INCORPORATION OF [Me-3H] METHIONINE INTO WOOL FOLLICLE PROTEINS: A BIOCHEMICAL AND ULTRASTRUCTURAL STUDY

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SUMMARY

The formation of the 'low-sulphur' group of proteins which constitutes more than half the cortical cell content of wool was investigated. Of the amino acids present in the low- and high-sulphur proteins isolated from wool, methionine is the only one which is exclusive to the low-S group. The incorporation of [Me-3H]methionine, administered intradermally to a sheep, was examined using both biochemical and autoradiographic procedures.

During the first hour after injection, most of the [PH]methionine appeared in the blood, but some was incorporated into the wool follicle proteins at the injection sites. Most of the radioactivity incorporated by the fibre during this period was subsequently retained. Initially, more than 90% of the activity was present in the prekeratin (8 M urea-soluble) portion of the fibre; 50 h elapsed before half of the radioactive material appeared in the keratinized (8 M urea-insoluble) part of the fibre. Methionine and its oxidation products accounted for more than 80% of the radioactivity present in hydrolysates of wool removed from injection sites after 4 h or 22 days. Analysis of labelled wool showed that the specific radioactivities of those fractions low in sulphur (α- and β-keratose) were 7 and 14 times greater than that of the high-S (γ-keratose) fraction.

Autoradiographic studies showed that the main site of formation of the low-S proteins was in the bulb region of the follicle. Here there was no specific intracellular site of low-S protein synthesis, the [PH]methionine being incorporated into the nucleus, cytoplasm, the various cell organelles and intercellular membranes. The amount of radioactivity incorporated by the cortical cells diminished at progressively higher levels in the fibre. In the keratinization zone, the various cell components showed limited protein synthesis, but 1-8 h after injection there was a localization of tritium over the keratin fibrils. It is suggested that a large proportion of the low-S proteins formed within the developing cortical cell ultimately contribute to the keratin microfibrils. The results provide further evidence for the formation of the low-S and high-S proteins at different sites in the follicle.

INTRODUCTION

It is generally accepted that the cortical cells of wool contain filamentous proteins, relatively poor in sulphur ('low-S' proteins), embedded in an amorphous matrix of sulphur-rich ('high-S') proteins (Crewther, Fraser, Lennox & Lindley, 1965; Mercer, 1961). The synthesis of the filaments is thought to occur in the lower regions of the follicle and to precede the synthesis of the matrix. This was indicated by autoradiographic studies which showed that [35S]cystine rapidly appeared in the keratinogenous
zone (Bern, Harkness & Blair, 1955; Downes, Lyne & Clarke, 1962; Ryder, 1956). As filaments are present below this level, the $^{35}S$ was presumed to be mainly incorporated into sulphur-rich proteins. Further evidence for this was obtained from specific activity measurements of the low- and high-S proteins of wool grown within 24 h of administering $[^{35}S]cystine$ to sheep (Downes, Ferguson, Gillespie & Harrap, 1966; Downes, Sharry & Rogers, 1963). The concept of a microfibril-matrix structure for wool has, however, been criticized on the grounds that it may be based on artifacts produced during the preparation of material for electron-microscopic and biochemical studies (Corfield, Fletcher & Robson, 1968; Forslind & Swanbeck, 1966). Recently Fraser (1969) has challenged the interpretation of the radiochemical evidence.

An alternative approach to the problem of identifying the sites of synthesis of these proteins is to trace the incorporation of an amino acid which is specifically incorporated into the low-S proteins. Analysis of the proteins isolated from wool by various extraction procedures shows that the low-S proteins have a higher concentration of aspartic acid, leucine, lysine, and phenylalanine than the high-S proteins (Crewther et al. 1965), but the only amino acid found in the low-S proteins and not in the high-S group is methionine. Crewther et al. (1965) found that the low-S fractions, SCMKA1 and SCMKA2, isolated from reduced wool contained 44 and 41 $\mu$mol of methionine per g respectively. No methionine was found in either of the high-S proteins, SCMKB1 and SCMKB2, or in the 'high-glycine' proteins, which are thought to be mainly derived from the fibre cuticle (De Deurwaerder, Dobb & Sweetman, 1964).

