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Migration and Keratinization of Cells in Wool Follicles

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Abstract

Migration of cells in wool follicles of an adult Merino sheep was studied autoradiographically in skin samples taken at intervals after an intravenous injection of [^{3}H]thymidine. Fibre and inner root sheath cells incorporated [^{3}H]thymidine in a cone-shaped region of the follicle bulb. Labelled inner sheath cells migrated out of the bulb ahead of contemporaneous cells in the fibre and remained in advance, although to a progressively lesser extent, until the inner sheath cells sloughed into the follicle lumen. Outer root sheath cells incorporated [^{3}H]thymidine along the length of the follicle. Cells in the proximal half of the outer sheath migrated inwards and distally and sloughed into the follicle lumen before contemporaneous inner sheath cells. Other cells in the distal half of the outer sheath migrated past the level where cells from the proximal population were shed and also sloughed into the lumen. In the most distal part of the outer sheath, which formed the epidermis-like lining of the follicle canal, little migration of cells was observed during 8 days of observation.

The specific activity of tritium in fibres plucked from the same sheep at intervals after the intravenous injection of [^{3}H]thymidine was determined by scintillation counting and assessed in terms of cell migration and hardening of the fibres. The time at which the specific activity of solvent-degreased fibres reached a maximum was found to give an estimate of the time for cells in the fibre to migrate to the upper limit of the keratogenous zone. When the plucked fibres were extracted with 8 M urea the times of the maximum specific activities of the urea-dispersible and urea-insoluble material provided respectively estimates of the times at which hardening of the fibres began and ended.

The effects of different planes of nutrition were examined in two other Merino sheep by radioassay of fibres plucked after intravenous injections of [^{3}H]thymidine given after an equilibration period of at least 2 months on each level of feeding. A high plane of nutrition increased the rate of cell migration and hastened the onset of hardening of the fibres, but prolonged the hardening process. The prolongation of the hardening process was confirmed by the specific activities of fibres plucked after intravenous injections of [^{35}S]cystine.

Introduction

The rate of formation of a wool fibre is determined by the proliferation of cells within the follicle bulb and by subsequent growth and migration of the cells. Various studies have been made of the effects of plane of nutrition on cell proliferation in wool follicles (Schinckel 1962; Fraser 1965; Short *et al.* 1965; Wilson and Short 1979), but there is relatively little information on the effects of plane of nutrition on cell migration (Chapman 1971) or keratinization in wool fibres (Marston 1946).

When [^{3}H]- or [^{14}C]thymidine is injected into animals, cells that are synthesizing deoxyribonucleic acid at the time of injection are labelled, and their proliferation and migration may be determined by autoradiography. This procedure has been used to study events within the proximal portions of hair follicles (Cattaneo *et al.* 1961; McCarter and Quastler 1962; Smoliar 1966; Epstein and Maibach 1969), and some

aspects of cell proliferation, migration and sloughing in wool follicles (Downes *et al.* 1966; Chapman 1971; Vsevolodov and Prusova 1976).

The kinetics of incorporation of various compounds by follicles has been studied by injecting radioactively labelled compounds into animals and measuring by liquid scintillation counting the specific activity of fibres plucked at various times after administration of the compounds (De Bersaques 1965; Downes 1965; Downes and Wilson 1971; Wilson *et al.* 1971). When this procedure is used after injection of labelled thymidine in sheep the specific activity of the wool root material rises to a maximum and then declines because much of the radioactive material is removed when cell nuclei in the fibre are degraded during keratinization (Downes *et al.* 1966). By contrast, when [^{35}S]cystine is injected, the specific activity of plucked fibres rises to an almost steady value during the next few days because the cystine is retained in the fibres (Downes 1965).

The passage of [^{35}S]cystine into keratinized fibres can be monitored by comparing the specific activities of plucked fibres before and after extraction with 8 M urea which removes most of the unhardened material (Mercer 1949). The time for the incorporated ^{35}S-labelled material to become non-extractable with urea has been used as an index of the rate of keratinization (Downes 1965).

In the present study the specific activity curves obtained by radioassay of wool fibres plucked from a sheep at intervals after an intravenous injection of [^{3}H]thymidine are evaluated in terms of cell migration and keratinization of the fibres by comparison with the information obtained from autoradiographs of follicles. In addition, the effects of high and low food intakes on the rates of cell migration and keratinization are examined by liquid scintillation counting of fibres plucked after the intravenous administration of [^{3}H]thymidine and [^{35}S]cystine to two other sheep.

Materials and Methods

Experimental Details

For the comparison of the scintillation counting of plucked fibres with the autoradiographic study of cell migration an adult Merino ewe (sheep 1), weighing *c.* 26 kg, was housed indoors and fed 600 g/day of a 1:1 mixture of chopped lucerne hay and whole oat grain. The wool was closely clipped from an area *c.* 30 by 15 cm on the dorsal region of the trunk to prepare an area of uniform staple length from which wool samples could be subsequently plucked for radioassay. Eight weeks later [6-^{3}H]thymidine (3·7 mCi; 5 Ci/mmol; from the Radiochemical Centre, Amersham) was injected intravenously. At the same time dyebands (Chapman and Wheeler 1963) were applied at skin level to the wool in the shoulder, midside and thigh regions on the right side, to enable measurement of fibre growth rate. At intervals from 2 h to 8 days after the thymidine injection, skin biopsies, 1 cm in diameter, were taken from the right side of the body, and wool samples consisting of 10–20 small staples plucked at random were collected from the previously clipped area. Subsequently, the dyebanded staples were carefully removed with fine animal clippers. The samples were processed and examined as described below.

The effects of two widely different planes of nutrition on the rates of cell migration and keratinization were studied in two other adult Merino sheep. These sheep were housed separately indoors and were initially fed 1200 g/day (sheep 2) and 400 g/day (sheep 3) of the same feed mixture as sheep 1. They were weighed weekly. Two areas, each 10 by 10 cm, were outlined by tattooed lines on the left midside region of the sheep, and the wool growth on these areas was harvested by clipping at weekly or fortnightly intervals throughout the period of study. The wool on the right midside was closely clipped to provide an area of uniform staple length for subsequent plucking. Eight weeks later [6-^{3}H]thymidine was injected intravenously at *c.* 30 μCi/kg body weight into each sheep (sheep 2, 1600 μCi; sheep 3, 900 μCi; 5 Ci/mmol). Fourteen days later, L-[^{35}S]cystine (from Schwarz BioResearch Inc, New York (5 μCi/sheep; 41·2 mCi/mmol)) was injected intravenously. At intervals between 1 and 7 days after each intravenous injection, wool samples weighing *c.* 200 mg were plucked

from the dorsal and ventral halves of the previously clipped area on the right midside. To provide samples for the measurement of length growth rate and fibre diameter, a series of three intradermal injections of DL-[3-^{14}C]cystine-HCl [from the Radiochemical Centre, Amersham (0·1 μCi; 39 mCi/mmol; in 0·5 ml 0·9% w/v saline)] was administered at 4-day intervals to two identified sites on each sheep, commencing 8 days after the injection of L-[^{35}S]cystine. The sites were located on the dorsal and ventral edges of the previously clipped region on the right midside.

After the last of these intradermal injections the rations of the sheep were reversed, sheep 2 receiving 400 g/day and sheep 3, 1200 g/day. Three weeks later the wool was again closely clipped from the right midside region of each sheep. After a further 8 weeks the same regime of intravenous and intradermal injections and plucking of wool samples was repeated, the amounts of [6-^{3}H]-thymidine injected being 1236 μCi (sheep 2) and 1250 μCi (sheep 3). Two weeks after the last series of intradermal injections the wool was plucked from the sites of the intradermal injections for measurement of fibre length growth rates and diameters.

