

Effects of Internally Administered *N*-[5-(4-Aminophenoxy)pentyl]phthalimide on Wool Follicles and Skin of Sheep

R. E. Chapman and R. D. G. Rigby

Division of Animal Production, CSIRO, P.O. Box 239, Blacktown, N.S.W. 2148.

Abstract

When *N*-[5-(4-aminophenoxy)pentyl]phthalimide was administered to sheep in sufficient quantities to permit manual removal of the fleece (400 mg/kg body weight orally, or 75 mg/kg body weight intravenously during a period of 48 h), cell division ceased in the wool follicle bulbs within 1 day. Dark-staining bodies (autophagic vacuoles) developed concomitantly in the cytoplasm of the bulb cells. The nuclei of cells in the keratogenous zones of the fibres became pycnotic 2 days after dosing and subsequent keratinization of these portions of the fibres was impaired. All the follicles retrogressed prior to day 7 after dosing, and the root ends which formed on the fibres moved towards the skin surface, reaching the level of the sebaceous glands by day 7. At this time mitotic activity recommenced around the dermal papillae in about 50% of the follicles. A small number of tips of new fibres emerged from the skin surface of some of the depilated sheep by day 14. The root ends on the fibres in the fleeces harvested at days 7-15 were fragmented with various degrees of taper. By 21 days, most follicles were growing emergent fibres. Thickening of the epidermis, increase in sebaceous gland size and decrease in skin thickness occurred in some of the depilated sheep.

Smaller doses of the compound (200 mg/kg body weight orally or 40 mg/kg body weight intravenously during 24 h) produced weakness in the wool. Fewer autophagic vacuoles were present in follicle bulbs 1 day after dosing and not all follicles regressed. The weakened region of the fleece contained a mixture of shed fibre ends and continuously growing fibres with thin regions proximal to poorly keratinized lengths of fibre. No change was observed in other components of the skin of the sheep with weakened wool.

The follicular changes produced by this compound are similar in some respects to those produced by other depilatory compounds or that occur during natural cyclic hair growth.

Introduction

Interest is being shown in the harvesting of wool from sheep by the internal administration of chemicals as an alternative to mechanical shearing. Accordingly, compounds reported to induce hair loss in various species are among those being administered to sheep and studied as potential depilating agents. One such compound is *N*-[5-(4-aminophenoxy)pentyl]phthalimide (or 1-*p*-aminophenoxy-5-phthalimido-pentane) which causes loss of wool from adult sheep and lambs (Hughes 1959).

Studies have been undertaken at this laboratory to examine the effects of this compound on wool follicles, fibres and skin of sheep. These studies include oral, intravenous and intraperitoneal administration of the compound.

Materials and Methods

Dosing of Sheep

N-[5-(4-Aminophenoxy)pentyl]phthalimide was prepared by slight modifications to the method of Barber *et al.* (1957). The identity and purity of the compound were established by (a) thin-layer chromatography on SiO₂, using a mixture of three parts benzene and one part ethyl acetate,

(b) melting point determination (m.p. 115–116°C), and (c) spectrometric methods (infra-red, nuclear magnetic resonance, mass spectrometry). The compound was administered to adult Merino ewes and Merino wethers either housed in individual pens and fed on a pelleted mixture of three parts lucerne hay and two parts oat grain or grazed at pasture. The routes of administration and dose rates of the compound, the numbers of sheep treated, and the daily feed intakes are listed in Table 1.

Histology of Skin

Skin biopsies, 1 cm in diameter, were taken from the midside region of each sheep before treatment and at 1, 2, 3 or 4, 6 or 7, 10, 13 or 14, and 20 or 21 days after administration of the compound. Some of the sheep were also sampled at 28, 35 and 42 days.

The biopsies were fixed for 6 h in Serra's fluid, dehydrated through increasing concentrations of ethanol, and embedded in paraffin. Serial sections, 8 µm thick, were cut longitudinal to the follicles and stained with haematoxylin, eosin and picric acid. The sections were examined by light microscopy for changes in the follicles, epidermis, sebaceous and sweat glands, and dermis. The size of the glands was assessed visually and the dermal thickness was measured with an eyepiece graticule.

Table 1. Dose rates of *N*-[5-(4-aminophenoxy)pentyl]phthalimide, numbers of sheep treated, daily feed intakes and resultant effects on fleeces

Route of administration	Dose rate (mg/kg body weight)	No. of sheep	Feed intake (g/day)	Result
Oral	400	12	600	All depilated
		5	1200	All depilated
		6	?(grazing)	All depilated
	200	7	600	Four depilated, one with weakened wool, no effect on two
		5	1200	One depilated, no effect on four
		5	600	No effect
	100	5	1200	No effect
		1	600	Depilated
Intravenous infusion (in propylene glycol)	75 ^A	1	600	Partly depilated
	40 ^A	1	600	Weakened wool
	40 ^B	1	600	No effect
Intraperitoneal injection	100	1	600	No effect
	50	1	600	No effect

^A Infused over 48 h.

