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MICRORADIOGRAPHIC AND AUTORADIOGRAPHIC STUDIES OF KERATIN FORMATION IN HUMAN HAIR

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Abstract. Quantitative microradiographic determinations of dry weight and sulphur content and autoradiographic investigations of S^{35}-L-cystine incorporation have been performed on human hair fibres. The main synthesis of the fibrous protein, keratin, and sulphur incorporation was found to take place in a region from about 200 μm to approximately 700 μm from the bottom of the follicle. This region has been shown to contain morphological entities indicating protein synthesis. The sulphur content of the cortex cells reached a value of roughly 5% at the upper border of the region of protein synthesis.

Keratin is an intracellular protein that constitutes the main part of human hair. It is synthesized and precipitated within a cortex cell while this is passing upwards in the growing hair follicle. Different stages of the keratin formation process are therefore represented in different levels of the hair follicle (12).

Because keratin is a rather insoluble protein, conventional biochemical studies are difficult to perform. However, keratin can be separated into various components having different chemical and physical properties (6, 7, 19, 20).

The role of sulphur and sulphur-rich keratin fractions has been discussed extensively (6, 7, 9, 10, 11, 14, 19, 20). In the hair, sulphur occurs mainly in the amino acid cystine and is probably incorporated in keratin in this form. The bulk of cystine is thought to be incorporated in an amorphous matrix of low molecular weight polypeptides surrounding the keratin filaments (2). This matrix is most conspicuous in electron micrographs of cross sections of fibres. Autoradiographic investigations with S^{35}-L-cystine at light microscopic resolution have shown that the tracer is incorporated into hair follicles mainly in a zone above the bulb of the hair root corresponding to a level where the keratin filaments form fibrils (8, 9, 10, 14, 23).

Investigations of cystine incorporation, sulphur content, and dry mass at different microscopic resolutions in the follicle may therefore give information about the sequential steps in the keratinization process.

Quantitative chemical analysis of particular regions in developing hair follicles is not feasible with conventional biochemical techniques. However, X-ray microradiography allows quantitative measurement, at a cellular level, of dry mass, as well as of the content of some specific elements in thin tissue sections. The present work is an investigation of sulphur incorporation in keratin fibres by means of microradiography and autoradiographic techniques at electron microscopic resolution (1, 3, 4, 13, 24).

MATERIAL AND METHODS

Material. For the microradiographic investigation, small biopsies of skin were collected from the scalp of adult males with no signs of skin disease. For the autoradiographic experiments, hairs were plucked from adult persons who similary showed no evidence of skin disorder.

Microradiographic procedures. The biopsies were fixed in formalin for 12 hours, dehydrated and embedded in paraffin according to standard histological routine. The tissue was sectioned parallel to the axis of the hair follicle in order to obtain longitudinal sections covering the protein synthesizing part of the follicle. The thickness of the sections was approximately 25 μm. Prior to X-ray exposure the sections were deparaffinized in xylene.

In a second series of experiments, sections were obtained with the freeze-sectioning technique previously described, so as to avoid fixation artefacts as far as possible (22).

