STUDIES ON THE DEVELOPMENT OF WOOL FOLLICLES IN TISSUE CULTURE

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Summary

Explants of skin from four sheep foetuses of different ages have been cultivated for periods of up to 38 days in a medium of fowl plasma and chicken embryo extract. The development of wool follicles of different types has been studied both in these living explants and in serial sections from them.

Explants from a 70-day foetus showed the complete development of primary wool follicles *in vitro* from epidermal plugs to fully differentiated follicles with emerging wool fibres. The histology of these follicles was normal and they developed at about the same rate as in the foetus *in utero*. Sebaceous glands of normal size and structure were differentiated *in vitro*, and, for the first time in any species, the formation of rudimentary sudoriferous glands *in vitro* has been reported.

In explants from an 80-day foetus, primary wool follicles which were at an early stage of development produced keratinized wool fibres at a slightly faster rate than in the foetus *in utero*, but secondary follicles did not form. Sebaceous glands were formed and sudoriferous glands underwent normal differentiation.

The primary follicles in explants from a 98-day foetus produced many large wool fibres but development was slower than in the foetus. There was some evidence for the formation of new secondary follicles *in vitro*.

There was little or no development in the wool follicles in explants from a 125-day foetus.

The potential value of this method for studying mechanical, nutritional, or hormonal influences on the initiation and early development of wool follicles and the production of wool is indicated.

I. INTRODUCTION

The study of living and growing organs in tissue culture has contributed much to the understanding of their development and function (Fell 1940, 1951). Hair follicles are suitable for cultivation because by virtue of their small size they might be expected to grow and function for some time without a blood circulation, and also their day-to-day changes in structure and output of hair should be more easily observable in explants than by any means available with the intact animal. Tissue culture has already been applied to the hair follicles of pre-natal guinea pigs (Strangeways 1931) and rats (Murray 1933) and has proved useful in studies of hair development in mice (Hardy 1949, 1951; Davidson and Hardy 1952). However, the sheep is a more suitable subject than any of the rodents for investigating some problems such as the formation of hair follicle groups (Carter 1943).

Only one reference has been found to the cultivation of tissues from the sheep. Thomas *et al.* (1948) removed large foetuses of cattle and sheep from the uterus and

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maintained them by perfusion for more than 2 days, but no differentiation of tissues was reported. The aims of the present study, a short account of which has already been published (Hardy and Lyne 1956*c*), were firstly to find a suitable technique for cultivating the skin of foetal sheep, secondly to observe the formation of individual wool follicles of different types, and thirdly to observe the formation of follicle groups and the effect of cultivation thereon. Such information is a prerequisite to assessing the potential value of tissue culture as a tool in investigations of the effect of hormonal, nutritional, or environmental factors on wool production.

II. Material and Methods

Skin for tissue culture was obtained in a fresh condition from four sheep foetuses. Two of these, believed to be Merino, were obtained *in utero* from the State Abattoir and Meat Works, Homebush, N.S.W. Their ages were estimated by the method previously indicated (Hardy and Lyne 1956*d*) to be 70 and 80 days of gestation. Skin samples were also obtained by biopsy, during intra-uterine operation, from two female foetuses of fine-woolled Merino (non-Peppin) parentage. The ages of the latter foetuses were known from mating records to be 98 and 125 days. Both the 80- and the 98-day foetuses were members of a pair of twins.

A series of hanging-drop cultures was prepared from skin from the midside of the 70-, 80-, and 98-day foetuses and from the shoulder of the 125-day foetus. The method of cultivation was similar to that used for the mouse (Hardy 1949). With the aid of a low-power binocular microscope the original skin sample was cut into small pieces with cataract knives. On the basis of a comparison, to be described in Section III, of the effectiveness of different techniques and media on explants from the 70-day foetus, all other cultures were prepared in the following way. Each explant consisted of the full thickness of skin with an epidermal surface area of 1–2 sq. mm, and was placed either on the surface or inside the medium, usually with the dermis nearest to the coverslip. The medium consisted of fowl plasma and chicken embryo extract in ratios ranging from 3:1 to 1:1. The extract was prepared from chicken embryos 10–12 days old and the same or twice the volume of Tyrode solution. The explants were transferred to fresh medium every 2–6 days as required, and a few large explants were divided at the time of transfer. All explants were examined daily under the microscope and many important features were sketched. In each of the four experiments some explants were fixed every few days to give a record of the progress made during cultivation, and the remainder were fixed when no further progress was discernible. All explants were fixed in Zenker's fluid with 3 per cent. acetic acid. Most were sectioned serially at 8 μ and stained with haemalum, eosin, and picric acid, and some were examined as whole mounts after staining with carmalum, borax carmine, or safranin. The 70- and 98-day control skin samples were fixed in Zenker with 3 per cent. acetic acid and the other control samples in 5 per cent. formalin. All control skin was sectioned serially at 7 or 8 μ parallel to the skin surface and also in the direction of the long axis of the follicles.

The terms and symbols used for follicle types are those proposed by the authors (Hardy and Lyne 1956*a*) and explained in a recent paper (Hardy and Lyne 1956*d*).

III. Results

The present authors have recently re-examined the development of different types of wool follicles in the Merino foetus (Hardy and Lyne 1956*b*, 1956*d*). Eight stages of follicle development in mammals (*F* stages) have been defined and their features in primary (*P*) and secondary (*S*) wool follicles have been described. These were then related to Carter's (1943) 18 stages of follicle group development (*G* stages) in the Merino. This study formed the basis for comparing follicle development in tissue culture with that in the foetus. Only an average rate of follicle development in the foetus is available (Hardy and Lyne 1956*d*), but some idea of the extent of variation between foetuses can be obtained from Carter and Hardy (1947) and Hardy and Lyne (1956*d*). There is no information on the rate of development in the foetus from day to day to compare with the observations of this kind which are possible in tissue culture.

Table 1

Summary of Experiments

Age of Foetus (days)	No. of Explants				Maximum Period of Cultivation (days)	Most Advanced *PCX* Follicle Stage Developed		
	Total	Discarded*	Successfully Cultivated	Showing Follicle Development†		Initial	*in vitro*	*in vivo*
70	29‡	1	28	28	38	*F*1	*F*8	*F*8
80	20	1	19	19	16	*F*2*a*	*F*6	*F*4
98	44	0	44	44	16	*F*6	*F*8	*F*8
125	42	1	41	0	11	*F*8	*F*8	*F*8

* Due to infection or failure to survive explantation.

