

RADIOAUTOGRAPHIC STUDIES OF THE INCORPORATION OF [³⁵S]CYSTINE INTO WOOL

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Summary

A study has been made of the distribution of radioactivity in skin samples and in plucked wool fibres taken at intervals after giving intravenous injections of DL- and L-[³⁵S]cystine respectively to two adult Corriedale sheep. The results indicated that radioactivity extends throughout the whole of the unkeratinized wool root 1 hr after the injection, appearing first in the suprabulbar region of the follicle and spreading distally to the keratinized region. Less than 3% of the spread of radioactivity during the first hour could be explained on the basis of fibre growth.

Possible mechanisms explaining the observed movement of ³⁵S in hair follicles are discussed.

I. INTRODUCTION

The rate of entry of intraperitoneally injected DL- and L-[³⁵S]cystine into hair follicles in the mouse has been studied by Bern, Harkness, and Blair (1955), Harkness and Bern (1957), and by Ryder (1958, 1959). The corresponding rate of entry into wool follicles has been studied in two Romney lambs (Ryder 1958, 1959). No analogous experiments in which adult sheep were used have been reported.

The rate of growth of wool can be measured by giving a sheep a series of intravenous doses of DL- or L-[³⁵S]cystine at known intervals of time, followed by radioautography of individual fibres and measurement of the distances between the clusters of silver grains corresponding to each dose (Downes and Lyne 1959, 1961). In developing this technique it was assumed that the radioactive label would always appear in the fully keratinized fibre at the same time after administering the dose. The actual time was unimportant provided it was constant. However, in certain applications of this technique, for example in studies of the influence of various factors on the crimping of wool, it may be important to have a precise measure of this time interval.

During the course of experiments on the fate of intravenous doses of DL- and L-[³⁵S]cystine in the sheep, the rate of arrival and mode of distribution of the ³⁵S in the wool follicles have been studied, with the results reported here.

II. MATERIAL AND METHODS

Two adult Corriedale sheep (wether, 40 kg body weight; ewe, 57 kg body weight) were studied. The doses of L-[³⁵S]cystine (from the Abbott Laboratories, Chicago) and of DL-[³⁵S]cystine (from the Radiochemical Centre, Amersham, England) were prepared and injected intravenously as described by Downes and Lyne (1961).

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Skin samples were taken with a 1-cm trephine from the mid-lateral region of the trunk of one sheep. The methods of Clarke (1960) were used. The samples were fixed in either neutral formalin, absolute ethanol, or acetone, and then embedded in paraffin and sectioned at $8\ \mu$ parallel to the long axes of the hair follicles. Radioautograms were prepared with Kodak AR 10 or AR 50 film, as described by Pelc (1956). The sections were stained through the film with either Mayer's haemalum and picric acid or with methyl green-pyronine. Another series of skin sections (not radioautographed) were stained with Barrnett and Seligman's (1952) reagent to show the sulphhydryl zone and were compared with adjacent sections stained with Mayer's haemalum and picric acid.

Wool fibres, in groups of 20–30, were plucked by hand. About 80% of the fibres possessed substantially complete wool roots, which had evidently broken off just above the bulb. Some of the fibres were stained with Mayer's haemalum and picric acid. Others were treated with the Barrnett and Seligman reagent: the reaction with 2,2'-dihydroxy-6,6'-dinaphthyl disulphide was stopped after 3 min at 50°C . With longer reaction times the staining was so intense that the overlying silver grains subsequently produced by radioautography could not be clearly seen. The fibres were radioautographed as described by Downes and Lyne (1961). Kodak AR 50 film was used.

III. EXPERIMENTAL

(a) *First Experiment*

A dose of DL-[^{35}S]cystine ($508\ \mu\text{c}$; $6.2\ \text{mg}$) was injected intravenously into the wether. Wool fibres were plucked at random from both flanks of the animal at times ranging from 30 sec to 4 days after the injection. Some of the fibres from each sample were radioautographed directly, while others were stained with Barrnett and Seligman's reagent and then radioautographed. Exposure times of 19 and 35 days were used.

(b) *Second Experiment*

A dose of L-[^{35}S]cystine ($356\ \mu\text{c}$; $6.7\ \text{mg}$) was injected intravenously into the ewe. Skin samples were taken 2, 5, 15, 45 min, 2, 5.5, 24 hr, and 5.5 days after injection. Wool fibres were plucked at the following times after the injection: 1, 2, 3, 4, 5, 6, 8, 10, 15, 30 min, 1, 2, 4, 7, 11, and 30.5 hr. Samples were radioautographed using exposure times ranging from 13 to 35 days.