For the determination of the total dry weight the

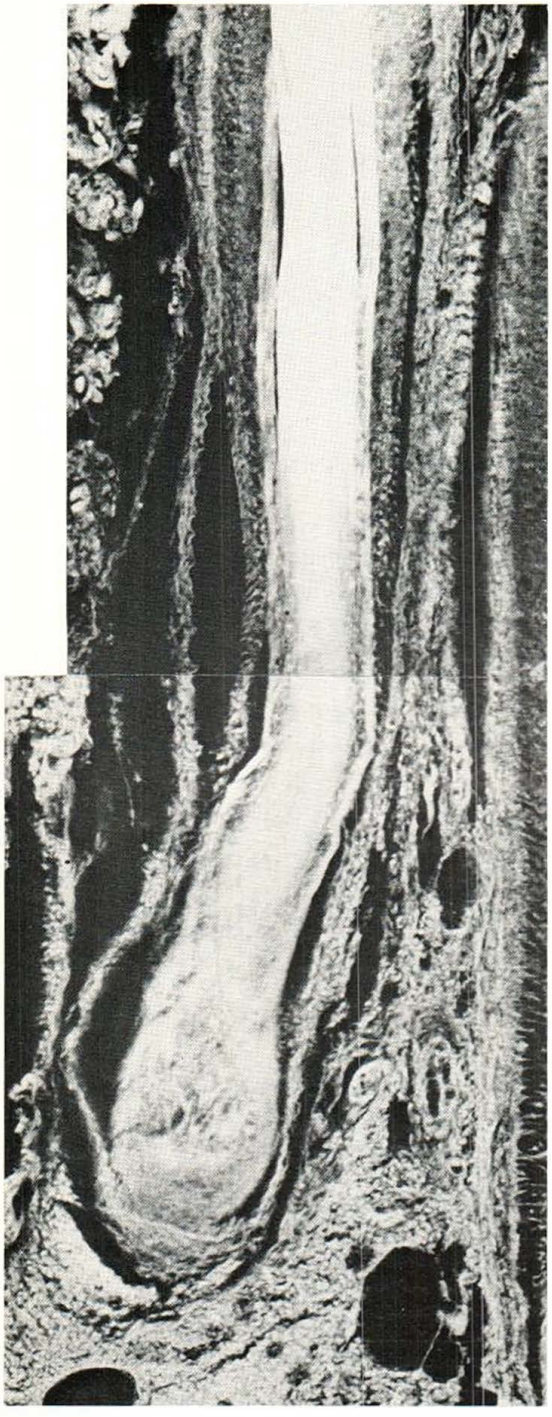


Fig. 1. Microradiograph of a human hair follicle. Paraffin section. Light areas indicate high absorption due to high mass per unit area. × 170.

specimen and the reference system, a stepwedge of Mylar® foils, are simultaneously exposed to continuous X-rays generated at less than 3 kV, corresponding to a wavelength range from 5 to 20 Å. The absorption images of the object and the reference system reflect the distribution of dry mass which can be quantitatively evaluated at the cellular level by photometric densitometry (15, 16, 17). Element analysis by microradiography is performed by exposing the specimen to strictly monochromatic X-rays. Two microradiographs of the specimen are recorded at different wavelengths chosen to lie on each side of an absorption edge characteristic of the element to be evaluated. The amount per unit area of the specific element is obtained from the quantitative measurements of the absorption difference in the two micrographs recorded. The percentage by weight of the specific element—in the present investigation, sulphur—can be determined in situ at the cellular level by combining the data of total dry weight and elementary analysis (15).

Autoradiographic procedures. For autoradiography, the plucked hairs were examined to ensure that at least the midbulb portion was included in anagen follicles. The hairs were then incubated for 20, 40, and 60 min respectively at 32°C (approximate skin temperature) in a physiological salt solution containing S^{35}-L-cystine with an activity of 150 μCi/ml. Autoradiography was performed both at light and electron microscopic resolution.

Light microscopy. The incubated hair fibres were rinsed in a physiological salt solution for a period equal to that of the incubation. The hair fibres were then air-dried, mounted on Scotch Tape which was subsequently pressed onto Ilford K4 plates and stored over a dehydrant (silica gel) during the exposure for 5, 7, and 9 days respectively. The photographic plates were then submerged in doubly distilled water for 2 min to allow swelling of the gelatin, developed in Kodak D-19B for 2 min, fixed for 8 min, and rinsed in running tap water for 30 min.

