EFFECTS OF FREEZING THE SKIN AND PLUCKING THE FIBRES IN SHEEP, WITH SPECIAL REFERENCE TO PIGMENTATION

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Summary

Macroscopic and microscopic changes in the skin of seven adult Suffolk and Merino sheep were studied for periods of up to 513 days following the application of solid carbon dioxide to hair- and wool-growing areas after clipping or plucking of the fibres. Observations were also made on the effect of plucking alone.

A colour change from black to white occurred in the coat subsequent to freezing skin which previously grew black hair in Suffolks and black hair or black wool in Merinos. In contrast, follicles which grew white wool in Suffolks and in an isolated white area on an otherwise "black" Merino sheep were induced to grow black or partially coloured fibres for periods ranging from a few days to several months when damaged by freezing the skin or plucking the fibres. The outer root sheaths of the follicles in the regions which normally grew white wool in these animals were the only source of the melanocytes which appeared near the dermal papillae of regenerating follicles and passed pigment into the new fibres. When the skin was frozen after the fibres had been plucked, the melanocytes in the follicle outer root sheaths were usually destroyed and the new fibres were non-pigmented.

Other changes which frequently resulted from freezing the skin were an increase in the thickness of the epidermis, destruction of the sweat glands, a reduction in the number of follicles per unit area of skin, and a reduction in the innervation of the skin. No follicle neogenesis was seen. An increase in the length of the fibres, due either to an increased growth rate or to an increase in the duration of the growth cycle, sometimes occurred.

I. INTRODUCTION

The effects of treatments such as freezing, X-irradiation, grafting, and plucking the hairs have received little attention in studies of the skin of the sheep although they have been investigated in other mammals. Taylor (1949) and Mikhail (1963) produced white hair in place of black hair in rats following intense cooling of the skin with solid carbon dioxide. This effect was similar to that produced in mice (Chase 1954) and rabbits (Straile 1959) with certain doses of X-irradiation. Butcher's (1945) studies of the rat showed that skin which normally grew black hair sometimes lost its capacity to produce pigment when interchanged with skin producing white hair. Plucking the hairs to initiate follicle activity has been extensively used in studies of hair growth cycles (e.g. Johnson 1965; Lyne 1965).

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The aim of the present study was to examine the effects of freezing the skin and plucking the fibres in both hair- and wool-growing regions in Merino and Suffolk sheep. A preliminary note on the pigmentation aspects of this work has been published (Lyne *et al.* 1967).

The only detailed study of pigmentation changes in the coat of Suffolk sheep appears to be that of Nichols (1927). He described the growth of pigmented fibres in non-pigmented areas following injury but his discussion on the mode of pigment deposition in the fibres did not refer to the role of the melanocytes.

II. Materials and Methods

Solid CO_2 was applied to hair-growing skin (pinna, scrotum, foreleg, cheek, and lower jaw) and wool-growing skin (midside and midback of trunk) of seven sheep (three Suffolks and four Merinos). The hair was closely clipped whereas the wool was either closely clipped or plucked before an applicator containing the CO_2 was applied to the skin. The applicator was cylindrical with a copper base 0·7 mm thick and 14·5 cm^2 in area. It was applied under gravity with a pressure of about 83 g/cm^2. The period of contact ranged from 15 sec to 8 min, usually 1–4 min. In several preliminary experiments, cylindrical blocks of solid CO_2, 2–3 cm in diameter, were applied directly to clipped regions of skin for periods ranging from 1 to 4 min. Subsequent to treatment, the areas were inspected at regular intervals during the next $2\frac{1}{2}$ years, and some were photographed. Skin biopsies were taken at various times up to 513 days after treatment. Control biopsies were taken from adjacent untreated skin. The activity of the follicles in hair-growing regions was checked at intervals by close clipping of the experimental areas. The effect of plucking alone was studied in skin biopsies taken subsequently at intervals up to 33 days after treatment.

The DOPA (3,4-dihydroxyphenylalanine) reaction was carried out using the method of Quevedo *et al.* (1966). Other histological and histochemical techniques used were those described by Lyne and Hollis (1968). Counts of the follicle population density (made on six fields of 1 mm^2 in area) were corrected for skin shrinkage as described by Carter and Clarke (1957). Measurements of the interfollicular epidermal thickness were obtained from sections (8 μ thick) cut at right angles to the skin surface and stained with haematoxylin, eosin, and picric acid (HEP). Using a microprojector at $\times 395$, three regions of the epidermis, each 0·5 mm long, were traced on cards of uniform thickness. The tracings were cut out and weighed, and the mean epidermal thickness was obtained from a graph relating card width (equivalent to epidermal thickness) to weight.

III. Observations

Immediately after the application of CO_2 (with or without the applicator), the skin, if frozen, was hard and whitish in colour. The period required to freeze the skin, usually from 1 to 4 min, varied in different body regions and was shorter when the fibres were plucked than when they were clipped. The full thickness of the pinna was frozen when the CO_2 was applied to only one surface for 4 min. In contrast, some wool-producing areas did not freeze even after 8 min. Subsequent to freezing, the skin usually thawed in 1–3 min.

(*a*) *Pigmentation of Untreated Skin*

Considerable variation was seen in the distribution of the melanocytes in control skin sections from both the hair- and wool-growing regions. Examples are shown in Figure 1. All sheep, including those growing only white fibres, had some melanocytes in the epidermis, either as a very sparse population (Fig. 1*A*), or as a

larger population extending into the upper parts of the follicles (Fig. 1*B*). Melanocytes in the outer root sheath were common in follicles growing pigmented fibres (Figs. 1*E*–1*I*), and in some sheep they were present in follicles which normally grew white fibres (e.g. in wool-growing regions of the Suffolks; Figs. 1*C*, 1*D*, and 19). Melanocytes capping the dermal papillae were present only in follicles growing pigmented fibres

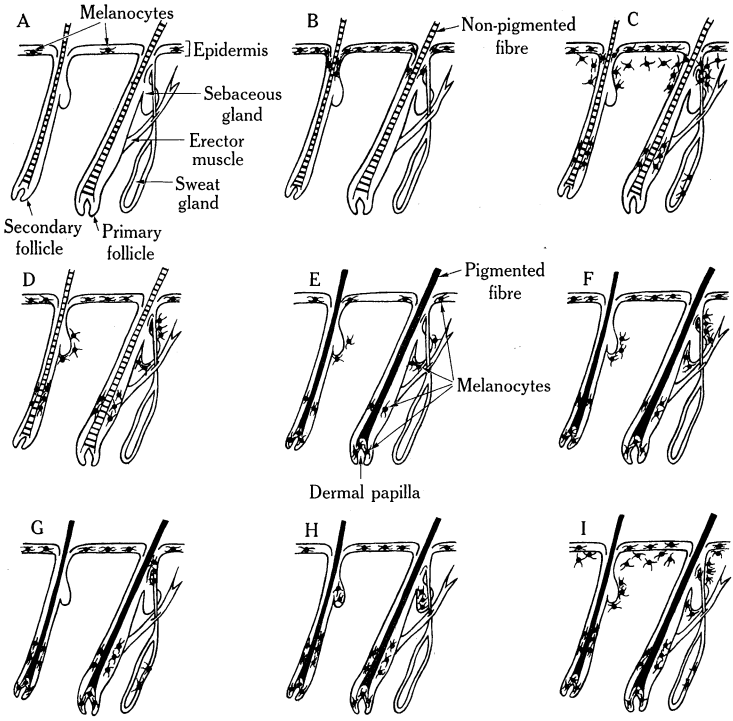

Fig. 1.—Diagram showing variations in the distribution of melanocytes in control skin samples from the sheep examined (for details see text).