† Excluding development of hair canals.

‡ Omitting 12 half-thickness explants.

Some of the differences between the results of the four tissue culture experiments are indicated in Table 1. Follicle development *in vitro* was observed in nearly all explants in the first three experiments but in none in the fourth experiment. The maximum period of cultivation, which was approximately the same as the period during which progress in wool follicle development was observed, was long in the first experiment but shorter in the second and third. The number of stages which the first-formed central primary (*PCX*) follicles passed through during cultivation was progressively smaller with increasing age of the foetus. This was partly due to the fact that stage *F*8, the emergence of a wool fibre, was the last stage recorded. However, the growth rate of fibres *in vitro* during stage *F*8 was lower than that *in vivo*, so that it is true that overall progress was less in the older foetuses. The last two columns in Table 1 show that in all experiments the stage reached *in vitro* was as advanced as the one at the corresponding age *in vivo*, and in explants from the 80-day foetus it was more advanced.

Another difference between the experiments, not expressed in Table 1, was that the older the foetal skin, the more frequently did degenerative changes occur after a period of cultivation.

(*a*) 70-*day Foetus*

(i) *Structure at Explantation.*—The epidermis consisted of a stratum germinativum of one cell layer, a stratum spinosum of two to three layers, and a periderm of one layer. The dermis consisted of a network of fibroblasts penetrated by small blood vessels. Wool follicle group development was at stage *G*2, with *PCX* and a few of the later-formed central primary (*PCY*) follicles present. The *PCX* follicles were at an advanced stage *F*1, not quite up to *F*2, (Plate 1, Figs. 1 and 2), and the *PCY* follicles were at a very early stage *F*1.

(ii) *Behaviour of Explants.*—Although most explants at first remained fairly flat on the surface of or inside the medium, after about 77 days (age of foetus+age of explant) they showed a tendency to curl up with the epidermis inside and the dermis spreading around it. The follicles therefore extended in all directions into the medium, and wool fibres grew towards the centre of the explant. This orientation, similar to that which develops in skin explants from embryonic mice (Hardy 1949, Text-fig. 2), has the advantage that follicles around the edge of the explant can be seen very clearly in the living state. The epidermis underwent normal differentiation, but usually keratinization took place about 10–15 days earlier than in the foetus, and a thick central mass of cornified cells was formed in some of the older explants. The dermal tissue proliferated and some connective tissue fibres were formed during cultivation. A healthy outgrowth of fibroblasts appeared at 71 or 72 days and new fibroblast outgrowth continued throughout the period of cultivation. After about 80 days there was frequently an epithelial outgrowth also.

(iii) *Effect of Treatments.*—In this first experiment with foetal sheep skin, the technique described in the preceding section was compared with a number of variations from it. The plasma of the medium was fowl plasma, sheep plasma, or a mixture of the two in equal quantities. Some explants were placed horizontally, i.e. with the skin surface parallel to the surface of the medium and the dermis nearest to the coverslip, or with the epidermis nearest to the coverslip, and others were placed vertically, i.e. with the larger cut edges parallel to the surface of the medium and the smaller edges perpendicular to the medium. The full thickness of skin was included in some explants, and others had the deeper half of the dermis removed with cataract knives. One large full-thickness explant with a surface area of about 12 sq.mm was prepared.

Explants in sheep plasma seldom showed any outgrowth before 75 or 76 days, while those in fowl plasma or fowl+sheep plasma had a good outgrowth of fibroblasts at 71 or 72 days. At 80 days explants in sheep plasma still had less outgrowth than those in the other two groups, but after 82 or 83 days there was no difference. Up to 87 or 88 days no differences in health or follicle development could be attributed to the type of plasma, but at this time all remaining explants in fowl+sheep and sheep plasma were fixed because of signs of early degeneration, while some of those in fowl plasma continued until 101 or 108 days with little degeneration. No important

differences were noted between explants placed on the surface and those within the medium, those with the dermis nearest the coverslip and those with the epidermis nearest, or those oriented horizontally and vertically. Owing to their tendency to curl up, most explants eventually assumed much the same form irrespective of their original orientation. Explants of half-thickness skin were much less healthy than those of full-thickness skin although the outgrowth from them was similar. Full-thickness explants had increased in volume at 74 days, but a decrease in volume of half-thickness explants was noted at 72 and 74 days. More than half of the half-thickness

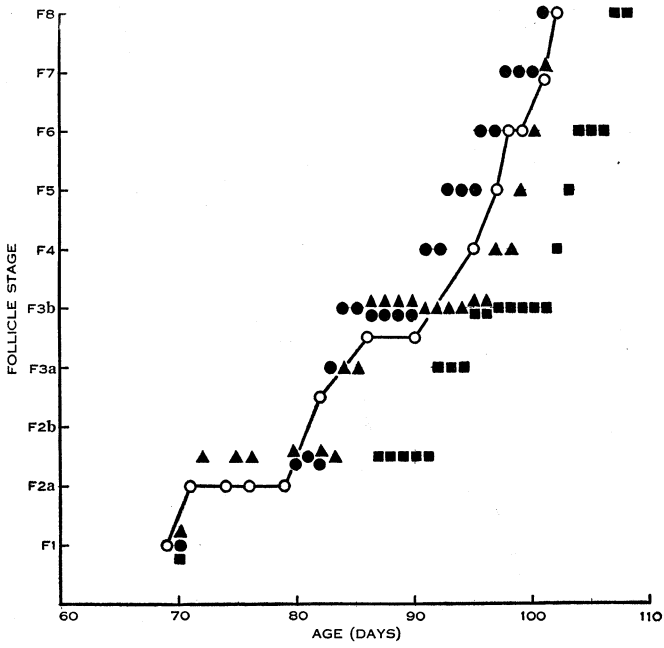

Fig. 1.—The rate of development of the most advanced primary follicles in three explants (●, ▲, ■) from the 70-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

explants showed degeneration at 74 days and only one of them showed any progress in follicle development, to stage *F*2 at 77 days. The large full-thickness explant progressed normally until 78 days and then underwent central degeneration, presumably due to inadequate diffusion.

