

## **Morphological and Protein Synthesis Changes in Skin during Wool Follicle Regeneration after Plucking and Mimosine Administration**

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### *Abstract*

The regeneration of wool follicles after plucking of the wool fibres is compared with the regeneration which occurs after treatment of sheep with the DNA synthesis inhibitor mimosine. The process has been examined by labelling skin proteins *in vitro* with [<sup>3</sup>H]leucine, separating them electrophoretically and measuring the radioactivity of the various proteins. The results show that keratin biosynthesis is not detectable during the early stages of follicle regeneration, and that the pattern of protein synthesis changes is similar whether regeneration follows plucking or mimosine treatment.

### **Introduction**

The amino acid mimosine when administered to sheep causes a temporary cessation of wool growth (Hegarty *et al.* 1964; Reis 1975; Reis *et al.* 1975) and in consequence has been suggested as an agent for chemical defleecing. The compound appears to act primarily by the inhibition of wool follicle DNA synthesis (Ward and Harris 1976), and morphological studies of sheep skin have shown a partial degeneration of the wool follicle structure after mimosine administration (Hegarty *et al.* 1964; Reis *et al.* 1975). Upon removal of the inhibitor, the wool follicles regenerate, but little information is available concerning the functional changes which occur in the follicle during this period of regeneration. Neither is it known whether the regeneration process differs from that which occurs following mechanical rather than chemical damage to the wool follicle.

In the present study some aspects of the regeneration of wool follicles were examined after plucking and after mimosine treatment. The hair growth cycle of most domestic breeds of sheep encompasses many years, with the result that essentially all follicles are in the anagen phase of the cycle (i.e. actively growing fibres), and a morphological study of the regeneration of such follicles after plucking was made. The results obtained supplemented the descriptions of earlier workers (Johnson 1965; Silver *et al.* 1967; Lyne and Hollis 1968; Lyne *et al.* 1974) and enabled an accurate comparison of the regenerative events with those described by Reis *et al.* (1975) for the regeneration of wool follicles after mimosine treatment. The protein synthesis activity of wool follicles during regeneration after plucking and after mimosine treatment was also examined by measuring the distribution of radioactivity in extractable skin proteins after they were labelled *in vitro* with [<sup>3</sup>H]leucine.

The results show that there are many similarities in the protein synthesis changes which occur during regeneration either after mimosine treatment or plucking, but

that additional qualitative and quantitative protein synthesis appears to be associated with the mimosine-induced regenerative process.

## Materials and Methods

The sheep used in this study were adult Dorset Horn × Merino crossbred ewes, 40–45 kg in weight, which were maintained indoors in individual pens and fed a diet of 6 parts chopped lucerne and 4 parts crushed oats (600 g per day).

For the study of follicle regeneration after plucking, four animals were used. An area of approximately 15 by 15 cm was selected on the midside of each animal and the wool fibres were plucked from it by the Araldite adhesive method described by Wilkinson (1970).

For the study of follicle regeneration after mimosine treatment, three animals were used. Mimosine was administered by intravenous infusion via the jugular vein at a dose rate of 80 mg per kg liveweight per 24 h for 2 days (Reis *et al.* 1975), and skin samples were taken from the midside of the treated animals at appropriate times.

Skin samples were processed for microscopic examination as described by Chapman and Gemmell (1971).

For the *in vitro* labelling of skin proteins, skin slices (1 by 10 mm approximately) were incubated in Waymouth's medium with L-[4,5-<sup>3</sup>H]leucine (57 Ci/mmol; 2–20  $\mu$ Ci/ml medium) for 30–120 min as described previously (Ward and Harris 1976). The skin slices were briefly rinsed in unlabelled medium at 4°C, suspended in 10 volumes of a buffer consisting of 0.01 M tris-HCl (pH 8.6)–0.01 M EDTA–8 M urea and homogenized in an Ultraturrax homogenizer rotating at maximum speed. Three homogenizations, each 10 s in duration, usually provided adequate homogenization, and an ice-bath placed around the homogenizing vessel maintained the temperature of the homogenate below 5°C. Collagen and keratin fibres and large tissue fragments were removed by filtration through cloth and the homogenate was then centrifuged at 105 000 *g* for 90 min. The supernatant solution was collected and the proteins reduced and alkylated as described by Downes *et al.* (1966). The solution was dialysed exhaustively against distilled water at 4°C and lyophilized.

Protein extracts from fully keratinized wool were prepared as described by Downes *et al.* (1966).

For electrophoresis, the lyophilized samples were dissolved in 0.05 M tris–0.038 M glycine buffer (pH 8.3) containing 8 M urea. Samples (20–300  $\mu$ g protein) were separated in 7.5% polyacrylamide gel slabs or rods in the presence of 8 M urea and using the buffer system of Davis (1964). The stacking gel was omitted from gel slabs. Protein bands were located by staining with 0.04% Coomassie blue G250 in 3.5% perchloric acid (Reisner *et al.* 1975).