^B Infused over 24 h.

Examination of Fibres

Samples of wool were collected either at the time of harvesting the wool, if the sheep were depilated, or by clipping a small area on the midside 1–2 months after treatment if the wool was not harvested. The samples were degreased in Shell X4 solvent. A portion of each was stained with eosin and picric acid or the Barnett and Seligman (1952) technique for sulphhydryl groups. After being rinsed and air-dried, the stained fibres were mounted on microscope slides and examined by light microscopy.

Portions of fibres approximately 1 cm long were cut from the basal ends of unstained portions of the wool samples from sheep depilated by the compound, and were mounted on scanning electron microscope stubs with thin double-sided adhesive tape. After being sputter-coated with gold, the fibre ends were examined in an I.S.I. Super IIIA scanning electron microscope.

Results

Depilating Activity

The effectiveness of the various dose levels and routes of administration of *N*-[5-(4-aminophenoxy)pentyl]phthalimide in removing the wool is summarized in

Table 1. Oral administration of the compound at 400 mg/kg body weight depilated all sheep irrespective of the level of feed intake. However, of 12 sheep dosed with 200 mg/kg only five were depilated and one other had a weakness in the wool. Oral administration of 100 mg/kg had no discernible effect.

Intravenous infusion of 75 mg/kg body weight over a 48 h period caused depilation (Table 1). A smaller dose of 40 mg/kg infused over 48 h partly denuded one sheep, whereas 40 mg/kg infused in a period of 24 h weakened the wool of another sheep but not sufficiently to permit harvesting of the wool. Intraperitoneal injection of as much as 100 mg/kg was ineffective.

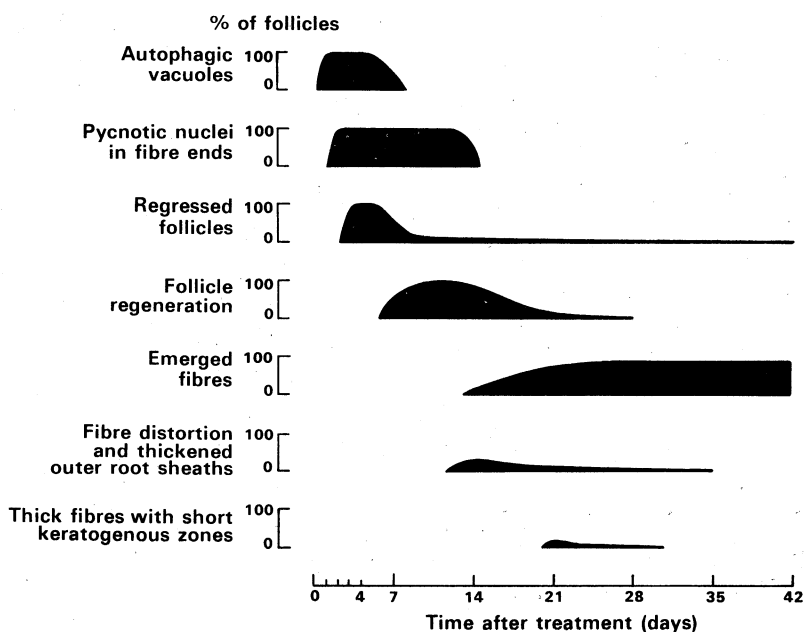
Effects on Follicles and Skin of Depilated Sheep