(iv) *Progress of* PCX *Follicles.*—A record was made of the most advanced stage of follicle development which could be seen each day in each explant. From the daily identification and sketching of many individual follicles it was concluded that the most advanced follicles in an explant at any time were predominantly the *PCX* follicles originally present. In one of the 25 explants for which complete records were available, the stage *F*1 follicles elongated without reaching stage *F*2 before fixation at 74 days, but in all others new *F* stages appeared *in vitro*. The most advanced follicles observed in explants were frequently at the same stage as in the

"average" foetus of the same age, but sometimes slightly ahead or slightly behind. At fixation, for example, 17 explants were at the same stage, four were ahead, and four were behind. Figure 1 illustrates the degree to which follicle development in three explants corresponded with the average rate in the foetus. The variation between explants in the most advanced stage of follicle development after some days of cultivation was greater than the variation between adjacent 1-sq.mm areas on a foetus, but probably much less than the variation between foetuses of the same age (Carter and Hardy 1947).

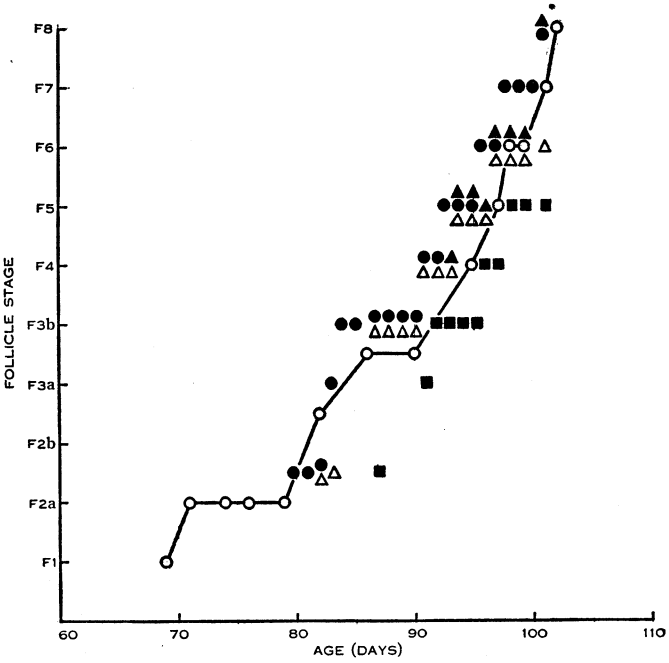

Fig. 2.—The rate of development of four follicles (● = 1, ▲ = 2, ■ = 3, △ = 4) in one explant from the 70-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

Figure 2 shows the rate of development of a few individual follicles in one explant. Follicles 1, 2, and 4 are almost certainly *PCX* follicles, but follicle 3 is possibly a *PCY* or a *PLx* (lateral primary associated with *PCX*) follicle. This figure illustrates the similarity between rates of development in early and later *P* follicles within an explant, comparable with that in the foetus (Hardy and Lyne 1956*d*).

(v) *Progress of Other Follicles.*—Information about follicles other than *PCX* was more difficult to obtain. The first new follicles which appeared during cultivation were identified and their development followed for several days. These were similar to the *PCY* follicles already present and appeared in the spaces between the *PCX* follicles. In 12 explants at 71 days the numbers of follicles ranged from nine to 19, with a mean of 12, and at 73 days the numbers ranged from 15 to 24 with a mean of 20, representing a 67 per cent. increase in follicle numbers, about what would be

expected to make the final numbers of *PCY* and *PCX* follicles roughly equal. By 80 days the numbers of follicles in these explants had increased by about 100–200 per cent. over the numbers at 71 days, and between 80 and 90 days there were further increases in the numbers of follicles in some explants. In one explant which had 11 *PCX* and three *PCY* follicles at 71 days, 37 follicles were observed at 80 days, 34 of these were at *F*3 by 94 days, and 12 were at *F*4 by 99 days. In another explant only one follicle was seen at 71 days and five at 72 days, but at 108 days 19 keratinized hairs were present.

From the above and from similar evidence the following conclusions were drawn. The *PCY* follicles present at 70 days continued their development, some to *F*6 or *F*8. New *PCY* follicles appeared at about the normal time (71–73 days) and in their typical position, and underwent further development. The additional new follicles which formed between 74 and 80 days were probably equivalent to the *PL* (lateral primary) follicles which appear in the foetus at this time, although in explants they were not arranged in the typical manner on either side of the *PC* (central primary) follicles. The skin surface area of explants did not expand as rapidly as that of the foetus, so that the follicles were tightly packed together and any tendency to grouping was likely to be obscured. Some at least of these "*PL*" follicles developed to stage *F*3 or further. There was no direct evidence for the formation of *S* (secondary) follicles, which begins in the foetus at about 86 days. A few follicles at stages *F*1 and *F*2 were noted in the explants fixed at 101–108 days, but since these particular ones could not be traced back to their origin in the living explants there was no means of determining whether they were new *S* follicles or retarded *P* follicles.

(vi) *Histology.*—All the histological features which distinguish the eight stages of hair follicles in mammals were observed *in vitro*. Many of these are illustrated in Plate 1, Figures 3–6, and in the comparison of Plate 2, Figures 1 and 5, with Plate 2, Figures 2 and 6, respectively. In explants cultivated for a sufficient period many wool fibres with a keratinized cuticle and cortex of normal structure were formed (Plate 1, Fig. 6). Medullated fibres were not found, but it is quite normal for *P* fibres on the midside of Merino foetuses between 100 and 110 days to be non-medullated. Apart from the absence of arrector pili muscles, and from variations in the sebaceous and sudoriferous glands to be described below, only the following minor differences were noted between primary follicles formed in explants and those in the Merino foetus. There was a tendency for the follicles in explants to be shorter in proportion to their width, and for the ental swelling of the outer root sheath to be more conspicuous. In explants the follicles were more frequently curved or twisted, and, because of the central position and precocious keratinization of the epidermis, the wool fibres had difficulty in penetrating it and frequently coiled beneath the surface.

The sebaceous gland rudiments made their first appearance in explants relatively late, when follicles were at stages *F*3*b* or *F*4 (92–102 days) instead of in the latter part of stage *F*2 (82 days) as in the foetus. However, they subsequently developed into glands comparable in size to those in foetuses of corresponding age (Plate 1, Fig. 6), and some were bilobed in the manner typical of those attached to *P* follicles (Plate 2, Figs. 1 and 2). Holocrine secretion from the normally differentiated sebaceous cells was observed (Plate 2, Fig. 4).

