SULPHUR IN DIFFERENT TYPES OF KERATOHYALIN GRANULES: A QUANTITATIVE ASSAY BY X-RAY MICROANALYSIS

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SUMMARY

The elemental composition of keratohyalin granules from the interpapillary and papillary lingual epithelium and the oesophageal epithelium of the rat were studied by X-ray microanalysis in an EMMA-4 analytical electron microscope, equipped with an energy dispersive detector. A quantitative assay of the sulphur concentration in keratohyalin granules was performed, using a suitable sulphur standard. The results demonstrate that different types of keratohyalin granule have different compositions. Single granules—a type of keratohyalin granule present in both nuclei and cytoplasm of the epithelial cells—are rich in sulphur, having a content of 3.6–3.8%. Another type of keratohyalin granule—composite granules—contains a sulphur-rich component and a sulphur-poor component. The sulphur-poor component contains 0.8–1.4% sulphur. It is suggested that the sulphur-rich keratohyalin granules are involved in the deposition of the peripheral envelope protein of cornified cells.

INTRODUCTION

The chemical composition of keratohyalin granules is a matter of considerable controversy. A salient point is their possible content of sulphur-amino acids. It has generally not proved possible to demonstrate sulphydryl and disulphide groups in keratohyalin granules by histochemical means (Barnett & Sognnaes, 1962). Furthermore, most recent biochemical studies do not report a significant content of cyst(e)ine in keratohyalin (Bernstein & Sibrack, 1972; Tezuka & Freedberg, 1972; Ugel, 1971). However, we have demonstrated that there are several types of keratohyalin granule and that some of these contain sulphydryl groups (Jessen, 1970, 1971, 1973).

This report will present observations on the elemental composition of various types of keratohyalin granule from different tissues. The study was performed by X-ray microanalysis in an EMMA analytical microscope, which allows detection and quantitation of elements in minute volumes of ultrathin sections. Special attention was paid to the detection of sulphur in keratohyalin granules, and an absolute quantitation of the sulphur concentration in the granules was made possible by the use of a suitable sulphur standard. It will be demonstrated that a type of keratohyalin granule, which is of widespread occurrence in squamous epithelia, is particularly rich in sulphur.
METHODS

Tissue preparation

Albino rats, weighing approximately 60 g, were perfused 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. There was no postfixation with osmium. Tissue blocks from interpapillary and papillary areas of the lingual epithelium and from abdominal oesophageal epithelium were dehydrated in graded ethanols and embedded in Araldite.

Sections 80–100 nm thick were mounted on 200-mesh copper grids coated with Formvar and carbon. Sections stained with uranyl acetate and lead citrate were examined in a Philips 300 electron microscope. Unstained sections were analysed in an AEI EMMA-4 analytical electron microscope.

Sulphur standard preparation

A quantitative elemental analysis of structures in ultrathin sections is possible only if appropriate standard sections are available for comparative measurements. Such standard sections should preferably have characteristics similar to the tissue sections and must be of known elemental composition. Standard sections were prepared as follows. The flexibilisator DY 041 (supplied by Ciba-Geigy, Plastic Division, Duxford, Cambridge, England) is a sulphur-based compound which contains 35–45% sulphur. The compound is homogeneously miscible with Araldite and can be added in concentrations up to 10% without adversely affecting the polymerization properties of Araldite. Approximately 2.5, 5, 7.5, and 10% of DY 041 were added to 4 portions of Araldite and the mixtures were thoroughly stirred. Samples of the 4 mixtures of DY 041 and Araldite were analysed by chemical methods to establish the percentage by weight of the elements present. Capsules with the 4 mixtures were polymerized and 80–100 nm sections were cut and mounted as above.

