KERATIN BIOSYNTHESIS

I. ISOLATION AND CHARACTERIZATION OF POLYSOMES FROM WOOL ROOTS

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Summary

Ribosomes and polysomes have been isolated from wool roots and fractionated into well-defined and consistently reproducible groups. Single ribosomes predominate, but evidence is presented which discounts enzymatic degradation or mechanical shear of polysomes as causes of this.

Two groups of polysomes are evident: a polydisperse group of light polysomes which sediments in eight discrete bands, and a homogeneous group of heavy polysomes which sediments as a single band, on sucrose density-gradient centrifugation.

The yield of polysomes is dependent on the concentrations of magnesium acetate and potassium chloride and on the pH; it is not improved by the use of sodium deoxycholate or ribonuclease inhibitors.

Ribosomes and polysomes have also been isolated from cells liberated from wool roots by incubation with the enzyme elastase.

A method is described for the collection of wool roots from live sheep in amounts which may be varied at will. The stripping technique enabled sequential collection of wool roots from the same animal for time-dependent studies on RNA and protein biosynthesis.

Hyaluronidase has been used to facilitate diffusion of radioisotopes in the skin. RNA synthesis studies, using [³H]uridine injected into the skin and sodium [³²P]-orthophosphate injected intravenously, indicate that messenger RNA in wool roots is probably stable.

I. INTRODUCTION

Studies with many bacteria, plant, and animal tissues (Risebrough, Tissieres, and Watson 1962; Gierer 1963; Warner, Knopf, and Rich 1963; Wettstein, Staehelin, and Noll 1963) have demonstrated the role played by polysomes (polyribosomes) in the synthesis of cytoplasmic proteins, but little is known about the size and function of polysomes in hair or wool follicles.

Electron microscope observations of hair follicles by Birbeck and Mercer (1961) and Rogers (1964), and of wool follicles (Orwin 1969) show clusters of ribosomes in developing cortical cells, while Rogers and Clarke (1965) and Clarke (1967) isolated polysomes from hair roots removed from the skin of guinea pigs by the wax sheet method of Ellis (1948). This method has the disadvantages of requiring the animal to be killed and skinned, the skin being subjected to temperatures of up to 70°C, with considerable time loss between skinning the animal and the final removal of the hair roots. Preparations from hair roots obtained by this procedure contained few polysomes. These were insensitive to magnesium ion concentration in the buffer within the range 0·005–0·1M and to pH within the range 6·0–7·4 (Clarke 1967).

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The present study was undertaken to improve the method of harvesting wool roots and to establish optimum conditions for isolation and characterization of polysomes with a view to further investigations on the role of wool root polysomes in the biosynthesis of keratin.

II. Materials and Methods

Wool follicle material (wool roots) was removed from coarse-woolled Romney wethers by a stripping procedure using a commercially available adhesive on a fibre-glass tape backing (Wilkinson *et al.* 1969). The area of the skin to be stripped was clipped of wool with Oster clippers (size 0000 head) and washed lightly with ethanol and allowed to dry. A thin layer of Araldite (Ciba Ltd.), consisting of 45 parts (v/v) resin AW106, 50 parts hardener HV953U, and 5 parts plasticizer 508, previously mixed thoroughly and left for 30 min, was applied to the skin by spatula. A woven fibre-glass tape, 2 in. in width, was applied over the adhesive and the bond between adhesive and backing secured by a further application of adhesive to the upper side of the permeable backing. The tape was left in position overnight and, with the sheep under light general anaesthesia, was then pulled free from one end as a continuous flexible strip. The wool roots were harvested with Oster clippers or a razor-blade.

Wool roots (2–5 mg/cm^2 skin area) were homogenized in 10 volumes of ice-cold standard buffer ($0\cdot02$M triethanolamine hydrochloride–NaOH, pH $7\cdot8$; $0\cdot2$M sucrose; $0\cdot25$M KCl; $0\cdot005$M magnesium acetate; $0\cdot006$M 2-mercaptoethanol) by 10 strokes in a loose-fitting Potter and Elvehjem homogenizer (Teflon–glass, $0\cdot18$ mm clearance) rotated mechanically at 500 r.p.m.

