J. Cell Sci. 3, 563-572 (1968)
Printed in Great Britain

X-RAY EMISSION MICROANALYSIS OF PROTEINS AND SULPHUR IN RAT PLANTAR EPIDERMIS

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SUMMARY

The distributions of proteins and protein-bound sulphur have been studied by X-ray emission microanalysis in the plantar epidermis of six albino rats. Plantar skin was fixed in 10% formaldehyde for 24 h and 5-μ paraffin sections obtained. Discrete and continuous measurements of the relative concentrations (mass/unit area) of proteins and sulphur were made along lines of scan perpendicular to the skin surface. The concentrations of proteins and sulphur almost double from the stratum basale across the stratum spinosum. There is a sharp twofold increase in their concentrations across the stratum granulosum to the stratum corneum where they become constant. The sulphur:protein ratio is constant across all layers of the epidermis.

Rough estimates of cell volumes give a ratio of 1:9:7·5 for the cells of the strata basale, granulosum and corneum respectively. Cell volume changes cannot be responsible for the changes in concentration so it is concluded that epidermal cells synthesize and accumulate proteins throughout their migration to the stratum corneum. The observations are regarded as circumstantial evidence for the incorporation of keratohyalin granules into cornified cells.

INTRODUCTION

A histological section of the epidermis cut in a plane perpendicular to the skin surface presents for examination several layers of cells. The deepest layer next to the dermis is the stratum basale where cell division occurs. Superficial to the stratum basale is the stratum spinosum in which the cells are joined by intercellular bridges. The next layer is the stratum granulosum where, as the name implies, the cells contain basophilic granules. These three layers of viable cells are called the Malpighian layers. Above the stratum granulosum are the stratum lucidum and stratum corneum made up of dead cells with no organelles. Cells generated in the stratum basale move through the layers in order and are shed at the surface of the stratum corneum. This means that the progressive changes in a series of cells in the space between stratum basale and the surface of the stratum corneum illustrate the progressive changes experienced by one cell during the time of its migration between the same regions. In the same way the differentiation of cells in the hair cortex is represented in a longitudinal section of an active hair follicle.

An experiment on the incorporation of [^{3}H]tyrosine into the hair of rats observed

by radioautography showed conclusively for the first time that protein accumulates in cortical cells during their keratinization (Sims, 1964). This conclusion was established readily because no loss of labelled proteins was detectable over a period of 36 h from cells actively synthesizing proteins. It was shown that the accumulation of protein is not accompanied by an increase in cell volume so the concentration of proteins in these cells must have increased.

Giroud, Bulliard & Leblond (1934) defined two fundamental types of keratinization which they termed 'soft' and 'hard'. One of the important differences between the two processes is that soft keratinization of cells involves granules of keratohyalin while hard keratinization does not. The cortical cells of hair are formed by hard keratization and this is associated with an increase in concentration of the cell proteins. The purpose of the present investigation is to discover if a similar increase in concentration is associated with soft keratinization in the plantar epidermis of the rat.

It is not possible to infer an increase in the concentration of proteins in epidermal cells from the demonstration of protein synthesis by radioautography because some investigators believe there is appreciable cytolysis in these cells (Jarrett, Spearman & Riley, 1966). A direct method of measuring concentration is required like X-ray emission microanalysis.

In the electron-probe X-ray microanalyser, information is obtained about the local composition of a specimen by the spectroscopic analysis of X-ray emissions which are excited by the impact of an electron beam focused on a spot 1 μ or less in diameter. The method is used primarily for the study of the weight fractions of chemical elements by the detection of their characteristic X-ray emission spectra (Birks, 1963; Hale, 1962). It has been pointed out (Hall, Hale & Switsur, 1966) that in thin sections of soft tissue the intensity of the X-ray continuum-radiation is generally proportional to the local mass per unit area in the section. Hence, in a section of uniform thickness, the variation of the continuum-intensity can give a direct measure of the variation of concentration (total mass per unit volume) along a line of scan.