Electron microscopy. The incubated hair fibres were fixed for 30 min in 1% osmium tetroxide solution buffered to pH 7.3 (21), rinsed, dehydrated and embedded in Epon according to standard procedure (18). Sections of 100 nm thickness were cut on an LKB Ultrotome. They were transferred to molybdenum grids and covered with a carbon support film. Subsequently, a carbon film approximately 60 Å thick was deposited on the specimens in order to protect the latent image (1). The grids were then covered with a monolayer of grains of the highly viscous photographic emulsion, Ilford Nuclear Research Emulsion L4 (1, 24). The emulsion thickness was controlled according to previously described techniques (11). The emulsion-coated grids were stored over silica gel for 2 months at +4°C in sealed tin cans. The emulsion was developed in Kodak Microdol X for 3 min, transferred to an acid stop bath for 10 sec, fixed for 5 min, and rinsed in three changes of doubly distilled water for 5 min each. Electron microscopy was performed at 50 kV in a Zeiss EM 9 instrument at a primary magnification of × 1 700. Survey pictures at × 600 primary magnification were registered in an Akashi Tronoscope operated at 50 kV.

Evaluation of amount of keratin per unit area of cytoplasm, and grain counting. The Chalkley method for eva-

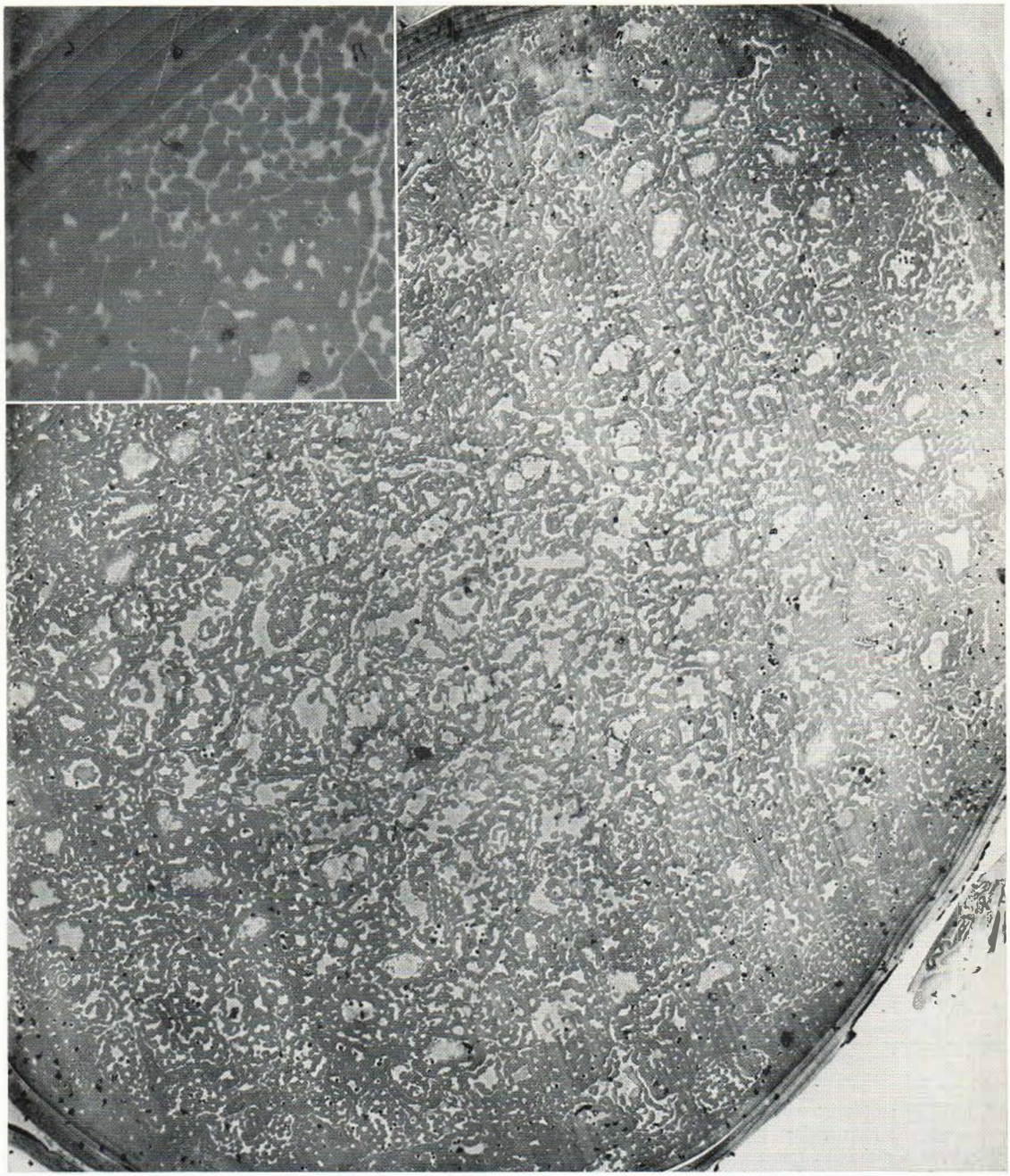


Fig. 2. Electron micrograph of a cross-sectioned hair fibre incubated with S^{35}-L-cystine for 40 min. Position of the incorporated tracer is indicated by the thread-like silver grain filaments. Nuclear remains are seen in cortex cells packed with fibrous keratin. × 1 900. Inset, × 5 800.

luation of the relative amount per unit area of a defined cell component (5) was used for the determination of the keratin content in cross sections of cortex cell (12). The Chalkley method is valid only if there is an homogeneous distribution of the cell content within the tissue under investigation (5). Since the keratin is not uniformly distributed over the entire cross section of human hair fibres (Fig. 1) two different zones with approximately homogeneous keratin distribution were studied separately. Thus a central and a peripheral zone, constituting the border area