(Figs. 1*E*–1*I*). Melanocytes in the sweat gland wall (Figs. 1*C*, 1*G*, and 1*I*) and in the sebaceous glands (Fig. 1*H*) were not uncommon. Dermal melanocytes (Figs. 1*C*–1*F* and 1*I*) were confined to the upper part of the dermis and were most prevalent near the follicles, sweat gland ducts, sebaceous glands, blood vessels, and nerves.

(*b*) *Pigmentation of Treated Skin*

(i) *Hair-growing Regions*

Figures 4–9 show changes in the colour of the skin and hair on the outer surface of the pinna of a Suffolk following the application of solid CO_2 to this surface. The hard scab present after 16 days was greatly diminished after 28 days, and white hairs were obvious at the periphery of the previously frozen area after 42 days. Repigmentation of the skin was complete after 56 days and the area was repopulated by hairs after 84 days. After 372 days inconspicuous pigmented hairs were common amongst the white hairs. On the inner surface of the pinna, i.e. the surface which was not in contact with the CO_2 applicator, colour changes occurred in the skin and hair similar to those seen on the outer surface.

Changes in the colour of the skin and hair on the outer surface of the pinna following the application of solid CO_2 to the inner surface are shown in Figures 10 and 11. Repigmentation of the skin spread from regions throughout this area (except the centre) as well as from the perimeter, and had almost ceased after 88 days (compare Fig. 10 with Fig. 11). In the centre of the region, presumably the area most severely affected, the follicles and melanocytes were permanently destroyed. After 186 days, some of the follicles were again growing pigmented hairs while the repigmented skin was paler than after 88 days (compare Fig. 11 with Fig. 10). Subsequent observations made at intervals up to 370 days showed no further changes in skin colour but there was a slight increase in the number of follicles which reverted to growing pigmented hairs. The inner surface of this pinna, i.e. the surface which was in contact with the CO_2 applicator, showed a greater and more uniform loss of skin pigmentation and less repigmentation. The follicle population was greatly reduced and hair pigmentation changes were similar to those seen on the outer surface.

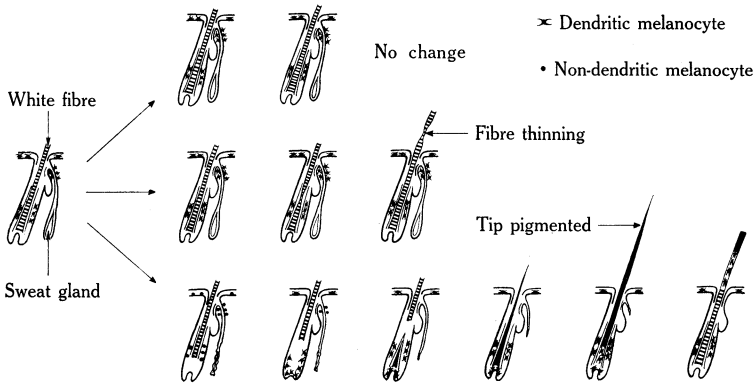

Fig. 2.—Diagram showing effects on the follicles of the application of solid CO_2 to clipped regions of Suffolk sheep which previously grew white wool (for details see text).

In similar experiments with two "black" Merinos, the pigmentation of the pinna skin was permanently reduced. White hairs in place of black ones were still present 357 days after freezing.

Freezing of other hair-growing regions also resulted in a regrowth of white hairs in place of black ones, and in most regions the change persisted during the period of observation of up to $2\frac{1}{2}$ years. During this time several hair growth cycles occurred as determined by sequential close clipping of the experimental areas.

When the junction of a black and white region on the lower jaw of a "black" Merino was frozen, the black skin which grew black hair was changed to white skin growing white hair, and the change persisted during the period of observation of $2\frac{1}{2}$ years. The white skin which originally grew white hair was apparently unchanged by the same treatment.

Freezing of the skin always resulted in at least a temporary reduction in the pigmentation of the epidermis of hair-growing regions. In areas with a sparse population of epidermal melanocytes (Fig. 39) repigmentation was not usually seen

(Fig. 40), whereas in areas with a large population repigmentation was common. In some areas, e.g. pinnae of Suffolks (Figs. 4–11), repigmentation spread mainly from "spots" which appeared within the epidermis, indicating that some epidermal melanocytes were not destroyed.

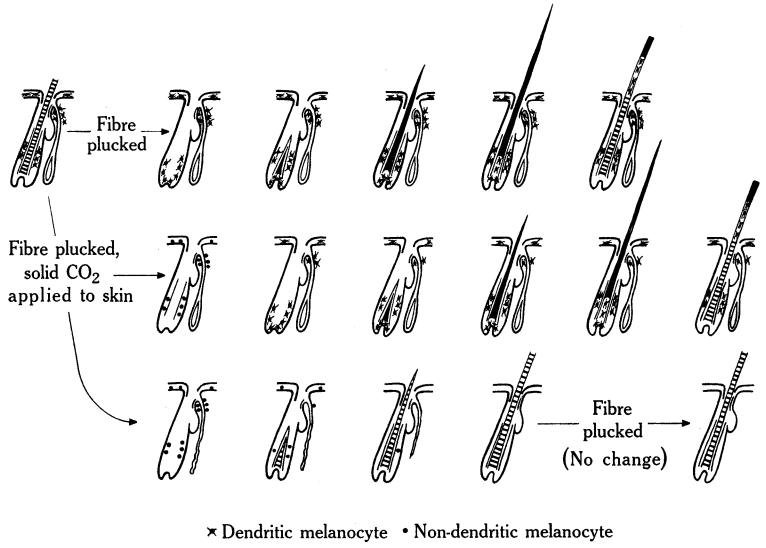

Fig. 3.—Diagram showing changes in the follicles after plucking the fibres, and after freezing the skin following plucking the fibres in white wool-growing areas of Suffolk sheep (for details see text). Further plucking of non-pigmented fibres grown by follicles in which the outer root sheath melanocytes were destroyed by freezing (lower row) did not produce any colour change in the regrowth.

(ii) *Wool-growing Regions*

The most interesting pigmentation changes occurred in the Suffolks. Figure 2 shows examples of changes in the follicles after clipping the fibres and applying solid CO_2 to white wool-growing areas in the Suffolks. No change in the follicles (Fig. 2, top row) was common when the skin did not freeze. A reduction in fibre diameter (Fig. 2, middle row, and Fig. 20), coinciding with the period following freezing, occurred on several occasions. When fibre growth ceased after freezing of the skin, the follicles usually regenerated as shown in the bottom row of Figure 2. The old fibre (Fig. 21) was shed and some of the outer root sheath melanocytes, which often went through a non-dendritic phase, were incorporated in the new bulb near the dermal papilla (Figs. 22 and 23). When the growth of a new fibre began, the melanocytes capping the dermal papilla and those in the outer root sheath were distinctly separate populations. The bulb melanocytes passed melanin granules into the fibres for periods ranging from a few days to several months before the melanocytes themselves entered the fibre (Fig. 24). This usually produced a fibre with a pigmented tip as shown in the bottom row of Figure 2 and in Figure 12.

Figure 3 shows examples of pigmentation changes after plucking the fibres and after plucking the fibres plus freezing the skin in white wool-growing areas of

the Suffolks. Pigmentation changes in the follicles after plucking alone (Fig. 3, top row, and Figs. 25–29) were similar to those after freezing following clipping the fibres (Fig. 2, bottom row, and Figs. 22–24). When the skin was frozen after plucking the fibres, the new fibres were pigmented (Fig. 3, centre row) unless the outer sheath melanocytes were destroyed by freezing, in which case the regrowth contained white fibres (Fig. 3, bottom row, and Fig. 13). If these white fibres were plucked, they were replaced by white fibres (Fig. 3, bottom row) in contrast to the black-tipped fibres which normally appeared after plucking (Fig. 3, top row).

Freezing the skin usually destroyed or greatly reduced the population of melanocytes in the dermis, including those commonly seen near the sweat gland ducts and sebaceous glands (Figs. 2 and 3).