In thin specimens the intensity of an element's characteristic radiation is known to be proportional to the element's mass per unit area (Sweeney, Seebold & Birks, 1960; Ichinokawa & Yamada, 1963). Hence, the simultaneous observation of the sulphur characteristic intensity and the continuum-intensity along lines of scan provides measurements of the variation of sulphur per unit volume, total mass per unit volume, and the sulphur weight-fraction, the last being the ratio of the other two data. Furthermore, in sections of formalin-fixed, paraffin-embedded skin, deparaffinized in xylol, the mass is virtually entirely protein.

MATERIAL AND METHODS

The main observations were made on material obtained from 6 albino rats that weighed between 100 and 120 g. Samples of plantar skin were fixed in 10 % formaldehyde for 24 h and embedded in paraffin wax in the usual way. Sections were cut perpendicular to the skin surface at a thickness of 5 μ. They were put on nylon film

for electron-probe microanalysis and on glass slides for staining with haematoxylin and eosin, or for treatment with thioglycollate followed by the dihydroxy-dinaphthyl-disulphide reagent (Barrnett & Seligman, 1952) or mercury orange (Bennett, 1951) for –SH groups.

Nylon film was prepared by squirting about 2 ml of a 10% solution of nylon in isobutyl alcohol at 60 °C on to a clean surface of distilled water. The film was removed from the water on a wire frame and dried in a desiccator. One end of a 1-cm diameter piece of aluminium tube was covered with film to make a carrier for a histological section. The ribbon of paraffin sections was flattened on a water bath then cooled and dried on filter paper. The paraffin was removed with xylol and the sections brought into water through descending grades of alcohol. They were transferred in water to the nylon film carriers and dried in a desiccator. While the sections were drying faults in the continuity of the stratum corneum occasionally developed. Measurements showed that this was not accompanied by shrinkage of the section.

Both surfaces of the preparations were coated with aluminium to a total thickness of about 800 Å by vacuum evaporation. The coating is necessary to conduct away from the specimen electrical charge, and heat transformed from the energy of the electron beam.

The preparations were photographed by transmitted light before aluminium coating, and by transmitted and reflected light after aluminium coating. When they were in the microanalyser their electron images on an oscilloscope were photographed before and after data were recorded (Fig. 4). All the pictures obtained were compared to ensure that point-to-point location of the analysis could be made in the layers of the epidermis. Breaks in the continuity of the stratum corneum were valuable guides for this purpose.

Microprobe analyses were carried out with the Cavendish Laboratory's probe, a forerunner of the Cambridge Instrument Company's 'Microscan' model. The probe voltage was near 30 kV and the specimen currents were in the range 0·2–0·5 μA. The sulphur characteristic radiation was detected with a quartz diffracting crystal.

The X-ray signals are proportional to the total and elemental masses per unit area only under certain conditions, which have been outlined by Hall *et al.* (1966). The electrons must pass through the specimen with an average loss of only a small part of their energy. For 30 keV electrons incident normally on to dried, 5-μ sections, the average loss in tissue, nylon support and aluminium-coating is calculated to be less than 2 keV. Self-absorption should be small; in our preparations the reduction in sulphur intensity due to absorption in tissue and aluminium is calculated to be less than 10%. The measurement of the sulphur weight-fraction is almost unaffected by this absorption because the continuum is measured in a band near the sulphur wavelength, so that the two signals are almost equally attenuated. Finally, the average atomic number, $\bar{Z}$, must be uniform, since the continuum-intensity from a thin specimen of mass per unit area, m, is in fact nearly proportional to $\bar{Z} \times m$. $\bar{Z}$ is reasonably uniform in skin, where the major constituents, carbon, nitrogen and oxygen, are present uniformly and the total weight-fraction of elements of higher atomic number may be expected to be less than 5%.