(c) *Examination of Material*

The skin sections and plucked fibres were examined to determine the position of the radioactive region relative to the different zones of the follicle. The terminology of Ryder (1958) was followed. In addition, the criteria of Hardy (1952) were used in assessing the limits of the various zones. The upper limit of the radioactive region, i.e. the point at which the density of silver grains was indistinguishable from the background, was independently determined by two observers without a knowledge of the sampling times of the specimens.

The length of the labelled zone on radioautograms of a number of plucked fibres was measured independently by three observers by means of a projection microscope (magnification $\times 215$). The lengths were corrected by subtracting the maximum distance which the β -rays from the ^{35}S in the fibre had penetrated into the film. This correction, which was assessed by measuring the length over which an increased silver grain density was detectable at right angles to the fibre, was usually of the order of $50\ \mu$

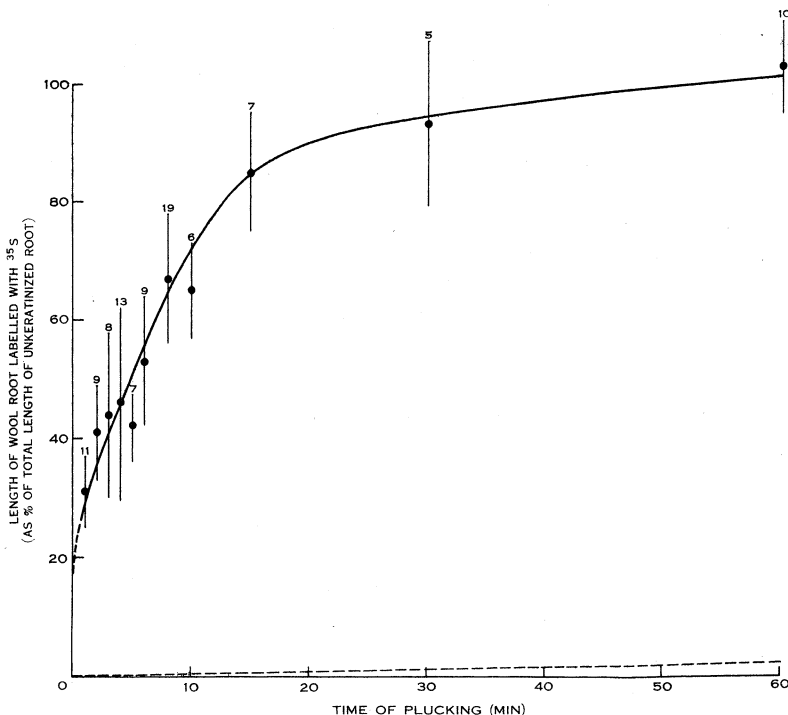


Fig. 1.—Rate of spreading of radioactivity in wool roots after an intravenous injection of L-[^{35}S]cystine ($356\ \mu\text{c}$; $6.7\ \text{mg}$) into a Corriedale ewe. Wool fibres were plucked at various time intervals after the injection, and were radioautographed. The distance from the lower end of each root to the upper limit of the radioactivity, corrected for the degree of penetration of the β -rays into the film (see text), was measured and expressed as a percentage of the total length of the unkeratinized root. The mean percentages for all of the fibres plucked at each time interval are shown on the graph. The vertical lines represent the standard deviation for a single fibre and the numerals above each vertical line indicate the number of wool roots measured in each group. The slope of the broken line indicates on the same length scale the mean rate of growth of the wool fibres from this animal at the time of the experiment (see text).

and corresponded to 3–4 hr wool growth. The corrected length of each radioactive region was then expressed as a percentage of the total length of the unkeratinized wool root, that is the distance from the end of the plucked root to the lower margin of the keratinized zone. This margin was identified from the shape of the fibre, which was often bent at the point where the diameter of the fibre became constant. In most of the plucked fibres examined this point was also shown to correspond to the boundary of the sulphydryl-staining zone. When this point was not obvious or

the wool root was not substantially intact, the fibre was not measured. Since plucked fibres break at different levels above the bulb, the estimated length of the labelled zone must be less than maximal.

IV. RESULTS

The radioautograms of the *skin sections* with both the AR 10 and AR 50 film showed that radioactivity was detectable in the suprabulbar region 2 min after the intravenous injection of L-[³⁵S]cystine. After 15 min radioactivity was also present in the fibrillary region. The 45-min and 2-hr samples showed that the radioactivity had reached the upper portion of the keratogenous zone. In the later samples the upper limits of the radioactivity appeared to have moved distances corresponding approximately to the growth rate of the fibres. In all specimens which produced a reasonably high silver grain density it was apparent that some radioactivity was present in the parts of the bulb surrounding the papilla, but in every case relatively larger amounts were present in the higher zones.