The distribution of radioactivity in gel rods was determined by freezing unstained gels and cutting them transversely into 1-mm slices according to the method of Matsumura and Noda (1973), and dissolving each slice in 0.6 ml of H<sub>2</sub>O<sub>2</sub> (30% w/v) by incubating in capped vials at 60°C overnight. Radioactivity was then measured after the addition of 8 ml of a toluene-based scintillation mixture containing 0.3% butyl-PBD–0.02% dimethyl POPOP (2 parts) : Triton X-100 (1 part). The patterns of radioactivity distribution obtained were highly reproducible both for duplicate runs of a single protein sample and for duplicate protein samples obtained from different animals. The electrophoretic mobilities of the stained protein bands and of radioactive peaks were determined with reference to the distance of migration of a bromophenol blue marker band.

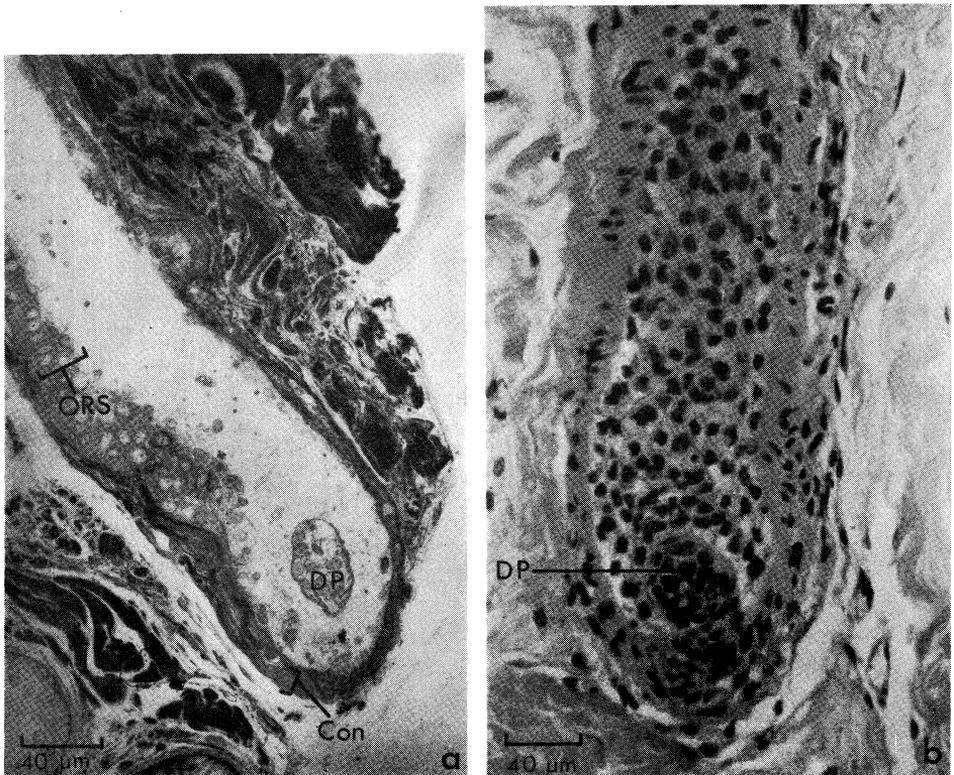
Protein concentrations were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