Specific labelling of the low-S proteins, however, would not occur following administration of $[^{35}S]methionine$, as a large proportion of the label is rapidly converted to $[^{35}S]cystine$ (Downes, Sharry & Till, 1964). In the present investigation we have used $[Me-^3H]methionine$, administered intradermally to a sheep, to indicate the sites of synthesis of the low-S proteins in the wool follicles. This assumes that there is a negligible transfer of tritium to other amino acids in the skin and that other metabolites such as choline, which might become labelled, are rapidly cleared from the site of injection. As most of the dose from intradermal injections is removed from the skin within 30 min (Downes et al. 1964), this technique was used in the investigation to produce a 'pulse' labelling of the tissue. Using both biochemical and autoradiographic procedures, the resulting distribution of radioactivity in the follicles at the site of $[Me-^3H]methionine$ injection was examined to establish the sites of synthesis of the low-S proteins.

MATERIALS AND METHODS

An adult Border Leicester sheep was maintained indoors in a metabolism cage and was provided with a constant daily ration (700 g of 1:1 mixture of chaffed lucerne hay and oat grain).

Seven weeks prior to the first experiment, the wool was uniformly clipped from the left and right mid-sides. Part of the right mid-side region was clipped again on the day before the experiment to facilitate the removal of skin samples from injection sites. Doses of $[Me-^3H]methionine$ (each 25.9 $\mu$Ci; 5 Ci/mM in 0.25 ml 0.9 % NaCl) were injected intradermally into 32 sites on the right mid-side region; 8 of these sites were in the wool plucking area, and the

* SCMK, sulphur-carboxymethyl keratine.
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remained were in the skin sampling region. In 4 of the latter sites the initial injection of \([^{3}H]\)methionine was followed 2 min later by an injection of unlabelled L-methionine (1 mg) in 0.25 ml 0.9% NaCl. The total time taken for the injections was 30 min.

Wool (approximately 20 mg) was plucked from 5 sites at 5, 15 min, 1, 4 and 8 h respectively after administering the \([^{3}H]\)methionine, and blood samples were taken from the jugular vein at frequent intervals. Wool was also plucked from non-injected regions on the left side of the sheep during the first 4 days post-injection. Skin biopsies (1 cm diameter) were removed from 2 sites and smaller skin samples (for electron microscopy) from another 2 sites at 5, 15 min, 1, 4 and 8 h after the injections. Also, a biopsy and sample were taken 5 and 15 min post-injection from the 4 sites which had received both \([^{3}H]\)methionine and L-methionine. After 22 days the wool was clipped from the 3 remaining injection sites and from the surrounding skin (total area of each site clipped, 25 cm²). The clipped wool was washed with light petroleum (b.p. 60–80 °C), ethanol, and water and air-dried.

In the second experiment, performed 11 months later, a further 26 doses of \([^{3}H]\)methionine (250 μCi; 11.1 Ci/mM) were injected intradermally into the same sheep. Sites on the left side, from which the wool had been clipped 10 days previously, were used. Duplicate skin samples were removed from these sites 5 min and 1 h after injection. Wool was plucked from each of 2 sites 5 min, 1, 4 (4 sites), 8, 24, 31, 48, 57, 70 and 94 h post-injection.

Histological preparations and autoradiography

The biopsies were immediately placed in 10% neutral buffered formalin containing L-methionine (2 mg/ml) for 24 h, then placed in a further 6 changes of fixative over a period of 18 days. Most of the extractable tritiated material was removed by the initial fixation, a negligible amount, less than 0.03% of the injected dose, appearing in the final changes of the fixative solution. The skin biopsies were then dehydrated, embedded in paraffin and serial sections (6 μm) cut parallel to the longitudinal axis of the follicles. The sections were covered with Kodak AR10 stripping film, and after 11 or 28 days’ exposure, were developed in Kodak D19b. The sections were stained with Ehrlich’s haematoxylin and picric acid and examined with the light microscope.

The skin samples for electron microscopy were fixed for 2 h in 2% OsO₄ buffered with veronal-acetate at pH 7.4, dehydrated in ethanol, and embedded in Araldite. By combustion and liquid scintillation counting techniques (Downes & Till, 1963) it was shown that this procedure removed about 62% of the \(^{3}H\) in skin samples taken 1 h after injection, compared with 46% extracted by 10% TCA. Autoradiography with Kodak AR10 of sections (1.2 μm) from the skin samples showed that the distribution of radioactivity was the same as that observed with skin biopsies.

