PROTEINS OF KERATIN AND THEIR SYNTHESIS

II.* INCORPORATION OF [^{35}S]CYSTINE INTO PREKERATIN AND KERATIN PROTEINS

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Summary

The presence within protein extracts from intact wool roots of a low-sulphur, non-microfibrillar protein masks the true specific activity values for microfibrillar proteins. As a consequence, the correct specific activity ratio of matrix to microfibrillar proteins at different time periods following a pulse of [^{35}S]cystine is also masked.

Evidence is presented which indicates that the non-microfibrillar, low-sulphur protein, extractable with Lockes solution, contains subunits of microfibrillar proteins. It is proposed that such subunits polymerize to form the three-dimensional helical structure of the microfibrillar proteins within cells. The attainment of the complex structural form is not instantaneous, as indicated by a pronounced "lag" greater than 5 hr in the incorporation of ^{35}S from a single pulse into microfibrillar proteins, as compared with matrix proteins in wool root cells.

The incorporation of matrix proteins into protein-synthesizing cells occurs independently of microfibrillar protein synthesis.

Though matrix proteins are incorporated faster into cells in the upper third of the prekeratin tissue than at lower levels, the results do not support a "two-stage" process of keratin synthesis. Microfibrillar proteins appear to be formed at much the same rate throughout the prekeratin tissue of wool follicles.

I. Introduction

Support for the "two-stage" synthesis of low- and high-sulphur-containing keratin proteins from wool as postulated by Rudall (1956) and Mercer (1961), was claimed as a result of [^{35}S]cystine incorporation studies undertaken by Downes, Sharry, and Rogers (1963). These workers showed that up to 17 hr after intravenous injection of [^{35}S]cystine into sheep, the high-sulphur proteins from prekeratin and keratin tissue had significantly higher ^{35}S specific activity than the low-sulphur proteins. The results were interpreted as indicating that the formation of matrix (high-sulphur) proteins occurred in the upper part of the prekeratin region of the wool root, as distinct from the formation of microfibrillar proteins (low-sulphur) in the lower part, as initially postulated by Rudall (1956) and Mercer (1961).

In a later study of wool root proteins, Downes *et al.* (1966) obtained results which they claimed added further support to the "two-stage" process.

A recent study of keratin proteins from wool and wool root tissue (Fraser 1969) refutes the "two-stage" process. Results indicate that both matrix and micro-

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fibrillar proteins are formed throughout the length of the prekeratin tissue above the level of cell proliferation. Though the fibrillar proteins appear to undergo a linear increase in cellular concentration throughout the length of the prekeratin tissue, there is evidence of an exponential increase in matrix proteins. It was proposed that approximately half of the final matrix protein content of cells is added in the upper third of the prekeratin tissue.

Though Downes, Sharry, and Rogers (1963) and Downes *et al.* (1966) claim that a differential in specific activity of the wool proteins indicates a difference in the site of synthesis, it is believed that such results could equally well indicate a difference in the time or rate of incorporation of the label into such proteins from a single pulse. In order to further investigate these possibilities, and to obtain more information on keratin synthesis, ^{35}[S]cystine incorporation studies were carried out in conjunction with the studies of prekeratin and keratin proteins reported earlier (Fraser 1969).

II. Materials and Methods

Animals 3 and 4, used in the study of prekeratin and keratin proteins reported in Part I of this series (Fraser 1969), and animals 5 and 6 were injected intravenously with 250 μCi of L-[^{35}S]cystine per 100 lb body weight, all within 8 min. At 5 and 24 hr after injection of the pulse, animals 3 and 4 respectively were slaughtered, flayed, and the wool roots stripped as previously reported (Fraser 1969).

An area (10 by 10 cm) on the side of each of animals 5 and 6 was prepared prior to injection of the isotope pulse. Fibre length growth was observed from this area by microscopic measurement, and sufficient time was permitted to elapse to ensure that activity from the isotope pulse was located in the keratinized fibre above skin surface. At 5 days after the pulse, length growth was 2·8 mm and at 7 days 4·0 mm. At each of these times wool was shaved from the skin surface.

Separation of prekeratin cells from wool roots, leaving residual keratinized fibres, was carried out as reported and illustrated earlier (Fraser 1969, fig. 1). Extraction, fractionation by chromatography on DEAE-cellulose, and gel filtration on Sephadex G-200 of high- and low-sulphur proteins from intact wool roots, root cells, wool, and keratinized wool root residues was carried out as previously described (Fraser 1969).