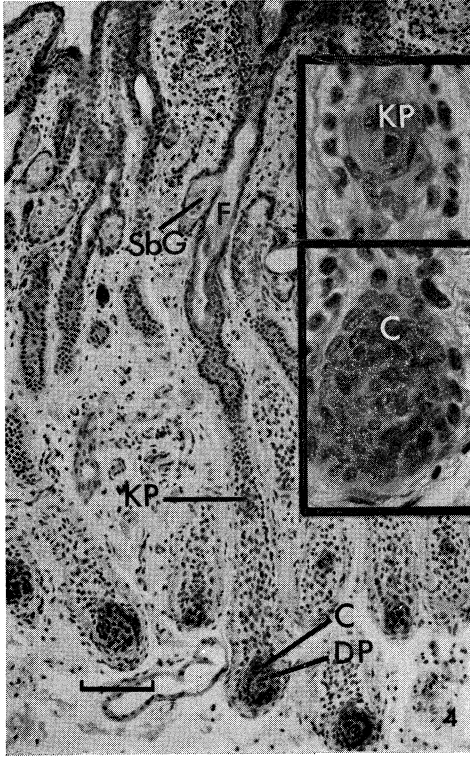
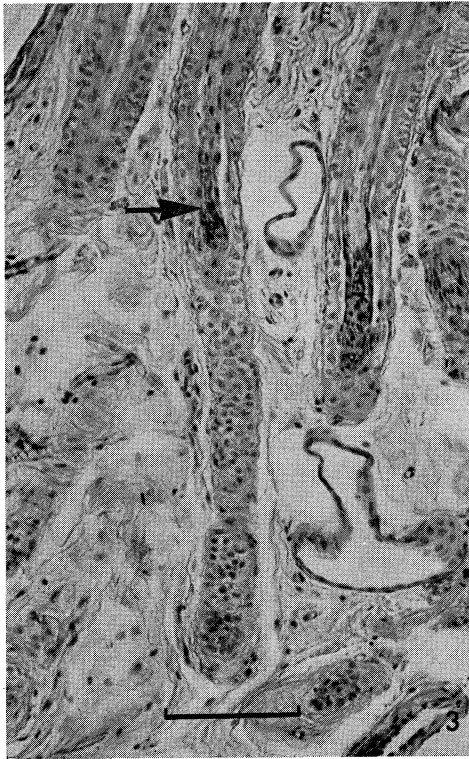
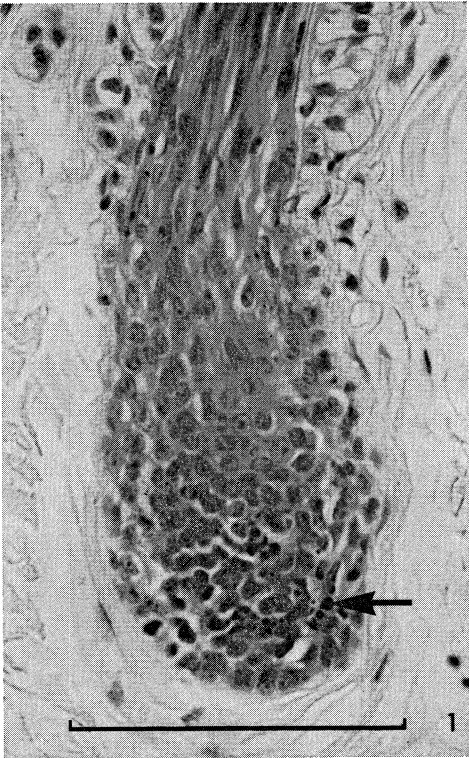
Follicles

The following follicular changes were observed in the 29 sheep denuded by the compound (Table 1):

- (1) development of dark-staining bodies in the cytoplasm of the cells in all follicle bulbs (Fig. 1); examination by transmission electron microscopy, as will be reported elsewhere, revealed that these bodies were autophagic vacuoles;
- (2) development of pycnotic nuclei in the keratogenous zones of the fibres in all follicles (Fig. 2);
- (3) regression of all follicles (Figs 2-4);
- (4) follicle regeneration (Fig. 5);
- (5) distortion of the tips of new fibres before emergence, with concomitant thickening of the surrounding outer root sheaths (Fig. 6), and penetration of the follicle walls by some of the new fibres (Fig. 7); and
- (6) thick fibres with short keratogenous zones in regenerated follicles (Fig. 8).

The following diagram is a schematic representation of the incidence and times of occurrence of various features associated with regression and regeneration of follicles in sheep depilated by the compound:





The autophagic vacuoles developed in the cytoplasm of cells throughout the proliferative zones of all follicle bulbs during the first day after the compound was administered (Fig. 1). Also during this time there was cessation of cell division in the bulbs, as judged by a paucity of mitotic figures. During regression of the follicles, autophagic vacuoles were present in cells around the dermal papillae and in the lower parts of the follicles (Fig. 2). The vacuoles persisted in decreasing numbers beyond day 7 after dosing, but could not be detected at day 14.

Nuclei in the keratogenous zones of the fibres became pycnotic during the second day after dosing (Fig. 2), and were retained in short, poorly keratinized lengths of fibre, distal to the root ends which formed as the follicles regressed (Fig. 3).

Regression of all follicles was proceeding on the second day after dosing, with fibre and inner root sheath cells withdrawing from around the dermal papillae (Fig. 2). By day 3, ends had formed on the fibres and were about 250 μm from the resting papillae to which they were connected by strands of outer root sheath cells. The ends of the fibres had moved half-way up the follicles by day 4 (Fig. 3) and were near the level of the sebaceous glands by day 7 (Fig. 4). Strands, 500–700 μm long, consisting of outer root sheath cells extended from the root ends to the dermal papillae (Fig. 4). In these strands groups of cells sometimes hardened and formed small keratinized 'pearls' (Fig. 4).

When the wool became loose and was harvested at days 7–15 after dosing, many of the fibres broke at the poorly keratinized region above the root end leaving fragments of the fibre ends in the upper parts of the follicles. These fragments were expelled from the follicles during the following week.