## Results

### *Follicle Regeneration after Plucking*

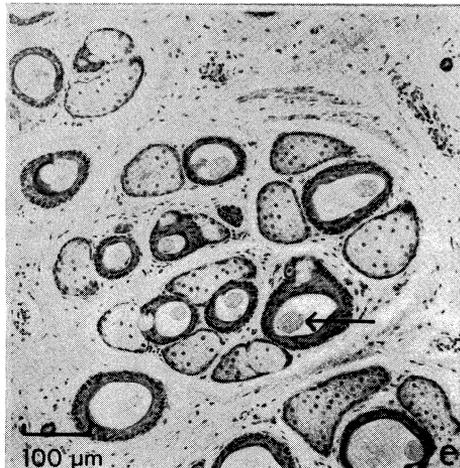
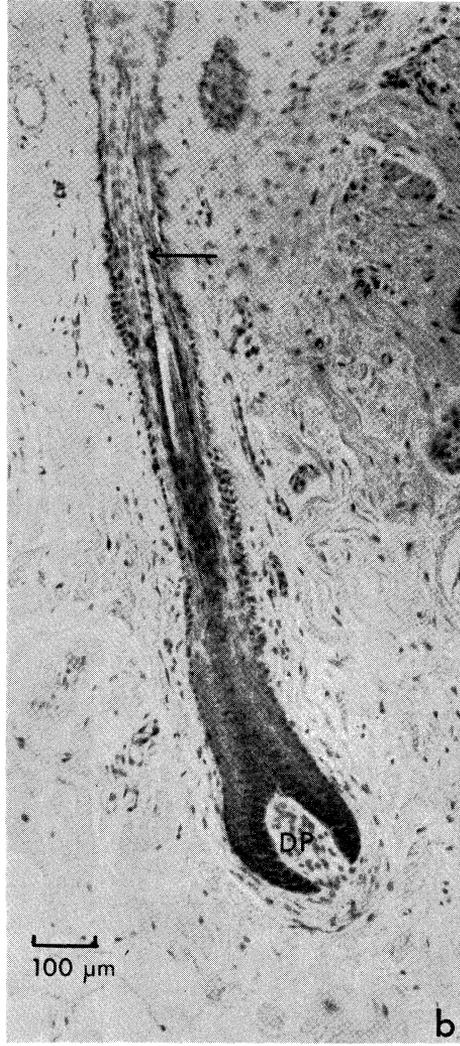
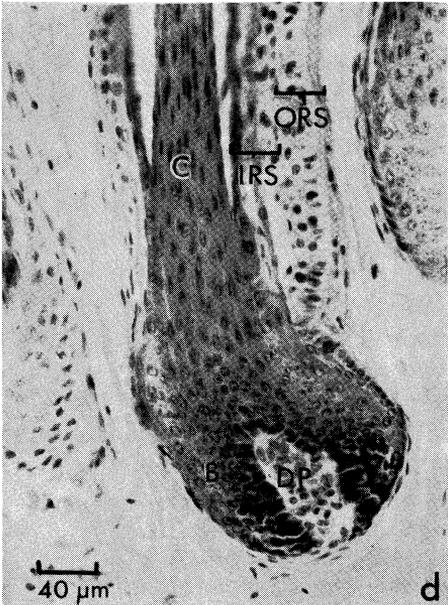
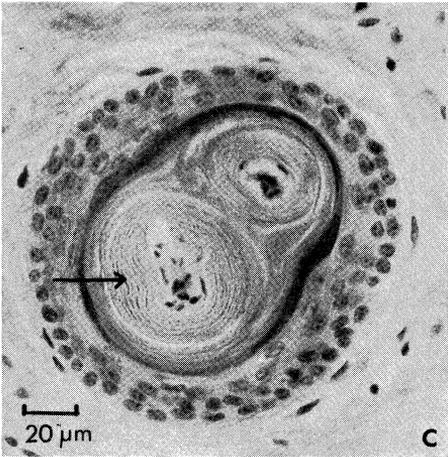
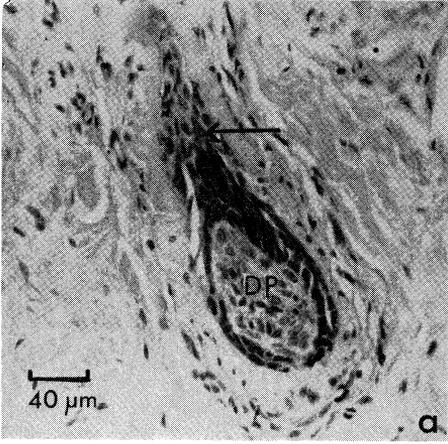
An example of the damage caused to wool follicles by plucking the keratinized fibres is shown in Fig. 1*a*. Most of the follicle bulb cells, differentiating keratinocytes and inner root sheath cells have been withdrawn with the fibre, leaving the outer root sheath and the connective tissue sheath in position. The amount of inner root sheath which remained after plucking was variable between follicles, but in all other respects the appearance of the follicle in Fig. 1*a* was typical of 90% of a total of 688 plucked

follicles examined by serial longitudinal sectioning. In the remaining follicles the fibre had broken higher up and the cells of the bulb were not removed. In all follicles examined a small number of keratinocytes could be seen in the bulb, generally located close to the dermal papilla, which was rarely removed.



**Fig. 1.** (a) Longitudinal section of the lower part of a follicle, immediately after plucking, showing the removal of most of the bulb cells, differentiating cortical cells, and the inner root sheath layers. The outer root sheath (*ORS*) remains intact. *DP*, dermal papilla; *Con*, connective tissue sheath. (b) Longitudinal section of the lower portion of a follicle 5 days after plucking, showing the dermal papilla (*DP*) and an apparently homogeneous population of cells filling the follicle.