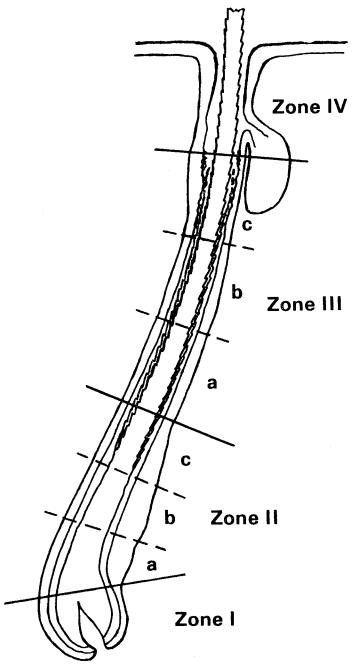


Fig. 1. Zones used for recording the positions of ^{3}H-labelled cell nuclei in the wool follicles at intervals after an injection of [^{3}H]thymidine. Zone 1 extends from the follicle bulb to the level where sulfydryl groups become detectable histochemically in the fibre. Zone II is the region of incorporation of sulfydryl groups in the fibre. Zone III extends from where the sulfydryl content has decreased markedly in the fibre to where root sheath cells slough into the follicle lumen. Zone IV extends from zone III to the skin surface. To enable more precise location of labelled cells in zones II and III, these zones are each subdivided in three subzones, *a–c*, of similar lengths.

Histology and Autoradiography

The skin samples taken for autoradiography were fixed for 6 h in acetic acid–formalin–ethanol (Davenport 1960) and processed into paraffin. Serial sections, 6 μm thick, were cut longitudinal to the follicles. Some of the sections were stained by the Barrnett and Seligman (1952) technique for protein-bound sulfydryl groups. All the sections were covered with Kodak AR10 stripping film, exposed for 9 weeks and developed. The unstained sections were then stained with Ehrlich's haematoxylin and picric acid.

The positions of the ^{3}H-labelled cells in the fibres and inner and outer root sheaths of *c.* 20 follicles per sample were determined microscopically. No distinction was made between cortical and cuticular cells in the fibres, nor of the individual layers of the inner sheath in which labelled cells were present. For the purpose of recording the locations of labelled cells, each follicle was subdivided visually into zones I, II*a–c*, III*a–c* and IV, as shown in Fig. 1, in which zone IV is added to the subdivision described by Chapman (1971). Labelled inner sheath cells upon hardening retained sufficient ^{3}H in their nuclei for their positions to be located autoradiographically until they sloughed into the follicle lumen in zone III*c*. By contrast, cells in zone III of the fibres did not retain sufficient ^{3}H in their nuclei to be detected autoradiographically, due to degradation of their nuclei in zone II*c*. To enable an estimate to be made of the time for cells in the fibres to traverse zone III, the lengths of this zone were measured in the follicles examined.

Radioassay of Plucked Wool

One portion of each plucked sample was degreased with light petroleum, and another was extracted with 8 M urea, water, ethanol and light petroleum, as described by Downes (1965). The samples were then dried and weighed. Those to be assayed for ^{3}H were combusted by the oxygen-flask technique (Downes and Till 1963), whereas the ^{35}S was assayed by direct liquid scintillation counting after addition of scintillation solution (Downes and Till 1963). The radioassays were performed with a Packard liquid scintillation spectrometer (model 3324). Corrections were made for the counting efficiencies of ^{3}H and ^{35}S when the specific activities (disintegrations per minute per milligram wool) were calculated.

Small subsamples of petroleum-washed and urea-extracted plucked fibres were stained with haematoxylin and examined by light microscopy to determine the percentages of fibres with adhering inner and outer root sheath material.

Measurement of Wool Growth, Length Growth Rate and Fibre Diameter

The lengths of staple between the dyebands and basal ends were measured in the dyebanded samples of wool from sheep 1. To these lengths were added 1·5 mm to correct for the pile of wool left by the clippers (Williams and Chapman 1966). The lengths from the dyeband to the basal ends of 50 randomly selected fibres were measured in each sample. The mean length was calculated and, together with the lengths of the shortest and longest fibres, was corrected for the clipper pile by multiplying by the ratio of the corrected to uncorrected staple lengths. The mean daily length growth rates of the shortest, average, and longest fibres in the dyebanded samples from the shoulder, midside and thigh were calculated for use in estimating the migration times of fibre cells through zone III.

The wool samples clipped at weekly or fortnightly intervals from the tattooed areas on the left sides of sheep 2 and 3 were cleaned in a warm aqueous solution of detergent, rinsed with water, dried and weighed. The mean weight of clean wool from the two clipped patches was calculated for each time of sampling.

At least 30 fibres from each of the wool samples plucked from the sites of the intradermal injections of [^{14}C]cystine-HCl on sheep 2 and 3 were mounted on microscope slides. Autoradiographs were prepared using X-ray film, as described by Downes *et al.* (1967). The length growth rates and diameters of those parts of the wool fibres delineated by the autoradiographs were measured by microprojection.

Results

Autoradiographic Determination of Cell Migration

The [^{3}H]thymidine injected intravenously into sheep 1 was incorporated by cells of the fibre and of the inner and outer root sheaths. The incorporation in the fibre and inner sheath was confined to the follicle bulb (zone I), the upper limit of incorporation being approximately cone-shaped with the apex near the tip of the papilla (Fig. 4). In the outer sheath the [^{3}H]thymidine was taken up by cells mainly in the outer layer of zones I-IIIa and zones III*c*–IV (Figs 2, 4, 5). More outer sheath cells were labelled in the proximal zones I and II than in the distal zones III*c* and IV (Fig. 2).

Fig. 3 indicates diagrammatically the percentage of follicles with labelled cells in the various zones at different times after injection of the [^{3}H]thymidine. Labelled cells of the inner sheath had moved into zone II in some follicles at 12 h, whereas labelled fibre cells were not detected in this zone until 24 h (Fig. 6). By this time the most distal labelled cells in the inner sheath had migrated some 50–120 μm ahead of the labelled fibre cells (Fig. 6). In a small proportion of follicles, in which migration was slowest, the difference in movement of cells of the inner sheath and fibre was less. From the data in Fig. 3 it was calculated that the average times for inner sheath and fibre cells in 50% of the follicles to reach the limit of zone I were 18 and 39 h respectively.

At 48 h labelled inner sheath cells in the majority of follicles had reached zone II*b* in advance of labelled fibre cells in zone II*a* (Figs 3 and 7). A difference still existed at 72 h (Fig. 8), at which time labelled inner sheath cells had migrated into zone III*a* in about two-thirds of the follicles, whereas fibre cells had reached the distal limit of zone II*c* in only about one-third of the follicles (Fig. 3). In these latter follicles some contemporaneously formed cells in the fibres may have migrated into zone III*a*, but could not be detected autoradiographically due to loss of the ^3H label during degradation of the fibre cell nuclei near the limit of zone II (Fig. 8). From Fig. 3, it was calculated that the average times for inner sheath and fibre cells to reach the distal limit of zone II were 67 and 74 h respectively.