The microfibrillar proteins from wool, keratinized wool root residues, and cells were separated by gel filtration. The matrix proteins from wool and keratinized wool root residues comprise the total high-sulphur protein extract. The matrix proteins from cells were obtained by chromatographic separation and pooling of fractions 0–95 as shown in earlier work [Fraser 1969, fig. 4(*b*)].

Liquid scintillation (Packard, Tri-Carb) using Carb-o-Sil (Gordon and Wolfe 1960) was used for measurement of ^{35}S specific activity of prekeratin and keratin wool proteins. Corrections were made for isotope decay and quenching, and a minimum of 500 counts/min was adopted for each analysis.

Sulphur contents were determined by the Schöniger (1954) oxygen-flash technique.

L-[^{35}S]Cystine (from the Radiochemical Centre, Amersham) of specific activity 135 mCi/m-mole was used.

III. Results

(*a*) *Proteins from Keratinized Wool Root Residues*

The keratinized wool root residues are the keratinized fibres from below the skin surface which remain after cells of the wool root have been removed [see Fraser 1969, fig. 1(*b*)]. The matrix proteins from the residual fibre shafts (Table 1) have considerably more ^{35}S activity per microgram of sulphur than the microfibrillar pro-

teins keratinized over the same periods of 5 and 24 hr. The specific activity ratio of matrix:microfibrillar proteins is reduced between 5 and 24 hr. At 5 and 7 days the specific activity ratios are 1·0:1 and 1·02:1 respectively for the activity contained in wool above the skin surface.

TABLE 1

SULPHUR CONTENT AND SPECIFIC ACTIVITY OF MATRIX AND MICROFIBRILLAR PROTEINS FROM KERATINIZED WOOL ROOT RESIDUES AND FROM WOOL AT SPECIFIED INTERVALS AFTER A SINGLE INJECTION OF [^{35}S]CYSTINE

Specific activity values are expressed as picocuries per microgram of sulphur, and sulphur contents as percentages

Source of Protein	Period after ^{35}S Pulse	Matrix Proteins (A)	Microfibrillar Proteins (B)	Ratio of Specific Activities (A/B)
Wool root residue				
Specific activity	5 hr	17·2	2·4	7·2:1
Sulphur content	5 hr	5·56	2·00	
Specific activity	24 hr	67·8	33·00	2·4:1
Sulphur content	24 hr	5·65	2·20	
Wool				
Specific activity	5 days	32·00	31·6	1·0:1
Sulphur content	5 days	5·20	2·78	
Specific activity	7 days	43·00	42·00	1·02:1
Sulphur content	7 days	5·52	2·12	

Similar results at 5 and 24 hr after a [^{35}S]cystine pulse were interpreted by previous workers (Downes *et al.* 1963) as indicating that matrix proteins are all formed at distal levels in the prekeratin tissue. Downes *et al.* (1963) found a ratio of low-sulphur:high-sulphur proteins of 11:1 after 4 hr and 4:1 after 17 hr. They inferred that at the time intervals studied after each pulse there would be little contribution of activity from the low-sulphur proteins assumed to be synthesized proximally in the follicle. The decreasing activity ratio with time could equally well imply a differential rate in the incorporation of the isotope into matrix and fibrillar proteins with time and not necessarily a "two-stage" process.

In Table 1 the specific activity ratio of matrix to fibrillar proteins tends to one with time. Whilst indicating that the activity per microgram of sulphur in the two proteins equilibrates, it does nothing to solve whether it results from differences in space, i.e. a "two-stage" process, or a differential in the rate of isotope incorporation with time.

(*b*) *Proteins from Intact Wool Root Tissue and Cell Groups*

(i) *Intact Wool Roots*

Extraction of intact wool root proteins was carried out immediately after stripping using urea–Tris solution. Animals 3 and 4 were used at 5 and 24 hr after the pulse as above. In Table 2 specific activity values and sulphur contents are given for both the low- and high-sulphur proteins from freshly stripped intact wool

follicle roots. High activity is present in both groups of proteins at 5 and 24 hr after the pulse. The specific activity ratio of 1·73:1 (high-sulphur:low-sulphur proteins) at 5 hr tends to 1 at 24 hr, almost entirely as a result of a large increase in the specific activity of the total low-sulphur protein extract at 24 hr. A small reduction in specific activity of the high-sulphur proteins has occurred between 5 and 24 hr.