A marked increase in the population of epidermal melanocytes was seen in sections of pigmented scars resulting from lethal freezing of wool-growing skin in Suffolk sheep (Fig. 14). Presumably, the additional melanocytes in the scar epidermis came from the outer root sheaths of adjacent follicles which remained viable during wound repair.

Freezing after clipping or plucking of regions which grew black wool in the Merinos always resulted in a regrowth of white fibres (Figs. 15–18) and there was no indication of repigmentation during a 2-year period of observation.

The effects of freezing the skin at the junction between a region of black wool and an isolated area of white wool on an otherwise "black" Merino are shown in Figures 15 and 16. Again, the regrowth on the black region was white. The regrowth on the white region, in contrast to the hair-growing region on the lower jaw described above, was mostly black except for white fibres which grew next to a non-pigmented scar in the centre. This was an unusual result since the skin and wool in normally white Merinos showed no pigmentation changes when the skin was frozen or the fibres were plucked.

(c) *Sebaceous Glands, Sweat Glands, and Erector Muscles*

The sebaceous glands, associated with all follicles, appeared to be remarkably resistant to freezing. However, the sweat glands and erector muscles were often destroyed by freezing, particularly in hair-growing regions.

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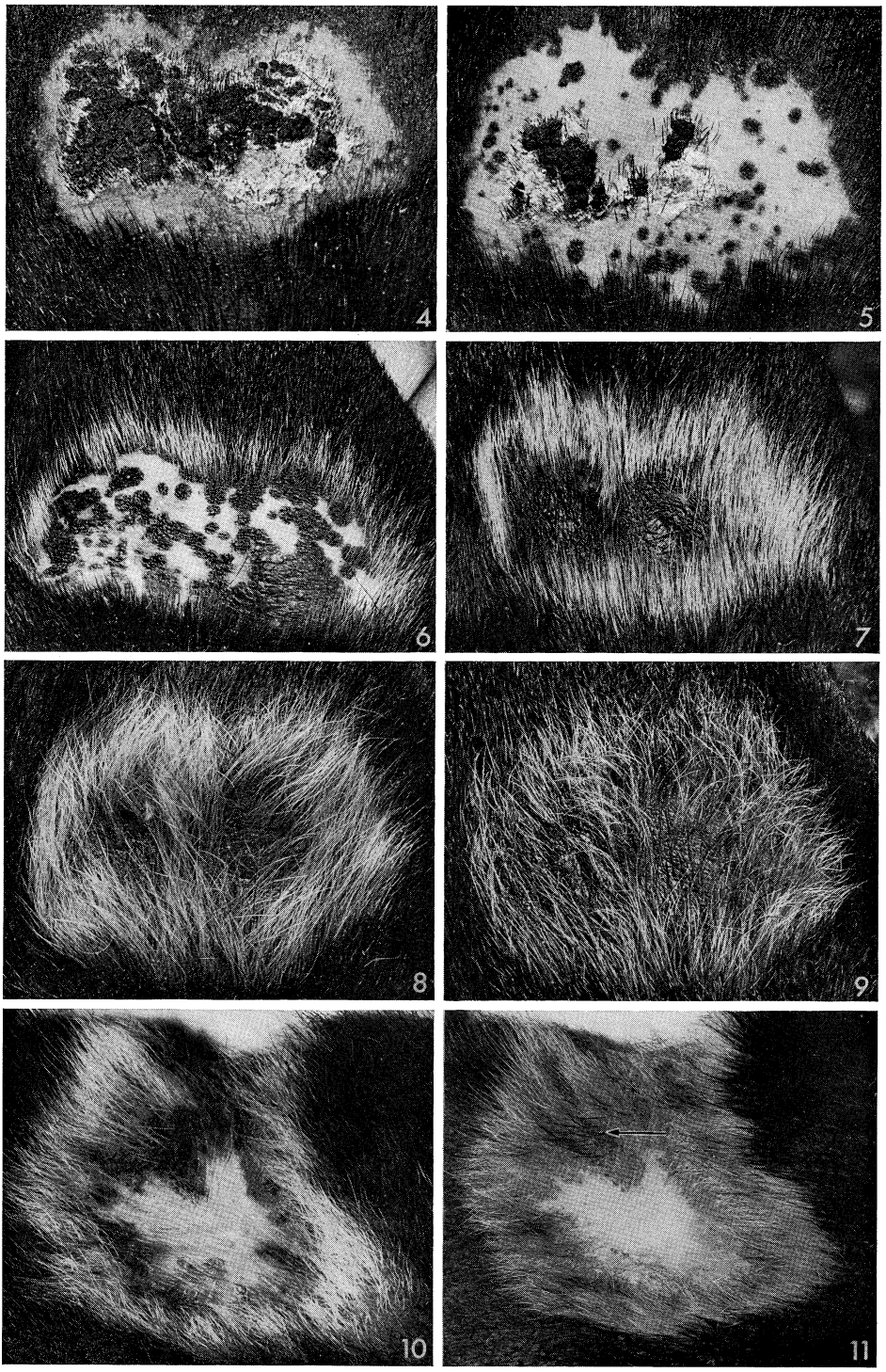
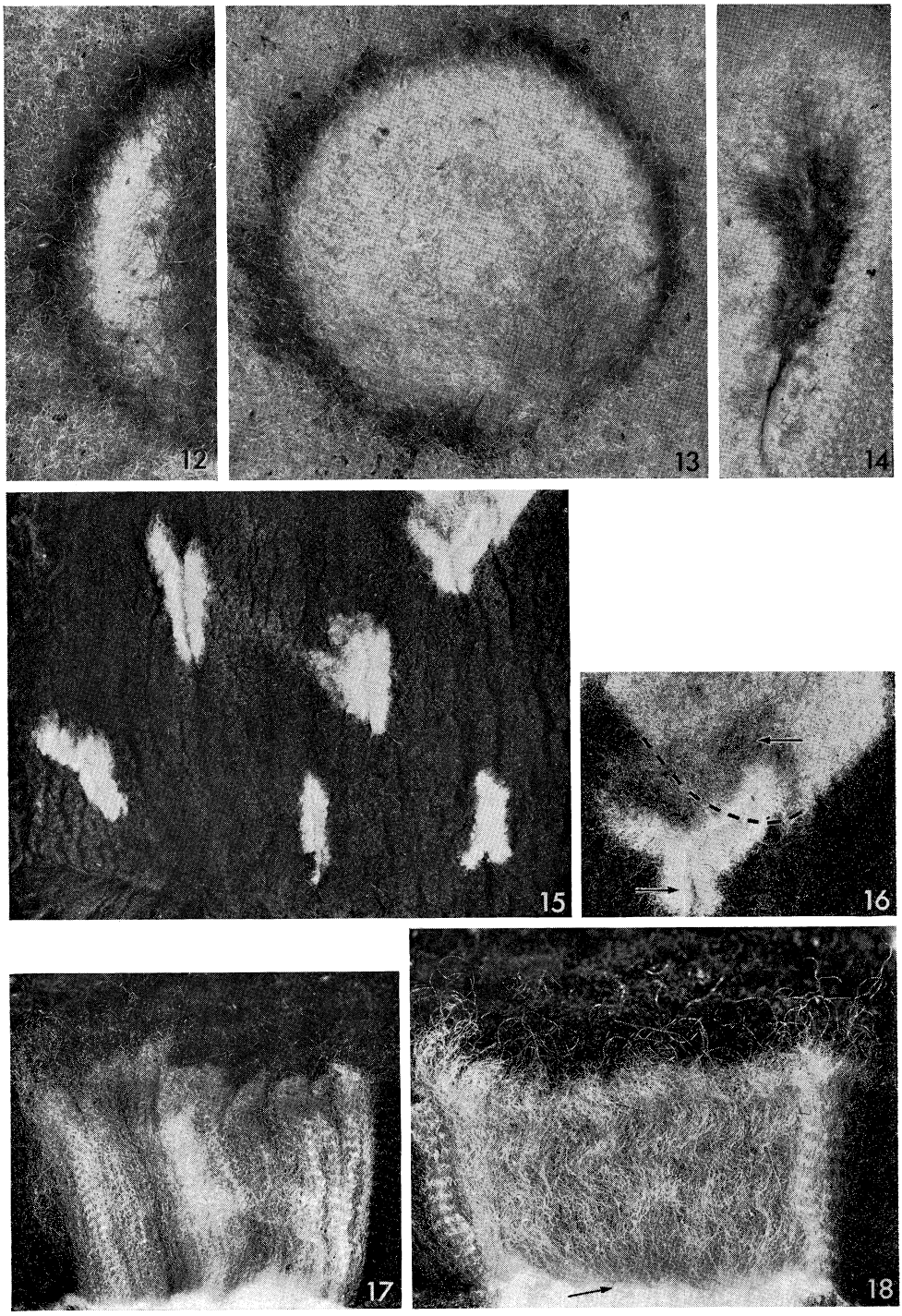
of CO_2 to the inner surface for 4·5 min (Figs. 10 and 11). *4*, Suffolk No. 13, day 16. The hairs have been shed from the frozen area except in the centre which is covered by a hard scab. *5*, Suffolk No. 13, day 28. The scab has reduced greatly in size. Repigmentation of the skin is spreading from centres within the frozen region as well as from the periphery. New non-pigmented hairs have emerged at the periphery but they are not visible in the photograph. *6*, Suffolk No. 13, day 42. Repigmentation of the skin is well advanced and white hairs are obvious around the outer part of the frozen area. *7*, Suffolk No. 13, day 56. Repigmentation of the skin is complete and the growth of white hairs has spread to the centre. *8*, Suffolk No. 13, day 84. Most of area is covered with white hairs which are longer than the pigmented hairs growing in adjacent control skin. *9*, Suffolk No. 13, day 372. Pigmented hairs are common amongst the white hairs but are not easily seen in this photograph. *10*, Suffolk No. 12, day 88. Repigmentation of the skin has almost ceased and the population of white hairs is maximal. *11*, Suffolk No. 12, day 186. The colour of the repigmented skin is lighter than at 88 days. Some of the follicles which were growing white hairs at 88 days are now growing black hairs (arrow).