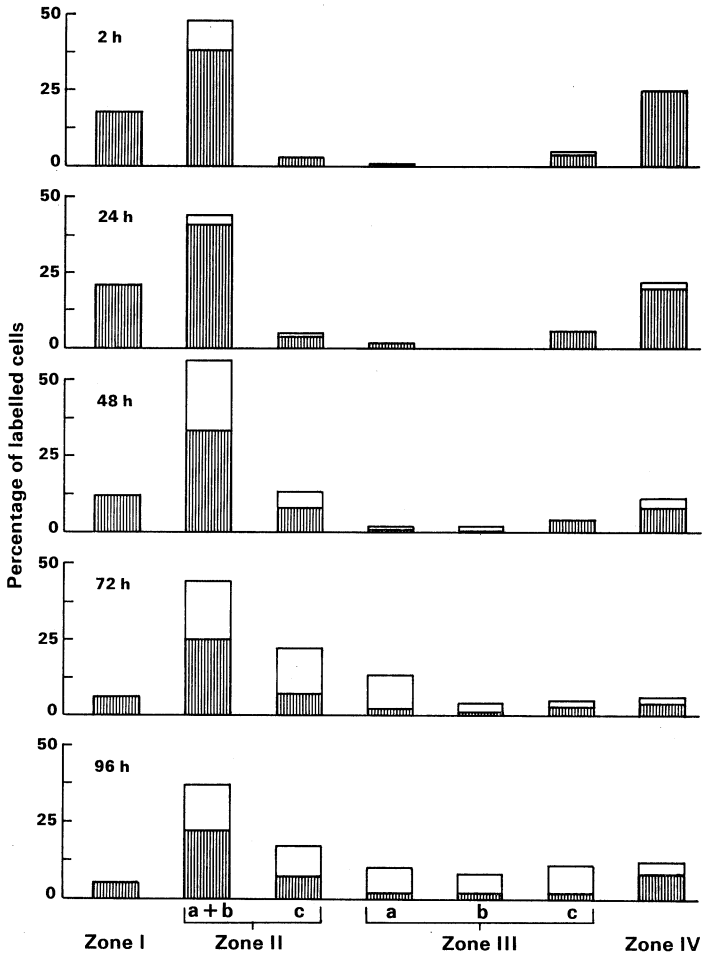


Fig. 2. Distribution of labelled cells in the outer (hatched) and inner (unhatched) layers of the outer root sheath at times after an intravenous injection of [^3H]thymidine, as determined by reconstruction of follicles from serial sections.

As mentioned previously, inner sheath cells retained sufficient ^3H on hardening in zone II to be detected autoradiographically until they were shed into the follicle lumen at the distal limit of zone III (Fig. 9). Shedding of inner sheath cells was occurring in 46% of follicles at 96 h and in all follicles at 120 h (Fig. 3). The average

time for inner sheath cells to be shed was calculated as 98 h. Estimates of the time for fibre cells to migrate through zone III were based on the assumption that the fibres with the slowest, average, and fastest length growth rates (196, 370 and 560 μm/day) grew in the follicles with the shortest, average, and longest lengths of zone III respectively (200, 410 and 640 μm). These values give estimates of 24, 26 and 27 h with a mean of 26 h. Adding this to the average time of 74 h for cells in the fibres to reach the distal limit of zone II gave 100 h as the average time for cells in the fibres to reach the distal limit of zone III.

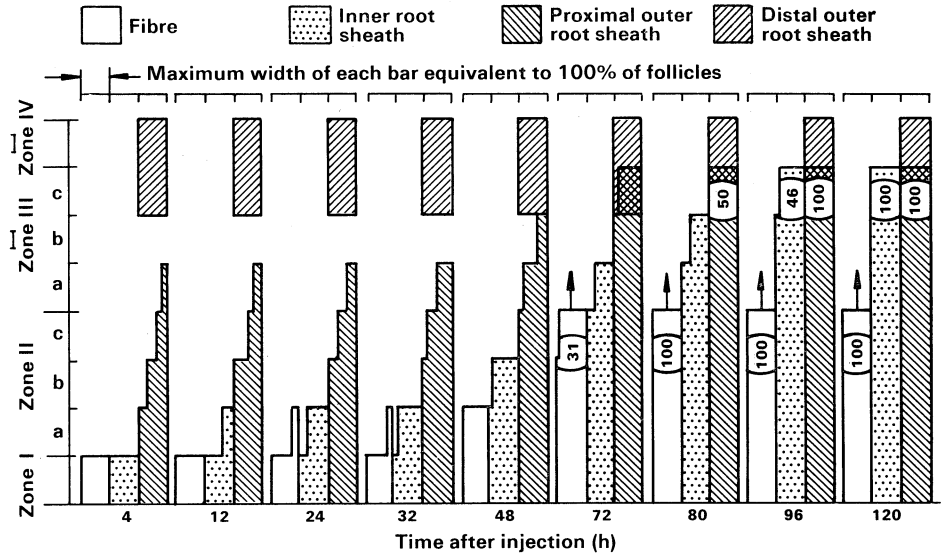


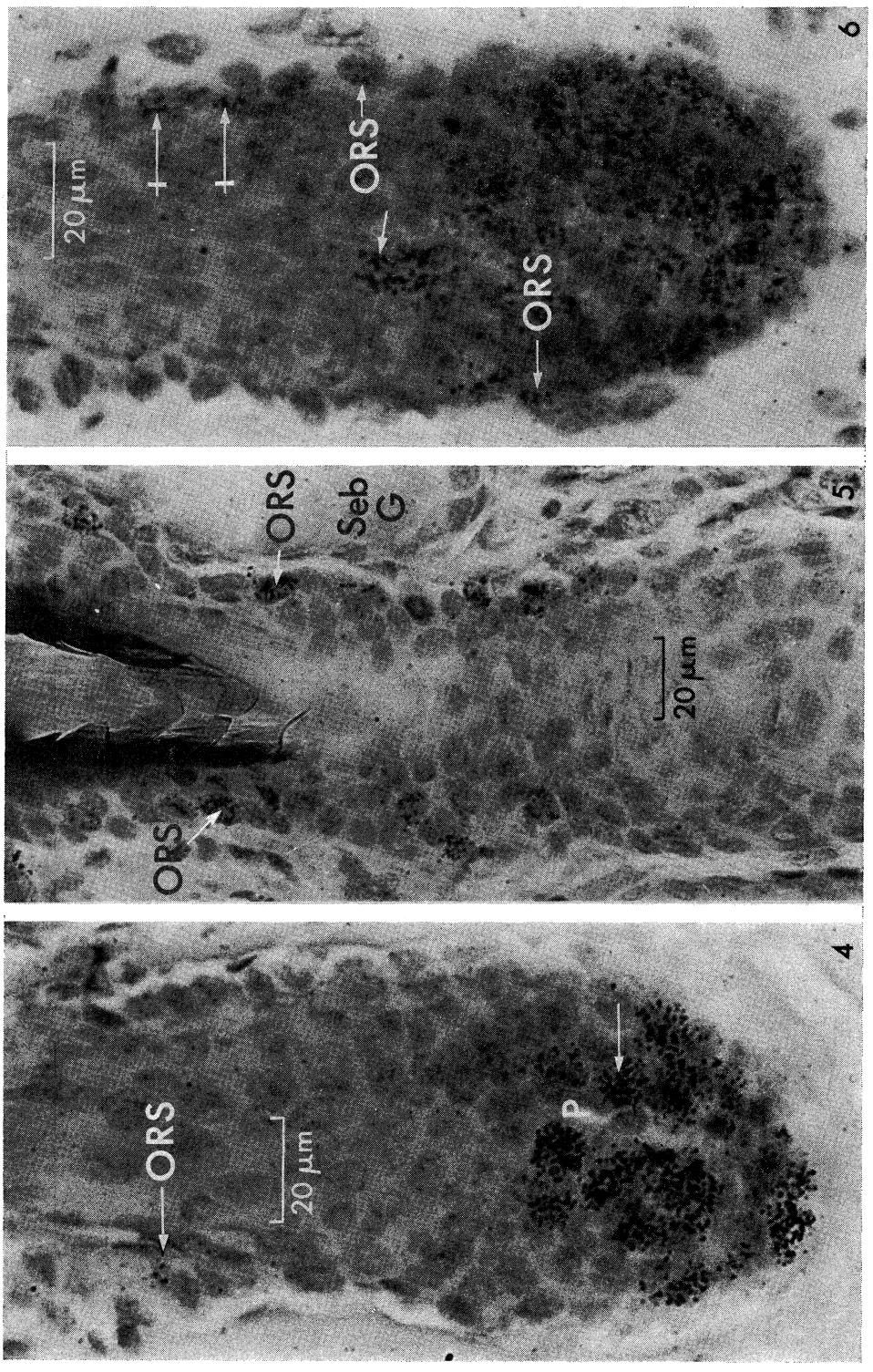
Fig. 3. Percentages of follicles with labelled cells in the various zones of the fibre, inner root sheath, proximal outer root sheath, and distal outer root sheath at times after intravenous injection of [^{3}H]thymidine. The numbers inserted in zone II*c* of the fibres represent the percentages of fibres with labelled cells at the distal limit of zone II at the times of sampling. In some of these fibres, cells may have already become undetectable autoradiographically and passed into zone III (↑). The numbers inserted in the zones III*c* of the inner and outer root sheaths represent the percentages of follicles in which labelled cells were being sloughed at the time of sampling.