The regeneration of the plucked wool follicles was found to take about 16–18 days from the time of plucking to the time that the tip of a new wool fibre reached the mid-sebaceous gland level of the skin. Each stage of the process showed considerable synchrony between follicles. About 2 days after plucking, the follicle, including much of the lower part of the hair canal, began to fill with a homogeneous population of cells similar in appearance to those of the outer root sheath. These cells continued to increase in number until 5 days after plucking, when they filled the follicle bulb (apart from the region of the dermal papilla) and also the lower hair canal (Fig. 1*b*). After 8 days of regeneration the characteristic cell layers of the wool follicle could be clearly recognized (Fig. 2*a*). The dermal papilla was greatly enlarged, and was surrounded by a layer of eosinophilic cells which extended part of the way up the follicle. Fourteen days after plucking, the reorganization of the follicle bulb was complete and new fibres were visible (Fig. 2*b*). At this time the upper portion of the



hair canal was filled with keratinized lamellar material (Fig. 2*c*) whose origin presumably was either from sloughing outer root sheath cells or from the cells which initially filled the hair canal immediately after plucking. The newly growing fibres were never observed to have reached the sebaceous gland level 14 days after plucking. Nineteen days after plucking, the follicles appeared identical to normal wool follicles (Fig. 2*d*), and transverse sections of the hair canals at mid-sebaceous gland level showed a new fibre in each hair canal (Fig. 2*e*).

The morphological changes which were observed during follicle regeneration suggested that substantial changes may occur in protein synthesis in the skin during this time.

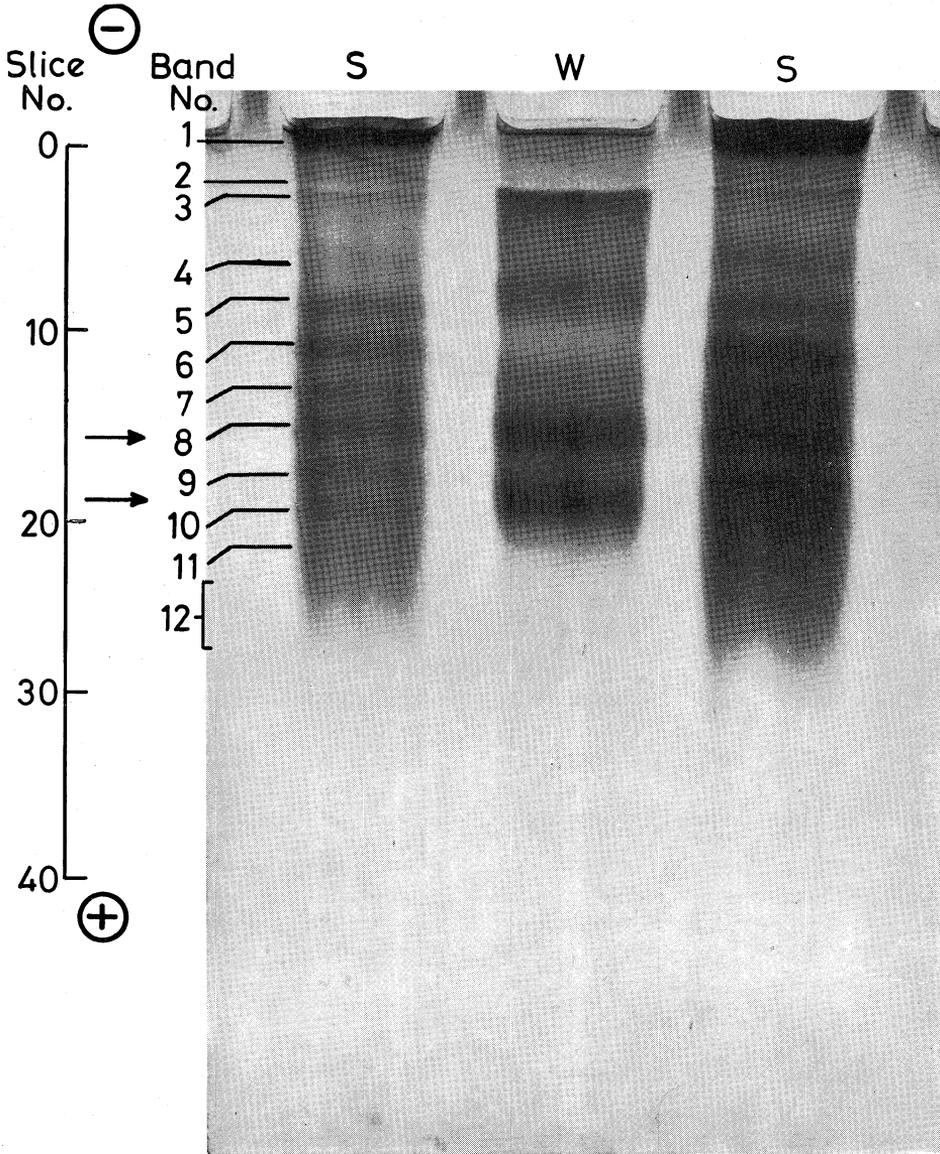
Protein extracts were prepared by homogenizing radioactively labelled skin slices in a solution of 8 M urea buffered at pH 8.6 and containing 10 M EDTA. This solution, which has proved very effective in earlier studies as a solvent for non-keratinized wool and hair proteins (Downes *et al.* 1966), extracted approximately 20% of the protein and between 70 and 80% of the radioactivity of skin slices. Polyacrylamide gel electrophoresis of the reduced and *S*-carboxymethylated proteins of a typical extract of normal sheep skin is shown in Fig. 3. Eleven bands can be observed together with a smear of stained material extending from the last observable band to the dye marker, suggesting the presence of additional non-resolved fast-migrating components. Comparison of the skin proteins with those of *S*-carboxymethylated wool proteins shows that band 8 has an identical electrophoretic mobility with one of the major low-sulphur wool proteins (Fig. 3). This may be coincidental, however, since the other major wool protein appears to run between bands 9 and 10, and the minor wool proteins show little correspondence with skin protein bands.

