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DISTRIBUTION OF S^{35}-L-CYSTINE IN MICE AFTER INTRAPERITONEAL INJECTION AS REVEALED BY WHOLE-BODY AUTORADIOGRAPHY

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Abstract. Analysis of whole-body autoradiograms from mice injected with S^{35}-L-cystine revealed a very rapid tracer elimination from the blood compartment and selective uptake in tissues of ectodermal origin. The pilary system was outstanding, in terms of label uptake, at each time interval studied. The findings are discussed with special interest focused on present knowledge of S^{35}-cystine incorporation in hair fibres.

Structural and chemical investigations of fibrous keratin have been focused, to a large extent, on the role and importance of sulphur as a factor responsible for the unique chemical and physical properties of keratinized tissues. In keratin, the major part of the sulphur is contained in the amino acid cystine.

A comprehensive review of chemical studies on keratin was published by Crewther et al. (2) and the relation between the chemical and the physical properties has been reviewed in the monograph by Mercer (15) and in the papers by Lundgren & Ward (12, 13). Autoradiographic investigations at the light microscope level (1, 14, 18) and studies of the uptake of labelled compounds as revealed in chemically separated fractions from plucked wool fibres have been reported (4, 5). At the electron microscopic level the S^{35}-L-cystine incorporation in mouse follicles has been studied by means of autoradiography (8, 17) and recently a histochemical study (20) on the cystine-containing proteins in transverse sections of the human hair was published. Results of a study of the H^3-cystine incorporation in epidermis of mice have been published (10). The results reported so far have not provided a complete picture of the sulphur incorporation process and have stimulated further research.

The present investigation aims at obtaining information about the distribution of S^{35}-L-cystine in various organs and tissues of mice selected for the experiments. Such information can be correlated to results obtained with autoradiography at the electron microscopic level, as well as to previous histochemical and autoradiographic investigations.

Properties of the S^{35}-L-isotope Relevant to Autoradiography

The S^{35}-isotope chosen which has a half-life ($T\frac{1}{2}$) of 87 days is a β-emitter with a maximal energy of 167 keV and a mean energy of 50 keV. The maximum range in water (hence approximately to the same degree in organic material) of the emitted β-radiation is 300 μm. The physical properties make the isotope suitable for autoradiography at the light microscopic level (3).

MATERIAL AND METHODS

White mouse litter (NMRI), 6 days old, received an intraperitoneal single dose of 15 μCi S^{35}-L-cystine (spec. act. 31.4 mCi/mmol) in 0.1 ml physiological saline solution. This roughly corresponded to 3 μCi S^{35}-L-cystine gram/body weight. The animals were sacrificed at four different times 1, 3, 6 and 22 hours after injection of tracer, to provide for an analysis of cystine distribution within the body as a function of time. The animals were anaesthetized, mounted on a microtome stage and dipped into a large volume of hexane cooled to $-76°C$. A metal frame can be fitted around the microtome stage, thus forming a basin which is filled with a semi-liquid mixture of carboxymethyl cellulose (CMC) and water. The animal is submerged in this mixture immediately before freezing. When completely frozen, a preliminary sagittal whole body sectioning was performed to determine op-

Table I. *Relative densities in whole body autoradiograms of mice injected intraperitoneally with S^{35}-L-cystine*

	1 hour	3 hour	6 hour	22 hour
Hair follicles[a]	10	> 10 (15)	> 15	> 15
Skin	1–2	1–2	1	< 1
Claws	> 10	> 10	No records	No records
Lens cortex	5	5–6	3	5–6
Lens centre	1	1–2	1–2	1–2
Retina	2	1–2	No records	> 1
Mouth epithelium	3	3	6	4
Tongue epithelium	8	8–10	6–7	6–7 (8)
Nasal cavity epithelium	4–5	3–4	4–5	2–3
Stomach epithelium[b]	8	3–4 & 5–6	5 & 8	4 & 6–7
Stomach content	—	1	1	< 1
Intestinal lining	2–(5)	3	1 & 4	2–3
Intestinal content	—	< 1–3	1–5	1–5–10
Oesophagus	6–7	6	5–6	5–6
Lungs	1	1–(2)	1	1
Liver	4–6	3	1	1
Spleen	3–4	3–4	5–6	3–4
Bladder content	15	8	9	2–3
Bladder epithelium[c]	1	2–3	3	1
Renal cortex	6	5–6	3–4	2–3
Renal medulla	1	1	1	1
Salivary glands	3	2	2	1
Pancreas, exocrine	> 10	> 10	> 10	5
Lymphatic glands	10	7-8	1	1
Lymph nodes of the peritoneum	10	8–< 10	9	1
Thymus	4–6	7–8	5–6	2–4
Bone	1	2	1–2	1
Bone marrow	1–2	3–4	(3)-4	(3)-4
Blood	0	< 1	< 1	< 1
Blood vessel linings	1–2	4–6	6	4
Myocardium	1	1	1	1
Muscle	1–2	< 1	< 1	< 1
Brown fat tissue	1	1	1	1
CNS	< 1	< 1	< 1	< 1
Plexi chorioides of the CNS[d]	2	2	1–2	2
Hypophysis	3–4	4	4	4