The movement of cells in the outer sheath differed from that in the fibre and inner sheath. Outer sheath cells initially in zones I–III*a* moved both distally and laterally towards the inner sheath in zones II–III*b* within 48 h (Figs 2, 3 and 7). Subsequently,

Fig. 4. Autoradiograph of the proximal portion of a follicle 2 h after an intravenous injection of [^{3}H]thymidine. Incorporation of [^{3}H]thymidine by fibre and inner root sheath cells, as indicated by silver grains over nuclei (←), was confined to the bulb, the upper limits of incorporation being approximately cone-shaped with the apex near the tip of the papilla (*P*). A labelled outer root sheath cell (*ORS*) was present in the suprabulbar region.

Fig. 5. Autoradiograph of the distal portion of a follicle 2 h after an intravenous injection of [^{3}H]thymidine. [^{3}H]thymidine was incorporated by cells in the outer layer of the outer root sheath (*ORS*). Seb G, sebaceous gland.

Fig. 6. Autoradiograph of the proximal portion of a follicle 24 h after an intravenous injection of [^{3}H]thymidine. Labelled inner root sheath cells (↔) have moved out of the follicle bulb ahead of labelled fibre cells (←). Labelled outer root sheath cells are also present (*ORS*).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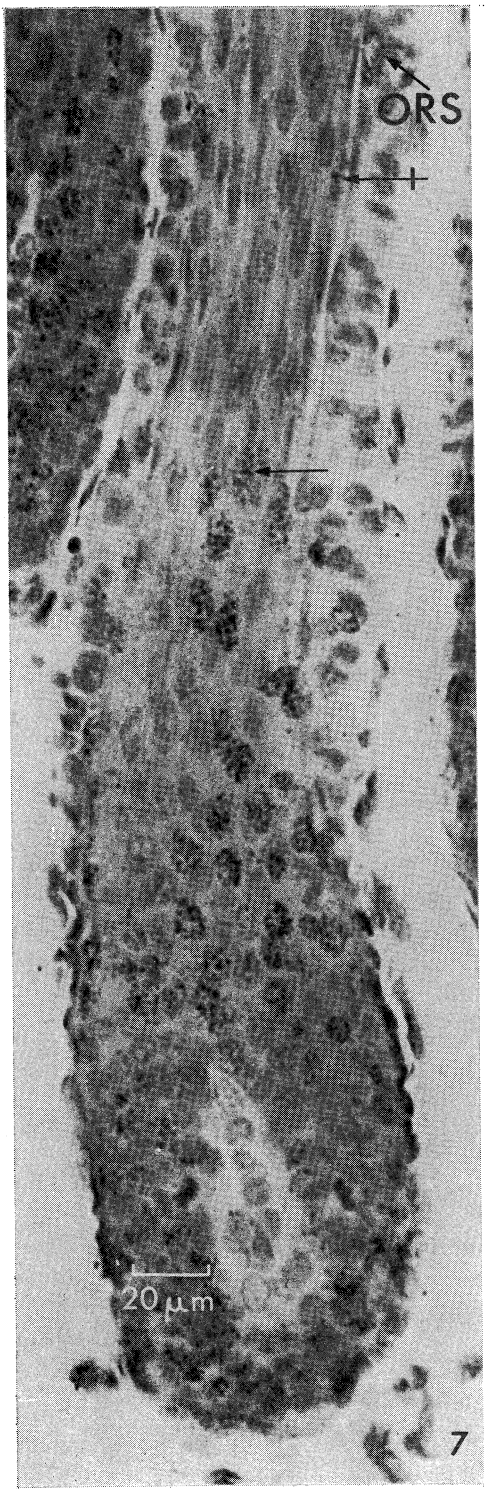
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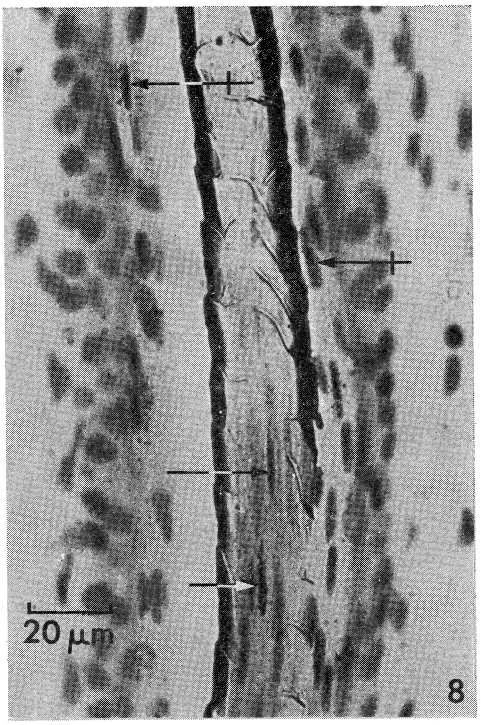
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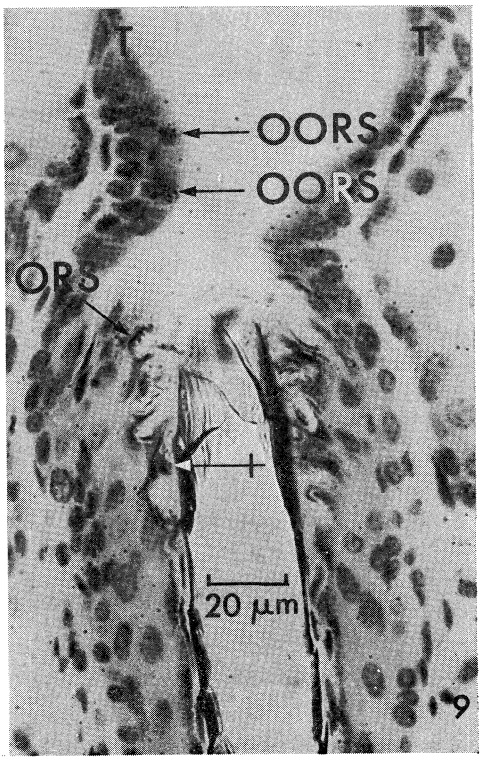
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labelled outer sheath cells moved through zone III in layers adjacent to the inner sheath, and were near the distal limit of zone III in 80% of follicles by 72 h. They were being shed into the lumen in half the follicles at 80 h and in all the follicles at 96 h (Figs 3 and 9).

The distal population of outer sheath cells which incorporated [^{3}H]thymidine in zone III*c* did not appear to move inwards and slough with cells from the proximal population at the limit of zone III. Instead they remained in the outer layers of the outer sheath and moved into the proximal part of zone IV (the transitional zone). At this level they moved inwards and were adjacent to the follicle lumen at 80 h (Fig. 9) into which they were subsequently shed. In the distal half of zone IV (the epidermis-like portion) the labelled outer sheath cells were still predominantly in the outer layers 8 days after injection of the [^{3}H]thymidine, with a few having migrated towards the follicle lumen. Labelled cornified cells were not observed sloughing into the lumen.

