THE INCORPORATION AND FATE OF H^3-TYROSINE IN THE HAIR CORTEX OF RATS OBSERVED BY RADIOAUTOGRAPHY

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ABSTRACT

The incorporation of H^3-tyrosine into the protein of the cells in the cortex of rat hair has been investigated by radioautography. In growing hairs, radioactivity is found in the matrix, the upper bulb, and the whole of the keratogenous zone up to the fully keratinized part of the shaft, 10 and 30 minutes after an injection of labelled tyrosine. This is unequivocal evidence of protein synthesis at these sites. There is a very precise relationship between the end of protein synthesis and the hardening of the cortical cells at the top of the keratogenous zone. The way in which the silver grains of the radioautographs are clustered indicates that at 30 minutes after the injection the isotope is distributed more evenly in the matrix and upper bulb than in the top of the keratogenous zone. Possibly this reflects a difference, at these sites, in the cell components engaged in protein synthesis, or in the proteins being synthesized. The fully keratinized and hardened part of the hair was not radioactive at 10 and 30 minutes after the injection of H^3-tyrosine. The rate at which the radioactivity moves into this region shows that the hair of rats grows 0.9 mm/24 hours. Comparison of the degree of radioactivity along the growing hair in the 30-minute, 12-hour, and 36-hour materials shows conclusively that protein accumulates in the cortical cells during their keratinization. An injection of a labelled amino acid does not behave as an ideal pulse dose; consequently, the grain density over the hair cortex at 36 hours is 100 per cent larger than would be expected if an ideal pulse dose situation existed.

INTRODUCTION

Hair follicles have an alternating pattern of activity and inactivity in terms of hair growth. A growing hair presents for examination a complete picture of its production from undifferentiated cells. A series of cells between the matrix and the top of the follicle, which forms one component of hair, represents the changes which occur in one cell during its conversion from the primordial to the fully differentiated state. Progressive differences between a number of cells in space illustrate the progressive changes experienced by one cell in time. This investigation was made to compare active and inactive follicles, and to compare different regions of the follicle. The results will give indications of the differences in one follicle when it is active and inactive, and the changes which take place in one cell during its differentiation from a primordial cell to a component of mature hair.

The cortex of mature hair is a dead structure composed of proteins. It is formed from cells which originate in the matrix of the hair bulb and move towards the skin surface. As they do so, they in-

crease in size and become filled with fibrils in a sulphur-rich ground substance (Rogers, 1959 *a* and *b*). The production of the fibrils and the ground substance is a convenient system for the study of protein synthesis. The manner in which the proteins of keratin are synthesized and the cell components involved are unknown.

The incorporation of S^{35}-cysteine into the keratogenous zone of hair cortex has been demonstrated in rats, mice, lambs, and sheep (Bélanger, 1956; Harkness and Bern, 1957; Ryder, 1959; Downes, Lyne, and Clarke, 1962). The cysteine could be incorporated into precursor proteins that are formed in the hair bulb and carried up to the keratogenous zone as suggested by Harkness and Bern (1957), or into new proteins as they are synthesized in the keratogenous zone. Braun-Falco (1958) states that protein synthesis does not occur in the upper part of the keratogenous zone, but refers to no relevant evidence. Leblond, Everett, and Simmons (1957) showed that S^{35}-methionine is incorporated into the proximal part of the keratogenous zone and concluded that the protein is synthesized there. The present investigation gives additional direct evidence on the amount of the keratogenous zone engaged in protein synthesis. The incorporation of tyrosine into protein at a given site is good evidence that protein synthesis takes place there.

