STUDIES ON THE SYNTHESIS OF HAIR KERATIN

A thesis submitted by

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CONTENTS

SUMMARY

The aim of this thesis was to investigate aspects of the mechanism of synthesis of the keratin proteins of the guinea pig hair follicle. Two different approaches were adopted. The first was to characterise in detail the properties of the hair follicle proteins. The second was to investigate protein synthesis in cell-free protein synthesis systems established from hair follicle tissue. The original findings of this work can be summarised as follows:

1 Procedures were developed for the isolation and characterisation of the different groups of proteins of hair and the hair follicle. Four groups of proteins were present in both. The major groups of proteins were the group-2 proteins which were defined as the low sulphur kerateine (LoS) proteins and the group-4 proteins which were defined as the high sulphur kerateine (HiS) proteins. Both the LoS and HiS proteins were heterogeneous and had amino-terminal N-blocked amino acids. One major LoS protein had an amino-terminal peptide of about 62 amino acid residues which was similar in chemistry to the HiS proteins. Although the properties of all corresponding proteins from both hair and hair follicle extracts were identical, there were two important quantitative differences. There were more LoS proteins but less HiS proteins in the follicle extracts than in the hair extracts and there were more HiS proteins of higher cysteine content in the hair extracts than in the follicle extracts. These differences were interpreted as indicative of the different rates of synthesis of the keratin proteins in the hair follicle.

2 Electron microscopic observations on intact hair follicles showed that densely-staining "cortical granules" are associated with groups of microfibrils (macrofibrils) in the cortical cells of the hair follicle.

The granules rapidly disappear as the macrofibrils adopt an appearance similar to that of mature keratin. It was postulated that the cortical granules contain HiS proteins. This implies that there is a precise point in follicle development at which the synthesis of HiS proteins is initiated and that this is after the stage when synthesis of the LoS proteins begins.

3 Procedures have been established for the isolation of polyribosomes from hair follicle tissue of very young guinea pigs of larger size and in higher yield than hitherto. These improvements were attributed to the low level of ribonuclease in the tissue homogenates and to the use of extraction buffers containing high salt concentration and dithiothreitol.

4 Cell-free protein synthesis systems were highly active in incorporation of amino acids into protein. The mechanism of cell-free protein synthesis was studied and was found to be very similar to that operative in other eukaryote systems. During incubation the polyribosomes degraded and released protein chains. Characterisation of these released protein chains by a number of techniques showed that they were completed LoS and HiS protein molecules identical to the native proteins. These observations imply that each of the keratin proteins is synthesised *in vivo* by the classical ribosomal-dependent mechanism.

5 Studies on the degree of *de novo* protein synthesis and rates of ribosomal subunit - polyribosome exchange showed marked differences between the LoS and HiS proteins. From these two observations it was considered that either the mechanism of initiation of HiS protein synthesis is different from that for the LoS proteins, or the rate of translation of the mRNA for HiS proteins is lower than that for LoS proteins. If the latter were the case, the level of cysteinyl-tRNA might be rate-limiting, and this could serve as an important *in vivo* control of HiS protein synthesis.

Two important conclusions arise from these studies. The first is that synthesis of the keratin proteins occurs by the classical ribosomal-dependent mechanism and not, as has been suggested previously, to any significant extent by non-ribosomal-dependent mechanisms. The second is that cell-free protein synthesis systems are potentially capable of answering several of the important outstanding problems of regulation of protein synthesis in the hair follicle.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

Signed:

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Peter M. Steinert.

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ABBREVIATIONS

Each abbreviation used in this thesis is defined in the text once before general use.

cAMP	3',5'-cyclic AMP
SCM-	S-carboxymethyl-
SCMK	S-carboxymethyl kerateine
LoS	low sulphur keratin *or* low sulphur kerateine
HiS	high sulphur keratin *or* high sulphur kerateine
PAGE	polyacrylamide gel electrophoresis
PCA	pyrrolidine carboxylic acid
dansyl- *or* DNS-	1-dimethylaminonaphthalene-5-sulphonyl-
TCA	trichloroacetic acid

With thon thaet mannes fex fealle, sing this thriva nigan sithan on aefen and on morgen on thaes mannes heafod ufan:

ic hit bebicge
cge hit bebicgan
thaes swearten wulle
and theysse sorge corn.

CHAPTER ONE

GENERAL INTRODUCTION

A INTRODUCTORY COMMENTS

The hair follicle synthesises several unique fibrous proteins that are deposited intracellularly. Since the principal group of proteins is the keratins, this system is potentially useful for studying the macromolecular events leading to the biosynthesis of the keratin proteins and their subsequent organisation in to an ordered form.

The aim of the work reported in this thesis was to investigate the mechanism of synthesis of the proteins of the hair follicle of the guinea pig with particular reference to the keratin proteins.

The present chapter is intended to provide the background necessary for the evaluation of the experimental work and is written in three parts. The first deals with the mechanism of gene expression and protein synthesis in eukaryote tissue as understood at this time. The second aspect deals with what is presently known of differentiation and protein synthesis in hair and wool follicle tissue and this is necessary for an evaluation of the usefulness of the hair follicle system for studying these mechanisms. In the third section prominent questions of differentiation in the hair follicle are discussed in relation to the aims of this work.

B MECHANISM AND CONTROL OF PROTEIN SYNTHESIS IN EUKARYOTE CELLS

(a) FACTORS INVOLVED IN GENE EXPRESSION

The potential function of an enzyme or protein resides in its amino acid sequence and the information for this is encoded in the DNA. The most obvious biochemical differences between different cells is in the content and function of their proteins. This can vary between cells as different proteins may have been synthesised during the life history of a cell-type or because certain intracellular conditions may have affected the activity of a

protein.

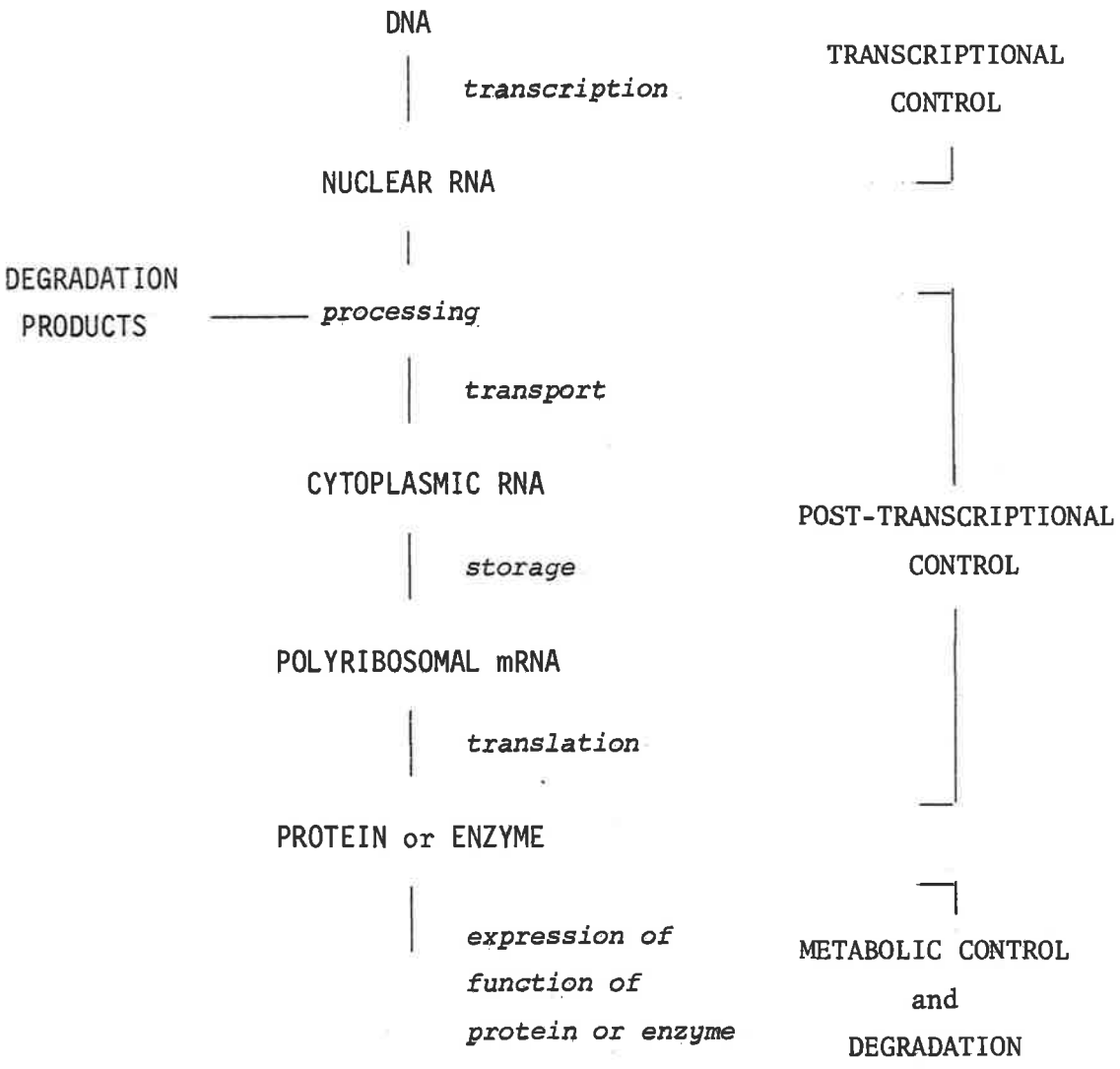
It is now generally acknowledged that the information in DNA is expressed by many consecutive steps. The main reactions by which the genetic information is converted into biologically active polypeptides is shown in Fig. 1.1. It is likely that such a scheme is a gross simplification as there could well be a number of other important steps which have not yet been elucidated. In addition, the scheme implies a uni-directional flow of information. That this may not be the universal case has been shown recently by the finding of an RNA-dependent DNA polymerase in HeLa cells infected with certain oncogenic viruses (Temin and Mizutani, 1970) and in normal human lymphocytes (Penner *et al.*, 1971).

In eukaryote organisms different populations of cells within the organism show stable phenotypic differences which have originated from the same genotype during development of the organism from its zygote. It can reasonably be assumed that the diversity of cell phenotypes in the organism must have been derived from the fact that each cell expressed only a limited amount of its genetic potential. Virtually every "differentiated" cell-type in a eukaryote organism has characteristic proteins not found in other cells (for examples; actin and myosin in muscle; collagen in dermis; keratins in epidermal tissues; haemoglobin in erythrocytes). Accordingly, regulation of the genetic information in eukaryote organisms during the processes of embryogenesis and differentiation must involve regulation of protein synthesis. It is conceivable that this could operate at the level of transcription or at some level after transcription but prior to expression of the function of the protein or by regulation of the activity or breakdown of the protein. The latter processes of metabolic control (for example, Stadtman, 1966) and degradation (for example, Ganschow and Schimke, 1969) will not be discussed further in this thesis. Further consideration will be given only to the mechanisms involved in the synthesis of proteins.

FIGURE 1.1

SCHEME OF GENETIC EXPRESSION

General scheme which summarises the steps involved in the flow of information encoded in the DNA into biologically active proteins (from Tomkins *et al.*, 1969). The three principal stages at which regulation of this scheme can occur are also given.


DNA
transcription
NUCLEAR RNA
DEGRADATION PRODUCTS
processing
transport
CYTOPLASMIC RNA
storage
POLYRIBOSOMAL mRNA
translation
PROTEIN or ENZYME
expression of function of protein or enzyme
TRANSCRIPTIONAL CONTROL
POST-TRANSCRIPTIONAL CONTROL
METABOLIC CONTROL and DEGRADATION

(b) REGULATION OF PROTEIN SYNTHESIS IN EUKARYOTE CELLS

(1) General concepts

The genome of prokaryotes and eukaryotes has the potential for the synthesis of a large number of gene products and it is clear that mechanisms must be operative to effect control over the expression of the genome. Eukaryotes are considered to be biologically more complex and contain DNA in quantities of several orders of magnitude greater than in prokaryotes (Ris and Kubai, 1970). Accordingly, the eukaryote cell may adopt alternative and different procedures for restriction of its genetic potential and it is conceivable that such restriction could occur at the levels of transcription and at some level(s) subsequent to transcription. Indeed, numerous examples of control at these levels have been cited in the literature. It is the purpose of this section to enumerate the various points in the scheme of Fig. 1.1 at which restriction of information flow has been experimentally observed in eukaryotes. This review will be concerned principally with regulation at levels subsequent to transcription.

(2) Transcriptional control

Initial concepts in bacteria

The first meaningful concepts on control of protein synthesis at the level of transcription in bacteria arose from the studies of bacteriophage infection and enzyme systems which were inducible and repressible by various metabolites (Jacob and Monod, 1961; Bretscher, 1968; Epstein and Beckwith, 1968).

In such studies it was found that many functionally-related genes were grouped in operons (for example; the *lac* system of *E. coli*; Jacob and Monod, 1961). These genes were transcribed sequentially by an RNA polymerase to yield a polycistronic mRNA which was then translated *in toto* on ribosomes in a co-ordinate manner. Control of these polycistronic

systems was found to be exercised at the level of transcription by a repressor specific for the operon in a negative manner. Positive control of the *lac* operon (and other catabolite-sensitive genes) can also occur. In these cases 3',5'-cylic AMP (*c*AMP) is responsible for normal initiation of mRNA synthesis by interaction with a specific activator protein which thereby allows proper interaction of the RNA polymerase with the promoter region of the operon (Pasten and Perlman, 1968; Zubay *et al.*, 1970). In the absence of *c*AMP transcription is inhibited by a non-functional RNA polymerase. (Emmer *et al.* (1970) and Eron *et al.* (1971) have shown that the *c*AMP-binding protein is distinct from the RNA polymerase σ factor.)

Further studies have shown that bacterial mRNA species are short lived (Jacob and Monod, 1961) and that translation occurs either simultaneously with or very shortly after transcription.

Regulation in eukaryotes

Chromosomes: In contrast to prokaryotes, the DNA of eukaryotes is subdivided into several chromosomes. Some evidence has shown that the DNA in the chromatid may be present in several subunits. These have been thought to exist as separate pieces (Cairns, 1966), as continuous lengths joined by alkali-unstable links (Lett *et al.*, 1970) and as tandemly-arranged replicating units analagous to replicons in prokaryotes (Prescott, 1970). The probable existence of a subunit structure of the DNA in eukaryote chromosomes has been used to explain such phenomena as DNA redundancy and the differential rates of replication during polytenisation of insect salivary gland chromosomes (Rudkin, 1969).

Chromatin: Eukaryote chromosomes do not exist as naked DNA as in prokaryotes but rather as chromatin, a nucleoprotein complex containing DNA, protein and RNA. The chromatin proteins can be divided into two types: the histone proteins, of which there are several subclasses (Bonner *et al.*, 1968; DeLange

et al., 1968); and the non-histone proteins which comprise a heterogeneous group of acidic proteins (MacGillivray *et al.*, 1971). An initial hypothesis on the role of histones suggested that they were specific repressors of the DNA (Huang and Bonner, 1962) but more recent studies conclude that this is inadequate since there is little sequence variation between histones of different tissues or species (Bonner *et al.*, 1968). The *in vitro* studies on RNA synthesis using chromatin as a template have illustrated the importance of non-histone proteins and chromosomal RNA components (Bekhor *et al.*, 1969; Huang and Huang, 1969; Paul and Gilmour, 1969). Histones may serve as structural proteins with non-specific masking functions. Specific activation of certain regions of the genome may occur on interaction of the histone proteins with chromosomal RNA species.

Operons: In eukaryotes, the genes of functionally-related proteins are not generally grouped together in operons, in marked contrast to prokaryotes. This conclusion has been reached in studies on fungal (Gross, 1969) and human (Dreyfus, 1969) genetics. In yeast, the six genes for galactose metabolism are scattered throughout the entire genome (Douglas and Hawthorne, 1964). The 28*S*, 18*S* and 5*S* rRNA genes are not grouped together in an operon (Brown, 1967; Birnstiel *et al.*, 1968; Brown and Weber, 1968).

DNA redundancy (gene duplication): Britten and Kohne (1968) showed that in many eukaryote organisms as much as 50 % (and in some cases, 90 %) of the DNA was present in the form of reiterated sequences with thousands of copies per cell. These authors postulated that this repetitious DNA was involved in the control of expression of various sets of genes within the organism. The different types of genes controlled by the common sequences could be activated by a single initiating event and could be responsible for the manifestation of a particular characteristic of a differentiated cell-type (Britten and Davidson, 1969).

Studies on rRNA synthesis in a number of different organisms have shown that there are many copies of rRNA genes within one cell (Ritossa *et al.*, 1966; Brown, 1967).

Gene amplification: Brown (1967) showed that multiplication of rRNA genes occurred in *Xenopus* on oogenesis. A selective replication of the chromosomal nucleolar organiser (where the rRNA genes are located) occurs at early stages of oogenesis which results in the formation of several hundred extra-chromosomal nucleoli (Brown and Dawid, 1968). This selective replication of rDNA in amphibian oocytes presumably reflects the need for extra templates to support the high rate of rRNA synthesis at early stages of oogenesis. Similar replication of rDNA occurs in other animal species (Brown and Dawid, 1968; Gall *et al.*, 1969).

Positive or negative control: Studies on inducible enzyme systems in fungi have shown that positive control predominates (Gross, 1969; Cove, 1970). No operator constitutive mutants have been detected in fungi and this suggests that the positive interaction of a regulatory substance is necessary to initiate gene function. A classical example of positive control in eukaryotes is the function of some hormones which act either directly or indirectly (*via* cAMP) on transcription (see later). Nuclear transplant experiments have demonstrated that modifications in the gene activity of transplanted nuclei most probably occur by positive control through cytoplasmic components of the host cell (Gurdon, 1970; Harris, 1970).

Stability of mRNA: Studies in numerous eukaryote systems have shown that the mRNA has a relatively much longer half-life than in prokaryotes. This has been established by the observations that protein synthesis can occur for long periods in the absence of RNA synthesis. Two classical examples of this are; (1) continued synthesis of globin in mammalian reticulocytes in the absence of a nucleus (Marks and Kovach, 1966); and (2) enzyme induction and

morphological changes in *Acetabularia crenulata* (Hämmerling, 1963; Spencer and Harris, 1964) in an enucleated cell. Such work has implied control of gene expression at post-transcriptional steps.

(3) Post-transcriptional control

Studies on a number of different differentiated systems have demonstrated that there is an actinomycin D sensitive step involved in protein synthesis (presumably due to mRNA synthesis) which may be hours or even days before synthesis of the proteins is first detected. This apparent lack of co-ordination of mRNA synthesis and translation led to the general notion of "translational control" of the expression of a preformed messenger. Examples are; haemoglobin synthesis in the blood islands of chick embryos (Wilt, 1965) and in foetal mouse erythroid cells (de la Chapelle *et al.*, 1969) and γ-crystallin synthesis in lens epithelial cells (Stewart and Papaconstantinou, 1967). The temporal syntheses of mRNA and protein were interpreted as in Fig. 1.2a. However, Gurdon (1971, personal communication[a]) pointed out that mRNA translation may occur simultaneously with mRNA transcription (Fig. 1.2b) but the levels of protein are not detected by the techniques used until some later time. Furthermore, a delay in translation due to transport of the mRNA out of the nucleus and attachment to ribosomes may occur. Thus when protein is first detected, during the exponential synthesis stage, sufficient mRNA has been synthesised to support protein synthesis even in the presence of actinomycin D.

However, examples exist where the delay between observed mRNA and protein synthesis is very long and involve major developmental changes; for example; in *Acetabularia* (Spencer and Harris, 1964); in the slime mould *Dictyostelium discoideum* (Newell *et al.*, 1971); and in pupal development of *Tenebrio molitor* (Ilan *et al.*, 1966). It would still be

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[a] Communicated in Adelaide, May 1971.

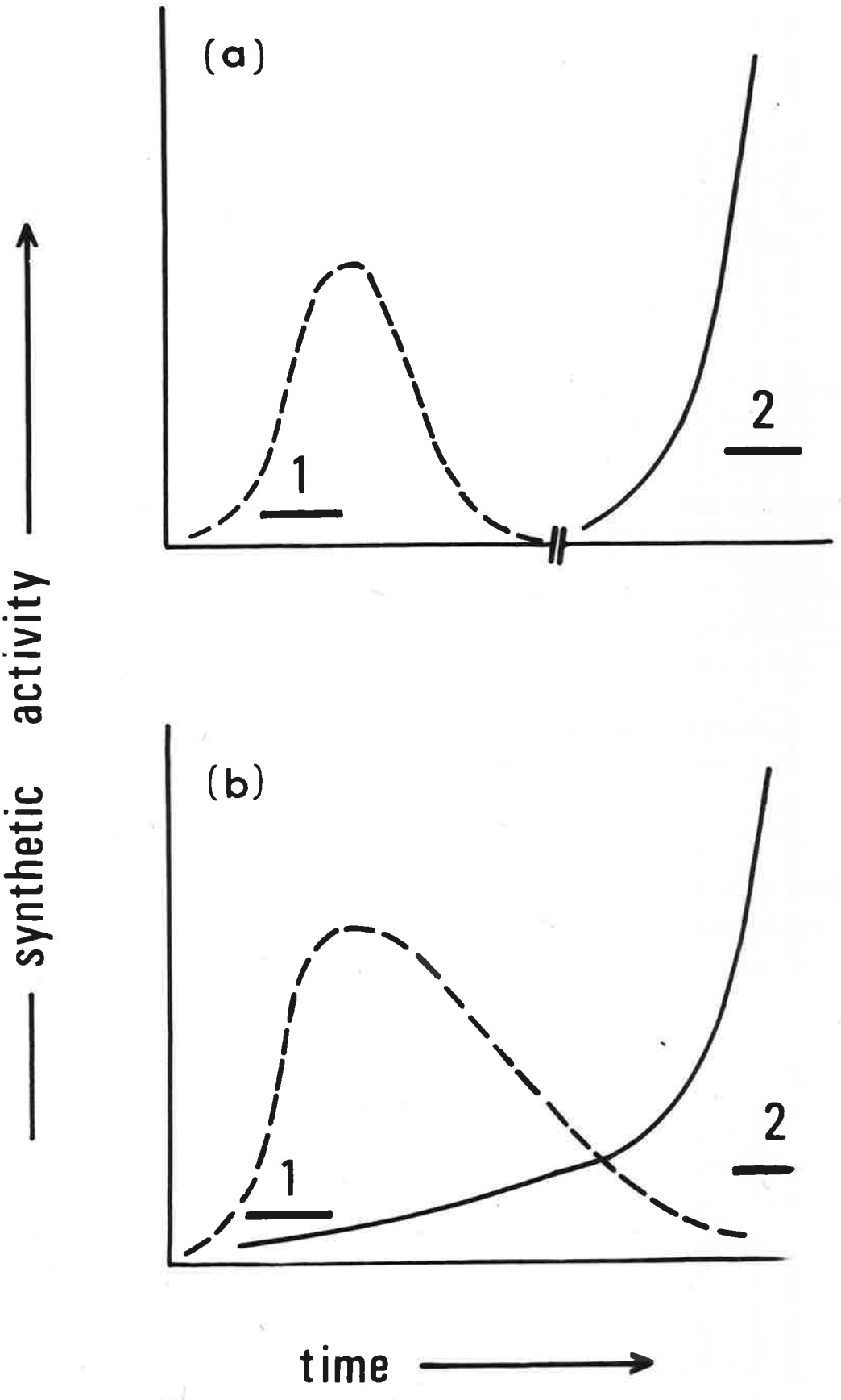
FIGURE 1.2

ALTERNATIVE MODELS FOR THE TEMPORAL SYNTHESES OF mRNA AND PROTEIN

(a) This is a diagrammatic representation on the widely-held view, in which mRNA transcription occurs at some time prior to mRNA translation.

(b) This is a diagrammatic representation of an alternative view expressed by Gurdon, in which the two processes of mRNA transcription and translation may occur simultaneously.

In each case (1) represents an actinomycin D - sensitive stage and (2) represents the stage when the protein is first detected.
----- , mRNA synthesis; ——— , protein synthesis.


(a)
1
2
(b)
1
2
synthetic activity
time

necessary in these systems to postulate the involvement of some mechanism which restricts free messenger translation, even if mRNA synthesis proceeds very slowly. There are a number of sites at which such restriction could occur in these systems (and others) and they will be discussed. Since the term "translational control" has been used loosely in the literature and generally implies control at steps subsequent to transcription, the term "post-transcriptional control" will be used in preference in this thesis.

mRNA synthesis

Incubation of eukaryote cells with radioactive precursors of RNA have demonstrated that most of the rapidly labelled RNA is located within the nucleus. This RNA has a short half-life and most never leaves the nucleus. It is polydisperse in size (hence the term heterogeneous nuclear RNA - HnRNA) and because of its similarity in base composition to that of DNA, it has been postulated to be a precursor of mRNA (Attardi *et al.*, 1966; Shearer and McCarthy, 1967; Scherrer and Macaud, 1968).

From the hybridisation experiments of the type of Scherrer and Macaud (1968), the notion arose that mRNA was edited from the HnRNA before release into the cytoplasm. To test this, Lindberg and Darnell (1970) and Wall and Darnell (1971) used cells transformed by the oncogenic virus SV40 which contain several viral genes in the cellular DNA (Westphal and Dulbecco, 1968). Hybridisation studies were conducted using cellular HnRNA and polyribosomal mRNA with viral DNA and homologous sequences were observed in both. This implies that the HnRNA serves as a precursor to mRNA.

An important precedent for an editing process exists in the processing of a 45*S* RNA precursor molecule to 28*S* and 18*S* rRNA where about 50 % of the molecule is degraded without any known function (Darnell, 1968).

A theory on how the editing process occurs has appeared. Studies on the polyribosomal mRNA species of mouse sarcoma ascites cells

(Lee *et al.*, 1971a) and HeLa cells (Darnell *et al.*, 1971) have shown that they contain polyadenylate (polyA) sequences of up to 200 nucleotides in length. These regions were also present in the HnRNA fraction. Furthermore, hybridisation studies showed that there were homologous sequences between the HeLa mRNA species and HnRNA (Darnell *et al.*, 1971). Edmonds *et al.* (1971) proposed that the polyA regions may have a role in HnRNA editing. They are resistant to ribonuclease (Lee *et al.*, 1971a; Darnell *et al.*, 1971) and could serve as recognition points for specific cleavage; only those regions bounded by or adjacent to polyA segments would be conserved while the remainder of the HnRNA is destroyed. Binding of proteins may also be involved. Sequences of nucleotides that do not code for amino acids in proteins have been described both in bacteriophage mRNA (Steitz, 1969) and the 9*S* globin mRNA (Gaskill and Kabat, 1971) and these may provide sites for protein binding (Henshaw and Loebenstein, 1970).

mRNA packaging

Spirin *et al.* (1965) described heterodisperse ribonucleoprotein (RNP) components in cytoplasmic extracts of loach embryos which were postulated to contain mRNA and were termed "informosomes". These particles were distinct from ribosomal subunits. They have subsequently been found in cytoplasmic extracts from many different eukaryote cell-types (Spirin, 1966 and 1969).