For electron-microscope autoradiography, the method of Salpeter & Bachmann (1964, 1965) was found to be the most satisfactory. Sections (approximately 100 nm thick) were cut parallel or perpendicular to the longitudinal axis of the follicle, and placed on parlodion-coated slides, using a stainless steel trough attached to an LKB microtome (Gemmell & Henrikson, 1970). After being stained with uranyl acetate and lead citrate, and coated with carbon, the sections were covered with a monolayer of Ilford L4 emulsion (Fig. 9, inset). Autoradiographs were exposed for periods from 26 to 160 days and developed with Microdol-X for 3 min at 22 °C. They were then examined with a Hitachi HU-11C electron microscope.

Analysis of autoradiographs

The distribution of grains was examined in autoradiographs of the undifferentiated region of the follicle, the fibrillar region and the keratinization zone of the fibre (zones A, C and D as defined by Mercer (1964); Fig. 7). The position in the follicle of the examined areas was confirmed from low-power electron micrographs (× 2000).

The grain counts were made on electron micrographs taken at an initial magnification of × 5800 or × 7500 and enlarged 3-fold. Similar results (within ±10%) were obtained by 2 methods, both applied by 2 independent workers. In the first method, the size of a developed grain was assessed visually, and if more than half fell within the boundary of an organelle, then the grain was assumed to be due to a β-particle originating in that organelle (Gibbs, 1968). In
the second, more precise method, the count was ascribed to the main organelle occurring within 160 nm of the grain origin (Salpeter, Bachmann & Salpeter, 1969).

The relative areas occupied by the various cell components were measured using the method of a regular pattern of points 0.5 cm apart and a relative standard error, i.e. standard error/relative area of component, of < 5% (Hally, 1964). This method was applied to all the electron micrographs used for grain counts.

Assay of \(^3\)H in plucked wool

Immediately after plucking, the prekeratin was removed from a portion of each wool sample (approximately 10 mg) with 8 M urea containing iodoacetate (0.073 M) buffered at pH 8.6 with tris (0.25 M). These samples were then washed successively with water, ethanol, and light petroleum (b.p. 60–80°C), dried, weighed, combusted and the radioactivity measured by liquid scintillation counting. A second portion of each plucked sample was washed 3 times with light petroleum, and the \(^3\)H contents of both this and the remaining untreated portion were determined as described above.

Identification of labelled compounds

The urea extracts of wool plucked from the injection sites were centrifuged at 7500 g for 10 min. Samples of the supernatant solutions were taken for liquid scintillation counting. For each supernatant, the free amino acids and small polypeptides were separated from proteins using Sephadex G 50 (Fine) which had been equilibrated with 8 M urea. The columns (6 x 1.7 cm) were eluted with 8 M urea and the \(^3\)H content of successive 1-ml fractions measured.

Samples of the plucked and clipped wool from the injection sites were hydrolysed with 6 N HCl in vacuo for 20 h at 110°C. To determine the proportion of the \(^3\)H still present as methionine, samples of the hydrolysates were analysed by chromatographic methods and by isotope dilution. A portion of each hydrolysate was rotary-evaporated, dissolved in sodium citrate buffer (pH 2.2, 0.2 M), and chromatographed on an Amberlite IR120 column (17 x 150 cm) (Moore, Spackman & Stein, 1958). Samples (1 ml) of the eluted fractions (2.8 ml) were taken for liquid scintillation counting. The amino acids present in the radioactive fractions were identified by paper chromatography in water-saturated phenol and butanol-acetic acid-water (4:1:5), and liquid scintillation counting was used to determine the location of the radioactivity. For dilution analysis, L-methionine (1 g), together with a known proportion of the hydrolysate or of the eluted fractions containing \(^3\)H, was dissolved in water, the solution evaporated to dryness, and a portion of the dried residue was recrystallized to constant specific activity from aqueous ethanol. The specific activity of the recrystallized samples was compared with that of the dried mixture.

A sample (1 g) of wool clipped from a site which had been dosed with \(^3\)H]methionine was oxidized with performic acid, as described by Thompson & O'Donnell (1959). The \(\alpha\) (low-S fraction), \(\beta\) (insoluble fraction) and \(\gamma\) keratoses (high-S fraction) were then separated using the procedure of Corfield, Robson & Skinner (1958), except that the \(\gamma\)-keratose was recovered by freeze-drying. The radioactivity of the keratoses was determined by liquid scintillation counting after combustion.

Measurement of radioactivity

The amount of radioactivity in the plasma and other aqueous solutions was determined after diluting to 7.5 ml with H\(_2\)O and adding 10 ml of scintillation solution. The latter contained 7 parts of a toluene scintillation fluid (toluene 1 l, PPO 4 g, POPOP 0.1 g) and 6 parts of Triton X-100 by volume. The radioactivity of the paper chromatograms was detected by suspending segments in toluene scintillation fluid. The \(^4\)H\(_2\)O from combusted samples was absorbed in ethanol (5 ml) and 4 ml of the solution mixed with 9 ml of the toluene solution.

