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ELECTRON MICROSCOPIC AND AUTORADIOGRAPHIC STUDY OF S^{35}-L-CYSTINE INCORPORATION IN MOUSE HAIR FOLLICLES

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Abstract. After intraperitoneal injection of a single dose, the incorporation of S^{35}-L-cystine in growing hair follicles was studied by means of autoradiography at the electron microscopic level. The experiments reveal a high tracer uptake associated with keratin filament formation. The results obtained are discussed in relation to the present knowledge of the sulphur incorporation process in hair follicles, and some critical viewpoints on the precision of tracer localization in sections for autoradiography at the electron microscopic level are set forth.

The importance of the disulphide bonds of the amino acid cystine in connection with the keratinization process is documented in the literature on keratinization. A review of the present knowledge of the chemistry of keratins was written by Crewther et al. (5) and the molecular structure and arrangement of the keratin macromolecules have been thoroughly dealt with by Lundgren & Ward (14, 15).

At the light microscopic level a number of investigations revealing the distribution of different amino acids and inorganic compounds marked with radioactive elements have advanced our knowledge of the keratinization process (12, 20). Because of its importance the metabolism and distribution of cystine has been the focus of interest for a considerable time (6, 7, 8). It has been shown that the time taken in sheep for labelled cystine (S^{35}-L-cystine) to diffuse into the hair follicles from the blood shortly after an intravasal injection is of the order of a few seconds and that appreciable signs of radioactivity begin to appear in the so-called prekeratinization region about 2 hours after such an injection (20). On the basis of autoradiographic experiments it is now generally believed that the bulk of sulphur is bound to the amino acid cystine which enters the hair follicle in what has been called the region of filament formation (the keratogenous zone) (8, 12, 20) rather than through the bulb. At the electron microscopic level the distribution of sulphur has previously been studied by autoradiography on longitudinal sections of mouse hairs (17) as well as by histochemical means (22). The distribution of sulphur and dry mass has also been studied in human hairs at optical resolution using quantitative microradiography (10).

The object of the present investigation was to demonstrate the localization of sulphur in cross sections at different levels of the hair follicle in mice and to elucidate the dynamics of the cystine incorporation into the keratin of hair fibres when administered as the amino acid S^{35}-L-cystine.

METHODS AND MATERIALS

Six-day-old white mice (NMRI) received 15 μCi of S^{35}-L-cystine (Schwartz Bio-Research Inc.) intraperitoneally. This dose roughly corresponds to 3 μCi gram/body weight. The animals were sacrificed by a lethal dose of ether 1, 3, 6, and 22 hours after injection of the tracer compound. Small pieces of the middorsal skin were taken for subsequent electron microscopy. Osmium tetroxide (1%) buffered to pH 7.3 according to Palade (18) was used as fixative and the fixation time was 30 min. The specimens were dehydrated in rising concentrations of ethyl alcohol and embedded in Epon according to Luft (13). Sections were cut on an LKB Ultrotome set for a section thickness of 1 000 Å. The sections were transferred to molybdenum grids (100 mesh Athenum) and stained in 1% uranyl acetate solution at 60°C for 30–60 min. Subsequently the grids carrying specimens were rinsed in doubly distilled water for 1–2 min and covered with a 60 Å carbon layer, to protect any latent image formed in the emulsion (cf. below).

Autoradiographic technique

Ilford Nuclear Research L4 emulsion was used for the autoradiographic experiments. The technique of forming

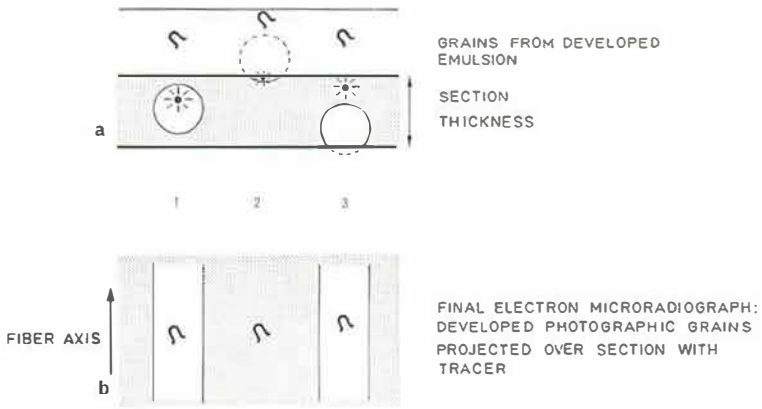

Fig. 1. Sources of error in tracer localization by electron microscopy. (*a*) Schematic section through autoradiographic sandwich normal to grid plane. (*b*) Representation of final image in the electron microscope.

