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Preparation of Metabolically Active Cell Suspensions from Wool Roots

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Abstract

A method is described for the preparation of metabolically active cell suspensions from plucked wool roots using the proteolytic enzyme trypsin. The suspensions consist of a heterogeneous population of cells which appear similar in morphology to follicle bulb cells, differentiating keratinocytes and possibly cells of the inner root sheath. The concentrations of trypsin and of inorganic ions for optimum activity of the suspensions have been determined, and the inclusion of EGTA was found to increase the yield of cells.

The cell suspensions incorporate [^{14}C]leucine, [^{3}H]uridine and [^{3}H]thymidine into acid-insoluble products, and are sensitive to the action of cycloheximide and emetine, but not to chloramphenicol or rifampin. Autoradiography has shown that the cells believed to be derived from the follicle bulbs show the greatest activity.

Introduction

The unique properties of the wool fibre are due mainly to the molecular organization of a complex mixture of keratin proteins, whose synthesis takes place in the 'pre-keratin' region of the wool follicle (for reviews, see Crewther *et al.* 1965; Rogers 1969; Fraser *et al.* 1972). The cellular control mechanisms which govern the biosynthesis of these proteins appear to be complex, since variations in nutritional status (Reis and Schinckel 1962; Reis *et al.* 1973) and hormone levels (Ferguson *et al.* 1965; Panaretto *et al.* 1974) are known to influence wool production *in vivo*. In addition, the wool follicle is acutely sensitive to the action of several inhibitors of cell function such as cyclophosphamide (Dolnick *et al.* 1969) and mimosine (Hegarty *et al.* 1964; Reis *et al.* 1974; Ward and Harris 1976).

The control mechanisms which operate during normal wool biosynthesis and also during its inhibition by mimosine and related compounds are at present only poorly understood. Detailed studies of these mechanisms can best be conducted by experimentation *in vitro*, either by using cell-free preparations or alternatively the *in vitro* incubation of intact wool follicle cells. The use of cell suspensions for such studies is particularly attractive since the complex cytoplasmic interactions believed to be involved in the control of keratin production may be relatively undisturbed within such cells.

The purpose of this paper is to define the optimum conditions for the preparation and short-term culture of cell suspensions from wool follicles using the proteolytic enzyme trypsin.

Materials and Methods

The sheep used were crossbred ewes (Merino × Dorset Horn), of body weight approximately 40 kg, maintained on a diet consisting of 6 parts chaffed lucerne and 4 parts crushed oats (600 g/day).

Chemicals

Cycloheximide, emetine–HCl, EGTA and soybean trypsin inhibitor (Type 11-S) were obtained from the Sigma Chemical Co. (U.S.A.); rifampin (B grade) and dithiothreitol were from Calbiochem (Aust.) Pty Ltd; Araldite monomer solutions were from Ciba Geigy (Aust.) Pty Ltd; trypsin (1 : 250) was from Difco Laboratories (U.S.A.); and the following labelled compounds were from the Radiochemical Centre, Amersham (U.K.): L-[U-^{14}C]leucine (311 mCi/mmol), [5-^{3}H]uridine (26 Ci/mmol) and [6-^{3}H]thymidine (26 Ci/mmol).

Isolation of Wool Roots

Wool roots were plucked from the mid-sides of animals by the Araldite stripping method described by Wilkinson (1970). The plucked wool roots were harvested with Oster clippers (size 40) as quickly as possible and placed directly into buffer I [calcium-free Krebs–Ringer–phosphate buffer, pH 7·2, containing 15 mM KCl, glucose (1 mg/ml), streptomycin (50 μg/ml) and penicillin (167 i.u./ml)] at 37°C.

Preparation of Cell Suspensions

Preliminary experiments were conducted comparing a number of different combinations of trypsin digestion, stirring of the wool root suspensions, and filtration. The following method was found to be optimum with regard to yield and activity of the resultant cell suspension.

Wool roots (0·5–1 g wet weight) were suspended in 20 ml of buffer I containing 1 mM EGTA and 0·01% trypsin and were incubated for 30 min at 37°C with gentle aeration with 95% O_2–5% CO_2 gas. The suspension was then stirred gently for 1 min, filtered twice through nylon cloth (170 μm mesh) and the cells collected from the filtrate by centrifugation at 500 *g* for 5 min. The wool roots and shafts retained by the nylon cloth were resuspended in a further 20 ml of the above buffer and the above treatment repeated. After collection, the cells were washed twice with buffer II (buffer I containing 2·5 mM $CaCl_2$) and used within 5 min.