The radioactivity was assayed with a Packard Liquid Scintillation Spectrometer (Model 3375). The counting efficiency was determined with \([1,2-\text{H}]\)-n-hexadecane. \([\text{Me-}\text{H}]\)-L-methionine and \([1,2-\text{H}]\)-n-hexadecane were obtained from the Radiochemical
Incorporation of \([\text{^{3}H}]\text{methionine into wool follicles}\)

Centre, Amersham, England. Immediately prior to use, the radiochemical purity of the \([\text{Me-}^{3}\text{H}]\)-L-methionine was found, by both paper chromatography and isotope dilution analysis, to be over 97%.

RESULTS

\(^{3}\text{H} \text{in plasma}\)

The amount of \(^{3}\text{H} \text{in the blood plasma increased rapidly after administering the doses of } [\text{^{3}H}]\text{methionine (Fig. 1). The concentration of }^{3}\text{H} \text{reached a maximum 1 h after completing the injections, and then fell by about 60\% during the next 4 h to a fairly constant level which was maintained up to at least 50 h after the injections.}\)

![Graph of \(^{3}\text{H} \text{in plasma} vs. time after injection (h)}\]

Fig. 1. \(^{3}\text{H} \text{in the blood plasma at various times after administering 32 intradermal doses of } [\text{^{3}H}]\text{methionine.}\)

\(^{3}\text{H} \text{in plucked wool}\)

About 1\% of each dose was incorporated into the wool at the site of injection, some radioactivity being present within 5 min of injection.

In the second experiment, the specific activity of wool plucked from injection sites after 4 h ranged from 15.8 to 37.4 nCi/mg; similar values were obtained for samples plucked 4-94 h after injection (Fig. 2).

In the first 8 h post-injection less than 5\% of the \(^{3}\text{H} \text{was present in the 8 M urea-insoluble fraction of wool (Fig. 3). During the next 4 days the proportion gradually increased to about 90\%.}\)

Two hours after the injections, \(^{3}\text{H} \text{was detectable in wool plucked from non-injected sites, but the specific activity of this wool was never more than 0.2\% of the specific activity of wool plucked from the injection sites at the corresponding times.}\)
Fig. 2. Relationship between the specific activity of plucked wool and the time of removal from injection sites. Correlation coefficient, \( r = -0.208 \).

Fig. 3. Rate of incorporation of \( ^3 \text{H} \) into the urea-insoluble portion of wool fibres plucked from injection sites on the sheep.

Fig. 4. Proportion of \( 8 \text{M} \) urea-soluble \( ^3 \text{H} \) associated with the protein fractions eluted from a Sephadex G 50 column.
Radiochemical composition of plucked and clipped wool

In the urea-soluble portion of wool plucked from an injection site after 5 min, a large proportion (44%) of the radioactivity was associated with proteins (Fig. 4). In samples plucked 1 h after injection the proportion had increased to 63%, and by 8 h to 89%.

Ion-exchange chromatography of the hydrolysate from the clipped wool showed that 71% of the total radioactivity was eluted with a single peak corresponding to methionine. In addition, small amounts of radioactivity were present in positions corresponding to methionine sulfoxide (4%), methionine sulfone (6%), and cystine (1%). The radioactivity in the main peak was shown, by paper chromatography and dilution analysis, to be present as L-methionine. Similar recoveries (50-74%) of the 3H as L-methionine were obtained in the hydrolysates of wool plucked 1 and 4 h after injection.

Separation of the α-, β- and γ-keratose fractions from the oxidized clipped wool and their subsequent assay showed that the specific activity of the 3 fractions was 390, 810 and 59 nCi/g (7:14:1) respectively. Further, the total amount of 3H present in each fraction, calculated from the specific radioactivities and the known relative proportions in wool (Crewther et al. 1965), was considerably greater in the α- and β-keratose than in the γ-keratose fraction (13:5:1 respectively).