For quantitative work it is necessary to subtract the constant backgrounds generated in the nylon and aluminium, and to subtract from the characteristic count the varying background due to continuum generated in the tissue. Under our conditions approximately one-eighth to one-third of the continuum came from the nylon and the aluminium, and up to one-third of the count from the diffracting crystal was due to continuum. The correction for background from nylon and aluminium is obtained simply by taking readings with the probe stationed on the supporting film away from the tissue. As to the correction for continuum accepted by the diffractor, this background is a constant *fraction* of the signal in the continuum-detector, and the fraction can be determined from any thin calibration spot which contributes only continuum-radiation to both signals. A slight dab of colloidal graphite serves the purpose. With the computer network described elsewhere (Hall *et al.* 1966; Marshall, 1967), after background settings are made with the probe positioned successively on support alone and on the colloidal graphite spot, the background corrections are made automatically during the study of the tissue itself.

While the line scan is the natural procedure for quantitative probe studies of our specimens, scanning images are also useful. Scanning images based on backscattered electrons, like Fig. 4, give a qualitative picture of the specimen. X-ray scanning images are usually based on the output of the X-ray spectrometer, which has been set for the characteristic radiation from some particular element, and the image should then be a map of the distribution of that element. However, one may readily form X-ray scanning images based on the intensity of the X-ray continuum, as in Fig. 5. When the local continuum-intensity is proportional to local total mass per unit area, this image is a map of the distribution of dry mass in the section.

Measurements were made from the stratum basale to the stratum corneum by two methods. First, a moving spot scan was made across the layers perpendicular to the superficial edge of the stratum corneum. Experience showed that an integrating time constant equivalent to about 10 μ on the sections was required to obtain a reasonably smooth curve. The variability of the curves obtained when shorter time constants were used is attributed to differences between intracellular structures; for example, nucleus and tonofibrils, and to statistical fluctuation.

The second method of making measurements was with a stationary spot at selected points on a line perpendicular to the superficial edge of the stratum corneum. This method has the advantage of accurate measurement and none of the variability obtained with moving-spot scans, but it has the disadvantage of haphazard intracellular localization. At present it is not possible to identify the position of cell organelles with this technique. In general the stationary-spot measurements corroborate the results of the moving-spot scans.

There are a number of factors which make the estimation of cell volumes in the epidermis difficult. The cells and their nuclei are not randomly distributed in space because the stratum basale is one cell thick and the cells of the stratum corneum are usually shaped like hexagonal plates. Abercrombie's (1946) formula cannot be used confidently because it assumes a random distribution of nuclei, an important point emphasized by Marrable (1962), who proved it by probability axioms. Other important

factors are that the cells of different layers have different shapes and volumes so there is no uniformity in the standard histological section.

A method was invented that gives rough estimates of changes in cell volumes from linear measurements. The basis of the method was measurement of width and thickness of cells in the sections stained with haematoxylin and eosin. Cell width is the measurement parallel to the superficial surface of the stratum corneum and cell thickness is the measurement at right angles to that surface. The cells were measured in groups of three situated within 150 μ of section, one from each of the stratum basale, deep stratum granulosum and stratum corneum. Those in the stratum basale had the centres of their nuclei in the section, those in the stratum granulosum and stratum corneum were the widest cells within the required distance. The measurements were made with an ocular micrometer and an oil-immersion objective on a Watson Bactil microscope. Calibration of the micrometer showed that one unit measured 0·52 μ.

OBSERVATIONS

Concentration of proteins

The electron images (Fig. 4) show where concentration changes occur in the sections but they cannot be quantified. It is possible to see the position of the dermo-epidermal junction, the boundaries of the stratum corneum, nuclei and nucleoli. Surface irregularities like microtome knife marks are visible on electron images.