Figs. 4–11.—Outer surface of pinna showing changes at various times after the application of solid CO_2 for 4 min to the closely clipped skin of the outer surface (Figs. 4–9), and after application



Figs. 12–18.—Wool-growing areas on the trunk of Suffolk and Merino sheep showing changes following the application of solid CO_2 to the skin after close clipping or plucking the fibres. *12*, A colour change from white to black wool in a Suffolk (No. 13). Photograph taken 28 days

(*d*) *Measurements of Follicles and Fibres*

The numbers of follicles per unit area in skin biopsies from untreated and treated regions are shown in Table 1. In general, application of solid CO_2 to the skin for either 4 or 8 min after clipping the fibres or for 0·5 or 1 min after plucking the fibres caused a marked reduction in the number of follicles per unit area and, in some instances, the formation of a naked area (Figs. 10–12). The reduction in the follicle density within a region was apparently related more to the severity of the treatment (that is, the extent of freezing within the skin) rather than to the period of application of CO_2. At least some of the differences in follicle density between the treated and untreated areas, shown in Table 1, were due to normal population differences within a body region. This appears to be the most likely explanation for the apparent increase in the follicle density on the frozen area of the pinna of Merino No. 17.

The presence of sweat glands and erector muscles enables primary follicles to be identified easily in untreated skin. However, because these structures were destroyed it was not possible to obtain counts of the secondary : primary follicle ratios in more than half of the biopsies from the frozen areas. When both primary and secondary follicle types could be identified, the secondary : primary follicle ratios in the control and frozen areas were similar, suggesting that the secondary and primary follicles were affected to the same extent by freezing. There was no evidence of follicle neogenesis, similar to that described by Lyne and Brook (1964), in any of the skin samples examined.

Occasionally, within frozen areas which had no appreciable change in follicle density, some of the fibres showed an increase in length growth rate and diameter. Measurements of wool fibres which grew on two treated regions and on an untreated control region of a normal white Merino (No. 2) are shown in Table 2. The increases in length growth rate and diameter were still present in one region in the period from 308 to 513 days after freezing.

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after freezing a clipped area of skin for 8 min. Very few fibres grew on the "naked" area. *13*, The effect of plucking white fibres in a Suffolk (No. 16) is shown by the circle of black fibres. The white region within the circle was also plucked but in addition it was frozen for 4 min. Photograph taken 25 days after freezing. Repeated plucking of the frozen region resulted in only white fibres. *14*, Pigmented scar in Suffolk (No. 16) 111 days after plucking the fibres and freezing the skin for 4 min. Scars of this type occurred only after lethal freezing of the skin. *15*, Colour changes from black to white wool in a "black" Merino (No. 19). Photograph taken 163 days after clipping the fibres and freezing areas of skin for 4 or 8 min. The treated area in the upper right corner was located across the junction with an isolated region growing white wool. *16*, Enlargement of the junction, indicated by the dotted line, of a white spot with a black region shown in Figure 15 (upper right) showing the effect of freezing. The regrowth on the black region was mostly white (lower arrow) and the regrowth on the white region contained many black fibres (upper arrow). *17*, A colour change from black to white wool in a Merino (No. 19). Photograph taken 245 days after plucking the fibres and freezing the skin for 1 min. The length growth rate and number of crimps per unit of length are similar in both black and white fibres. *18*, A colour change similar to that shown in Figure 17 in the same sheep. Photograph taken 190 days after clipping the fibres and freezing the skin for 8 min. The fibres adjacent to the scar (arrow) showed a reduction in the crimp frequency but the mean length growth rate was the same as that of the black fibres growing on adjacent control skin.