Light-microscopic autoradiography

The autoradiographs of skin samples removed at various times after injection of [3H]methionine showed that the radioactivity was concentrated in the wool follicles, with smaller amounts in the epidermis and sebaceous glands. In the wool follicle, the

![Fig. 5. Density of silver grains at different levels in the fibre. A section from an 8-h (△), and 2 sections from a 4-h follicle (●, ◦) were examined. These sections were through the medial longitudinal axis of the follicle. The arrows denote the commencement of the keratinized part of the fibre. The sections, from OsO4-fixed samples, were coated with L4 emulsion (diluted 1:2), and exposed for 10 days.](image-url)
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relative distribution of label was similar at all time intervals (Figs. 7, 8), the greatest concentration being in the bulb and fibrillar zone. From the bulb to the distal region of the keratinization zone, there was a gradual decrease in the amount of label present in the cortical cells (Figs. 5, 7, 8). No radioactivity was detected in the keratinized portion of the fibre, even at 8 h after injection. Some $^3$H was also incorporated into the outer root sheath and the unkeratinized region of the inner root sheath (Figs. 7, 8).

The distribution of label in follicles from sites which received both $[^3]$Hmethionine and unlabelled L-methionine was similar to that in follicles from sites which received $[^3]$Hmethionine alone. However, the amount of $^3$H present was considerably reduced following the injection of unlabelled methionine.

**Electron-microscopic autoradiography**

The number of grains over the various cell components in each of the regions examined is given in Table 1. In all 3 regions, 7–20% of the grains were observed near cell membranes (Figs. 9–12), but these grains were attributed to radioactivity in the adjacent cytoplasm or fibrils, as cell membrane width was less than the resolution of the autoradiographs.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total number of grains</th>
<th>Percentage of grains in</th>
<th>Nucleus†</th>
<th>Cytoplasm</th>
<th>Mitochondria</th>
<th>Golgi</th>
<th>Fibrils</th>
</tr>
</thead>
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<td>Undifferentiated bulb</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min*</td>
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<td>40</td>
<td>50</td>
<td>7</td>
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<td>42</td>
<td>51</td>
<td>6</td>
<td>0.8</td>
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</tr>
<tr>
<td>1 h</td>
<td>373</td>
<td></td>
<td>43</td>
<td>48</td>
<td>8</td>
<td>0.5</td>
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<tr>
<td>4 h</td>
<td>602</td>
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<td>49</td>
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<tr>
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<td>8</td>
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* Received both $[^3]$Hmethionine and unlabelled L-methionine.
† Label was present over the nucleolus at all times, but is included in the nuclear grain counts.

In the bulb region of the follicle, the relative distribution of silver grains was similar at all time intervals (Table 1). Examination of the autoradiographs showed that the label was not located specifically over any cell component (Fig. 9), although it would appear from Table 1 that more label had been incorporated into the cytoplasm than into the nucleus, mitochondria or Golgi apparatus. However, in the bulb such differences in the
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The relative distribution of label merely reflected variations in the relative areas of the cell components (Table 2). When the results are expressed in terms of grain concentration (number of grains/100 \(\mu m^2\) of each cell component), it is apparent that in the bulb there was a similar concentration of label in the nucleus, cytoplasm, mitochondria or Golgi at any of the time intervals studied (Table 3).

Table 2. Areas occupied by cell components at 2 stages of cortical cell differentiation

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total area examined, (\mu m^2)</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Mitochondria</th>
<th>Golgi*</th>
<th>Fibrils</th>
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<tr>
<td>5 min</td>
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<td>52</td>
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* Measured by planimetry.

Table 3. Grain concentration in the cell components of cortical cells at 2 stages of differentiation

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<tr>
<th>Stage</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Mitochondria</th>
<th>Golgi</th>
<th>Fibrils</th>
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</tr>
<tr>
<td>1 h</td>
<td>17</td>
<td>32</td>
<td>0</td>
<td>—</td>
<td>51</td>
</tr>
<tr>
<td>4 h</td>
<td>23</td>
<td>27</td>
<td>0</td>
<td>—</td>
<td>50</td>
</tr>
<tr>
<td>8 h</td>
<td>24</td>
<td>26</td>
<td>0</td>
<td>—</td>
<td>50</td>
</tr>
</tbody>
</table>

Autoradiographs of the fibrillar zone also showed a general distribution of label within the cells, although at this level in the follicle 9–21% of grains were located within 160 nm of the numerous thin fibrils, or filament bundles, present in the cytoplasm (Fig. 10, Table 1). A comparison of the grain concentration over fibrils and other cell components was, however, not possible due to the difficulties in measuring fibril area in this region.
In the keratinization region of the fibre, much less label was present but there was a further increase in the proportion of grains associated with the fibrils (Table 1). The amount of fibrillar material in the cortical cells had also increased, the area of tissue occupied by the fibrils varying from 24 to 37% (Table 2). Examination of the grain concentrations showed a similar amount over fibrils and cytoplasm at 5 min but after 1 h the label was mainly present over the fibrils (Figs. 11, 12, Table 3). At 1, 4 and 8 h, the concentration of label in the fibrils was in fact approximately twice that of the nuclear and cytoplasmic components (Fig. 6, Table 3).

