules (sg) are seen in both the nucleus (n) and the cytoplasm. Several single granules are seen in the upper periphery of the granular cell (unmarked arrows). A transitional cell (tc) is seen to the left in the micrograph. A peripheral envelope (pe) is deposited along its plasma membrane. eg, composite keratohyalin granule. Glutaraldehyde/osmium-fixed, stained section. × 10,500.
RESULTS

Morphology

Newborn rat epidermis. This contains several types of keratohyalin granules (Fig. 1). The granules in the lower part of the granular layer are globular and dense after double fixation. They are dense also after glutaraldehyde fixation alone. These granules may reach a considerable size in the uppermost granular cells. In the upper part of the granular layer a number of small keratohyalin granules appear in the cytoplasm as well as in the nuclei of the cells. These granules are very dense after double fixation but are of low density after glutaraldehyde fixation alone (Fig. 2), and they will be termed single keratohyalin granules (Jessen, 1970, 1973). Single granules are often associated with the periphery of the dense keratohyalin granules, giving these granules the appearance of composite bodies (Figs. 3, 4, 7). The keratohyalin granules of newborn epidermis show no particular association with filament bundles of the epithelial cells.

Adult rat epidermis. This contains 2 types of keratohyalin granules. As in newborn epidermis, single keratohyalin granules are present in the nucleus and in the cytoplasm (Fig. 11). In the cytoplasm they may occur either free or associated with the periphery of another type of keratohyalin granule which consists of dense, stippled material intimately associated with filament bundles (Fig. 10). The shape of the filament-associated granules varies from globular to highly irregular. Often the dense keratohyalin material does not form distinct granules but may cover extensive areas of filament bundles. The filament-associated keratohyalin is dense after glutaraldehyde fixation alone, in contrast to single-granule material (Fig. 14).

X-ray microanalysis

X-ray spectra were obtained with the energy-dispersive spectrometer to establish the elemental composition of the different types of epidermal keratohyalin, filament bundles and the interior part of cornified cells. The spectrometer was pre-set to record identical ‘white’ counts to allow a relative estimation of the elemental concentrations in the different structures.

Newborn rat epidermis. A spectrum, which is representative of intranuclear and cytoplasmic single granules as well as the single granule component of composite
granules, is shown in Fig. 9. A prominent sulphur peak is present. Fig. 8 is a spectrum which is representative of the dense granules and the dense component of composite granules. It includes a prominent phosphorus peak and minor sulphur and chlorine peaks. A spectrum from the interior part of a cornified cell is shown for comparison in Fig. 5. Moderate sulphur and chlorine peaks are present.

Adult rat epidermis. Fig. 16 shows a spectrum obtained from a cytoplasmic single granule. The spectrum is typical for single granule material irrespective of its position. It contains a prominent sulphur peak. A spectrum from the keratohyalin granules associated with filament bundles shows a prominent phosphorus peak. In addition, minor sulphur and chlorine peaks are present (Fig. 15). Fig. 13 shows a spectrum obtained from a filament bundle of a granular cell and Fig. 12 from the interior part of a cornified cell. The 2 spectra are almost identical, both showing minor sulphur and chlorine peaks.

A spectrum obtained from Araldite is illustrated in Fig. 6. No peaks are present. The spectra demonstrate that single keratohyalin granules from newborn and adult rat epidermis contain a sulphur-rich material. In contrast, the dense granules of newborn rat epidermis and the filament-associated keratohyalin of adult epidermis contain phosphorus but are poor in sulphur. The interior parts of cornified cells have a minor sulphur content but phosphorus is not detected there.

Quantitation

The sulphur concentration in keratohyalin was analysed according to the continuum-method of Marshall & Hall (see Hall, 1971). This method allows calculation of weight fractions of elements in thin sections. The method is based on normalization of the measured intensity of a characteristic radiation against simultaneously measured continuum radiation, in both tissue specimens and standards. The continuum radiation serves as a measure of the local mass in the analysed volumes.

