[8] See R. Salem, "On Some Singular Monotonic Functions Which Are Strictly Increasing," *Trans. Am. Math. Soc.*, **53**, 427–439, 1943, particularly pp. 431–433, where the notations are slightly different. The fact that the infinite product is the Fourier-Stieltjes series of dG in the Walsh-Paley system is an immediate consequence of the uniform convergence of G_n and of the fact that each function of the system is a step function.

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*RADIOAUTOGRAPHIC STUDIES OF KERATIN FORMATION**

BY HOWARD A. BERN, DONALD R. HARKNESS, AND SIDNEY M. BLAIR

DEPARTMENT OF ZOÖLOGY AND ITS CANCER RESEARCH GENETICS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

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Some general aspects of the problem of keratin formation in vertebrate tissues have been discussed briefly in a recent note.[1] One of the questions raised therein was concerned with whether keratin occurred as the result of a specific synthesis of fibrous protein or of the degradation of normal protoplasmic proteins. Data relevant to this question have been selected from some detailed radioautographic studies of the distribution of S^{35}-labeled *l*-cystine in the mouse, with special attention to the production of "hard" and "soft" keratins.[2] Additional experiments with P^{32}-labeled phosphate and C^{14}-labeled carbonate and protein-hydrolyzate were also conducted. Unless otherwise indicated, young mice of the C_{57} strain were employed. Tissues were fixed in Bouin's fluid, and paraffin sections were cut at 10 μ. The radioactive materials thus demonstrated are considered to be protein-bound. The radioautographic methods were essentially those of Bélanger and Leblond[3] (emulsion-coating technique) and of Doniac and Pelc[4] (stripping-film technique).

It was reported[1] that during the active growth phase (anagen) in the hair cycle of the mouse there occurred a striking concentration of cystine (and/or its derivatives)[5] in the hair follicles evident in radioautographs of skin removed 8 hours after the administration of S^{35}-labeled cystine. A similar high uptake was seen in the conical papillae of the mouse tongue[1] and has also been found in the claw bed. The intensive concentration of radiocystine occurs in the so-called "keratogenous zone," characteristic of "hard" keratinization.[2] This concentration could conceivably result from either specific uptake of cystine from the body pool by the keratogenous zone itself or uptake in the hair bulb associated with protein synthesis accompanying cell proliferation and subsequent retention of the cystine as the living cells keratinize and die. Radioautographs of skin made 8 hours after radiocystine injection (approximately 3 μc/gm body weight) show a definite though minimal uptake in the hair bulb (Fig. 1). However, any increase in cellularity in the keratogenous zone resulting from a decrease in volume of the cells which have proliferated from the hair bulb is insufficient to account for the manifold difference in radioactivity.

Examination of radioautographs made 1, 2, 4, and 6 hours after radiocystine administration reveals that there is an appreciable uptake of isotope by the keratogenous zone even 1 hour after administration, when the hair-bulb region is only

slightly active radioautographically (Fig. 4). The intensity of the radioautograph increases with the time after administration up to 8 hours, and the radioactive region of the hair follicle also becomes somewhat greater in extent (explicable on the basis of the growth of the hair shaft and the incorporation of the cystine therein). Essentially, however, the radioautograph appears much the same 1 hour after administration as it does 8 hours after. In mice (C_{57} and A strains) wherefrom skin and tongue sections are made one or more days after radiocystine injection, the radioactive zone can be followed out distally as the hair shaft or conical papilla grows (Figs. 2 and 3).

Sections of skin biopsied or necropsied 1–8 hours after cystine injection were treated before being covered with stripping film with various agents to determine the mode of binding of the cystine taken up by the keratogenous zone. Extraction of tissue sections with 1 and 2 per cent thioglycollate solution (at pH 10 and 11) and 0.5 *M* mercaptoethanol (at pH 4 and 7), all at 50° C. for 2 hours, revealed no apparent decrease in radioactivity when compared with distilled water–treated control sections (Figs. 5 and 6). Hence the cystine is evidently not bound to side groups by disulfide linkages but is presumably incorporated (as cysteine?) into the main chains of the keratin molecule by peptide linkages.

Eight hours after injection of about 40 μc of C^{14}-labeled algal protein-hydrolyzate into a young (15-gm.) mouse in anagen, skin sections produced lightly positive radioautographs when exposed to film for 8 or more weeks. The C^{14}-labeled amino acids were not specifically concentrated in the keratogenous zone. Activity was seen in the hair bulb and extended up the hair follicle into the keratogenous zone region with qualitatively little difference in concentration (Fig. 9). Similar results were obtained in another mouse 6 hours after injection with 300 μc of radiocarbonate (after exposure of sections to film for 9 weeks).