MATERIAL AND METHODS

Observations were made on material obtained from 12 hooded rats. When they were 1 month old, the hair was clipped from one side of the animals at the level of the forelimb where the black and white regions meet. The second wave of hair growth was followed by observing the colour change, from pink to black, over the naked area of skin which carried black hair. The wave begins ventrally and progresses dorsally. When the colour change reached the dorsal skin, each rat was given radioactive DL-tyrosine, 10 μc/gram body weight, by intraperitoneal injection. The tyrosine was generally labelled with tritium and had a specific activity of 434 mc/mM. At the time of injection the rats weighed between 60 and 80 gm. They were killed in pairs 10 minutes, 30 minutes, 12 hours, 36 hours, 7 days, and 30 days after injection. Two pieces of skin with white hair were taken from all the animals, one piece with growing hair, the other with no growing hair.

The samples of skin were fixed in Bouin's solution and embedded in paraffin wax. Radioautographs of 5-μ sections stained with hematoxylin and eosin were prepared by the coating technique of Kopriwa and Leblond (1962). The photographic emulsion was exposed to the sections for 7, 22, and 44 days before it was developed.

Grain counts were made, on the material exposed for 22 days, with a Whipple micrometer in the ocular of the microscope. The length of the sides of the squares on the micrometer grid were measured against a stage micrometer and found to correspond to 7.1 μ on the field seen with the oil immersion 2-mm objective used for the counts. Approximately 1,000 grains were counted for each observation which was recorded as the number of grains per 100 μ^2.

Observations on the way the grains are distributed in clusters were made on areas of 500 μ^2, which is 10 squares on the micrometer grid. The grains present singly, in clusters of two, in clusters of three, and in clusters of more than three, were counted separately in individual squares. Then the frequency with which grains were present in each size of cluster was recorded for 500 μ^2 as a percentage of the total number of grains.

An estimate was made of the difference in relative volumes of cortical cells in the matrix, in the upper bulb, at the junction of the upper bulb and shaft, and at the top of the keratogenous zone. The nuclear fragments were counted in an area of 5,000 μ^2 of the section. The number of nuclear fragments was converted into an estimate of the number of nuclei in a corresponding volume of tissue by Abercrombie's formula (Abercrombie, 1946). The relative volume of the cells at each site was obtained with the following formula:

$$\frac{\text{Number of nuclei in the standard volume of the matrix}}{\text{Number of nuclei in the standard volume of the site}}$$

OBSERVATIONS

The terminology used in this account is based on that of Montagna and Scott (1958) and is summarized in the diagram at the top of Fig. 14. The dilated lower end of a follicle is the hair bulb which comprises the matrix and the upper bulb. The matrix, which is composed of cells that are constantly dividing (Fig. 2), extends from the base of the follicle to the level of the widest part of the papilla. The upper bulb is the region between the matrix and the shaft which is taken to begin where the thickness and number of cell layers of the outer root sheath increases (Figs. 1 and 3). The lower part of the shaft which stains with hematoxylin and eosin is referred to as the keratogenous zone. The fully keratinized and hardened region of the shaft does not stain with hematoxylin and eosin (Fig. 4).

Hair that was not growing at the time of injec-

tion shows no evidence of radioactivity in the cortex of any of the samples, regardless of the time interval between the injection and the death of the animal.

The radioactivity in hair that was growing at the time of injection is different for each time interval between the injection and the death of the animal; so separate accounts are given below for each time interval.

10-Minute Specimens

These preparations show that radioactivity is present from the matrix of the bulb to the junction of the keratogenous zone and fully keratinized part of the shaft. There is no radioactivity in the fully keratinized part of the hair cortex.

30-Minute Specimens

The distribution of radioactivity (Figs. 5 to 8) is exactly the same as that in the 10-minute specimens, but the grain density is about double.

Complete longitudinal sections along the axis of 3 different hairs were chosen for each animal. The widths of the fully keratinized part of their shafts were all between 50 and 65 μ. A complete series of grain counts was made at 70 μ intervals along the inner part of the cortex of each of the 6 hairs. While the results from only one hair are presented as a histogram in Fig. 13, the level and distribution of grain density was the same in the other 5 hairs. The grain densities over the matrix and adjoining part of the upper bulb are similar. The grain density over the upper bulb increases as the hair shaft is approached until the highest density is reached over the keratogenous zone. The keratogenous zone has about the same density over its whole length. There is a rapid reduction in the density of grains at the top of the keratogenous zone near the fully keratinized and hardened part of the hair.