The analysis was made on unstained sections of glutaraldehyde-fixed epidermis to avoid contributions to the continuum radiation from heavy elements present in metal stains and osmium tetroxide. The keratohyalin granules were analysed with the probe focused to the minimum attainable diameter of about 150 nm (Fig. 7). In newborn epidermis the keratohyalin granules are of sufficient size to accommodate the probe fully. In adult epidermis, some difficulties were encountered in limiting the probe to single keratohyalin granules, because of their relatively modest size in this tissue.

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**Fig. 10.** Micrograph showing cell from the granular layer of adult rat epidermis. Two types of keratohyalin are present. Dense keratohyalin material (dm) is seen on tonofibrils (tf). Single keratohyalin granules (sg) are seen free in the cytoplasm and at the edge of the dense material (unmarked arrow). cc, cornified cell. Glutaraldehyde/osmium-fixed, stained section. × 21 500.

**Fig. 11.** Micrograph showing cell from the granular layer of adult rat epidermis. Several single keratohyalin granules (sg) are seen in both the nucleus and the cytoplasm. Glutaraldehyde/osmium-fixed, stained section. × 17 000.
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The results of the quantitative analysis of keratohyalin granules, tonofibrils and the interior parts of cornified cells are summarized in Table 1. The single keratohyalin granules from newborn epidermis contained the highest sulphur concentration, namely 3.6%. Single keratohyalin granules from adult epidermis contained 2.5% sulphur. The other type of keratohyalin granules of newborn epidermis and the filament-associated keratohyalin of adult epidermis were considerably poorer in sulphur (0.6-0.9%). Tonofibrils and the interior parts of cornified cells in both types of epidermis contained about 1% sulphur.

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<tr>
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<th>Newborn rat epidermis</th>
<th>Adult rat epidermis</th>
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<tr>
<td>Single granules</td>
<td>3.6 ± 0.3</td>
<td>2.5 ± 0.14</td>
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<tr>
<td>No. of areas analysed</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Dense component of</td>
<td>0.6 ± 0.05</td>
<td>0.9 ± 0.05</td>
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<td>composite granules and</td>
<td>10</td>
<td>10</td>
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<tr>
<td>dense granules</td>
<td>10 ± 0.05</td>
<td>0.9 ± 0.08</td>
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<tr>
<td>Tonofibrils</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Cornified cells</td>
<td>1.2 ± 0.09</td>
<td>1.2 ± 0.05</td>
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<td>No. of areas analysed</td>
<td>4</td>
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DISCUSSION

During the differentiation of epidermal cells two major products are synthesized, namely filaments and keratohyalin granules. These two products interact during cornification and are generally assumed to constitute the main content of the dead, cornified cells.

There are different types of epidermal keratohyalin granules. One type of keratohyalin granule found in both newborn and adult epidermis as well as in other epithelia is the single keratohyalin granule. One of us has previously described a number of characteristic features of single keratohyalin granules (Jessen, 1970, 1973).

The other types of epidermal keratohyalin granules differ in newborn and adult epidermis. In newborn epidermis the granules are spherical to ovoid and show only occasional association with filament bundles. In adult epidermis, however, the granules are irregular and intimately associated with the prominent filament bundles of this epithelium.

Fig. 12. Elemental spectrum from analysis of the interior of a cornified cell. Adult rat epidermis.

Fig. 13. Elemental spectrum from analysis of a tonofibril from a granular cell. Adult rat epidermis.

Fig. 14. Micrograph showing cornified cells (cc) and a granular cell of adult rat epidermis in a glutaraldehyde-fixed, stained section (compare with Fig. 10). Dense keratohyalin material (dm) is seen on tonofibrils (tf). Single keratohyalin granules (sg) are seen free in the cytoplasm and at the edge of dense keratohyalin material. × 23,000.

Fig. 15. Elemental spectrum from analysis of dense keratohyalin material. Adult rat epidermis.