Informosomes have been ascribed several regulatory roles (Spirin, 1969). They may serve as; (1) passive protectors of mRNA against degradation in the cytoplasm; (2) temporary stores of inactive mRNA ("masked mRNA"; Spirin, 1966) which are later "activated" by interaction with specific proteins for translation (for example, after fertilisation of an egg) or "deactivated" for degradation; (3) structural and regulatory components involved in transport of mRNA from the nucleus into the cytoplasm and (or) attachment to ribosomes in the cytoplasm.

Supportive evidence for these theories has been obtained. Perry and Kelley (1968) and Henshaw and Loebenstein (1970) have demonstrated that some HnRNA species rapidly associate with proteins within the nucleoplasm and these RNP particles appear later in the cytoplasm, suggesting a role in transport. In addition, similar RNP particles can be dissociated from polyribosomes when disaggregated either *in vitro* (Perry and Kelley, 1968; Infante and Nemer, 1968; Henshaw, 1968; Henshaw and Loebenstein, 1970; and Lee and Brawerman, 1971) or *in vivo* (Lee *et al.*, 1971b). For example, Lee *et al.* (1971b) showed that heterodisperse RNP particles are released from polyribosomes on amino acid starvation which not only resemble informosomes but also could be re-utilised on addition of amino acids. These results have provided circumstantial evidence that informosomes serve as precursors to functional polyribosomal mRNA and have a possible regulatory role.

Transport of mRNA into the cytoplasm

Proteins may be involved in the transport of mRNA species across the nuclear membrane and the polyA regions may direct the binding of transport proteins (Lee *et al.*, 1971a). The experiments of Harris *et al.* (1969) and Sidebottom and Harris (1969) using hybrid cells have implicated the nucleolus in the transport mechanism.

Availability of ribosomes

The role of single ribosomes in eukaryote cells is unknown but it is known that they do not participate in the ribosomal subunit - polyribosomal cycle (Grubman and Nakada, 1969; Kabat and Rich, 1969). It has been suggested that they may serve as a storage form for later use (Brown, 1967; Hogan and Korner, 1968; Kabat and Rich, 1969, Fan and Penman, 1970). Their inactivity can be reversed by a number of processes such as fertilisation of sea urchin (Vittorelli *et al.*, 1969) and frog (Rinaldi and Monroy, 1969) eggs and hormones (Wool *et al.*, 1968).

Interestingly, Kabat (1970) has demonstrated that several reticulocyte ribosomal proteins can be modified by phosphorylation of serine and threonine residues. In particular, one protein which was present in all ribosomes could be phosphorylated only in single ribosomes. Thus phosphorylation may provide a molecular basis for the inactivity of single ribosomes, and indeed, experimental evidence for this was obtained (Kabat, 1970). This process may be a general phenomenon for regulation of the use of ribosomes.

Initiation of protein synthesis

Preformed mRNA and ribosomes: Development in sea urchin embryos immediately following fertilisation involves the synthesis of proteins for which the mRNA species were transcribed during oogenesis (Gross, 1967). In addition, large numbers of inactive ribosomes are present in the unfertilised oocyte (Brown, 1967). The mechanism by which utilisation of these messengers and ribosomes is restricted has been investigated. Upon fertilisation, there is a rapid increase in the percentage of active ribosomes (Rinaldi and Monroy, 1969). Metafora *et al.* (1971) showed that ribosomes of unfertilised eggs were less efficient in translating artificial messenger (polyuridylic acid) *in vitro* than were those of the fertilised egg. Moreover, they showed that upon fertilisation a protein was removed from the "inefficient" ribosomes which was a powerful inhibitor of polypeptide synthesis *in vitro* . The inference from these results is that, upon fertilisation, a factor is produced which releases the inhibitor and permits translation of the messengers. This factor could be a phosphatase in view of the Kabat (1970) experiments.

Mechanism of initiation: The initiation of protein synthesis in prokaryote-type organisms (70*S* ribosomes) is now well understood. The first step is the binding of N-formylmethionyl-tRNA to a 30*S* ribosomal subunit in response to an initiating codon (AUG) in the mRNA (Nomura and Lowry, 1967). A 50*S*

ribosomal subunit then binds to form a functional ribosome. The N-formyl-methionine initiates the synthesis of each protein by reacting with the next amino acid encoded in the message and is later cleaved to expose the amino terminal amino acid of the protein. This initiation process also involves the participation of certain protein initiation factors (Ochoa, 1968; Iwasaki *et al.*, 1968). In eukaryote organisms (80*S* ribosomes) there is now clear evidence that a major initiating tRNA species is methionyl-tRNA which serves in a manner identical to the initiator in prokaryote-type systems. This has been established in reticulocytes (for examples; Jackson and Hunter, 1970; Yoshida *et al.*, 1970; Wilson and Dintzis, 1970), chick embryo muscle (Heywood, 1970a) and wheat embryo tissue (Marcus *et al.*, 1970). In the case of histone synthesis in liver, N-acetylseryl-tRNA has been described as a possible initiator (Liew *et al.*, 1970).

Initiation factors: Specific regulatory roles have been described for eukaryote initiation protein factors. For example, Heywood (1970b) has shown that the synthesis of globin in a cell-free system shows an absolute requirement for the proteins that can be removed from reticulocyte polyribosomes by washing with buffers of high ionic strength. Globin synthesis could be demonstrated *in vitro* using reticulocyte mRNA and these factors with embryonic chicken muscle polyribosomes. Similar factors tissue-specific to liver have been described (Naora and Kodaira, 1969; Naora and Pritchard, 1971). Other studies have shown that the factors are tissue-specific but not species specific (Lockard and Lingrel,1969 and 1971; Neinhus *et al.*, 1971). These observations suggest that specific initiation factors are required for the translation of different mRNA species. The factors could provide a mechanism for the stabilisation of a differentiated state and limit at any time the amount of a protein that could be synthesised in a cell, regardless of its content of mRNA species (Heywood, 1970b; Naora and Kodiara, 1969).

The experiments of Lane *et al.* (1971) show, however, that when globin mRNA from rabbit reticulocytes is injected into frog oocytes, large amounts of globin are synthesised. This is in direct contrast to the predictions of the above hypothesis and promoted the alternative hypothesis that every mRNA molecule which enters the cytoplasm of a cell is translated. A similar conclusion was reached in the experiments of Mathews *et al.* (1971) using globin mRNA in mouse ascites tumour cells.

No reconciliation of these points is possible at this stage. Oocytes and tumour cells are "less differentiated" that reticulocytes in the sense that they synthesise a wider range of proteins and therefore might be expected to contain initiation factors for a large number of different proteins (Mathews *et al.*, 1971). On the other hand, the translation efficiency of the *in vivo* system of Lane *et al.* (1971) was several hundred times greater than that of the *in vitro* experiments of Heywood (1970b) and thus a low degree of translation of the globin mRNA in the absence of initiation factors *in vitro* might not be detected.

Stage specific initiation factors: Ilan and Ilan (1971) demonstrated the presence of initiation factors on ribosomes from *Tenebrio molitor* which could discriminate between mRNA species obtained from different stages of insect development. This work implies the existence of developmental stage specific initiation factors.

Compartmentalisation

Evidence has arisen that synthesis of certain proteins may occur at specific sites within the cell. Bulova and Burka (1970) showed that globin protein is synthesised on free polyribosomes whereas non-globin proteins are synthesised on membrane-bound polyribosomes. The proteins synthesised on the membrane-bound polyribosomes were identified as membrane proteins (Burka and Bulova, 1971). Similar evidence of "compartmentalisation"

has been found for the synthesis of fibrous proteins; ribosomes appear to be localised at the site of fibril deposition. Examples are; keratin synthesis (Rogers, 1969) and myosin synthesis (Larson *et al.*, 1969). This association of the synthetic machinery with the finished product suggests control at the level of translation as proposed by Cline and Bock (1966).

Rates of translation

tRNA: Models for the regulation of protein synthesis in which a prominent role is ascribed to multiple isoaccepting species of tRNA have been described (Ames and Hartman, 1963; Stent, 1964; Sueoka and Kano-Sueoka, 1964 and 1970). A general prediction of these theories is that any change in the abundance of an individual tRNA or amino acyl-tRNA species is potentially capable of changing the rate of translation of mRNA. Since multiple amino acyl-tRNA synthetases have been described for different amino acids, the possibility exists that the synthetases may also play a role in regulation. Experimental systems in both plants and animals in which such regulation may be involved have been described (Kanabus and Cherry, 1971). Two examples of this control are discussed.

There is a direct linear relationship between the amino acid contents of the major proteins and the levels of the corresponding tRNA species in the systems of crystallin synthesis in lens and fibroin synthesis in silk gland (Garel *et al.*, 1970). In the latter system, the levels of the amino acid synthetases are proportional to the levels of the specific tRNA species (Garel *et al.*, 1970). On secretion of the fibroin proteins, changes in the levels of various amino acyl-tRNA and synthetase isoaccepting species occur (Garel *et al.*, 1971). These changes are postulated to be responsible for the preferential translation of the fibroin mRNA.

Ilan *et al.* (1970) investigated the effects of juvenile hormone on insect pupation in *Tenebrio molitor* and showed that it activated

the appearance of a unique species of tRNA and activating enzyme during metamorphosis. These tRNA species were essential for the translation of the specific mRNA species involved in metamorphosis; tRNA from earlier developmental stages did not support translation.

Termination: Incubation of rat liver microsomes with *c*AMP stimulated the release of polypeptides containing tyrosine aminotransferase (TAT) activity (Oliver, 1971). It was postulated that *c*AMP could act at the termination step of translation to regulate TAT (and possibly other metabolic enzymes) synthesis.

(4) Other factors

Hormones

Hormones are a class of chemical effectors produced by one cell-type of a eukaryote organism which can evoke a physiological response in other cells (Tomkins and Martin, 1970). Hormones may act either with the membrane of a target cell whereupon they initiate a series of chemical events within the cell, or they may be transported into the cell and interact directly with the synthetic apparatus.

Those hormones which function at the cell membrane are thought to do so by activation of the membrane-bound enzyme adenyl cyclase to produce *c*AMP (Robison *et al.*, 1968; Tomkins and Martin, 1970). The intracellular effects of the *c*AMP are responsible for the action of the hormone. Specificity of action of these hormones is exerted through interaction withhormone-specific membrane receptors (Kono, 1969). Precisely how the intracellular concentrations of *c*AMP effect cellular functions is not known but the bacterial precedent may apply (*see* page 4). Also, *c*AMP phosporylates various proteins in several eukaryote tissues (Kuo and Greengard, 1969). Langan (1969) showed that glucagon stimulates the phosphorylation of liver histones, presumably resulting from *c*AMP stimulation

of liver phosphokinases, and postulated that histone phosphorylation permits increased transcription of specific genes.

Post-transcriptional sites of *c*AMP action have been described. An example at the level of termination was observed by Oliver (1971). In addition, Garren *et al.* (1965) showed that synthesis of proteins and not RNA is involved in the ACTH stimulation of adrenal steroidogenesis. Since ACTH response is probably mediated by *c*AMP (Gill and Garren, 1969), the *c*AMP was thought to stimulate protein synthesis at a post-transcriptional level (Tomkins and Martin, 1970). *c*AMP may regulate translation of mRNA by phosphorylation of certain components such as ribosomes and initiation factors.

Other hormones exert their action after entry of the target cells. For example, sex steroid hormones may react initially with specific receptor proteins in the cytoplasm (Bailieu and Jung, 1970) or nucleus (DeSombre *et al.*, 1969) or by a two-step process involving first the cytoplasm and then the nucleus (Fang *et al.*, 1969). Tomkins and Garren (1970) suggest that the primary site of action of these hormones is stimulation of RNA synthesis. Ecdysone-induced chromosomal puffing is an example of this (Clever *et al.*, 1969). Regulation at post-transcriptional levels has also been described. The action of juvenile hormone on insect pupation (Ilan *et al.*, 1970) was described above. Also, studies on inducible enzymes such as TAT in liver have suggested that hormone-sensitive labile repressors regulate mRNA translation (Tomkins *et al.*, 1969).

Growth factors

Many differentiated cell-types appear to have characteristic chemical effectors (hormones) which regulate development in the tissue. For example, erythropoietin is now well established as an inducer of haemoglobin synthesis in mammalian bone marrow. It appears to exert its action at the level of transcription by stimulation of the synthesis of 9*S* globin mRNA

(Gross and Goldwasser, 1969 and 1971). Protein factors from mouse submaxillary gland have been characterised which stimulate nerve (Levi-Montalcini, 1964) and epidermal (Cohen, 1962) growth. Hoober and Cohen (1967) have demonstrated that the epidermal growth factor alters the protein synthesising capacity of polyribosomes from epidermal cells.

(5) Unifying concepts

Differentiation was defined earlier as a process of cellular specialisation in which cells express only a portion of their total genetic potential. That is, differentiation relies on precise expression of certain sets of genes. The onset of cell specialisation must initially be controlled at the level of transcription of DNA into RNA and the most obvious choice for such a control is through hormone action. Once cells have become committed by differentiation, chemical effectors are frequently produced which maintain the cells in the specialised state while development towards the terminal state proceeds.

Eukaryote organisms employ unique processes for regulation of transcription during periods of rapid growth. A striking example of this is the amplification of rRNA genes during development of the oocyte. An abundance of evidence has been summarised which implicates control at various post-transcriptional levels of genetic expression and there can be little doubt that such control is important in differentiation and cytodifferentiation. Despite this wealth of information, it is as yet unwise to evaluate the relative importance of any one of these controls within a system as different cell-types may regulate their development at several control points. Such evaluations must await a more detailed understanding of the molecular mechanisms involved.

C PROTEIN SYNTHESIS IN THE HAIR FOLLICLE

(a) THE KERATINS

The term "keratin" is usually applied to a whole tissue; for examples; hair, horn, quill, feather; or more specifically to the fibrous structural proteins synthesised and retained in the cells (keratinocytes) of these tissues (Mercer, 1961). The keratins are a heterogeneous group of proteins which are characteristically high in cystine content and are insoluble in the normal solvents for globular proteins. This insolubility is attributable largely to the extensive cross-linking of their constituent protein chains by disulphide bonds. There are major differences between the keratins of different tissues both in ultrastructure and complexity.

In this section the major developmental features of only hair (and wool) keratins will be considered. These have been classed as the α-keratins, on the basis of the characteristic X-ray diffraction patterns the keratinised structure (Mercer *et al.*, 1964).

(b) THE NATURE OF THE KERATIN PROTEINS OF THE HAIR AND HAIR FOLLICLE

(1) Hair Keratin

Hairs are columns of dead epidermal cells which develop from tubes of invaginated epidermis, the hair follicles, which protrude into the underlying dermis (Mercer, 1961). Although hairs and hair follicles vary in size between animals and between different regions of the same animal, their basic structure is the same (Montagna and Van Scott, 1958).

(2) Morphology of the hair follicle

This has been described in detail on several occassions (for examples; Auber, 1952; Mercer, 1961; Rogers, 1964; Epstein and Maibach, 1969)[a].

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[a] All unreferenced information in this section was taken from these reviews.

The hair follicle develops embryonically by a downward growth of the basal layer of the epidermis. These cells envelop the dermis to form a cap, the future hair bulb, and the mesodermal cells within form the follicle papilla. The cells around the papilla and at the base of the follicle bulb undergo mitosis and force the column of epidermal cells above them laterally where they form an outer root sheath. The rising column of cells comprises the developing hair follicle.

In a diagrammatic representation of a hair follicle shown in Fig. 1.3, the processes of follicle development are divided into three regions.

The bulb (level 1)

About the base and the papilla of the follicle, where mitosis occurs, the cells are undifferentiated, spherical in shape and have a large nuclear volume in comparison to the total cell volume. This is characteristic of rapidly dividing cells. Within a few cell layers differentiation into several discrete cell layers occurs. Ribosomes and polyribosomes are common in the cells and mitochondria are small but numerous. Endoplasmic reticulum is not present but there is a well-defined Golgi region, which is a typical feature of cells that synthesise and deposit their proteins intracellularly (Birbeck and Mercer, 1961). Desmosomes are present on adjacent cell membranes and appear to act as points of attachment between neighbouring cells.

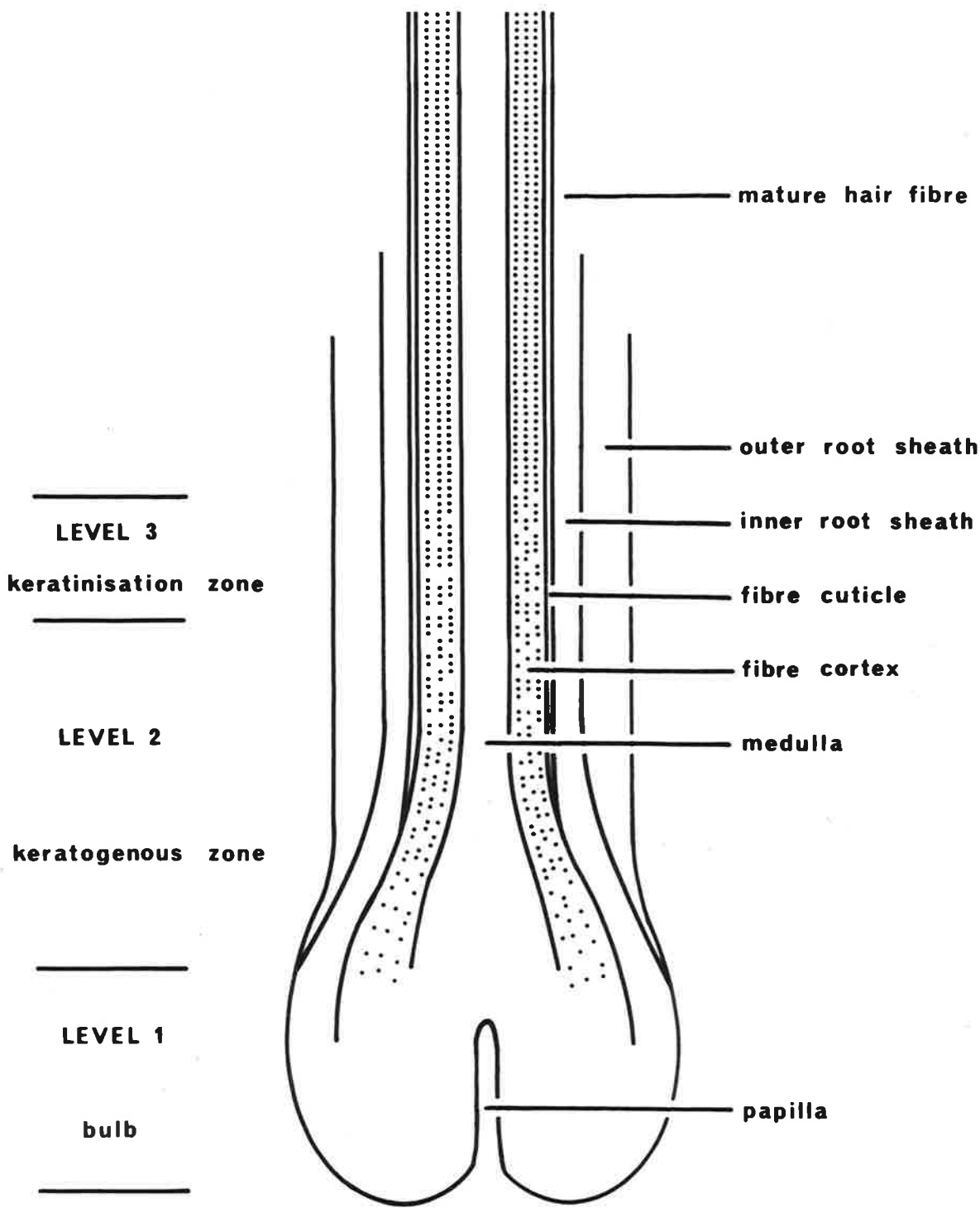
Keratogenous zone (level 2)

Above this point of differentiation each cell-type can be readily characterised by its content of distinctive proteins and as well by variations in cell morphology. The follicle comprises a number of concentric sheaths of cells which are, from the outside proceeding inwards, the inner root sheath, the hair fibre cuticle, the cortex and the central column of cells, the medulla. The inner root sheath is further subdivided

FIGURE 1.3

THE HAIR FOLLICLE

Diagrammatic representation of a hair follicle showing the major features considered in the text. The approximate positions of the three developmental levels described are shown.


mature hair fibre
outer root sheath
inner root sheath
fibre cuticle
fibre cortex
medulla
papilla
LEVEL 3
keratinisation zone
LEVEL 2
keratogenous zone
LEVEL 1
bulb

into three uni-cellular layers, the Henle layer, the Huxley layer and cuticle, which is adjacent to the hair fibre cuticle.

The inner root sheath cells produce distinct electron-dense granules termed trichohyalin droplets, the amorphous proteins of which appear to become closely associated with filaments of protein. As these cells move to higher levels of the follicle the amount of filamentous material increases and the trichohyalin droplets disappear. The medulla, which is present in many larger hairs and quills, develops from the cells of the bulb above and around the papilla. The cytoplasm of these cells also develops trichohyalin droplets but they do not become associated with fibrous material, but eventually disperse into a quasi-fibrous form. The neighbouring cell layers of the fibre cuticle and fibre cortex each produce a specific type of keratin. The cells of the presumptive cortex fill with microfibrils which aggregate into macrofibrils by continual lateral addition of microfibrils. At a somewhat higher level, densely-staining matrix proteins gradually become prominent between the microfibrils and the macrofibrils assume an appearance similar to that of mature keratin. At about the same level at which matrix protein first appears in the presumptive cortical cells, dense droplets of protein appear in the cytoplasm of the fibre cuticle cells, which appear to migrate to the outermost edges of each cell to form a consolidated layer.

Major morphological changes in the cells occur at this stage. The process of fibrillation within the inner root sheath cells is more advanced than that in the cortical cells. The role of the inner root sheath appears to be a mechanical one in that it leads to shaping of the hair by acting as a solid retaining sheath (Straile, 1965). The cortical cells begin to elongate in the direction of fibre growth and the macrofibrils within these cells also become well-aligned in the direction of fibre growth. The hair fibre cuticle cells are flattened by the shearing forces set up in

this way. Microtubules within the cortical cells are thought to contribute to these structural changes (Rogers, 1970, unpublished observations).

As these developmental processes occur, resorption of cell nuclei and mitochondria begins. The characteristic proteins and ribosomes predominate in the cytoplasm. The ribosomes frequently appear in the form of clusters closely associated with the deposited proteins.

Keratinisation zone (level 3)

At the higher regions of the follicle the cells of all layers are filled with their characteristic protein end-products. The remnants of nuclei and ribosomes are restricted to only a small volume of the cells. The intercellular δ layer which is probably concerned with adhesion becomes prominent at this stage. Dehydration of the cells, oxidation of the cysteine-rich proteins and cell death finally occur.

Thus the life of all follicle cells as they gradually move along the follicle is programmed toward the synthesis of specific proteins to the exclusion of all other cell components, leading to eventual cell death.

Further considerations of this thesis will be concerned principally with the developmental processes in the cell-types that comprise the hair fibre.

(3) Ultrastructure of hair keratin

The notion has arisen that α-keratin consists of a complex of microfibrils of 70 - 80 Å in diameter embedded in an amorphous matrix. This is based on X-ray diffraction studies (Dobb *et al.*, 1965) and electron microscopy on developing (Birbeck and Mercer, 1957) and mature keratins (Rogers, 1959a; Filshie and Rogers, 1961; Rogers and Filshie, 1962). An ordered substructure of protofibrils within the microfibrils was also postulated (Filshie and Rogers, 1961; Fraser *et al.*, 1962 and 1967; Dobb

and Rogers, 1967). These protofibrils were thought to consist of "coiled-coils" containing two or three polypeptide chains in an α-helical form. In contrast, there appears to be little or no structural order in the matrix. The affinity for heavy metal stains suggested that the constituent proteins are rich in cystine and are poorly-oriented (Rogers and Filshie, 1962). Thus, microfibrils appear to be responsible for the characteristic X-ray diffraction pattern. The matrix probably consists of relatively - disorganised protein chains that interconnect adjacent microfibrils and stabilise the structure.

(4) The nature of the keratin proteins

Isolation of proteins by reduction

Methods of solubilisation of the keratin proteins have depended upon rupture of the disulphide bonds either by oxidation or by reduction in the presence of denaturing reagents such as urea (see Crewther *et al.*, 1965). The favoured method is by reduction which is followed by conversion of the sulphydryl groups on the proteins to more stable derivatives. This is usually done by treatment with iodoacetic acid which converts the cysteine residues to S-carboxymethyl- (SCM) cysteine residues, although other reagents have been used (Frater, 1966). The term SCM-kerateine (SCMK) is used for the proteins solubilised and derivatised in this manner. Early studies showed that these proteins could be fractionated into two groups, one lower in SCMcysteine content than the total proteins (the low sulphur kerateine, or LoS, proteins) and the other of higher SCMcysteine content than the total proteins (the high sulphur kerateine, or HiS, proteins). These LoS and HiS protein fractions were also termed the SCMK-A and SCMK-B fractions, respectively.

Properties of the LoS proteins

The wool LoS proteins have been studied by Thompson and

O'Donnell (1964, 1965 and 1967), O'Donnell and Thompson (1964 and 1968), O'Donnell (1969) and Frater (1966). They constitute about 60 % of the total proteins and appear as two main components when separated by electrophoresis (Thompson and O'Donnell, 1964). These can be separated by chromatography (O'Donnell and Thompson, 1964; Thompson and O'Donnell, 1965) and have been termed components 7 and 8 (Thompson and O'Donnell, 1965). The molecular weights are about 45 000 daltons (Thompson and O'Donnell, 1965) and evidence which suggested that the molecular weights might be half this value (DeDeurwaerder and Harrap, 1964; Jeffrey, 1968) now appears to be incorrect (Jeffrey, 1969). The helical content of both components is near 50 % which strongly suggests that they arise from the microfibrillar moeity of the original fibre. Component 8 was shown to be microheterogeneous on the bases of amino-terminal sequence analyses (O'Donnell and Thompson, 1968; O'Donnell, 1969), peptide mapping (Thompson and O'Donnell, 1965), quantitative estimations of the yields of fragments produced by cyanogen bromide cleavage (Thompson and O'Donnell, 1967; O'Donnell, 1969) and immunological studies (Frater, 1968 and 1969). It was concluded from this data that components 7 and 8 consisted of families of closely-related proteins.

Studies on their physical structures showed that there were regions of high helical content along the proteins and non-helical "tails". These "tails" could be removed by partial enzyme digestion (Crewther and Harrap, 1967) and were thought to be responsible for the propensity of the LoS proteins for aggregation *in vitro* and involved in the organisation of the microfibril-matrix structure *in vivo*.

Properties of the HiS proteins

The wool HiS proteins have been studied by Gillespie (1963, 1965 and 1967). They constitute about 30 % of the total wool proteins and are very heterogeneous with respect to both molecular size and charge.

The molecular weights vary within the range of 10 - 28 000 daltons, and interestingly, there is a linear relationship between increases in molecular weight and SCMcysteine content of different proteins (Gillespie, 1963). The HiS proteins do not have any detectable ordered structure in solution and this provides firm evidence that they constitute the matrix moiety of the wool fibre. This low structural order is presumably due to their high contents of the amino acids serine, threonine, SCMcysteine and proline which inhibit the formation of helical structures (Gillespie and Harrap, 1963).