Table 1. *Greatest width and thickness (μ) of 10 cells situated in each of 3 layers of the rat plantar epidermis*

Stratum basale		Stratum granulosum		Stratum corneum	
Width	Thickness	Width	Thickness	Width	Thickness
5	15	34	7	35	4·8
6	17	35	7	37	5
10	18	36	7	36	4·5
10	15	32	6	37	5·5
7	15	37	7	33	5·5
7	15	33	6	32	4·5
5	16	30	7	34	5
7	16	32	7	36	4·5
7	18	30	7	38	4·5
7	16	33	7	32	5

The images formed from the X-ray continuum give a better indication of the degree of concentration variation even though the relationship between tissue density and brightness in the image is not linear. Observations by this method (Fig. 5) show that the protein concentration of the Malpighian layers increases from the stratum basale to the stratum granulosum and the density of the stratum corneum is constant. Nuclei are obviously less dense than the cytoplasm of the epidermal cells.

The results of the moving-spot scans are exemplified by that illustrated in Fig. 1. The concentration of proteins almost doubles from the cells of the stratum basale across the stratum spinosum, then it doubles again across the stratum granulosum to a maximum in the contiguous stratum corneum. Across the stratum corneum the concentration of proteins is usually constant. The increase in protein concentration in the tissue from the stratum basale to the stratum corneum is by a mean factor of 4·4 with a standard error of 0·45.

Concentration of sulphur

The results of the moving-spot scans are similar to that illustrated in Fig. 2. The concentration of sulphur almost doubles from the stratum basale across the stratum spinosum. There is a sharp twofold increase in concentration across the stratum granulosum to a maximum in the stratum corneum. Across the stratum corneum the concentration of sulphur is usually constant.

Sulphur:protein ratio

Observations are illustrated in Fig. 3. The sulphur:protein ratio is constant across all the layers of the epidermis.

Cell volumes

The measurements of cell widths and lengths made on histological sections stained with haematoxylin and eosin are presented as Table 1. The widths of the cells in the stratum granulosum and stratum corneum are roughly three times greater than those of the cells in the stratum basale. Cell thickness decreases by half between the stratum basale and stratum granulosum and by about a quarter from the latter to the stratum corneum.

DISCUSSION

Continuous measurements of the concentration (mass per unit volume) of proteins in epidermal cells by a line scan across the cell layers in histological sections have not been performed before. The demonstration of a higher concentration of proteins in the stratum corneum than the Malpighian layers was expected but the extent of the difference was not predictable. The line scans and the stationary spot measurements show conclusively that the concentration of proteins in epidermal cells of rat plantar skin increases twofold from the stratum spinosum across the stratum granulosum to the stratum corneum.

Continuous measurements of the concentration of sulphur in epidermal cells by line scans have not been performed before. Electron emission microanalysis is the only method available for such observations at present. The results presented in this work confirm the impression gained from histochemical techniques of a higher concentration of sulphur in the stratum corneum than in the stratum basale. The line scans and the stationary spot measurements quantitate this impression to a twofold increase of sulphur concentration across the stratum granulosum.

Considered together, the protein and sulphur measurements suggest that the sulphur to protein ratio remains approximately constant from the stratum basale to the stratum corneum. This is confirmed by the estimates of the sulphur-to-protein ratios made directly by X-ray emission microanalysis. The first chemical analysis of sulphur in layers of the epidermis was made by Giroud, Bulliard & Giberton (1929). They found virtually no difference in the sulphur-to-protein ratio of the stratum corneum (0·5 %) and Malpighian layers (0·49 %). They were able to obtain sufficient amounts of the dead and viable cell layers for volumetric analysis from horse burrs. Rudall (1952) obtained a similar result on the stratum corneum (1·13 %) and the Malpighian layers (1·15 %) from the epidermis of the cow's nose. Human skin has been analysed by Van Scott & Flesch (1954). They were unable to isolate pure preparations of each layer so they analysed mixed layers. Their values for the amount of sulphur in proteins of the stratum corneum with Malpighian layers, Malpighian layers with dermis, and dermis alone are 0·345 %, 0·410 % and 0·035 % respectively. It is to be expected that specimens of epidermis of different origins will have different sulphur-to-protein ratios. All the chemical analyses are compatible with the X-ray emission analysis presented here and with the general statement of Giroud & Leblond (1951) that the sulphur content of proteins is not increased during soft keratinization. This is a curious fact when it is considered with the extensive morphological changes that occur.