All procedures were performed at 4°C. Cells were freed from wool roots by incubation for 20 min at 4°C in 5 ml calcium-free Locke's solution containing pancreatic elastase (Sigma; twice crystallized—30 μg/100 mg of wool roots). The cells were separated by filtration through nylon bolting cloth (Nybolt No. 11, 118 μm), washed in standard buffer, and homogenized as above.

Alternatively, follicle material was removed from the fibre-glass strip directly by a brushing procedure. A test-tube brush was rotated mechanically in a horizontal plane and the strip fed over the wet brush through a Perspex container containing a small volume of standard buffer.

The homogenates were centrifuged at 12,000 *g* for 10 min at 4°C to remove mitochondria, cell debris, and residual wool root material, and cell supernatant fractions were withdrawn by pipette. Aliquots of cell supernatant fractions were layered on 26 ml of 15–40% linear sucrose density gradients in the standard buffer and centrifuged at 23,000 r.p.m. for $2\cdot5$ hr at 4°C in a Spinco SW 25·1 rotor of an L4 ultracentrifuge. Ribosomes and polysomes were also separated from the cell supernatant fraction by pelleting, either through layers of sucrose in standard buffer (2 ml of 1M sucrose, 2 ml of 2M sucrose, or 4 ml of 1M sucrose) or without sucrose layers, at 60,000 r.p.m. for 2 hr at 4°C in a Spinco type 65 rotor. The clear pellets readily dispersed by gentle stirring in standard buffer and were analysed by sucrose density-gradient centrifugation.

Sucrose gradients were emptied by upward displacement using an ISCO model D density-gradient fractionator. Continuous optical density readings at 254 nm were obtained with an ISCO model 222 ultraviolet analyser and recorded on an ISCO model 170 chart recorder, with the chart speed setting at 20 in/hr. Individual fractions collected from the gradients were measured manually at 260 and 280 nm on a Beckman DGB spectrophotometer.

Sucrose gradient fractions containing ^{3}H radioactivity were counted in a Beckman LS–200 spectrometer either (1) directly in Bray's solution, or (2) after being co-precipitated with bovine serum albumin with 10% trichloroacetic acid and washed on to Millipore filters (HAWP 02500; HA $0\cdot45$ μm), in toluene scintillating fluid (toluene, 1 litre; dimethyl POPOP, $0\cdot2$ g; PPO, 4 g). The methods of Mans and Novelli (1961) and of Heywood, Dowben, and Rich (1968) were used for counting [^{14}C]amino acid incorporation.

Radioisotopes (^{14}C and ^{3}H) were introduced into wool roots by injection into the skin under the fibre-glass strips. Diffusion of the isotopes through the dermis was facilitated by pretreatment of the area with hyaluronidase (Hechter 1950). Hyaluronidase (ovine testes, B grade; Calbiochem.) dissolved in isotonic saline (200 μg/ml) was injected into the skin ($0\cdot5$ ml/injection) just above the top edge of a fibre-glass strip positioned on the side of the animal. Two injections of $0\cdot5$ ml isotonic saline were then given at 30-min intervals. The bleb spread downwards and outwards, usually

covering an area of approximately 10 cm² of skin underneath the strip. The isotope was injected at or near the point of injection of the enzyme and saline, 30 min after the second saline injection. The area covered by this procedure was checked by the injection of India ink into the treated area, after the strip had been removed.

Actinomycin D (Dactinomycin; Merck, Sharp, and Dohme) was injected into the skin (200 μg/injection) by the same procedure.

Sodium [^{32}P]phosphate was injected into the jugular vein of the experimental animals.

The procedures and the choice of standard buffer detailed above were determined as optima by varying as many parameters as possible. The merits of the selected conditions, the effects of varying homogenization procedures, buffer components, and pH, and the use of ribonuclease inhibitors and sodium deoxycholate, will be considered fully in Section III. Gentle homogenization was considered to be produced by five strokes with the Potter and Elvehjem homogenizer at half speed. Severe homogenization was accomplished by using a manually operated glass–glass homogenizer in which glass beads (for gas chromatography, approx. 100 mesh), which were introduced with the wool roots, shattered during homogenization, completely disintegrating the tissue.