Cells were routinely tested for viability as measured by their ability to exclude the dye eosin. About 0·1 ml of cell suspension in buffer II containing 10% calf serum [CSL (Aust.)] was mixed with an equal volume of 1% eosin and the number of stained cells counted under a phase contrast microscope. For most preparations about 60–70% of the round-shaped cells excluded the stain. The elongated cells were always fully stained in all preparations examined.

Assay of Isotope Incorporation

Cells (approximately 200 μg protein) were suspended in 0·5 ml of buffer II containing either 1 μCi of [^{14}C]leucine, 10 μCi of [^{3}H]uridine or 10 μCi of [^{3}H]thymidine and incubated at 37°C. Reactions were terminated with 0·5 ml of cold 5% trichloroacetic acid (containing where appropriate 0·1% leucine) and the acid-insoluble material collected by centrifugation, washed twice with 5% trichloroacetic acid, once with ethanol–ether (2 : 1), once with ether and then air-dried. The dried precipitate was dissolved in 1·0 ml of formamide by heating for 10 min at 180°C and 0·05 ml taken for the determination of protein (Lowry *et al.* 1951). To the remainder was added 4 ml of a toluene-based scintillation solution [0·3% butyl-PBD–0·02% dimethyl POPOP (2 parts) : Triton X-100 (1 part)], and radioactivity was determined by means of a Packard Model 2235 liquid scintillation spectrometer.

Microscopy and Autoradiography of Cell Preparations

For light microscopy, cell suspensions or undissociated plucked follicles were fixed for 16 h in Serra's fixative (Davenport 1960), dehydrated with graded alcohol solutions and embedded in paraffin. Sections were cut to a thickness of 7 μm and stained with haematoxylin and picric acid.

For autoradiographic studies, samples were fixed for 2 h in 1·3% OsO_4 buffered with 0·067 M s-collidine (pH 7·2) (Bennett and Luft 1959), dehydrated with graded alcohol solutions and embedded

in Araldite essentially as described by Chapman and Gemmell (1971). Sections (1 μm) were coated with Kodak AR10 stripping film, exposed for 20–60 days and developed with Kodak D10 developer. The autoradiographs were examined by phase contrast microscopy after which the emulsion was removed and the sections stained with toluidine blue to enable more positive identification of individual cell types.

Results

Morphology of Cell Suspensions

The cell preparations obtained from wool roots consist of a heterogeneous population of single and clumped cells (Fig. 1*a*). The cells which clump in clusters of up to 20 cells are approximately cuboidal in shape with a round nucleus and prominent nucleoli, whereas the cells which are elongated and contain an elongated nucleus are rarely found in clusters. The round clumped cells are probably derived from the region of the follicle bulb, and the elongated cells from the supra-bulbar region of the follicle. The latter cells appear striated under phase contrast microscopy, and it is possible that the striations represent the forming macrofibrils of cortical cells.