There are three regions in which the grain densities over the cortex are about the same: the matrix, the proximal part of the upper bulb, and the distal part of the keratogenous zone. The way in which the grains are distributed in clusters was investigated in these three regions of the 6 hairs. The results from all the hairs are similar to those shown in Table I. The percentage of single grains is lower, and the percentage of grains in clusters of three is higher, for the distal part of the keratogenous zone than for the matrix and the upper bulb. The constant difference between the shaft and the other two regions is shown to be significant at the 5 per cent level by the sign test ($p = 0.016$).

Grain counts were made over the inner (near the medulla) and outer (near the cuticle) limits of the cortex at exactly the same level along the axis of the follicles. The regions investigated in this manner were the matrix, the upper bulb, the middle of the keratogenous zone, and the distal part of the keratogenous zone. The density of grains is lower over the outer limit than over the inner limit of the matrix and the distal part of the keratogenous zone (Fig. 8). There was no such difference over the upper bulb and middle of the keratogenous zone.

12-Hour Specimens

These preparations show that radioactivity is present in the hardened cortex beyond the distal limit of the keratogenous zone. This radioactivity of the fully keratinized part of the shaft extends to a level which is just below that of the duct of the sebaceous gland.

The mean grain densities over the hair bulb and proximal keratogenous zone (Table II) are lower than those for the animals killed 30 minutes after the injection (Fig. 13).

36-Hour Specimens

Radioactivity extends 1.3 mm along the fully keratinized part of the cortex from the top of the keratogenous zone to a level just above the skin surface (Fig. 12). This distance is constant for the hair on both animals.

Complete longitudinal sections along the diameter of 3 different hair shafts were chosen in the preparation from one animal. The widths of their fully keratinized shafts were all between 50 and 65 μ. A complete series of grain counts was made at 70-μ intervals along the inner part of the cortex of each hair. The results from one hair are presented as a histogram in Fig. 14. A similar level and distribution of grain density was found in the other hairs. The grain density gradually increases along the hair from the matrix to the fully keratinized part of the shaft (Figs. 9 to 11). Then there is a more rapid increase in density which corresponds to the increase over the upper bulb of the 30-minute material.

No complete longitudinal sections of follicles which are suitable for serial grain counts are present in the material from the second animal.

The hair matrix, the junction of the upper bulb and keratogenous zone, the middle, and the distal part of the keratogenous zone were chosen as four levels which could easily be identified in transverse and oblique sections of follicles. Grain counts were made over the cortex at each level in 3 different follicles. The results were combined with those obtained from the first animal and are presented in Fig. 14.

An estimate was made of the relative volumes

FIGURE 1 A longitudinal section of a growing hair stained with hematoxylin and eosin. The boundaries of the hair matrix (*M*), upper bulb (*U*), keratogenous zone (*K*), and fully keratinized part of the hair are indicated by the arrows. Magnification, 40.

FIGURE 2 A longitudinal section of the lower part of a growing hair bulb stained with hematoxylin and eosin. The line indicates the boundary between the hair matrix (*M*) and the adjacent portion of the upper bulb (*U*). Magnification, 300.

FIGURE 3 A longitudinal section of a growing hair stained with hematoxylin and eosin, showing the junction of upper bulb and keratogenous zone. The arrows indicate the increase in thickness and number of cell layers in the outer root sheath which marks the boundary between the upper bulb (*U*) and the keratogenous zone (*K*). Magnification, 300.