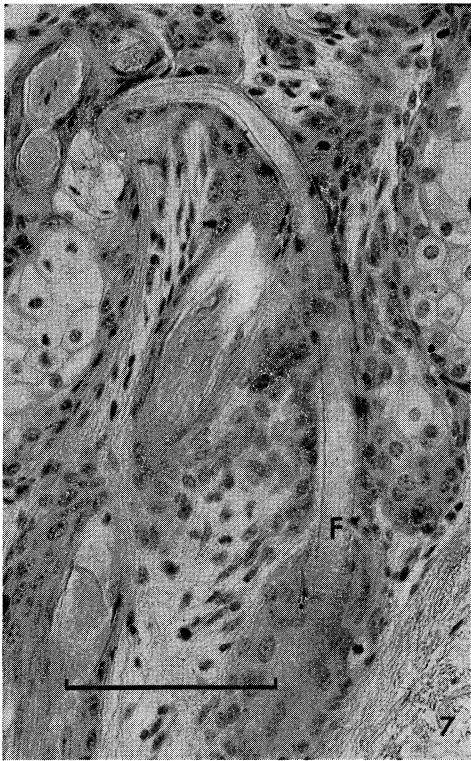
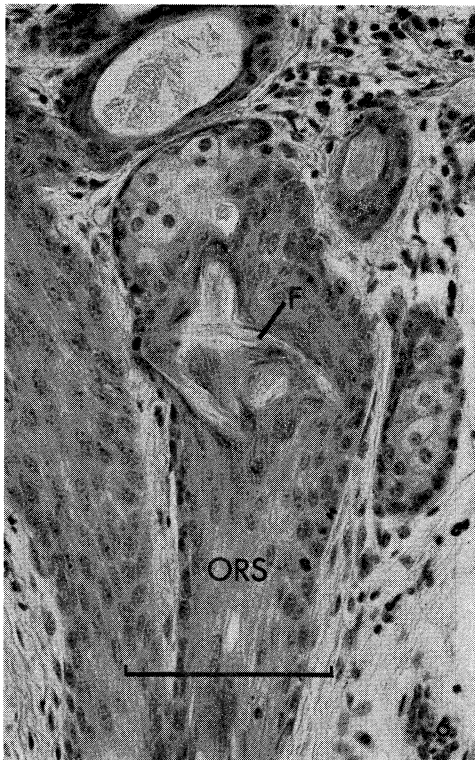
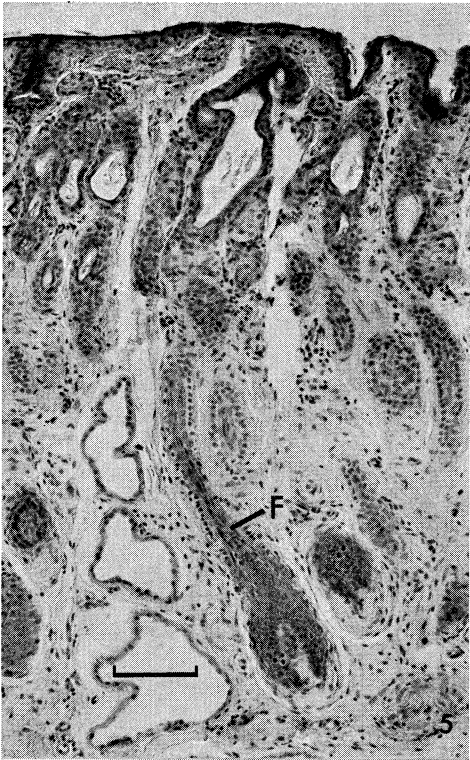
Regeneration of about 50% of follicles had commenced by day 7 with resumption of mitotic activity in cells around the dermal papillae and formation of small cones of inner root sheath cells (Fig. 4). By day 14 about 90% of follicles were in various stages of regeneration from the early cone stage to producing fibres either unkeratinized (Fig. 5) or with keratinized tips almost at skin level. In 4 of the 29 denuded sheep, fibres had emerged above the skin level by day 14, but in only about 10% of the follicles. More than 90% of the follicles were growing emergent fibres in all the sheep at day 21, with the remaining follicles at earlier stages of regeneration, except for 2–3% which were still inactive. All except 1–2% of follicles had fully regenerated by day 28.

Fig. 1. Wool follicle bulb with dark-staining bodies (autophagic vacuoles) (\leftarrow) at day 1 after an oral dose of 400 mg *N*-[5-(4-aminophenoxy)pentyl]phthalimide/kg body weight. Bar = 100 μm .

Fig. 2. Regressing wool follicle with autophagic vacuoles (\rightarrow) in the proximal part of the follicle and pycnotic nuclei (\leftrightarrow) in the keratogenous zone of the fibre at day 2 after the same dose as in Fig. 1. Bar = 100 μm . Inset: Higher magnification of autophagic vacuoles (\rightarrow) in the bulb. Bar = 55 μm .

Fig. 3. Regressed wool follicle with a poorly keratinized root end (\rightarrow) with retained pycnotic nuclei half-way up the follicle at day 4 after the same dose as in Fig. 1. Bar = 100 μm .