X-ray microanalysis

EMMA-4. Sections were analysed for elemental composition in an AEI EMMA-4 analytical electron microscope, equipped with a Kevex Si(Li) energy dispersive detector, as well as the standard EMMA-4 analytical equipment consisting of 2 diffracting crystal spectrometers. The electron beam is focused to a spot reducible in diameter to about 150 nm, by a minilens. X-rays generated within the irradiated microvolume are transmitted through Al-coated Makrofol windows 2 μm thick, into the spectrometers. X-rays incident on the crystal face at the Bragg angle are diffracted into a gas-flow proportional counter. The electrical output from the counter is recorded on a scaler, which can be set to record the integral count over a preset time.

Quantitation. A peak count \( P \) is recorded for sulphur with the spectrometer set at the Bragg angle. A background count \( b \) is recorded with the spectrometer to specimen distance offset from that corresponding to the Bragg angle by 2.5 mm. The quantity \( b \) is a measure of the proportion of the peak count attributable to extraneous sources of radiation, such as X-rays generated by backscattered electrons incident on the interior of the microscope column. A continuum or 'white' count \( W \) is recorded on the Kevex Si(Li) energy dispersive detector over a band of energies typically 6 keV wide, excluding any peaks due to characteristic radiation. This quantity is approximately proportional to the thickness of the section. Radiation produced in the supporting Formvar film, and by stray electrons, will contribute to the value of \( W \), so a correction is made by subtracting from \( W \) a quantity \( W_b \), recorded over the same energy band, with the electron beam incident on a neighbouring piece of supporting film. The 4 measured quantities are used to determine a ratio \( R \),

\[
R = \frac{P - b}{W - W_b}.
\]

To obtain a quantitative estimate of the local sulphur concentration in an analysed micro-
X-ray microanalysis of keratohyalin granules

volume, the value of $R$ is estimated for the tissue specimen and for a standard specimen of known composition. Quantitation is performed by the method of Hall, Anderson & Appleton (1973), in which the values of $R$ for tissue and standard specimens are linked by quantities relating to the composition of the tissue and of the standard. The relationship between $R$ and elemental concentration is very nearly linear, and the method of Hall et al. (1973) corrects for the small deviation from direct proportionality which occurs when there is a small difference in mean atomic number between the specimen and the standards. Apart from this slight correction, concentrations can be read directly from a calibration curve of the type shown in Fig. 1 (p. 363).

An accelerating voltage of 40 keV was used to obtain the maximum possible contrast in the unstained sections. A beam current of between 30 and 50 nA and counting times of 40 s were selected as a compromise between a low count rate and excessive radiation damage to the sections. All keratohyalin granules were probed with the minimum attainable spot diameter of about 150 nm, in which case the spot was entirely contained within the granules. It was therefore known that the X-rays generated were from the keratohyalin granules, and not from surrounding structures.

RESULTS

X-ray microanalysis was performed on keratohyalin granules from lingual inter- papillary epithelium, epithelium of the lingual filiform papillae, and oesophageal epithelium.

Morphology

**Lingual interpapillary epithelium.** This contains 2 types of keratohyalin granule - single granules (sg) and composite granules (cg) (Fig. 2). The 2 types of granule show morphological and cytochemical differences (Jessen, 1970, 1973). Single granules occur in the nucleus (Fig. 7) as well as in the cytoplasm (Fig. 6) and they appear electron-lucent in glutaraldehyde-fixed specimens. Composite granules occur only in the cytoplasm and contain 2 components. One component is similar to single granules. The other component appears electron-dense in glutaraldehyde-fixed specimens (Fig. 6).

**Oesophageal epithelium.** This is similar to interpapillary lingual epithelium (Fig. 3). Both single and composite keratohyalin granules are present (Fig. 11).

**Lingual filiform papillae.** These contain 2 populations of epithelial cells. A granular layer, containing keratohyalin granules, is present only in the anterior part of the papillae (Fig. 16). The majority of the keratohyalin granules are homogeneous and appear electron-dense in glutaraldehyde-fixed specimens. In the uppermost part of the granular layer, however, an increasing number of single granules occur both in the cytoplasm and nucleus. Some of the single granules are associated with the periphery of the dense granules, thus resembling the composite granules of interpapillary and oesophageal epithelia (Fig. 19).