FIGURE 4 A longitudinal section of a growing hair stained with hematoxylin and eosin. The boundary between the keratogenous zone (*K*) and the fully keratinized and hardened part of the hair (*F*) is shown by large arrows for the inner portion and by small arrows for the outer portion of the cortex. Keratogenesis is completed at a lower level in the outer cortex than in the inner cortex. Magnification, 300.

The tissues shown in Figs. 5 to 8 were fixed 30 minutes after injection of the H^3-tyrosine

FIGURE 5 Radioautograph of the *matrix* of a growing hair cut in a longitudinal plane. Magnification, 1,000.

FIGURE 6 Radioautograph of the *upper bulb* of a growing hair cut in a longitudinal plane. The grain density is similar to that of the hair matrix (Fig. 5). Magnification, 1,000.

FIGURE 7 Radioautograph of the *keratogenous zone* of the *cortex* (*C*) of a growing hair cut in a longitudinal plane. The grain density is higher than that of the matrix (Fig. 5) and the upper bulb (Fig. 6). Magnification, 1,000.

FIGURE 8 Radioautograph of the junction of the keratogenous zone and fully keratinized part of a growing hair cut in a longitudinal plane. Compare with Fig. 4. The grain density over the part of the cortex (*C*) near the cuticle (*Cu*) is lower than that near the medulla (*Me*). Magnification, 1,000.

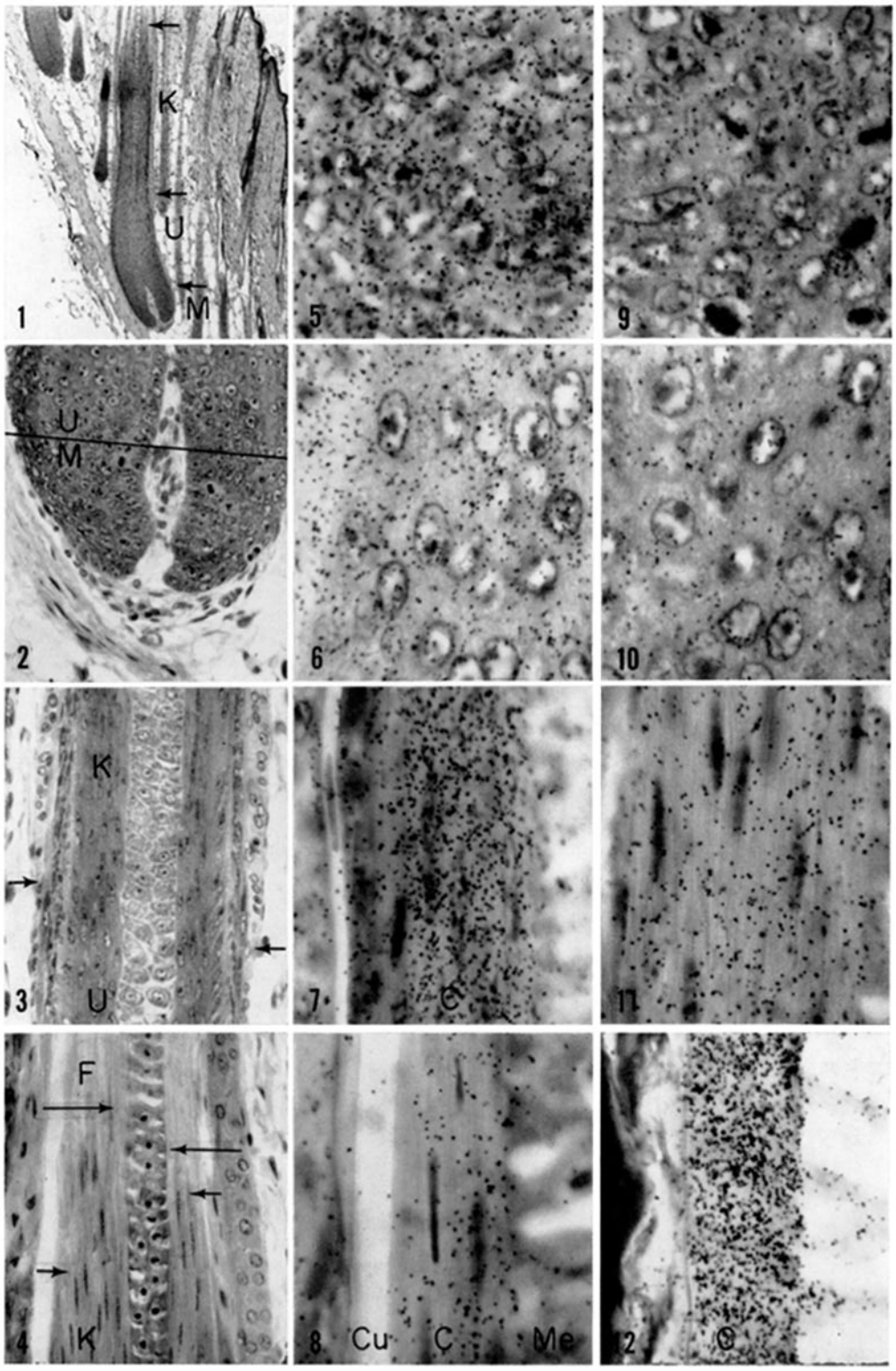
The tissues shown in Figures 9 to 12 were fixed 36 hours after injection of the H^3-tyrosine.