Fig. 4. Previously regressed follicle showing signs of regeneration with the formation of a cone of inner root sheath cells (C) on the dermal papilla (DP) at day 7 after the same dose as in Fig. 1. A long strand of outer root sheath cells, frequently containing a keratinized 'pearl' (KP), separates the dermal papilla from the old fibre end (F), which is near the level of the sebaceous glands (SbG). Bar = 100 μm . Insets: Higher magnifications of the keratinized 'pearl' (KP) and cone of inner root sheath cells (C). Bar = 21 μm (upper inset) and 25 μm (lower inset).



Distortion of the tips of new fibres in the upper parts of follicles was a common feature in about 30% of follicles at day 14 (Fig. 6). In the follicles in which this occurred there was concomitant thickening of the surrounding outer root sheath (Fig. 6). In 2–3% of follicles the distorted tips penetrated through the follicle wall into the adjacent dermis (Fig. 7). The proportion of follicles with distorted fibres progressively decreased and was negligible at day 42.

Another feature of the regenerating follicles was the occurrence at day 21 of 5–8% of follicles with large bulbs growing thick fibres with very short keratogenous zones (Fig. 8). This was a transient feature and was observed in only 2–3% of follicles at day 28 and in none at day 42.

Fibre ends

The fibres in the wool harvested from sheep depilated by the compound had fragmented ends with various degrees of taper (Figs 9 and 10). The fracturing appeared to have occurred both between and within cortical cells.

Epidermis

Autophagic vacuoles were not detected in epidermal cells of any of the dosed sheep. In 17 of the 29 depilated sheep, thickening of the epidermis from 2–4 cell layers below the stratum corneum to 5–6 layers became evident around follicle orifices 1–4 days after dosing. A further 10 sheep exhibited this phenomenon after 14 days, particularly in follicles with retained fragments of old root ends or with distorted tips on regenerating fibres. This thickening was accompanied by a considerable increase in the amount of stratum corneum in the follicle orifices. Further general thickening of the epidermis by 1–2 cell layers occurred in seven of the affected sheep at 14–21 days after dosing and persisted with the thickening around the orifices for 2–3 weeks.

Sebaceous and sweat glands

The sebaceous glands in two of the depilated sheep increased slightly in size 21 days after dosing. This increase persisted for 1–2 weeks, after which time the glands returned to their pretreatment sizes. No change was evident in the sweat glands of any of the sheep.

Skin thickness

In 25 of the 29 depilated sheep, skin thickness decreased by 10–50% (mean 27%). The thickness started to decrease as early as day 4 after dosing, was minimal at days 7–10, and generally returned to its pretreatment state 1–2 weeks later.

Fig. 5. Regenerating follicle with a new, unkeratinized fibre (*F*) reaching about half-way to the skin surface at day 14 after the same dose as in Fig. 1. Bar = 100 μ m.

Fig. 6. Distorted tip on a new fibre (*F*) and thickening of the surrounding outer root sheath (*ORS*) at day 14 after the same dose as in Fig. 1. Bar = 100 μ m.

Fig. 7. New fibre (*F*) penetrating through the follicle wall at day 14 after dosing in one of the sheep depilated with an oral dose of 200 mg of the compound/kg body weight. Bar = 100 μ m.

Fig. 8. Portion of a follicle growing a thick fibre (*F*) with a short keratogenous zone at day 21 after dosing in a sheep depilated with the same dose as in Fig. 7. Bar = 100 μ m.

Subdermal fat

No change in the amount of subdermal fat beneath the follicle bulbs was detected in any of the sheep during either regression or regeneration of the follicles.

Effects on Follicles and Skin of Sheep with Weakened Wool

Follicles

In the one sheep that was partly denuded after an intravenous infusion of the compound at 40 mg/kg body weight over 48 h (Table 1), autophagic vacuoles were present in all follicle bulbs at day 1 after dosing, but the vacuoles appeared to be fewer in number than in sheep completely depilated. In the two other sheep with weakened wool after an oral dose of 200 mg/kg and an intravenous infusion of 40 mg/kg over 24 h, autophagic vacuoles were also present at day 1, but not in all follicles. Vacuoles were no longer discernible at day 7 in the sheep with weak wool after oral dosing. However, vacuoles were still present in small numbers at this time in the two sheep infused intravenously.

Reduction in the size of the follicle bulbs was evident in the three sheep at days 1 and 2, and trichohyalin formation appeared to have ceased in the inner root sheath cells in the suprabulbar region and around the lower part of the keratogenous zone of the fibres. This inhibited hardening of the layers of inner root sheath cells and there was concomitant thickening of the fibres at this level (Fig. 11). Partial withdrawal of bulb cells from around the dermal papillae was evident at day 3.