Elemental spectra

Elemental spectra of the different types of keratohyalin granule were obtained in the EMMA-4. All the spectra were obtained with the Kevex detector preset to record identical 'white' counts over the different structures. This procedure gives a
relative estimation of the concentration of an element in the different types of kerato-
hyalin granule, as well as allowing identification of the elements present.

**Interpapillary lingual epithelium.** Figs. 8 and 9 illustrate spectra obtained from a
cytoplasmic and from an intranuclear single keratohyalin granule. The spectra are
almost identical, both demonstrating a prominent sulphur peak. A silicon peak,
which is of instrumental origin, is also present. The spectrum obtained by analysing
the dense component of a composite granule is seen in Fig. 5. A prominent phosphorus
peak is present. Only a minor sulphur peak is present. A spectrum from the interior
of a cornified cell demonstrates a minor sulphur peak (Fig. 4).

**Oesophageal epithelium.** Fig. 12 shows a spectrum from a cytoplasmic single
keratohyalin granule. A prominent sulphur peak and a minor chlorine peak are
seen. The spectrum from the dense component of a composite granule demonstrates
a prominent phosphorus peak and minor sulphur and chlorine peaks (Fig. 13). A
spectrum from the interior part of a cornified cell is shown for comparison in Fig. 10.
It contains minor sulphur and chlorine peaks.

**Lingual filiform papilla.** Fig. 17 shows a spectrum obtained from a cytoplasmic
single granule. As in the previous 2 types of epithelia a prominent sulphur peak is
observed. In addition, minor chlorine and potassium peaks are also seen. Fig. 18
shows a spectrum obtained by analysing a dense keratohyalin granule. A minor
sulphur peak is noted as well as a chlorine and a potassium peak. A spectrum obtained
over the interior part of a cornified cell adjacent to the granular layer of the papilla
is seen in Fig. 14. Minor sulphur and chlorine peaks are present.

Finally, a spectrum obtained over Araldite is shown in Fig. 15. No peaks are
apparent. (The Araldite presumably contains some chlorine, but not enough to
appear in the counting time employed for the figure and for the spectra obtained from
the tissues.)

The spectra clearly demonstrate that a sulphur-rich material is present in single
keratohyalin granules in all 3 types of tissue. The dense component of composite
keratohyalin granules in interpapillary tongue and oesophageal epithelium is sulphur-
poor, but contains phosphorus. The dense keratohyalin granules of filiform papillae
are poor in both sulphur and phosphorus.

**Quantitation**

A quantitative analysis of the sulphur concentration in keratohyalin was performed
according to the continuum-method of Marshall & Hall (see Hall, 1971). A detailed
presentation of the scheme of data collection has previously been published (Hall et
al. 1973) and is summarized in Methods above.

**Standard specimens.** To allow quantitation of the local concentration of sulphur in
the different types of keratohyalin granules it is necessary to know the value of \( R \)
(see Methods) for a sulphur-containing standard of known composition.

To test the suitability of Araldite/DY 041 mixtures as sulphur standards a series
of measurements was performed on 80- to 100-nm sections of the 4 mixtures of
Araldite/DY 041 with different known sulphur contents. Fig. 1 illustrates the values
of \( R \) obtained for the 4 sets of sections plotted against the sulphur concentration,
known from chemical analysis. There is a linear relationship between the measured values of $R$ and the known sulphur content of the standard sections, with the regression line passing close to the origin. The homogeneity of the standard sections is confirmed by the small coefficient of variation (2–3.5%) with repeated measurements from different areas of the sections.

Fig. 1. Graph illustrating measured values of $R$ (ratio of sulphur peak to continuum) versus known sulphur concentration in sections from the 4 mixtures of Araldite/DY 041 used as calibration standards.