[a] Only the maximal intensity values over the hair roots are given.
[b] The lower values are obtained at the oesophagal part of the stomach.
[c] A differentiation between the muscular wall and the lower part of the stratified epithelium was not possible.
[d] Differentiation between the ependymal tissue and the blood vessels of this tissue was not possible at the resolution allowed by the method used.

timal thickness of sections and times of exposure. In the final experiments sagittal whole body sections (20 μm) were cut and subsequently freeze-dried at -10°C according to the "tape-method" of Ullberg (21, 22). The tape-mounted sections (tape no. 810, 3M, Minnesota) were pressed against X-ray films (Structurix, Gevaert) and stored in press for 3, 4 and 5 days. After removal of the tape-mounted sections, the films were processed in a fine grain developer. Most of the sections were kept on the adhesive tape, unstained and covered with transparent plastic tape strips for protection. A few sections representing the different time intervals of isotope exposure were stained with haematoxylin-eosin and mounted in Euparal® under cover glass for microscopic identification of different organs. In addition, a number of sections were taken from the same animals, mounted on tape 688 (3M, Minnesota), freeze-dried, and were subsequently mounted on Ilford G5 nuclear plates (11). About 25 sections were autoradiographed in each time group. After an exposure time of 4 to 5 days the tape was removed from the specimen-film sandwich by immersion in xylene. The section-film units were subsequently passed through a decreasing concentration series of ethanol, developed, fixed, and rinsed in water. The sections, adhering to the nuclear plate, were then stained with haematoxylin-eosin and mounted under cover glass in Euparal® (Flatters & Garnett Ltd., Manchester, England).

RESULTS

The concentration of tracer shown by the developed photographic grain distribution and density in the autoradiographic film, was measured on a

relative scale, i.e. ocular judgement of density in relation to a step wedge of 21 steps covering a nominal density range of 0.05–3.05. This step wedge thus constituted a density function which could be related to the autoradiographic densities. The nominal increment per step is 0.15, so chosen for differentiation of grain density over different organs. Due to different causes, such as variations in section thickness and/or development, a slight fluctuation of the order of two steps sometimes was recorded in consecutive autoradiograms. Although this classification was a crude one, it served as a means to analyse the dynamics of cystine incorporation of different tissues. Table I shows the evaluation data obtained referring to various organs in mouse litter, 6 days old, at different time intervals after the intraperitoneal injection of S^{35}-L-cystine.

The highest levels of tracer uptake were preferentially located to organs of ectodermal origin. The hair follicles were the most active organs in this respect. A general impression was that of a correlation between the protein synthetizing activity of the cells and the degree of tracer incorporation (cf. Fig. 1 *d* where the lens centre was relatively inactive and lens cortex which was composed of dividing cells (7) revealed a higher density in the autoradiograms).

Hair follicles

After 1 hour the autoradiographic image of the hair follicles revealed a very high grain density compared with all other sites in the body, with the exception of claws. In the hair follicles both the lower and the upper (proximal and distal) border of the zone of incorporation were relatively well defined. The region of isotope uptake corresponds to what has been called the suprabulbar and fibrillar regions (18) or the region of filament formation (9) (Fig. 2). A maximum grain density was recorded over this zone 6 hours after the injection of tracer. This maximum grain density remained at the same density level after 22 hours. However, in the 6 hour animals a tapering tail of the grain density over the follicles was recorded which increased in length in the 22 hour animals corresponding to the increment of the hair fibre length produced between 6 and 22 hours. During the 22 hour experiment the labelled fibrillar area moved from a position low in the dermis to reach the level of the cornified epidermis with its topmost part.