FIGURE 9 Radioautograph of the *matrix* of a growing hair cut in a longitudinal plane. The density of the grains is lower than that in the same region 30 minutes after injection of the H^3-tyrosine (Fig. 5). Magnification, 1,000.

FIGURE 10 Radioautograph of the *upper bulb* of a growing hair cut in a longitudinal plane. The density of the grains is lower than that in the same region 30 minutes after injection of the H^3-tyrosine (Fig. 6). Magnification, 1,000.

FIGURE 11 Radioautograph of the *keratogenous zone* of a growing hair cut in a longitudinal plane. The density of grains is higher than that over the matrix (Fig. 9) and upper bulb (Fig. 10), but lower than that over the same region 30 minutes after injection of the H^3-tyrosine (Fig. 7). Magnification, 1,000.

FIGURE 12 Radioautograph of the *fully keratinized part* of a growing hair at the level of the skin surface. The density of grains over the hair cortex (*C*) is higher than that at the 3 levels illustrated in Figs. 9 to 11. Magnification, 1,000.


K
U
M
1
5
9
U
M
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6
10
K
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C
11
F
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8
Cu
C
Me
12
C

of the cortical cells in the matrix, the upper bulb, the junction of the upper bulb and shaft, and the top of the keratogenous zone next to the fully keratinized part of the hair. This was possible only with the longitudinal sections of follicles because of the elongated shape of the nuclei. The relative cell volume increases at these sites from matrix to keratogenous zone in the ratios 1:2:3:4,

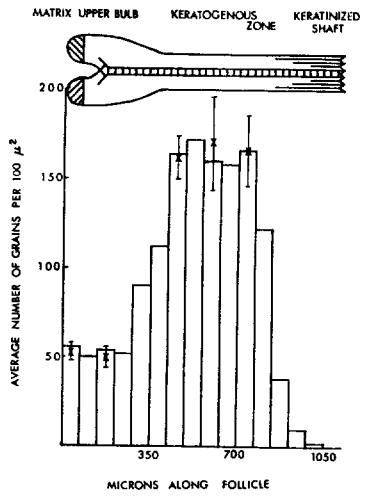

FIGURE 13 Histogram of serial grain counts at 70-μ intervals along the inner part of the cortex of a hair which was fixed *30 minutes* after the rat received H^3-tyrosine, 10 μc/gram body weight. The diagram of a growing hair above the graph is given so that the measurements can be related to their position along the hair. The average background count is 0.3 grains/100 μ^2.