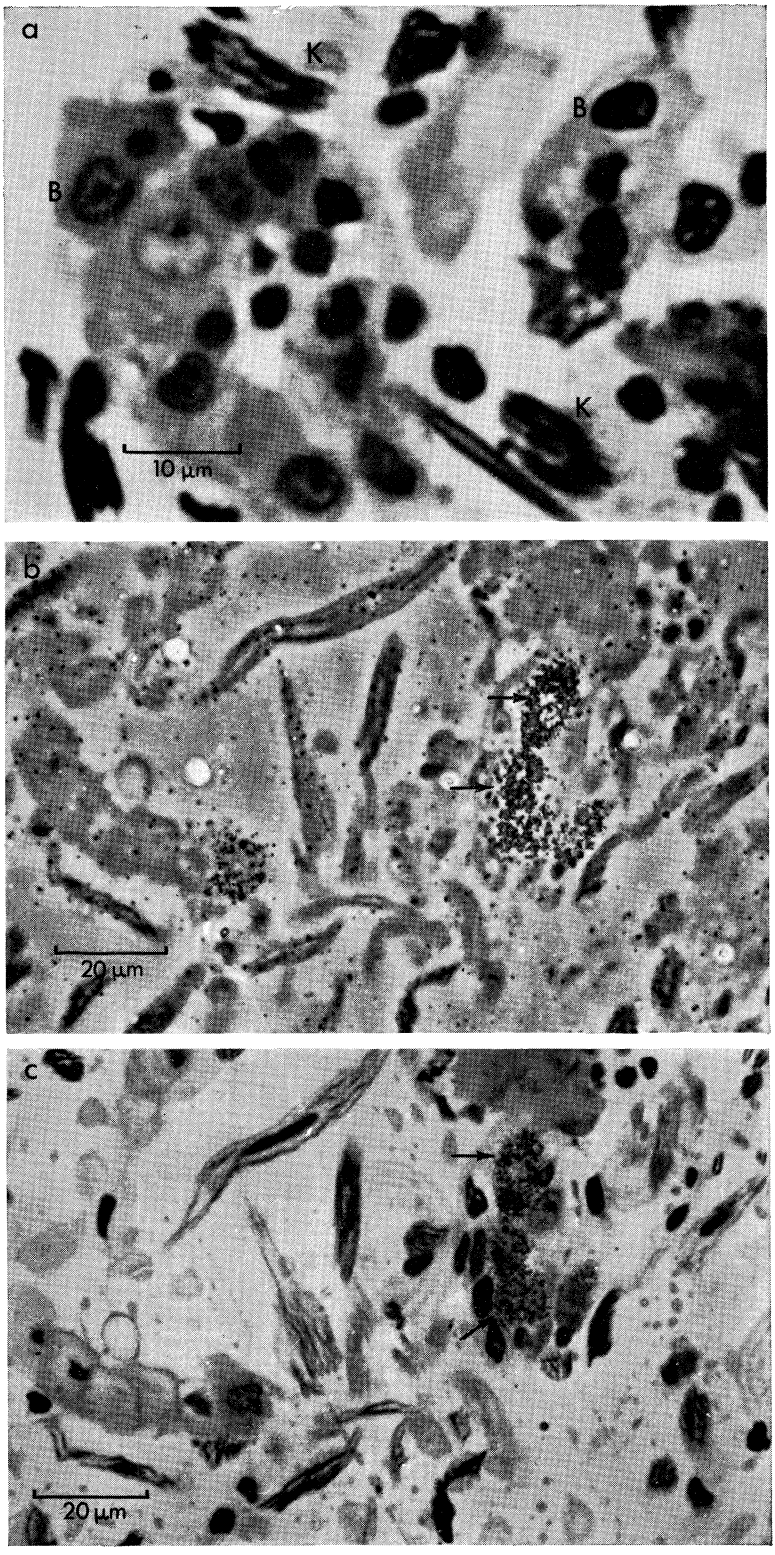
Factors Influencing the Yield and Activity of Wool Follicle Cells during Isolation

Preliminary experiments showed that the yield and the activity of the cell preparations were subject to considerable variation between different breeds of sheep. However, within the group of sheep used in the present experiments, acceptable reproducibility was obtained between preparations for any particular set of conditions of isolation. As shown in Table 1, variations in the extent of trypsin digestion have a pronounced effect on both the yield and the activity of the resultant cells. Thus digestion with 0·1 % trypsin gave a yield of about 4 mg cell protein per gram of wool roots. The yield decreased to about 1 mg/g at the low trypsin concentration of 0·01 %. However, the cells isolated at the latter trypsin concentration showed a 10-fold increase in the values for incorporated [^{14}C]leucine, expressed in terms of total cell protein. Much of this increase is probably due to the very low contamination of the 0·01 % trypsin preparations by fully keratinized, metabolically inert cortical cells, which can be seen in large numbers in preparations utilizing higher trypsin concentrations. It is also possible that the use of 0·1 % trypsin resulted in some damage to the active cells of the suspensions, although no overt damage could be detected in such preparations.

The yield of cells obtained from wool roots was increased almost twofold by the inclusion of either of the chelating agents EGTA or EDTA in the follicle dissociating medium (Table 1). The use of EDTA, however, resulted in a substantial lowering of the values obtained for the incorporation of [^{14}C]leucine by the cell suspensions. This loss of activity was not observed when EGTA was used.

In several experiments the addition of soybean trypsin inhibitor was included in the first washing solution after the initial isolation of the cells in order to inhibit any residual trypsin activity. The values obtained for the incorporation of [^{14}C]leucine by the cell suspensions treated with trypsin inhibitor were reduced by more than 50 % compared with untreated cell suspensions (Table 1).

The optimum concentrations of the inorganic ions in the incubation buffer were determined for maximal activity of the wool root cell suspensions, and were found to correspond essentially to those of a Krebs–Ringer phosphate buffer with the exception of the potassium ion requirement which was 15 mM.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Incorporation of Radioactive Amino Acids and Nucleic Acid Precursors

The biosynthetic activity of wool root cell suspensions was initially tested by autoradiographic examination of cells which had been incubated in a medium containing [^{3}H]leucine. Heavy deposition of silver grains was seen above groups of round cells, while nearby elongated cells were associated with very few grains (Figs 1*b* and 1*c*).