TABLE 1

EFFECT OF FREEZING THE SKIN ON THE NUMBER OF FOLLICLES PER UNIT AREA

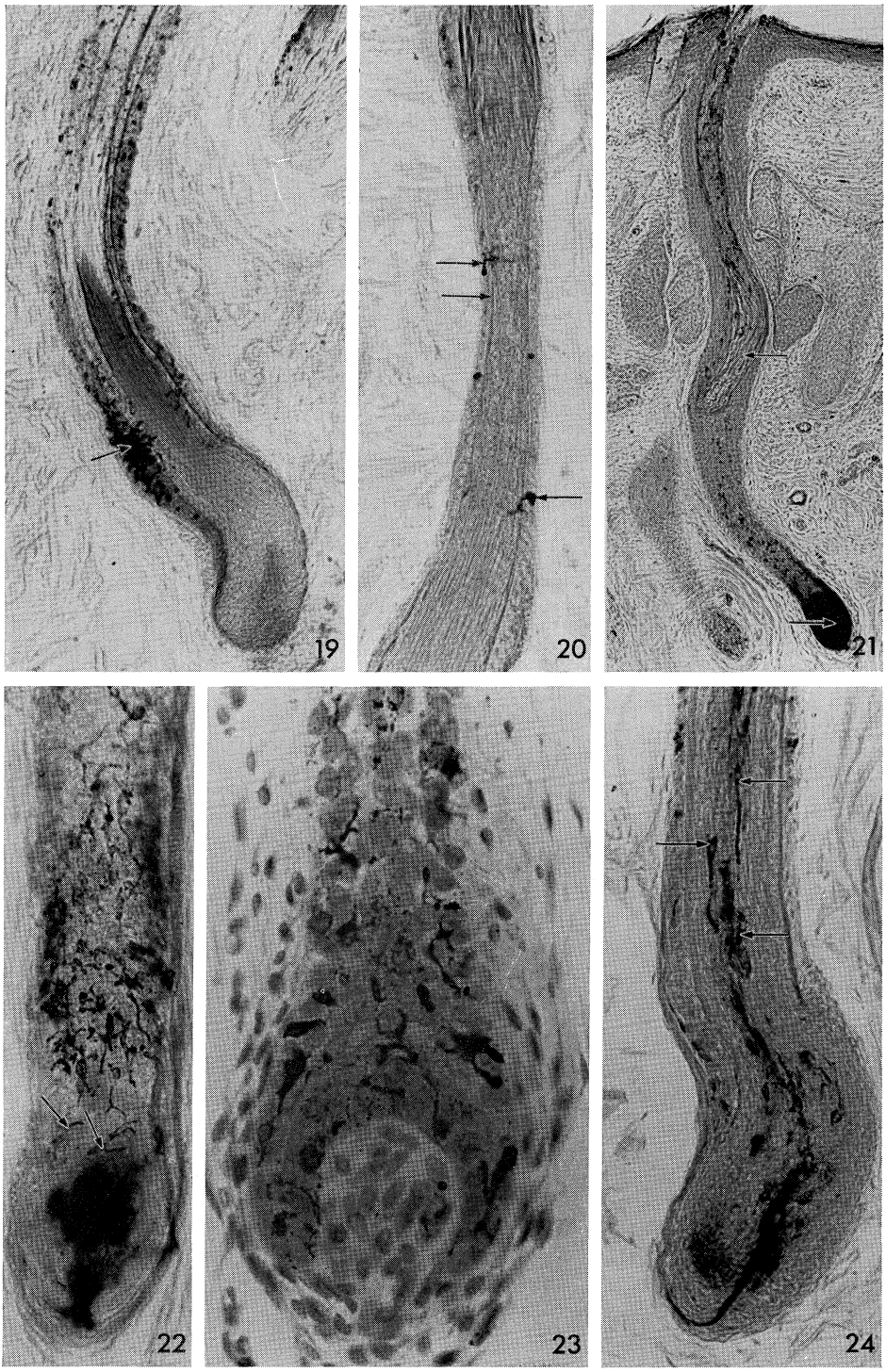
Biopsies were performed once only on each frozen area

Breed	Sheep No. and Sex	Body Region	Time (min) of Freezing*	Days to Biopsy†	Follicles/mm² of Skin	
					Control Area	Frozen Area
Suffolk	12♀	Cheek	4	370	14·2	11·0
		Inner pinna	4	438	3·8	1·4
		Outer pinna	4	370	9·9	0·0
		Foreleg	4	370	9·0	9·9
	13♂	Cheek	4	372	7·4	1·7
		Outer pinna	4	372	6·0	6·7
		Scrotum	4	372	3·8	0·1
		Midside	8	288	12·3	1·9
	16♂	Midside	4	111	19·3	13·9
			4	169	19·3	15·2
			4	255	19·3	17·8
Merino	2♀	Midside	4	513	41·6	38·1
	17♀	Inner pinna	4	296	19·2	28·8
		Midside	8	245	69·2	13·6
			1	296	69·2	73·7
			8	296	69·2	61·4
			8	296	69·2	67·6
	18♀	Midside	0·5	245	59·1	16·3
			8	245	59·1	31·7
	19♀	Lower jaw	4	357	37·1	30·2
		Inner pinna	4	357	26·8	19·4
		Midside	4	245	35·0	14·6
			1	357	35·0	31·6
			4	357	35·0	30·2
			8	357	35·0	17·8

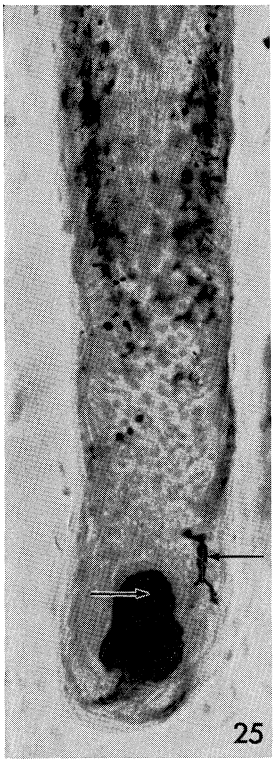
* Minutes of application of solid CO_2 to skin.

† Days after application of solid CO_2.

growing regions of Suffolk sheep (Nos. 13 and 16). Compare with Figures 25–29. *19*, Section of the lower part of a follicle in control skin showing melanocytes (arrow) in outer root sheath. Note absence of melanocytes in follicle bulb. Butyrylcholinesterase. ×104. *20*, Middle part of a follicle showing a marked reduction in the diameter of the fibre and follicle (middle arrow) following the application of CO_2 for 4 min, 12 days previously. Melanocytes (upper and lower arrows) are present in the outer root sheath. Alkaline phosphatase. ×175. *21*, Follicle damaged by freezing the skin for 8 min, 7 days previously. The old fibre (upper arrow) is in the process of being shed. Regeneration of the follicle has commenced and intense alkaline phosphatase activity is seen in the dermal papilla (lower arrow). ×71. *22*, Lower part of a follicle, 6 days after freezing for 4 min, showing large population of outer root sheath melanocytes, some of which (arrows) are near the dermal papilla. Regeneration of the follicle has commenced and alkaline phosphatase activity is seen in the dermal papilla. ×201. *23*, Lower part of a follicle 21 days after freezing the skin for 4 min. At this stage of regeneration, the population of melanocytes in the outer root sheath is continuous with those capping the dermal papilla. Haematoxylin. ×455. *24*, Lower part of a follicle 42 days after freezing the skin for 4 min. The follicle has grown a black-tipped fibre and most of the melanocytes previously in the bulb have entered the fibre (arrows). Alkaline phosphatase. ×181.



Figs. 19–24.—Changes in the distribution of melanocytes in longitudinal sections of follicles following the application of solid CO_2 to the skin after close clipping of the fibres in white wool-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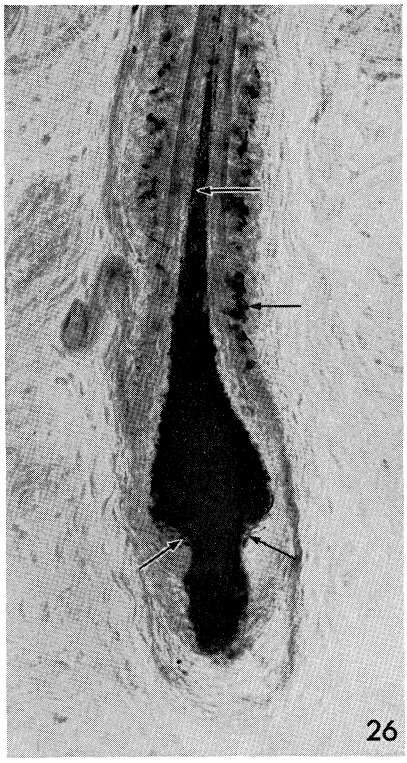
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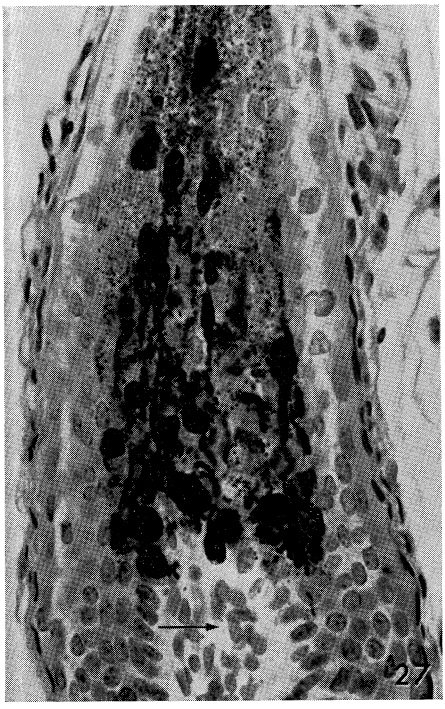
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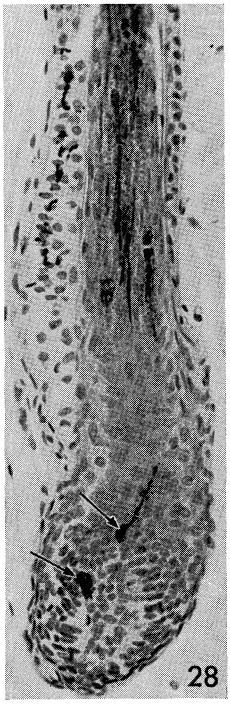
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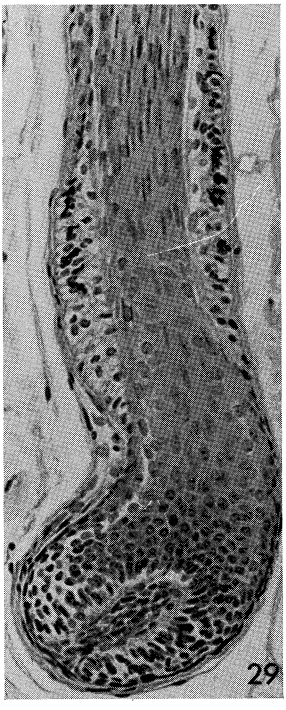
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A conspicuous increase in hair length following freezing occurred on the outer surface of the pinna of Suffolk No. 13 (see Fig. 8). The mean length of 140 fully-grown hairs (i.e. hairs with clubs) plucked from the centre of the area 3 months after freezing was 9·6 mm whereas the mean length of 190 fully-grown adjacent control hairs was 7·3 mm. This increase (over 30%) was due either to an increased growth rate or to an increase in duration of the growth cycle. The follicle population density 372 days after freezing was similar to that of the control skin (Table 1).