Eight hours after administration of massive doses of P^{32} (200–500 μc per animal), the amount of radioactivity is high in the hair bulb and decreases toward the keratogenous zone (Fig. 7). The radioautographic distribution is in direct contrast to the picture of cystine localization in the hair follicle. The radioactivity with P^{32} is concentrated in the region of active cell division and, hence, nucleic acid synthesis. Autographs made 18 and 24 hours after P^{32} injection show a generalized uptake by the lower part of the follicle, including the keratogenous zone, which is still less active than the hair bulb.

We have previously reported[1, 6] that there is little evidence of difference in the localization of cystine in the androgen-stimulated vagina and uterus as compared with the estrogen-stimulated organs, beyond that related to the degree of hyperplastic response induced in the epithelium by the steroids used. As a part of a study of the distribution of radiocystine in the female genital tract under various hormonal stimuli, 6 virgin female Heston A mice, 5 months of age and castrated for 90 days, were injected intramuscularly with estradiol in sesame oil, 0.01 mg. daily for 3 days. Eight hours before sacrifice, 3 μc of radiocystine per gram of body weight were injected subcutaneously. Segments of vagina and uterus were removed from each animal, fixed together in Bouin's fluid, and treated together thenceforth. Paraffin sections of a single block containing both tissues were made at 6 μ and were covered with stripping film. After exposure for 3 weeks, the slides were developed and the contact radioautographs studied.

Grain-counting on the superimposed film was performed at 1,500× with an ocular grid, the counts being made from twenty areas of each tissue region and recorded as mean count per grid square. Among other regions, counts were made over the stratum basale and the mid-stratum spinosum of the keratinized hyperplastic vaginal epithelium and over the lining and the glandular epithelium of the hypertrophic uterus. Table 1 gives the mean ratios of some of the regions compared. Statistical analysis of paired data shows that the radiocystine concentration in the uterine lining or glandular epithelium is not significantly different from that in the vaginal stratum basale. However, the vaginal stratum spinosum contains significantly more cystine than the other regions, although the difference between this region and the uterine lining epithelium is not great. An appreciable concentration of radiocystine is evident immediately below the keratin layer in the vagina (Fig. 8).

TABLE 1

MEAN RATIO OF GRAIN COUNTS OF RADIOCYSTINE UPTAKE BY VARIOUS REGIONS OF THE ESTROGEN-STIMULATED FEMALE MOUSE REPRODUCTIVE TRACT

Areas Compared	Mean Ratio of Counts ±S.E.$_m$, n = 6
Vaginal stratum basale / Uterine lining epithelium	0.92 ± 0.03
Vaginal stratum spinosum / Vaginal stratum basale	1.28 ± 0.08
Vaginal stratum spinosum / Uterine lining epithelium	1.14 ± 0.04
Uterine lining epithelium / Uterine glandular epithelium	1.19 ± 0.06

From these data it appears that there is no specific uptake of cystine by the basal layer of the keratinizing vaginal epithelium, the radioactivity of which is indistinguishable from the nonkeratinizing uterine epithelium. However, the higher concentration in the lower and middle stratum spinosum cannot be explained on the basis of increased cellularity, since the cells of this region are at least as large as those of the stratum basale. Radioactivity is least in the stratum basale and greatest immediately below the keratin layer. Such a gradient is also seen in the "soft" keratinization occurring on the surface of the tongue (between the papillae)[1], in the foot pad, and in the lining of the mouse esophagus and forestomach. Furthermore, examination of autographs of the vagina made 4 hours after radiocystine injection into estrogen-treated female mice reveals a distribution of the cystine similar to that seen 8 hours after injection. Twenty-four hours after radiocystine administration, the basal region of the keratin layer becomes radioactive, and vaginal smears taken at 48 hours show considerable activity.

Additional information on the radioautographic localization of protein-synthetic regions with radiocystine will be published elsewhere. The technique has permitted the acquisition of some suggestive data on the mechanism of keratin formation not heretofore available. Although the differences between "hard" and "soft" keratinization are considerable[2] (recent work indicates that intermediate situations also exist[7]), the highly radioactive zone beneath the stratum corneum

in the vagina and elsewhere may be analogous to the keratogenous zone in "hard" keratinization. The higher radioactivity in this region could be a reflection of the flattening of the cells and decrease in their volume, prior to keratinization. However, this activity is present within a short time after radiocystine administration, suggesting that it need not be ascribed to the S^{35} content of cells which have proliferated from the basal layer. Less than 8 hours after isotope administration, the entire stratified epithelium is radioactive, not just the more basal layers. As has been stated previously[1] in regard to the hair follicle, the region of cystine concentration corresponds to the region of high concentration of protein-bound sulfhydryl groups (as detected cytochemically). This is also the case in the lingual papilla, as well as in the analogous area in "soft" keratinization. It would seem, then, that much of the S^{35} is present as protein-bound cysteine in the keratogenous zones.