a suitable monolayer of silver halide grains to be positioned on top of the specimen section followed in detail the description given by Salpeter & Bachmann (1, 21). The gelling film was formed in a copper wire loop of 40 mm diameter and transferred to the grids mounted on plastic pegs for ease of operation (16). Plain copper grids with a carbon support film were coated with emulsion chosen by the criteria given by Salpeter & Bachmann (21) and examined in the electron microscope parallel to the experimental procedure. The emulsion film was checked by means of the Chalkley method (4) to contain a monolayer of silver halide particles with such a density that approximately 40–50% of the surface was covered by grains. In order to make sure that the properties of the gelling emulsion did not change during the procedure, a number of specimen-free molybdenum grids were similarly covered with emulsion and stored under identical conditions. The final accepted background registered on the grids contained no more than 2 grains in 1 000 μm^2.

In order to ascertain that the background of developed grains was not due to radiation from uranium isotopes (i.e. U-234 $T\frac{1}{2}$: 2.48×10^5 years; U-235 $T\frac{1}{2}$: 7.13×10^8 years; U-238 $T\frac{1}{2}$: 4.51×10^9 years) included in the uranyl acetate used for contrast staining, bacterial flagella known to be of protein origin were negatively contrasted in a heavy background of uranyl acetate on carbon-coated molybdenum grids. These specimens were covered with emulsion and stored under conditions identical to those of the hair sections and for an identical exposure time. A grain count no higher than the permitted background was observed in these specimens after 2 months of exposure. Thus there was no recorded contribution to the background from the uranyl acetate. Furthermore, the uranyl acetate concentration in these negatively contrasted grids with bacterial flagella was considerably higher than that of the keratin studies.

During the exposure to the β-radiation from the S^{35}-isotope the emulsion-covered molybdenum grids were kept at 4°C in sealed tin cans over dehydrant agent (silica gel) for 2 months. The emulsion was developed in Kodak Microdole X for 3 min followed by an acid stop bath (1% acetic acid) for 10 sec and then fixed for 5 min followed by three rinses in doubly distilled water for 5 min each.

Electron microscopy

The electron microscopy was performed at 50 kV on a Zeiss EM 9 at an instrumental magnification of × 1 700.

Quantitative measurements

Grain counting was performed on the negatives in a low powered light microscope. The determination of the relative amount of keratin in the cytoplasm of the cross section was performed by means of the Chalkley principle (4) on prints enlarged × 4 as described by the author (9) elsewhere.

RESULTS

For different hair fibres the depth in dermis of cross sections containing a defined amount of keratin differs in the same area of the skin. This fact complicates the localization of a specific cross section level in any given follicle. Technical difficulties during serial sectioning for electron microscopy meant that it was not possible to identify a specific hair fibre in different cross sections. Therefore the relative amount of keratin in the cytoplasm of the cross section was chosen as a substitute for a precise level determination which allows the estimation of the tracer (S^{35}-L-cystine) incorporation at different levels of the hair fibre development (cf. 9). The data is con-

Table I. *Grain counts over cortex cells in cross sections of mice hair after intraperitoneal injection of S^{35}-L-cystine*