![Relative concentrations of silver grains over the fibrils (•●), cytoplasm (▲▲), and nuclei (○○) in autoradiographs of the keratinization zone of the fibre.](image.png)

**DISCUSSION**

The results show that there was a significant localized incorporation of $^3$H into the wool grown at the injection sites and most of this $^3$H was still present as methionine. Radioassay of the $\alpha$-, $\beta$- and $\gamma$-keratoses separated from the oxidized wool showed that the specific activity of both the $\alpha$- and $\beta$-fractions was considerably higher than that of the $\gamma$-keratose. Thus, as expected from the chemical composition of the high- and low-S fractions, most of the [$^3$H]methionine incorporated was present in the low-S fraction ($\alpha$-keratose). Although the $\alpha$-keratose fraction is heterogeneous (Corfield et al. 1968), it is considered to be derived from keratin filaments (Crewther et al. 1985; Mercer, 1961; Rogers, 1959). On this basis, the results indicate that considerable [$^3$H]methionine had been incorporated into the filaments. In addition, [$^3$H]methionine
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was present in the insoluble $\beta$-keratose fraction, which is also low in sulphur and consists largely of cellular membranes (Asquith & Parkinson, 1966; Corfield et al. 1968). However, the relatively high specific radioactivity of the $\beta$-keratose fraction is not surprising as the cell membranes also contain a high concentration of methionine relative to that of other histological components of wool (Bradbury, Chapman & King, 1968).

The specific activities of the wool samples plucked from injection sites during the period from 4 to 94 h show a large variation ($r = -0.2076$) due to the considerable sampling errors associated with intradermal dosing. The greater variations in samples plucked during the first 50 h were presumably caused by the varying amount of prekeratin and associated radioactivity removed from the follicle; this effect is less pronounced in the later samples as an increasing proportion of the radioactivity is present in the keratinized part of the fibre (Fig. 3). As the sites had been clipped only 10 days prior to the experiment, and as about 5 days' growth would have been left in the skin after clipping, the further increase in fibre length and mass during the 4-day sampling period would have produced an apparent fall of about 25% in the specific activity of the plucked wool. This value is close to that observed for the mean results in Fig. 2. Thus, the results indicate that the amount of radioactivity present in the fibre remained relatively constant. That is, most of the $^3H$ present in the plucked fibres at 4 h was incorporated into the keratinized fibres during the following 4 days.

Of the $^3H$ in the hydrolysates of the plucked wool, more than 80% was accounted for as methionine and its oxidation products, and this proportion was no less than that recovered from hydrolysates of the clipped wool. A large proportion of the injected methionine was rapidly removed by the blood and the remaining methionine was presumably demethylated to a large extent (Downes et al. 1964), but the above evidence indicates that most of the tritium-labelled metabolites was also removed from the injection sites in the first hour. The results in Fig. 4 confirm that a large proportion of radioactivity in the follicles was confined to proteins within 1 h. Thus, the radioactivity detected by autoradiography of skin samples taken 1 or more hours after injection was due to $[^3H]$methionine residues incorporated in proteins.

The incorporation of methionine, as revealed by autoradiography both at the light- and electron-microscope level, was maximal in the bulb region of the follicle, and diminished in the cortical cells at progressively higher levels. In view of the results obtained from the radioassays of the keratoses, we assume that the observed distribution of radioactivity indicates that the low-S $\alpha$- and $\beta$-keratoses are mainly synthesized in the bulb region of the follicle, the rate of synthesis progressively decreasing during cell migration and keratinization. Within the undifferentiated bulb cells there was no specific intracellular site of protein synthesis, the incorporation of $[^3H]$methionine being similar for nucleus, cytoplasm, mitochondria and Golgi. This lack of localization of label in the autoradiographs cannot be attributed to the presence of free amino acids in the fixed tissues, as 10% trichloroacetic acid removed a similar amount of radioactivity to that removed during fixation. Also the intracellular distribution was similar in the samples from the sites which had received the chaser dose of unlabelled methionine. Thus the non-specific incorporation of $[^3H]$methionine
within the bulb cells must indicate general protein synthesis occurring in this region of the follicle.