Table I. *Cumulated data of Chalkley measurements and grain counts in plucked human hair incubated in S^{35}-L-cystine*

K_b, keratin in border zone. C_b, cytoplasm in border zone. K_c, keratin in central zone. C_c cytoplasm in central zone. Column marked (1) represents hits on keratin material; (2) hits on cytoplasm; (3) hits on nuclei and other prominent cell organelles; (4) ratio between hits on keratin and hits on keratin + hits on cytoplasm; (5) grain count over keratin material; (6) grain count over cytoplasm; (7) grain count over nuclei.

		Peripheral zone						
		Chalkley measurements				Grain counts		
Incubation time	Level	Keratin (1)	Cyto-plasm (2)	Nuclei (3)	$\frac{K_b}{K_b+C_b}$ (4)	Keratin (5)	Cyto-plasm (6)	Nuclei (7)
20 min	3	507	1 872	171	0.213	4	6	—
		545	1 766	189	0.234	23	45	—
20 min	1	572	814	114	0.413	25	13	—
		551	830	119	0.399	17	13	—
	2	649	782	69	0.454	40	18	—
		519	662	69	0.439	25	15	—
	3	530	661	59	0.445	16	14	1
		521	655	74	0.443	9	7	—
40 min	1	582	383	35	0.603	295	82	—
		536	414	50	0.564	163	36	—
	2	559	425	16	0.568	192	94	2
		573	386	41	0.598	185	50	5
	3	620	363	17	0.631	102	34	1
		596	381	23	0.610	124	42	2
40 min	1	625	356	19	0.637	301	61	—
		622	365	13	0.630	204	49	1
	2	646	349	5	0.649	387	85	—
		670	320	10	0.677	250	48	—
	3	518	224	8	0.698	128	23	—
		509	231	10	0.688	34	23	—
60 min	2	529	1 352	119	0.281	94	121	16
		497	1 356	147	0.268	85	133	15
	3	543	1 331	126	0.290	55	65	8
		547	1 068	135	0.339	79	57	11
60 min	1	668	294	38	0.694	32	7	—
		714	251	35	0.740	38	13	—
	2	631	107	12	0.855	11	—	—
		630	106	14	0.856	7	—	—
	3	654	91	5	0.878	3	—	—
		636	105	9	0.858	—	—	—

of the fibre cortex immediately inside the cuticula, were explored. The intermediate zone is equivalent to the region used for the determination of the keratin content at different levels of the hair follicle (12).