Time of sacrifice after injection	Ratio[a]	Cortex				Cuticles							Comments
		Total of grains	Nucleus	Fibrils	Cyto-plasm	of Cortex	of IRS	Huxley layer	Henle layer	ERS	Medulla		
	0.11	7		1	6	1		5		2			
	0.17	5		3	2								
	0.26	4		1	3	2			2	1			
	0.28	3		1	2			1	1	2			
1 hour	0.30	3		1	2	1	2	4	3	4			
	0.34	3			3			6	2	6			
	0.42	5		1	4			2	1	2			
	0.23	6	1	1	3		2				1		
	0.24	8	2	3	3	2		2	1	1	2		
3 hours	0.25	3		1	2		2	3		4			
	0.35	3		2	1					1			
	0.41	4		3	1	1		1		5			
	0.42	3	1	1	1		1	1		2			
	0.50	3		1	2								
	0.51	9		4	5		1	1	1	3			[b]
	0.51	11		4	7	1		2		2			
	0.71	3		3		1		1		1			
	0.12	1			1	1		1	1	1			
	0.15					1		1					
	0.16	9		3	6	3	3	4	5	6			
	0.27	7		2	4	3	2			4			
6 hours	0.27	5		3	2	3	3	3	2	1			[c]
	0.27	9	2	6	1	3	2	3	1	2			
	0.42	13		7	5	1			1	1	1		
	0.43	17		8	8	2	1		2	2			
	0.44	11		6	5	1			2	2			
	0.48	8		6	2	4		1	3		3		
	0.50	21		14	7	7	3	1		4	6		
	0.54	5		3	2	2	4	3					
	—[d]												[e]
	—[d]												
	—[d]												
	—[d]												
	0.1												
	0.1								8	1			
22 hours	0.1	3	2		1		2	1		2			
	0.11									1			
	0.25	2		2			1		2	4			
	0.35												
	0.68												
	0.89												
	0.92	3	2		1			3					
	1.00												

[a] Keratin/keratin + cytoplasm ratio.
[b] Two of a series of sections at same level.
[c] Series of section at same level.
[d] These levels are low down in the bulb under the top level of the dermal papilla where matrix cell differentiation has not begun or is initiated.
[e] Nuclei 13, Cytoplasm 8, Plasma membrane 1.

sequently presented according to this principle (Table I). In the present experiments the grain counts indicating a registered amount of radioactive tracer were recorded in animals sacrificed 1, 3, 6, and 22 hours after the intraperitoneal injection of S^{35}-L-cystine.

At early stages of keratinization, i.e. few filaments in the cytoplasm and hence a very low

Chalkley ratio in the cross section, the tracer grains did not appear over or in the vicinity of the keratin filaments in a systematic way at the resolution used in the present investigation. When the number of grains recorded was related to the relative amount of keratin present in the cross section, the overall uptake of tracer appeared to increase as a function of the time passed between the injection of the radioactive compound and the time of sacrifice. During the first hour the majority of grains indicating presence of tracer began to appear in the cells of the root sheaths (Table 1). In the 3 and 6 hour experiments this balance was shifted, and the majority of the grains were then found in the cortex cells. With a 22 hour period between injection and sacrifice very few grains were found over cortex cells at levels of active protein synthesis.

In longitudinal sections the first few keratin filaments were observed in the cytoplasm of the cortex cells roughly 80 to 100 μm from the proximal end of the bulb. However, grains were found in the hair fibre cells at all levels. In the matrix cells of the bulb a conspicuous grain site was located over the large nuclei. The quotient of grains found over nuclei to grains found over cytoplasm was > 1.2 whereas the quotient of nuclear area to cytoplasm area was roughly 0.7 as determined by the Chalkley method. Below a level roughly 40 μm from the proximal end of the bulb there are few characteristics of the cells which made it possible to identify cortex cells in the sections.

In all the present experiments very few grains were found in the medullary cells and grains were effectively not found over trichohyalin granules of the medullary cells or of the inner root sheath cells. The Henley layer cells were observed to be fully consolidated at roughly 100 μm from the bottom of the hair follicle.

DISCUSSION

The resolution obtained in the present autoradiographic experiments at the electron microscopic level is of the order 100–200 nm (1, 3, 11) provided the section thickness is roughly 100 nm and the emulsion thickness is of the same magnitude as is the diameter of the average silver halide crystal, i.e. roughly 100 nm. The diameter of a keratin filament bundle (or fibril) is of the same order of magnitude or even less at low levels in the hair follicle. This means that a precise localization of tracer *within* individual keratin fibril is not feasible with the present autoradiographic techniques.