Fig. 16. Elemental spectrum from analysis of a single keratohyalin granule. Adult rat epidermis.
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The present X-ray microanalytical results clearly demonstrate that the morphological difference between epidermal keratohyalin granules corresponds to a difference in elemental composition. Single keratohyalin granules are particularly rich in sulphur, in contrast to the other types of keratohyalin granules present in epidermal cells. Furthermore, the sulphur content of single granules is significantly higher than that of filament bundles of granular cells and of the interior parts of cornified cells.

The values of sulphur concentration, calculated for the different types of keratohyalin granules from rat epidermis, are consistent with the values obtained previously for similar types of keratohyalin granules of the rat mucosa (Jessen et al. 1974). The sulphur-rich single granules of rat mucosa contained 3.6–3.8% sulphur. Single granules of newborn rat epidermis contain 3.6%. The sulphur content of single granules from adult epidermis is 2.5%. We suspect that the somewhat lower figure for single granules of adult epidermis reflects difficulties in probing these granules with optimal spatial resolution. The sulphur-poor keratohyalin in the different types of epithelia analysed thus far has sulphur concentrations of the same order of magnitude, significantly lower than the sulphur-rich single granules (0.8–1.4% for lingual and oesophageal epithelia and 0.6–0.9% for epidermis). Moreover, the sulphur-poor keratohyalin of the various epithelia are rich in phosphorus, except for the dense keratohyalin granules of the lingual filiform papillae (see fig. 18 of Jessen et al. 1974).

In spite of the similarities as to sulphur and phosphorus content in sulphur-poor keratohyalin, the heterogeneous morphology of these granules in different epithelia may well reflect a heterogeneity in composition not detectable by X-ray microanalysis.

Our findings may reconcile the controversy in the literature regarding sulphur-containing amino acids in keratohyalin. It has been demonstrated by several techniques that keratohyalin granules are histidine-rich. Keratohyalin granules have thus been stained by a histochemical method for histidine (Reaven & Cox, 1965) and 3H-labelled histidine has been shown to be incorporated into keratohyalin granules (Cox & Reaven, 1967; Fukuyama & Epstein, 1967). Furthermore, a histidine-rich (and cystine-poor) protein has been isolated and characterized by different biochemical methods (Sibrack et al. 1974; Tezuka & Freedberg, 1974). This protein appears to be of keratohyalin origin. In contrast to these findings are those of Matoltsy & Matoltsy (1970, 1972). They extracted keratohyalin granules from newborn rat epidermis and found a low content of histidine in the isolated protein, but a high cystine content. The sulphur content of the protein isolated by Matoltsy & Matoltsy is comparable to the sulphur concentration demonstrated in situ in single keratohyalin granules by X-ray microanalysis. The other types of keratohyalin granules, which are poor in sulphur, may well contain the histidine-rich (and sulphur-poor) protein isolated by Sibrack et al. (1974) and Tezuka & Freedberg (1974).

There is no agreement on the function of keratohyalin granules. It is generally assumed that keratohyalin granules disperse and participate in the formation of the interfilamentous matrix of cornified cells. We have stressed the point that the sulphur-rich single granules appear to be involved in the deposition of the peripheral envelope protein of cornified cells (Jessen, 1970, 1973; Jessen et al. 1974). This suggestion is based on the observation that single granules accumulate along the periphery of the
upper granular cells and appear to fuse along the cytoplasmic aspect of the plasma membrane during the transformation of granular cells into cornified cells. The deposition of the peripheral envelope appears to be an initial and rapid event in this transformation. However, transitional cells with the features of granular cells – except for the presence of peripheral envelope and absence of single keratohyalin granules – can be observed. These morphological observations and the previously demonstrated cytochemical similarities between the peripheral envelope and single keratohyalin granules strongly indicate that the sulphur-rich single granules are the source of the peripheral envelope protein(s).

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REFERENCES


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