A detailed study of the sequence of incorporation over the bulb (level of matrix cells) reveals an initial, relatively high, grain density after 1 hour of label incorporation which subsequently diminished so as to become almost negligible after 22 hours.

Skin and claws

The skin was characterized by constant and evenly distributed tracer (grain density) which appeared within the first hour. After only 1 hour of tracer incorporation, located spots of higher density were seen in the Malpighian layer of the epidermis. These spots were not found at other times. The grain density pattern over the claws showed the same general trend and intensity response as found in the case of the hair follicles.

Lens

The autoradiograms of the lens cortex reached a medium density after 1 hour in contrast to the low density of the centre. Exactly the same density pattern was found at all times investigated.

The alimentary tract

Over the epithelium of the tongue a high grain density was recorded. Over the soft palate epithelium a weak to moderate density was recorded which changed little during the 22 hour interval. The oesophagus revealed the same sort of steady state pattern at a slightly higher density level. This was also true for the upper (oesophageal) part of the stomach whereas the lower (pyloric) end had a still higher level of tracer incorporation. The stomach content density was more or less constant from (in and after) the 3 hour interval. Over the intestinal tract the grain density increased with time in distal direction.

Liver, pancreas and salivary glands

The time series of autoradiograms from the liver revealed a steadily decreasing grain density pattern. Exocrine pancreas primarily showed a high uptake which seemed constant for 6 hours, to decrease after 22 hours. As expected, due to the cystine content of insulin, the islets of Langerhaans had considerable tracer uptake although lower than the exocrine gland. Salivary glands