The number of silver grains over the keratin and over other cell constituents was recorded in those zones where the relative keratin content was determined. A developed photographic grain was considered to have its radiation source at the centre of the smallest possible circumscribed circle (24). When the "radiation source" thus defined falls on a keratin fibril, the grain is attributed to a radioactive tracer within the keratin.

RESULTS

Microradiography. The microradiography of a hair follicle reflects the dry weight distribution, i.e. light areas in the micrograph have a higher mass than darker ones. When the dry weight was plotted as a function of the distance from the bottom of the follicle the distribution of points was approximately the same for the two preparation methods. At the bottom of the follicles the sulphur content was low, but increased mainly in a region between 300 to 600 μm from the bot-

Table II. *Cumulated data of Chalkley measurements and grain counts in plucked human hair incubated in* S^{35}-L-*cystine*

Explanations of symbols see Table I

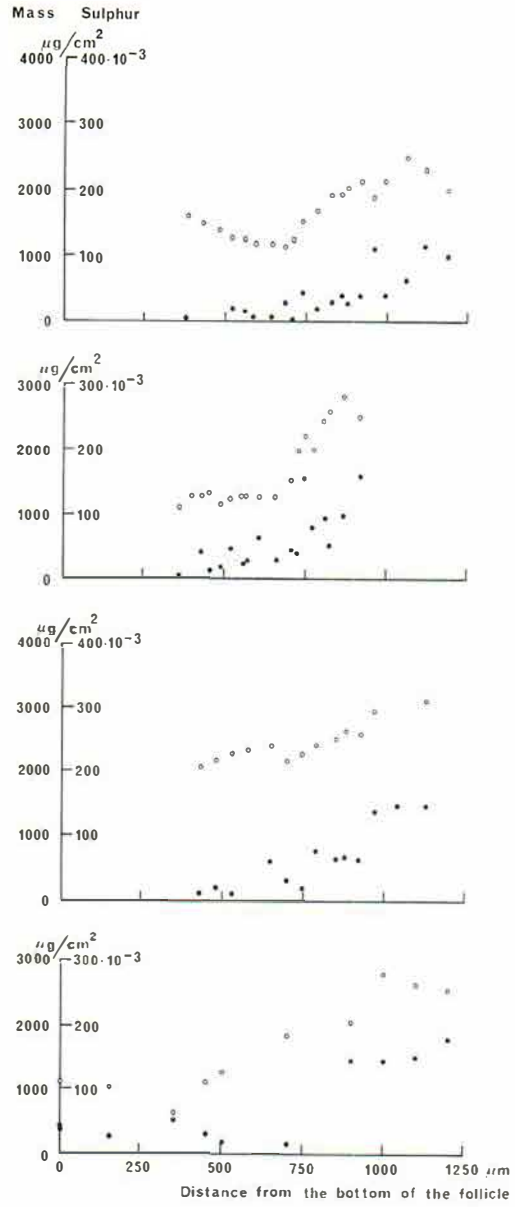
		Central zone						
		Chalkley measurements				Grain counts		
Incubation time	Level	Keratin (1)	Cyto-plasm (2)	Nuclei (3)	$\frac{K_c}{K_c+C_c}$ (4)	Keratin (5)	Cyto-plasm (6)	Nuclei (7)
20 min	3	478	2 229	293	0.177	3	7	1
		507	2 170	323	0.189	16	43	4
20 min	1	453	946	101	0.324	7	4	1
		427	951	122	0.310	9	3	4
	2	536	845	119	0.388	6	9	—
		502	899	99	0.358	7	6	—
	3	522	914	64	0.364	2	1	—
		551	842	107	0.396	9	2	—
40 min	1	496	901	103	0.355	20	11	1
		540	908	32	0.381	19	21	—
	2	568	878	54	0.393	51	34	2
		559	845	96	0.398	33	24	—
	3	582	619	49	0.485	8	3	1
		542	673	35	0.446	13	6	—
40 min	1	530	946	24	0.359	57	39	4
		507	950	43	0.348	35	25	—
	2	566	675	9	0.456	37	21	—
		559	678	13	0.452	16	9	—
	3	573	407	20	0.586	7	4	1
		560	413	27	0.576	6	5	—
60 min	2	500	1 640	110	0.234	39	51	—
		538	1 783	179	0.232	37	65	9
	3	543	1 616	91	0.252	24	34	1
		582	1 834	84	0.241	67	69	4
60 min	1	560	365	75	0.605	8	2	1
		546	360	94	0.603	9	1	—
	2	619	369	12	0.627	5	—	—
		617	367	16	0.627	8	1	—
	3	618	366	16	0.628	2	—	—
		641	335	24	0.657	1	—	—