It is apparent from a superficial examination of a histological section of the epidermis cut in a plane perpendicular to the skin surface that the shapes and sizes of the cells are different in the various layers. The differences in shape make the size changes difficult to estimate. The only figures for the size of cells for all layers that we have found in the recent literature are those of Pinkus (1958). He states that for human skin the widths of cells in the basal, spinous, granular and cornified layers are 6, 10–15, 25 and 30 μ respectively. In the last century Wagner (1844) published ranges for the sizes of human epidermal cells measured in Paris lines. When converted to the metric system Wagner's values are similar to those of Pinkus. Both authors give no indication of how the measurements were made, the number of cells measured or the standard deviations. It is essential to have estimates of the volume changes of cells in rat plantar epidermis to discovery whether the changes in protein concentration across the stratum granulosum result from a change in cell volume or a change in cell content. If it is assumed that all the cells are circular in a plane parallel to the skin surface, then from the figures in Table 1 cell volumes in the stratum basale, deep stratum granulosum and stratum corneum are of the order of 600, 5900 and 4600 μ^3. It can be stated with confidence that there is a gross increase in cell volume from the stratum basale to the deep aspect of the stratum granulosum. Since the concentration of proteins in those cells also increases, the granulosum cells have accumulated proteins during their migration from the basale layer. There is some reduction in volume between the cells in the stratum granulosum and those in the stratum corneum but it is not sufficient to give a twofold increase in the concentration of cell proteins. Therefore, proteins are synthesized and accumulate in cells as they migrate through the stratum granulosum. Although there are no estimates of the rates

of protein synthesis and lysis in the cells of the Malpighian layers it is possible to state that the rate of synthesis is greater than the rate of lysis.

The synthesis and accumulation of proteins in the cells of the stratum granulosum could be circumstantial evidence on the nature of the so-called keratohyalin granules characteristic of these cells. Observations with optical and electron microscopy both show that the size and number of keratohyalin granules in the cells increase as the stratum corneum is approached. Occasional transition cells that appear to be full of keratohyalin can be found against the stratum corneum. This distribution and the similar appearance of keratohyalin and the contents of cornified cells seen by electron microscopy led Brody (1960) and others to support the hypothesis that keratohyalin is incorporated into the cornified cells. The observations presented here are compatible with this hypothesis. The accumulation of keratohyalin could be associated with the increase in the concentration of proteins in the cells. Other investigators dismiss the electron-microscopy results as artefacts and on the basis of the histochemical properties of the granular cells support the hypothesis that keratohyalin granules are partially lysed cellular components (Jarrett *et al.* 1966). While the demonstration of an increase in total cell mass does not exclude the possibility of concomitant cytolysis it is unlikely that the occurrence of cells apparently full of areas of cytoplasmic lysis can exist with a twofold increase in cell density. It is known that the cell organelles are destroyed during cornification, so loss of other material should be minimal.

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(*Received* 10 *January* 1968)

Figs. 1–3. Scans across the epidermis which show the concentration of proteins, concentration of sulphur and the sulphur:protein ratio respectively. There is a fault in the section at the superficial limit of the stratum granulosum which is identified by the incisura on the trace. The line above the trace is 100 μ long. The stained section below the trace shows its localization.

Figs. 4, 5. Images of the same section recorded from electrons and continuum X-rays respectively. The electron image shows nuclei and nucleoli in the viable layers of the epidermis and knife marks on the stratum corneum. The X-ray image shows the gradual increase in protein concentration from the stratum basale to the stratum corneum where it is constant. The light areas correspond to the position of nuclei and the dark areas at the top and bottom of the picture are the stratum corneum and dermis respectively. $\times 550$.