In contrast to these results, the white wool which grew on frozen areas in the black Merinos was usually normal in length growth rate and crimp formation (Fig. 17). On some occasions, after lethal freezing and the formation of a scar, there was an increase in staple length of the fibres adjacent to the scar, due to a reduction in the crimp frequency (Fig. 18). Measurements of these fibres and adjacent control fibres showed that their mean length growth rates were the same.

TABLE 2

LENGTH GROWTH RATES AND DIAMETERS OF FIBRES FROM TWO FROZEN REGIONS COMPARED WITH CONTROL FIBRES ON THE MIDSIDE OF A MERINO

Each sample measured contained approximately 200 fibres. Values are means ± standard deviations

Region	Period after Skin Frozen (days)	Length Growth Rate of Fibres (μ/day)		Diameter of Fibres (μ)	
		Control	Frozen Region	Control	Frozen Region
1	0–161	240± 59	280± 61	20·5±5·7	21·0±5·7
	28–189	310± 62	340±102	21·2±4·8	21·3±4·6
2	189–308	290±139	430±143	23·3±5·1	26·6±5·4
	308–513	320± 42	420± 54	22·9±5·5	24·9±4·1

(e) *Thickness and Structure of the Epidermis*

Measurements of the thickness of the epidermis in biopsies taken at 3–513 days after freezing the skin are compared with control measurements in Table 3. Examples of changes in epidermal structure are shown in Figures 30–38.

Figs. 25–29.—Changes in the distribution of melanocytes in longitudinal sections of follicles following plucking of the fibres in white wool-growing regions of a Suffolk (No. 16). Compare with Figures 19–24. *25*, An early stage of regeneration, three days after plucking. An outer root sheath melanocyte (right arrow) is near the alkaline phosphatase-positive dermal papilla (left arrow). ×227. *26*, A regenerating follicle 12 days after plucking showing a new pigmented fibre (upper arrow). The melanocytes near the dermal papilla (two lower arrows) and those in the outer root sheath (second arrow from top) are now separate populations. Alkaline phosphatase activity is present in the dermal papilla. ×187. *27*, Regenerating follicle 21 days after plucking. Melanocytes and melanin granules are in the fibre and adjacent to the upper part of the dermal papilla (arrow). DOPA plus alcoholic carmine. ×420. *28*, Follicle which has grown a fibre with a pigmented tip 21 days after plucking. Two melanocytes (arrows) remain near the dermal papilla. The other melanocytes have entered the fibre. DOPA plus alcoholic carmine. ×200. *29*, Follicle which has reverted to growing a non-pigmented fibre 21 days after plucking. There are no melanocytes in the vicinity of the dermal papilla but some are present in the outer root sheath. DOPA plus alcoholic carmine. ×207.

TABLE 3

EFFECT OF FREEZING THE SKIN ON EPIDERMAL THICKNESS

Biopsies were performed once only on each frozen area

Breed	Sheep No. and Sex	Body Region	Time (min) of Freezing*	Days to Biopsy†	Epidermal Thickness (μ)	
					Control Area	Frozen Area
Suffolk	12♀	Cheek	4	370	27	41
		Inner pinna	4	438	46	60
		Outer pinna	4	370	32	45
		Foreleg	4	370	22	27
	13♂	Cheek	4	372	29	54
		Outer pinna	4	372	33	57
		Scrotum	4	372	79	122
		Midside	4	7	22	27
			8	7	22	45
			4	14	22	35
			8	14	22	75
			1	21	22	25
			2	21	22	22
			4	21	22	30
			8	21	22	83
			1	42	22	45
			2	42	22	30
			4	42	22	27
			8	288	22	25
	16♂	Midside	4	3	25	36
			4	6	25	38
			4	12	25	28
			4	111	25	25
			4	169	25	21
			4	255	25	22
Merino	2♀	Midside	4	7	14	13
			8	7	14	13
			8	14	14	18
			8	14	14	17
			4	21	14	13
			8	21	14	13
			4	513	14	12
	17♀	Inner pinna	4	357	30	35
		Midside	8	245	18	14
			1	357	18	15
			8	357	18	14
	18♀	Midside	0·5	245	16	16
			8	245	16	14
	19♀	Lower jaw	4	357	22	30
		Inner pinna	4	357	25	39
		Midside	0·5	245	16	20
			4	245	16	27
			8	245	16	26
			1	357	16	32
			4	357	16	41
			8	357	16	35

* Minutes of application of solid CO_2 to skin.

† Days after application of solid CO_2.

In general, the thickness of the epidermis was increased by freezing the skin, and this increase, seen in samples taken after 3 days (compare Fig. 32 with Fig. 30), was still clearly evident in later biopsies (compare Fig. 34 with Fig. 33 and Fig. 36 with Fig. 35). The increase in epidermal thickness was usually greater in hair-growing than in wool-growing regions. Complete, or almost complete, destruction of the follicles was often accompanied by the production of a thicker epidermis with distinct dermal papillae (Fig. 38) in place of an epidermis with a relatively smooth junction with the dermis (Fig. 37).

Plucking the fibres without applying CO_2 always resulted in at least a temporary increase in the thickness of the epidermis in one of the Suffolks (No. 16) examined. This increase, shown in a biopsy taken after 3 days (compare Fig. 31 with Fig. 30), was still evident in biopsies taken up to 33 days after treatment.

(*f*) *Branched Cells in the Epidermis*

The acetylcholinesterase-positive branched cells (Lyne and Chase 1966) in the epidermis and upper parts of the follicles (Figs. 41 and 42) were almost as resistant to freezing as the epidermal cells. A small population of these branched cells always survived severe freezing and, although counts were not made, the numbers appeared to return to normal within approximately 2 weeks.

(*g*) *Innervation of the Skin*

The innervation of both hair- and wool-growing skin was usually greatly reduced by freezing. However, follicle nerve networks were found in some biopsies, even in those from areas which were subjected to CO_2 treatment for 4 or 8 min (compare Fig. 44 with Fig. 43).