Attempts to fractionate the wool HiS proteins for sequence analyses using various chromatographic procedures (Gillespie, 1965; Joubert *et al.*, 1967) have shown that the HiS proteins exist as a large number of families of proteins of similar size and charge. More recent sequence analyses of purified homogeneous proteins (Gillespie *et al.*, 1968; Haylett and Lindley, 1968; Lindley *et al.*, 1971; Haylett and Swart, 1969; and Haylett *et al.*, 1971) have documented curious features such as repeated sequences and frequent di- and tri-peptide repeats of the same amino acid.

Other proteins

A protein rich in glycine, tyrosine, phenylalanine and tryptophan can be extracted from wool and appears to be derived from the proteins of the cuticle cells (Harrap and Gillespie, 1963). Gillespie (1971) has suggested that these proteins might also originate in part from the cortical cells.

(5) Prekeratin

The term prekeratin has been used widely to describe the protein complex in developing keratinocytes at stages before it becomes insoluble. Rudall (1968) described this term as representing a state of development of the proteins and probably not a unique class of proteins.

However, a number of studies in both wool and hair follicle tissue have not yet convincingly established this point.

The first studies were those of Rogers (1959b) on wool roots. Proteins could be extracted with urea buffers and resolved into LoS and HiS protein fractions. Rogers and Clarke (1965) and Clarke and Rogers (1970a) extended these studies to guinea pig hair follicles and showed that similar proteins could be extracted with aqueous buffers in the absence of urea. Other more detailed studies were made in wool follicle tissue. Downes *et al.* (1966a), Frater (1966) and Fraser (1969) compared a number of properties of the follicle and wool proteins and found several similarities and several important differences. The SCMcysteine content of the HiS proteins and the HiS content of the follicle proteins were less than the contents of the wool proteins. In addition, it appeared that some components present in the follicle proteins were not present in the wool proteins and *vice versa*.

(6) Other proteins of the hair follicle cells

The medulla and inner root sheath cells of the hair follicle contain trichohyalin droplets. In the case of the inner root sheath, these amorphous masses are believed to be precursors of the inner root sheath filaments (Birbeck and Mercer, 1957; Rogers, 1959b, 1963 and 1964) although alternative views have been expressed (Charles, 1959; Parakkal and Matoltsy, 1964; Parakkal, 1969). The mature inner root sheath and medulla proteins contain large amounts of protein-bound citrulline (Steinert *et al.*, 1969) which is believed to arise from an arginine-rich precursor protein by desimidation of some of the arginine residues (Rogers, 1959b, 1963 and 1964). Whether in fact the trichohyalin droplets comprise this arginine-rich precursor is not yet clear.

The mature medulla and inner root sheath proteins contain ε-(γ-glutamyl)lysine cross-links and these are thought to be responsible for

the insolubility of these proteins (Harding and Rogers, 1971). Partial sequence studies on peptides from the medulla protein have shown frequent repeats of single amino acids such as citrulline and glutamic acid (Steinert *et al.*, 1969). The mature inner root sheath cells contain protein filaments about 70 - 80 Å in diameter which contain citrulline (Steinert *et al.*, 1971).

(c) BIOSYNTHESIS OF THE KERATINS OF THE HAIR FOLLICLE

(1) Control and onset of keratin synthesis

Mitosis and DNA synthesis

Mitosis occurs only in the basal layers of the follicle (Short *et al.*, 1965; Fraser, 1965; Epstein and Maibach, 1969).

Autoradiographic studies using {^{3}H}thymidine have shown that DNA synthesis is also restricted to the basal cell layers of the rat hair (Epstein and Maibach, 1969) and wool (Downes *et al.*, 1966b) follicle. Rogers and Kemp (1970, unpublished studies) using a similar technique have demonstrated that keratin fibrils are not present in hair follicle cells that rapidly incorporate {^{3}H}thymidine. It would therefore appear that keratin synthesis does not begin until the cells have lost the ability to synthesise DNA and divide.

RNA synthesis

Sims (1967) investigated RNA synthesis in rat hair follicles by autoradiography after labelling with {^{3}H}cytidine. Within 3 h all label was associated with the nucleus, up to the low regions of the keratogenous zone of the follicle, although the basal cells were the most highly labelled. However, by about 12 h, most label was localised within the cytoplasm and by about one day, no radioactivity could be detected in the nucleus. By this time, and at 3 days, radioactivity was also present in level 3 of the follicle. The low region of the keratogenous zone at which radioactivity could no longer

be detected at the shortest times of incorporation presumably indicated where the cells had lost the ability to synthesise RNA. Interestingly, cells above the low regions of the keratogenous zone had also lost their nucleoli. Similar observations using {^{3}H}uridine have been made by Rogers (1970, unpublished studies).

In both studies, most of the radioactivity in the cytoplasm was associated with the developing keratin fibrils of the cortical cells and this has been used as evidence that protein synthesis occurs at the surface of the microfibrils. Although the nature of the RNA species labelled in these experiments was unknown, it is likely that they were rRNA and mRNA involved in keratin synthesis.

Wilkinson (1970a) investigated the species of RNA that could be labelled *in vivo* in wool follicles using {^{3}H}uridine. After a short pulse, radioactivity was present in high molecular weight species greater than 28*S* which were probably rRNA precursors and HnRNA. Within one hour, most radioactivity was detected in the 28*S* and 18*S* rRNA species. No radioactive mRNA species were detected. In addition, Wilkinson (1970b) investigated the distribution of radioactivity in polyribosomes after various times of *in vivo* incorporation and showed that radioactivity was present in rRNA only.

Stability of mRNA

Wilkinson (1970b) showed that actinomycin D did not affect the polyribosome profile from wool follicle tissue for times up to 4 h which implied that protein synthesis could continue in the absence of mRNA synthesis for at least 4 h. Since no labelled mRNA could be detected in polyribosomes after an *in vivo* incorporation it is likely that most of the mRNA must have been synthesised at a very early stage in the keratogenous zone. The studies of Sims (1967) and Rogers support this conclusion.

Pathway of cytodifferentiation

These observations on DNA and RNA synthesis suggest a general scheme of cytodifferentiation in the hair follicle as shown in Fig. 1.4 (from Fraser *et al.*, 1972). After a critical mitotic step, the ability of the basal cells to synthesise DNA and divide ceases. The daughter cells differentiate and the synthesis of the specific proteins begins. Also, some factor(s) instructs different cells to develop into the different cell-types of the hair follicle. Synthesis of mRNA presumably occurs very early, followed by synthesis of the specific proteins either immediately or after a short delay. Later, synthesis of RNA and other general cellular proteins ceases and degradation of the nucleus occurs, but synthesis of the specific proteins continues for a longer time. Eventually, as synthesis dwindles, stabilisation of the keratin proteins begins and cell death finally takes place.

(2) Other factors affecting development in the hair follicle

Hormones

The dependence of hair and wool growth on adrenal, gonadal, thyroid and pituitary levels have been investigated by Rouget (1965), Houssay *et al.* (1965), Ferguson *et al.* (1965) and Downes and Wallace (1965). The stimulatory effects of throxine on normal wool growth after thyroidectomy and the suppression of wool growth by administration of adrenal corticosteroids suggest that hormones play an important role (Rogers, 1969). However, no definitive studies describing the molecular mechanism or sites of action of these hormones have been done.

Nutritional status

The synthesis and formation of wool keratin can be varied according to the nutritional status of the animal. When the diet of a sheep is supplemented with either cystine or methionine or proteins or compounds rich in

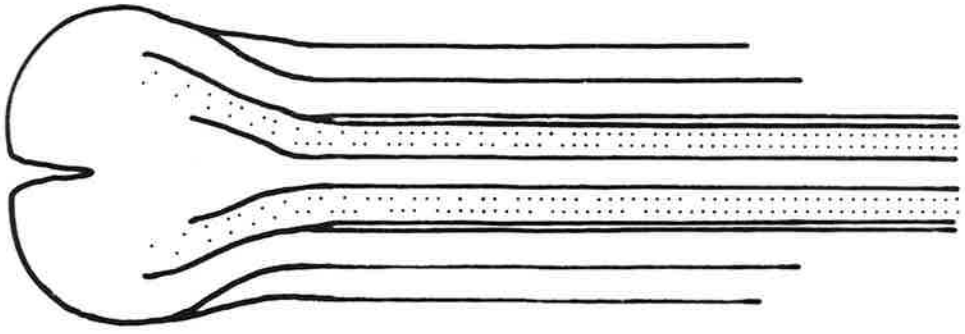
FIGURE 1.4

PATHWAY OF CYTODIFFERENTIATION IN THE HAIR FOLLICLE

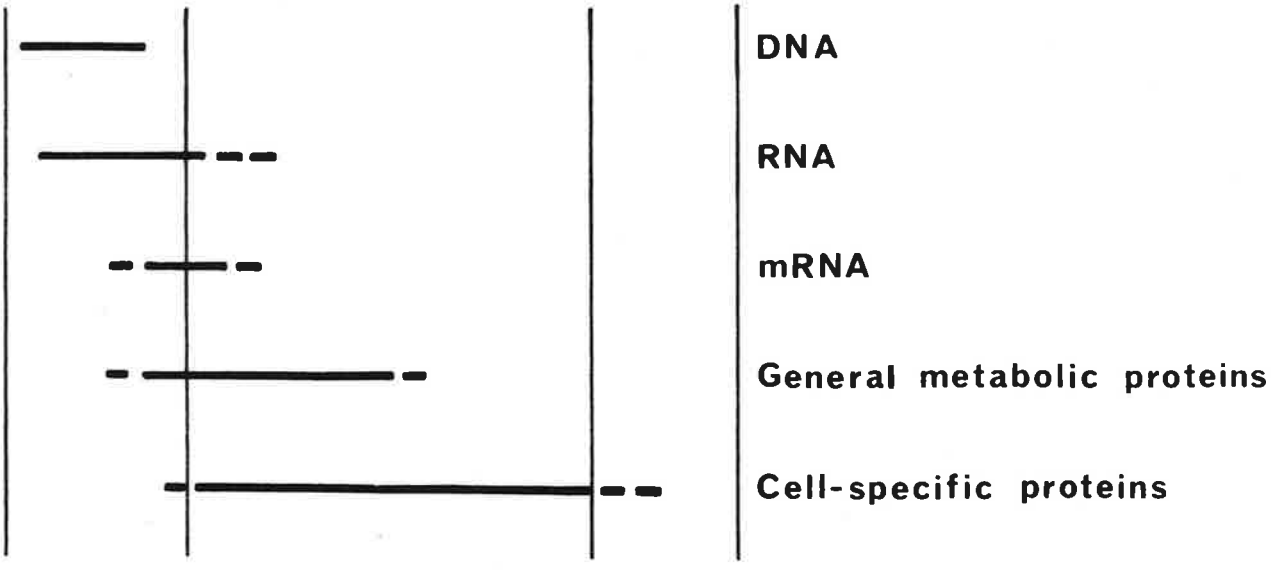
The approximate stages of follicle development at which syntheses of the various macromolecules are thought to occur are represented by the lines (from Fraser *et al.*, 1972).

Level

| 1 | 2 | 3 |



Synthesis of



these amino acids, the rate of wool growth and the cystine content of the wool is markedly increased (Reis and Schinckel, 1963; Downes *et al.*, 1970). Analysis of the keratin produced by such sheep revealed that there is an increase in the relative amount of matrix (HiS) proteins (Reis, 1965; Gillespie, 1965). Furthermore, these HiS proteins have a higher SCMcysteine content than normal which is due to the appearance of a new class of proteins which have the highest SCMcysteine contents of all the HiS proteins (and were termed the "ultra-HiS" proteins) (Reis, 1965; Gillespie and Reis, 1966; Gillespie *et al.*, 1969), On the other hand, when sheep are maintained on a very low but not lethal diet of protein or cystine, the rate of wool growth declines and a "steely" wool is produced. This wool contains little matrix protein and the HiS proteins have a lower SCMcysteine content (Gillespie, 1965). Thus sulphur-containing amino acids or sources of them can influence the follicle protein synthesis machinery in some marked way.

(3) Kinetics of synthesis of the keratin proteins

The ultrastructural evidence presented earlier indicated that the microfibrils appear in the follicle cortex before the matrix. Autoradiographical studies were undertaken to investigate this further. Bern *et al.* (1957) showed that {^{35}S}cystine incorporates rapidly into the basal follicle cells but at later times, radioactivity could be detected throughout the keratogenous zone. Similar observations were made by Downes *et al.* (1962) using {^{35}S}cystine, Sims (1964) using {^{3}H}tyrosine and Rogers and Kemp (1970, unpublished studies) using several different radioactive amino acids. These findings were initially explained by correlating the location of the radioactivity with the appearance of the matrix proteins. The notion arose that keratin proteins were synthesised in two stages; the microfibrillar (LoS) proteins in the lower regions and the matrix (HiS) proteins in the higher regions of the follicle (Mercer,

1961; Rogers, 1964). This hypothesis was tested (Downes *et al.*, 1963) by measurement of the specific activities of SCMcysteine in the two groups of prekeratin proteins after administration of {^{35}S}cystine into sheep. The specific activity was higher in the HiS proteins which conformed with the two-stage hypothesis. This work was repeated (Downes *et al.*, 1966a) and confirmed.

Recently, a more definitive approach to this problem was adopted by Fraser (1969) in which the proteins of three levels comprising the entire keratogenous zone of the wool follicle were extracted and characterised. His results suggested a "dual-stage" synthesis mechanism in which simultaneous synthesis of both LoS and HiS proteins occurred throughout the follicle. Deposition of the LoS proteins appeared to be approximately constant throughout the follicle but the HiS proteins were deposited in an "exponential" manner such that about half were synthesised in the upper third (near the zone of keratinisation) portion of the follicle.

The notion of a dual-stage mechanism is consistent with the electron microscope studies on wool and hair follicles which showed that keratinising cells at similar levels in the follicle are frequently at markedly different stages of development. However, cells low in the keratogenous zone of the follicles do not appear to have any detectable matrix associated with the microfibrils. This means that there must still be a stage in the development of the hair follicle cells at which synthesis of the HiS proteins begins, which is subsequent to the initiation of LoS protein synthesis.

(4) Cell-free protein synthesis

Attempts to establish cell-free protein synthesis systems have been made. Initial procedures for the isolation of guinea pig hair follicle polyribosomes met with limited success (Rogers and Clarke, 1965;

Freedberg *et al.*, 1967; Clarke and Rogers, 1970b; Freedberg, 1970) since the yields of polyribosomes were low. Furthermore, the ability of these polyribosomes to incorporate amino acids into high molecular weight material was poor. These problems were attributed to the presence of an active ribonuclease in the tissue homogenates. More recently, Wilkinson (1970b) used better isolation procedures and obtained larger polyribosomes in high yields from wool follicles.

D MAJOR QUESTIONS OF DIFFERENTIATION IN HAIR FOLLICLE TISSUE: AIMS OF THIS THESIS

(a) NATURE OF THE PREKERATIN PROTEINS

It is important to determine whether there are any significant differences between the proteins isolated from the follicle and keratinised fibre, as it would indicate whether any post-synthetic modifications to the proteins occur before or during keratinisation.

The properties of the prekeratin proteins from wool follicle tissue were described before (*see* pages 24 and 25) and it was noted that several differences existed. Since both the SCMK-A and SCMK-B protein fractions of this tissue contained many protein species, the differences noted could be due to the presence of different amounts of the various proteins within each fraction. The lower SCMcysteine content of the wool follicle SCMK-B proteins could be due to the presence of proteins that are not typically high in SCMcysteine content. Alternatively, there may be major differences in the properties of the follicle and wool HiS proteins. Therefore, it is necessary to define what is a HiS protein. The same argument also applies with the LoS proteins.

(b) MECHANISM OF SYNTHESIS OF THE KERATIN PROTEINS

There is no known exception to the general rule that proteins

are synthesised by mechanisms involving ribosomes and mRNA, apart from certain peptide antibiotics (Perlman and Bodzansky, 1971). However, certain developmental questions in the wool follicle have prompted the suggestion that some proteins may be synthesised by non-ribosomal-dependent mechanisms. These concerned; (1) the mechanism by which the cystine content of the HiS proteins changes on dietary variations; (2) the reason why follicle HiS proteins contained lower levels of SCMcysteine than wool HiS proteins; and (3) the origin of the heterogeneity of the proteins.

Gillespie (1965) postulated that precursor "HiS" proteins of lower cysteine content might exist to which are added sequentially cysteine and other amino acid residues either as free amino acids or as short peptides rich in these amino acids. This random addition mechanism could occur in terminal stages of development during dehydration and cell death. In dietary situations where cysteine was abundant more extensive addition of cysteine would be expected to take place and *vice versa*. This model could accomodate each of the questions listed above.

On the basis of more detailed characterisation of the HiS proteins produced during maintainence of sheep on high cysteine diets, Gillespie (1967) and Broad *et al*. (1970) proposed the alternative mechanism that the follicle cells have the potential to synthesise new groups of HiS proteins in cellular conditions of high concentrations of cysteine. The inference of this conclusion is that the intracellular cysteine levels can act either directly or indirectly on the protein synthesis machinery to synthesise new classes of HiS proteins. Thus the possibility exists that the genome contains information for all the various types of HiS and LoS proteins and that certain intra- or extracellular functions (including cysteine) can determine the relative rates of expression of these genes. It is also possible that both the non-ribosomal-dependent mechanisms and

the classical ribosomal-dependent mechanism may operate simultaneously in protein synthesis in the hair follicle.

(c) EXPERIMENTAL APPROACHES TO THESE QUESTIONS

In order to resolve the theories mentioned above, several experimental approaches are possible. Firstly, it would be necessary to determine whether the follicle keratin proteins are in fact the same as or different to the hair fibre keratin proteins. Included in this is the question: are the "ultra-HiS" proteins that are produced on dietary supplements of cysteine also present in small amounts in normal wool? Secondly, the establishment of cell-free protein synthesis systems that can direct the synthesis of the keratin proteins would determine whether the proteins are made by a ribosomal or non-ribosomal-dependent mechansim, or a combination of both. Thirdly, characterisation of the RNA species could show if mRNA species exist for entire keratin protein molecules and if they are heterogeneous. The translation of these mRNA species in homologous and heterologous cell-free systems would establish these points firmly. Furthermore, these cell-free protein synthesis systems have the attractive potential of providing answers to several key control mechanisms that might operate at post-transcriptional levels in the follicle. One important aspect that could be investigated for example, is the question of the temporal sequence of synthesis of the LoS and HiS proteins.

It is assumed in this thesis that the developmental features that have been described for the wool follicle system are also applicable to the guinea pig hair follicle system. While some aspects of the wool follicle system such as dietary regulation of HiS protein synthesis have not been described for the guinea pig system, the experimental approaches foreshadowed should enable clarification of the questions discussed.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

A MATERIALS

(a) ENZYMES AND PROTEINS

Albumin: bovine serum (fraction V); Sigma Chemical Co., St. Louis, U.S.A.

Carboxypeptidase A: hog pancreas, crystallised; Sigma Chemical Co.

Carboxypeptidase B: hog pancreas, in 0.1 M NaCl solution; Sigma Chemical Co.

α-Chymotrypsin: bovine pancreas, 3-times crystallised; Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chymotrypsinogen A: bovine pancreas, 3-times crystallised; Worthington Biochemical Corp.

Deoxyribonuclease: bovine pancreas, ribonuclease-free; Sigma Chemical Co.

Insulin: crystalline beef; Commonwealth Serum Laboratories, Melbourne, Australia.

Lysozyme: egg white, 3-times crystallised; Sigma Chemical Co.

Mercuripapain: papaya latex, crystallised, in 70 % ethanol suspension; Sigma Chemical Co.

Myoglobin: sperm whale; Pierce Chemical Co., Rockford, N.Y., U.S.A.

Ovalbumin: egg, 2-times crystallised, grade V; Sigma Chemical Co.

Pepsin: hog stomach, 3-times crystallised; Sigma Chemical Co.

Pronase: B grade; Calbiochem, Los Angeles, Calif., U.S.A.

Pyruvate kinase: rabbit skeletal, type II-A, in 2.1 M $(NH_4)_2SO_4$; Sigma Chemical Co.

Ribonuclease A: bovine pancreas, type II-A; Sigma Chemical Co.

Trypsin: bovine pancreas, 2-times crystallised, type III; Sigma Chemical Co.

Trypsin: minimal chymotrypsin content, Mann Research Laboratories, New York, U.S.A.

Trypsin: bovine pancreas, trypsin-TPCK: Worthington Biochemical Corp.

(b) RADIOACTIVE COMPOUNDS

{5-^{3}H}uridine: specific activity, 24.9 C/mmole: Radiochemical Centre, Amersham, England.

$^{32}PO_4^{3-}$: carrier-free; Australian Atomic Energy Commission, Lucas Heights,

Australia.

L-{U-^{14}C}phenylalanine: specific activity, 455 C/mole; Schwarz BioResearch, New York, U.S.A.

L-{2-^{3}H}alanine: specific activity, 52.0 C/mmole; Schwarz BioResearch.

L-{4,5-^{3}H}leucine: specific activities, 58.1 C/mmole, 53.9 C/mmole and 2.0 C/mmole; Schwarz BioResearch.

L-{2-^{3}H}serine: specific activity, 3.0 C/mmole; Schwarz BioResearch.

^{3}H-labelled reconstituted algal hydrolysate; Schwarz BioResearch.

(c) FINE CHEMICALS FOR SPECIFIC PROCEDURES

(1) Measurement of radioactivity

2,5-diphenyloxazole: scintillation grade; Packard Instruments Co. Inc., La Grange, U.S.A.

1,4-*bis*-(5-phenyloxazolyl)-benzene: as above.

Ethylene glycol: extra-pure; B.D.H. Ltd., Poole, England.

Dioxan: analytical grade; Merck, Darmstadt, Western Germany.

Methanol: AR grade; May and Baker Ltd., Dagenham, England.

Naphthalene: micro-analytical reagent; B.D.H. Ltd.

Toluene: reagente puro; Carlo Erba, Milan, Italy.

(2) Polyacrylamide gel electrophoresis

Acrylamide: Fluka, Buchs, Switzerland.

N,N'-methylenebisacrylamide: Eastman Organic Chemicals, New York, U.S.A.

N,N,N',N'-tetramethylethylenediamine: Eastman Organic Chemicals.

Riboflavin: B.D.H. Ltd.

Ammonium persulphate: analytical reagent; Univar, Sydney, Australia.

Coomassie Brilliant Blue: Mann Research Laboratories.

(3) Column chromatography

Urea: reagente puro; Carlo Erba; and AnalaR, B.D.H. Ltd.

Sephadex: grades G-200, G-100, G-75, G-50 and G-25; Pharmacia, Uppsala, Sweden.

DEAE-cellulose: Whatman DE-50; W. and R. Balston Ltd., England.

Dowex ion-exchange resins: grades 50W-X2 (100-200 mesh); 50W-X8 (20-30 mesh); 1W-X8 (*minus* 400 mesh); and 2W-X8 (100-200 mesh); Calbiochem.

(4) Antibiotics

Chloramphenicol: Sigma Chemical Co.

Cycloheximide: Sigma Chemical Co.

Puromycin dihydrochloride: grade II; Sigma Chemical Co.

(d) MISCELLANEOUS FINE CHEMICALS

Amino acids: Mann Research Laboratoris.

Cyanogen Bromide: AnalaR: B.D.H. Ltd.

Dansyl-chloride[a]: B.D.H. Ltd.

Dansyl-amino acids[a]: Mann Research Laboratories

disodium ATP: grade II; Sigma Chemical Co.

disodium GTP: grade II; Sigma Chemical Co.

Dithiothreitol: Sigma Chemical Co.

Glycerol: AnalaR; B.D.H. Ltd.

Ninhydrin: Pierce Chemicals Co.

Polyuridylic acid: high molecular weight; Sigma Chemical Co.

Pyrrolidine carboxylic acid: Aldrich Chemical Co., Milwaukee, U.S.A.

Sodium fluoride: AnalaR; B.D.H. Ltd.

Sucrose: ribonuclease- and metal-ion free; Schwarz BioResearch.

Trizma base: reagent grade; Sigma Chemical Co.

All other reagents used were of the highest possible standard of purity.

(e) PREPARED CHEMICALS

Phosphoenolpyruvate was prepared as its dipotassium salt (Clark and Kirby, 1966).

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[a] "Dansyl-" is an abbreviation for 1-dimethylaminonaphthalene-5-sulphonyl-

Diallyltartardiamide was prepared with diethyltartrate and allylamine (Anker, 1970).

N-acetylalanine and N-acetylserine were prepared by the procedure of Akabori *et al.* (1959).

Iodoacetic acid (Sigma Chemical Co. and B.D.H. Ltd.) was recrystallised from boiling petroleum ether before use.

2-mercaptoethanol (Sigma Chemical Co.) and N-ethylmorpholine (Eastman Organic Chemicals) were distilled under reduced pressure before use.

(f) MISCELLANEOUS MATERIALS

Cellophane dialysis tubing: Visking; B.D.H. Ltd.

Chromatography paper: Whatman 3MM; W. and R. Balston Ltd.

Collodion membrane filters: Sartorius-Membranfilter GmbH, Göttingen, Germany.

Glass fibre circles: Whatman GF/C; W. and R. Balston Ltd.

Polyamide layers: Cheng Chin Trading Co. Ltd., Taipei, Taiwan.

Resin: South American, grade G; W. and G. Dean Pty. Ltd., Melbourne, Australia.

Shell-sol A: Shell Chemicals of Australia Ltd.

(g) GIFTS

Chicken SCM feather keratin protein: Mr. D.J. Kemp.

Washed and ground (milled to through-40 mesh size) guinea pig hair: Mr. H.W.J. Harding.

N-acetylserine, N-acetylalanine and N-acetylserine-SCMcysteine: Mr. D.J. Kemp.

Various rabbit antisera (*see* Fig. 8.4 of this thesis): Mr. D.J. Kemp.

B GENERAL METHODS

Only those procedures that were used routinely throughout this work are described in the present chapter. Other more specific procedures will be described in the Methods sections of the following chapters.

(a) SOURCE OF HAIR FOLLICLE TISSUE

Albino guinea pigs were used as the source of hair follicle tissue throughout this work. Very young animals (age less than 3 weeks, or body weight less than 150 g) were used since these animals had high proportions (about 90 - 95 %) of actively-growing hair follicles in their skin (Bosse, 1965). The use of guinea pigs had two principal advantages over other animals. Firstly, one or a small number of young animals provided sufficient tissue for the types of experiments to be reported in this thesis, compared with, for example, rats or mice. Secondly, the small size of the animal represented considerable economy in animal husbandary, compared with, for example, sheep.

No differences in any properties of the hair follicle tissue could be attributed to the sex of the animals and therefore animals of both sexes were used.

(b) PREPARATION OF HAIR FOLLICLE TISSUE

The wax-sheet procedure was employed to separate the hair follicle tissue from the skin of the animals. The method was originally established by Ellis (1948) and subsequently modified by Rogers (1959b and 1964) and more recently by Clarke and Rogers (1970a). All procedures, including killing and flaying the animals, were performed at 2°. After exposure of the hair follicles with the wax mixture (consisting of crude beeswax and resin, 2 : 7, w/w) as described by Clarke and Rogers (1970a), the follicles were removed with animal clippers (model A-2, John Oster, Wisconsin, U.S.A.) fitted with size 40 cutters and placed immediately into an appropriate buffer to prevent dehydration and deterioration. Unless otherwise stated all subsequent procedures were conducted at 0 - 2°.