Sudoriferous glands appeared late in stage *F*2 (76–83 days) instead of at the beginning of *F*2 (71 days). The gland rudiments were observed in the living state in only five out of 18 explants cultivated after 80 days. Of these five explants, only one showed sudoriferous glands in stained sections after fixation (Plate 1, Fig. 4). The rudiments disappeared from the other living explants after a few days and it was assumed that they had regressed. Three other explants showed sudoriferous gland rudiments in sections. Unlike the sebaceous glands, the sudoriferous glands did not attain a size or degree of development comparable with that in the foetus of 100 days or more, but always remained in a rudimentary state. The most advanced sudoriferous gland found in any explant (at 101 days) was a short blind tube with a small distal lumen, typical in histology though shorter than the gland of a *PC* follicle in the foetus at *F*4 or *F*5 (95–97 days). It was attached to a follicle at *F*7 (Plate 2, Figs. 1 and 3).

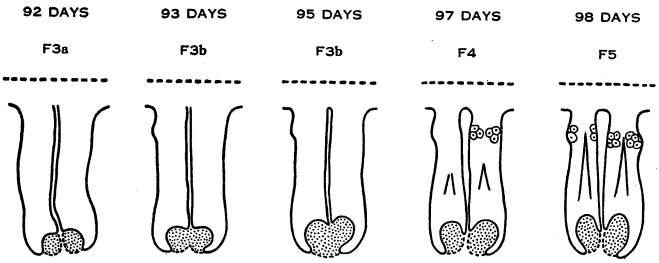

Fig. 3.—Diagram showing the progress from stage *F*3*a* to *F*5 of "twin" follicles in an explant from the 70-day foetus. Note the separation, fusion, and subsequent separation of the dermal papillae.

One unusual arrangement of developing follicles was noted in three of the five explants cultivated after 88 days. Five pairs of "twin" follicles were found with their dermal papillae joining. Three pairs were noted only in sections but the other two pairs were observed also in a living explant from 91 to 101 days (Fig. 3). In one pair the follicles were contiguous along their whole length and in the other, only at the base. The follicles were at stage *F*3*a* when first observed, and by 101 days one pair was at *F*4 and the other at *F*5. The rates of development of the members of a pair were very similar but not always identical.

(*b*) 80-*day Foetus*

(i) *Structure at Explantation.*—The skin contained follicle groups at stage *G*5. Long *PCX* follicles were at stage *F*2*a*, with sudoriferous glands extending to about two-thirds of the depth of the follicles (Plate 3, Fig. 1). The *PCY* follicles were shorter, also at *F*2*a*, with sudoriferous glands extending to about half the depth of the follicles. The *PLx* follicles were still shorter; some were at *F*2*a* with sudoriferous gland rudiments beginning, and others at *F*1. Some *PLy* follicles were at *F*1 and the remainder had not yet formed. This stage of follicle group development was slightly earlier than that in the average Merino foetus at 80 days, which is stage *G*6 with all four types of follicles at stage *F*2*a*.

(ii) *Behaviour of Explants.*—The explants remained flat until about 85 days, and then about half of them began to curl up like those from the 70-day foetus with the dermis surrounding the epidermis (Plate 3, Fig. 3), while the remainder began to curl in the opposite direction with the epidermis surrounding the dermis and obscuring the view of living follicles. There was no appreciable difference between the two groups in the health of explants or the development of follicles. In both groups keratinization of the epidermis began about 10–15 days earlier than in the foetus. There was a good outgrowth of fibroblasts and a small outgrowth of epithelial cells.

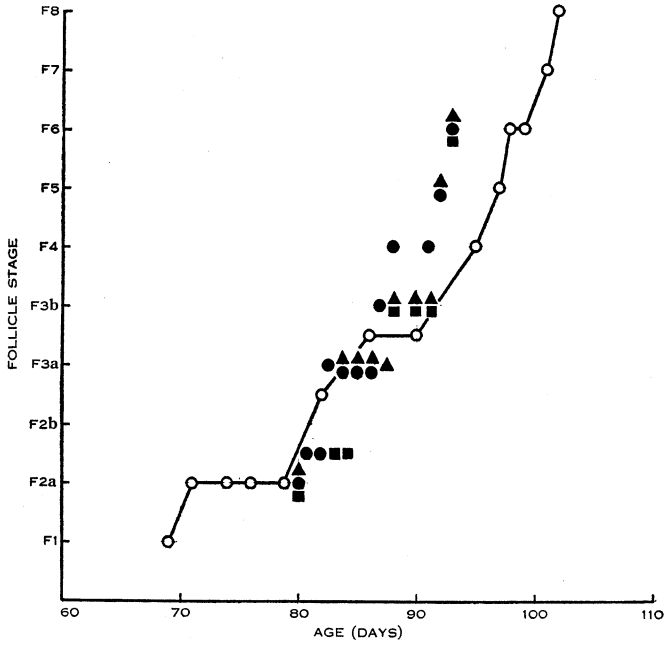

Fig. 4.—The rate of development of the most advanced primary follicles in three explants (●, ▲, ■) from the 80-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

(iii) *Progress of Follicles.*—In every explant, some *PCX* follicles underwent further development, to at least stage *F3a* and in some cases to stage *F6*. Unlike the 70-day explants, many of the 80-day explants showed *PCX* follicle development at a more rapid rate than in the foetus. Thus, at fixation, eight explants were ahead, eight were at the expected stage, and three were retarded. The follicles in three explants which reached stage *F6* at 93 days were about 5 days or about 2·5 *F* stages ahead of those *in vivo*. Figure 4 shows their progress compared with that in the foetus. The *PCY* follicles began to develop in the same way as *PCX* follicles but their final stage could not be determined because they could not be distinguished from *PCX* follicles throughout cultivation. However, the use of 80-day explants made possible some observations on the development of *PL* follicles which could be

identified from their position on either side of the *PC* follicles. The *PLx* and *PLy* follicles already present were seen to elongate *in vitro*, and some at least reached stages *F3a* and *F3b* by 84 days, much earlier than in the foetus. In five explants during the first few days of cultivation some new *PLy* follicles appeared. The trio arrangement was preserved during cultivation in most of the explants. There was no sign of the formation of *S* follicles.