*Tissue specimens.* Analysis was made on unstained, 80- to 100-nm sections of the 3 types of epithelia. Only sections which were placed centrally on grids were used for measurements. Although sections of glutaraldehyde-fixed epithelia showed poor contrast, this was sufficient to centre the probe on selected areas (Fig. 20). Such areas included single keratohyalin granules in all 3 epithelia, the dense component of composite keratohyalin granules in the 3 epithelia, and the dense keratohyalin granules of filiform papillae. A few cornified cells in oesophageal epithelium were also analysed. Only keratohyalin granules of sufficient size to accommodate the probe fully were chosen. Particular attention was paid during analysis of composite kerato-
hyalin granules to ensure that the probe was exclusively situated over their dense component.

After each series of measurements to obtain the values of $R$ for keratohyalin granules, measurements were repeated on one of the sulphur standards to ensure that the data from tissue sections and standard sections were collected under the same instrumental conditions.

**Table 1. Sulphur concentration (%) of keratohyalin granules in the various areas analysed**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Single granules</th>
<th>Dense component of composite granules and dense granules</th>
<th>Cornified cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interpapillary lingual epithelium</td>
<td>$3.7 \pm 0.1$</td>
<td>$0.82 \pm 0.07$</td>
<td>—</td>
</tr>
<tr>
<td>Oesophageal epithelium</td>
<td>$3.8 \pm 0.4$</td>
<td>$1.4 \pm 0.2$</td>
<td>$1.7 \pm 0.2$</td>
</tr>
<tr>
<td>Lingual filiform papillae</td>
<td>$3.6 \pm 0.2$</td>
<td>$0.8 \pm 0.1$</td>
<td>—</td>
</tr>
<tr>
<td>Number of areas analysed</td>
<td>36</td>
<td>42</td>
<td>4</td>
</tr>
</tbody>
</table>

The final calculations of the sulphur concentration in the analysed areas of keratohyalin granules were done according to a method previously presented (Hall *et al.* 1973). The results are presented in Table 1. The highest sulphur concentration was found in single keratohyalin granules, which contained 3.6–3.8% sulphur. Analyses were done on both intranuclear and cytoplasmic single granules as well as single granule material associated with composite granules. No variation in sulphur concentration, related to the situation of single granules, was observed. The dense component of composite granules of lingual and oesophageal epithelia and the dense granules of filiform papillae contained substantially less sulphur, namely 0.8–1.4%. Cornified cells of oesophageal epithelium contained 1.7% sulphur.

**DISCUSSION**

The nature of keratohyalin granules is not well known. Biochemical studies to characterize keratohyalin have given conflicting results. Keratohyalin granules have been reported to contain a histidine-rich protein (Bernstein & Sibrack, 1972), a ribonucleoprotein (Ugel, 1971) and a glycine and glutamic acid-rich protein (Tezuka & Freedberg, 1972). None of these authors found sulphur-amino acids to be a substantial component of keratohyalin. In contrast, Matoltsy & Matoltsy (1970, 1972) have reported high values for half-cystine in isolated keratohyalin granules from newborn rat epidermis.

The conflicting biochemical results may be due to the differences in preparative procedures, but may also reflect heterogeneity of keratohyalin granules from different tissues as well as within individual keratinizing cells. The combination of X-ray microanalysis and electron microscopy offers a valuable method for investigation of heterogeneity of keratohyalin granules by allowing an accurate correlation of chemical and morphological information.
X-ray microanalysis of keratohyalin granules

The present study clearly demonstrates that several types of keratohyalin granules are present in different keratinizing epithelia. The granules differ in sulphur content. The single keratohyalin granules are rich in sulphur. These granules are of widespread occurrence in squamous epithelia and they occur in the nucleus as well as in the cytoplasm of granular cells (Jessen, 1970, 1971, 1973). No differences in sulphur concentration related to the situation of single granules could be observed, confirming the identity of these granules. Single keratohyalin granules have a sulphur content (3.6–3.8%) comparable to the high-sulphur proteins from hard mammalian keratins such as wool and hair. The high-sulphur proteins are thought to be present primarily in the interfilamentous matrix of cortical cells (see Fraser, MacRae & Rogers, 1972). It is interesting that differentiating cells of the hair cuticle contain granules similar to the single keratohyalin granules of squamous epithelia (Jessen, 1972). X-ray microanalysis of cuticular single granules demonstrates a sulphur content of 4.1% (Jessen, Peters & Hall, unpublished observations).