Other methods for the isolation of hair follicles from the skin

of the animals, for example, using "Araldite" (Wilkinson, 1970b) were not investigated.

(c) PREPARATION OF STANDARD S-CARBOXYMETHYL- PROTEINS

The enzymes and proteins were converted to their S-carboxymethyl- (SCM) derivatives since they were used throughout this work for the estimation of the molecular weights of SCM proteins from keratin tissues. The types and molecular weights of the proteins and enzymes used for this work are listed in Table 2.1. Each protein was dissolved in a buffer of 8 M urea, 0.10 M tris-HCl (pH 7.6) and 1mM EDTA (20 mg/ml) and made to 0.1 M 2-mercaptoethanol and pH 10 by addition of 5 N NaOH. The solution was flushed with N_2 and the reduction continued for 16 h at 37^{o}. Alkylation was effected at pH 8.5 by addition of iodoacetic acid dissolved in 3 M tris-HCl (pH 8.5) (200 mg/ml) to 0.15 M. Once the solution had become sulphydryl-negative (Feigl, 1947), excess iodoacetic acid was destroyed with 2-mercaptoethanol and the solution was dialysed exhaustively against water and freeze-dried. The purity of each protein was examined by polyacrylamide gel electrophoresis at pH 9 and was used only if homogeneous.

Polypeptides of varying sizes were obtained from some proteins. The SCM insulin A and B chains were separated by acid precipitation (Thompson and O'Donnell, 1966). The haem group of myoglobin was removed by the method of Teale (1959) prior to reduction and alkylation of this protein. SCM-lysozyme was cleaved at its two methionine residues with cyanogen bromide (*see* Methods of Chapter Four, page 67) and the cyanogen bromide fragments were separated on a column of Sephadex G-50 using 1 N acetic acid as eluant.

(d) COLUMN CHROMATOGRAPHY USING SEPHADEX

Several different grades of Sephadex were used during this work and different procedures were employed for the preparation of them.

TABLE 2.1

TYPES AND MOLECULAR WEIGHTS OF STANDARD PROTEINS USED FOR THE CALIBRATION OF SEPHADEX COLUMNS IN THIS WORK

In most references cited the molecular weight values were for the unmodified proteins. Appropriate changes have been made here to the values to allow for the addition of the S-carboxymethyl- (SCM) groups at the rate of 60 daltons per SCM group.

Protein	Molecular weight (daltons)	Reference
Albumin	68 000	Putman (1965)
Ovalbumin	45 500	Warner (1954)
Pepsin	35 500	Blumenfeld and Perlman (1959)
Chymotrypsinogen A	25 500	Dayhoff and Eck (1967-8)
Apomyoglobin	17 200	"
Lysozyme	14 700	"
Lysozyme peptide 1	10 500	"
2	3 000	"
3	1 300	"
Feather keratin	10 500	Harrap and Woods (1965)
Insulin A	2 550	Dayhoff and Eck (1967-8)
B	3 450	"

Dry Sephadex G-200 (medium) was washed in sodium-dried ether to remove fine beads (Kawata and Chase, 1968), soaked in water with several changes for 1 week to ensure complete swelling and then in the urea buffer (*see* Chapter Three) for 2 days before packing the column by established procedures. The direction of flow in these columns was upwards with a net hydrostatic pressure head of 15 - 20 cm. The columns equilibrated and operated in this manner maintained a constant flow rate of about 2 ml/h/cm^2 for periods in excess of 2 years. The prior removal of fine beads by the ether process was essential as the flow rate of the columns rapidly decreased in their presence. Preparative columns of Sephadex G-200 were 175 x 5.5 cm and analytical columns were 165 x 1.6 cm.

Less stringent procedures were used for equilibrating lower grades of Sephadex. When 50 % aqueous formic acid was used as eluant, the columns were operated at a flow rate of about 3 ml/h/cm^2 by means of a Beckman Accu-flow pump. Teflon stoppers and tubing which were resistant to corrosion were used.

For all Sephadex columns used, the volume of the sample loaded and the fraction size collected was about 1 % of the total column volume (V_t). The columns were calibrated when necessary with dextran blue (for V_o), $K_3Fe(CN)_6$ (for V_t) and at least five proteins of known molecular weight which were well fractionated by the grade of Sephadex used. A plot of $\log_{10}$ of the molecular weight of these standard proteins against their elution volume (V_e) from the columns yielded a straight line which could be used for determination of the molecular weights of proteins of unknown size (Whitaker, 1963).

(e) COLUMN CHROMATOGRAPHY USING DEAE-CELLULOSE

Before use the DEAE-cellulose powder was soaked in water for three days with numerous changes to remove fine particles and then washed

successively with 0.5 N NaOH, water, 0.5 N HCl and water to convert it completely to the Cl^--form. The washed product was equilibrated in the desired buffer for one day before use. In all experiments the operating temperature was about 23°. Protein samples were dialysed against 100 volumes of starting buffer before loading the column.

(f) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

7.5 % acrylamide gels at pH 9.0 containing 5 M urea were made by established procedures (Davis, 1964) except that riboflavin was used for polymerisation of the gels. Samples (up to 200 µg), dissolved in buffer containing 8 M urea, were applied to the top of the "stacking" gel and electrophoresed at 1 mA/gel until the tracking dye band (of methylene blue) had entered the "separation" gel and then at 3 mA/gel for the desired length of time.

In some gels the normal cross-linking reagent (N,N'-methylene-bisacrylamide) was replaced by N,N'-diallyltartardiamide (Anker, 1970). The advantage of these gels was that they could be rendered soluble with periodic acid. While these gels were not as "firm" as the normal gels, the separation of proteins on them was as good.

After electrophoresis in all cases, the gels were washed in 10 % (w/v) trichloroacetic acid (TCA) to remove urea and then stained with 0.05 % (w/v) coomassie brilliant blue (Chrambach *et al.*, 1967) in 10 % TCA for 2 days. Excess stain was removed from the gels by rinsing with 50 % aqueous ethanol. It was found that this stain was the most convenient to use and it stained adequately all the types of proteins studied in this work.

(g) DIALYSIS

Dialysis tubing (Visking) was boiled in 1 % (w/v) $NaHCO_3$ for 2 min to remove impurities and then washed in water (or buffer, where applic-

able) before use (Thompson and O'Donnell, 1965; Hughes and Klotz, 1956).

(h) AMINO ACID ANALYSIS

Protein samples were thoroughly desalted by extensive dialysis against water and freeze-dried. Samples (about 1 - 2 mg) were hydrolysed in 6 N HCl *in vacuo* at 110° for times varying up to 30 h. Hydrolysates contained 0.1 % (w/v) phenol to prevent modification (by chlorination) of tyrosine residues (O'Donnell and Thompson, 1968; Sanger and Thompson, 1963). Samples were freed of HCl by rotary film evaporation prior to analysis. Samples suspected of containing homoserine were treated with 0.1 M K_2CO_3 after removal of HCl to convert all homoserine lactone to homoserine (Moore and Stein, 1951).

Two column chromatographic procedures were employed for amino acid analysis. The two-column system (for the acidic and neutral amino acids and the basic amino acids) of the Beckman 120C amino acid analyser were used for most analyses. About 400 µg of hydrolysate was applied to each column. In some experiments, a 10 x scale expanding device was used, and only 40 µg was loaded on each column. The single column procedure described by Harding and Rogers (1971) which was attached to the Beckman 120C analysing system was used when the separation of homoserine was desired. About 80 µg of hydrolysate was applied when the scale expanding device was employed.

In both chromatographic systems, citrulline and proline co-eluted. Citrulline was deemed to be present whenever the ratio of the peak areas of the 570 : 440 nm traces exceeded 1 : 6 (Harding, 1971, personal communication).

(i) ELECTRON MICROSCOPY

All specimens were examined in a Siemens Elmiskop 1 electron microscope operated at 80 kV with a 50 µ aperture.

Specimens were negatively-stained on the grid with either 1 % or 2 % (w/v) uranyl acetate or 1 % (w/v) phosphotungstic acid (pH 4). Specimens for sectioning were firstly fixed in 2 % (w/v) glutaraldehyde, post-fixed in 1 % (w/v) osmic acid, dehydrated in acetone and embedded in "Araldite" by standard procedures. Occassionally, samples were stained with 1 % (w/v) uranyl acetate prior to embedding. Sections of the appropriate thickness (usually 100 - 800 Å) were prepared with an LKB Ultramicrotone using glass knives and when necessary were stained on the grid before examination.

(j) SUCROSE DENSITY GRADIENT CENTRIFUGATION

The only grade of sucrose used for the preparation of gradients in this work was that specified ribonuclease and metal ion free.

Discontinuous gradients were formed by superimposing layers of less dense sucrose solutions in the required tube.

Continuous linear gradients were prepared using a coupled-vessels apparatus similar to that originally described by Bock and Ling (1954). For gradients in the 33 ml cellulose-nitrate tubes for the Spinco SW25.1 rotor, the gradients were formed by over-layering of progressively less dense sucrose solutions. For gradients in the 65 ml polypropylene tubes for the MSE swinging bucket rotor, the method of Noll (1967) was used in which progressively denser sucrose solutions were layered at the bottom of the tube by means of a fine capillary tube.

(k) MEASUREMENT OF RADIOACTIVITY

All measurement of radioactivity was conducted in a Packard Tricarb Liquid Scintillation Spectrometer.

Two types of scintillation fluid were used in this work: that of Bray (1960) for samples in aqueous solution and a toluene - based fluid

containing 0.3 % (w/v) 2,5-diphenyloxazole and 0.03 % (w/v) 1,4-*bis*-(5-phenyloxazolyl)-benzene for samples dried on glass fibre circles. It was found that the toluene in the latter fluid could be replaced by a cheaper solvent "Shell-sol A" without significant alteration to measurement efficiencies.

CHAPTER THREE

ISOLATION AND CHARACTERISATION OF THE HAIR AND HAIR FOLLICLE PROTEINS OF THE GUINEA PIG

A INTRODUCTION

The principal aim of the studies to be described in this chapter is to provide information about two aspects. The first is to establish whether there are any important differences in the types and properties of the proteins that can be isolated from hair follicle tissue and those from fully keratinised hair. This is an important question as it would indicate whether any post-synthetic modifications of the proteins occur before or during keratinisation. A number of studies to answer this question have been made in the wool follicle system (O'Donnell and Thompson, 1964; Frater, 1966; Downes *et al.*, 1966a; Rogers, 1959b; Fraser, 1969), but none of these established this point clearly. A second and concurrent aspect of this work is to provide basic procedures for the isolation and characterisation of the various types of hair and hair follicle proteins. These techniques are necessary before meaningful studies on the mechanism and control of synthesis of specific proteins, both *in vivo* and *in vitro*, can be made.

B METHODS

(a) EXTRACTION OF PROTEINS

(1) Hair follicle proteins

The procedure used for the extraction of proteins from the guinea pig hair follicle tissue in the present work was based on methods established by other workers; for example; O'Donnell and Thompson (1964); Frater (1966); Fraser (1969); Clarke and Rogers (1970a); and has been published (see Kemp and Rogers, 1970). Hair follicle tissue was suspended in a buffer of 8 M urea, 0.1 M tris-HCl (pH 7.6), 1 mM EDTA and 25 mM 2-mercaptoethanol (25 ml/g of follicle tissue) and disrupted by homogenisation in a loose-fitting vessel with a motor-driven teflon plunger (Potter-Elvehjem). The solution was stirred for 10 min to ensure complete reduction of the sulphydryl

groups on the proteins. The pH was raised to 8.5 and the solution was treated with iodoacetic acid dissolved in 3 M tris-HCl (200 mg/ml, pH 8.5) to a final concentration of 50 mM. The alkylation reaction was allowed to proceed until free sulphydryl groups could no longer be detected (usually about 10 min) (Feigl, 1947), whereupon excess iodoacetic acid was destroyed by addition of 2-mercaptoethanol. The solution was filtered through nylon gauze (pore size about 0.2 mm), centrifuged at 38 000 x *g* for 10 min to remove debris and then dialysed against 10 mM tris-HCl (pH 7.6) and 1 mM EDTA buffer with four changes of 100 volumes. All procedures, except the alkylation step, were conducted at 0^{o}.

(2) Hair proteins

Guinea pig hair was cleaned by standard procedures of solvent and water extraction and ground in a mill to through-40 mesh size. The proteins were then extracted by the procedure of O'Donnell and Thompson (1964) and alkylated with iodoacetic acid as described above.

(b) PREPARATION OF THE pH 4.4 SOLUBLE AND INSOLUBLE PROTEIN FRACTIONS

This was performed by the procedure of Crewther and Dowling (1968, personal communication). The dialysed extracted proteins were made to 0.5 M KCl and centrifuged at 38 000 x *g* for 10 min to remove any precipitated material. The pH was lowered to 4.4 by addition of 0.5 volumes of 0.2 M sodium acetate (pH 4.4). The solution was stirred for 15 min and the precipitate (*fraction A*) was collected by centrifugation at 1000 x *g* for 10 min. This was redissolved in 50 mM sodium tetraborate (pH 9.2) (about 10 mg/ml), made to 0.5 M KCl and reprecipitated. This process was repeated a third time. The pH 4.4 soluble proteins (*fraction B*) from the first precipitation were dialysed, concentrated and reprocessed two more times. Both pH fractions were finally concentrated to about 20 mg/ml and dialysed exhaustively against

a buffer of 20 mM tris-HCl (pH 7.6) and 1 mM EDTA.

(c) PREPARATION OF UREA BUFFERS

Wherever possible, 8 M urea solutions were used to prevent aggregation of the keratin proteins. However, constant exposures to urea solutions increased the possibility of the side reaction of cyanate ions, that exist in equilibrium with urea in solution, with protein amino groups (Thompson and O'Donnell, 1965). Therefore, precautions were adopted to remove cyanate ions at all stages. Solid urea was dissolved in bidistilled de-ionised water to 8.5 M and stirred with mixed ion-exchange resin for 1 h to remove ionic impurities. The resin was filtered off and the solution made to pH 3 with 5 N HCl to hydrolyse remaining traces of cyanate. Solid "tris" base and other components were then added to the desired concentrations and pH corrected to pH 7.6. "Tris" was used as a buffer throughout since this acts as a scavenger for cyanate ions (Thompson and O'Donnell, 1965). The final urea concentration was 8.0 M.

(d) CONCENTRATION OF PROTEIN SOLUTIONS IN 8 M UREA

Solutions of proteins in 8 M urea buffers were first dialysed against a buffer of 10 mM tris-HCl (pH 8) and 1 mM EDTA with several changes of 100 volumes. Three methods were routinely used for concentration: (a) rotary-film evaporation under reduced pressure (about 100 μ of Hg) at 25 - 35°; (b) ultrafiltration using collodion membrane filters, under reduced pressure (about 20 mm of Hg) at 4°; and (c) freeze-drying after a further dialysis against water. It was found during the course of this work that the LoS proteins became aggregated on freeze-drying and these aggregates were difficult to disperse on redissolving and so this procedure was used infrequently. Of the former two procedures, (a) was the most frequently used as it was the most rapid. However, some proteins (for example, LoS

component I) became aggregated and in general, procedure (b) was used only if procedure (a) led to the aggregation of a protein.

(e) STORAGE OF PROTEINS

Proteins were stored frozen in solution in a buffer of 10 mM tris-HCl (pH 8) and 1 mM EDTA at -15° at a concentration of 10 - 20 mg/ml in tightly-sealed containers to prevent freeze-drying.

(f) ANALYTICAL ULTRACENTRIFUGATION

This was performed in a Beckman Model E ultracentrifuge equipped with a temperature control unit and schlieren optics. Protein samples were dissolved in a buffer of 50 mM tris-HCl (pH 7.6) and 50 mM KCl. A titanium rotor (An-F) was used for all experiments at a speed of 59 780 rev./min. Values for the sedimentation coefficient $s_{20,w}$ were calculated from the equation described by Schachman (1957):

$$s_{20,w} = s_{obs} \cdot \left(\frac{\eta_t}{\eta_o} \right) \cdot \left(\frac{\eta}{\eta_o} \right) \cdot \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t} \right)^a$$

Distances of the boundary from the axis of rotation were measured photographically. Values for the viscosity and density of the buffer used were calculated from appropriate tables in *International Critical Tables* Vols. 3 and 5 (1929 and 1930; New York; McGraw-Hill) and in the *Handbook of Chemistry and Physics* (1968; Cleveland; Chemical Rubber Co.). Values for $\frac{1}{\omega^2}$

---

[a] $s_{obs} = \frac{1}{\omega^2} \cdot \frac{dx}{dy}$; where x = distance of the boundary from the axis of rotation; y = time; ω = angular velocity; $\frac{\eta_t}{\eta_o}$ = viscosity of water at t^o relative to that at 20°; $\frac{\eta}{\eta_o}$ = viscosity of solvent relative to that of water; $\rho_{20,w}$ = density of water at 20°; ρ_t = density of water at t^o; $\bar{v}$ = partial specific volume.

were obtained from tables supplied by Dr. J.C. Wallace. Values for $\bar{v}$ were calculated from the amino acid composition of the proteins studied (Schachman, 1957). For each protein, $s_{20,w}$ was determined for at least five different concentrations of protein. Plots of $s_{20,w}$ *vs* concentration yielded a straight line which on extrapolation to zero concentration, yielded $s^{o}_{20,w}$. Values for the diffusion coefficient $D_{20,w}$ were evaluated in a sedimentation velocity experiment at a speed of 4908 rev./min by means of the equation:

$$\left(\frac{A}{H}\right)^2 = 4\pi D_{obs} t \qquad a,b$$

Plots of $\left(\frac{A}{H}\right)^2$ *vs* t fitted a straight line, the slope of which was $4\pi D_{obs}$.

The values of $s^{o}_{20,w}$ and $D_{20,w}$ were then used for calculation of molecular weights from the Svedberg equation:

$$M = \frac{RTs}{D(1 - \bar{v}\rho)} \qquad c$$

---

a From Kawahara (1969). This equation is an approximation of that of Lamm (1929) and is reasonably valid providing, *inter alia*, a sufficiently low speed of rotation is employed.

b where A = the area enclosed by the sedimentation boundary curve above its base line; H = its maximum height; t = time measured from the start of centrifugation; $D_{20,w}$ was calculated from:

$$D_{obs} \cdot \left(\frac{\eta_t}{\eta_o}\right) \cdot \left(\frac{\eta}{\eta_o}\right) \cdot \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t}\right)$$

c where R = gas constant; T = absolute temperature; $s = s^{o}_{20,w}$; $D = D_{20,w}$.

C RESULTS

(a) PREPARATION AND NOMENCLATURE OF THE HAIR AND HAIR FOLLICLE PROTEINS

(1) Preparation of proteins

In preliminary studies on the extraction of the hair follicle proteins, iodoacetic acid was added to the extracting buffer instead of 2-mercaptoethanol (*see* Clarke and Rogers, 1970a) to enable alkylation of the sulphydryl-rich proteins during extraction. Analysis of the proteins extracted in this way by polyacrylamide gel electrophoresis (PAGE) indicated the presence of numerous bands, but they were diffuse and the electrophoretic patterns obtained were not reproducible. This suggested that some oxidation of the sulphydryl groups had occurred during extraction which had led to aggregation of the proteins. Therefore, to minimise this problem, 2-mercaptoethanol was added to the extraction buffer and the alkylation procedure described in Methods was utilised.

(2) Nomenclature

In Fig. 3.1a is shown PAGE patterns of protein extracted from guinea pig hair and hair follicle tissue. The S-carboxymethyl kerateine (SCMK) proteins were termed the H-SCMK and F-SCMK protein extracts, depending on whether they were prepared from hair (H) or hair follicle (F) tissue. The patterns were divided operationally into the four groups of increasing electrophoretic mobility as shown. It can be seen from Fig. 3.1a that although there were quantitative differences in the proteins extracted from both sources, there were few significant differences in the types of proteins extracted. There were more group-1 proteins in the F-SCMK protein extract but less group-3 and group-4 proteins than in the H-SCMK protein extract. The background of stain in the F-SCMK gel was denser than the H-SCMK gel, presumably due to the presence in the former of numerous cytoplasmic proteins.

FIGURE 3.1

POLYACRYLAMIDE GEL ELECTROPHORESIS OF HAIR AND HAIR FOLLICLE PROTEINS

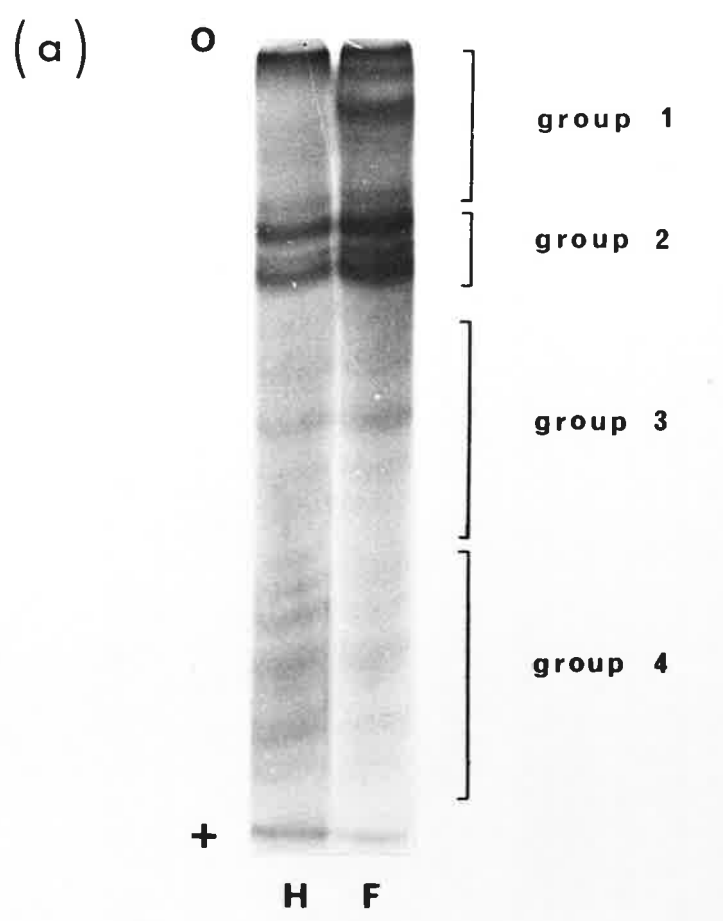
The gels were prepared, used and stained as described in General Methods (*see* page 41). The samples were prepared as described in Methods and were electrophoresed for 3 h.

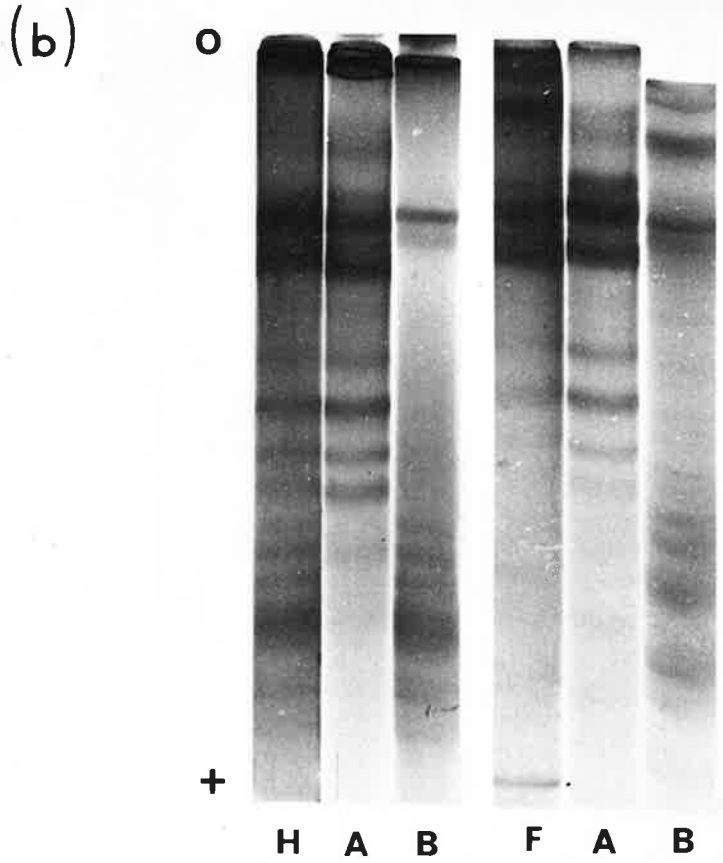
(a) Electrophoresis of SCMK protein extracts

Each gel was loaded with 90 - 100 μg of sample. 0 = origin; + = anode end of gel. The band at the anode end is the band of bromophenol blue used as a tracking dye. The bands of the gels were operationally divided into the four groups as described in the text. H = H-SCMK; F = F-SCMK.

(b) Electrophoresis of SCMK-A and SCMK-B protein fractions

Each gel was loaded with 80 μg of sample. H = H-SCMK; F = F-SCMK; A = SCMK-A; B = SCMK-B.


(a)
O
group 1
group 2
group 3
group 4
+
H
F


(b)
O
+
H
A
B
F
A
B

The PAGE patterns of the pH 4.4 insoluble (SCMK-A) and soluble (SCMK-B) protein fractions are shown in Fig. 3.1b. Both of the SCMK-A and SCMK-B protein fractions contained group-1 proteins and these are termed the group-1A (from SCMK-A) and the group-1B (from SCMK-B) proteins. Apart from differences in the hair and hair follicle group-1B proteins, the corresponding A and B fractions of both extracts were very similar. In each case the fractions A contained group-2 and group-3 proteins and the fractions B contained group-4 proteins, as well as group-1 proteins. The bands which appear in the SCMK-B gels of Fig.3.1b in the group-2 region were due to nucleic acid material (see later).

In studies on wool kerateine proteins (for example, Thompson and O'Donnell, 1964) the SCMK-A fraction was termed the low sulphur kerateine (LoS) proteins and the SCMK-B fraction was termed the high sulphur kerateine (HiS) proteins. Since each of these fractions in this work contained other proteins as well (Fig. 3.1b), the guinea pig LoS and HiS proteins were operationally defined to be the group-2 and group-4 proteins, respectively, since each was the major component of each fraction.

(3) Amino acid composition

The amino acid compositions of the proteins extracted from both sources as well as the pH 4.4 soluble and insoluble protein fractions from both sources are given in Table 3.1. The values given were representative only since the contents of the amino acids SCMcysteine, threonine and serine in especially the unfractionated and SCMK-B samples varied by more than 20 % in different batches prepared from different guinea pigs. The analyses of the H-SCMK-A and F-SCMK-A samples were similar but the analyses of the unfractionated and H-SCMK-B and F-SCMK-B samples showed marked differences in their contents of the amino acids SCMcysteine, serine, threonine, glutamic acid, proline and leucine. These differences were similar to those observed

TABLE 3.1

AMINO ACID COMPOSITION OF THE UNFRACTIONATED AND pH 4.4 - FRACTIONATED HAIR AND HAIR FOLLICLE PROTEINS

The values are expressed as residues per 100 residues and represent only one analysis of each sample because of variability between different batches of proteins. Amounts indicated by "trace" were present in < 0.05 residues percent.