Marked differences are observed in the comparison of the specific activity ratios of the high- to low-sulphur proteins from intact roots (Table 2) with the ratio for newly synthesized wool matrix and microfibrillar proteins (Table 1) at the same time periods after the pulse. The preponderance of activity in the matrix proteins of newly keratinized protein is not as apparent in the high-sulphur protein extracts of wool roots.

TABLE 2

SULPHUR CONTENT AND SPECIFIC ACTIVITY OF HIGH- AND LOW-SULPHUR PROTEIN EXTRACTS OF INTACT WOOL ROOTS, 5 AND 24 HR AFTER A SINGLE INJECTION OF [^{35}S]CYSTINE

Specific activities are expressed as picocuries per microgram of sulphur, and sulphur contents as percentages. Results from two animlas are recorded, together with mean values

Measurement	Period after ^{35}S Pulse	High-sulphur Extract (A)	Low-sulphur Extract (B)	Ratio of Specific Activities (A/B)
Specific activity	5 hr	138	72	
		156	98	
Mean		147	85	1·73:1
Sulphur content	5 hr	4·21	1·77	
		4·22	1·59	
Mean		4·22	1·68	
Specific activity	24 hr	142	154	
		131	140	
Mean		136	147	0·93:1
Sulphur content	24 hr	3·61	1·76	
		4·22	1·40	
Mean		3·91	1·58	

(ii) *Cell Groups*

As in Tables 1 and 2, greater ^{35}S specific activity is associated with matrix than with microfibrillar proteins from isolated cells, 5 hr after the pulse (Table 3). After 24 hr a substantial increase in specific activity of microfibrillar protein is again apparent, with only a small reduction in the specific activity of matrix proteins.

The increased specific activity ratio of matrix:microfibrillar proteins at 5 hr (5·4:1) compared with the ratio for proteins of the intact root extract (1·73:1) occurs as a result of the lower specific activity of microfibrillar proteins from isolated cells.

The ratio is reversed at 24 hr (mean 0·44:1), rather than tending to 1 as in Tables 1 and 2. The change in the specific activity ratio shown in Table 3 between 5 and 24 hr, though extreme, is almost entirely due to increased specific activity of

microfibrillar proteins, with little change in matrix proteins. This is also the case in Table 2.

The mean specific activity ratio of matrix:microfibrillar proteins from root cells at 5 hr (5·4:1, Table 3) is more in accord with the value of 7·2:1 for the same proteins from newly keratinized proteins at 5 hr (Table 1). The value of the ratio for proteins in cell group 3 (8·8:1, Table 3) is also in accord with the above value.

TABLE 3

SPECIFIC ACTIVITY AND SULPHUR CONTENT OF MATRIX AND MICROFIBRILLAR PROTEINS FROM EACH OF THREE CELL GROUPS AT 5 AND 24 HR AFTER A SINGLE INJECTION OF [^{35}S]CYSTINE

Specific activities are expressed as picocuries per microgram of sulphur, and sulphur contents as percentages

Measurement	Period after ^{35}S Pulse	Matrix Proteins (A)	Microfibrillar Proteins (B)	Ratio of Specific Activities (A/B)
Specific activity	5 hr			
Cell group 1		126	42	3·0:1
Cell group 2		100	22	4·5:1
Cell group 3		114	13	8·8:1
Mean		113	26	5·4:1
Sulphur content	5 hr			
Cell group 1		5·26	1·87	
Cell group 2		5·43	1·72	
Cell group 3		5·49	2·01	
Specific activity	24 hr			
Cell group 1		56	126	0·44:1
Cell group 2		80	201	0·40:1
Cell group 3		145	276	0·54:1
Mean		94	201	0·44:1
Sulphur content	24 hr			
Cell group 1		*	1·94	
Cell group 2		*	1·98	
Cell group 3		5·38	1·98	

* Sulphur content as for animal at 5 hr after pulse.