tom. Roughly 700 μm from the bottom of the follicles the sulphur content reached values about 5% of the total dry weight. At greater distances from the bottom the curves of the dry mass and the sulphur content were closely linked.

Autoradiography. Light microscope autoradiography of plucked human hairs incubated in physiological saline solution with S^{35}-L-cystine showed a tracer distribution that conforms with previous data of sulphur incorporation. The autoradiographic density recorded indicated that tracer incorporation increased with incubation time for the short period used. This experimental technique is therefore suitable for sulphur incorporation studies at higher levels of resolution. The electron micrographs of plucked hairs were recorded so as to show the medulla and the cortex. In the cross sections, the autoradiographic grains were found mainly over the keratin and the cytoplasm of the cortex cells. Few grains appeared over nuclei and the tracer was not seen in intercellular spaces or over cell membranes.

The tracer incorporation pattern followed that revealed by light microscope autoradiography and the results are summarized in Tables I and II.

There are three groups of specimens for which defined numbers are not given. These represent sections at such low levels in the hair follicles



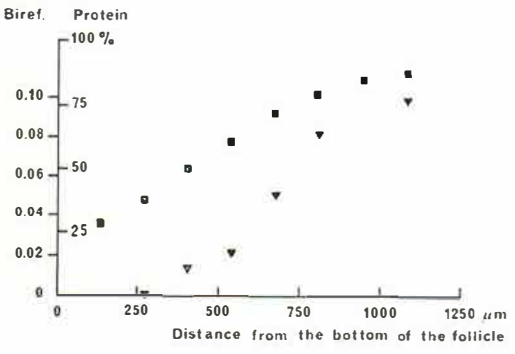


Fig. 3. Dry mass per unit area (open rings) and sulphur content (dots) of human hair follicles plotted against distance from the bottom of the bulb. Freeze-sectioned follicles are represented in the left hand column; formalin-fixed specimens in the right hand column. At the foot of the left hand column the interferometrically-determined protein content (solid squares) and the birefringence (triangles) in a representative hair follicle are plotted against the distance from the bulb.

that the small diameter of the keratin fibrils made a precise location of the level impossible. In the peripheral zone the grain count rose with increasing content of keratin in the cross sections up to a level roughly represented by a Chalkley ratio (K/K+C) of about 0.6 and then decreased. This was true not only for grain counts over the keratin but also for the total grain counts recorded over cortex cells.