The specific concentration ability of the keratogenous zone may be restricted to cysteine-cystine and/or their derivatives (and possibly other sulfur-containing amino acids[8]). The radiocarbon studies indicate an activity at 8 hours in this zone, which is no higher than that of the hair bulb and, hence, which could result in part from amino acids absorbed by the hair bulb and there incorporated into protein and in part by concentration of C^{14}-labeled cystine from the body pool. The radiophosphate studies show slight grain deposition over the mitotically inactive keratogenous zone 8 hours after injection of P^{32}, which may be accounted for largely by the residual DNA and RNA carried up by the cells deriving from the hair bulb.

"Enrichment in sulfur"[7] certainly occurs in the course of "hard" keratinization. That it is the one generally valid criterion for this phenomenon, as suggested by Van Scott and Flesch,[7] may be open to some question, since the "soft" keratinization in the vagina and elsewhere may also involve cystine concentration immediately beneath the keratin layer in a region analogous to the keratogenous zone. That the

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hb = Hair bulb; *kz* = Keratogenous zone; *hs* = Hair shaft; *m* = Melanin.

FIG. 1.—Skin from female C_{57} mouse to show intense uptake in keratogenous zone and minimal uptake in hair bulb 8 hours after injection of S^{35}-cystine. Distinguish dense melanin in core of hair bulb and shaft from granular-appearing radioactive areas in this figure and in Figures 4–7 and 9. Overexposed emulsion coating, unstained. ×65.

FIG. 2.—Tongue from female Heston A mouse to show intensely active conical papillae 3 days after injection of S_{35}-cystine. Stripping film, unstained. ×130.

FIG. 3.—Skin from old estrogen-treated female Cal A mouse to show radioactive zone emerging from hair follicle 5 days after injection of S^{35}-cystine. Emulsion coating, unstained, uncleared. ×65.

FIG. 4.—Skin from female C_{57} mouse to show active keratogenous zone 1 hour after injection of S^{35}-cystine. Stripping film, unstained. ×65.

FIG. 5.—Hair follicles from female C_{57} mouse 8 hours after injection of S^{35}-cystine. Control for treatment with disulfide-reducing agents (Fig. 6). Note active keratogenous zones (*at right*). Stripping film, unstained, uncleared. ×65.

FIG. 6.—Hair follicles from skin section adjacent to that shown in Fig. 5. Treated with sodium thioglycollate (see text). Note retention of activity in keratogenous zones. Stripping film, unstained, uncleared. ×65.

FIG. 7.—Hair follicles from skin of male C_{57} mouse 8 hours after injection of P^{32}-phosphate. Note concentration in hair bulb decreasing toward keratogenous zone. Stripping film, unstained ×65.

FIG. 8.—Section of keratinized vagina from estrogen-treated female Heston A mouse 8 hours after injection of S^{35}-cystine. Somewhat tangential plane of section emphasizes zone of cystine concentration below stratum corneum. Stripping film, unstained. ×265.

FIG. 9.—Hair follicles from skin of male C_{57} mouse 8 hours after injection of 40 μc C^{14}-labeled algal protein-hydrolyzate. Note moderate activity of hair bulb extending into keratogenous zone. Stripping film (exposure 11 weeks), unstained. ×130.