By day 7 about 30% of follicles had regressed in the orally dosed sheep with weakened wool, while about 70% had regressed in the intravenously infused sheep with weakened wool and about 90% in the partly denuded animal. A feature of the regressed follicles in the intravenously infused sheep was the cessation of production of fibre cells before the inhibition of inner root sheath cells. This resulted in strands of inner root sheath cells extending towards the dermal papillae proximal to the somewhat enlarged root ends on the fibres (Fig. 12). The remaining active follicles at this time contained fibres which were very thin proximal to thickened regions near mid-follicle level (Fig. 13). By days 10–14 some of these thin portions of the fibres were kinking in the distal halves of the follicles, inducing thickening of the surrounding outer root sheaths.

Regeneration of the regressed follicles had commenced by day 14. New fibre tips which had almost reached the level of the sebaceous glands were also distorted and the surrounding outer root sheaths thickened. A small proportion (*c.* 5%) of the regenerating follicles were producing thick fibres with short keratogenous zones, similar to Fig. 8, at this time.

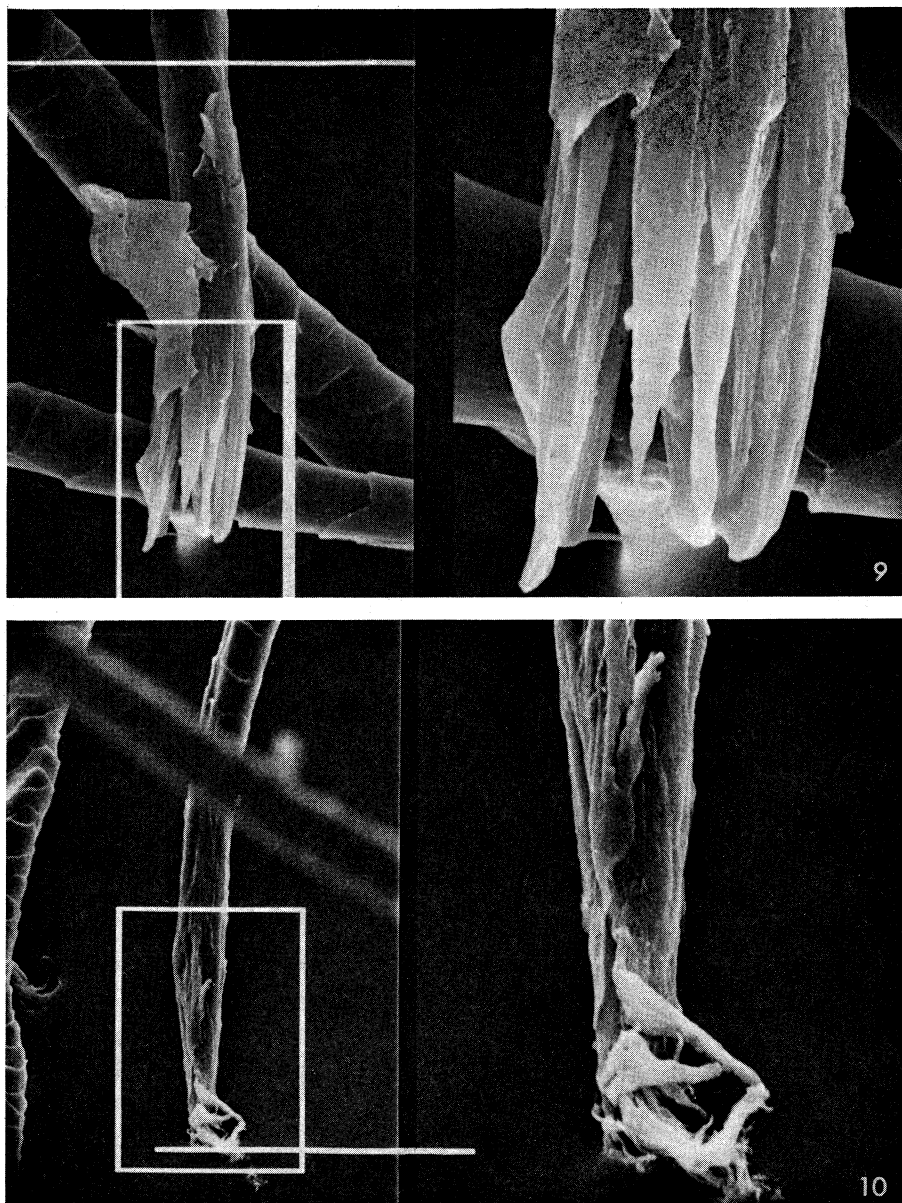
More than 90% of the follicles were active at day 21. Of these about 10% had non-emergent fibres many of which were distorted within thickened outer root sheaths in the distal parts of the follicles. The remains of 1–2% of atrophied follicles were also present. Fibre distortion and outer root sheath thickening persisted in about 3% of follicles beyond day 28.