The composite keratohyalin granules of interpapillary and oesophageal epithelia contain a sulphur-rich, single granule component as well as a sulphur-poor component. The sulphur-poor component contains phosphorus, which may possibly relate to the presence of ribonucleoproteins in this component (Ugel, 1971). The dense granules of filiform papillae are poor in both sulphur and phosphorus.

The finding of a high sulphur content in single keratohyalin granules is in good agreement with a recent cytochemical study (Jessen, 1973), which demonstrated that single granules can be stained by silver-methenamine. Control experiments indicated that the stainability could be attributed to the presence of sulphydryl groups in single granules. Our observations are also in agreement with those of Matoltsy & Matoltsy (1970, 1972), that keratohyalin granules may contain a sulphur-rich protein. However, their isolated keratohyalin granules from newborn epidermis appeared to be a homogeneous group. We have evidence that keratohyalin granules are heterogeneous in newborn epidermis. The granules may have a high sulphur content similar to single granules in the present material. But a sulphur-poor component is also present in epidermal keratohyalin granules (Jessen, Peters & Hall, in preparation).

The absolute values calculated for the sulphur content of different keratohyalin granules (3.6–3.8% for single granules and 0.8–1.4% for the sulphur-poor component of composite granules) should be treated with a certain reserve, because of complexities inherent in the analytical method. The quantitative, continuum method for calculation of weight fractions of elements in thin specimens is based on assumptions which are valid in conventional microprobe analysis (see Hall, 1971). The method is based on normalization of the measured intensity of a characteristic radiation against the simultaneously measured continuum radiation in tissue and standard specimens, using the continuum radiation as a measure of the local mass in the analysed microvolumes. In application of the method to ultrathin sections a major concern is the validity of the collected data on the characteristic and continuum radiation. Biological tissue sections are not stable during the period of analysis (Hall & Gupta, 1974) and loss of material from the sections may therefore influence both the characteristic and continuum radiation. Furthermore, the continuum radiation may contain artificial
components originating from a variety of sources (Hall et al. 1973). In spite of these possible sources of error we feel that the method is sufficiently accurate, because of the good correlation between the measured data and the known sulphur concentration of the 4 standard specimens tested.

It should be noted that the calculated figures for the sulphur concentration in keratohyalin granules are minimal, since the data were obtained on embedded material. The figures refer to the analysed microvolumes containing both tissue and embedding material.

The functions of keratohyalin granules are not well known. The present data provide conclusive evidence for a high sulphur concentration in single keratohyalin granules. This implies that the granules are functionally important in the keratinization process. An initial event in the keratinization of squamous epithelial cells is the deposition of a protein layer 15 nm thick along the cytoplasmic face of the plasma membrane. It has previously been suggested that single keratohyalin granules are involved in the deposition of this peripheral envelope (Jessen, 1970, 1973). This suggestion was based on the finding that single granules were preferentially localized along the periphery of granular cells. In addition, single granules and the peripheral envelope of cornified cells showed similarity in several cytochemical respects. Some attempts were made in the present study to elucidate whether differences in sulphur concentration could be demonstrated by X-ray microanalysis between the peripheral and the interior parts of cornified cells. The data indicated a higher sulphur concentration along the periphery of cornified cells. However, since the analysed microvolumes (probe size approximately 150 nm diameter) included more than peripheral envelope, a meaningful calculation of the sulphur percentage in this material could not be made. Other evidence has been presented which indicates a high sulphur content in the peripheral envelope of cornified cells. The presence of sulphhydryl groups in the peripheral envelope, especially in the lowermost cornified cells, has been demonstrated (Jessen, 1973). Matoltsy & Matoltsy (1966) found a high cystine content in isolated plasma membranes of cornified cells. It has also been demonstrated that protein-bound [H]cyst(e)ine accumulates along the plasma membrane of cornified cells (Fukuyama & Epstein, 1969). Since single keratohyalin granules are sulphur-rich, it appears possible that their function is the deposition of the peripheral envelope of cornified cells.