Amino acid	Unfractionated		pH 4.4 - fractionated			
	SCMK		SCMK-A		SCMK-B	
	F	H	F	H	F	H
SCMcysteine	6.10	10.75	6.15	6.25	12.10	19.10
Aspartic acid	7.10	6.05	7.95	7.85	4.85	3.30
Threonine	4.80	6.95	5.15	5.05	5.35	8.85
Serine	8.25	11.30	8.20	8.15	8.30	11.05
Glutamic acid	17.65	12.55	18.65	18.35	18.25	13.45
Citrulline [a]		0.65				1.55
Proline	3.95	7.15	3.35	3.25	8.05	11.85
Glycine	8.90	9.25	8.45	8.55	5.70	5.45
Alanine	5.65	4.90	6.25	6.45	4.10	3.35
Valine	4.85	4.85	4.90	5.15	4.65	4.45
Half-cystine			trace		trace	
Methionine	0.75	0.65	0.75	0.70	0.45	0.20
Isoleucine	2.95	3.15	3.45	3.65	2.65	2.60
Leucine	9.45	6.95	9.45	9.65	6.05	4.15
Tyrosine	3.75	3.85	3.65	3.80	1.60	1.40
Phenylalanine	2.45	2.30	2.40	2.30	1.90	1.25
Lysine	2.95	2.60	3.45	3.75	2.60	1.45
Histidine	0.75	0.65	0.75	0.75	0.80	0.80
Arginine	7.40	7.65	7.40	7.90	6.95	6.60

[a] During acid hydrolysis, citrulline undergoes partial decomposition to ornithine. The citrulline value given is the sum of the citrulline remaining and the ornithine produced during acid hydrolysis.

between the wool and wool follicle proteins (Rogers, 1959b) (*see* Chapter One). Further experiments were designed to characterise these differences.

(b) FRACTIONATION OF THE DIFFERENT GROUPS OF PROTEINS FROM HAIR AND HAIR FOLLICLE TISSUE

(1) Chromatography on Sephadex G-200

The elution profiles of the F-SCMK and H-SCMK protein extracts on a calibrated Sephadex G-200 column are shown in Fig. 3.2a. The profiles were similar but there was notably more material in peak 3 in the former which was presumably due to the presence of nucleic acid material. The PAGE patterns of peaks 1 - 3 are shown in Fig. 3.2b and it is seen that whilst there were differences in the types of proteins of peak 1 from the two samples (*compare* gel F-1 *with* gel H-1), similar proteins were present in peaks 2 and 3. Peak 1 contained mostly group-1 and some LoS proteins. Peak 2 contained mostly LoS proteins and peak 3 contained group-3 and HiS proteins. The dense stain at the top of the gel H-1 arose because the protein material migrated down the *outside* of the gel.

The molecular weights of the proteins were; group-1 (peak 1), > 95 000 daltons (eluted at V_o); LoS (peak 2), 40 - 50 000 daltons; group-3 and HiS (peak 3), 10 - 30 000 daltons.

(2) Chromatography on DEAE-cellulose

O'Donnell and Thompson (1964) showed that appreciable fractionation of the wool kerateine proteins was possible by stepwise elution from DEAE-cellulose using urea buffers and increasing KCl concentrations. The present studies showed that this procedure was also effective in resolving the components of the H-SCMK and F-SCMK protein extracts and the elution profile of such an experiment using the F-SCMK proteins only is shown in Fig. 3.3a. The elution profiles of both extracts were in fact similar but

FIGURE 3.2

(a) CHROMATOGRAPHY OF SCMK PROTEIN EXTRACTS ON SEPHADEX G-200

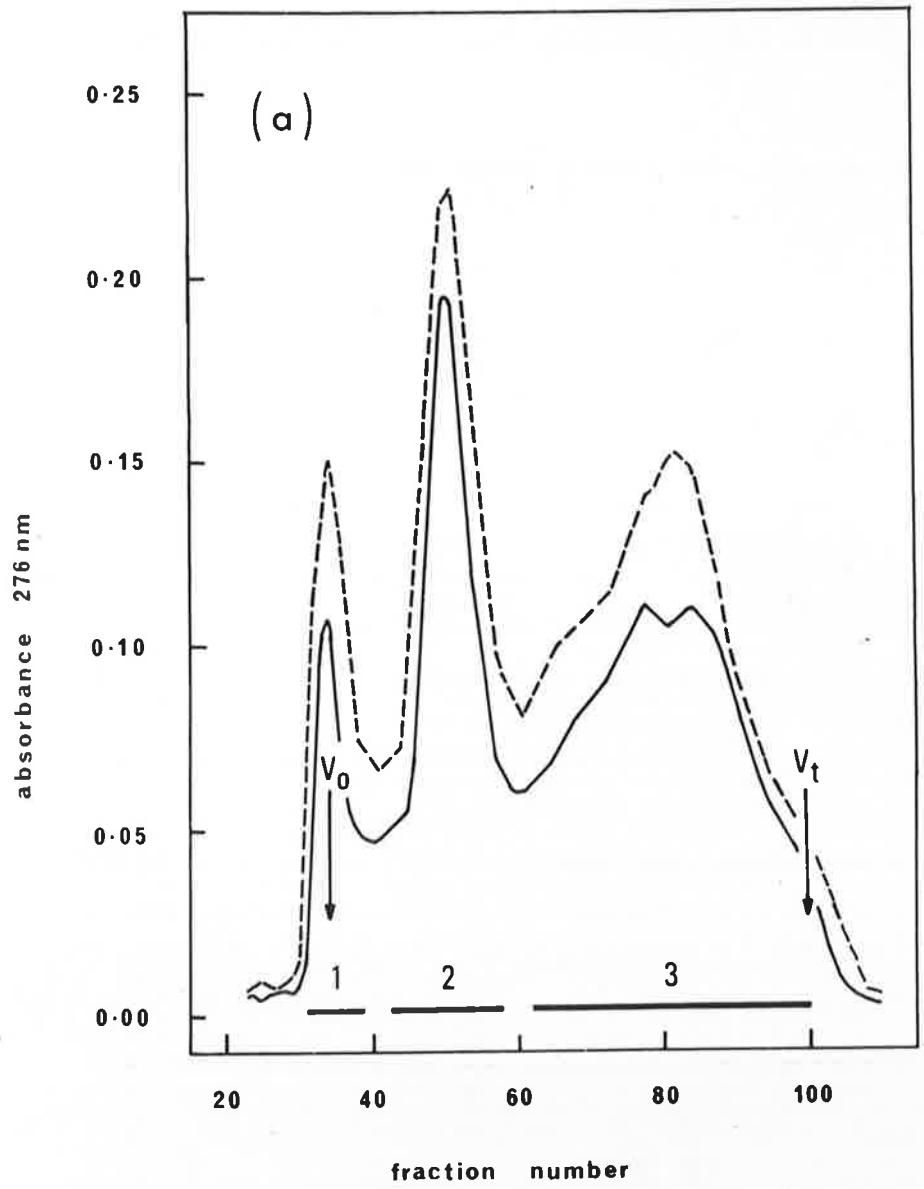
A Sephadex G-200 column 165 x 1.6 cm was prepared as described in General Methods (*see* page 40) and was equilibrated using the buffer of 8 M urea, 0.2 M KCl, 50 mM tris-HCl (pH 7.6) and 1 mM EDTA. The flow rate was 4 ml/h and fraction size 3.0 ml. The column was calibrated before use (see below). In each case about 40 mg of protein was chromatographed. ——, H-SCMK; - - - - - , F-SCMK. The bars 1 - 3 refer to the tubes that were pooled for characterisation of their contents.

CALIBRATION PROTOCOL

Protein	Molecular weight (daltons)	V_e (fraction number)
Dextran blue	2.10^6	34.0 (V_o)
Albumin	68 000	38.5
Ovalbumin	45 500	49.5
Pepsin	35 500	59.0
Chymotrysinogen A	25 500	69.5
Apomyoglobin	17 200	81.5
Lysozyme	14 700	88.0
Insulin B	3 450	100.5 (V_t)

(b) PAGE OF FRACTIONS FROM COLUMN

Samples from the pooled tubes of *(a)* were applied to the gels without prior desalting. The volume of sample loaded was adjusted so that it contained about 50 µg of protein and the gels were electrophoresed for 3 h. H = H-SCMK; F = F-SCMK. 1 - 3 are from the pooled fractions enumerated as such in *(a)*.


(a)
0·25
0·20
0·15
0·10
0·05
0·00
absorbance 276 nm
V_0
V_t
1
2
3
20
40
60
80
100
fraction number

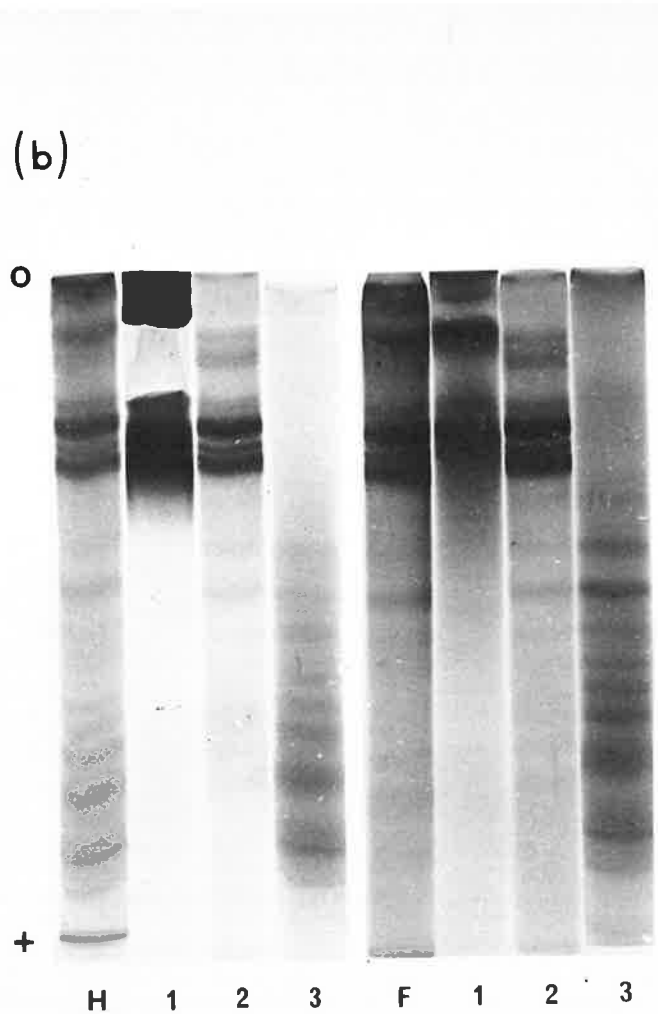

(b)
O
+
H
1
2
3
F
1
2
3

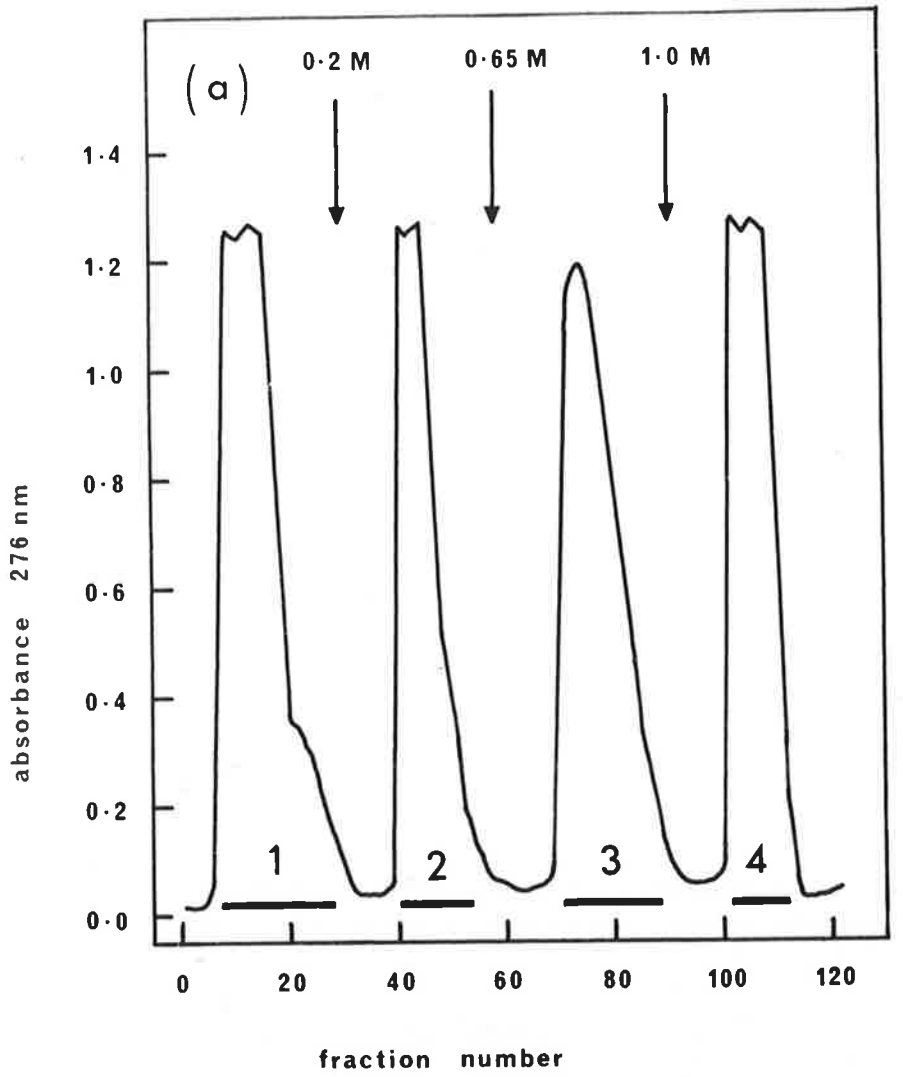
FIGURE 3.3

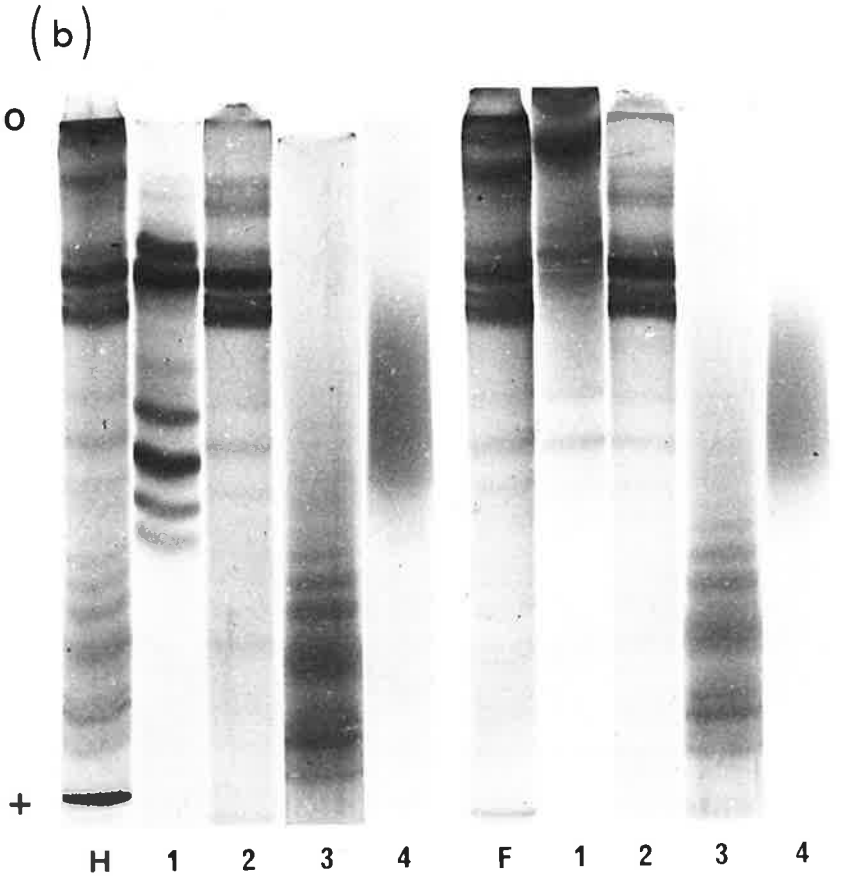
(a) CHROMATOGRAPHY OF THE F-SCMK PROTEIN EXTRACT ON DEAE-CELLULOSE

A 50 x 2.2 cm column of DEAE-cellulose was prepared as described in General Methods (*see* page 40) and was equilibrated with a starting buffer of 8 M urea, 50 mM KCl, 20 mM tris-HCl (pH 7.6) and 1 mM EDTA. The flow rate was 100 ml/h and the fraction size was 20 ml. Approximately 1 g of F-SCMK protein was loaded. Buffers of the same composition but with the increased KCl concentrations of 0.2 M, 0.65 M and 1 M were applied at tubes 31, 62 and 93, respectively. ———, absorbance at 276 nm. The bars 1 - 4 refer to the tubes that were pooled for characterisation of their contents. An identical experiment was performed on a sample of an H-SCMK protein extract.

(b) PAGE OF FRACTIONS FROM COLUMN

Samples from the pooled tubes were applied to the gels without prior desalting. The volume of sample loaded in each case, except 4, was adjusted so that it contained about 80 µg of protein. The samples in high salt electrophoresed more slowly than others and so gels were stopped when the tracking dye band reached the end of the gel. Electrophoresis was for 3 - 4.5 h. H = H-SCMK; F = F-SCMK. 1 - 4 are from the pooled fractions enumerated as such in *(a)*.


(a)
0·2 M
0·65 M
1·0 M
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
absorbance 276 nm
1
2
3
4
0
20
40
60
80
100
120
fraction number


(b)
O
+
H
1
2
3
4
F
1
2
3
4

there was markedly more peak 4 material in the F-SCMK extract. This material was most likely nucleic acid since it absorbed maximally at 260 nm. PAGE patterns of the peaks 1 - 4 from the F-SCMK sample and from a similar experiment using an H-SCMK protein sample are shown in Fig. 3.3b. Peak 1 contained group-1 and group-3 proteins as well as some LoS proteins. The protein material of gel H-1 in Fig. 3.2b did not elute from the DEAE-cellulose column (*see* H gels of Fig. 3.3b). Peaks 2 and 3 contained mostly the LoS and HiS proteins, respectively. It can be seen that the only significant differences were in the group-1 and group-3 proteins of peak 1 (*compare* gel F-1 *with* gel H-1). In addition, the background of the stain in gel F-1 was denser than that of gel H-1 presumably due to the presence in the former of numerous cytoplasmic proteins.

(3) Complete fractionation of the protein extracts

The three procedures of acid precipitation at pH 4.4 and chromatography on Sephadex G-200 and DEAE-cellulose provided convenient means of fractionation of the hair and hair follicle protein extracts into the four groups as summarised in Table 3.2. These techniques were used routinely for the preparation of the various groups of proteins.

The amino acid composition of the groups from both extracts are given in Table 3.3. The analyses of the group-1A, group-2 (LoS), group-3 and group-4 (HiS) proteins from both extracts were the same within experimental error (± 5 %). However, marked differences existed in the group-1B proteins and these will be discussed in more detail in the next section of this chapter.

The amounts of the various groups of proteins present in both hair and hair follicle extracts were determined during these experiments and are given in Table 3.4. Some variations in yields were apparent between different batches of tissue extracts but these may have been due to the experimental conditions used. Nevertheless, the F-SCMK extracts contained

TABLE 3.2

METHODS OF PREPARATION OF THE DIFFERENT GROUPS OF HAIR AND HAIR FOLLICLE PROTEINS

Protein group	Protein fraction source	Chromatographic method
1	SCMK	Sephadex G-200 (peak 2)
1A	SCMK-A	"
1B	SCMK-B	"
2 (LoS)	SCMK	DEAE-cellulose (peak 2)
		or
		Sephadex G-200 (peak 2)
	SCMK-A	"
3	SCMK	DEAE-cellulose (peak 1) followed by Sephadex G-200 (peak 3)
	SCMK-A	Sephadex G-200 (peak 3)
4 (HiS)	SCMK	DEAE-cellulose (peak 3)
	SCMK-B	Sephadex G-200 (peak 3)

TABLE 3.3

AMINO ACID COMPOSITION OF THE DIFFERENT GROUPS OF HAIR AND HAIR FOLLICLE PROTEINS

The values are expressed as residues per 100 residues and represent the average of at least two different analyses on different batches of proteins. Amounts indicated by "trace" were present in < 0.05 residues percent.

Amino acid	Group-1A		Group-1B		Group-2		Group-3		Group-4	
	F	H	F	H	F	H	F	H	F	H
SCMcysteine	5.70	5.55	2.45	1.95	6.25	6.15	12.85	12.30	21.65	23.40
Aspartic acid	8.55	8.40	7.35	6.95	8.15	7.95	3.70	3.65	2.30	2.35
Threonine	5.05	4.95	2.65	2.85	5.00	4.90	4.25	4.40	9.95	10.40
Serine	7.45	7.60	5.00	4.85	8.60	8.75	11.25	12.00	12.45	12.55
Glutamic acid	18.65	19.05	26.85	27.85	18.05	18.40	3.45	3.15	9.90	10.20
Citrulline [a]			trace	7.95						
Proline	3.10	3.05	2.70	2.20	3.40	3.45	6.00	5.85	13.45	13.05
Glycine	7.15	6.95	5.60	5.35	8.95	9.15	23.75	24.15	5.85	5.45
Alanine	7.05	6.70	4.85	4.65	6.45	6.15	1.95	1.75	3.00	2.95
Valine	5.35	5.50	4.55	4.15	5.00	4.95	3.00	2.85	4.90	4.45
Half-cystine	trace	trace	trace	trace						
Methionine	0.90	0.75	0.85	0.90	0.80	0.70	0.05	trace		
Isoleucine	3.80	3.85	2.65	2.45	3.60	3.70	1.15	1.05	2.70	2.55
Leucine	9.50	9.30	9.75	9.95	9.15	8.85	5.75	5.85	3.10	2.80
Tyrosine	3.25	3.25	2.15	2.20	3.95	4.15	11.05	11.45	1.15	1.20
Phenylalanine	2.20	2.35	3.00	3.05	2.50	2.60	5.05	4.85	0.75	0.75
Lysine	3.70	3.85	4.70	4.90	3.35	3.50	0.35	0.35	0.50	0.55
Histidine	0.90	0.85	0.95	0.95	0.85	0.80	0.40	0.40	0.65	0.65
Arginine	7.75	7.60	11.95	5.65	7.10	7.90	4.50	4.75	6.30	6.80

[a] See footnote to Table 3.1.

TABLE 3.4

YIELDS OF GROUPS OF PROTEINS FROM THE HAIR AND HAIR FOLLICLE EXTRACTS

The groups of proteins were fractionated as described by the methods summarised in Table 3.2. Protein samples were dialysed exhaustively against water and the weight of protein was determined after freeze-drying. The values are expressed as a percentage of the total weight of proteins extracted. The absolute values given were from single batches of tissue extracts and the limits of variations were determined by comparison of each value from several different batches.

Protein group	Percentage content from F-SCMK	Percentage content from H-SCMK
1A	7 ± 2	2 ± 1
1B	9 ± 3	5 ± 1
2 (LoS)	71 ± 5	57 ± 3
3	3 ± 1	8 ± 2
4 (HiS)	10 ± 3	28 ± 2

relatively more group-1 and LoS proteins but less group-3 and HiS proteins than the H-SCMK extracts. Now, there were marked differences in the amino acid compositions of the different groups of proteins (*see* Table 3.3). Therefore the differences in the amino acid compositions between the hair and hair follicle SCMK and SCMK-B proteins (*see* Table 3.1) can be adequately accounted for by the differences in the amounts of the groups of proteins present in these two samples. This consideration had not been recognised in the previous studies of other workers in the wool system (Rogers, 1959b; Downes *et al.*, 1966a).

(c) FURTHER CHARACTERISATION OF GROUP-1 PROTEINS

Fig. 3.1b showed that group-1 proteins were present in both the SCMK-A and SCMK-B protein fractions, and this implied that the group-1A proteins are different from the group-1B proteins. The different electrophoretic mobilities (*see* Fig. 3.1b) and amino acid compositions (*see* Table 3.3 columns 1 - 4) support this conclusion.

The amino acid analyses of the group-1A proteins from both sources are very similar (Table 3.3) and were similar to those of the LoS proteins (*see* Table 3.3; *compare* columns 1 and 2 *with* columns 5 and 6). This suggested that the group-1A proteins were high molecular weight aggregates of the LoS proteins by analogy with the studies on wool proteins (Thompson and O'Donnell, 1964; Frater, 1966). In the group-1B proteins, the contents of arginine and citrulline were different. The presence of citrulline in the H-group-1B proteins suggested that they might have arisen from contaminating medulla (or inner root sheath) proteins, as there are marked similarities in the amino acid contents of these proteins (*see* Table 3.5). The arginine content of the F-group-1B proteins is approximately the same as the sum of the contents of arginine and citrulline in the H-group-1B and medulla proteins. These observations can be interpreted to mean that the F-group-1B

TABLE 3.5

AMINO ACID COMPOSITION OF THE GROUP-1B PROTEINS AND MEDULLA AND INNER ROOT SHEATH PROTEINS

The values are expressed as residues per 100 residues

Amino acid	Group-1B proteins [a]		Tryptic peptides of [b] guinea pig:	
	F	H	Hair medulla	Inner root sheath
SCMcysteine	2.45	1.95		
Aspartic acid	7.35	6.95	5.95	7.25
Threonine	2.65	2.85	1.80	3.20
Serine	5.00	4.85	3.35	7.20
Glutamic acid	26.85	27.85	33.95	21.55
Citrulline [c]	trace	7.95	9.85	3.35
Proline	2.70	2.20	2.15	3.50
Glycine	5.60	5.35	4.00	7.40
Alanine	4.85	4.65	4.80	6.25
Valine	4.55	4.15	4.25	4.90
Half-cystine	trace	trace	trace	trace
Methionine	0.85	0.90	0.60	2.30
Isoleucine	2.65	2.45	1.75	3.55
Leucine	9.75	9.95	9.20	9.70
Tyrosine	2.15	2.20	2.25	2.40
Phenylalanine	3.00	3.05	2.80	3.15
Lysine	4.70	4.90	8.75	9.00
Histidine	0.95	0.95	1.25	1.40
Arginine	11.45	5.65	3.35	3.75

[a] These values are from Table 3.3.

[b] These values are from Steinert *et al.* (1969).

[c] See footnote to Table 3.1.

proteins are precursors of the medulla (and inner root sheath) proteins that are also synthesised in the hair follicle and which presumably were solubilised by the urea extraction procedure. The H-group-1B proteins may have arisen from contaminating small pieces of medulla tissue which was broken up during grinding of the guinea pig hair prior to extraction. No further experiments were performed on either group during this work.

(d) FURTHER CHARACTERISATION OF GROUP-2 (LoS) PROTEINS

(1) Quantitative estimations by PAGE

The LoS proteins consisted of three main protein bands and these were termed components I, II and III, of increasing electrophoretic mobility as shown in Fig. 3.4.

In Fig. 3.4 is a PAGE experiment designed to determine the relative amounts of each component in the F-LoS and H-LoS protein fractions. It can be seen that the densitometer traces of the two samples were almost identical and this implies that each component was present in approximately the same amount in both extracts. If it can be assumed that the ratio of stain to protein was the same for each protein component, then in each case, component I represented about 52 % of the total protein and components II and III represented about 8 % and 20 % respectively. While these absolute values varied between different batches of proteins due to varying degrees of contamination by group-1 and group-3 proteins, the absolute ratios of 13 : 2 : 5 were maintained.