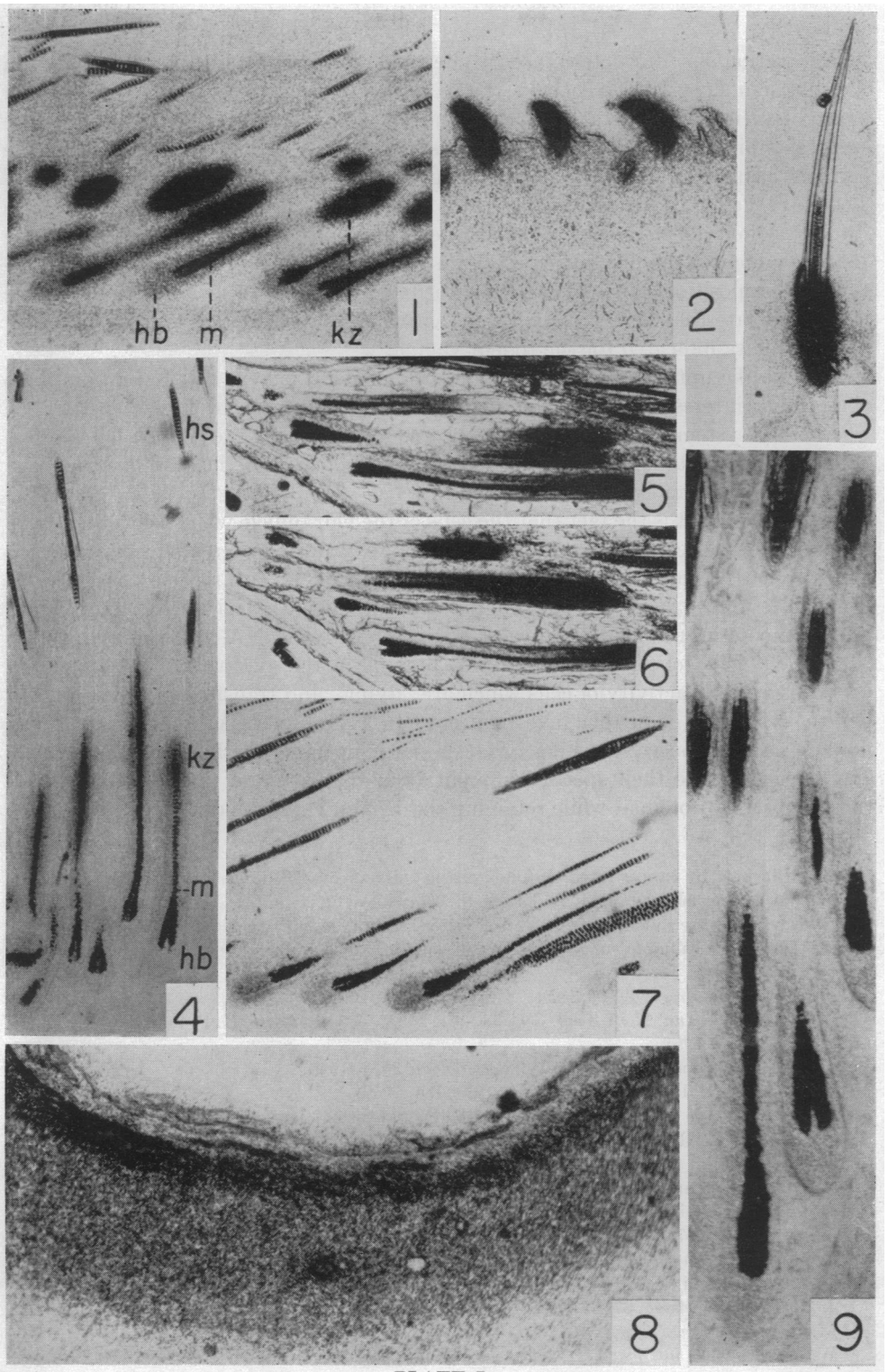

PLATE I

concentration of sulfur determined by chemical means in the stratum germinativum (Malpighian layer) is essentially the same as that in the stratum corneum in "soft" keratinization[7] could result from the fact that this cystine-concentrating zone (corresponding to the stratum granulosum-lucidum region) is included in the former. The results from our cystine studies would not support the suggestion of Leblond[2] that keratin itself may be the radiocarbon-tagged protein which moves "up with the cells of the skin towards the outside" (p. 472). Much of the cystine may be added to the precursor protein(s) (which presumably undergo prior hydrolytic decomposition[9]) just below the stratum corneum. In general, the evidence would indicate that keratinization is an active synthetic process, as Biggers' histochemical data indicated for the rodent vagina.[10]

The specific uptake of cystine by the keratogenous zone in hair formation raises some other interesting questions. The path of transport of the cystine from the circulatory system to the zone is not known. Absorption through the hair bulb is conceivable, but the possibility of a lateral transport from the capillaries around the follicle shaft[11] must also be considered.

Of great interest is the fact that the cells of the hair follicle which concentrate the cystine and synthesize the keratin evidently *de novo* are located well above the mitotically active hair bulb (in Hardy's zones D and E[11]). Hardy's extensive cytochemical studies[11] have shown that the spindle-shaped nuclei in the "moribund" cells of the keratogenous zone are still Feulgen-positive (DNA-containing) and that ribonucleic acid concentrations are also present in the cytoplasm. The residual protein-synthetic activity of cells which are "dying" and transforming thereby into keratin is worthy of further investigation. In avitaminosis-A, wherein squamous metaplasia and consequent keratinization occur in many epithelia, epithelial cells can be said to lose their specific differentiation capacity and their ability to synthesize specific proteins[12] while retaining the residual synthetic ability to produce keratin.

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[5] This is implied in subsequent discussion wherever "cystine" is used.

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