IV. Discussion

The most obvious changes in the skin and coat following local freezing of the skin after clipping or plucking the fibres were seen in pigmented animals. The growth of a white coat (hair or wool) in place of a black one after freezing was similar to that described in rodents (Taylor 1949; Mikhail 1963) but different from the regrowth response of two marsupials (bandicoot *Perameles nasuta* and possum *Trichosurus vulpecula*) which showed no change in the colour of the hair which grew on previously frozen areas of skin (Lyne, unpublished data).

Melanocytes in the outer root sheath appeared to be essential for a colour change from white to black following injury to the follicles (e.g. by freezing the skin or plucking the fibres). In other species, e.g. chinchilla (Lyne 1965), the outer root sheath likewise appeared to be an important source of melanocytes, supplying the bulb at the beginning of each hair cycle. No "clear" cells (amelanotic melanocytes) which could become pigmented, similar to those described by Silvers (1958) in albino mice, were seen in the vicinity of the dermal papillae in the sheep used in these experiments.

The loss of melanocytes from the follicle bulbs and their incorporation into the fibres when the follicles reverted to growing non-pigmented fibres was similar to

that described in certain genotypes of mice during the active phase of hair growth (McGrath and Quevedo 1965). As the length of the growth cycle in wool-growing regions of these sheep lasts a long time, at least 1 year, the follicles seldom go through a phase of regeneration. However, the present study has shown that the follicles have the potential to regenerate many times and, in Suffolks, grow fibres with pigmented tips similar to those of the first generation of fibres in the birthcoat (Nichols 1927).

There is a paradox concerning the effects of freezing on melanocytes in Suffolk sheep. When hair-growing regions were frozen, mostly white hairs replaced pigmented hairs whereas in wool-growing regions fibres with pigmented tips usually replaced white fibres. It is not known why the melanocytes in the bulb and in the outer root sheath were destroyed in hair-growing regions, whereas in wool-growing regions the outer root sheath population was usually not destroyed. But when destruction of the outer root sheath melanocytes did occur in wool-growing regions, as sometimes happened when the fibres were plucked prior to freezing, the regrowth consisted of mostly white fibres. At the time of treatment, the wool-growing regions contained virtually all active follicles, whereas the hair-growing regions contained approximately 50% inactive follicles (Lyne, unpublished data). Perhaps the effect of freezing on the melanocytes was inversely related in some way to follicle activity. Chase (1946) found that loss of pigmentation following X-irradiation in the mouse was less in active than in quiescent follicles.

The epidermal melanocytes, however, were usually not entirely destroyed when the follicle melanocytes were destroyed. Although the pigmentation of the skin, especially that of the epidermis, was greatly reduced following freezing, repigmentation of areas, originally darkly pigmented, usually occurred within 1–2 months. The follicles, however, usually continued to grow non-pigmented hairs in place of pigmented ones for periods of observation of up to $2\frac{1}{2}$ years.

The present experiments showed that non-pigmented skin could become pigmented following freezing if melanocytes were originally present in the outer root sheaths of the follicles. A similar effect can be produced by surgical removal of the upper part of the skin in areas growing white wool of Suffolk sheep (Lyne, unpublished data). What was claimed by Hardy, Fraser, and Short (1952) from their experiments

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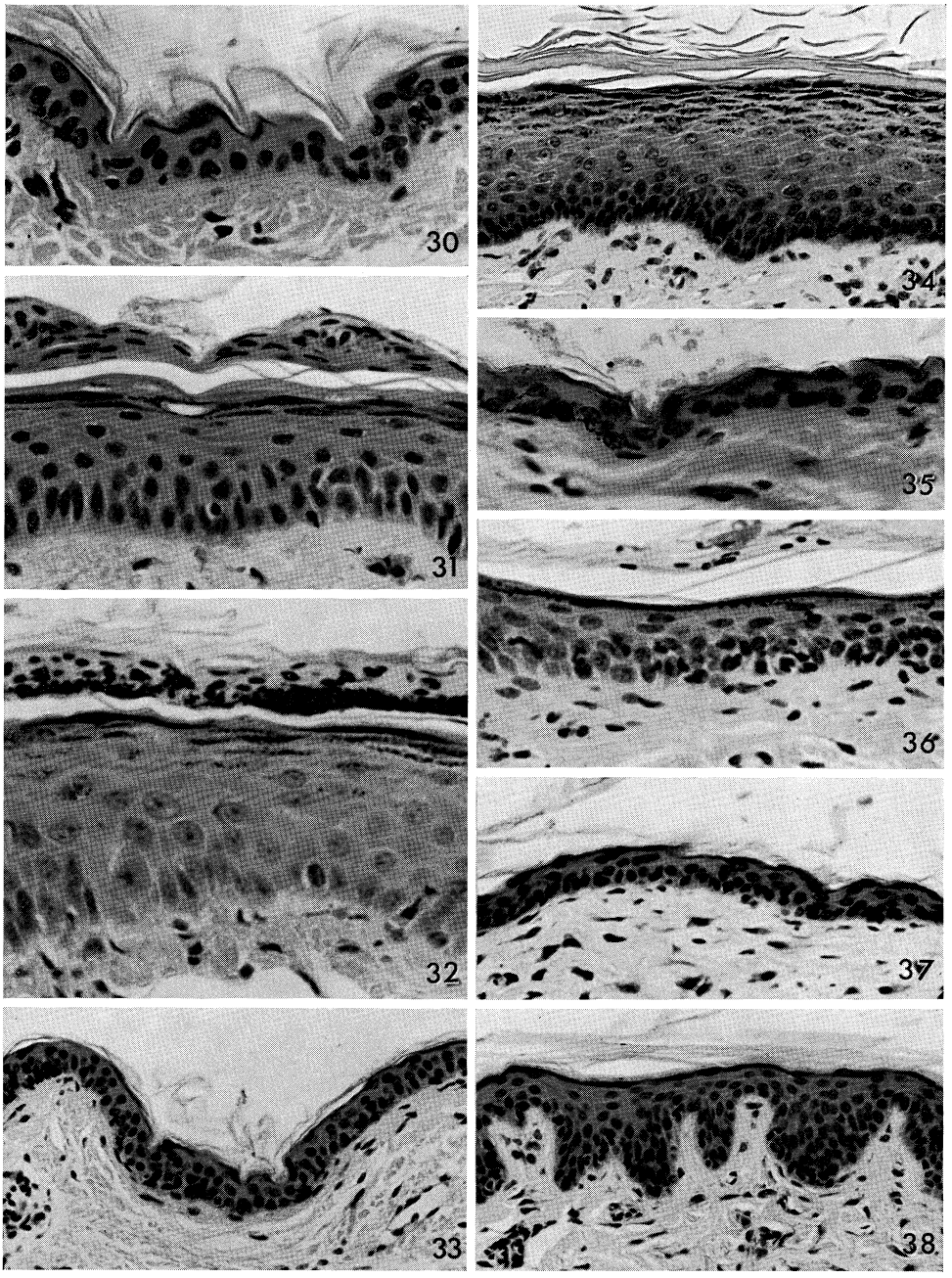
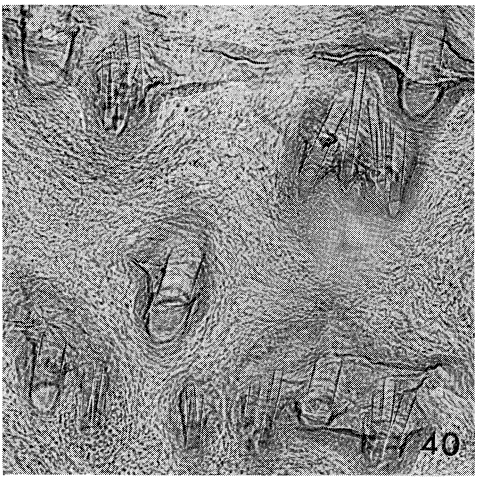
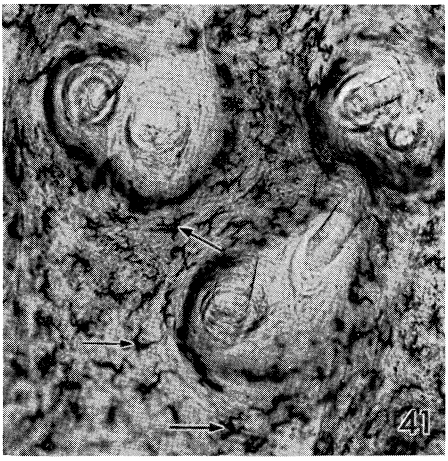
after plucking the fibres. The stratum spinosum and stratum granulosum are conspicuous below a sloughing parakeratotic layer. HEP. ×387. *32*, Greatly thickened epidermis from an area adjacent to the control shown in Figure 30, 3 days after clipping the fibres and freezing the skin for 4 min. The effect is similar to that seen after plucking the fibres. A layer of dead cells is seen above the stratum granulosum. HEP. ×387. *33*, Thin epidermis of the untreated midside of a Suffolk (No. 13). HEP. ×207. *34*, Epidermis from an area adjacent to the control shown in Figure 33, 14 days after clipping the fibres and freezing the skin for 8 min. Note greatly thickened stratum spinosum and stratum granulosum. HEP. ×207. *35*, Thin epidermis of the untreated midside of a Merino (No. 19). HEP. ×367. *36*, Thickened epidermis from an area adjacent to the control shown in Figure 35, 357 days after plucking and freezing for 1 min. HEP. ×367. *37*, Thin epidermis of the untreated outer surface of the pinna of a Suffolk (No. 12) showing a relatively smooth dermo–epidermal junction. HEP. ×220. *38*, Thickened epidermis from the bare area shown in Figures 10 and 11 adjacent to the control shown in Figure 37, 370 days after freezing the inner surface of the pinna for 4·5 min. HEP. ×220.