Wool fibres

Examination of the wool samples clipped from the partly denuded sheep and those with weakened wool revealed that in the zone of weakness there were both shed, thickened fibre ends and continuously growing fibres with very thin regions proximal to poorly keratinized, thickened lengths of fibre (Fig. 14).

Epidermis, sebaceous and sweat glands, skin thickness and subdermal fat

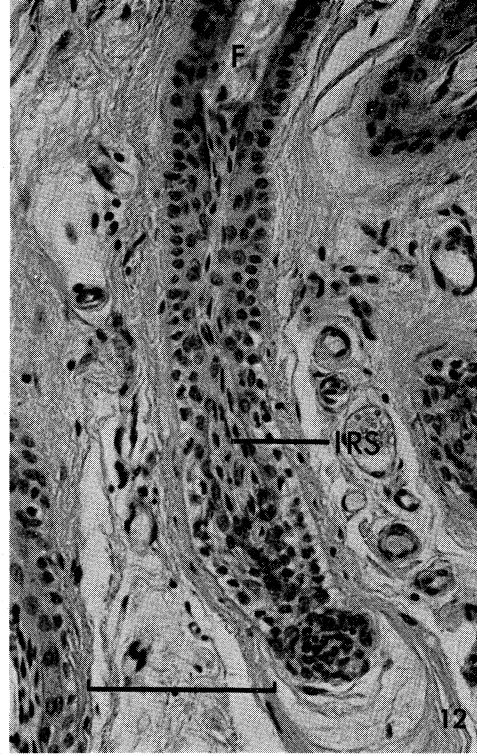
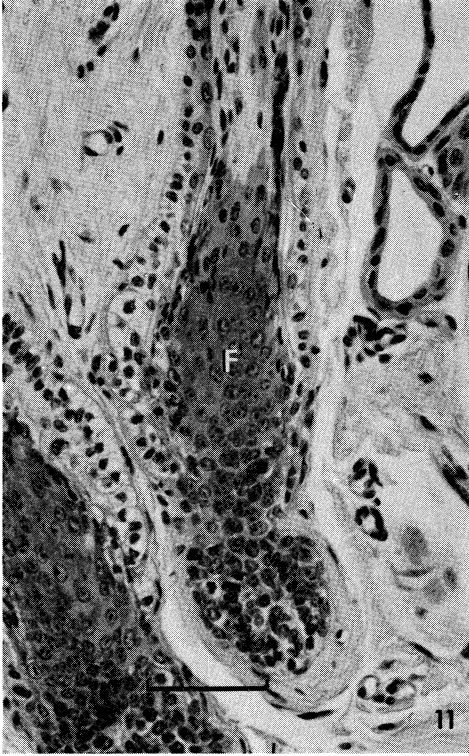
No changes were observed in any of these components of the skin of the partly denuded sheep or of those with weakened wool.



Figs 9 and 10. Magnifications of fibres with a fragmented end (Fig. 9) and tapered and fragmented end (Fig. 10) from a sheep depilated with the same dose as in Fig. 1. Bar = 79 and 26 μm in the left-hand and right-hand micrographs respectively in Fig. 9, and 109 and 36 μm respectively in Fig. 10.

Discussion

Dark-staining bodies (autophagic vacuoles, Fig. 1) which developed in the follicle bulbs following administration of *N*-[5-(4-aminophenoxy)pentyl]phthalimide to sheep



are similar to those reported in wool follicles of sheep dosed with cyclophosphamide (Brinsfield *et al.* 1972; Reis and Chapman 1974) and mimosine (Reis *et al.* 1975). They also develop following administration of glucocorticoid analogues to sheep (R. E. Chapman and B. A. Panaretto, unpublished observations). However, vacuoles are not only produced by exogenous chemicals, but also occur during the catagen (or regression) phase of normal cyclic hair growth in rats and mice (Parakkal 1970) and develop in the bulb cells of rat vibrissae after ionizing irradiation (Pearson and Malkinson 1969).

Regardless of how they are induced, these vacuoles exhibit acid phosphatase and acid esterase activity (Pearson and Malkinson 1969; Parakkal 1970; Brinsfield *et al.* 1972), indicative of lysosomal involvement (de Duve and Wattiaux 1966). As such, they would promote autolysis of cell contents during regression of the follicles. The factor initiating the formation of the autophagic vacuoles by compounds of such widely different chemical compositions as those above is unknown. However, in liver their formation is stimulated by amino-acid deprivation (Mortimer and Schworer 1977). It is of interest that along with the development of these vacuoles there is concomitant inhibition of mitotic activity in the cells of the follicle bulbs.

The development of pycnotic nuclei in the keratogenous zone of fibres of the depilated sheep at day 2 after administration of *N*-[5-(4-aminophenoxy)pentyl]-phthalimide (Fig. 2) occurred in cells which would have been formed in the follicle bulbs prior to dosing of the sheep, because it takes about 2 days for cells to migrate from the proliferative region of the bulb to the keratogenous zone (Chapman 1971). Whether the synthesis of keratin proteins in the fibres was affected and the reason for the impairment of keratinization of the portion of the fibre with retained pycnotic nuclei are unknown.