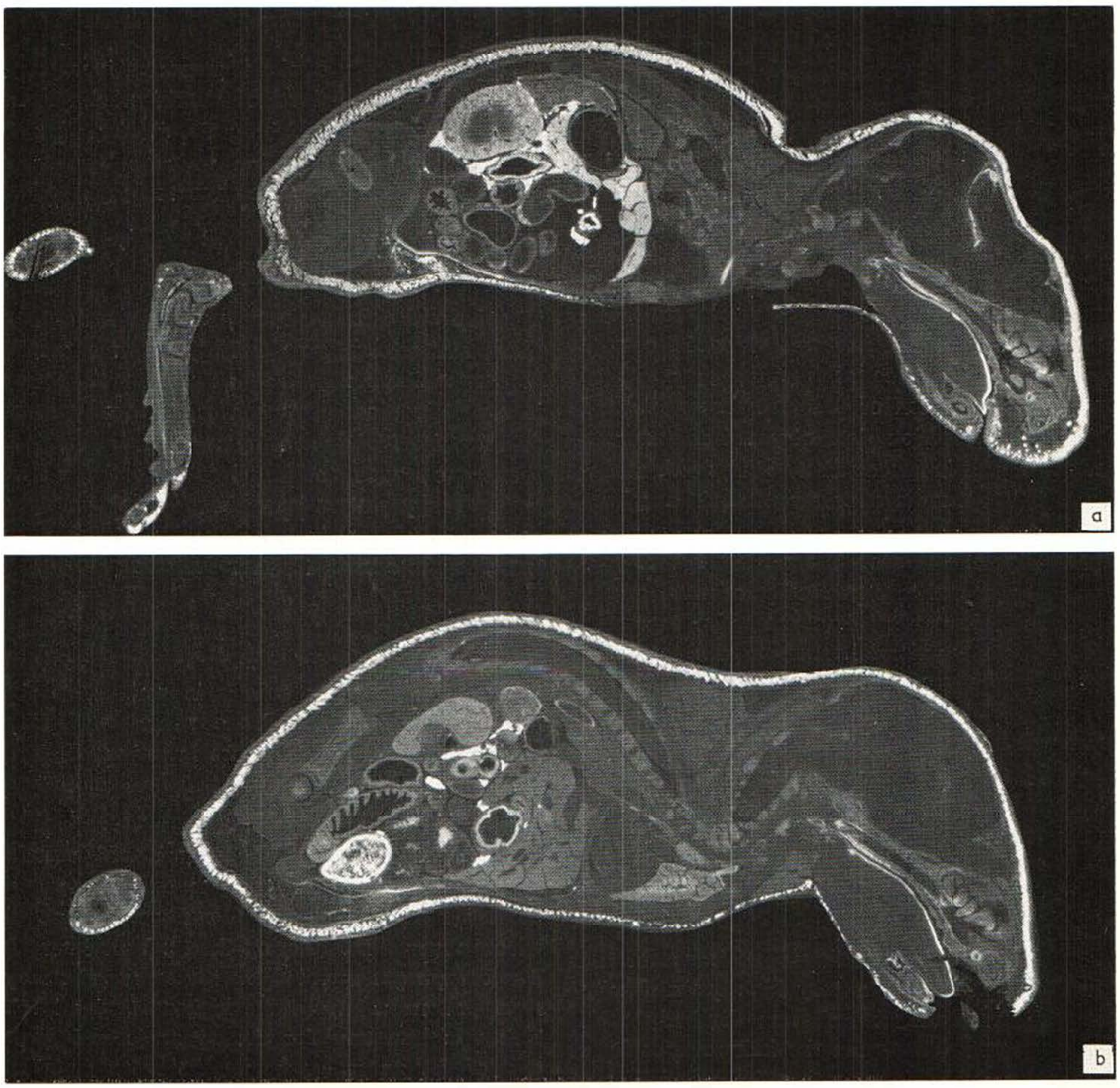


Fig. 1. Whole-body autoradiograms from 20 μm sections through 6-day-old mice after intraperitoneal injection of S^{35}-L-cystine. (*a*) Section taken 1 hour after injection. Exposure time: 4 days. (*b*) Section taken 3 hours after the injection. Exposure time: 4 days. (*c*) Section taken 6 hours after the injection. Exposure time: 4 days. (*d*) Section taken 22 hours after the injection. Exposure time: 4 days. × 2.7.

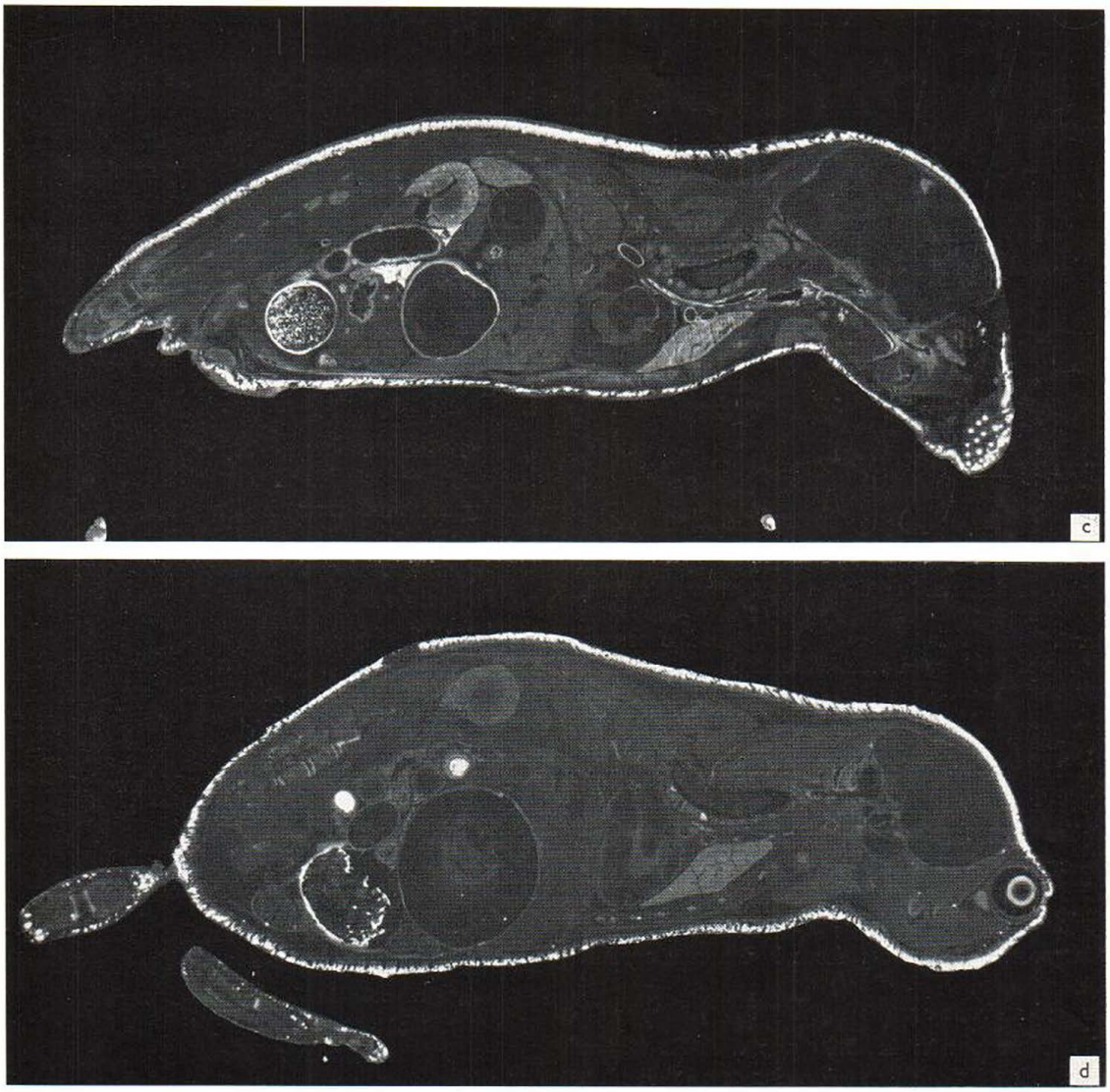
starting on a low density level decreased in grain density progressively.