The radioautograms of *fibres* from the first experiment also revealed that radioactivity had penetrated throughout the whole of the unkeratinized root during the first hour. Radioactivity was detectable at the base of some of the wool roots plucked 30 sec after the injection. A more detailed study of this penetration was made in the second experiment. The results (Fig. 1) again showed that the whole of the unkeratinized root became labelled during the first hour. For comparison the average length growth rate of the fibres is also shown in Figure 1. This was calculated from the average length of the wool roots referred to in Figure 1 (0.59 mm) and the average length growth rate of the fibres on this animal at the time of the experiment (0.34 mm/day). Thus the average length growth rate was 14.2 μ /hr, equivalent to 2.4% of the average length of the unkeratinized roots.

In samples taken up to 1 hr after the intravenous injection of DL- or L-[³⁵S]cystine, the greater part of the ³⁵S present in the follicles was still in the suprabulbar region or the lowest third of the unkeratinized roots. At 2 hr the ³⁵S had spread more uniformly over the whole of the unkeratinized root but with the maximum density of silver grains now in the fibrillary zone. At 4 hr the maximum had shifted to the keratogenous zone, and decreased towards the bulb. Later samples also showed an almost uniform band of radioactivity, corresponding approximately to the unkeratinized region of the wool root with decreasing activity towards the bulb. Radioactivity appeared to accumulate in the follicles for at least the first 7 hr after the dose. These features are illustrated in the series of photomicrographs of plucked fibres shown in Plate 1.

V. DISCUSSION

When DL- or L-[³⁵S]cystine is injected intravenously into adult sheep the radioactive label penetrates throughout the whole of the unkeratinized wool root during the following hour. Thus, when DL- or L-[³⁵S]cystine is used to measure the rate of wool growth, as described by Downes and Lyne (1959, 1961), the leading edge of each cluster of silver grains must represent the point where the fibre was becoming fully keratinized approximately 1 hr after the time of injection of labelled

cystine, provided due allowance is made for the penetration of the β -particles into the film.

In the sheep studied here, the time taken for the ^{35}S to spread over the unkeratinized root was intermediate between the times observed for the mouse and the lamb. Bern, Harkness, and Blair (1955) and Harkness and Bern (1957) found that, although the intensity of the radioautograms increased for 8 hr after administration of L-[^{35}S]cystine to mice, each radioautogram showed essentially the same distribution of radioactivity, i.e. a relatively large amount in the keratogenous zone, trailing off towards the bulb. A similar labelling pattern was seen after approximately 4 hr in the follicle of our sheep, and after approximately 8 hr in the follicles of the lamb (Ryder 1958, 1959).

There are several possible explanations for the observed difference in the rate of movement of ^{35}S in the follicles of lambs and those of adult sheep. Ryder injected labelled cystine intraperitoneally whereas the intravenous route was used here. The absorption rate following intraperitoneal injection may be the reason for the differences between the two sets of results. The film exposure time also has an important effect on the silver grain density produced by a labelled specimen. In the samples of skin taken from lambs 30 min after the administration of the dose, the radioactivity extended from the base of the follicle into the lower part of the fibrillary region (Ryder 1958). If longer exposure times had been used the finding that the greatest concentration of ^{35}S was in the suprabulbar region would doubtless not have been altered, but smaller amounts of ^{35}S , if present further up the growing fibre in the fibrillary and keratogenous zones, would have been recorded on the film. Harkness and Bern (1957) have previously pointed out the effect of longer exposure times on the interpretation of radioautograms of hair follicles labelled with ^{14}C . Finally the mass of labelled cystine injected relative to the mass of cystine with which it is mixed in the animal must be important. However, in the absence of values for the cystine levels in sheep and lambs, no comment can be made on this aspect.

Ryder (1958, 1959) showed that ^{35}S from DL-[^{35}S]cystine first appears in the suprabulbar region of the follicle, and our results support this. As shown in Figure 1, the label then appears to move rapidly up the unkeratinized part of the fibre. The rate of this apparent movement is much faster than the length growth rate of the keratinized fibre (Fig. 1). Radioautographic evidence does not, however, show how [^{35}S]cystine or cysteine enters the follicle. All of the cyst(e)ine could enter the fibre by incorporation in the cells of the bulb or suprabulbar region and then move along the unkeratinized root by diffusion, with the thiol-disulphide exchange reaction possibly playing a part (Jensen 1959). Alternatively, the later appearance of radioactivity in the fibrillary and keratogenous zones might be due to a slower rate of entry of cyst(e)ine from the blood vessels immediately adjacent to these regions. Bern, Harkness, and Blair (1955) extracted tissue sections with thioglycollate or with mercaptoethanol and found no decrease of the radioactivity by comparison with control sections treated with water. This result would appear to rule out the thiol-disulphide exchange reaction as a possible mechanism, but apparently applied only to ^{35}S in the keratogenous zone and therefore does not rule out the possibility that

half-cystine residues are bound by disulphide bonds to proteins in the lower regions of the follicle.