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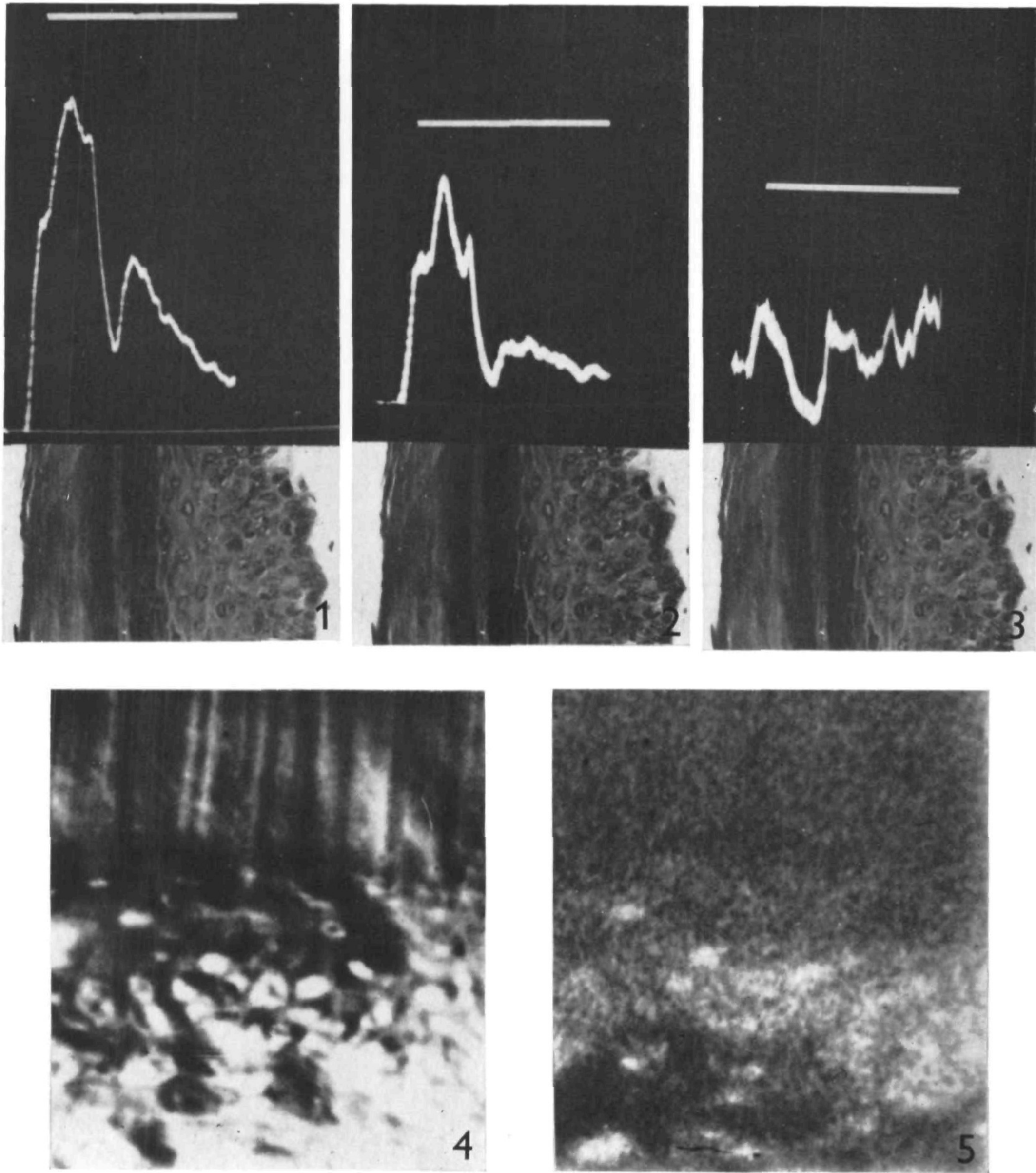

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