Radioisotopes were obtained from the Radiochemical Centre, Amersham, England. The synthesis of RNA was studied using [^{3}H]uridine(G), 2·70 Ci/m-mole and sodium [^{32}P]orthophosphate, 5 mCi/ml, with pulse times from 15 min to 16 hr. A [^{14}C]amino acid mixture, 52 mCi/m-atom labelled (CFB104), was injected 15, 30, and 60 min before stripping to study polypeptide synthesis.

III. RESULTS

(a) Properties of Polysomes from Wool Roots

Typical sucrose density-gradient profiles of polysomes from the cell supernatant fraction of wool roots isolated by the standard procedure are shown in Figure 1, at two concentrations. Some resolution is lost at the higher concentration but the profiles may be divided readily into three groups: a predominant ribosome peak, a group of polysomes with marked heterogeneity in eight bands, and a further group of polysomes sedimenting as a broad band which is not further resolved at low concentration. The E_{260nm}/E_{280nm} ratio of both groups of polysomes is 1·8 and that of the ribosome peak 1·9.
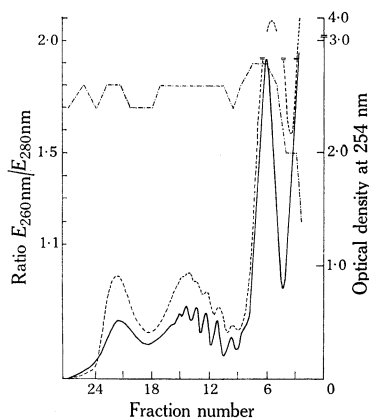

Fig. 1.—Sucrose density-gradient profile of wool root cell supernatant fraction at two concentrations. Profiles show a large single ribosome peak, a heterogeneous group of "light" polysomes, and a characteristic peak of "heavy" polysomes. ———, – – – – Optical density at 254 nm. – · – · Ratio E_{260nm}/E_{280nm}. Sedimentation is to the left (for conditions see text).

The polysomes were sensitive to ribonuclease (2 μg/ml for 10 min at 4°C), and to a lesser extent to mechanical shear as a result of severe homogenization [Fig. 2(a)], and to freezing and thawing.

In spite of ready resuspension of the ribonucleoprotein pellet with gentle agitation in standard buffer, the heavier polysomes were sheared after centrifugation at 230,000 g (av.) for 2 hr. This was particularly evident when sucrose layers were omitted, and was quite apparent when 1M sucrose was used alone [Fig. 2(b)]. When both 1M and 2M sucrose layers were used, shear of the heavy polysomes was less while the yield of light polysomes and ribosomes was depressed because of their inability to form pellets under the conditions used [Fig. 2(c)]. Longer centrifugation resulted in a polysome profile similar to Figure 2(b). It is apparent that it was the stresses imposed with high centrifugation rather than resuspension of the nucleoprotein pellet that caused shear.
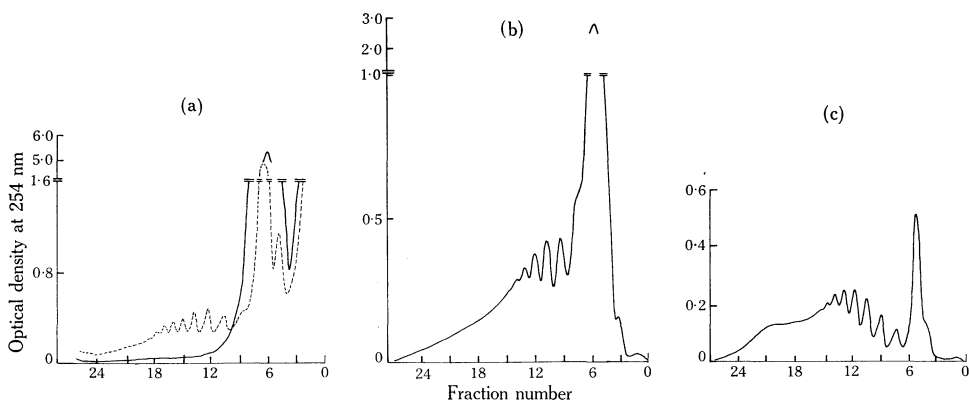

Fig. 2.—Sucrose density-gradient profiles. Sedimentation is to the left in all cases. (a) Profile of wool root cell supernatant fraction showing the effect of incubation with ribonuclease (2 μg/ml) for 10 min at 4°C (———) and of severe homogenization (– – –). (b) Profile of wool root ribosomes and polysomes after pelleting through 1M sucrose and gentle resuspension in standard buffer. Loss of the heavier polysomes is evident. (c) Profile of wool root ribosomes and polysomes after pelleting through 1M and 2M sucrose and gentle resuspension in standard buffer. Partial loss of the heavier polysomes is evident.