Table 1. Influence of trypsin concentration and chelating agents on wool follicle cell suspensions

Values expressed are means ±s.e. of five experiments except those involving the trypsin inhibitor which are for three experiments

Trypsin concn (% w/v)	Chelating agent (1 mM)	Cell yield (mg cell protein/g wet wool roots)	[^{14}C]Leucine incorporation [net DPM h^{-1} (mg protein)$^{-1}$][A]
0·10	EGTA	4·04±0·23	550±15
0·01	EGTA	1·04±0·16	5320±240
0·01	—	0·56±0·05	5550±100
0·01	EDTA	0·99±0·11	1860±160
0·01 (t.i.)[B]	EGTA	—	2140±150

[A] [^{14}C]Leucine incorporation is expressed as net DPM per 60 min incubation per milligram of cell protein.

[B] Trypsin inhibitor (7·5 mg) was included in the first wash (buffer II) after initial collection of the cells.

This suggests that the round cells are far more active than the elongated cells in the incorporation of [^{3}H]leucine.

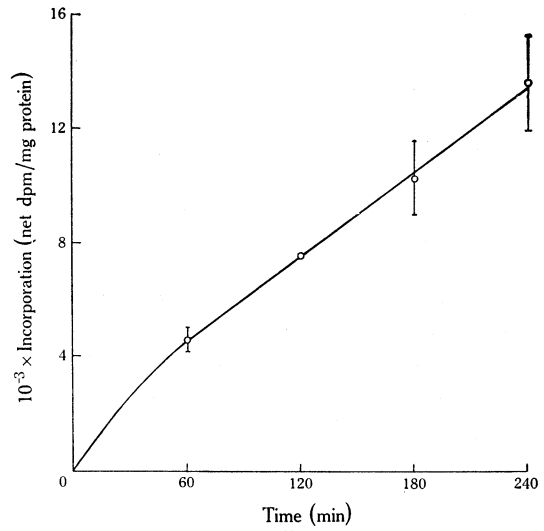

Fig. 2. Incorporation of [^{14}C]leucine by wool follicle cell suspensions. Cells (*c.* 200 μg protein) were incubated with 1 μCi of [^{14}C]leucine for the times indicated and the amount of radioactivity in acid-insoluble material was measured. Each point is the mean ±s.e. of three experiments.

Fig. 1. (*a*) A section of a wool root cell suspension. Groups of round (possibly follicle bulb) cells (*B*) can be seen together with numerous elongated cells (*K*) which are probably keratinocytes at various stages of differentiation. (*b*), (*c*) Autoradiography of a wool root cell suspension. Cells were incubated for 2 h with 10 μCi of [^{3}H]leucine and autoradiographs prepared as described in Methods and exposed for 28 days. A heavy concentration of silver grains is located over a group of round-shaped cells (shown by arrows). (*b*) Phase contrast micrograph. (*c*) After partial removal of photographic emulsion and staining with toluidine blue.

The protein synthetic activity of wool root cell suspensions was further examined by measuring the rate of incorporation of radioactively labelled precursors into acid-insoluble material during incubation at 37°C. Protein synthesis, measured with [^{14}C]leucine, proceeded linearly for at least 4 h (Fig. 2). The amount of radioactivity in the acid-insoluble material was not affected either by 1 M NaOH or hot 5% trichloroacetic acid.

Nucleic acid synthesis in wool root cell suspensions was measured by the use of the RNA precursor [^{3}H]uridine and the DNA precursor [^{3}H]thymidine. Both compounds were incorporated by the cell suspensions into acid-insoluble material, although the rate of incorporation, particularly of [^{3}H]thymidine, decreased with time of incubation (Fig. 3). The possibility of a bacterial contribution to the measure of follicle cell nucleic acid synthesis would appear unlikely since a concentration of 100 μg/ml of the bacterial RNA polymerase inhibitor rifampin was found to have a negligible effect on the incorporation of [^{3}H]uridine.

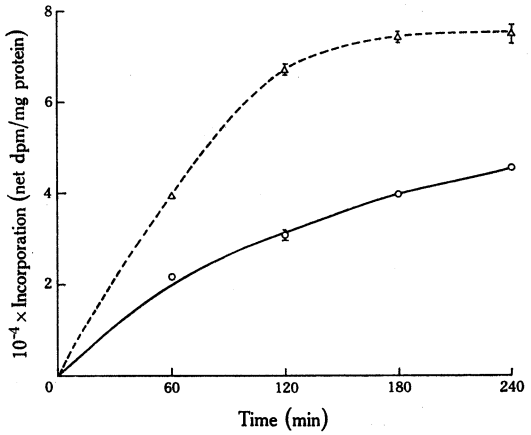

Fig. 3. Incorporation of [^{3}H]uridine and [^{3}H]thymidine by wool follicle cell suspensions. Cells (*c.* 200 μg protein) were incubated with 10 μCi of [^{3}H]uridine (○) or 10 μCi of [^{3}H]thymidine (△) for the times indicated and the amount of radioactivity in acid-insoluble material was measured. Results are the means ±s.e. of three experiments for each isotope.