The distribution of radioactivity in protein extracts of normal skin after *in vitro* labelling with [<sup>3</sup>H]leucine is shown in Fig. 4*a*. The pattern is dominated by two large peaks of radioactivity which correspond with the electrophoretic mobilities of the two major wool proteins. Several smaller peaks of radioactivity could be observed, particularly in the upper portion of the gel, but were not sufficiently clearly resolved to be assigned with certainty to corresponding stained protein bands. The amount of radioactivity was low in the region of the gel containing proteins which migrated faster than the two main wool proteins.

The effect of plucking on the distribution of radioactivity is shown in Fig. 4*b*. The radioactivity associated with the two main wool proteins was very low and instead a broad, poorly resolved distribution of radioactivity was observed in the upper part of the gel.

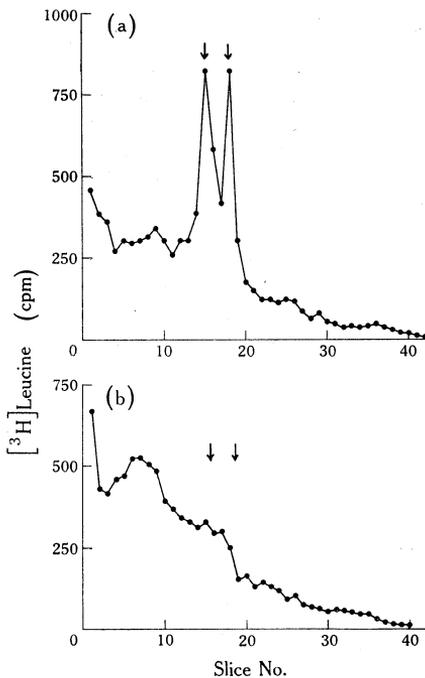
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**Fig. 2.** Morphology of plucked wool follicles at various stages of regeneration. (*a*) Longitudinal section of a follicle 8 days after plucking. The large dermal papilla (*DP*) is capped by a hair cone (→). (*b*) Longitudinal section of a plucked follicle 14 days after plucking, showing the tip of the newly formed fibre (→), the reorganized follicle bulb and enlarged dermal papilla (*DP*). (*c*) Transverse section of a follicle at the same stage of regeneration as shown in (*b*), cut slightly above the mid-sebaceous gland level. Keratinized material (→) in the form of annulate lamellae is clearly visible. (*d*) Longitudinal section of the lower part of a plucked wool follicle 19 days after plucking, showing the structure of the fully regenerated bulb region (*B*) and lower follicle. *DP*, dermal papilla; *IRS*, inner root sheath; *ORS*, outer root sheath; *C*, differentiating cortical cells of fibre. (*e*) Transverse section of a follicle group at the same stage of regeneration as (*d*), cut at the mid-sebaceous gland level showing a new fibre (→) within each hair canal.



**Fig. 3.** Comparison of reduced alkylated skin extract proteins and reduced alkylated wool proteins from control sheep. Skin was homogenized in extraction buffer and the soluble proteins reduced, alkylated, and lyophilized. Samples were dissolved in electrophoresis buffer containing 8 M urea. Skin extract proteins (*S*) (25 and 50  $\mu$ g) and wool proteins (*W*) (50  $\mu$ g) were separated by electrophoresis on a polyacrylamide gel slab and stained with Coomassie blue. Arrows indicate the positions of the two major wool keratin proteins. The correspondence between stained bands and the slice number of fractionated gel rods is also indicated.

A number of changes in the radioactivity distribution occurred during follicle regeneration (Figs 5a–5d). Five days after plucking (Fig. 5a), a large radioactive peak was observed corresponding with band 5, and a second large radioactive peak was associated with band 1. These two peaks were also prominent 8 days after plucking (Fig. 5b), together with a number of clearly resolved smaller peaks of radioactivity in the upper portion of the gel. Very little radioactivity was observed at this time in the region of the two major wool proteins. Fourteen days after plucking, however, these two proteins were associated with clearly resolved peaks of radioactivity (Fig. 5c) which corresponded with histological observations, indicating the formation of the new wool fibres at this time. The two large peaks of radioactivity in bands 1 and 5 were also prominent and a substantial peak of radioactivity was observed in the region of the gel slightly lower than that of the two main wool proteins, in the position of band 12. Nineteen days after plucking (Fig. 5d) the distribution of radioactivity approximated that observed in normal skin, although the peaks associated with the two main wool proteins did not appear as large in proportion to the remaining peaks as was observed in normal skin.