(iv) *Histology.*—The histology of follicles developed *in vitro* was similar to that of follicles in the 70-day explants (Plate 3, Figs. 2 and 3). Some of the wool fibres were of normal structure but some were incompletely keratinized. As in the 70-day explants, sebaceous gland development was retarded. Gland swellings, some with differentiated sebaceous cells, were first noted in living explants at 87 days or later, in follicles at stage *F3a* or *F3b*. However, sebaceous glands were found in all 19 of the explants fixed between 84 and 96 days.

Sudoriferous gland development was more extensive than in explants from the 70-day foetus. In all explants the glands originally present elongated together with the follicles to which they were attached, and underwent some distal enlargement. In 14 explants at fixation the sudoriferous glands showed a distinct distal lumen. The glands were thus as advanced in their development as the follicles and as sudoriferous glands in foetuses of similar ages. During cultivation a number of new sudoriferous glands appeared in association with *PLx* and *PLy* follicles and underwent some development, although not every *PL* follicle was found to possess a sudoriferous gland at the time of fixation.

In each of three living explants one or more follicles which had become separated from the epidermis were observed. No epidermal tissue other than the follicle wall itself was in contact with these "isolated" follicles, which were completely surrounded by dermis. These follicles were first noted at stage *F2* or *F3a* and progressed to *F3a*, *F4*, and *F6* respectively. One developed a sudoriferous gland rudiment.

(*c*) 98-*day Foetus*

(i) *Structure at Explantation.*—The follicle groups were at stage *G13*, which appears at 99 days *in vivo*. Only a few *PCX* follicles had reached the beginning of stage *F6* and the remainder of the *PCX* and the *PCY* follicles were at *F5*. The *PL* follicles were at *F5*, *F4*, or *F3b*. The original secondary (*SO*) follicles were at *F3a*, *F2*, or *F1*, and a few of the most advanced ones had some differentiated sebaceous cells. On a few of the *SO* follicles at *F3a* and *F2*, a single derived secondary (*SD*) follicle at *F1* was present as a bud. There was usually not more than one *SD* follicle per follicle group, but occasionally two were present.

(ii) *Behaviour of Explants.*—These explants remained flatter during cultivation than those from younger foetuses. There was a healthy outgrowth of fibroblasts and usually a smaller healthy epithelial outgrowth. The explants, however, were less healthy than those from 70- and 80-day foetuses, and degenerative changes took place at the base of many of the larger follicles.

(iii) *Progress and Histology of Follicles.*—During the first few days of cultivation there was an increase in the number of *PCX* follicles at *F6*, and many *PCY* follicles also reached this stage. The longer the period of cultivation, the more likely was an

explant to reach *F*8, but the usual rate of progress, unlike that in 70- and 80-day explants, was slower than *in vivo*. Before cultivation the diameter of keratinized fibres varied from 3 to 8 μ (average 4 μ), but after cultivation many fully keratinized, non-medullated fibres of 16–25 μ were found. Other thick fibres in the explants were incompletely keratinized, having areas in which the cuticle was fully keratinized and stainable with picric acid, while the remainder of the fibre was of abnormal structure and stained only with eosin. Healthy sebaceous glands were found with *PC* follicles in this as in other experiments, and the lumina of sudoriferous glands usually enlarged during cultivation. Further progress of *PL* follicles was observed in this experiment, to stage *F*6 or beyond in 12 explants and to *F*5 in most others, but the rate of progress was slower than *in vivo*. Some of the *PL* fibres formed *in vitro* were fully keratinized and others partly keratinized. The sebaceous and sudoriferous glands progressed slightly.

This experiment provided the first opportunity for observations on *S* follicles *in vitro*. It was not possible to determine the extent of development of individual *SO* follicles in 12 whole-mount preparations. In the explants which were sectioned, some *SO* follicles reached stage *F*3*b*, and one possibly *F*4, which indicates a slower rate of development than *in vivo*. However, in skin sections from the foetus used in this experiment (dead) and from its larger twin (alive), when aborted at 118 days, the follicle groups were only at stage *G*16 (instead of *G*17, as in the average foetus), and no *SO* follicles were seen at stages more advanced than *F*3*b*. In some explants the sebaceous glands attached to the *SO* follicles had undergone further development.

Individual *S* follicles were not identified during the course of the experiment, but evidence for any changes during cultivation in the numbers of *S* follicles of either type was sought from follicle counts in sectioned material. The numbers of *S* follicles per follicle group in transverse sections (1) at the mid-sebaceous gland level of *P* follicles, and (2) above the sebaceous glands, in control skin were compared with the numbers at these levels in explants. In control skin the mean number was 2·4 (range 1–3) at level (1) and 4·4 (range 4–5) at level (2). In each of nine explants fixed at 100–111 days there was an increase in the upper limit of the range and usually also in the mean value at level (1). In eight out of nine explants there were corresponding increases at level (2). The greatest increases were found in an explant fixed at 110 days, in which the ranges of counts were 5–8 at level (1) and 5–10 at level (2).

In order to interpret the above results, some reconstructions were made of the *S* follicles in three entire follicle groups from the explant referred to, and in five groups in all from three other explants. Each of these groups contained a number of short *SO* follicles which did not extend as deep as level (1). Some longer *SO* follicles had *SD* follicles budding from them above or occasionally at level (1), but not more than one *SD* was found with any *SO* follicle, and not more than two *SD* follicles were found in any group. In two explants other than those from which reconstructions were made, a few *SO* follicles each with two *SD* follicles were seen, but this was apparently an infrequent occurrence. In one whole mount of an explant fixed at 107 days, follicle groups containing four to eight *S* follicles at level (2) were clearly seen. The numbers of *S* follicles were lower at level (1) and this appeared to be due to the shortness of some *SO* follicles.

It was concluded that in many explants there was development of *S* follicles during cultivation. The increased numbers of *S* per group above sebaceous gland level could be explained by the formation of new *SO* follicles, and the increased number of *S* at the sebaceous gland level could be due to the increase in length of the *SO* follicles originally present and perhaps of some of the new ones. In two explants at least there was probably an increase in the number of *SD* follicles.

(*d*) 125-*day Foetus*

(i) *Structure at Explantation.*—Follicle groups were at stage *G*18, and all *P* and some *SO* follicles were at stage *F*8. Other *SO* follicles were at stages *F*4 to *F*7. The *SD* follicles were numerous, as many as eight *SD* per group being present, and some of them had reached stage *F*3*b* or *F*4.