Some sources of error must be avoided when autoradiography at the electron microscopic level is used for studying the distribution of a radioactive compound in particles or oriented fibres which have a diameter smaller or roughly equal to the thickness of the section. To demonstrate the principles involved, a case with only one cell type in the section is described. This cell type contains uniform particles or fibrils oriented in the plane of the section representing cell constituents which are targets for the tracer compound. Fig. 1 demonstrates two of the possible clear-cut sources of error in tracer localization. Case 1 represents the correct relation between the developed photographic grain and the source of radioactivity. The tracer is incorporated in the cell particle or fibril (Fig. 1 a_1) and the corresponding grain is found over this cell component in the electron autoradiograph that is produced (Fig. 1 b_1). In case 2 the tracer is incorporated in a superficial part of a cell particle or fibril which is only just included in the section (Fig. 1 a_2). Since the thickness of the part of this particle that is included in the section is small, compared with that of the cell content forming the background, its contrast becomes negligible in comparison with the background. Hence, in the autoradiograph the developed grain appears to be located over the background (Fig. 1 b_2). In case 3 the tracer is located in the cytoplasm. A particle or fibril with a coinciding vertical projection is sectioned to a thickness which gives good contrast in the electron micrograph although the particle or fibril is not completely included in the section (Fig. 1 a_3). Consequently the developed grain may be attributed to a radiation source in the particle or fibril (Fig. 1 b_3). This type of localization error is avoided when sections perpendicular to the fibre axis are used for the study of biological fibrous materials with autoradiography at the electron microscopic level.

Using the criterion for the localization of the radiation source as indicated by the smallest possible circle circumscribing the grain (21) the grains attributed to the cytoplasm in cross sections of cortex cells were seen to predominate over those