The renal system

Initially the kidney cortex had a rather high radioactivity in the 1 and 3 hour animals. Subsequently the recorded radioactivity decreased progressively in the 6 and 22 hour animals. The amount of tracer grains over the renal medulla was constant at all times and represented a very low density level. The bladder epithelium exposed a low but relatively constant level of grain density during the experiments.

Lymphatic and haematopoetic tissue

The lymph nodes of the peritoneum showed a very high grain density after 1 hour and there was a faint increase in activity during the first 6



hours. However, the autoradiograms showed that at 22 hours the release of label was almost complete. Lymphatic tissue at other sites in the body showed the same general pattern although the decrease in radioactivity was faster. On the other hand the autoradiograms representing the thymus region slowly built up the grain density to reach a maximum at 3 hours followed by a decrease in density that slowly ebbed to a low density value at 22 hours. The bone marrow reached a low grain density maximum after 3 hours which remained constant during the experimental period. The blood was immediately deprived of any appreciable amounts of tracer and the grain density over the blood in the large vessels could hardly be differentiated from the general background.

Blood vessel linings

The tissue had comparatively high uptake that reached a peak at 6 hours followed by a slight decrease at 22 hours.

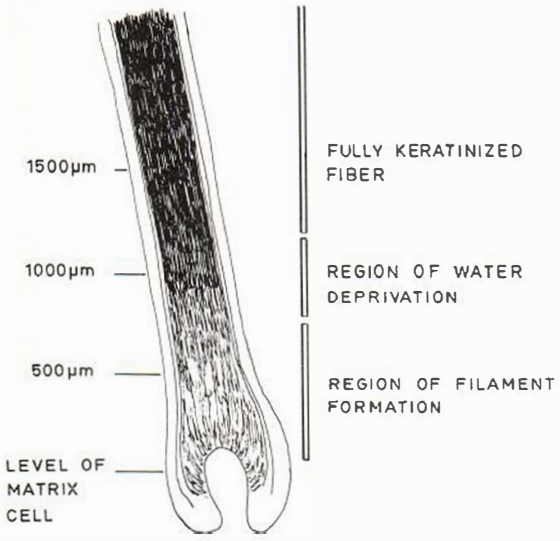


Fig. 2. Schematic representation of growing hair fibre. Region of filament formation denotes the region where protein synthesis occurs. In Region of water deprivation morphological signs of protein synthesis, i.e. ribosomes, are not at hand and a constriction of the fibre diameter may be recorded.

Muscles and brown fat tissue

The isotope uptake of skeletal muscles was slightly higher than the background density in the autoradiograms. The tracer uptake over the myocardium was more conspicuous although the density was barely recognizable in comparison with the background. Brown fat tissue had a low uptake.

Central nervous system

The tracer uptake in CNS was a shade higher than that of the general background at all time intervals. The plexi chorioides of the CNS revealed a grain density slightly higher than that of the blood. This grain density was constant within the experimental period.