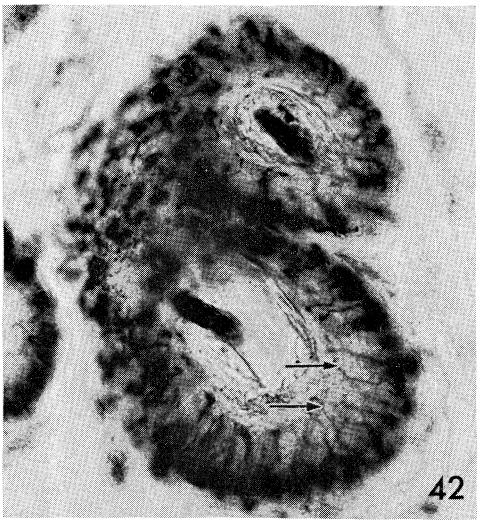


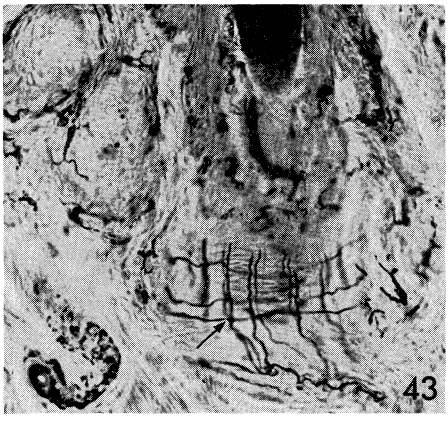
Figs. 30–38.—Vertical sections of the epidermis showing the effects of plucking the fibres alone and of applying solid CO_2 to the skin after close clipping or plucking of the fibres. *30*, Thin epidermis of the untreated midside of a Suffolk (No. 16). The stratum spinosum is one to two cells thick and the stratum granulosum is absent, except around the follicles (not shown). HEP. ×387. *31*, Thickened epidermis from an area adjacent to the control shown in Figure 30, 3 days

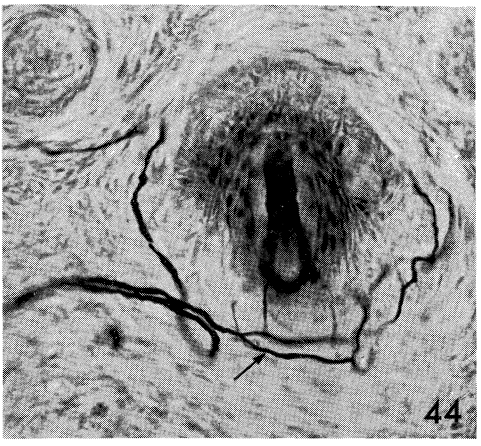

39


40


41


42


43


44

with sheep to have been a spread of pigment from autografts of black skin into the surrounding white skin might have actually been a wounding response of melanocytes in the white skin. In similar experiments with guinea pigs, Pepper (1954) found that white-skinned regions subsequently became pigmented when the upper part of the skin was surgically removed and the lower part of the skin, including the follicle bulbs, was "seeded" with a cell suspension containing melanocytes from the epidermis of the same animals. Perhaps the pigmentation changes described in these guinea pig experiments were due mainly to a wounding response of the melanocytes, which were said to be present in the follicle walls, presumably in the outer root sheath, and not due to melanocyte "seeding".

The destruction of sweat glands following freezing the skin does not appear to have been reported in other species. No doubt this was because the animals most commonly studied (mice, rats, and rabbits) did not possess sweat glands on the general body surface. As the destruction of the sweat glands in the sheep appeared to be permanent, the freezing technique might be useful in physiological studies of the thermoregulatory importance of sweat glands in this mammal. The applicability of the technique to other species would depend on the response of their sweat glands to freezing.

An interesting finding in this study was the increased growth of wool or hair on several of the frozen areas of skin, even when the number of follicles per unit area was not markedly changed. Perhaps the faster length growth rate, or increased length of the hair growth cycle, was partly due to the availability of more nutrients following the destruction of the sweat glands and many of the cutaneous nerves. Ferguson (1949) reported an increase of 36% in wool growth rate following sympathectomy and suggested that it was brought about by vasodilation of the denervated blood vessels. Further studies are needed in order to determine the influence of the various skin structures on the growth of hair or wool.

Fig. 39.—Tangential section of the epidermis from a control black hair-growing region of the lower jaw of a Merino (No. 19) showing a sparse population of melanocytes (arrows) in the basal layer of the epidermis. Alkaline phosphatase. ×70.

Fig. 40.—Tangential section of the epidermis from an area adjacent to that shown in Figure 39, 357 days after clipping the hairs and freezing the skin for 4 min. Melanocytes are absent from the epidermis and all the hairs are white. Alkaline phosphatase. ×70.

Fig. 41.—Tangential section of the epidermis from the midside of a Suffolk (No. 13) 42 days after clipping the fibres and freezing the skin for 1 min. The distribution of the acetylcholinesterase-positive branched cells (arrows) is similar to that in control skin. ×89.

Fig. 42.—Transverse section of upper part of two follicles on the midside of a Suffolk (No. 13) 21 days after clipping the fibres and freezing the skin for 8 min, showing acetylcholinesterase-positive branched cells with conspicuous dendrites (arrows). ×160.

Fig. 43.—Slightly oblique section of the upper part of a primary follicle in untreated skin on the inner surface of the pinna of a Suffolk (No. 12) showing a nerve network (arrow). Silver impregnation. ×173.

Fig. 44.—Transverse section of a primary follicle with a follicle nerve network (arrow) in a treated area of the inner surface of the pinna adjacent to the control shown in Figure 43, 438 days after freezing the skin for 4 min. Silver impregnation. ×237.

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