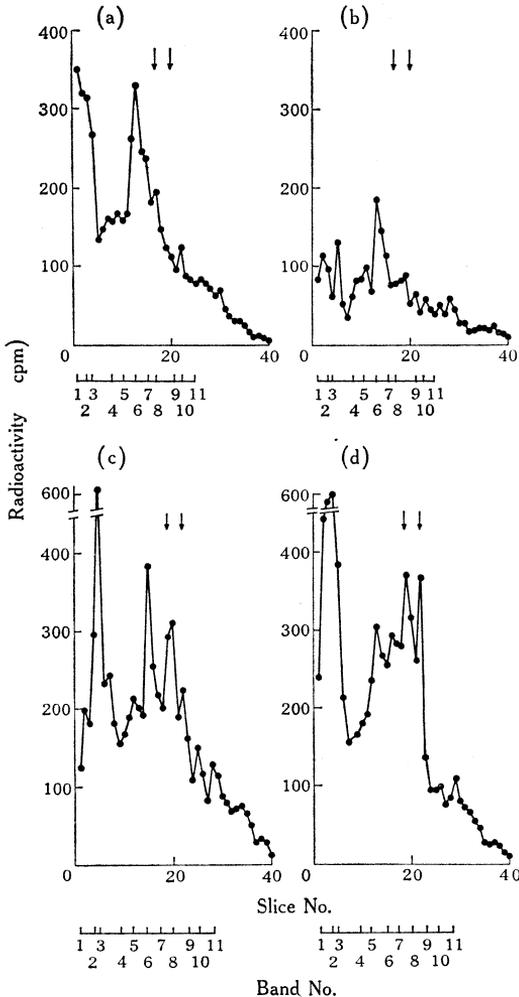
**Fig. 4.** Distribution of [<sup>3</sup>H]leucine in skin protein extracts (a) from normal sheep skin and (b) from sheep skin obtained immediately after plucking. The skin slices were incubated for 2 h in Waymouth's medium containing 20  $\mu$ Ci/ml of [<sup>3</sup>H]leucine and protein extracts were then prepared as described in Methods. Approximately 300  $\mu$ g of protein were subjected to polyacrylamide gel electrophoresis, after which the gels were frozen, cut transversely into 1-mm slices and radioactivity determined. Arrows indicate the positions of the two major wool keratin proteins.

#### *Follicle Regeneration after Mimosine Administration*

The intravenous infusion of mimosine into sheep resulted in the rapid degeneration of the wool follicles, followed by regeneration and the formation of new wool fibres. Although the sheep used in this study were Dorset Horn  $\times$  Merino crossbred animals, the morphological changes which were observed after mimosine treatment were indistinguishable from those described previously for Merino sheep (Hegarty *et al.* 1964; Reis *et al.* 1975). Furthermore, the morphology of the regeneration process appeared very similar to that observed for follicle regeneration after plucking, except

that in most follicles the changes, measured from the time of mimosine administration, were delayed by 3–4 days compared with the equivalent changes observed after plucking.

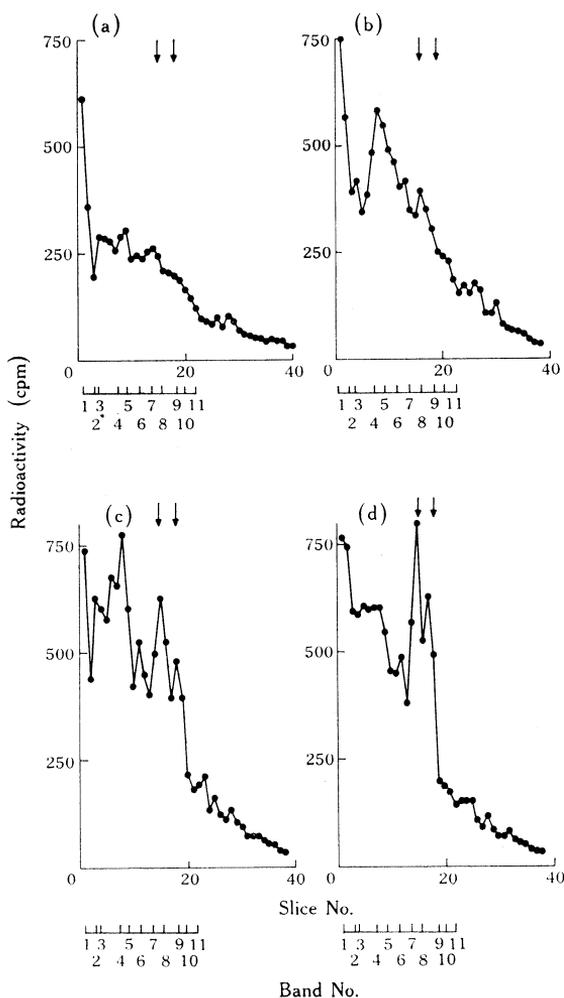
When allowance was made for this time lag, the distribution of radioactivity in electrophoresis gels of skin proteins obtained during regeneration after mimosine administration was similar to that observed during regeneration after plucking. Two days after mimosine administration the radioactivity associated with the main wool