(b) Yield of Polysomes

The yield of polysomes and the ratio of polysomes to ribosomes were not increased by the use of sodium deoxycholate (0·5%), or ribonuclease inhibitors such as polyvinyl sulphate (2, 5, 10, or 20 μg/ml) and the 230,000 g supernatant fraction. The use of wool root ribosomal RNA as a competitive inhibitor for endogenous ribonuclease during isolation of the polysomes did not alter the sucrose gradient profile, or increase yield.

There was some indication that the method of harvesting the wool roots was of importance with regard to polysome integrity. Cutting by razor-blade introduced contaminants into the preparation in the form of small pieces of adhesive and skin fragments sloughed off in stripping. Also the area immediately beneath the strip was liable to be contaminated by sweat on the skin. When the wool roots were cut immediately against the strip, much of the keratinized portion of the roots was included in the preparation, making homogenization difficult because of clogging of the plunger. The combined effect of these characteristics was to cause a deterioration in the polysome profile and so electric clippers were used routinely.

The brushing procedure also suffered from the earlier disadvantages mentioned above, the effect in all cases being mainly on the heavier polysomes.

The standard homogenization procedure was adopted after extensive trials. Gentle homogenization only served to depress the yield of ribosomes and polysomes and severe homogenization disrupted the heavier polysomes, the apparent increase in the light polysomes and ribosomes being at the expense of the heavier polysomes. Re-homogenization of the cell debris after standard homogenization produced only a slightly increased yield. Methods using pestle and mortar with or without alumina, and a blade-type homogenizer (Virtis 45) did not increase yield and heavy polysomes were sheared.

(c) Buffer Components

The components and pH for the standard buffer were determined from a number of trials in which component concentrations were varied individually. No change in yield or profile of polysomes was observed with variation in sucrose concentration within the range $0-0.4$M. Whole nuclei were more numerous in the cell debris when sucrose was included in the buffer above 0.1M, but there was no advantage to be gained from higher concentrations. Using 0.2M sucrose (6.8% w/v) in the standard buffer enabled the cell supernatant fraction to be layered on 15–40% sucrose density gradients without mixing.

No difference in yield or profile of polysomes was apparent when triethanolamine hydrochloride–NaOH was replaced with Tris–HCl, but the former buffer was employed so that electron microscope studies could be undertaken using formalin as a fixative.

(i) pH Dependence

Triethanolamine hydrochloride (1M) in distilled water at $4°C$ was adjusted with sodium hydroxide to a range of pH values from 6.8 to 8.6. The pH of the prepared buffer (standard concentrations) and those of the cell supernatant fractions were recorded. The concentrations and profiles of polysomes per unit volume of cell supernatant fraction were related to the starting weight of wool roots over six pH values (6.8, 7.2, 7.6, 7.8, 8.2, 8.6). The yield of heavy polysomes increased with increasing pH up to 7.8 and then decreased, while the overall yield of polysomes and ribosomes followed a similar, though less pronounced, trend. The buffer made up with 1M triethanolamine hydrochloride–NaOH, pH 7.8, had a pH of 7.76 at $4°C$ while that of the cell supernatant fraction was 7.67.

(ii) Potassium Chloride Dependence

Similar trials were conducted with potassium chloride concentrations in the buffer of 0, 0.05, 0.1, 0.25, 0.3, and 0.5M (sucrose 0.2M; triethanolamine hydrochloride–NaOH 0.02M, pH 7.8; magnesium acetate 0.01M; 2-mercaptoethanol 0.006M). Potassium chloride was essential for the isolation of ribosomes and polysomes from wool roots. There was a marked improvement in resolution in sucrose density gradients up to 0.25M KCl: the profile at zero potassium chloride concentration was completely disrupted throughout while that at 0.3 and 0.5M showed progressive decay in the light polysome region. Yield was maximal at 0.3M KCl.