Change in Specific Activity of Plucked Wool Fibres

The specific activities of the petroleum-washed fibres and urea-insoluble material in the samples of wool which were plucked from sheep 1 at various times after the injection of [^{3}H]thymidine are shown in Fig. 10. The difference between these specific activities at each time of sampling represents the specific activity of the urea-dispersible material in the fibres, which is also shown in Fig. 10. The specific activities of the petroleum-washed fibres, the urea-insoluble fibres and the urea-dispersible material reached peaks at 78, 87 and 60 h respectively.

Examination of subsamples of the plucked fibres by light microscopy revealed that 45% of the root ends of the petroleum-washed fibres were encased in inner and outer root sheath material. Agitation in 8 M urea dispersed the unconsolidated cells of the lower inner sheaths, some of the distal inner sheath cells, the outer sheath material and the unhardened portion of the fibre roots. After this treatment 27% of the root ends still had hardened inner sheath material adhering to them. Each specific activity is therefore a composite value, and this has to be taken into account in evaluating the changes in the specific activities in Fig. 10.

Fig. 7. Autoradiograph of the proximal portion of a follicle 48 h after an intravenous injection of [^{3}H]thymidine. A labelled inner root sheath cell (↔) is still in advance of the most distal labelled fibre cell (←). A labelled outer root sheath cell (*ORS*) has migrated inwards and is adjacent to the inner root sheath.

Fig. 8. Autoradiograph of the mid-portion of a follicle 72 h after an intravenous injection of [^{3}H]thymidine. At the top of zone II, about two-fifths the distance up the follicle, the nuclei of fibre cells (→) are being degraded and are losing their label. Inner root sheath cells (↔), by contrast, retain their label, and are still in advance of the most distal labelled fibre cell. Some contemporaneous fibre cells may have already lost their label and moved distally undetected. (It was difficult to obtain satisfactory compromise focus of silver grains and underlying tissue at this level in the follicle.)

Fig. 9. Autoradiograph of the distal portion of a follicle as seen in some follicles at 96 h and in all follicles at 120 h after an intravenous injection of [^{3}H]thymidine. Outer root sheath cells (*ORS*) from the proximal population were shed into the follicle lumen immediately above where inner root sheath cells (↔) were shed. Other outer root sheath cells (*OORS*) from the distal population migrated past the zone of sloughing of outer root sheath cells from the proximal population into the lower (transitional) part (*T*) of zone IV. They were adjacent to the follicle lumen at 80 h and were subsequently shed.

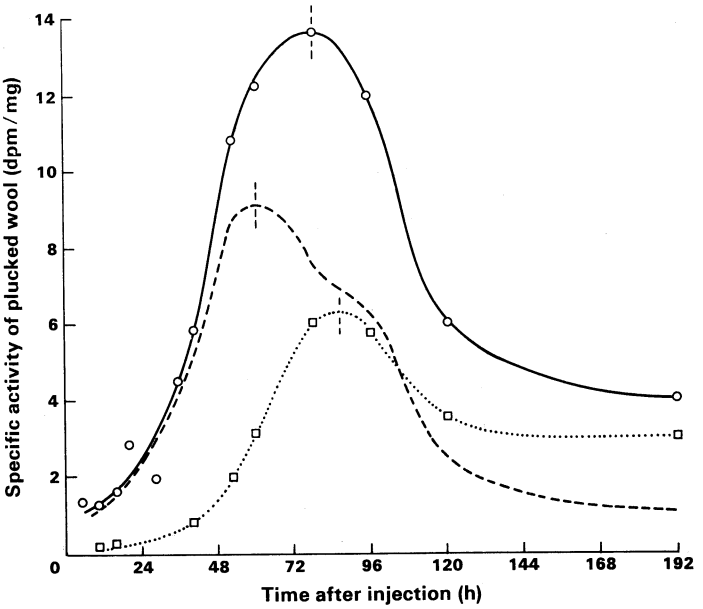

Fig. 10. Specific activity curves of fibres plucked from the dorsal region of sheep 1 at various times after an intravenous injection of [^{3}H]thymidine. ○Fibres degreased with light petroleum. □Fibres extracted with 8 M urea, water, ethanol and light petroleum. ---- Urea-dispersible material (by difference).

Table 1. Effects of different planes of nutrition on the wool growth of Merino sheep

The sheep were fed on the amounts shown of a 1:1 mixture of chopped lucerne hay and whole oat grain

Parameter	Sheep 2		Sheep 3	
Daily feed intake (g)	1200	400	400	1200
Mean body weight (kg)[A]	52	44	30	44
Daily mean wool growth (mg/100 cm^2)[A]	95	45	38	71
Mean fibre length growth rate, L (μm/day)[A]	414	352	290	357
Mean fibre diameter, D (μm)[A]	32·3	25·0	18·7	23·8
$L:D$	12·8	14·1	15·5	15·0
After [^{3}H]thymidine injection:				
Time (h) to reach maximum specific activity in				
(*a*) petroleum-washed fibres	72	84	81	72
(*b*) urea-insoluble material	90	96	98	92
(*c*) urea-dispersible material	66	79	77	66
Time (h) between (*b*) and (*c*)	24	17	21	26
After [^{35}S]cystine injection:				
Time (h)				
(*d*) to reach maximum specific activity in urea-dispersible material	9	11	13	8
(*e*) for ^{35}S in fibres to become urea-insoluble[B]	23	18	20	23

[A] During the periods of sampling after the intravenous injections of [^{3}H]thymidine and [^{35}S]cystine.

[B] Based on the level of ^{35}S present in petroleum-washed fibres when the specific activity of the urea-dispersible ^{35}S is maximal.

The decline in the specific activity of the *petroleum-washed* samples after 78 h resulted from the loss of some of the ^3H from the fibres during degradation of the nuclei near the distal limit of zone II (Fig. 8) (average time 74 h) and from sloughing of labelled outer sheath cells at the top of zone III (Fig. 9) (average time 80 h). The effect of the presence of inner sheath material, in which initially labelled cells were still migrating distally at this time (Fig. 3), would have been to increase slightly the time at which the peak specific activity occurred.

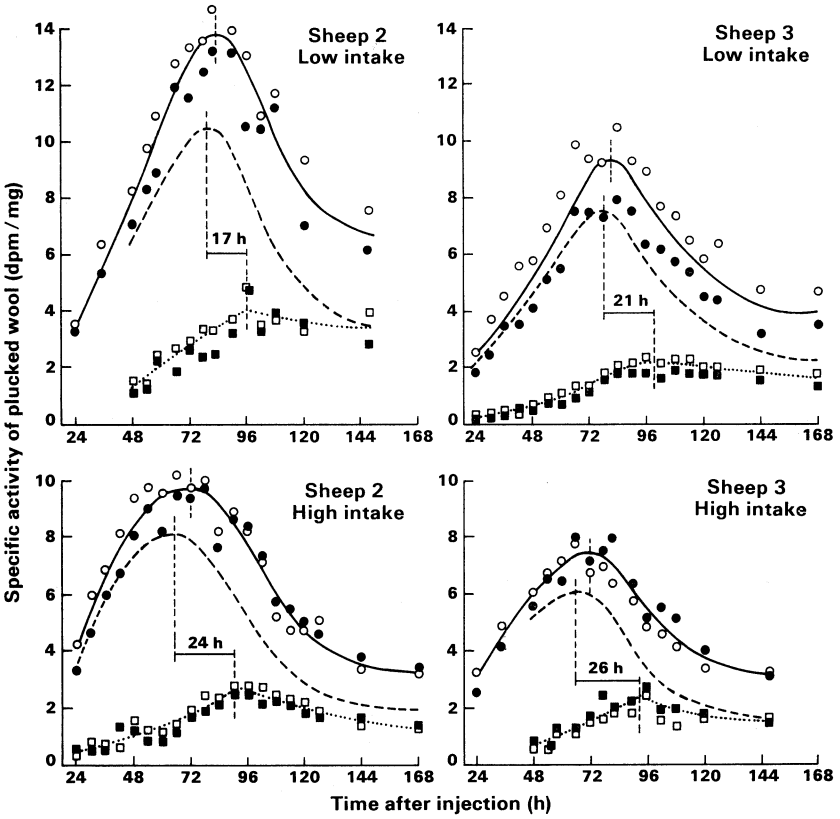

Fig. 11. Specific activity curves of fibres plucked from sheep 2 and 3 at various times after intravenous injections of [^{3}H]thymidine administered during periods of low and high food intake. —— Mean for samples from dorsal (○) and ventral (●) regions after degreasing with light petroleum. ···· Mean for samples from dorsal (□) and ventral (■) regions after extraction with 8 M urea, water, ethanol and light petroleum. – – – – Urea-dispersible material (by difference).