The ratios associated with proteins from cell groups 1 and 2, i.e. in the proximal two-thirds of the prekeratin region [see fig. 1(*a*), Fraser 1969], at 5 hr (Table 3) are again higher than values recorded for the proteins extracted from intact wool roots. A decreasing specific activity gradient is evident from cell group 1 to cell group 3 for microfibrillar proteins, whilst the matrix proteins in all cell groups have similar values. At 24 hr after the pulse, though the specific activity of microfibrillar proteins has increased, there is a decreasing specific activity gradient from cell group 3 to cell group 1. This is also the case for matrix proteins, with a reduction in specific activity below the values at 5 hr except in cell group 3.

As already described (Fraser 1969), washing freshly collected wool roots in Lockes solution extracts a considerable quantity of non-dialysable proteins. Though such proteins are fractionated into those low in sulphur and those of higher sulphur

content by the same procedure for fractional precipitation of wool proteins at pH 4·4, they are distinct chromatographically and electrophoretically from matrix and microfibrillar proteins. At 5 hr after the pulse the low-sulphur proteins extractable with Lockes solution from intact wool roots have a higher specific activity (98 pCi/μg sulphur) than the average value for microfibrillar proteins from cell groups (26 pCi/μg sulphur) as shown in Table 3. At 24 hr a greatly reduced specific activity was recorded for this fraction (12 pCi/μg sulphur).

IV. Discussion

The low-sulphur, non-microfibrillar proteins, which have a high specific activity at 5 hr after the ^{35}S pulse, and a markedly reduced one at 24 hr, are present in total extracts of intact wool roots, but are removed from isolated root cells with Lockes solution during cell separation. This removal explains the differences between the specific activity ratios for high-sulphur:low-sulphur and matrix:microfibrillar proteins from within the same follicle population from such tissues. Because of the mixture of both proteins in extracts of intact wool roots at 5 and 24 hr after the pulse the large differential in microfibrillar protein specific activity between each of the time periods is almost totally masked, as evidenced by the specific activity ratios of 1·83:1 to 0·93:1 (Table 2) as compared with 5·4:1 to 0·44:1 (Table 3).

The large increase in specific activity of microfibrillar proteins from wool root cells (Table 3) between 5 and 24 hr after the ^{35}S pulse, indicates a time "lag" in the incorporation of the label into microfibrillar proteins as compared with matrix proteins. A small decrease in specific activity is recorded for matrix proteins over the same period. This decrease, and a decreasing specific activity gradient for matrix proteins from cell group 3 to cell group 1 at 24 hr (Table 3) indicates a fall-off in the activity of the circulating pulse.

The ratio of mean specific activities of root cells of 5·4:1 (matrix:microfibrillar proteins) and the ratio of 8·8:1 for cell group 3 (Table 3) 5 hr after the pulse, are of a similar order to the ratio for newly formed proteins keratinized over the 5-hr period (7·2:1, Table 1). Such ratios, when compared with the ratios in Table 2 at 5 hr, indicate that the non-microfibrillar, low-sulphur protein is not incorporated as such into the keratinized protein. The progressive incorporation of ^{35}S into microfibrillar proteins leads to a reversal of the specific activity ratio of the two types of proteins in the protein-synthesizing root cells with time. The lag in incorporation of ^{35}S into microfibrillar proteins explains the progressive decrease of the ratio to unity in newly formed wool proteins as keratinization proceeds, i.e. from 5 hr to 5 days (Table 1). On the basis of the degree of reduction in specific activity ratio within newly keratinized proteins between 5 and 24 hr (Table 1) attainment of the 1:1 ratio within keratinized protein could be at approximately 31 hr. It has clearly occurred by 5 and 7 days (Table 1). The wool grown over such a 31-hr period will have a distal margin of high matrix protein activity and a proximal margin of high microfibrillar protein activity. The results from root cells (Table 3) at 5 and 24 hr indicate both ends of the range (i.e. 5·4:1 to 0·44:1). The higher specific activity in the low-sulphur, non-microfibrillar proteins compared with microfibrillar proteins at 5 hr, falling to considerably less than the values for microfibrillar proteins at