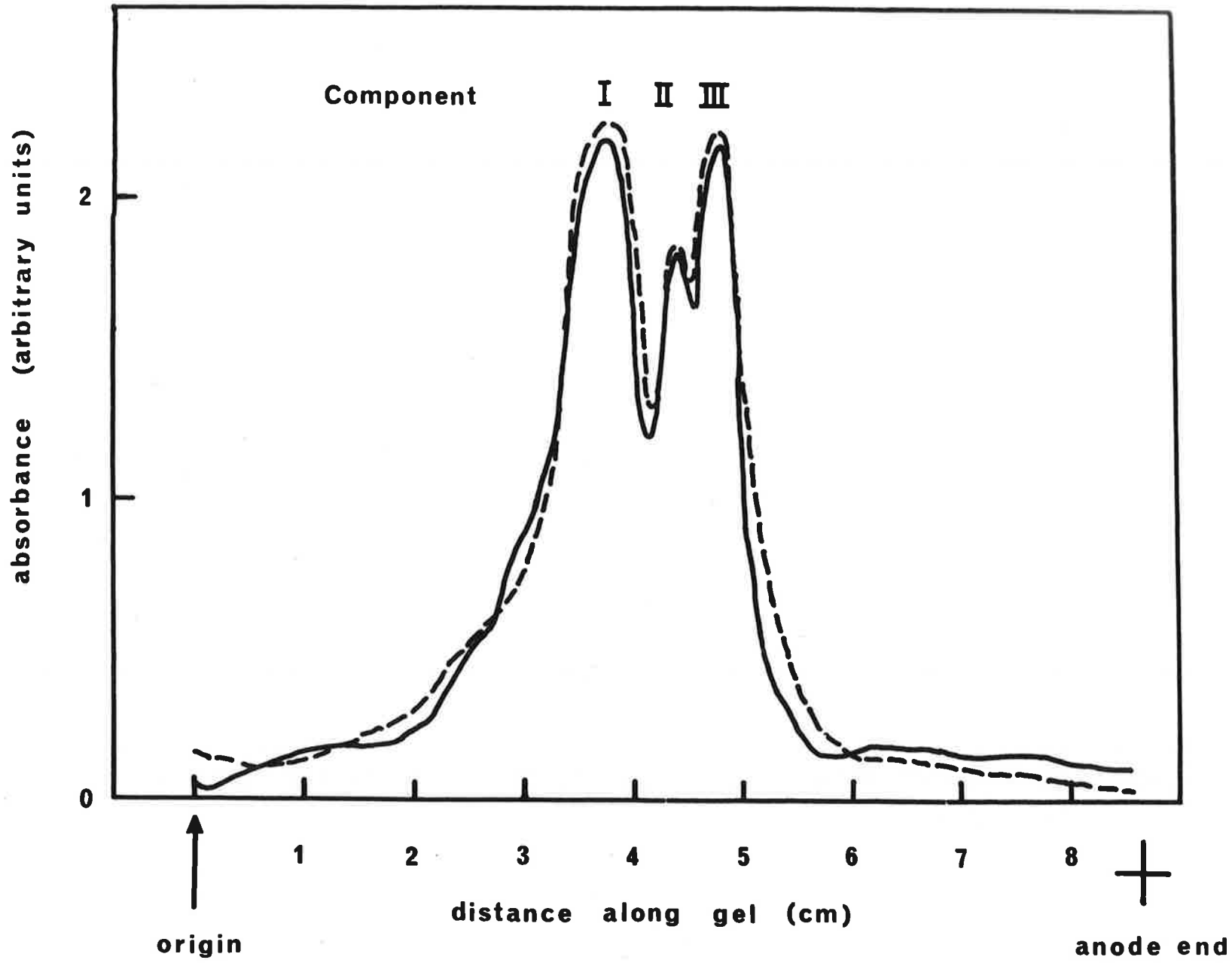
(2) Fractionation

The experiment shown in Fig. 3.3a showed that the LoS proteins could be separated from other protein groups by chromatography on DEAE-cellulose between the KCl concentration range of 0.05 M and 0.2 M (*see* Fig. 3.3a, peak 2). Further studies on the LoS proteins showed that each

FIGURE 3.4

QUANTITATIVE ESTIMATIONS OF THE RELATIVE AMOUNTS OF THE LoS COMPONENTS OF BOTH TISSUE EXTRACTS BY PAGE

Approximately 50 µg samples of the LoS proteins prepared from both extracts by the procedures described in Fig. 3.3 were electrophoresed for 4 h. The gels were stained and the densitometer traces were determined using a Uvicord apparatus. ——— , H-LoS, ----- , F-LoS. I, II and III are the different components of increasing mobility as described in the text.


Component
I
II
III
absorbance (arbitrary units)
2
1
0
1
2
3
4
5
6
7
8
origin
distance along gel (cm)
anode end

of the components could be separated simply by utilising smaller steps of increasing KCl concentration within this range. The elution profile of the H-LoS proteins is shown in Fig. 3.5a and the PAGE patterns corresponding to the peaks 1 - 6 are shown in Fig. 3.5b. Each component was completely separated from each other (peaks 3, 4 and 5). Similar fractionation was obtained under identical conditions for the F-LoS protein fraction.

(3) Purification

The group-1 and group-3 protein impurities that were still present in the separated components could be removed by repeated re-chromatography on Sephadex G-200. The protocol for the purification of H-I is illustrated in Fig. 3.6 in which case three chromatographic steps were employed. This component (and F-I) was the most difficult to purify by this procedure: the high and low molecular weight protein contaminants diminished only slightly on each re-chromatographic step, despite judicious pooling of fractions from the previous step. Amino acid analysis was used to check the nature of these peak 1 and peak 3 proteins as well as the main peak of H-I protein obtained from each chromatographic step. The amino acid compositions of the peak 1 samples were very similar and generally similar to the amino acid composition of the H-I samples, which implied that they were group-1A proteins and had arisen from H-I by aggregation during the concentration and subsequent chromatographic procedures. The amino acid composition of the peak 3 samples were constant and very similar to the composition of the group-3 proteins (*see* Table 3.3). The amino acid composition of the H-I samples before chromatography and the samples at the chromatographic steps (a) and (b) showed progressive decreases in their SCMcysteine, serine, glycine and tyrosine contents, but the analyses of the H-I (b) and (c) samples were constant. These observations suggested that on re-chromatography group-3 proteins were being successively removed, and after

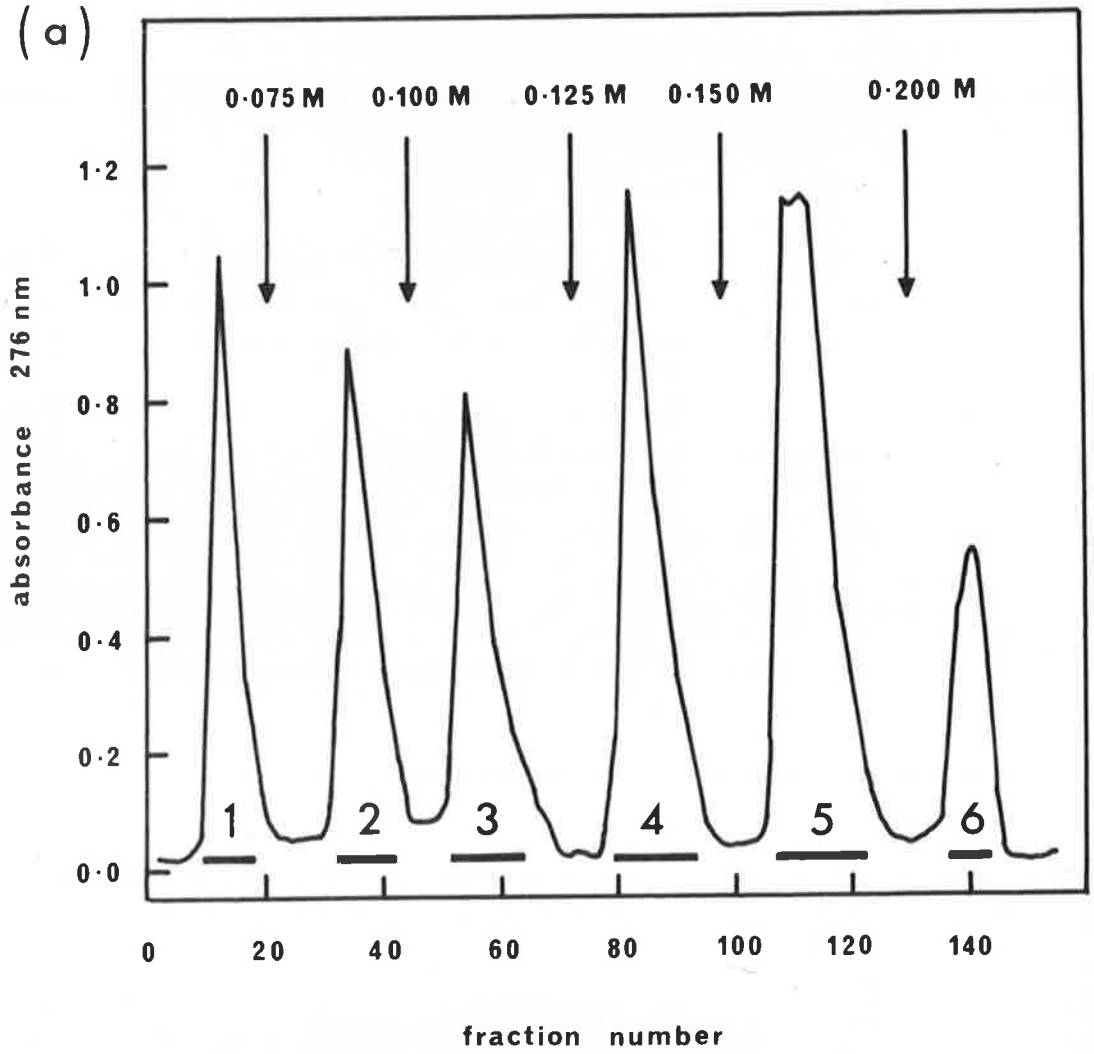
FIGURE 3.5

(a) CHROMATOGRAPHY OF THE H-LoS PROTEINS ON DEAE-CELLULOSE

The LoS proteins that eluted from DEAE-cellulose between the KCl concentrations of 0.05 M and 0.2 M (*see* Fig. 3.3a) were chromatographed on DEAE-cellulose using the smaller steps of increasing KCl concentrations of 0.075 M, 0.10 M, 0.125 M, 0.15 M and 0.20 M and were applied as shown. A column 20 x 2.5 cm was loaded with about 0.5 g of H-LoS proteins in a starting buffer of 8 M urea, 50 mM KCl, 20 mM tris-HCl (pH 7.6) and 1 mM EDTA. The flow rate was 80 ml/h and fraction size 15 ml. ——— , absorbance at 276 nm. The bars 1 - 6 refer to the tubes that were pooled for characterisation of their contents.

(b) PAGE OF FRACTION FROM COLUMN

Samples from the pooled tubes of *(a)* were applied to the gels without prior desalting. The volume of sample was adjusted so that it contained about 50 µg of protein. The gels were stopped when the tracking dye band reached the end of the gel. Electrophoresis was for 3 - 4 h. H = H-SCMK; 1 - 6 are gels of the pooled fractions enumerated as such from *(a)*.


(a)
0·075 M
0·100 M
0·125 M
0·150 M
0·200 M
absorbance 276 nm
1·2
1·0
0·8
0·6
0·4
0·2
0·0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
fraction number

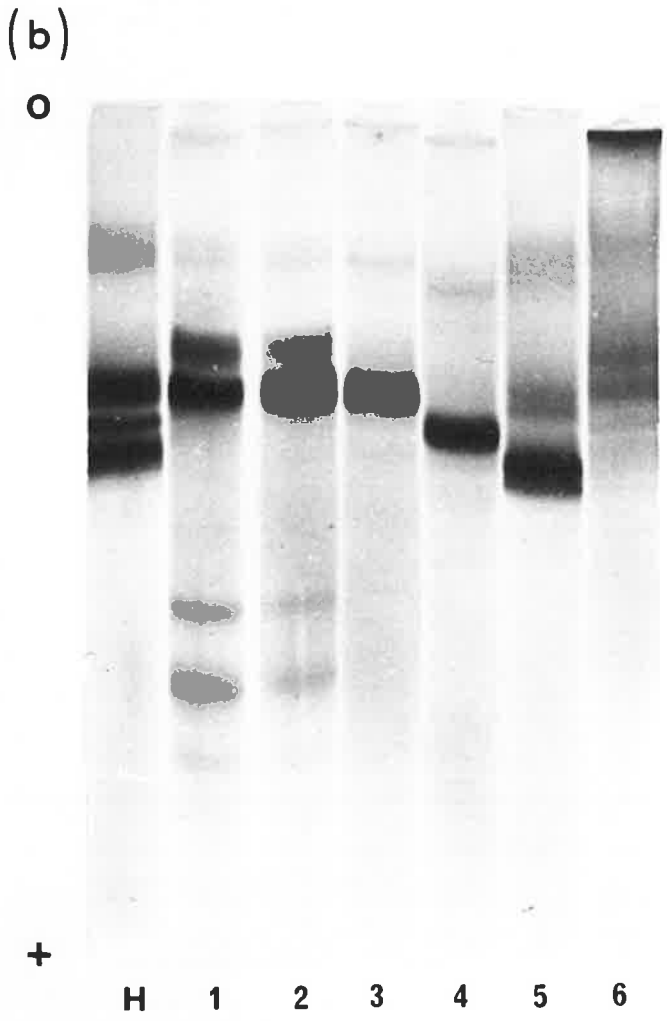
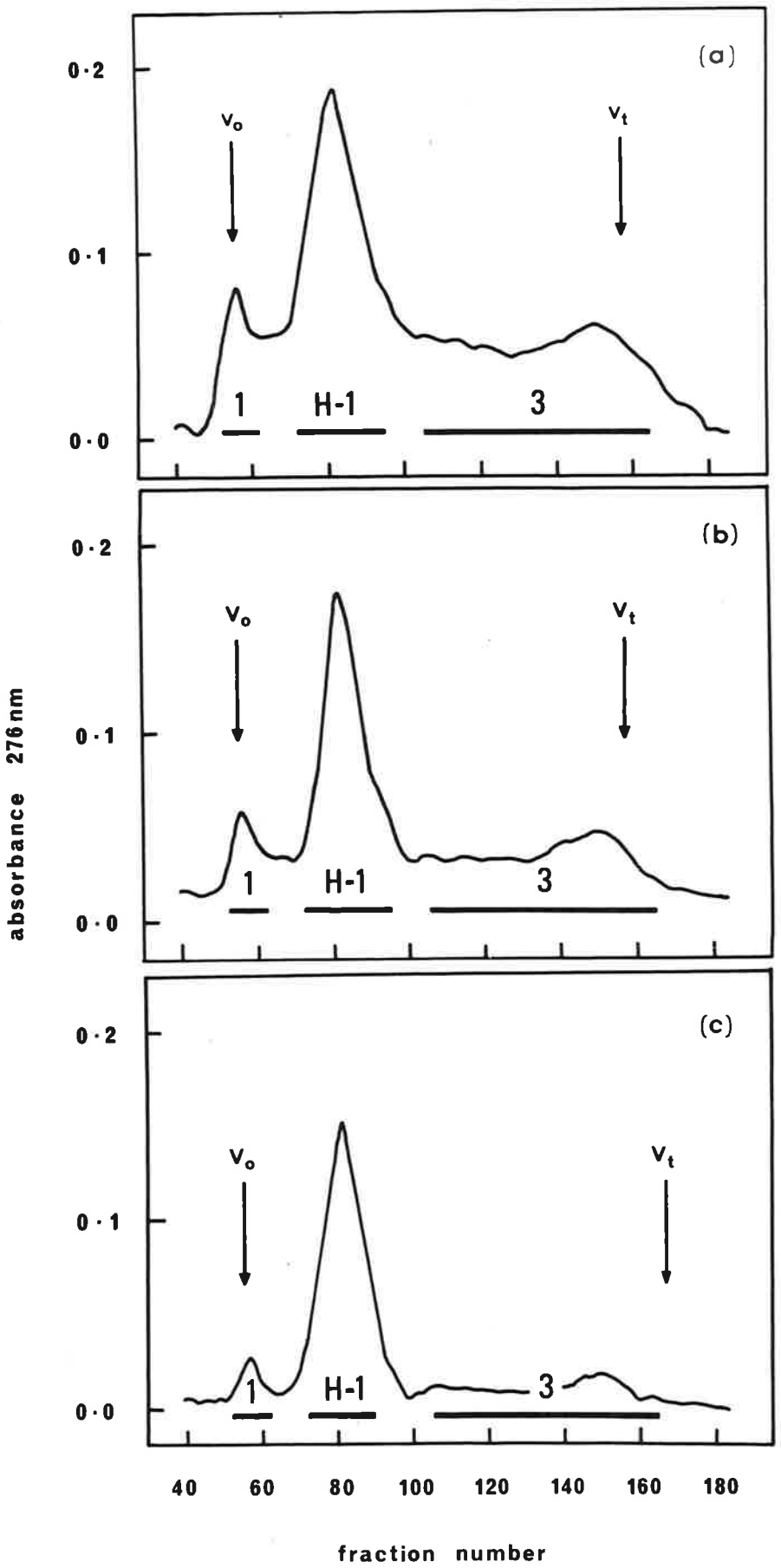

(b)
O
+
H
1
2
3
4
5
6

FIGURE 3.6

PURIFICATION OF COMPONENT H-I BY RE-CHROMATOGRAPHY ON SEPHADEX G-200

A 175 x 5.5 cm Sephadex G-200 column was prepared as described in General Methods (*see* page 40) and was equilibrated with the buffer of 8 M urea, 0.2 M KCl, 50 mM tris-HCl (pH 7.6) and 1 mM EDTA (*see* Fig. 3.2). The flow rate was 45 ml/h and fraction size 25 ml. The column was first loaded with about 400 mg of crude H-I (in 30 ml of buffer). The tubes containing the main peak of H-I were pooled, dialysed, concentrated by ultrafiltration and then re-chromatographed on the same column. The resulting H-I sample was re-chromatographed a third time. The bars labelled 1 and 3 at each chromatographic step refer to the tubes that were pooled for amino acid analyses of their contents. ——— , absorbance at 276 nm.


(a)
(b)
(c)
V_o
V_t
1
H-1
3
0·2
0·1
0·0
absorbance 276nm
40
60
80
100
120
140
160
180
fraction number

three re-chromatographic steps, near-complete removal had been achieved. These observations are similar to those of Jeffrey (1969) where several chromatographic steps were necessary to prepare wool LoS proteins of homogeneous molecular weight.

On the other hand, components II and III were more amenable to purification by the re-chromatographic procedures. These components gave constant amino acid compositions after two chromatographic steps.

The yields of the various purified protein components are given in Table 3.6 and have been corrected relative to 1.0 g of the crude LoS preparations. Component III was obtained in highest yield, despite the fact that component I was more abundant in the original extracts (*see* Table 3.4). Indeed, component H-III was the easiest to prepare in quantity since hair proteins were more readily prepared than hair follicle proteins.

(4) Properties of the purified LoS components

PAGE

PAGE patterns of the purified protein components are shown in Fig. 3.7 and it is seen that the respective components from both H and F origin co-electrophoresed precisely indicating close similarity. Of interest also is the observation that on extended electrophoresis, component III from both sources appeared as two distinct bands. It was not possible to separate these two protein species by any procedures. No further fractionation of either component I or II was observed.

Attempts to characterise the H and F protein fractions or purified components on acrylamide gels at acid pH values in order to investigate further the similarities and complexities of individual components were not successful since the proteins were insoluble.

Amino acid analysis

TABLE 3.6

YIELDS OF THE PURIFIED LoS PROTEIN COMPONENTS

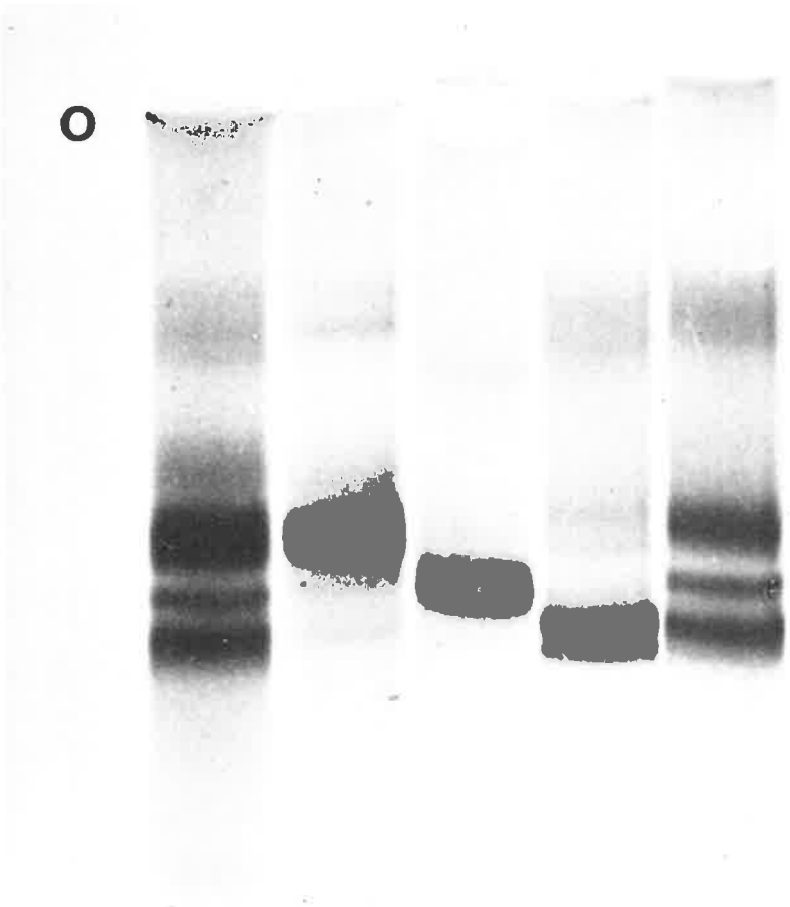
The values are expressed as mg of the purified components per 1.0 g of the F-LoS and H-LoS protein fractions. The values are representative only since there were marked variations in yields between different batches.

Protein fraction	I	II	III
F-LoS	186	98	204
H-LoS	194	100	210

FIGURE 3.7

PAGE PATTERNS OF THE PURIFIED LoS PROTEIN COMPONENTS

Each gel was loaded with 50 µg of sample and was electrophoresed for 7 h. H = mixture of equal amounts of H-LoS and F-LoS proteins. Gels I, II and III were loaded with equal mixtures of the respective H and F components. M = mixture of each component from both sources.



+ H I II III M

The amino acid analyses are summarised in Table 3.7. The amino acid composition of the corresponding H and F components are very similar. Differences that are observed were not greater than the differences obtained on duplicate analyses of the same preparation or different preparations of a single component. The components II and III are very similar, although the latter had a slightly higher content of acidic amino acid residues. In addition, there are marked similarities in the amino acid composition of the guinea pig component I and wool "component 7" and guinea pig components II and III and wool "component 8" proteins (Thompson and O'Donnell, 1965; Frater, 1966), suggesting that there may be close chemical and structural homologies between these proteins.

Despite the long exposures of the proteins isolated in the present work to solutions containing urea, no modifications of protein amino groups by cyanate ions present in the urea solutions were detected by amino acid analysis.

Molecular weight and physicochemical properties

Samples of the purified components were chromatographed on calibrated columns of Sephadex G-200. The molecular weight estimates of the proteins obtained by this method are summarised in Table 3.8. It is noteworthy that these values can be subject to errors of ± 10 % (Whitaker, 1963), despite the use of rigorously controlled experimental conditions. Nevertheless, it is clear that component I from both sources had a significantly higher molecular weight (about 48 000 daltons) than components II and III (about 43 000 daltons each). The estimated molecular weights of the H-SCMK-A and F-SCMK-A protein fractions were 45 000 daltons each.

The molecular weight of the group-1 proteins obtained from H-I (*see* Fig. 3.6) was 90 - 100 000 daltons, suggesting that this material was in fact a simple dimer of component H-I.

TABLE 3.7

AMINO ACID COMPOSITION OF THE PURIFIED LoS PROTEIN COMPONENTS

The values are expressed as residues per 100 residues and are the averages of analyses on three different batches of proteins. Amounts indicated by "trace" were present in < 0.05 moles percent.

Amino acid	F components			H components		
	I	II	III	I	II	III
SCMcysteine	5.85	5.70	5.45	5.60	6.00	5.70
Aspartic acid	8.25	9.40	10.15	8.50	9.45	9.95
Threonine	4.85	5.85	4.85	4.65	5.65	4.90
Serine	7.00	7.80	7.90	6.90	8.05	8.00
Glutamic acid	19.10	21.90	22.75	18.65	22.05	22.75
Proline	3.00	3.25	3.65	2.90	3.40	3.55
Glycine	8.05	5.35	5.05	8.10	5.30	4.70
Alanine	8.05	5.85	6.10	8.35	6.00	5.80
Valine	5.80	4.80	4.50	5.60	5.00	4.75
Half-cystine	trace	0.05	trace	0.05	trace	0.05
Methionine	1.15	0.25	0.25	1.10	0.25	0.25
Isoleucine	4.00	3.25	3.40	3.85	3.25	3.45
Leucine	9.15	10.45	11.55	9.05	10.15	11.50
Tyrosine	3.15	3.25	3.05	2.85	3.40	3.15
Phenylalanine	2.70	1.75	1.80	2.65	1.95	1.75
Lysine	4.60	2.35	2.90	4.45	2.15	2.75
Histidine	0.90	0.90	0.75	0.90	0.85	0.80
Arginine	7.90	8.15	7.75	7.85	8.55	7.85

TABLE 3.8

MOLECULAR WEIGHTS OF PURIFIED LoS PROTEINS ESTIMATED BY CHROMATOGRAPHY ON SEPHADEX G-200

These values were determined using a 165 x 1.6 cm column of Sephadex G-200 that had been calibrated with standard proteins of known molecular weight. The calibration protocol and all other details are as in Fig. 3.2.

Protein component	Molecular weight (daltons)
F-I	48 000
F-II	43 000
F-III	43 000
H-I	48 500
H-II	43 500
H-III	43 000

Certain physicochemical parameters of several components were determined (Table 3.9). The molecular weight values calculated from the Svedberg equation are also given (Table 3.9). The values obtained for the corresponding H and F components are identical within the limits of experimental error. In addition, the molecular weights estimated by analytical ultracentrifugation are in good agreement with the values estimated by Sephadex G-200 chromatography (*see* Table 3.8).

(e) IDENTIFICATION OF THE GROUP-3 PROTEINS

The amino acid analyses of the group-3 proteins from both hair and hair follicle extracts were given in Table 3.3 and it was seen that there were no significant differences. The high contents of the amino acids serine, glycine and tyrosine suggested that they were of the same class of proteins described in wool protein extracts. Thus the proteins may have arisen in part from cuticle or membrane components of the hair fibre (Crewther *et al.*, 1965) as well as from some cortical component (Gillespie, 1971). The molecular weights of these proteins were not determined accurately but were of the order of < 10 - 14 000 daltons. No further studies were conducted on them.