(ii) *Behaviour of Explants.*—These explants remained fairly flat. The outgrowth of fibroblasts and epithelial cells appeared more slowly than in explants from younger foetuses and was frequently less extensive, but usually appeared healthy. However, the explants showed some degeneration at the base of the larger follicles even at 129 days, and progressive degeneration in the dermis and larger follicles thereafter. The explants fixed at 136 days were very unhealthy.

(iii) *Progress and Histology of Follicles.*—No evidence was found of progress in any of the types of follicles present. The *P* wool fibres did not increase in length, and there was no indication of an increase in the number of keratinized *SO* fibres per group. It was not possible to test whether the number of *SD* follicles had increased.

(*e*) *Development of Follicle Groups in All Experiments*

Although the study of follicle group development was one of the objects of the experiments, Carter's (1943) definitions of group stages were found unsuitable for recording the changes in explants because they referred in turn to differentiation in different types of follicles. Consequently the results of the present experiments have so far been described in terms of the differentiation of each type of follicle separately, but the extent of follicle group development will now be indicated. In the explants from 70-day skin at stage *G*2, stage *G*3 developed as *in vivo* when sudoriferous gland buds appeared with the *PCX* follicles, and new "groups" were initiated by the appearance of new *PCY* follicles. Although the *PCX* follicles continued their development to the stage which they reach at *G*15 *in vivo*, it could not be said that the explants reached even a typical stage *G*4, because the trio arrangement of *P* follicles was not observed, or a stage *G*8, because *S* follicles did not appear. Explants from the 80-day foetus progressed from *G*5 to *G*7, and new *PLy* follicles completed some trio groups, but there was no *G*8, although the development of *PCX* follicles at 93 days corresponded to that of stage *G*12. In the 98-day foetus all follicle types had been initiated, and explants proceeded from stage *G*13 to *G*16 as *in vivo*. The 125-day foetus had already reached the most advanced stage of group development.

IV. Discussion

(*a*) *Technique*

The technique used was found to be reasonably satisfactory for skin from the younger foetuses but unsuitable for the older ones. The surprising superiority, in the

first experiment, of plasma from the fowl may be due to mechanical factors, such as the production of a firmer clot. In view of the disappointing results with skin from older foetuses, it would be worth testing the effects on those of semi-homologous, homologous, and autologous media. The finding that the full thickness of dermis was necessary for successful follicle development in skin from the 70-day foetus was also surprising, since the dermis appeared to be histologically homogeneous at this stage, and was very much deeper than necessary to accommodate the extending follicles. In the mouse all that was required for successful follicle development, in addition to epidermis, was a portion of dermal tissue (or subepidermal mesoderm) (Hardy 1949).

(*b*) *Comparison of Sheep and Mouse Hair Follicles* in vitro

The development of the first pelage hair follicles from stage *F*1 to stage *F*8 occupied about 9 or 10 days in the mouse (Hardy 1949) but about 38 days in the sheep, so that tissue cultures from sheep had to be maintained over four times as long to include the same sequence of development. Nevertheless, the maximum degree of progress achieved *in vitro* in a single set of explants was almost as great with the sheep as with the mouse. In mouse skin, follicles initiated *in vitro* progressed to stage *F*8, while in sheep skin, follicles explanted at *F*1 progressed to *F*8. The rate of development of follicles in explants from the mouse was at best very similar to that *in vivo*, and the rate in explants from the 70-day sheep foetus was also similar to that *in vivo*, but the rate in some explants from the 80-day foetus appeared to be significantly faster. The fractions of the total follicle development time occupied by the several stages *in vivo*, or the shapes of the stage-age curves, were different for the two species, and the explants behaved in this respect like the species from which they were taken.

In all essentials, the histogenesis of follicles was normal in explants of both mouse skin and sheep skin. Two abnormalities found in explants of sheep skin are of morphogenetic interest. The "twin" follicles, or structures like them, could perhaps give rise to the multiple fibres of sheep (Auber and Ryder 1956) or other species (Pinkus 1951), the origin of which has not been studied. Moreover, their orientation suggests that the influence of dermal papilla cells on hair matrix cells, in palisade formation, for example, may extend from one follicle to the next. The occurrence of "isolated" follicles indicates that, at certain stages at least, the differentiation of follicles does not require continuity with the epidermis.

The sebaceous glands produced in sheep skin explants were much larger than those in explants of mouse skin, and were nearer to the normal in size and extent of differentiation. They developed *in vitro* from follicles initially at stage *F*1. This seems to be the best differentiation of sebaceous glands obtained *in vitro* for any species, since other tissue cultures of sebaceous glands (Zymbal 1933) have not shown any growth of glands or differentiation of gland cells.

There are no sudoriferous glands on the general body surface of the mouse, and the present work includes what is apparently the first report of the origin and differentiation of those structures *in vitro* for any species, since other authors have obtained only undifferentiated epithelial growth (e.g. Pinkus 1938). Sudoriferous glands formed *in vitro*, elongated, and formed a small distal lumen, although develop-

ment was slower than normal. Glands already present at explantation differentiated at a normal rate and developed a large distal lumen.

(c) *Development of Follicle Groups* in vitro

It was concluded that the capacity of sheep skin *in vitro* for building up a follicle group by the addition of new types of follicles in a particular arrangement was more limited than its capacity for the development of individual follicles. This may or may not be related to the failure of the skin surface area to increase at a normal rate *in vitro*. The abnormally rapid rate of development of *PCX* follicles, together with the failure of initiation of *S* follicles, in skin from the 80-day foetus, is of particular interest. This process in an intact foetus would probably result in a fleece not unlike that of primitive or carpet-wool breeds, in which the *P* fibres are very large and the *S* fibres are very much smaller and relatively few in number (Carter 1955). It is not known whether these breeds show such a delay in the initiation of *S* follicles or rapid rate of progress of *P* follicles; Ross (1954) compared the follicle group development in normal Romneys and *N*-type Romneys which produce a carpet-wool type of fleece and found no differences up to 88 days foetal age, but after this, development in *N*-type appeared to be more rapid: "*N*-type is judged to be slightly ahead, by 2 days, in the 92-, 96-, and 104-day samples . . ." Thus it is possible, but by no means certain, that more precise observations would show a more rapid rate of *P* follicle development and possibly a delay in *S* follicle initiation at this period in carpet-wool types of sheep. If so, Dry's (1934) "prenatal check" or the theory of competition for available nutrients (Fraser 1951) or both of these might be demonstrated in Merino skin *in vitro* by choosing different conditions in which *S* follicle initiation was normal, delayed, and inhibited respectively.