Within the cortical cells of the fibrillar zone, there was likewise no specific intracellular site of protein synthesis, although label was often located in the vicinity of fibrils. In the keratinization region of the follicle, methionine was again incorporated into proteins of the cytoplasmic, nuclear and fibrillar components of the cell, although the extent of incorporation was greatly reduced compared to that in the bulb. Examination of the relative concentration of label in the keratinization zone indicates that 5 min after injection the rate of incorporation in the fibrils was similar to (or slightly faster than) that in the cytoplasm. At subsequent time intervals, however, there was an increased concentration of label over the fibrils, and a decreased concentration over the nucleus and cytoplasm. This implies that in addition to protein synthesis being directly associated with fibrils, labelled proteins synthesized by the cytoplasm and nucleus were also incorporated into fibrils. A similar situation occurs in mitochondria, both in vivo and in vitro (Beattie, Basford & Koritz, 1966; Haldar, Freeman & Work, 1966; Kadenbach, 1970), the transferred protein being detected in mitochondria 2 h after injection of labelled amino acids (Beattie et al. 1966). The distribution of label within the cortical cells was not studied beyond 8 h after injection, but the specific activities of the plucked wool show that most of the [3H]methionine incorporated by the cells was retained, moving into the urea-insoluble portion of the fibre during keratinization. This further indicates that a considerable proportion of the 3H-proteins formed in the various cell components of the undifferentiated bulb cells must ultimately be transferred to the filaments which form approximately 50% of the protein of the keratinized fibre (Fraser, 1969).

Besides the formation of keratin filaments, it is possible that some of the labelled low-S proteins of the various cell components may also contribute to the cytoplasmic and nuclear remnants of the cortical cell. At the 3 levels of cortical cell differentiation that were examined the proportion of label associated with cell membranes was similar and although it was assigned to the adjacent cytoplasmic or fibrillar component of the cell, the presence of [3H]methionine in the /β-keratose fraction shows that such label had in fact originated from the cell membranes. These results clearly indicate that the low-S proteins of wool are derived from various sources within the developing cortical cells.

The incorporation of both [3H]methionine and labelled low-S proteins into fibrils would imply that filament formation may occur by the aggregation of low-S proteins in association with further protein synthesis. Alternatively, the labelled low-S proteins may undergo a complete or partial hydrolysis, with immediate incorporation into filaments. In either instance, by comparison with the amount of [3H]methionine incorporated by the undifferentiated bulb cells, only a relatively small amount of additional protein synthesis is directly associated with filament formation. The contribution of labelled cytoplasmic and nuclear proteins to the filaments is presumably confined to the non-helical sections of the protofibril polypeptide chain, as it has been shown that methionine is absent from the helical sections (Crewther, Dobb, Dowling & Harrap, 1968). The proposal that non-microfibrillar low-S proteins of prekeratin
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contain subunits which polymerize to form microfibrillar proteins (Fraser, 1969) is consistent with the present finding of incorporation of [³H]methionine into non-specific proteins within the cells, but the precise mechanism of microfibrillar formation is still to be elucidated.

The incorporation of 2 other amino acids, [³⁸S]cystine (Nakai, 1964) and [³H]proline (Rogers, 1970), into growing hair fibres has already been examined at the electron-microscope level. Unlike [³H]methionine, cystine was intensely localized in the keratogenous zone of the follicle even at the earliest time intervals after injection, while the amount of label in the bulb remained very low. With both cystine and proline, however, the label in the keratogenous zone was clearly associated with the keratin fibrils soon after injection. Due to the limits of resolution of the autoradiographic method this localization over the fibrils in the keratogenous zone could indicate matrix and/or filament protein synthesis, although both proline and cystine are present in higher concentration in the high-S fraction (Crewther et al. 1965) which is considered to be derived from the matrix. Moreover, in contrast to the results with [³H]methionine, 6 h after [³⁸S]cystine administration very little label (< 7%) was present in the cytoplasm or nucleus of the hair cortex in the keratogenous zone (Nakai, 1964). In view of our present findings, the site of incorporation of [³⁸S]cystine into proteins within the cortical cells is markedly different from that for methionine.