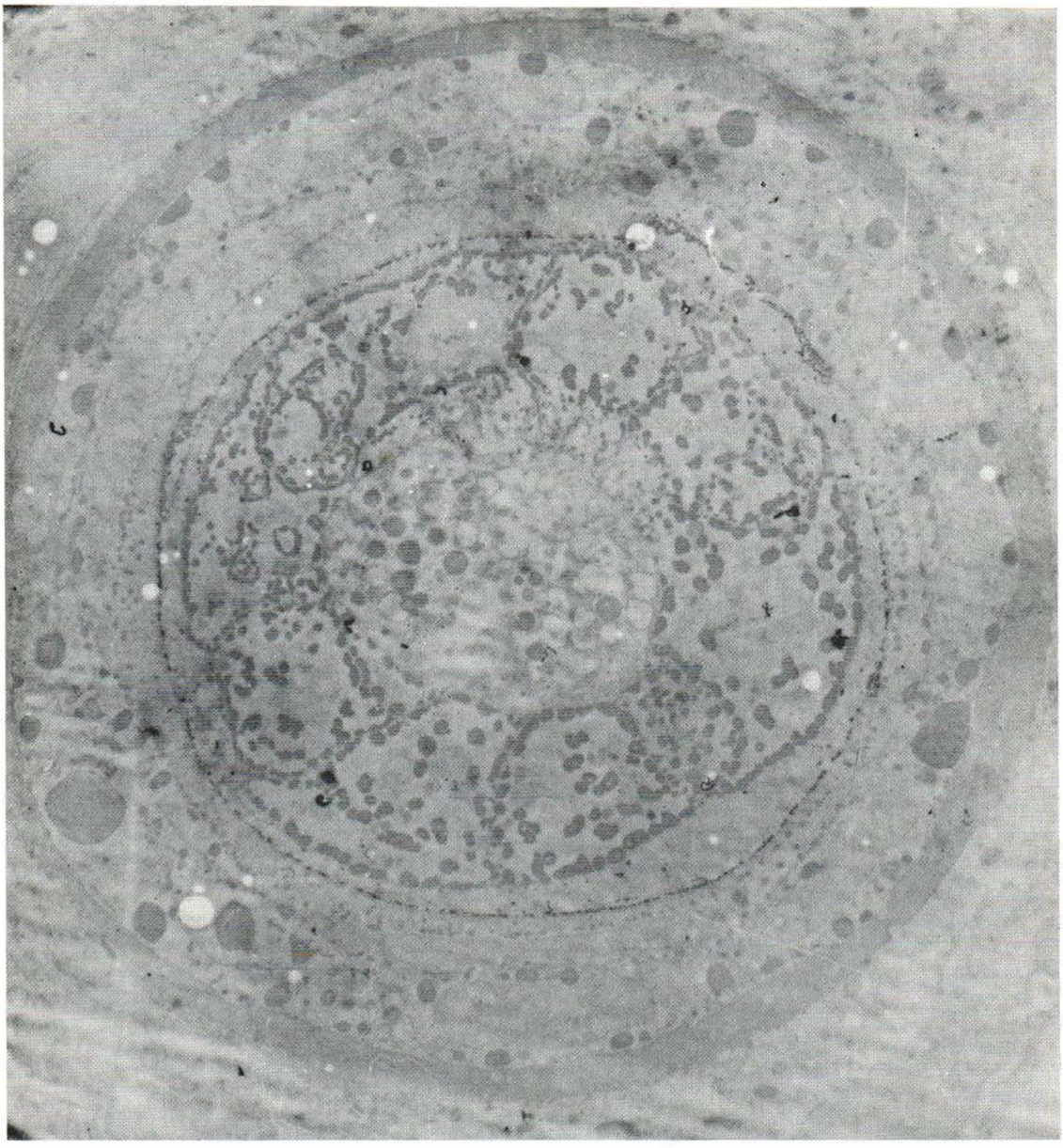

Fig. 2. Cross section of a mouse hair follicle 3 hours after intraperitoneal injection of S^{35}-L-cystine (150 μCi/ml). The sites of the radioactive isotope incorporation are indicated by filamentous silver grains superposed on section. Primary magnification, × 1 700; total magnification, × 5 800.

attributed to the keratin material up to 6 hours after the intraperitoneal injection of the radioactive compound (Table I). Considering the fact that the grain count numbers were too small for statistical analysis, the total amount of tracer recorded over the cortex cells appeared to increase with time up to the 6 hour period after the injection of tracer. In the 22 hour experiment little evidence of radioactive events was recorded over cortex cells expected to be in a phase of active protein synthesis. The results suggest a close correlation to previous histochemical results (2, 20) and to whole body autoradiographic experiments (8). When the cells are in a phase of active pro-

tein synthesis the incorporation of cystine is expected to be related to the amount of keratin produced. Hence, with higher keratin content in the cross section higher grain counts are expected. The present results indicate that this expected relation does in fact occur (Table I).

Histochemical investigations (2) have shown that trichohyalin gives no thiol reaction. The Henle layer is thiol-positive with Bennett's reagent at lower regions than the Huxley layer, both of which are negative at high levels (20). In electron micrographs of longitudinal sections, the Henle layer is seen to be consolidated in the suprabulbar region whereas the Huxley layer is consolidated in the fibrillar region or even in the keratogenous zone. The grain counts over the internal and external root sheaths were subject to fluctuations as can be expected for cellular systems through which a tracer compound is transported without any appreciable accumulation due to a specific uptake of the compound in question. This concept is supported by the findings that the internal root sheath contains practically no cystine (19). The cells of the external root sheath do not undergo keratinization and are therefore not expected to accumulate any cystine.

In an autoradiographic experiment of S^{35}-L-cystine incorporation in mouse hair follicles at the electron microscopic level (17) it is believed that the amorphous keratin granules in the cuticula cells of the cortex contain significant amounts of tracer. This result must, however, be seen in relation to the problem of tracer location in longitudinal sections as discussed above. Until experiments have been performed that cover the incorporation sites of S^{35}-L-cystine at fully mature levels, i.e. in the consolidated hair after an experimental period of more than 24 hours, the possibility cannot be ruled out that high grain counts over the cuticula cells are recorded only during a transient phase of sulphur incorporation. When grain counts are given unrelated to the level in the hair follicle or to some equivalent measure such as the amount of keratin in the cross section, it is difficult to evaluate the importance of such data.

The increase in electron density observed in osmium-fixed sections of the internal root sheath cells due to coalescence of the trichohyalin granules has been interpreted as a sign of hardening of these cells. The present investigation showed that the Henle cell layer appeared to be permeable to the amino acid cystine when completely hyalinized. Therefore, although this cell layer may be physically hard, it still appeared to be permeable to amino acids.

The results of the present investigation are also related to the question of amino acid transportation through the cells of the external and internal root sheaths and the cuticles. It seems unlikely from the present data that the transportation through the Henle layer cells is an active process, since the cells appear hyalinized and no cytoplasmic organelles are seen. The relative accumulation of developed grains over the cuticle of the cortex (Table I) may be due either to a real high cystine incorporation or to a transportation mechanism operating in these cells. Autoradiographic studies of fully keratinized hair fibres taken 24 hours or more after the injection of label might provide an answer to this question. Whole body autoradiography (8) indicates that some of the incorporated tracer has then moved with the growing hair fibre up to the level of the skin surface where the fibre is fully developed. The few grains observed over the medullary cells might be related to the small quantities of fibrous keratin produced by these cells. The lack of tracer grains over trichohyalin in the medullary cells conforms to previous histochemical and chemical results (20, 22).

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