Potassium chloride could be replaced by ammonium chloride but to no advantage.

(iii) *Magnesium Dependence*

The isolation of polysomes from wool roots was dependent upon magnesium. No polysomes were isolated in the presence of EDTA ($0\cdot001$M) or at magnesium concentrations of zero or $0\cdot001$M magnesium acetate ($0\cdot2$M sucrose; $0\cdot02$M triethanolamine hydrochloride–NaOH, pH $7\cdot8$; $0\cdot25$M KCl; $0\cdot006$M 2-mercaptoethanol). Dissociation of ribosomes into subunits was apparent with EDTA or zero magnesium acetate. The yield of polysomes was depressed at $0\cdot001$M, maximal at $0\cdot005$M, and slightly depressed at $0\cdot01$M magnesium acetate.

(iv) E_{260nm}/E_{280nm} *Ratio of Ribonucleoprotein Pellet*

The E_{260nm}/E_{280nm} ratio of resuspended ribonucleoprotein was depressed when sucrose layers were omitted, when potassium chloride concentration was less than $0\cdot2$M, and when 2-mercaptoethanol was omitted from the standard buffer.

(d) *Polysomes from Wool Root Cells*

Preliminary trials have precluded the use of trypsin, chymotrypsin, papain, pronase, and lysozyme as enzymes useful for freeing wool root cells. Nonidet P40 (Shell) at 1% had little action, while shaking with glass beads of different sizes for various times caused cell breakage.

The most useful agent for freeing cells from the base of the bulb to the keratogenous zone was the enzyme elastase. Gentle agitation was required and correct enzyme–substrate proportions were essential. A magnetic stirrer, held in the wool root suspension by a platinum wire, was useful for stirring and avoided pronounced cell breakage. Ribosomes and polysomes isolated from the cell brei appeared little affected by the enzyme treatment as shown by sucrose density-gradient centrifugation.

(e) *Radioisotope Labelling*

(i) *Hyaluronidase and Spread*

Hyaluronidase provided a means of spreading the isotope through the skin to the wool follicles under the strip. It was not always successful and while the explanation for this is not known, it is, at least in part, associated with the positioning of the hypodermic needles in the skin. The shape and size of the bleb were also governed by position on the animal. Isotope studies were restricted to the sides of the sheep so that the bleb was narrow and elongated. No difference could be shown between the yield and profile of ribosomes and polysomes from wool roots from hyaluronidase-treated or from normal skin.

Other agents (5% dimethyl sulphoxide; collagenase) were not successful for increasing spread within the skin.

(ii) *RNA Labelling*

(1) [^3H]*Uridine*.—This was injected into the skin after pretreatment with hyaluronidase and wool roots were collected from several strips after 15, 30, 45 min, 1, 2, 4, 8, and 16 hr. No activity was distinguishable in sucrose gradients of the cell

supernatant fractions except at the top of the gradients at times less than 1 hr. Activity in the top of the gradients, even at short times, was high when counted directly in Bray's solution and was markedly reduced when fractions were precipitated with trichloroacetic acid and counted on Millipore filters. At 1 and 4 hr, essentially the same result was obtained (Fig. 3). At 8 hr there was some evidence of ribosomal RNA labelling, with activity appearing in the ribosome peak and in the polysome region. At 16 hr, ribosomal RNA labelling was quite apparent (Fig. 3) with the radioactivity profile following the optical density profile.
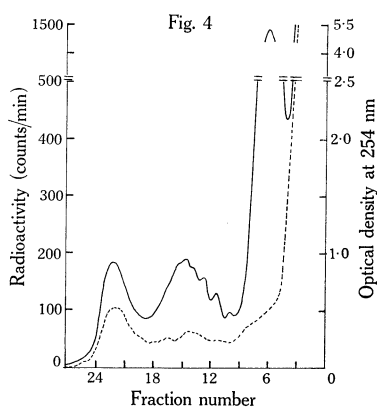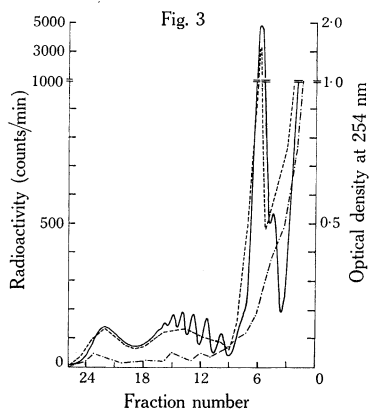