The function of other types of keratohyalin granules (composite granules, dense granules) may be to contribute to the interfilamentous matrix of cornified cells.

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REFERENCES


(Received 26 November 1973)
Fig. 2. Micrograph showing cells from the granular layer of interpapillary epithelium from the rat tongue. Single keratohyalin granules (sg) are seen in both the nucleus and the cytoplasm. Composite keratohyalin granules (eg) are seen in the cytoplasm. Cornified cells (cc) are seen in the upper part of the micrograph. Glutaraldehyde-fixed, stained section. ×6000.

Fig. 3. Micrograph showing cells from the granular layer of oesophageal epithelium from the rat. Single keratohyalin granules (sg) are present in both the nuclei and the cytoplasm. Composite keratohyalin granules (eg) are present in the cytoplasm. Cornified cells (cc) are seen in the upper part of the micrograph. Glutaraldehyde-fixed, stained section. ×6000.
X-ray microanalysis of keratohyalin granules
Fig. 4. Elemental spectrum from analysis of the interior of a cornified cell. Interpapillary tongue epithelium.

Fig. 5. Elemental spectrum from analysis of the electron-dense component of a composite granule. Interpapillary tongue epithelium.

Fig. 6. Micrograph showing the 2 types of keratohyalin granules in interpapillary tongue epithelium. The single granules (sg) show low electron density. The composite granule (eg) is composed of a single granule component and an electron-dense component. cc, cornified cell. Glutaraldehyde-fixed, stained section. × 23,500.

Fig. 7. Micrograph showing an intranuclear single granule (sg) from interpapillary tongue epithelium. Glutaraldehyde-fixed, stained section. × 29,500.

Fig. 8. Elemental spectrum from analysis of a cytoplasmic single granule. Interpapillary tongue epithelium.

Fig. 9. Elemental spectrum from analysis of an intranuclear single granule. Interpapillary tongue epithelium.
X-ray microanalysis of keratohyalin granules
Fig. 10. Elemental spectrum from analysis of the interior of a cornified cell. Oesophageal epithelium.

Fig. 11. Micrograph showing single granules (sg) and a composite granule (eg) in oesophageal epithelium. cc, cornified cell. Glutaraldehyde-fixed, stained section. $\times 38000$.

Fig. 12. Elemental spectrum from analysis of a cytoplasmic single granule. Oesophageal epithelium.

Fig. 13. Elemental spectrum from analysis of the dense component of a composite granule. Oesophageal epithelium.
X-ray microanalysis of keratohyalin granules
Fig. 14. Elemental spectrum from analysis of the interior of a cornified cell adjacent to the granular layer of a filiform papilla.

Fig. 15. Elemental spectrum from analysis of Araldite.

Fig. 16. Micrograph showing a filiform papilla of the rat tongue. Keratohyalin granules are seen in the anterior part of the papilla. cc, cornified cell; dg, dense keratohyalin granules; sg, intranuclear and cytoplasmic single granules. Glutaraldehyde-fixed, stained section. ×4000.
X-ray microanalysis of keratohyalin granules
Fig. 17. Elemental spectrum from analysis of a single granule from a filiform papilla.
Fig. 18. Elemental spectrum from analysis of a dense granule from a filiform papilla.
Fig. 19. Micrograph showing the different types of keratohyalin granules in the filiform papilla of the rat tongue. *cg*, composite granule, consisting of a single granule component and a dense component; *dg*, dense granule; *sg*, single granules. Glutaraldehyde-fixed, stained section. × 20 500.
Fig. 20. Micrograph showing the 3 types of keratohyalin granules in the filiform papilla in an unstained section. *cg*, composite granule; *dg*, dense granule; *sg*, single granule. Contamination rings – deposited during analysis – are seen over the composite and the dense granules (arrows). × 25 000.
X-ray microanalysis of keratohyalin granules