The occurrence of the peak specific activity of the *urea-insoluble* material at 87 h resulted from both the retention of residual ^3H in the fibre after degradation of the nuclei as the cells hardened and the subsequent removal of distal inner sheath cells sufficiently degraded during their migration through zone III to be dispersed by urea.

The decline in the specific activity of the *urea-dispersible* material after 60 h was the combined effect of the commencement of hardening of the fibre and hardening of the layers of adhering inner sheath. The time at which the peak specific activity occurred would have been increased slightly by the presence of dispersed labelled outer sheath cells and dislodged labelled inner sheaths in the urea-dispersible material, because labelled root sheath cells were still migrating distally at this time (Fig. 3).

Effects of Different Planes of Nutrition

The mean body weights, wool growth rates, fibre length growth rates and fibre diameters during the periods of observation for sheep 2 and 3 while on the two planes of nutrition are listed in Table 1. Reduction in the feed intake of sheep 2 decreased its body weight from 52 to 44 kg, daily wool growth from 95 to 45 mg/100 cm^2, fibre length growth rate from 414 to 352 μm/day and fibre diameter from 32·3 to 25·0 μm.

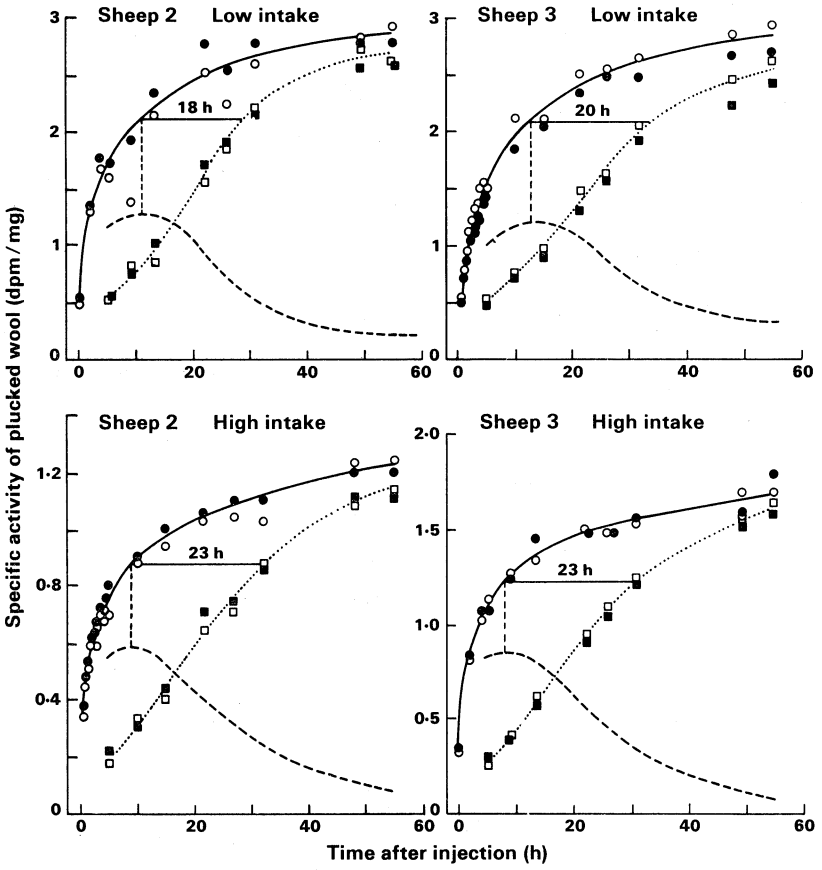

Fig. 12. Specific activity curves of fibres plucked from sheep 2 and 3 at various times after intravenous injections of [^{35}S]cystine administered during periods of low and high food intake. —— Mean for samples from dorsal (○) and ventral (●) regions after degreasing with light petroleum. ···· Mean for samples from dorsal (□) and ventral (■) regions after extraction with 8 M urea, water, ethanol and light petroleum. – – – – Urea-dispersible material (by difference). Also indicated are the times taken for the ^{35}S present when the urea-dispersible ^{35}S is maximal to become urea-insoluble.

Increase in the feed intake of sheep 3 increased its body weight from 30 to 44 kg, daily wool growth from 38 to 71 mg/100 cm^2, fibre length growth rate from 290 to 357 μm/day and fibre diameter from 18·7 to 23·8 μm. Based on the values while the sheep were fed 1200 g/day, these changes represent decreases of 15, 53, 15 and 23% respectively in sheep 2 and increases of 32, 46, 19 and 21% respectively in sheep 3. The ratio of length growth rate to fibre diameter (L:D) increased on the lower feed intake by 10% (12·8 to 14·1) in sheep 2 and by 3% (15·0 to 15·5) in sheep 3.

The specific activities of the petroleum-washed fibres and urea-insoluble and urea-dispersible material in the wool samples plucked at various times after the intravenous injections of [^{3}H]thymidine and [^{35}S]cystine in sheep 2 and 3 are shown in Figs 11 and 12. The times at which maximum specific activities occurred are listed in Table 1. Following injection of [^{3}H]thymidine the specific activity in the petroleum-washed fibres from the two sheep was maximal at 72, 72 h on the high plane of nutrition and at 84, 81 h on the low plane. The corresponding times for the urea-insoluble material were 90, 92 h and 96, 98 h, and for the urea-dispersible material 66, 66 h and 79, 77 h. Following intravenous injection of [^{35}S]cystine the urea-dispersible material reached a maximum specific activity at 9, 8 h on the high plane of nutrition and at 11, 13 h on the low plane (Fig. 12; Table 1). The time taken for the ^{35}S present in the petroleum-washed fibres at these times to become urea-insoluble, which is an estimate of the average time for fibres to keratinize, was 23, 23 h on the high plane of nutrition and 18, 20 h on the low plane (Fig. 12; Table 1).

Discussion

Cell Migration

Cell migration in wool follicles is a well-ordered procedure. Cells destined to enter the inner root sheath and fibre are produced in the follicle bulb, while outer root sheath cells proliferate along the length of the follicle. The pattern of incorporation of [^{3}H]thymidine in the outer sheath indicates that the cells in this sheath behave as two different populations. One, a proximal population, forms the outer sheath of the lower half of the follicle and undergoes renewal in an elongate region extending from around the bulb to half-way up the follicle; the other, a distal population, comprises the outer layers of the outer root sheath in zone III*c* and the epithelium around the follicle lumen in zone IV. The greatest proliferation occurs in the proximal population and, as in other species and breeds (Straile 1962; Epstein and Maibach 1969; Chapman 1971), is mainly in the suprabulbar region and around the keratogenous zone of the fibre where the outer sheath is thickened.