Fig. 3.—Sucrose density-gradient profile (———) of wool root cell supernatant fraction showing the incorporation of [³H]uridine 1 and 4 hr (– · – · – · –) and 16 hr (– – – – – –) after injection into the skin. Sodium [³²P]orthophosphate injected intravenously gave essentially the same result (for details see text). Sedimentation is to the left.

Fig. 4.—Sucrose density-gradient profile (———) of wool root supernatant fraction showing incorporation of [¹⁴C]amino acid mixture, 15 min after injection into the skin (– – – – – –) (for details see text.) Sedimentation is to the left.

(2) *Sodium Orthophosphate.*—To check the validity of the [³H]uridine incorporation studies, the same series of experiments was conducted with sodium [³²P]-orthophosphate injected into the jugular vein. The sucrose gradient profiles from these studies are not shown as they mimic precisely the [³H]uridine profiles qualitatively, though the incorporation of the [³²P]-orthophosphate into RNA of the ribosomes and polysomes gave low specific activity. Trichloroacetic acid precipitation and millipore filtration were essential prerequisites for following the trends of ³²P activity in the sucrose gradients as the "noise" level in unwashed fractions, counted in Bray's solution, was so high that the profiles were obliterated, even at 16 hr.

(3) *Actinomycin D.*—This was injected in normal saline (200 μg/injection) into hyaluronidase-treated areas at times of 15, 30, 60, and 240 min following [³H]uridine injection (500 μCi/injection). No change in polysome profiles on sucrose gradients was observed at any of the times.

(iii) *Polypeptide Labelling*

A [¹⁴C]amino acid mixture (25 μCi) was injected into the skin following hyaluronidase treatment and the wool roots harvested 15 min later.

The cell supernatant fraction was fractionated by sucrose density-gradient centrifugation and fractions counted by the method of Heywood, Dowben, and Rich (1968).

No radioactivity was associated with the ribosomes (Fig. 4), and the specific activity was significantly higher in the heavy polysome peak than in the light polysome region. The counting method of Mans and Novelli (1961) gave the same profile.

IV. DISCUSSION AND CONCLUSIONS

Polyribosomes have been described in many tissues, for instance by Warner, Rich, and Hall (1962); Gierer (1963); Wettstein, Staehelin, and Noll (1963); Warner, Knopf, and Rich (1963); Staehelin *et al.* (1963); Clark, Matthews, and Ralph (1963); Howell, Loeb, and Tomkins (1964); Wilkinson and Kirby (1966); Heywood, Dowben, and Rich (1967; 1968); Chen and Young (1968); Earl and Morgan (1968). However, comparatively little is known about polysomes in wool and hair follicles. Electron microscope observations by Birbeck and Mercer (1961), Rogers (1964), and Orwin (1969) showed clusters of ribosomes in developing cortical cells, while Rogers and Clarke (1965) isolated polysomes by sucrose density-gradient centrifugation from homogenates of guinea pig hair roots. Clarke (1967) extended this work to include a study on several aspects of protein synthesis and showed electron micrographs of polysomes varying in size from two to eight units.

No work reported to date has been aimed at elucidating the optimum conditions for the isolation of polysomes from wool roots. The sucrose gradient profiles of ribosomes and polysomes from guinea pig hair root homogenates (Clarke 1967) show a paucity of polysomes and a lack of sensitivity to pH within a range 6·0–7·4 (cf. Petermann and Pavlovec 1961; Arnstein *et al.* 1965; Earl and Morgan 1968) and to magnesium concentration within the range of 0·005–0·1M (cf. Lamfrom and Glowacki 1962).