By measuring the silver grain density produced by different parts of the follicle at different times after injecting labelled cystine into lambs, Ryder (1958) found that the bulb seemed to be "out of step" with the rest of the follicle; the rise and fall of radioactivity in the bulb was independent of that elsewhere in the follicle. This was taken to indicate that sulphur possibly enters the follicle by two routes—through the bulb and through the network of blood vessels surrounding the suprabulbar region. However, some of the [^{35}S]cystine was probably converted to [^{35}S]sulphate. Montagna and Hill (1957) and Ryder (1958) have shown that radioactivity accumulates slowly in the bulb and outer root sheath after doses of [^{35}S]sulphate. Since this may well explain the later peak of radioactivity in the bulb after injecting [^{35}S]cystine, there seems to be no need, at least on the basis of the radioautographic results, to postulate two paths of entry of sulphur into the follicle.

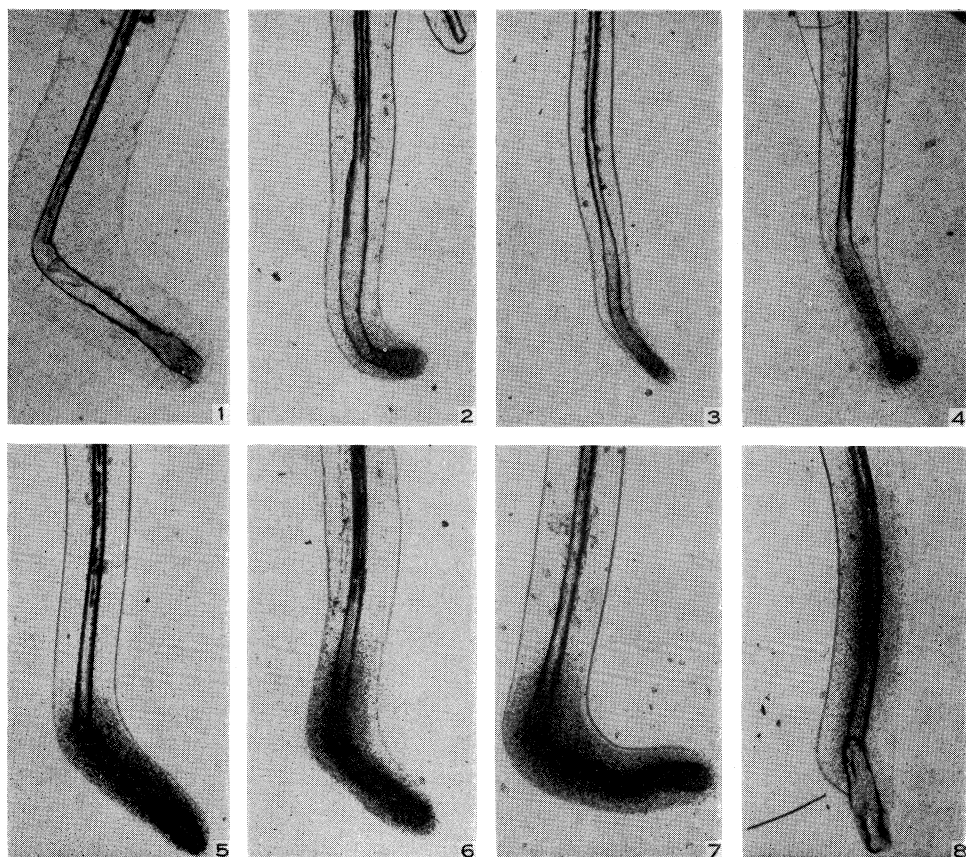
Ryder (1959) compared the rates of entry of DL-[^{35}S]cystine into the follicles of the rat, mouse, and lamb and stated that the rapid entry of sulphur into the follicles of the rat and mouse and the apparent movement upwards would seem to be associated with the faster growth rate in these animals. Alternatively, Downes (1961) postulated the existence of a metabolic pool of cystine associated with wool follicles. This pool which probably also contains other amino acids could be relatively smaller in the rat and mouse than in the sheep and could have a faster turnover. Thus the pool size could determine the rate of appearance of radioactivity in the keratogenous zone, even if the length growth rates of hair and wool fibres were not very different.

VI. ACKNOWLEDGMENTS

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Figs. 1-8.—Typical radioautograms of wool fibres plucked at various time intervals after injection of a Corriedale ewe with L- ^{35}S cystine ($356\ \mu\text{c}$; $6.7\ \text{mg}$). Unstained. Magnification $\times 35$. The time of each plucking was: 1, 3 min; 2, 8 min; 3, 15 min; 4, 1 hr; 5, 2 hr; 6, 4 hr; 7, 7 hr; 8, 30.5 hr.

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