**Fig. 5.** Distribution of radioactivity in skin protein extracts obtained from sheep skin at various stages of wool follicle regeneration after plucking. Protein labelling, electrophoresis and radioactivity determination are as for Fig. 4. (a) 5 days after plucking; (b) 8 days after plucking; (c) 14 days after plucking; (d) 19 days after plucking. Arrows indicate the positions of the two major wool keratin proteins.

proteins was greatly reduced (Fig. 6a) and the distribution of radioactivity appeared similar to that obtained from skin immediately after plucking. This distribution of radioactivity remained essentially unchanged until about 10 days after the start of the mimosine treatment, at which time a peak of radioactivity began to appear in the region of the gel corresponding to band 5. This peak continued to increase in size as regeneration proceeded but, in addition, by 14 days regeneration small peaks of radioactivity corresponded with the positions of the two main wool proteins (Fig. 6b).

After 21 days regeneration (Fig. 6c) the major wool keratin proteins were associated with clearly identifiable radioactivity peaks, but in addition four large peaks of radioactivity were located in positions corresponding with bands 1, 3, 5, and 6. As regeneration proceeded further, these latter peaks became reduced in size relative to those of the main wool proteins, and also were not clearly resolved, so that by 28 days after mimosine administration (Fig. 6d) the radioactivity profile approximated that found for untreated skin samples and also for skin samples 21 days after plucking of wool fibres.



**Fig. 6.** Distribution of radioactivity in skin protein extracts obtained from sheep skin at various stages of wool follicle regeneration after the administration of mimosine (80 mg per kg body weight per day for 2 days). Protein labelling, electrophoresis and radioactivity determination are as for Fig. 4. (a) 2 days after start of infusion; (b) 14 days after start of infusion; (c) 21 days after start of infusion; (d) 28 days after start of infusion. Arrows indicate the positions of the two major wool keratin proteins.

The rate of protein synthesis in skin slices during regeneration was determined *in vitro* as shown in Table 1. No detectable changes were observed during regeneration after plucking. However, the rate varied substantially during the period of follicle regeneration following mimosine administration. The incorporation of radioactivity decreased slightly 9 days after the administration of mimosine, and then rapidly increased to almost 250% of control values by 21 days after mimosine infusion.

## Discussion

The regeneration of wool follicles after the physical damage of plucking and their regeneration after chemical disruption by mimosine appear to be similar morphologically and functionally. The 3–4-day lag between the stage of regeneration after mimosine treatment and the equivalent stage after plucking reflects the period of inhibition of the follicle bulb cells by mimosine, together with their subsequent recovery when the inhibitor concentration falls. Once regeneration commences, however, it proceeds in a well-defined manner, as indicated by the similarities in the protein synthesis patterns after mimosine treatment or plucking. This is not surprising, since the follicle is a complex structure, and the developmental events involved in its formation would be expected to be under tight constraint. The process commences with the bulb and lower follicle filling with an apparently homogeneous population of cells of unknown origin. Similar observations have been made previously in the study of plucked mouse

**Table 1.** Incorporation of [ $^3\text{H}$ ]leucine into sheep skin slices during wool follicle regeneration after plucking or after mimosine treatment

Skin slices were obtained at various times after plucking or mimosine administration, incubated for 60 min in 5 ml of Waymouth's medium containing 2  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]leucine, and trichloroacetic acid-insoluble radioactivity was then measured. Results are the means of triplicate (mimosine) or quadruplicate (plucked) determinations ( $\pm$  s.e.) from single animals, and are representative of three experiments