The sucrose gradient profiles of wool root polysomes clearly show two broad classes: a heterogeneous group of light polysomes and a broad band of heavy polysomes. The proteins of keratin can also be fractionated into two major classes (Goddard and Michaelis 1935; Gillespie and Lennox 1953; O'Donnell and Thompson 1959; Fraser 1969): the high molecular weight, low-sulphur microfibrillar protein fraction is made up of proteins of molecular weight 40,000–50,000, and represents 50–60% of wool protein; the high-sulphur, lower molecular weight protein fraction is composed of proteins of widely differing molecular weights (7,000–30,000) and represents 20–30% of wool protein.

As in general there is a relation between polysome size and the size of polypeptides synthesized (Heywood, Dowben, and Rich 1967), a higher concentration of large polysomes would be expected in wool roots. Also of importance are the results of Heywood, Dowben, and Rich (1967, 1968) who worked with skeletal muscle. Previous work had indicated a low concentration of polysomes in muscle, but these workers, using buffers of high ionic strength, were able to obtain a high yield of polysomes, approximately equivalent to that from the liver of the same organism.

Several factors are of importance for isolating wool root polysomes:

(1) Harvesting of wool roots is best accomplished by clipping to avoid contamination by skin fragments and sweat.

(2) The stripping procedure enables wool roots to be removed from live animals directly into cold media thus limiting the chance of degradation of cell components.

(3) Homogenization is best achieved using a Potter-type homogenizer. Other methods either cause shear of the heavier polysomes or limit yield. Brushing directly from the strip is a facile procedure and would be particularly useful for bulk isolation of polysomes. However, it appears that this method introduces contaminants in a similar way to shaving the strip by razor-blade and requires re-examination.

(4) The ionic concentration and pH of the isolating medium are important. Magnesium is essential and the concentration is optimum at 0·005M (cf. Ts'o, Bonner, and Vinograd 1956; Tissieres *et al.* 1959; Dass and Bayley 1965). Other divalent cations may be satisfactory but were not tested.

Heywood, Dowben, and Rich (1967) and Chen and Young (1967) have shown that muscle polysomes bind to material sedimenting at 10,000 *g* unless potassium chloride is present in the homogenizing medium at high concentration. Potassium chloride was essential in the homogenizing medium in the present work and the optimum concentration was similar to that found for muscle polysomes. The full explanation for this is not known, but it was observed that cell supernatant fractions prepared in low potassium chloride (0·025M) became cloudy within a short time if left to stand at 4°C. Dintzis, Borsook, and Vinograd (1958) and Petermann and Pavlovec (1961) have discussed binding of proteins to ribosomes and dissociation of the complex at high ionic strength. Ammonium chloride was of no particular advantage in the homogenizing medium instead of potassium chloride (cf. Earl and Morgan 1968) but it may be useful in cell-free systems.

The pH of the homogenizing medium was optimum at 7·8. Ribosomes and polysomes precipitate at pH 6·0 (Arnstein *et al.* 1965) and those from wool roots are no exception (cf. Clarke 1967).

Triethanolamine hydrochloride–NaOH buffer was used instead of Tris because of the proposed expansion of this investigation to include electron microscopy of polysomes. Formalin may be used for fixing ribosomes for electron microscopy (Dass and Bayley 1965) and it was observed in the present study that the addition of formaldehyde to Tris buffer solutions at pH 7·6 resulted in a marked lowering of pH with resultant precipitation of the ribonucleoprotein. This was no doubt due to reaction of the free amino group of the Tris with formaldehyde, causing loss in buffering capacity. Tris buffers therefore cannot be used when fixation with formalin is proposed.

Electron micrographs of intact hair follicle cells have shown that there is little or no endoplasmic reticulum (Rogers and Clarke 1965). It is not unexpected that sodium deoxycholate did not increase the yield of ribosomes or polysomes. Ribonuclease inhibitors offered no advantage, either during harvesting of wool roots, subsequent homogenization, or during sucrose gradient centrifugation.