DISCUSSION AND CONCLUSIONS

The fate of S^{35}-L-cystine after intraperitoneal injection into mouse litters was followed in whole-body autoradiograms. By observing the distribution and the density of the photographic grains in whole-body autoradiograms information is obtained about the uptake in different tissues as well as a rough estimate of the incorporation of the isotopically labelled compound in the tissues during a 22 hour experimental period. There is evidence from previously reported autoradiographic experiments that once cystine enters the circulating blood it appears in the hair follicle within a matter of seconds (18). From the blood the amino acid freely (actively or passively) and preferentially diffuses into organs of ectodermal origin with the exception of the central nervous system. As measured by the present technique the blood was quickly deprived of any recognizable amount of the S^{35}-L-cystine; hence the tracer grains associated with the blood produced a very weak grain density in the autoradiograms. After an intraperitoneal injection it is likely that the tracer amino acid was absorbed, at least partly, by the mesenteric blood vessels. The initial high activity measured over the liver conforms to this idea. As registered in the present investigation the lymphatic vessels of the mesenterium were also actively involved. This is shown by the high activity seen over the mesenteric lymph nodes.

In mice at an age of 6 days all hair follicles are actively producing hair fibres (in anagen). In agreement with previously reported results (1, 5, 14, 18) a high concentration of tracer was found in the keratogenous zone (region of filament formation) whereas the tracer concentration in the bulb was low. An uptake of S^{35}-L-cystine in nuclei is shown by previous studies of nuclear protein metabolism (19) and a recent autoradiographic study at the electron microscopic level (8) also reveals such an uptake of S^{35}-L-cystine in the nuclei and cytoplasm of cells in the bulb region.

The present experiments indicate that the hair follicle uptake of tracer following a single dose of radioactive S^{35}-L-cystine is continuous for at least 6 hours. This result agrees with an autoradiographic experiment at the electron microscopic level (8) and confirms data previously published. In the hair follicles the top end of the actively incorporating zone is relatively well defined whereas the lower end revealed a tapering pattern. This probably indicates that the blood concentration of tracer decreases *slowly* during the experimental period. Such a phenomenon can be expected when the uptake of the labelled compound from the injected deposit is low, when a re-utilization process is involved and/or when the target organs can be saturated below the blood level of amino acid present. At 3 hours and later, no indication of labelled compound in

the peritoneal cavity was observed. The cystine dose given in these experiments corresponds to a total of only 0.15 mg cystine per animal. Thus the second alternative given above seems to be the most plausible.

The main pathway of the metabolized cystine most probably goes via a reabsorption of the tracer amino acid from the content of the intestine which contains a significant amount of labelled cell remains emanating from the intestinal mucous membranes. The present investigation indicates that the reabsorption through the renal tubuli contributed to maintaining the slowly decreasing blood level of tracer. The possible error of cystine-feeding, through the milk by the mother mouse which might have comsumed excrements from the litter containing tracer, is considered negligible due to the dilution factor caused by the discriminating uptake of cystine when the tracer compound passes her body.

A rough estimation of the hair growth per unit time is obtained from a registration of the position of the top end of the zone of incorporated tracer at different time intervals in comparison with the bulb bottom, bearing in mind that individual differences may occur. The value obtained was of the order 0.3–0.4 mm per 24 hours, a figure which is consistent with results obtained by other authors (16).

When all hair follicles are in anagen, the uptake of tracer is low in the thin epidermis as compared with the uptake of the hair follicles (1). Only during the first hour were spots of rather high activity seen in the Malpighian layer. Otherwise, the epidermis had a homogeneous grain density distribution that remained constant during the time periods under investigation. In contrast, an increasing tracer cystine uptake in the anagen hair follicle was registered during a period between injection and autopsy of roughly 6 hours. Although all cell layers with the exception of stratum corneum contained tracer 1 hour after injection, stratum corneum is known to show signs of tracer incorporation only about 4–6 days (6) after injection, whereas the fast-growing anagen hair follicles produce a fully mature hair fibre segment with incorporated tracer that reaches the surface of the stratum corneum in about 24 hours. The density relation between the mature hair fibre and the epidermal cell layer was exaggerated (15/1) in comparison with the known data of sulphur content of these tissues (2/1). This is partly explainable because the horny layer is very thin and reliable measurements are not obtained with the technique used.

It has been observed previously (6, 10) that some tracer is distributed over the entire epidermis with the exception of stratum corneum within 1 hour of injection of the labelled compound. As revealed by previous reports and the present study, a high uptake of tracer was recorded in the cortex cells of hair fibres shortly (within 1 hour) after intraperitoneal injection of tracer. There is presently no indication that the tracer amino acid might be transported over the external and internal root sheaths via the intercellular spaces of these cellular layers (8). The (tracer) amino acid transportation through these cellular layers might therefore be a complex passage via membranes and cytoplasm, by means of a possible carrier(s), active or passive diffusion, or combinations of such mechanisms. The fact that the Henle layer of the inner root sheath is fully consolidated at the levels in the hair follicle (8), where the tracer incorporation mechanism occurs, underlines the need for information about the transportation of amino acids to the cortex cells.