The symbol $\bar{X}$ shows the mean of the values for 6 hairs, with its 95 per cent Confidence Interval.

1:2:4:4, and 1:2:4:5, in the three follicles on which grain counts were made.

7- and 30-Day Specimens

No evidence of radioactivity was found over the hair cortex in the preparations from these animals with the exposure times used.

DISCUSSION

Sites of Protein Synthesis in Hair

After tissues have been fixed in Bouin's solution and processed through alcohol, paraffin wax, xylol, and water, to prepare stained sections, only protein remains in them. There are no free amino acids. The presence of radioactivity in the protein at 10 and 30 minutes after the injection of H^3-tyrosine reveals the sites at which protein is synthesized (Droz and Warshawsky, 1963). The regions in which the hair cortex is radioactive at 10 and 30 minutes after the injection are the matrix, the upper bulb, and the whole of the keratogenous zone. Therefore, these are sites of protein synthesis. No radioactivity is present in the fully keratinized and hardened part of the hair; so no protein synthesis takes place there. The ratios of the grain densities over the cortex in the various regions along the hair are the same in the 10- and 30-minute specimens; so the intense radioactivity over

TABLE I

The Percentage of the Total Number of Grains Present, in the Size of Cluster Indicated, over Three Regions of One Growing Hair

	Matrix	Upper bulb	Distal part of keratogenous zone
	per cent	*per cent*	*per cent*
Single grains	38	53	26
Grains in cluster of 2	24	20	26
Grains in cluster of 3	14	16	34

the whole of the keratogenous zone must indicate the synthesis of proteins there, and not the transfer of protein from other parts of the hair. Since the greatest density of grains is found over the keteratogenous zone at short time intervals after the injection of cysteine as well as tyrosine, it is not necessary to postulate a different mechanism for the incorporation of these two amino acids. The simplest hypothesis is that they are incorporated together in the synthesis of new proteins. Cysteine is not incorporated into precursor proteins carried up by cells from the matrix, as Harkness and Bern (1957) suggested. At the top of the keratogenous zone the density of grains is lower over the outer part of the cortex than over its inner part (Fig. 8) because the fall in grain density takes place at a slightly lower level on the outside of the cortex than on the inside. This can be associated with the completion of keratogenesis and the hardening of the cells which occurs at a slightly lower level on the outside of the cortex than on the inside (Fig. 4). There must be a very precise relationship between the end of keratin synthesis and the harden-

ing of these cells. Braun-Falco's (1958) statement that protein synthesis does not take place at the top of the keratogenous zone is not compatible with the evidence presented here.

The analysis of the way the grains are distributed in clusters, performed on the 30-minute material, shows that the isotope is distributed more evenly in the matrix and upper bulb than at the top of the keratogenous zone (Sims, 1963). On this material it is not possible to discover what is responsible for the difference. The isotope could be in different cell components or in the same component under different spatial conditions. The distribution of RNA, which is known to be involved in protein synthesis, could be related to this problem. Braun-Falco (1958) found that RNA is limited to the lower part of the keratogenous zone and hair bulb. If it is absent from the top of the keratogenous zone, then protein synthesis must be performed by other components of the cells there. It is possible that this difference in the distribution of the isotope reflects the synthesis of different proteins at these sites, as shown by Downes, Sharry, and Rogers (1963).

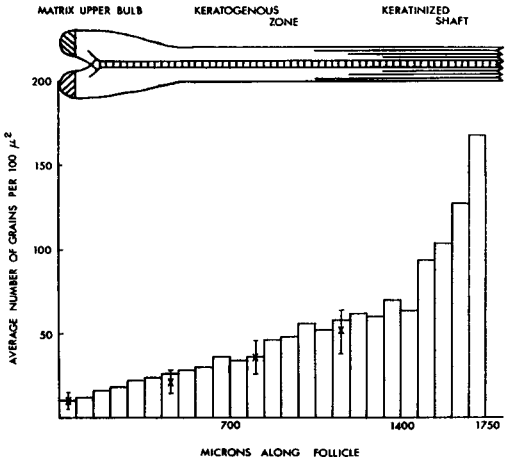

FIGURE 14 Histogram of serial grain counts at 70-μ intervals along the inner part of the cortex of a hair which was fixed *36 hours* after the rat received H^3-tyrosine, 10 μc/gram body weight. The diagram of a growing hair above the graph is given so that the measurements can be related to their position along the hair. The average background count is 0.3 grains/100 μ^2.