In the depilated sheep in which follicle regression was well advanced at day 2 after dosing (Fig. 2) there was no obvious differential inhibition of inner root sheath and fibre cells. By contrast, in regressing follicles in the partly denuded sheep and those with weakened wool, production of fibre ceased before that of inner root sheath (Fig. 12). However, in these sheep trichohyalin formation in the inner root sheath cells was inhibited by day 1 after dosing. Consequently, the cells of Henle's layer did not harden in the suprabulbar region of the follicle, and thickening of the fibre occurred at this level (Fig. 11). This was evident in both regressing follicles and those which continued to grow a thin fibre below the thickened region in the sheep with weakened wool.

Fig. 11. Wool follicle with thickening of the fibre in the lower part of the keratogenous zone (*F*) at day 2 after an intravenous dose of 40 mg of the compound/kg body weight over 24 h which weakened the wool. Bar = 100 μ m.

Fig. 12. Regressed wool follicle with a strand of inner root sheath cells (*IRS*) proximal to the root end on the fibre (*F*) at day 7 after an intravenous dose of 40 mg of the compound/kg body weight over 48 h, which partly denuded the sheep. Bar = 100 μ m.

Fig. 13. Wool follicle in which the wool fibre is very thin (\rightarrow) proximal to a distorted, thickened region (\leftrightarrow) at day 7 in the same sheep as in Fig. 11. Bar = 100 μ m.

Fig. 14. Fibres from the weakened region in the fleece of a sheep dosed orally with 200 mg of the compound/kg body weight. There is a mixture of shed, thickened fibre ends (\rightarrow) and continuously growing fibres with thin regions (\leftrightarrow) proximal to poorly keratinized thickened lengths ($\leftarrow\ast$). Bar = 100 μ m.

Likewise the structure of the ends of the fibres shed from the sheep depilated by the higher dose rates differed from that of the fibre ends in the weakened region of the fleeces of sheep dosed at lower rates. Most of the fibres in the wool from the depilated sheep had broken at a weakness immediately distal to the root end (Figs 9 and 10), whereas in the sheep with weakened fleeces the ends were tapered below a poorly keratinized thickened zone (Fig. 14).

The time at which regeneration of regressed follicles commenced was not influenced greatly by dose rate. Mitotic figures were present around dermal papillae of regressed follicles at day 7 after dosing (Fig. 4) and most follicles were regenerating by day 14 (Fig. 5), some producing emergent fibres. This agrees with an observation (D. A. Tunks, personal communication) that regrowth above the skin surface was first visible at 14 days after dosing in sheep depilated by this compound. It took almost 21 days for most follicles to produce emergent fibres in both the depilated sheep and those with weakened wool. Even so, the follicles recovered more quickly than after dosing with mimosine (Reis *et al.* 1975) or cyclophosphamide (Reis and Chapman 1974), and much more quickly than after dosing with cortisol (Chapman and Bassett 1970).

Distortion of fibres in the distal parts of follicles, whether it be of tips on new fibres (Fig. 6) or of very thin regions of continuously growing fibres, induced proliferation and thickening of the surrounding outer root sheath. A similar phenomenon has been observed in sheep given abomasal supplements of methionine while on a diet of wheaten grain (Chapman and Reis 1978).

The inhibition of follicle bulb cells by *N*-[5-(4-aminophenoxy)pentyl]phthalimide contrasts with the thickening of the epidermis. While the latter suggests a stimulatory effect of the compound on epidermal cells, it may have resulted from increased availability of nutrients to the epidermis during regression and inactivity of the follicles.

The reduction in skin thickness of the depilated sheep indicates that catabolism of the connective tissue also occurred during the time of follicle regression. The extent of the reduction approached that induced by prolonged administration of cortisol (Chapman and Bassett 1970). Whereas accumulation of subdermal fat accompanies follicle regression which is induced in sheep by cortisol (Chapman and Bassett 1970) or mimosine (Reis *et al.* 1975), and which occurs naturally in fur-bearing animals (Dolnick 1965), there was no accumulation of subdermal fat in the sheep depilated in the present study.

N-[5-(4-Aminophenoxy)pentyl]phthalimide administered orally at 400 mg/kg body weight enables harvesting of fleeces and comparatively quick recovery of the follicles. However, it has been found to cause blindness in sheep when administered at dose rates greater than 700 mg/kg (D. A. Tunks, personal communication), although retinotoxic effects had previously only been reported in cats (Collins *et al.* 1959). Consequently, other compounds are being examined in the search for a depilating agent without retinotoxicity (Rigby *et al.* 1980). In conclusion, it is becoming apparent that the follicular changes produced by internal administration of various compounds with depilatory activity mimic to some degree the changes which occur during natural cyclic hair growth.

Acknowledgments

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