(d) *Potential Applications*

Among the many problems of follicle development which could be studied by tissue culture, it seems that the following might be profitable: the effect of skin expansion on follicle initiation and group formation (provided that a suitable means can be devised for mechanically stretching the skin); the effect of various conditions of cultivation on the origin of *SO* follicles and the extent of their branching to form *SD* follicles; and the effect of the retardation of follicles of one type on the development of those of other types.

The effect of temperature on wool follicle and fibre development could be tested simply *in vitro*. With a basic medium of plasma and embryo extract, it would also be possible to test the effect of adding to the skin substances such as glucose, cystine, protein hydrolysates, or copper, thus helping to separate direct from indirect effects on wool growth. The development of synthetic media suitable for differentiated growth, which is expected in the near future, will enable these and other nutritional experiments to be carried out in a more satisfactory manner. The present technique would also be adequate for examining the direct effects of vitamins or hormones on the skin and wool follicles; other keratinizing epidermal structures have recently been studied in this way (Fell and Mellanby 1953; Hardy, Biggers, and Claringbold 1953). It should be emphasized, however, that these methods are unsuitable for

highly quantitative work or for examining factors which produce only small differences in the rate of follicle development or wool production.

V. Acknowledgments

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EXPLANATION OF PLATES 1–3

All figures are of sections through follicles or associated glands or both in skin of the midside from sheep foetuses directly or after cultivation. The staining is with haemalum, eosin, and picric acid. Sections shown in Plate 2, Figure 2, and Plate 3, Figure 1 were fixed in formalin, and all others in Zenker's fluid. *B*, basement membrane; *Cu*, cuticle of wool fibre; *D*, dermis; *DP*, dermal papilla of wool follicle; *G*, stratum germinativum; *H*, keratinized wool fibre; *He*, Henle's layer; *Hu*, Huxley's layer; *HN*, hair cone; *I*, inner root sheath; *ICu*, inner root sheath cuticle; *L*, lumen of sudoriferous gland; *O*, outer root sheath; *P*, periderm; *PP*, pre-papilla; *S*, stratum spinosum; *SG*, sebaceous gland with differentiated cells; *SudG*, sudoriferous gland; *SudGD*, sudoriferous gland duct; *SudGR*, sudoriferous gland rudiment.

PLATE 1

Development of wool follicles in tissue culture

Fig. 1.—Vertical section of skin from 70-day foetus with a *PCX* follicle at stage *F*1.
Fig. 2.—Horizontal section of skin from 70-day foetus with a *PCX* follicle at stage *F*1.
Fig. 3.—Longitudinal section of *PCX* follicle at stage *F*2 in explant of skin from 70-day foetus at 74 days.
Fig. 4.—Longitudinal section of *P* follicle at stage *F*3*b* showing sudoriferous gland rudiment in explant of skin from 70-day foetus at 101 days.
Fig. 5.—Longitudinal section of *P* follicle at stage *F*4 in explant of skin from 70-day foetus at 88 days.
Fig. 6—Longitudinal section of *P* follicle at stage *F*7 in explant of skin from 70-day foetus at 108 days.

PLATE 2

Development of wool follicles in tissue culture

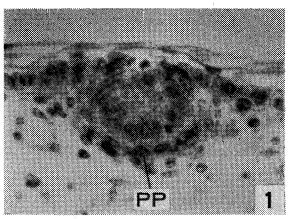
Fig. 1.—Oblique section of *P* follicle at stage *F*7 at level of sebaceous gland in explant of skin from 70-day foetus at 101 days.
Fig. 2.—Transverse section of *PC* follicle at stage *F*7 at level of sebaceous gland in skin from from 100-day Merino foetus.
Fig. 3.—Transverse section of sudoriferous gland associated with the *P* follicle shown in Plate 2, Figures 1 and 5.
Fig. 4.—Section of sebaceous gland showing secretory process in explant of skin from 70-day foetus at 108 days.
Fig. 5.—Longitudinal section of base of *P* follicle shown in Plate 2, Figure 1.
Fig. 6.—Longitudinal section of base of *PC* follicle at stage *F*5 in skin from 98-day Merino foetus.

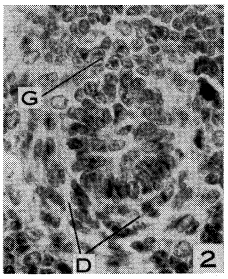
PLATE 3

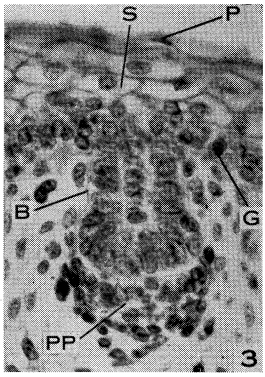
Development of wool follicles in tissue culture

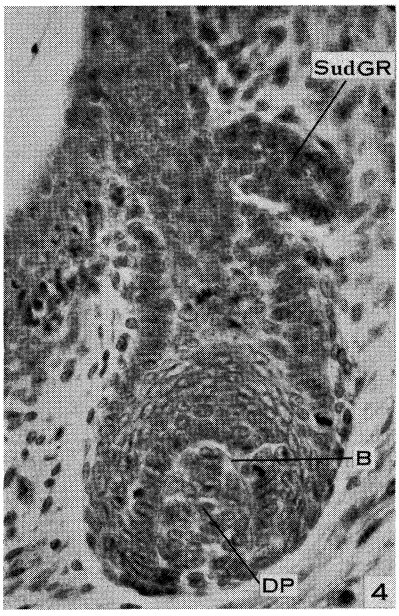
Fig. 1.—Vertical section of skin from 80-day foetus with a *PCX* follicle at stage *F*2*a*.
Fig. 2.—Part of explant of skin from 80-day foetus at 84 days, showing follicle on left at stage *F*3*a* and follicle on right at stage *F*1.
Fig. 3.—Horizontal section of entire explant of skin from 80-day foetus at 84 days, showing development of follicles around central epidermis.