Effect of Protein Synthesis Inhibitors

The influence of several inhibitors of protein synthesis on [^{14}C]leucine incorporation by cell suspensions is shown in Table 2. The antibiotic cycloheximide, which is a potent inhibitor of eucaryotic protein synthesis, strongly inhibited the activity of wool root cells. Thus, the incorporation of [^{14}C]leucine was inhibited by almost 90% at an inhibitor concentration of 1 μg/ml. Another inhibitor of eucaryotic protein synthesis, the antibiotic emetine, also inhibited protein synthesis in wool root cells (Table 2), although somewhat higher concentrations were required than for cycloheximide.

In contrast to cycloheximide and emetine, the antibiotic chloramphenicol is a specific inhibitor of procaryotic protein synthesis. When follicle cells were incubated in the presence of chloramphenicol the rate of protein synthesis was virtually unaffected, indicating that any bacterial contribution to the incorporation of [^{14}C]leucine can be considered insignificant in comparison with that of the active follicle cells.

The thiol reducing agent dithiothreitol, which was tested in view of the known high thiol content of prekeratinized wool follicle cells, was found to inhibit the activity

of the cell suspension at concentrations above about 10 μg per ml (Table 2). Below this concentration, the compound had no apparent effect on cell activity.

Discussion

Suspensions of wool root cells appear to be useful for the *in vitro* study of some of the mechanisms which control the biosynthesis of wool. The cell suspensions have the ability to continue the biosynthesis of proteins for many hours *in vitro*, and show a reproducible response to the action of several metabolic inhibitors. The conditions described in this paper for the preparation of the cell suspensions appear to be optimum with regard to the combination of yield and activity of the cells. Although greater yields of cells may be obtained by the use of higher concentrations of trypsin, these cells consist mainly of keratinized cortical cells, which are metabolically inert. Furthermore, the possibility of damage to the cells is increased by higher trypsin levels. Soybean trypsin inhibitor cannot be used to remove residual proteolytic activity, since it inhibits the activity of the cells. The reason for this is not known, although trypsin inhibitors have been reported to reduce the activity of cultured cells by causing cell-surface alterations (Collard and Smets 1974). The residual trypsin activity in isolated wool follicle cells may possibly be removed by the use of other trypsin inhibitors, but this has not been investigated.

Table 2. Effect of several metabolic inhibitors on the activity of follicle cell suspensions

Values expressed are means ±s.e. of five follicle cell preparations. Each experiment was run in triplicate

Inhibitor	Concentration (μg/ml)	[^{14}C]Leucine incorporation[A]	Inhibition (%)
—	—	5420±310	—
Cycloheximide	1·0	670±40	87
	10·0	590±30	89
Emetine–HCl	1·0	2265±100	58
	10·0	1895±50	65
Chloramphenicol	100	5210±270	4
Dithiothreitol	18·5	4020±210	26
	185	2820±170	48

[A] Measured as in Table 1.

The decreased rate of thymidine incorporation which occurs in the cell suspensions after several hours' incubation indicates that continued DNA synthesis is not supported by the present incubation conditions. The decrease in thymidine incorporation takes place in a variety of incubation media, including Waymouth's medium MB752 containing up to 20% foetal calf serum. Such a finding is not unexpected, since those cells of the wool follicle which are in the S-phase of the cell division cycle are located at the base of the follicle bulb and only a small proportion would be removed from the animal by the Araldite stripping procedure (Wilkinson 1970; Downes and Wilson 1971). Thus the cells which constitute the majority of the suspension are those already committed to differentiate into the components of the developing wool fibre. Recent evidence would suggest that in such cells, DNA synthesis and keratin synthesis are mutually exclusive (Kemp *et al.* 1974).

The continued synthesis of RNA in follicle cell suspensions suggests that these cells may be of value in studies of the RNA synthesis involved in wool keratin production. The messenger RNA templates for wool keratins are believed to be synthesized in the follicle bulb and to have a relatively long half-life (Wilkinson 1970). If this is correct, the two-stage synthesis of wool keratins (Downes *et al.* 1963) implies the existence of a translational control in addition to a transcriptional control over wool biosynthesis. Studies of protein and RNA synthesis in fractionated follicle cell suspensions may provide an answer to this question.

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