The symbol X shows the mean of the values for 6 hairs, with its 95 per cent Confidence Interval.

Migration of Label with Hair Growth

Because none of the labelled tyrosine is incorporated into the fully keratinized part of the hair, it is possible, on this material, to estimate the rate at which hair increases in length. As the time interval between the injection and death of the animals increases, the most distal part of the shaft which is radioactive is found to move away from the hair bulb. At 30 minutes after injection, it is at the top of the keratogenous zone; at 12 hours, it is in the fully keratinized part of the hair just below the level of the duct of the sebaceous gland; at 36 hours, it is just above the skin surface. The hair formed in any given time interval is that between the top of the keratogenous zone and the most distal part of the shaft which is radioactive. The length of this region was found to be 1.3 mm in the two animals killed at 36 hours. Therefore, the rate of hair growth is 0.9 mm/24 hours. This estimate agrees well with that of 0.92 mm/24 hours made by Fleischer, Vidaver, and Haurowitz (1959). The rate of hair growth in sheep (Downes, Lyne, and Clarke, 1962), and man (Myers and Hamilton, 1951), is about 0.35 mm/24 hours. The faster rate in the rat is associated with the distribution of growing hairs in a wave, while the slower rate in sheep and man is associated with the distribution of growing hairs in a mosaic.

Information on the fate of the proteins synthesized in the growing hair can be obtained by consideration of the possible loss of protein from the cells as they travel up the follicle. If protein is broken down at the same rate as it is formed, the amount of it in the cells would remain constant as they travel up the follicle. If protein is broken down more slowly than it is formed, the amount of it in the cells would increase as they travel up the follicle. If protein is broken down more rapidly than it is formed, the amount of it in the cells would decrease as they travel up the follicle. Such a decrease would result in a uniform and low level of grain density along the shaft at 36 hours. The histogram of the 36-hour material (Fig. 14) shows that there is no decrease in the amount of protein in the cells as they pass up the follicle.

The gradual increase of grain density as the shaft is ascended in the 36-hour specimens can be expected if the amount of isotope contained by the matrix cells is halved each time they divide. Thus, the cells produced last at the bottom of the shaft would contain less isotope than those produced earlier towards the top of the shaft.

Comparison of the histogram of the 36-hour specimen with that of the 30-minute specimen suggests that there is more radioactivity in the bulb and keratogenous zone at 36 hours than could have been carried up from the matrix. This was checked by comparison of the actual density of grains in the 12- and 36-hour material with the expected density calculated from the 30-minute material. For this calculation, the movement of the cells along the follicle with time was estimated from the growth rate of the hair shaft. The expected densities of grains at the bottom of the shaft were calculated as follows:

12 hours:

$$\frac{\text{Average density of grains over lower part of upper bulb at 30 minutes}}{\text{Increase in volume of cells from upper bulb to bottom of shaft}}$$

36 hours:

$$\frac{\text{Expected density of grains over matrix at 12 hours}}{\text{Increase in volume of cells from matrix to bottom of shaft}}$$

The expected density of grains over the matrix at 12 and 36 hours was calculated as follows:

$$\frac{\text{Average density of grains over matrix at 30 minutes}}{\text{Number of hours between 30-minute and relevant sample}} \times \frac{13}{2}$$