(f) FURTHER CHARACTERISATION OF GROUP-4 (HiS) PROTEINS

(1) Quantitative estimates by PAGE

The HiS proteins consisted of a complex number of components when separated by PAGE. In Fig. 3.8 is shown a PAGE experiment comparing the densitometer traces of the HiS proteins prepared from both hair and hair follicle extracts. It is seen that while the traces of both samples were similar, there were marked differences in the amounts of the bands: F-HiS proteins contained less slower-moving but more faster-moving bands than the H-HiS proteins. The ratios of the amounts of these bands varied between

TABLE 3.9

PHYSICOCHEMICAL PROPERTIES OF THE LoS PROTEIN COMPONENTS

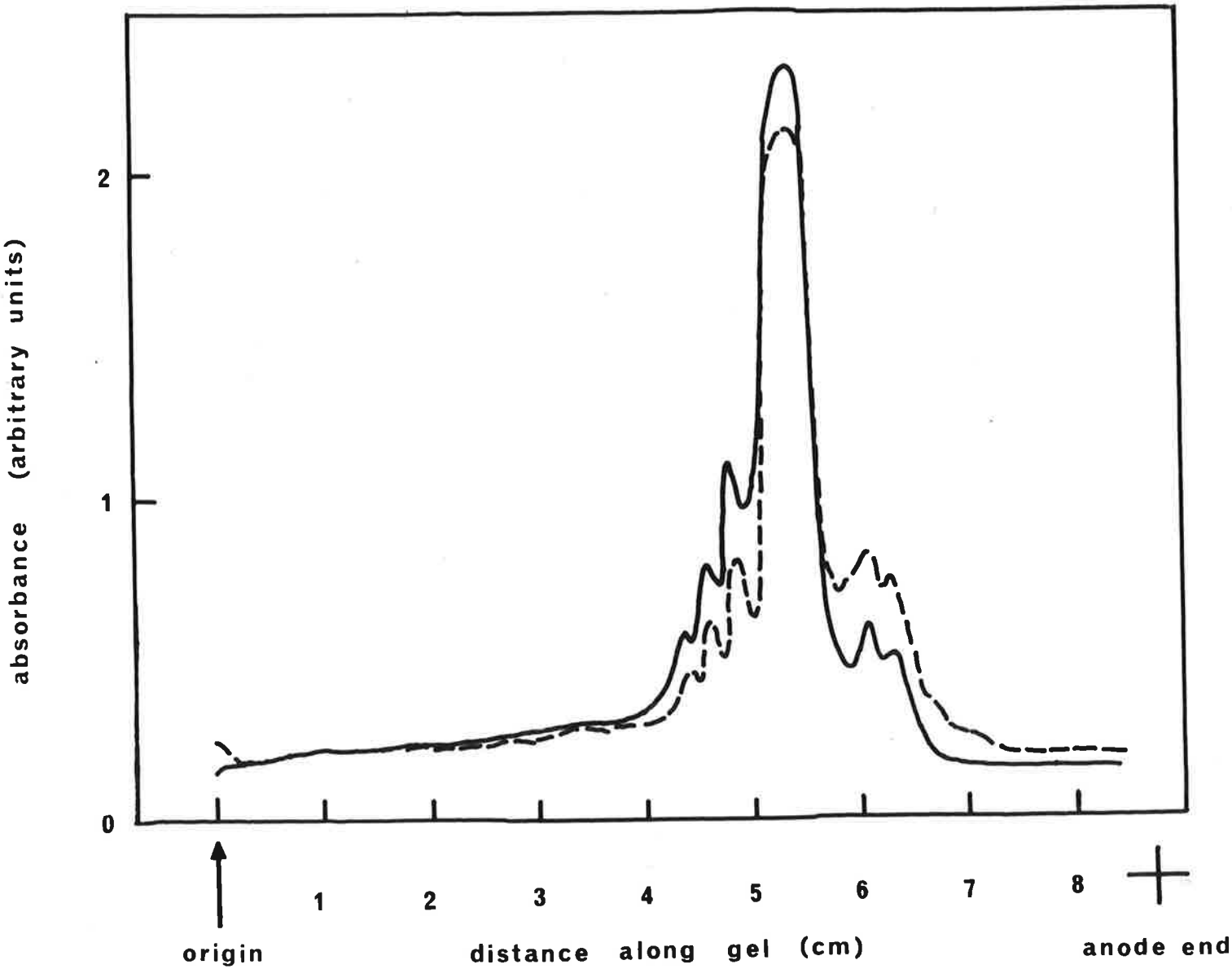
These were determined by analytical ultracentrifugation. The $s^{o}_{20,w}$ and $D_{20,w}$ values were determined by sedimentation velocity experiments as described in Methods. The partial specific volumes $\bar{v}$ were determined from the amino acid composition (Schachman, 1957). The molecular weights were then calculated from these values using the Svedberg equation.

Property	Component				
	F-I	F-III	H-I	H-II	H-III
$s^{o}_{20,w}$ (sec^{-1} x 10^{-13})	3.10	3.00	3.18	2.96	2.98
$D_{20,w}$ (cm^2/sec x 10^{-7})	5.54	6.06	5.78	5.92	6.02
$\bar{v}$ (cm^3/g)	0.724	0.723	0.727	0.720	0.725
Molecular weight (daltons)	49 000	43 000	48 500	43 000	43 000

FIGURE 3.8

QUANTITATIVE ESTIMATIONS OF THE RELATIVE AMOUNTS OF THE HiS PROTEINS OF BOTH TISSUE EXTRACTS BY PAGE

Approximately 50 µg samples of the HiS proteins prepared from both extracts by the procedure described in Fig. 3.3 were electrophoresed for 3 h. The gels were stained and the densitometer traces were prepared (*see* Fig. 3.4).
——— , H-HiS; ----- , F-HiS.


absorbance (arbitrary units)
2
1
0
origin
1
2
3
4
5
6
7
8
distance along gel (cm)
anode end

different batches of proteins, but the same relationship was always evident.

(2) Chromatography on Sephadex G-100

The elution profiles of the F-HiS and H-HiS proteins from a calibrated column of Sephadex G-100 are shown in Fig. 3.9a. The profiles were similar but marked quantitative differences were apparent: the F-HiS proteins contained fewer high molecular weight proteins but more low molecular weight proteins than the H-HiS proteins. The PAGE patterns of the peaks 1 - 3 of both samples were similar and the patterns for the H-HiS proteins are shown in Fig. 3.9b and it is seen that the high molecular weight proteins electrophoresed least rapidly. Therefore, the quantitative differences seen in this experiment were the same as those of the previous experiment (*see* Fig. 3.8).

(3) Chromatography on DEAE-cellulose

The experiments of Gillespie (1963) showed that wool HiS proteins could be fractionated most satisfactorily on DEAE-cellulose at pH 4.5. When guinea pig HiS proteins were chromatographed under similar conditions the elution profiles of Fig. 3.10a were obtained. Again, the profiles were similar but quantitative differences were observed. The PAGE patterns of the peaks 1 - 4 are given in Fig. 3.10b and in conjunction with Fig. 3.10a it is seen that the F-HiS proteins of greatest mobility were more abundant and the proteins of lowest mobility were less abundant than in the H-HiS protein fraction.

Since the four peaks of Fig. 3.10a contained different proteins, the peaks 1 - 4 were classified as subgroups of HiS proteins. The relative amounts of the subgroups were determined (Table 3.10). Although variations were evident between different batches of proteins, there were always 2 - 3 times more subgroup-1 proteins in F-HiS than in H-HiS, but only 0.5 - 0.3 of the subgroup-4 proteins.

The amino acid compositions of the subgroups are given in

FIGURE 3.9

(a) CHROMATOGRAPHY OF HiS PROTEINS ON SEPHADEX G-100

A 92 x 1.0 cm column of Sephadex G-100 was prepared as described in General Methods (*see* page 40) and was equilibrated using 50 % aqueous formic acid. The flow rate was 5 ml/h and fraction size 1.25 ml. The column was calibrated before use (see below). In each case about 25 mg protein was chromatographed. The absorbance was measured at 276 nm. ——— , H-HiS; ----- , F-HiS. The bars 1 - 3 refer to the tubes that were pooled for characterisation of their contents.

CALIBRATION PROTOCOL

Protein	Molecular weight (daltons)	V_e (fraction number)
Albumin	68 000	21.0 (V_o)
Pepsin	35 500	27.5
Chymotrypsinogen A	25 500	31.0
Apomyoglobin	17 200	34.5
Lysozyme	14 700	37.5
Feather keratin	10 500	42.5
Insulin A	2 450	55.5 (V_t)

(b) PAGE OF FRACTIONS FROM COLUMN

Samples from the pooled fractions of the H-HiS experiment of *(a)* were evaporated to dryness *in vacuo* and a sample equivalent to about 50 µg of protein was electrophoresed for 3 h. H = H-HiS; 1 - 3 are from the pooled fractions enumerated as such in *(a)*.

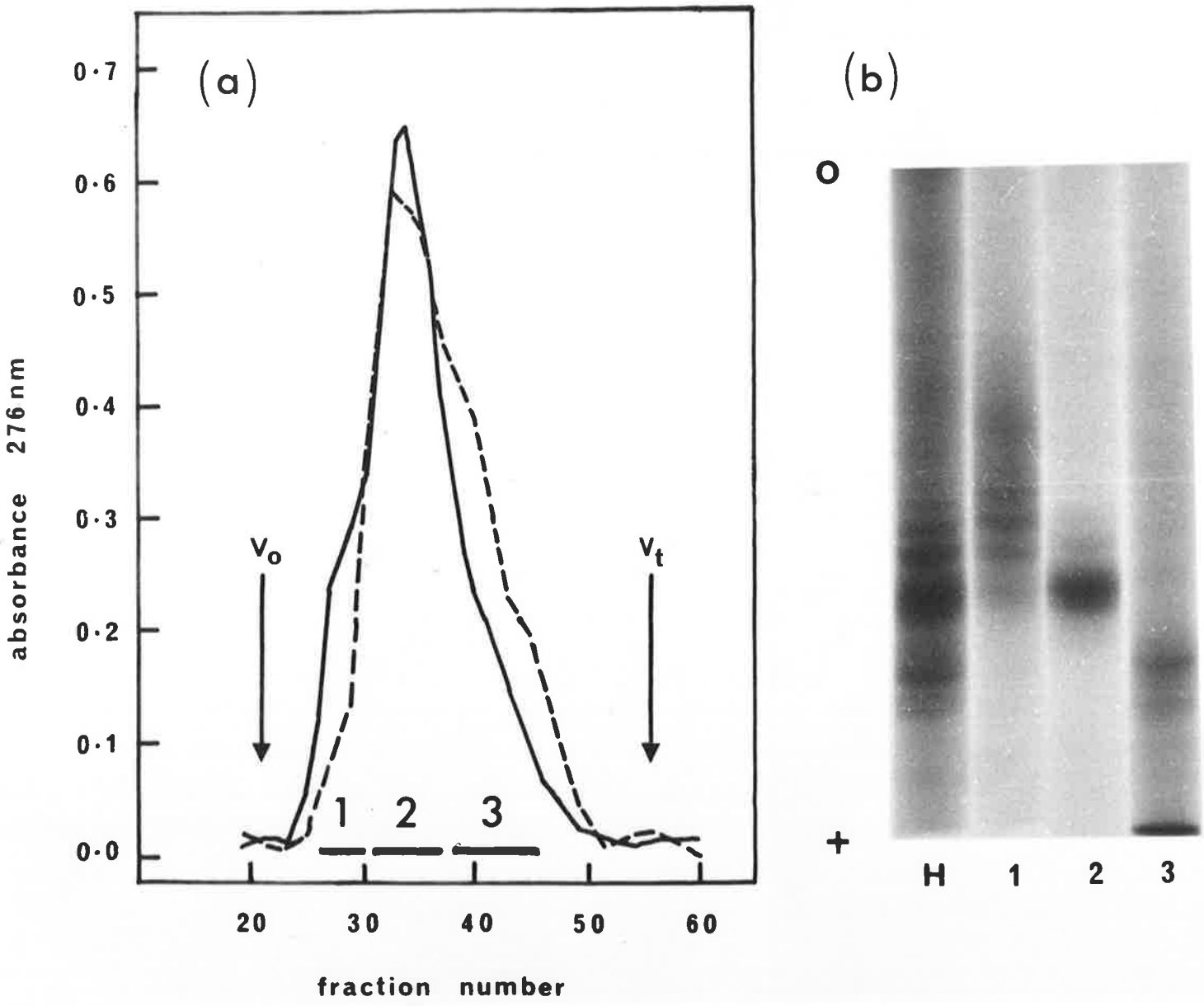

(a)
(b)
absorbance 276nm
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
V_o
V_t
1
2
3
20
30
40
50
60
fraction number
O
+
H
1
2
3

FIGURE 3.10

(a) CHROMATOGRAPHY OF HiS PROTEINS ON DEAE-CELLULOSE

A 15 x 1.6 cm column of DEAE-cellulose was prepared and equilibrated in a starting buffer of 20 mM sodium acetate (pH 4.5) and 0.2 M NaCl (Gillespie, 1963). The flow rate was 10 ml/h and fraction size 3.0 ml. Approximately 50 mg of each HiS protein was loaded. At tube 10 a linear gradient of increasing NaCl concentration was applied (from 0.2 M to 0.75 M; 2 chambers, 100 ml in each). The aborbance was measured at 276 nm. ——— , H-HiS; ----- , F-HiS. The bars 1 - 4 refer to the tubes that were pooled for characterisation of their contents. The numbers 1 - 4 referred to HiS subgroups 1 - 4, repectively.

(b) PAGE OF FRACTIONS FROM COLUMN

Samples from the pooled fractions of the F-HiS experiment were applied to the gels without prior desalting. The volume of sample was adjusted so that each gel was loaded with about 50 µg of protein. Gels were stopped when the tracking dye reached the end of the gel. Electrophoresis was for 3 - 3.5 h. F = F-HiS. 1 - 4 are from the pooled fractions enumerated as such in *(a)*.

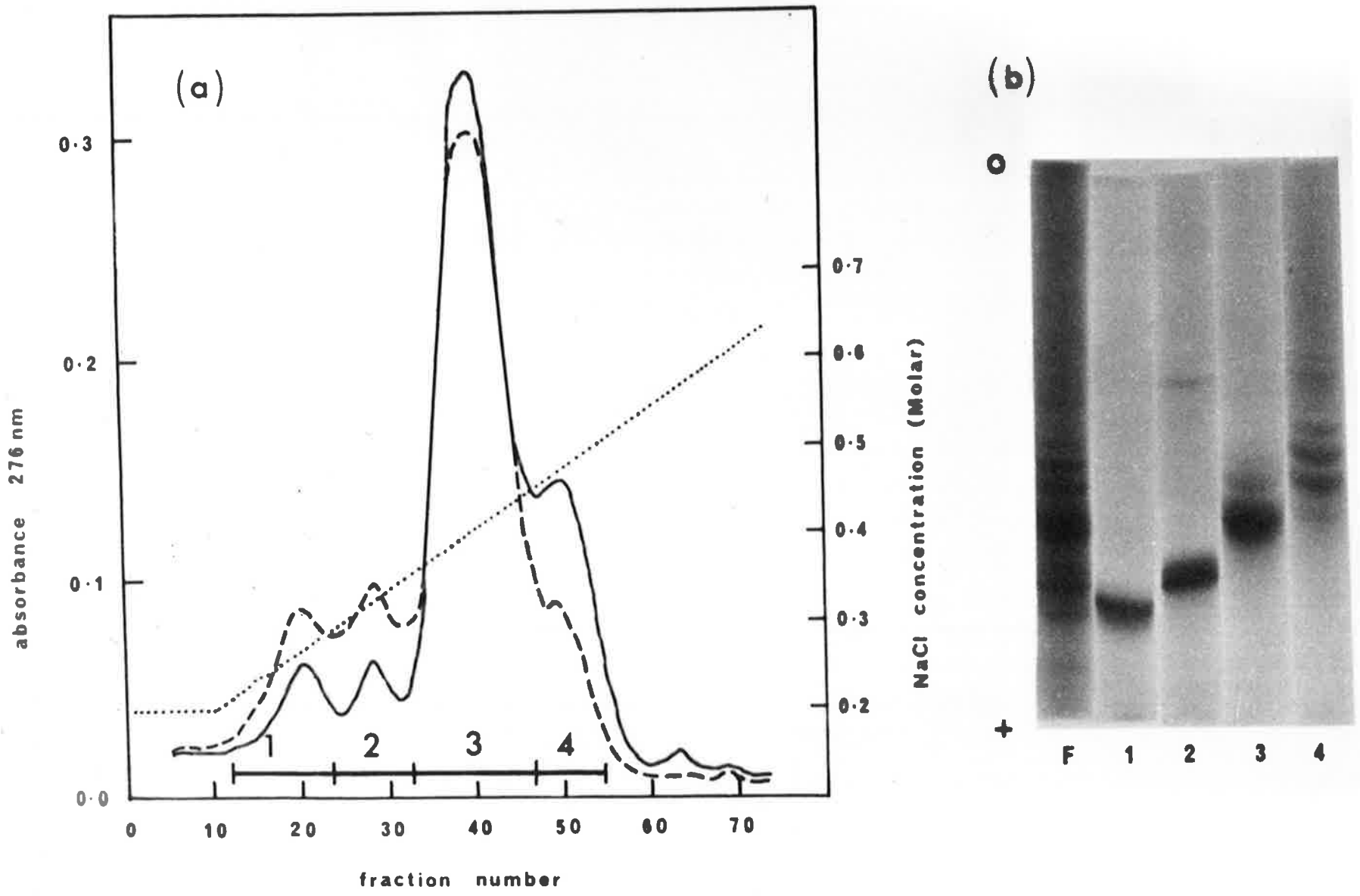

(a)
absorbance 276 nm
0·0
0·1
0·2
0·3
0
10
20
30
40
50
60
70
fraction number
0·2
0·3
0·4
0·5
0·6
0·7
NaCl concentration (Molar)
1
2
3
4
(b)
o
+
F
1
2
3
4

TABLE 3.10

YIELDS OF SUBGROUPS OF PROTEINS FROM HAIR AND HAIR FOLLICLE HiS PROTEINS

The subgroups of proteins were fractionated on DEAE-cellulose as shown in Fig. 3.10. The pooled fractions were dialysed exhaustively against water and the weight of protein was determined after freeze-drying. The yields are expressed as percentages of the total weight of the proteins used before fractionation. The absolute values given are from single batches of proteins and the limits of variations were determined by comparison of each value from at least three different batches.

Protein Subgroup	Percentage content of F-HiS	H-HiS
1	13 ± 3	5 ± 2
2	18 ± 4	8 ± 1
3	61 ± 5	69 ± 6
4	8 ± 1	18 ± 3

Table 3.11 and the molecular weights, which were determined by chromatography on Sephadex G-100, are also given. Several observations are apparent. Firstly, the corresponding hair and hair follicle HiS protein subgroups had the same amino acid compositions. When this observation is considered together with the PAGE and chromatographic properties of the HiS proteins of both sources, it is concluded that there are no significant differences in the properties of the HiS proteins from both hair and hair follicle origin. Secondly, the proteins of lowest molecular weight and lowest affinity for DEAE-cellulose (that is, of the lowest negative charge), contained the lowest levels of SCMcysteine and the SCMcysteine content of the subgroups increased as their molecular weights and charges increased. Significant gradations in the contents of other amino acids are also evident: the levels of serine, proline and arginine increased as the SCMcysteine content and molecular weight increased, but the levels of aspartic acid, glycine, alanine, valine, leucine, phenylalanine and lysine showed a reversed relationship. The subgroup-4 proteins contained the highest levels of the four amino acids SCMcysteine, threonine, serine and proline (64.7 moles percent) compared with the subgroup-1 proteins (50.7 moles percent). Since these amino acids inhibit the formation of helical structures in proteins, it is possible that the subgroup-4 proteins had less structural order than the subgroup-1 proteins. Studies on wool HiS proteins showed that there was a linear relationship between the SCMcysteine content of the proteins and their molecular weight (Gillespie, 1963) and such a relationship also existed with the guinea pig HiS proteins (Fig. 3.11).

D DISCUSSION

In this chapter methods have been established for the fractionation of the different groups of proteins that can be extracted from guinea pig hair and hair follicle tissue. Three different procedures were

TABLE 3.11

AMINO ACID COMPOSITION OF THE HiS PROTEIN SUBGROUPS FROM BOTH EXTRACTS

The values are expressed as residues per 100 residues and are the averages of analyses on at least two different batches of proteins.

Amino acid	Group-4 [a]		Subgroup-1		Subgroup-2		Subgroup-3		Subgroup-4	
	F	H	F	H	F	H	F	H	F	H
SCMcysteine	21.65	23.40	18.90	19.10	20.90	20.80	24.60	23.85	26.80	27.45
Aspartic acid	2.30	2.35	4.05	3.90	3.20	3.20	2.25	2.10	1.10	1.20
Threonine	9.95	10.40	9.55	9.90	10.35	10.25	10.75	10.50	9.55	8.95
Serine	12.45	12.55	11.20	11.00	11.95	11.90	11.75	12.00	14.55	14.25
Glutamic acid	9.90	10.20	9.20	9.50	10.35	10.30	10.35	10.25	10.25	10.70
Proline	13.45	13.05	11.00	11.55	12.65	12.45	13.05	13.05	14.20	13.85
Glycine	5.85	5.45	8.05	7.70	7.10	6.75	5.45	5.55	3.95	3.70
Alanine	3.00	2.95	3.85	3.70	3.50	3.25	2.65	2.95	1.95	1.90
Valine	4.90	4.45	6.95	6.60	5.75	5.40	4.55	4.50	3.00	3.35
Methionine			0.05	0.10						
Isoleucine	2.70	2.55	3.05	2.95	2.60	2.65	2.30	2.60	2.35	2.40
Leucine	3.10	2.80	3.55	3.45	2.95	2.95	2.65	2.85	1.75	1.95
Tyrosine	1.15	1.20	1.20	1.15	1.45	1.30	1.10	1.20	0.90	0.85
Phenylalanine	0.75	0.75	1.20	1.35	0.95	1.00	0.70	0.75	0.55	0.65
Lysine	0.50	0.55	0.75	0.70	0.60	0.60	0.45	0.45	0.40	0.35
Histidine	0.65	0.65	1.20	0.95	0.70	0.80	0.55	0.55	0.40	0.45
Arginine	6.30	6.80	4.85	5.05	6.20	6.15	6.40	6.35	7.30	7.35
Molecular weight (daltons): [b]			10 000		14 000		22 000		28 000	

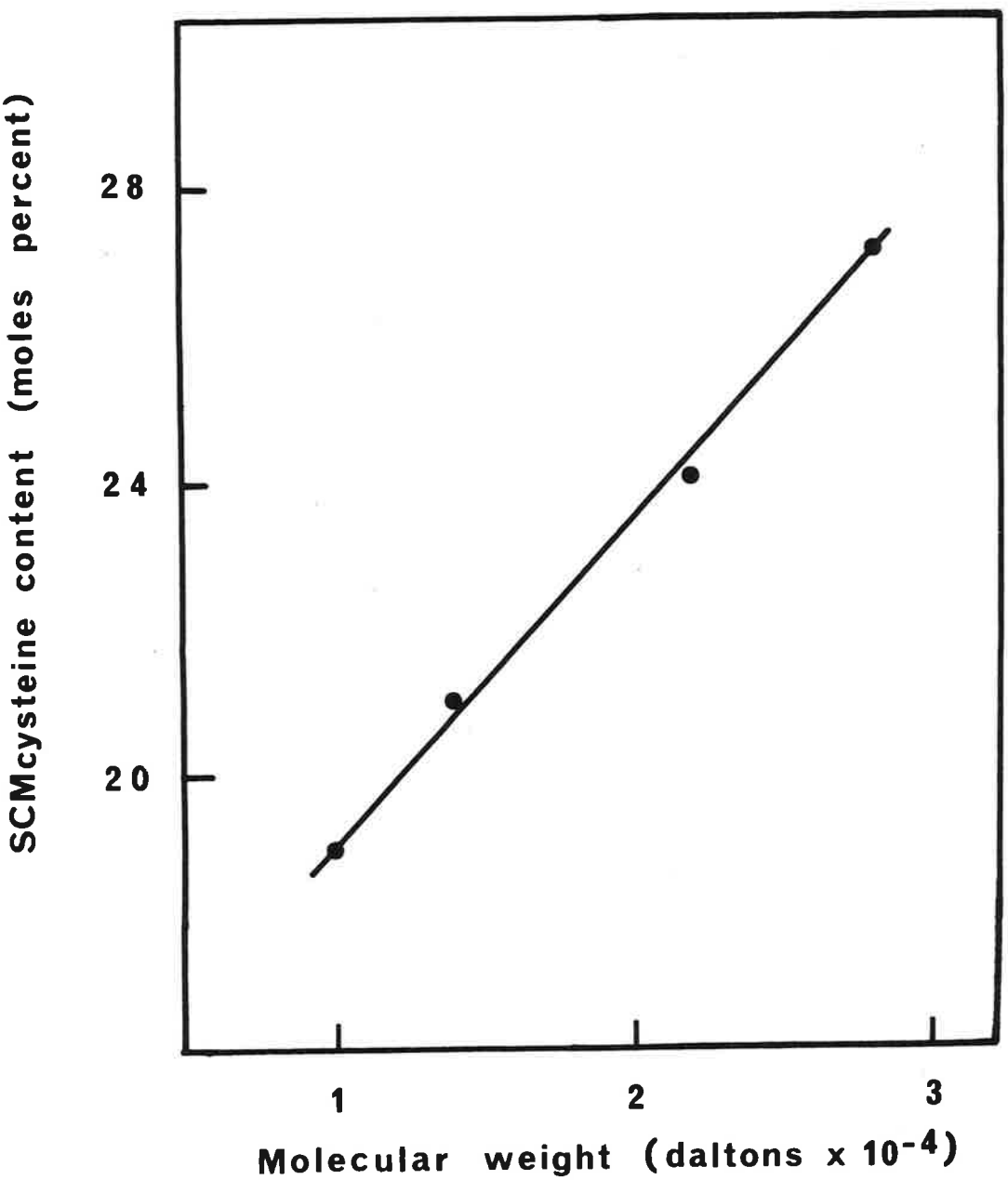
[a] These values are from Table 3.3.

[b] These were determined by chromatography on the calibrated Sephadex G-100 column (*see* Fig. 3.9).

FIGURE 3.11

RELATIONSHIP BETWEEN SCMCYSTEINE CONTENT OF THE HiS PROTEINS AND MOLECULAR WEIGHT

The data for the figure were taken from Table 3.11


SCMcysteine content (moles percent)
28
24
20
1
2
3
Molecular weight (daltons x 10-4)

employed: chromatography on Sephadex G-200 and DEAE-cellulose and acid precipitation at pH 4.4. Four different groups of proteins were delineated and each group was characterised using several different properties.