The present investigation gives support to the concept that the amino acid cystine is preferentially incorporated into growing hair follicles and claws. In other forms of epithelia the incorporation appears to be related to the cell turnover and the protein synthesis activity in specialized epithelial cell forms such as glandular cells.

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REFERENCES

1. Bern, H. A., Harkness, D. R. & Blair, S. M.: Radioautographic studies of keratin formation. Proc Nat Acad Sci *111*: 55, 1955.
2. Crewther, W. G., Fraser, R. D. B., Lennox, F. G. & Lindley, H.: The chemistry of keratins. *In* Advances in Protein Chemistry XX, p. 191. Academic Press, New York, 1965.

3. Doniach, I. & Pele, S. R.: Autoradiograph technique. Brit J Radiol *23:* 184, 1950.
4. Downes, A. M.: A study of the incorporation of labelled cystine into growing wool fibres. *In* The Biology of Skin and Hair Growth (ed. Lyne-Short), p. 345. Angus & Robertson, Sydney, 1965.
5. Downes, A. M., Sharry, L. F. & Rogers, G. E.: Separate synthesis of fibrillar and matrix proteins in the formation of keratin. Nature *199:* 1059, 1963.
6. Downes, A. M., Matoltsy, A. G. & Sweeney, T. M.: Rate of turnover of the stratum corneum in hairless mice. J Invest Derm *49:* 400, 1967.
7. Duke-Elder, S.: System of Ophthalmology, vol. XX. The Lens, p. 309. Henry Kimpton, London, 1961.
8. Forslind, B.: Electron microscopic and autoradiographic study of S^{35}-L-cystine incorporation in mouse hair follicles. Acta Dermatovener (Stockholm) *51:* 9, 1971.
9. Forslind, B. & Swanbeck, G.: Keratin formation in the hair follicle. An ultrastructural investigation. Exp Cell Res *43:* 191, 1966.
10. Fukuyama, K. & Epstein, W. L.: Sulphur containing proteins and epidermal keratinization. J Cell Biol *40:* 830, 1969.
11. Hammarström, L., Appelgren, L.-E. & Ullberg, S.: Improved method for light microscopy autoradiography with isotopes in watersoluble form. Exp Cell Res *37:* 608, 1965.
12. Lundgren, H. P. & Ward, W. M.: Levels of molecular organization in α-keratins. Arch Biochem Biophys, Suppl. *1:* 78, 1962.
13. — The Keratins. *In* Ultrastructure of Protein Fibers (ed. Borasky), p. 39. Academic Press, New York, 1963.
14. Harkness, D. R. & Bern, H. A.: Radioautographic studies of hair growth in the mouse. Acta Anat *31:* 35, 1957.
15. Mercer, E. H.: Keratin and Keratinization. Pergamon Press, London, 1961.
16. Monroe, D. D.: Hair growth measurements using intradermal sulphur-35 cystine. Arch Derm (Chicago) *93:* 119, 1966.
17. Nakai, T.: A study of the ultrastructural localization of hair keratin synthesis utilizing electron microscopic autoradiography in a magnetic field. J Cell Biol *21:* 63, 1964.
18. Ryder, M. L.: Investigations into the distribution of thiol groups in the skin follicles of mice and sheep and the entry of labelled sulphur compounds. Proc Roy Soc Edinburgh, *67 B:* 65, 1957/58.
19. Schultze, B.: Chapt. 3 & 6 *in* Physical Techniques in Biological Research (ed. A. W. Pollister), 2nd ed. Academic Press, New York, 1969.
20. Swift, J. A.: The electron histochemistry of cystine-containing proteins in thin transverse sections of human hair. J Roy Micr Soc *88:* 449, 1968.
21. Ullberg, S.: Studies on the distribution and fate of S^{35}-labelled benzylpenicillin in the body. Acta Radiol, Suppl. *118:* 1, 1954.
22. — Autoradiographic studies on the distribution of labelled drugs in the body. 2nd UN Int. Conf. Peaceful Uses of Atomic Energy *24:* 248, 1958.

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