The fraction $13/2$ in the formula represents the time taken for the cells of the matrix to reproduce themselves. This value has not been estimated for rats; so it was assumed that the value for mice given by Bullough and Laurence (1958) could be used. The expected density of grains for the top of the keratogenous zone was calculated for 36 hours because no assumptions about the matrix are necessary. The calculation was made as follows:

$$\frac{\text{Average density of grains over lower part of upper bulb at 30 minutes}}{\text{Increase in volume of cells from upper bulb to top of keratogenous zone}}$$

The expected and actual densities of grains for the various regions are shown in Table II. Because the actual density of grains over the two positions of the shaft is always greater than the expected density, there can be no breakdown of protein in the cells as they move up the follicle. It has been shown from the 10- and 30-minute material that protein is synthesized throughout the keratogenous zone, so protein must accumulate in the cells as they pass from the primordial state in the matrix to the mature state in the fully keratinized part of the hair. The accumulation of protein during keratogenesis of hair cortex has not been demonstrated before, although it is required by the description of keratin fibres, accumulating and then being surrounded by a matrix, in the accounts of the process such as those of Auber (1950) and Birbeck and Mercer (1957). The possibility may have been obscured by the slight degree of the increase in cell size from the bottom to the top of the keratogenous zone. It seems that the accumulation of protein is not accompanied by an equivalent increase in cell size.

TABLE II

An Estimate of the Error Introduced by the Assumption that a Single Injection of H^3-Tyrosine Behaves as a Pulse Dose

Site	Time after injection	Expected number of grains/100 μ^2	Actual number of grains/100 μ^2 with the 95 per cent confidence interval	Error
	hrs.			*per cent*
Matrix of hair bulb	12	30	39 ± 10	30
	36	10	11 ± 6	10
Bottom of hair shaft	12	30	42 ± 5	40
	36	10	20 ± 7	100
Top of keratogenous zone	36	26	52 ± 15	100

The actual grain densities over the hair shaft are greater than the expected density by 40 per cent at 12 hours, and 100 per cent at 36 hours. These discrepancies are too large to be disregarded. They cannot be the result of an increase in the protein concentration in the cells produced by loss of water, because the cell volumes show no decrease along the keratogenous zone. They must be the result of the incorporation of isotope into the protein of hair after the dose injected has been cleared from the body fluids. The calculations of the expected grain densities assume that the H^3-tyrosine injected behaves as an ideal pulse dose.

Borsook, Deasy, Haagen-Smit, Keighley, and Lowry (1950) showed that less than 3 per cent of C^{14}-labelled amino acids remained in the blood at 10 minutes after its injection. An ideal pulse dose of a tracer is available to the tissues of an animal which receives it for one short period: none of the tracer is available before or after the pulse. A single injection of a labelled amino acid does not behave as an ideal pulse dose. Small amounts of it continue to be available to the tissues after the pulse has gone (Shemin and Rittenberg, 1944). The effect can be ignored in tissues with a rapid protein turnover only if observations are made over short time periods. It cannot be ignored in tissues which have a slow protein turnover if observations are made over long periods, because the error produced becomes too great. The increase of the error with time in this experiment confirms that there is no loss of protein from the system. The interpretations of experiments to estimate rates of protein turnover with labelled amino acids, especially on tissues with slow rates of protein turnover, must be regarded with caution if this error is not considered.

There is no difference between the actual grain densities over the matrix and the expected densities. If the assumed rate of cell turnover is correct, the absence of any accumulation of radioactivity, like that of the shaft, needs comment. It can be postulated that any incorporation of isotope which occurs just balances the loss which results from protein turnover.

This experiment shows that the presence of small amounts of radioactive amino acids in the body fluids can be detected by the examination of growing hair. The possibility of detecting the accidental exposure of laboratory staff at risk to such compounds, by examination of their hair, needs to be explored. Hair is easy to biopsy, and the position of radioactivity along its length would allow the date of exposure to be estimated.

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