The loss of heavy polysomes after pelleting and resuspension must have been due to shear during centrifugation. The loss was marked when sucrose layers were omitted or when 1M sucrose was used alone [Fig. 2(*b*)]. Heavy polysome yield was less affected when 2M sucrose was used [Fig. 2(*c*)]. From these results it could not be

discounted that the heavy polysomes were merely aggregates of smaller units, but the marked reproducibility of the profile, the ^{14}C labelling data (Fig. 4), and the observation under the electron microscope (D. F. G. Orwin, personal communication) of large polysomes of the same order of size in sectioned wool roots indicate that this is unlikely.

The ribosome content is particularly large compared with that of polysomes, but the radioisotope data (Fig. 4) discount the possibility of this being due to shear or degradation. Nevertheless, it is interesting to consider the probable role of the large single ribosome complement in wool roots in the light of the observations of Mangiarotti and Schlessinger (1966) that single ribosomes in bacterial preparations can only arise from polysome degradation. Their results show that ribosomes in bacteria dissociate into subunits between rounds of protein synthesis and that single ribosomes do not exist *in vivo*. There are conflicting reports from other workers who have observed single ribosomes in bacterial preparations in which degradation of polysomes was carefully excluded (Flessel, Ralph, and Rich 1967; see also Phillips, Hotham-Inglewski, and Franklin 1969). The lack of radioisotope label from a single short [^{14}C]amino acid pulse, over the ribosome peak in the present study, supports a model of ribosome function in wool roots (and in reticulocytes: Colombo, Vesco, and Baglioni 1968), in which single ribosomes are liberated from polysomes at the end of a round of translation. The ribosomes then dissociate into subunits and a recombination of subunits into ribosomes occurs just before or at the time that the synthesis of a new peptide chain is initiated.

The ratio of single ribosomes to polysomes, or more correctly the size of the monomer pool, has been implicated in the control of ribosome synthesis (Morris and DeMoss 1966). The monomer pool of rat liver is decreased 14 hr after partial hepatectomy (Rizzo and Webb 1968) and there is a linear and inverse relationship between the rate of ribosome synthesis and the concentration of monomeric ribosomes. It would be of interest to investigate the ribosome–polysome profile of wool roots stripped from an area previously depilated, particularly as there appears in the present study to have been a trend towards a reduction in the relative size of the ribosome complement since the standard technique for isolation was first established. Wool production varies both seasonally and between sheep. As several animals were used in this work, the possible production significance of this trend escaped attention.

The RNA labelling studies were undertaken initially as a check on the validity of the polysome profile (Fig. 3). The results obtained with [^{3}H]uridine after pretreatment of the skin with hyaluronidase were supported by the sodium [^{32}P]orthophosphate trials. There was some concern that hyaluronidase may have upset wool follicle metabolism and that the lack of incorporation of [^{3}H]uridine into messenger RNA (*m*RNA) may have been due to this. However, the ^{32}P data support the view that as the products of protein synthesis in wool follicles are retained within the cell and the synthesis of both high- and low-sulphur proteins is a repetitive function throughout the length of the keratogenous zone (Fraser 1969), the major proportion of the *m*RNA moiety is probably stable.

The maturation of undifferentiated cells in the wool root into mature keratinized cells of the fibre is a process comparable with erythropoiesis. Haemoglobin *m*RNA is

stable (Marks *et al.* 1962) and there is evidence that stable *m*RNAs may be present in other mammalian tissue (Revel and Hiatt 1964).

Actinomycin D had no effect on the polysome profile at 4 hr and autoradiographic studies (D. F. G. Orwin, personal communication) have shown that the uptake of [³H]uridine into wool follicles is restricted to near the base of the follicle while protein synthesis is evident right through the keratogenous zone. These results provide evidence to support the postulate of Rogers and Clarke (1965) for hair follicles, that the predominant species of *m*RNA in wool and hair roots are stable.

It is apparent that a more precise delineation of function of the ribosomes and polysomes in keratin biosynthesis will follow from fractionation of wool roots into respective cell types and the identification of protein synthesized by these cells.

The cellular and subcellular fractionation of wool roots is difficult because of the close association and heterogeneity of cells throughout their differentiation and maturation, but it was gratifying to observe in the present study that ribosomes and polysomes isolated from cell brei, derived from elastase digestion of wool roots, had not been adversely affected at least in terms of their sedimentation behaviour.

Studies on polysomes from isolated and fractionated wool root cells are in progress.

V. Acknowledgments

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