Inner root sheath cells move out of the follicle bulb ahead of contemporaneously formed fibre cells, an occurrence which has also been observed in follicles of the human scalp (Epstein and Maibach 1969). As the cells move distally the difference in the times at which labelled inner sheath and fibre cells reach any particular level becomes progressively less, the time differences at the distal limits of zones I, II and III being 21, 7 and 2 h respectively in the present study. Factors which contribute to the lessening of the difference are marked elongation of fibre cortical cells in zone II (Auber 1950) and progressive degradation and distortion of the inner sheath cells in zone III before they are shed (Gemmell and Chapman 1971). The times for inner sheath and fibre cells to migrate through zones II and III of follicles in the Merino sheep in the present study are of the order of half-a-day less than observed previously in Corriedale sheep on a comparable feed intake (Chapman 1971) and in Khazakh sheep (Vsevolodov and Prusova 1976).

At the commencement of their migration, outer root sheath cells in the proximal population move inwards to the layers adjoining the inner root sheath. A similar inward movement has been observed in follicles of the human scalp (Epstein and Maibach 1969) and Corriedale sheep (Chapman 1971). When labelled outer sheath cells are adjacent to the inner sheath, they migrate upwards and slough into the

follicle lumen immediately distal to where the inner sheath cells slough. However, the outer and inner sheath cells that are sloughing at any one time were not formed contemporaneously because labelled outer sheath cells from the proximal population slough into the follicle lumen *c.* 18 h ahead of contemporaneous inner sheath cells. This is a consequence of the proliferation of the inner and outer root sheath cells at different levels in the follicle.

Within the distal population of the outer root sheath there are two further patterns of cell migration. Cells in the outer layers of zone III*c* migrate into the lower part of zone IV and slough into the follicle lumen. By contrast, in the upper half of zone IV, where the outer sheath cells form the epidermis-like epithelium around the follicle lumen, migration towards the lumen is very slow and shedding of labelled cells was not observed. This may have resulted from either loss of ^{3}H during cornification of the cells before they were shed, as occurs in epidermis (Messier and Leblond 1960; Fukuyama and Bernstein 1961; Epstein and Maibach 1965; Potten 1975), or their migration time exceeded the 8-day period of the present observations.

Radioassay of Plucked Fibres

The time at which the specific activity of the *petroleum-washed* fibres from sheep 1 reached a maximum after an intravenous injection of [^{3}H]thymidine (78 h, Fig. 10) is only 5% longer than the time determined autoradiographically for cells in the fibres to reach the top of zone II (74 h). The time of this maximum specific activity therefore provides a reasonable estimate of the average time for cells in the fibres to migrate to the distal limit of zone II, i.e. to the level where the intensity of staining for sulfydryl groups decreases markedly. This is also the level at which synthesis of the bulk of the proteins in the cortical cells of the fibres ceases, because the cells are replete with the microfibril-matrix complex at this level, although not completely hardened (Chapman and Gemmell 1971).

It can be estimated from Fig. 3 that at the time when the specific activity of the *urea-dispersible* material was maximal (60 h, Fig. 10) labelled inner sheath cells would have reached zone II*c*. By comparison with previous electron-microscopic studies (Chapman and Gemmell 1971), the three layers of inner sheath cells (Henle's layer, Huxley's layer and cuticle) would have hardened respectively on leaving zones I, II*b* and II*a*, and so would have hardened prior to 60 h (Fig. 3). Labelled fibre cells, on the other hand, would have been in zone II*b* at this time (Fig. 3). The upper part of zone II*b* is the region of most intense staining for sulfydryl groups in the fibres (Chapman and Gemmell 1971) and is the level where oxidation of sulfydryl to disulfide bonds commences (Mercer 1961). The time of the maximum specific activity of the urea-dispersible material in fibres plucked after an intravenous injection of [^{3}H]-thymidine therefore provides a reasonable estimate of the average time at which fibre cells start to harden.

Factors which have to be considered in the interpretation of the time at which the specific activity of the *urea-insoluble* material in the fibres and adhering inner sheath cells reaches a maximum are the lack of autoradiographic detection of ^{3}H-labelled nuclei in the fibres in zone III (Fig. 8) and the gradual degeneration of inner sheath cells as they move up zone III (Gemmell and Chapman 1971). The lack of auto-radiographic detection of ^{3}H in the fibres in zone III does not necessarily mean that all of the ^{3}H was removed at the top of zone II. The form of the residual ^{3}H in the

fibres is unknown, but it is anticipated that this tritium-labelled material would become increasingly urea-insoluble as the fibres harden and that the specific activity of the fibres would tend to plateau when hardening is complete. Added to this is the increasing specific activity of the adhering inner sheath cells until such time as they become urea-dispersible. As observed microscopically, this occurs at a level below the top of zone III. Hence they would become urea-dispersible prior to the time of 98 h when they would have normally sloughed. This accounts for the peak in the specific activity of the urea-insoluble material at 87 h and the subsequent decline. But also at 87 h cells in the fibres would have been moving up zone III for 13 h (i.e. 87–74 h), and so would be 13/26, i.e. half-way, up zone III. This is the level at which previous electron-microscopic studies have revealed that hardening of wool fibres is complete (Chapman and Gemmell 1971). Therefore, the time at which the specific activity of the urea-insoluble material reaches a maximum in fibres plucked after an intravenous injection of [^{3}H]thymidine provides an estimate of the time at which the fibres are fully hardened. Consequently, the difference between this and the time of the maximum specific activity in the urea-dispersible material is an estimate of the time taken for the fibres to keratinize .

From the above it can be seen that radioassay by scintillation counting of the wool fibres plucked after injection of [^{3}H]thymidine in sheep does provide information on aspects of cell migration and keratinization in the fibres. Being a simpler and less time-consuming technique than autoradiography, it would be useful in studies with larger numbers of sheep than could be studied autoradiographically.

Effects of Nutritional Change

The differences in wool production by sheep 2 and 3 on the two planes of nutrition (Table 1) resulted from changes in both fibre length growth rate (L) and diameter (D). The $L:D$ ratio remained approximately constant for each sheep, as has been observed in previous nutritional studies (Downes and Sharry 1971).

The maximum specific activity in the petroleum-washed wool after injection of [^{3}H]thymidine (Fig. 11; Table 1) occurred earlier on the higher plane of nutrition by 9–12 h (11–14%) than on the lower plane, indicating that cells in the fibres migrated to the top of zone II more rapidly on the higher plane. The ratio of the times of the maximum specific activities on the two planes of nutrition is rather similar to the inverse ratio of the fibre length growth rates. Hence it would appear that the protein synthetic processes in the fibre and length growth rate are closely related and change in like manner with change in feed intake. The extent of the change in the times for cells in the fibre to reach the top of zone II, however, is small compared with the threefold difference in feed intake and twofold difference in wool production. This is in agreement with previous cell migration times determined autoradiographically in Corriedale sheep on various planes of nutrition (Chapman 1971).

The commencement and completion of hardening of the fibres, as judged by the times of the maximum specific activities of the urea-dispersible and urea-insoluble material after injection of [^{3}H]thymidine (Fig. 11; Table 1) also occurred earlier on the higher plane of nutrition than on the lower. However, the actual hardening process was longer on the higher feed intake (24, 26 h *cf.* 17, 21 h). This is substantiated by data obtained on fibres plucked after intravenous injection of [^{35}S]cystine (Fig. 12; Table 1).

In interpreting Fig. 12, the mode of incorporation of [^{35}S]cystine in wool follicles differs from that of [^{3}H]thymidine. The [^{35}S]cystine is incorporated into the keratogenous zone of wool fibres (Ryder 1958; Downes *et al.* 1962; Chapman and Gemmell 1973) and, in contrast to [^{3}H]thymidine, is retained in the fibres as indicated by the increase in the specific activity of the petroleum-washed fibres with time (Fig. 12). The time of the maximum specific activity of urea-dispersible ^{35}S (Table 1) represents the average time after injection of [^{35}S]cystine for the conversion of sulfydryl groups to disulfide bonds to commence and for fibres to start to harden. The length of time required for the ^{35}S, which is present in the petroleum-washed fibres at the time they start to harden, to become urea-insoluble (Table 1) is an estimate of the average time for the fibres to keratinize. No information relating to cell migration can be obtained from the changes in the specific activity of fibres plucked after an intravenous injection of [^{35}S]cystine.