DISCUSSION

Electron microscopic studies have shown that measurable amounts of keratin material in the form of filament bundles appear in the cytoplasm of cortex cells at a level about 200 μm from the bottom of the follicle (12). In a region from about 200 to approximately 700μm from the bottom of the follicle there is a continuous increase in the relative amount of keratin. This increase is closely correlated to the morphological findings of a wealth of ribosomes in the cytoplasm of cortex cells. At higher levels where ribosomes are scarce, the increase of the relative amount of keratin is continuous as registered by electron microscopy, though this increase seems to be related to coalescence of filament bundles, an effect probably due to water deprivation (12). The developing hair fibre cortex may thus be divided into three main regions: an undifferentiated region (0-$\sim$200 μm) from the bottom, a region of filament formation ($\sim$200–$\sim$700 μm), and a water deprivation region (above $\sim$700 μm).

The present microradiographic investigation of dry weight distribution in the human hair follicle confirms previous electron microscopic data (12). The dry mass curves were parallel to the sulphur content curves and the undifferentiated regions showed a fairly constant dry mass distribution which increased slowly in the region of filament formation. A steep increase in dry mass occurred in the water deprivation region, a fact which is consistent with the electron microscopic picture.

It is interesting to note that the general pattern of birefringence and mass determined interferometrically follows closely the dry mass curves obtained by microradiography.

The picture of sulphur recorded by microradiography closely followed the incorporation pattern revealed by autoradiography at the electron microscopic level. Thus very small amounts of sulphur were recorded over the undifferentiated region. In the region of filament formation the microradiographic analysis indicated an increase in sulphur content up to a level of about 700 μm where the sulphur content amounts to about 5% of the total dry weight. In the autoradiographic experiments the main label uptake was confined to the region of filament formation. The experiments showed that isotope uptake was almost negligible in the cortex part of the cross section at levels about 700 μm or more from the bottom of the follicle. Here, the keratinization process is completed, as can be judged from the fact that ribosomes are absent (12). The two independent techniques thus favour the concept that most of the sulphur bound to amino acids enters the fibre above and not through the bulb in the region of filament formation (23). The fact that the relative sulphur content seems to be fairly constant as measured by microradiography, once the region of water deprivation is reached, corresponds to negligible isotope uptake at those levels.

The analysis of autoradiographic experiments at the electron microscopic level cannot at the present time be performed on a strictly quantitative basis due to a number of complicating factors inherent in the technique (13, 24). Possible differences in isotope diffusion and uptake, or variations in section thickness and emulsion thickness might contribute to the deviations recorded (11).

In the autoradiographic experiments it was observed that the distribution of grains was uneven over the cross section of the hair fibre. The greatest accumulation of grains was found close to the periphery of the cross-section. It is conceivable that the depth of labelled amino acid penetration was at least partly a function of time, as the time interval of the experiments was short ($\leqslant$60 min).

The combination of thin sections, roughly 100 nm in thickness, and a photographic emulsion, chosen to have a thickness corresponding to the average diameter of the constituent silver halide crystals, allows a resolution of the order 100–200 nm (13, 24). The average diameter of a filament bundle (fibril) in the cross section of human hairs is of the same order of magnitude. Hence quantitative evaluation of the tracer localization within the keratin fibril was not feasible. From electron microscopic investigations, keratin in hair has been described as a two-phase system, the phases

of which have been correlated to two main fractions obtained at chemical analysis. One of these fractions constitutes a high molecular weight fibrous protein which is poor in sulphur, whilst the other fraction, rich in sulphur and of low molecular weight, is believed to stabilize the former fraction. The resolution of the techniques used in the present investigation is thus not capable of giving conclusive data to support such an hypothesis.

From the present investigation it is not possible to deduce whether the label uptake was related to an active transport of the amino acid (in this case S^{35}-L-cystine) or to a passive transportation function. However, once the protein synthesizing capacity of the keratinizing cell decreased, the tracer uptake was considerably reduced. The conspicuous decrease of the hair fibre diameter has been interpreted as a sign of water deprivation (12). Such a process is likely to involve irreversible changes of the proteins in the cortex cells as well as of the proteins in the cell membranes.

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