It has been suggested that the association of labelled amino acids with fibrils indicates that keratin synthesis occurs at the surface of fibrils and that polypeptide chains are synthesized directly on the fibril (Rogers, 1970). Other proposals based on similar findings with the epidermis suggest that ribosomes near fibrils are involved in the synthesis of fibrillar proteins (Fukuyama & Epstein, 1967; Rhodin & Reith, 1962). While such mechanisms may apply to the completion of microfibrillar proteins, and to the formation of matrix proteins, the present results indicate that other pathways are responsible for the synthesis of low-S proteins prior to their incorporation into fibrillar proteins.

Besides differences in the intracellular sites of incorporation of [³H]methionine and [³⁸S]cystine, a difference in the region of incorporation within the follicle is demonstrated by autoradiography and by specific activity measurements on plucked wool. The latter shows that about 50 h were required for half of the incorporated [³H]methionine to pass to the urea-insoluble portion of the fibre; the corresponding time for [³⁸S]-cystine was about 22 h (Downes, 1965). This lag in the conversion of ³H low-S proteins is in accord with the time taken for matrix cells to move from the bulb to the keratinization zone (Downes, Chapman, Till & Wilson, 1966) and further substantiates the fact that the main site of formation of such low-S proteins is in the bulb region of the follicle. Fraser (1969), however, claims to have refuted the hypothesis of a '2-stage' synthesis of keratin, and suggests that both matrix and microfibrillar proteins are formed simultaneously throughout the length of the prekeratin tissue, but at different rates of synthesis. In particular, he has suggested that the microfibrillar proteins increase linearly in concentration proximo-distally up to the level of keratinization. The results obtained from the present study show, however, that neither a discrete '2-stage' nor a 'dual' synthesis of keratin occurs. Rather it is a 'complementary'
synthesis, with the low-S proteins being formed predominantly in the follicle bulb and at a diminishing rate towards the keratogenous zone, while the synthesis of the high-S proteins reaches a maximum in this latter zone. Such a merging of the 2 processes was proposed by Downes et al. (1966) as a possible explanation of their results. Between these 2 main sites of synthesis, the filamentous proteins, or specialized structural low-S proteins, are formed, possibly by the modification of existing low-S proteins. The rate of formation of such filamentous proteins is not necessarily the same as that of low-S proteins; evidence from ultrastructural studies (Forslind & Swanbeck, 1966) indicates that the synthesis of fibrillar material may in fact be a linear process.

From this investigation, it can be concluded that the production of low-S proteins within the developing cortical cell results from protein synthesis occurring in the cytoplasm, nucleus, and the various cell organelles, and from the synthesis of intercellular membranes. These low-S proteins ultimately contribute to the components of filaments, cell membranes and possibly nuclear and cytoplasmic remnants of the keratinized cortical cell.

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REFERENCES


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Fig. 7. Autoradiograph of a wool follicle from a biopsy removed 1 h after an intradermal injection of \[^{3}H\]methionine. The undifferentiated bulb region, the fibrillar region and the commencement of the keratinization zone of the fibre are indicated by the lower, middle and upper arrows respectively. Radioactivity in the fibre decreases from the bulb to the distal region of the keratinization zone. \(^{3}H\) has also been incorporated by the cells of the inner and outer root sheaths. \(\times 240\).

Fig. 8. Autoradiograph of a follicle 8 h after an intradermal injection of \[^{1}H\]methionine. The main concentration of radioactivity is still in the lower part of the follicle. \(\times 240\).
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Fig. 9. Autoradiograph of cells in the undifferentiated region of the follicle bulb 5 min after injection of [3H]methionine. Grains are present over all cell components. A similar distribution of grains in this region was present at 1, 4 and 8 h. × 6000.

Inset: monolayer of Ilford L4 emulsion (purple interference colour) used in the preparation of autoradiographs. The distribution of the silver halide crystals was checked for close, even packing prior to covering the sections with emulsion. × 7000.
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Fig. 10. Autoradiograph of a section through the fibrillar zone 4 h after injection of $[^3H]$methionine. In the cortical cells, numerous grains are associated with the fibrils. The nuclei, mitochondria, cell membranes, and cytoplasm are also labelled. Label is present in the cuticle cells (c). $\times 22,000$. 
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Fig. 11. Autoradiograph of the keratinization zone 4 h after injection of [3H]-methionine. In the cortical cells, the label is mainly associated with the fibrils. Silver grains are also present in the cuticle (c) and inner root sheath. × 17000.
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Fig. 12. Autoradiograph of the keratinization zone 4 h after injection. Numerous grains are associated with the fibrils. Grains are also present over the cell membranes (arrows). × 17000.