The maximum specific activity of the ^{35}S in the urea-dispersible portion of the fibres was reached more quickly on the higher plane of nutrition that on the lower (9, 8 h *cf.* 11, 13 h) (Fig. 12; Table 1). But the time taken for the fibres to keratinize was longer on the higher feed intake (23, 23 h *cf.* 18, 20 h) in agreement with the finding following injection of [^{3}H]thymidine. Hence it would appear that the hardening process in the fibres is rate-limited and the time required depends largely on the amount of fibre to be hardened.

It can be concluded from the above data provided by scintillation counting of fibres plucked after intravenous injections of [^{3}H]thymidine and [^{35}S]cystine that a high plane of nutrition increases the rate of cell migration and hastens the onset of hardening of the fibres, but prolongs the hardening process.

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References

Auber, L. (1950). The anatomy of follicles producing wool-fibres, with special reference to keratinization. *Trans. R. Soc. Edinb.* **62**, 191–254.

Barrnett, R. J., and Seligman, A. M. (1952). Histochemical demonstration of protein-bound sulphydryl groups. *Science* **116**, 323–7.

Cattaneo, S. M., Quastler, H., and Sherman, F. G. (1961). Proliferative cycle in the growing hair follicle of the mouse. *Nature (London)* **190**, 923–4.

Chapman, R. E. (1971). Cell migration in wool follicles of sheep. *J. Cell Sci.* **9**, 791–803.

Chapman, R. E., and Gemmell, R. T. (1971). Stages in the formation and keratinization of the cortex of the wool fiber. *J. Ultrastruct. Res.* **36**, 342–54.

Chapman, R. E., and Gemmell, R. T. (1973). An ultrastructural autoradiographic study of the incorporation of [^{35}S]cystine in the wool fibre cortex. *J. Cell Sci.* **13**, 811–9.

Chapman, R. E., and Wheeler, J. L. (1963). Dyebanding: a technique for fleece growth studies. *Aust. J. Sci.* **26**, 53–4.

Davenport, H. A. (1960). 'Histological and Histochemical Techniques.' p. 165. (Saunders: Philadelphia.)

De Bersaques, J. (1965). 'Over de Synthese van Keratine.' (Arscia: Brussels.)

Downes, A. M. (1965). A study of the incorporation of labelled cystine into growing wool fibres. In 'Biology of the Skin and Hair Growth'. (Eds A. G. Lyne and B. F. Short.) pp. 345–64. (Angus and Robertson: Sydney.)

Downes, A. M., Chapman, R. E., Till, A. R., and Wilson, P. A. (1966). Proliferative cycle and fate of cell nuclei in wool follicles. *Nature (London)* **212**, 477–9.

Downes, A. M., Clarke, W. H., and Dagg, T. C. (1967). Use of radioisotopes in the measurement of wool growth. *Atom. Energy, Sydney* **10**(2), 2–7.

Downes, A. M., Lyne, A. G., and Clarke, W. H. (1962). Radioautographic studies of the incorporation of [^{35}S]cystine into wool. *Aust. J. Biol. Sci.* **15**, 713–19.

Downes, A. M., and Sharry, L. F. (1971). Measurement of wool growth and its response to nutritional changes. *Aust. J. Biol. Sci.* **24**, 117–30.

Downes, A. M., and Till, A. R. (1963). Assay of tritium, carbon-14 and sulphur-35 in wool by liquid scintillation counting. *Nature (London)* **197**, 449–50.

Downes, A. M., and Wilson, P. A. (1971). The incorporation of ^{14}C-leucine into the proteins of plucked wool follicles. *J. Invest. Dermatol.* **57**, 285–94.

Epstein, W. L., and Maibach, H. I. (1965). Cell renewal in human epidermis. *Arch. Dermatol.* **92**, 462–8.

Epstein, W. L., and Maibach, H. I. (1969). Cell proliferation and movement in human hair bulbs. In 'Advances in Biology of Skin'. Vol. 9 (Eds W. Montagna and R. L. Dobson.) pp. 83–97. (Pergamon Press: Oxford.)

Fraser, I. E. B. (1965). Cellular proliferation in the wool follicle bulb. In 'Biology of the Skin and Hair Growth'. (Eds A. G. Lyne and B. F. Short.) pp. 427–46. (Angus and Robertson: Sydney.)

Fukuyama, K., and Bernstein, I. A. (1961). Autoradiographic studies of the incorporation of thymidine-H^3 into deoxyribonucleic acid in the skin of young rats. *J. Invest. Dermatol.* **36**, 321–6.

Gemmell, R. T., and Chapman, R. E. (1971). Formation and breakdown of the inner root sheath and features of the pilary canal epithelium in the wool follicle. *J. Ultrastruct. Res.* **36**, 355–66.

Marston, H. R. (1946). Nutrition and wool production. Proc. Symp. on Fibrous Proteins. pp. 207–14. (Soc. Dyers and Colourists: Leeds.)

McCarter, J. A., and Quastler, H. (1962). Effect of dimethylbenzanthracene on the cellular proliferation cycle. *Nature (London)* **194**, 873–4.

Mercer, E. H. (1949). The formation of keratin during growth. *J. Text. Inst.* **40**, T640–9.

Mercer, E. H. (1961). 'Keratin and Keratinization.' pp. 218, 220. (Pergamon Press: Oxford.)

Messier, B., and Leblond, C. P. (1960). Cell proliferation and migration as revealed by radioautography after injection of thymidine-^{3}H into male rats and mice. *Am. J. Anat.* **106**, 247–85.

Potten, C. A. (1975). Epidermal transit times. *Br. J. Dermatol.* **93**, 649–58.

Ryder, M. L. (1958). Investigations into the distribution of thiol groups in the skin follicles of mice and sheep and the entry of labelled sulphur compounds. *Proc. R. Soc. Edinb.* B **67**, 65–82.

Sckinckel, P. G. (1962). Variation in wool growth and of mitotic activity in follicle bulbs induced by nutritional changes. *Anim. Prod.* **4**, 122–7.

Short, B. F., Wilson, P. A., and Schinckel, P. G. (1965). Proliferation of follicle matrix cells in relation to wool growth. In 'Biology of the Skin and Hair Growth'. (Eds A. G. Lyne and B. F. Short.) pp. 409–26. (Angus and Robertson: Sydney.)

Smoliar, V. (1966). Effects of ionizing radiation on the hair-bulbs of young rats. *Int. J. Radiat. Biol.* **11**, 21–6.

Straile, W. E. (1962). Possible functions of the external root sheath during growth of the hair follicle. *J. Exp. Zool.* **150**, 207–23.

Vsevolodov, E. B., and Prusova, L. S. (1976). Kinetics of the hair follicle cell population in the sheep. [In Russian.] *Tsitologiya* **18**, 324–8.

Williams, O. B., and Chapman, R. E. (1966). Additional information on the dye-banding technique of wool growth measurement. *J. Aust. Inst. Agric. Sci.* **32**, 298–300.

Wilson, P. A., Henrikson, R. C., and Downes, A. M. (1971). Incorporation of [Me-^{3}H]methionine into wool follicle proteins: a biochemical and ultrastructural study. *J. Cell. Sci.* **8**, 489–512.

Wilson, P. A., and Short, B. F. (1979). Cell proliferation and cortical cell production in relation to wool growth. *Aust. J. Biol. Sci.* **32**, 317–27.

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