Plucking	Days after:		Net dpm per mg tissue per 60 min		
	Plucking	Mimosine	Control	Plucked	Mimosine
		-5			439 $\pm$ 17
0		0	481 $\pm$ 27	502 $\pm$ 15	435 $\pm$ 20
2		2	432 $\pm$ 54	662 $\pm$ 39	471 $\pm$ 25
7		7	433 $\pm$ 43	325 $\pm$ 6	
9		9	570 $\pm$ 20	558 $\pm$ 33	264 $\pm$ 36
		14			688 $\pm$ 19
16		16	551 $\pm$ 21	520 $\pm$ 7	
		21			1087 $\pm$ 25
		28			1043 $\pm$ 6

follicles (Silver *et al.* 1967) suggesting that the process may apply generally to regenerating follicles. The cells have a morphological similarity to outer root sheath cells which suggests that they may have migrated from this layer in much the same fashion as other epithelial cells move in response to a wound stimulus (Gillman 1970). The migration of melanocytes from the outer root sheath to the follicle bulb in response to plucking has been clearly demonstrated in Suffolk sheep (Lyne and Hollis 1968) and the capacity for outer root sheath cells, in conjunction with the dermal papilla, to regenerate functional hair follicles has been shown (Oliver 1967). The regeneration of the plucked wool follicle subsequent to its cellular replenishment appears to follow a developmental sequence of sheath and fibre formation similar to that described for the latter stages (stages F4–F8) of wool follicle embryogenesis (Hardy and Lyne 1956).

It is clear that during the early stages of regeneration, keratin biosynthesis is precluded, and its re-initiation does not occur until the follicle is largely restructured. The biochemical mechanism which controls this process probably operates at a fundamental level within the cell and its elucidation constitutes a major area for research in hair growth studies.

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## References

- Chapman, R. E., and Gemmell, R. T. (1971). Stages in the formation and keratinization of the cortex of the wool fibre. *J. Ultrastruct. Res.* **36**, 342–54.
- Davis, B. J. (1964). Disc electrophoresis—II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404–27.
- Downes, A. M., Ferguson, K. A., Gillespie, J. M., and Harrap, B. S. (1966). A study of the proteins of wool follicles. *Aust. J. Biol. Sci.* **19**, 319–33.
- Gillman, T. (1970). Dermo-epidermal interplay with special reference to the healing of cutaneous injuries and to carcinogenesis. In 'An Introduction to the Biology of the Skin'. (Eds R. H. Champion, T. Gillman, A. J. Rook and R. T. Sims.) pp. 318–41. (Blackwell Scientific Publications: Oxford.)
- Hardy, M. H., and Lyne, A. G. (1956). The pre-natal development of wool follicles in Merino sheep. *Aust. J. Biol. Sci.* **9**, 423–41.
- Hegarty, M. P., Schinckel, P. G., and Court, R. D. (1964). Reaction of sheep to the consumption of *Leucaena glauca* (Benth) and to its toxic principle mimosine. *Aust. J. Agric. Res.* **15**, 153–67.
- Johnson, E. (1965). Inherent rhythms of activity in the hair follicle and their control. In 'Biology of the Skin and Hair Growth'. (Eds A. G. Lyne and B. F. Short.) pp. 491–505. (Angus and Robertson: Sydney.)
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–75.
- Lyne, A. G., and Hollis, D. E. (1968). Effects of freezing the skin and plucking the fibres in sheep, with special reference to pigmentation. *Aust. J. Biol. Sci.* **21**, 981–1000.
- Lyne, A. G., Downes, A. M., and Chase, H. B. (1974). Growth of vibrissae in the marsupial *Trichosurus vulpecula*. *Aust. J. Zool.* **22**, 117–29.
- Matsumura, T., and Noda, H. (1973). A simple device for transverse slicing of polyacrylamide gel rods. *Anal. Biochem.* **56**, 571–5.
- Oliver, R. F. (1967). Regeneration of the dermal papilla and its influence on whisker growth. In 'Advances in Biology of Skin'. Vol. 9. (Eds W. Montagna and R. L. Dobson.) pp. 19–33. (Pergamon Press: Sydney.)
- Reis, P. J. (1975). Effects of intravenous infusion of mimosine on wool growth of Merino sheep. *Aust. J. Biol. Sci.* **28**, 483–93.
- Reis, P. J., Tunks, D. A., and Chapman, R. E. (1975). Effects of mimosine, a potential chemical defleecing agent, on wool growth and the skin of sheep. *Aust. J. Biol. Sci.* **28**, 69–84.
- Reisner, A. H., Nemes, P., and Bucholtz, C. (1975). The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focussing on polyacrylamide gels. *Anal. Biochem.* **64**, 509–16.
- Silver, A. F., Chase, H. B., and Arsenault, C. T. (1967). Early anagen initiated by plucking compared with early spontaneous anagen. In 'Advances in Biology of Skin'. Vol. 9. (Eds W. Montagna and R. L. Dobson.) pp. 265–86. (Pergamon Press: Sydney.)
- Ward, K. A., and Harris, R. L. N. (1976). The inhibition of wool follicle DNA synthesis by mimosine and related 4(1*H*)-pyridones. *Aust. J. Biol. Sci.* **29**, 189–96.
- Wilkinson, B. R. (1970). Keratin biosynthesis. I. Isolation and characterization of polysomes from wool roots. *Aust. J. Biol. Sci.* **23**, 127–38.