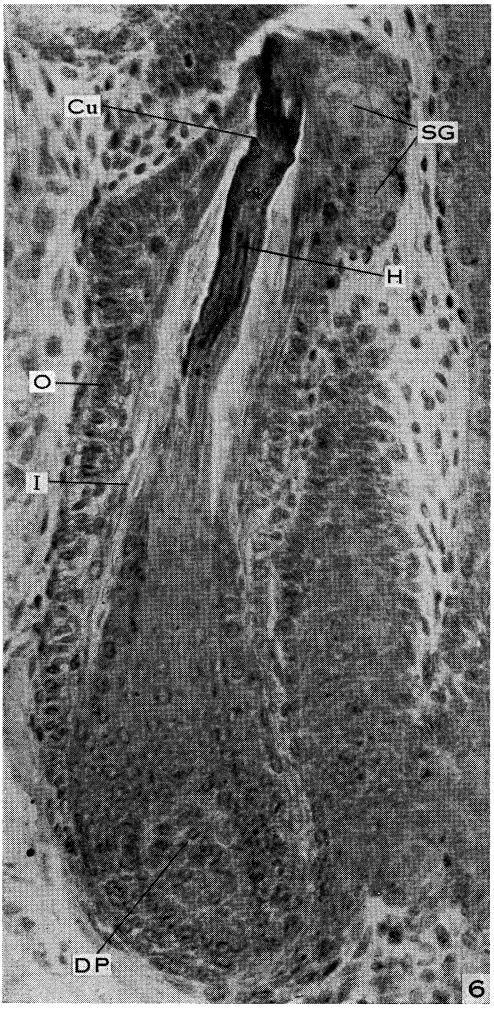
WOOL FOLLICLE DEVELOPMENT IN TISSUE CULTURE

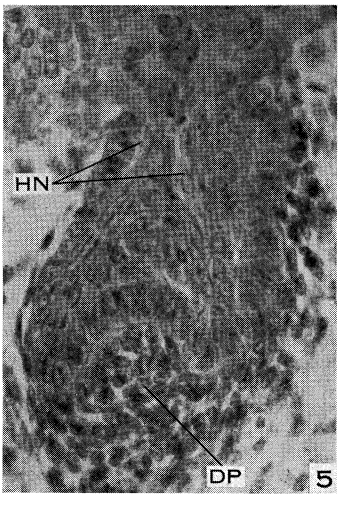






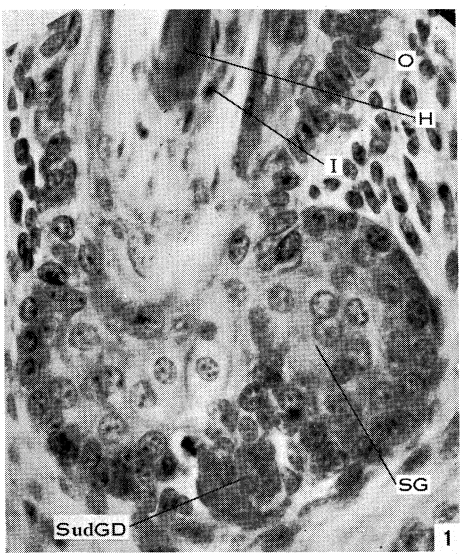


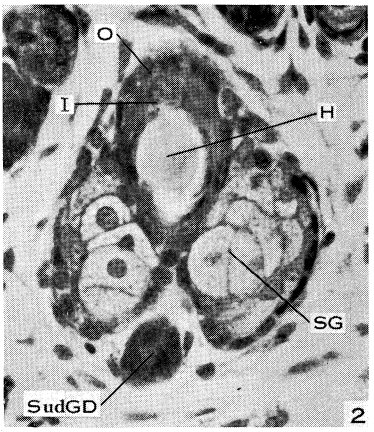


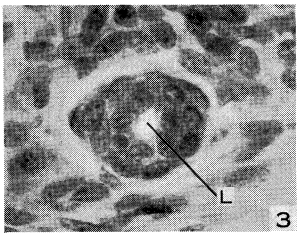


100μ

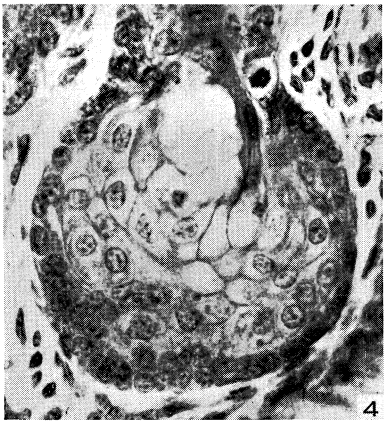
WOOL FOLLICLE DEVELOPMENT IN TISSUE CULTURE

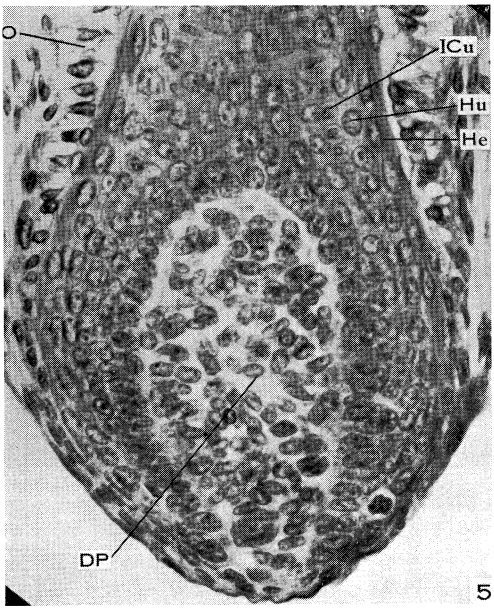


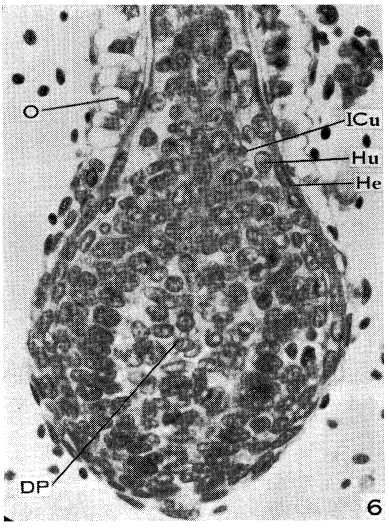




50μ







WOOL FOLLICLE DEVELOPMENT IN TISSUE CULTURE