24 hr, occurs concurrently with a marked increase in microfibrillar protein specific activity between 5 and 24 hr. Such a transfer of activity, together with the fact that these non-microfibrillar proteins are not incorporated as such into keratinized protein, suggests that they are precursor proteins requiring hydrolysis before the ^{35}S is incorporated into structural fibrous proteins, or are subunits which in time polymerize to form microfibrillar proteins. The latter interpretation is considered more likely for the following reason. If non-specific sulphur-containing proteins are involved as precursors within the cells, the intermediary hydrolytic products would be expected to be equally available for incorporation into matrix proteins. This is clearly not the case, because between 5 and 24 hr a large increase in ^{35}S incorporation into microfibrillar proteins is accompanied by a small reduction in the specific activity of matrix proteins. This same argument also indicates that matrix proteins are not involved in total microfibrillar protein synthesis. Both matrix and microfibrillar proteins thus appear to be independently formed, contrary to the thesis that a single protein is synthesized, to which high-cystine-containing polypeptides are grafted during keratinization, as suggested by Corfield, Fletcher, and Robson (1967).

No evidence is presented that suggests the presence, in synthesizing cells, of precursor matrix proteins. Though precursors with a fast turnover may occur, the final matrix proteins may equally well be synthesized directly from monomers entering the follicle cells. The proposal that subunits of microfibrillar proteins are contained in those proteins first labelled with ^{35}S, and extractable with Lockes solution from keratin-synthesizing tissue, is in accord with work on the isolation of subunits from other fibrous proteins, notably collagen. The lag in the incorporation of ^{35}S into the complex three-dimensional structure of the fibrillar proteins is considered to correspond with the time taken for polymerization of subunits to occur. The high helical content of microfibrillar proteins is believed to be responsible for the X-ray diagram of wool (Thompson and O'Donnell 1965) which indicates a coiled-coil structure of two to three chains forming the protofilament (Fraser, McRae, and Miller 1964).

Whilst evidence indicating the existence of low molecular weight components in fibrillar proteins has been reported (de Deurwaerder and Harrap 1964, 1965), different disaggregating agents have failed to separate them. Recently, immunophoretic techniques (Frater 1968) have indicated heterogeneous composition of the fibrillar proteins. Though interpretation of the results is consistent with the concept that the complex structure of fibrous proteins occurs spontaneously after synthesis of components (Epstein, Goldberger, and Anfinsen 1963), attainment of the complex structure of keratin fibrillar proteins is not immediate after synthesis. A cell concentration effect of subunits may be involved. Further, current concepts postulating instantaneous synthesis of the complete fibrous protein complex (Kretsinger *et al.* 1964; Priestly and Speakman 1966) from amino acids on polyribosomal aggregates seems less likely to be applicable to the fibrillar proteins of wool keratin.

The specific activity values for proteins from the different levels within the prekeratin tissue (cell groups 1–3) at 5 hr after the pulse, in association with earlier estimations of matrix and microfibrillar protein content of such cells (Fraser 1969), allows for an approximation of the rate of protein synthesis. The decreasing specific activity of microfibrillar proteins proximodistally within the prekeratin tissue (cell

groups 1–3 at 5 hr after the pulse) does not indicate a differential in the rate of incorporation of ^{35}S but rather a dilution effect. Earlier work indicates that cells from cell group 3 have three times the microfibrillar protein content of cells from cell group 1 (Fraser 1969), and Table 3 shows that the former have one-third the specific activity of the latter. Similarly, cells of group 2 have been shown to contain approximately double the protein content of cells of group 1, and Table 3 shows that group 2 cells have approximately half the specific activity of cells of group 1. Thus, on correcting for ^{35}S dilution effects within cells at 5 hr after the pulse, the rate of incorporation of label is the same at all prekeratin levels. Such results corroborate the earlier concept that there is a linear increase in cell microfibrillar protein content throughout the prekeratin tissue. It is also clear that substrate availability must be similar throughout the columns of synthesizing cells.

The similar specific activity values for matrix proteins at all three cell-group levels indicates that the rate of ^{35}S incorporation must be approximately proportional to the differential in the matrix protein content of cells. That is, as for protein content of cells from the same population (Fraser 1969), there is likely to be an exponential increase in matrix protein synthesis proximodistally. Results suggest that approximately half of the final matrix protein content of the wool cells is formed in the upper third of the prekeratin tissue.

The results of the study reported here and in Fraser (1969) refute the hypothesis of a "two-stage" process of keratin synthesis. However, though both matrix and microfibrillar proteins are formed throughout the length of the prekeratin tissue of wool follicles, evidence is presented indicating a more rapid incorporation of matrix proteins into cells in the upper third than at lower levels of the prekeratin tissue.

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