Group-2 proteins were defined as the main LoS proteins. Group-3 proteins were similar to a class of proteins that exist in wool extracts that contain high levels of the amino acids glycine and tyrosine. Group-4 proteins were defined as the HiS proteins. Group-1 proteins contained two distinctly different types of proteins: the group-1A proteins (insoluble at pH 4.4) appeared to be aggregates of the main LoS proteins and the group-1B proteins (soluble at pH 4.4) were thought to originate from the medulla and inner root sheath layers of the hair follicle (see later discussion).

With the sole exception of the group-1B proteins, the types and properties of all hair and hair follicle proteins were identical as far as could be determined in the present experiments.

There were, however, two significant quantitative differences in the types of proteins. The LoS proteins were more abundant in the F-SCMK extracts than in the H-SCMK extracts and HiS proteins were markedly less abundant. Therefore, either the rate of LoS protein synthesis decreases or the rate of HiS protein synthesis increases towards the terminal stages of development in the hair follicle. The present experiments are unable to distinguish these possibilities. Secondly, there were quantitative variations within the HiS proteins themselves: there were more subgroup-1 proteins (of lowest molecular weight and SCMcysteine content) in the F-HiS fraction than in the H-HiS fraction and less subgroup-4 proteins (of highest SCMcysteine content and molecular weight). Therefore, as before, the relative rates of synthesis of the proteins of highest molecular weight and SCMcysteine content must increase at terminal stages of development. Again, the present experiments cannot clarify this point.

One definitive approach to these problems was adopted by Fraser (1969) in which studies were made on the properties of the proteins isolated from various levels of the developing wool follicle with those extracted from fully keratinised wool. In addition, Fraser (1969) investigated {^{35}S}cystine incorporation into the HiS proteins of various levels of the wool follicle. The method Fraser (1969) used for the separation of the wool follicle cells could be criticised as it involved use of proteolytic digestion. It is possible that significant damage to the cellular contents could have occurred, especially to those of the highest levels of the follicle, since these cells were exposed to the highest concentrations of enzyme for the longest periods of time. Notwithstanding this criticism, Fraser (1969) showed that the wool LoS proteins were synthesised at an approximately constant rate throughout the follicle. The rate of HiS protein synthesis was not constant but increased "exponentially" in the higher levels of the follicle such that about 50 % of the HiS proteins were deposited at the terminal stages of development. These observations would therefore appear to explain the differences mentioned earlier if they are applicable to the guinea pig hair follicle system.

As discussed in Chapter One, hair follicle proteins could be synthesised by the classical ribosomal-dependent mechanism or by a non-ribosomal-dependent mechanism, or a combination of both (*see* page 32). Thus at the terminal stages of development, the HiS proteins of high molecular weight and SCMcysteine content could be synthesised by any one or more of these mechanisms. However, it was shown in the present chapter that the subgroups of F-HiS proteins are chemically indistinguishable from the subgroups of H-HiS proteins using several different criteria for the basis of comparison. This means that all of the proteins must have been synthesised by a process involving the ordered addition of amino acids so as to

maintain the properties of the proteins. The only mechanism which could satisfy this requirement is the classical ribosomal-dependent one. It is considered likely therefore that non-ribosomal-dependent mechanisms of protein synthesis are of little significance in development in the hair follicle.

The cell-free protein synthesis experiments to be described in the later chapters of this thesis will clarify the questions on the mechanisms of the differential rates of synthesis of the LoS and HiS proteins and of the subgroups of HiS proteins.

Fraser (1969) reported the presence in wool follicle HiS protein fractions (that is, proteins soluble at pH 4.4) of a major group of proteins of low SCMcysteine content which he termed "low sulphur non-microfibrillar proteins". Together with the *in vivo* {^{35}S}cystine incorporation studies, he postulated that these were "precursor proteins requiring hydrolysis before ^{35}S is incorporated into structural fibrous protein or are subunits which in turn polymerise to form microfibrillar protein". No protein species that could fulfil the role of this "subunit" protein was found in the present work using guinea pig follicle proteins in any protein fraction. Therefore, in the absence of more detailed information on the molecular weight and chemical structure of this protein of Fraser (1969), it is not possible to speculate on this difference observed between the wool and hair follicle systems.

There were some other minor differences between the F-SCMK and H-SCMK protein extracts. The former contained numerous other protein bands of low electrophoretic mobility and which eluted from DEAE-cellulose at KCl concentrations < 0.05 M. It is likely that these are cytoplasmic proteins. The F-SCMK protein extracts also contained nucleic acid. Similar types of components were present in wool follicle extracts (Downes *et al.*,

1966a; Fraser, 1969).

The only significant difference between the proteins extracted from hair and hair follicle tissue was in the group-1B proteins. The amino acid analyses of the H-group-1B proteins were very similar to the medulla and inner root sheath proteins and it was suggested that the F-group-1B proteins might be precursors of them. The F-group-1B protein fraction represented about 10 % of the total extractable proteins. Few, if any, other cytoplasmic proteins would be present in such a high level: ribosomal and histone proteins are likely to be present in large amounts, but their molecular size and amino acid compositions are grossly different from those of the F-group-1B proteins. The other major group of proteins present in hair follicle cells is those of the trichohyalin droplets of both the medulla and inner root sheath layers and it is possible that the F-group-1B proteins originated from the trichohyalin droplets. If this assertion is true, then it implies that the trichohyalin droplets may serve as the precursor protein of the mature proteins of the medulla and inner root sheath. However, this theory must await characterisation of isolated trichohyalin droplets before validation. Nevertheless, from the amino acid data in which citrulline and arginine are prominent, there exists some support for the hypothesis of Rogers (1959b, 1963 and 1964) that desimidation takes place for the formation of citrulline. The F-group-1B proteins should be investiagted further with regard to the possibility that they are precursor proteins of the citrulline proteins.

CHAPTER FOUR

PARTIAL SEQUENCE STUDIES ON PURIFIED LOW SULPHUR AND HIGH SULPHUR KERATIN PROTEINS FROM GUINEA PIG HAIR AND HAIR FOLLICLE TISSUE

A INTRODUCTION

This chapter provides information on the amino acid sequence at the amino-terminal regions of LoS and HiS proteins that were isolated and purified by methods established in Chapter Three. This information is an important pre-requisite to studies on the synthesis of hair follicle proteins *in vitro* and *in vivo*.

B METHODS

(a) PROTEOLYTIC DIGESTIONS

The procedures described here refer only to the use of enzymes for amino acid sequence studies.

Digestions with trypsin (TPCK-treated, minimal chymotrypsin activity) were conducted in 0.2 M N-ethyl-morpholine acetate buffer (pH 8.3) (10 mg of protein/ml) using an enzyme : protein ratio of 1 : 50 at 37°. Digestions that were continued for more than 3 h contained 0.1 % phenol to inhibit bacterial growth. The reaction was terminated by freeze-drying.

Digestions with carboxypeptidase B were also performed in 0.2 M N-ethyl-morpholine acetate buffer (pH 8.3) (1 mg of peptide/ml) using an enzyme : peptide ratio of about 1 : 10 at 37°. The amino acid(s) released by this procedure were collected after 15 min by addition of about 100 mg of Dowex AG 50W-X8 (20-30 mesh) (H^+-form) and mixing (Thompson, 1952). The beads were washed with water by centrifugation and the absorbed amino acid(s) were released with 5 N NH_4OH and dried *in vacuo*.

(b) ISOLATION OF N-ACETYL AMINO ACIDS

The procedure for the digestion of the protein or peptide samples was based on the methods established by Narita (1958) and O'Donnell and Thompson (1968). The sample was dissolved in 0.2 M N-ethyl-morpholine acetate buffer (pH 8.3) (about 10 mg/ml) and digested with either pronase using an enzyme : protein ratio of about 1 : 10 or a mixture of pronase

(ratio 1 : 10), carboxypeptidase A (ratio 1 : 100) and mercuripapain (ratio 1 : 100) at 37° for 3 days. Additional similar amounts of enzyme were added at 24 h intervals. The reaction also contained 0.1 % phenol to inhibit bacterial growth. The digestions were stopped by deproteinisation by addition of 10 volumes of 1 % (w/v) aqueous picric acid (Stein and Moore, 1954). Insoluble material was removed by centrifugation at 500 x *g* for 10 min and the picric acid was removed from the supernatant on a 10 x 1 cm column of Dowex AG 2W-X8 (100-200 mesh) (Cl^--form) in 0.02 N HCl. The amino-terminal blocked acidic species were recovered from this eluate on a 10 x 1 cm column of Dowex AG 50W-X8 (100-200 mesh) (H^+-form) in water and the material which eluted was collected and dried by rotary film evaporation. The products were chromatographed on a 40 x 0.6 cm column of Dowex AG 1W-X8 (-400 mesh) (formate-form) using a gradient of increasing formic acid concentration (Table 4.1) after Offer (1965). The flow rate was maintained at 30 ml/h and effluent was continuously monitored with a Technicon Peptide Analyzer analysis system. One tenth of the effluent was analysed whilst the remainder was collected into 2 ml fractions. The chromatographic system was calibrated with the amino acid and N-blocked species as shown in Fig. 4.1.

(c) CLEAVAGE OF PROTEINS WITH CYANOGEN BROMIDE

The procedure employed was that of Gross and Whitkop (1962) and Thompson and O'Donnell (1967). Protein samples were dissolved in 90 % aqueous formic acid (30 mg/ml) and an equal weight of solid cyanogen bromide dissolved in a small volume of formic acid was added. The reaction was continued for 24 h at 4° and stopped by dilution with water and freeze-dried.

(d) REACTION WITH DANSYL-CHLORIDE [a]

Protein or peptide samples containing the equivalent of 2 - 5 nmoles of amino-terminal amino acid were reacted with dansyl-chloride by the method of Woods and Wang (1967). The isolated products were analysed by two-

---

[a] Dansyl- and DNS- are abbreviations for 1-dimethylaminonaphthalene-5-sulphonyl-.

TABLE 4.1

COMPOSITION OF THE GRADIENT FOR THE TECHNICON PEPTIDE ANALYZER SYSTEM

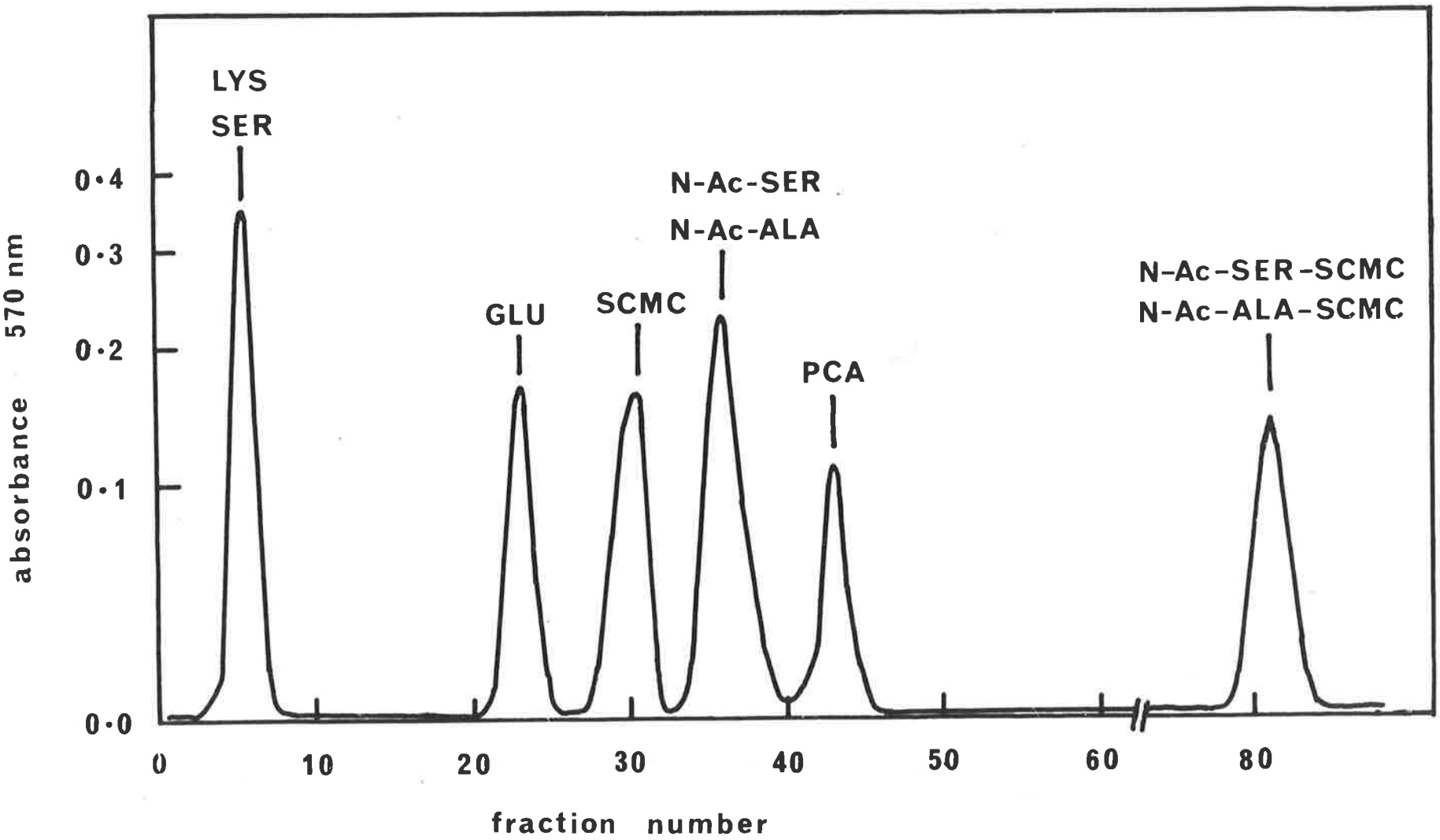
Each chamber contained 30 ml of formic acid. Other details are given in the text. (From Kemp, 1971, personal communication.)

Autograd chamber number	Molarity of formic acid
1	0.10
2	0.25
3	0.25
4	1.00
5	1.00
6	1.00
7	2.50
8	2.50
9	5.00

FIGURE 4.1

SEPARATION OF STANDARD COMPOUNDS ON THE TECHNICON PEPTIDE ANALYZER

The details of the column and gradient system used are given in the text and Table 4.1, repsectively. A mixture containing approximately 125 µg each of lysine, serine, glutamic acid, SCMcysteine, N-acetylserine, N-acetylalanine, pyrrolidine carboxylic acid (PCA) and N-acetylserine-SCMcysteine was chromatographed on the column. One tenth of the effluent was analysed and the remainder was discarded. The compound N-acetylalanine-SCMcysteine was not used as a standard, but its position of elution was found to be the same as that of N-acetylserine-SCMcysteine (see later experiments).


LYS
SER
N-Ac-SER
N-Ac-ALA
N-Ac-SER-SCMC
N-Ac-ALA-SCMC
GLU
SCMC
PCA
absorbance 570 nm
0·4
0·3
0·2
0·1
0·0
0
10
20
30
40
50
60
80
fraction number

dimensional thin layer chromatography on 5 x 5 cm sheets of polyamide layers using solvents of: first dimension: water - 90 % aqueous formic acid (200 : 3, v/v) and the second dimension: benzene - acetic acid (9 : 1, v/v) (Woods and Wang, 1967). Occasionally, single dimension chromatography was performed on 5 x 10 cm sheets using a solvent of *n*-butyl acetate - methanol - acetic acid (20 : 1 : 1, v/v/v) (Milne, 1970; personal communication) to achieve optimal separation of DNS-serine and DNS-threonine and DNS-aspartic acid and DNS-glutamic acid.

(e) NINHYDRIN ANALYSIS

This was used for analysis of peptide samples in eluates from Sephadex chromatography column experiments. The samples (containing up to 0.3 µmoles of amino acid) were hydrolysed with 10 % NaOH for 3 h at 100° and neutralised to pH 5 with 40 % aqueous acetic acid. Determination with ninhydrin reagent was carried out by the procedure of Yemm and Cocking (1955) as described by Chibnall *et al.* (1958).

C RESULTS

(a) AMINO-TERMINAL AMINO ACIDS OF LoS PROTEINS

Reaction of each of the purified LoS protein components from both hair and hair follicle extracts with dansyl-chloride as described in Methods indicated the presence in each case of only ε-DNS-lysine and O-DNS-tyrosine and traces of DNS-gly and DNS-leu. It was therefore concluded that these proteins had blocked amino-terminal groups. Accordingly, a search was made for N-acetyl blocked amino acids since these have been found in wool LoS proteins (O'Donnell *et al.*, 1962; Thompson and O'Donnell, 1967). 220 mg of purified component H-III (5.0 µmoles) were digested with TPCK-trypsin for 3 h and then with pronase for 3 days as described in Methods. The digest was deproteinised as described and the acidic fraction which eluted from Dowex 50

was chromatographed on the Dowex 1 column of the Technicon Peptide Analyzer (Fig. 4.2a). The amino acid composition of the material contained in the peak indicated was determined and was found to contain principally serine with traces of SCMcysteine, glutamic acid and proline. The material was considered to be N-acetyl-serine since it was unreactive both with ninhydrin before alkaline hydrolysis and dansyl-chloride before acid hydrolysis, and co-chromatographed with an authentic sample of N-acetylserine. Assuming that the serine content of this material was due only to N-acetylserine (and not in part due to other seryl-peptides), the total yield of N-acetyl-serine isolated as such from component H-III was 0.53 moles per mole of H-III. Thus the major amino-terminal group of this protein was N-acetylserine. Similar experiments were performed on components H-I and H-II and F-III and N-acetylserine was again found in each case: H-I, 0.47 moles/mole; H-II, 0.39 moles/mole; F-III, 0.43 moles/mole.

In other experiments (not described here in detail) two peptides of amino acid composition ser, glux (probably PCA-ser) and ser, glux, pro (probably PCA-(ser, pro)) were recovered from tubes 28 - 40 from the Peptide Analyzer. In addition, the material of the peak which eluted at tubes 49 - 52 (Fig. 4.2a) contained only glux and on the basis of its position of elution, its probable sequence was PCA-glu.

(b) AMINO-TERMINAL AMINO ACIDS OF HiS PROTEINS

Reaction of the HiS proteins from both hair and hair follicle extracts with dansyl-chloride indicated the presence of only ε-DNS-lysine. It was therefore concluded that these proteins had blocked amino-terminal groups as found with wool HiS proteins (Gillespie *et al.*, 1968; Haylett and Lindley, 1968). Accordingly, 44 mg of H-group-4 proteins (2.2 μmoles, assuming an average molecular weight of 20 000 daltons) were digested with pronase and N-blocked species were recovered and characterised as described

FIGURE 4.2

CHROMATOGRAPHY OF AMINO-TERMINAL BLOCKED PEPTIDE SPECIES DERIVED FROM DIGESTS OF LoS AND HiS PROTEINS ON THE TECHNICON PEPTIDE ANALYZER

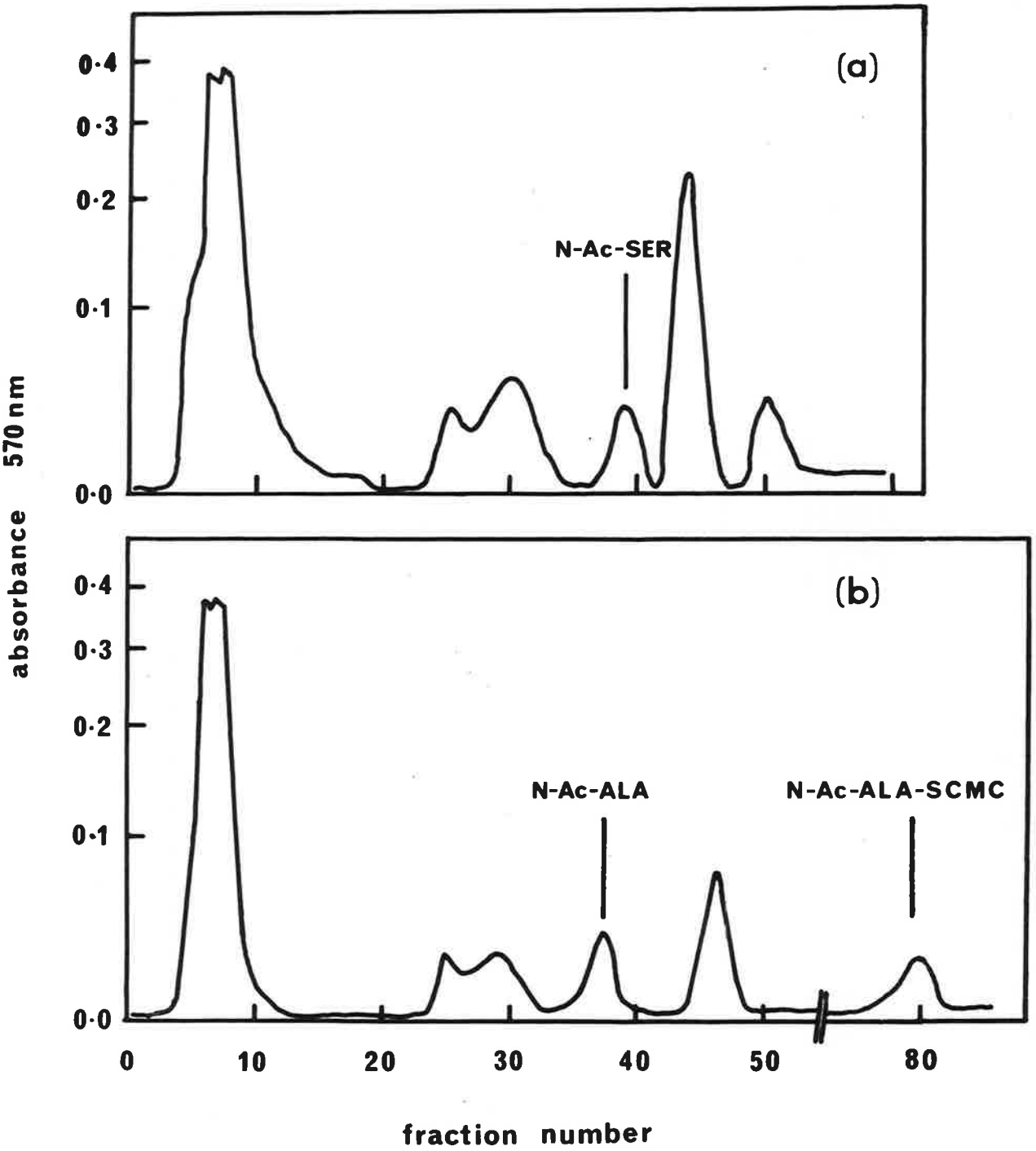
The chromatographic procedures used were as described in the text and Fig. 4.1. When the yields of material in the peaks were determined, appropriate corrections were made for all sampling steps.

(a) DIGEST OF THE LoS COMPONENT H-III

5.0 μmoles of H-III were digested with trypsin and pronase as described in the text and the Dowex 50 eluate was chromatographed on the Dowex 1 column. The tubes containing the peak indicated were pooled for characterisation of their contents.

(b) DIGEST OF THE H-GROUP-4 PROTEINS

2.2 μmoles of H-group-4 proteins were digested using the *unmodified* procedure (as above). The tubes containing the peaks indicated were pooled for characterisation of their contents.


(a)
N-Ac-SER
(b)
N-Ac-ALA
N-Ac-ALA-SCMC
absorbance 570 nm
0·0
0·1
0·2
0·3
0·4
0
10
20
30
40
50
80
fraction number

previously (Fig. 4.2b). Amino acid analyses of the peaks indicated showed that they contained principally alanine, and SCMcysteine and alanine, respectively. On the bases of the positions of elution of these peaks it was considered that they contained N-acetylalanine and N-acetylalanine-SCMcysteine and they were recovered in the yields of 0.13 moles/mole and 0.07 moles/mole, respectively.

An alternative digestion procedure was adopted in order to increase these yields. Sequence studies on the wool HiS proteins have shown that all proteins so far examined have the common amino-terminal sequence N-acetyl-alanine-SCMcysteine-SCMcysteine-serine (Haylett and Lindley, 1968). The SCMcysteine-SCMcysteine bond was resistant to proteolytic cleavage and this was the probable reason for the low yields of amino-terminal peptide fragments. This bond could, however, be cleaved with mercuripapain (Haylett and Lindley, 1968). Therefore, 64 mg (about 3.2 μmoles) of H-group-4 proteins were digested using a modified procedure involving pronase, mercuripapain and carboxypeptidase A as described in Methods and the N-blocked species were characterised as before. Both N-acetylalanine and N-acetylalanine-SCMcysteine were recovered but in the increased yields of 0.47 moles/mole and 0.11 moles/mole, respectively. Therefore, it was considered that the major amino-terminal sequence of the proteins was N-acetylalanine-SCMcysteine.

Similar experiments were performed on the F-group-4 proteins and the yields of N-acetylalanine and N-acetylalanine-SCMcysteine were 0.56 moles/mole and 0.09 moles/mole, respectively.

(c) PARTIAL SEQUENCE ANALYSIS OF PURIFIED COMPONENT H-III

It was shown in Chapter Three that the LoS protein component H-III could be prepared in highest yield from the H-SCMK-A protein fraction and that it was comparatively easy to purify. Therefore, this component was chosen for further characterisation with a view to partial sequence analysis of the amino-terminal region. However, it was also shown before that H-III

was composed of two sub-components, one present in about 85 % and the other 15 % of the total. Since these must have had generally similar properties, it was considered that the amino acid sequence of the polypeptide moeities of H-III would also be similar.

(1) Cleavage with cyanogen bromide

Component H-III contained 1.0 residues of methionine per 400 amino acid residues in the total protein (Table 3.7), that is, about 1 residue/mole. In one experiment, 875 mg (20 μmoles) of H-III were treated with cyanogen bromide as described in Methods and the products were separated on a column of Sephadex G-75 in 50 % aqueous formic acid (Thompson and O'Donnell, 1967) (Fig. 4.3). Two peaks were obtained. The Sephadex column was calibrated with standard S-carboxymethyl-proteins and the approximate molecular weight values of the two fragments were; CNBr-1, > 30 000 daltons (eluted at V_o); and CNBr-2, 6000 - 7000 daltons. The CNBr-2 moiety was therefore about 14 - 16 % of the total protein. The yield of CNBr-2 was routinely 13 - 15 % over several preparations, which suggested that cleavage at the methionine residue was near quantitative.

It was possible that exposure of the proteins to 50 - 90 % aqueous formic acid solutions at room temperature for 24 - 48 h might have led to random breakage of peptide bonds. However, no control experiments were performed in this work since Thompson and O'Donnell (1967) were unable to demonstrate random peptide bond cleavage of similar proteins from wool after treatment with formic acid solutions.

(2) Characterisation of the CNBr fragments

The amino acid composition of the CNBr fragments is shown in Table 4.2. CNBr-2 had 1.0 homoserine residues per 62 amino acid residues. This suggested that CNBr-2 contained the amino-terminus of the protein H-III and indicated that its molecular weight was 7000 daltons. Since the

FIGURE 4.3

CHROMATOGRAPHY OF CYANOGEN BROMIDE CLEAVAGE PRODUCTS OF COMPONENT H-III ON SEPHADEX G-75

The fragments resulting from the cleavage of H-III with cyanogen bromide were fractionated on a 125 x 1.7 cm column of Sephadex G-75 in 50 % aqueous formic acid. The loading sample contained about 200 mg of protein in 10 ml of 50 % formic acid. The flow rate was 30 ml/h and the fraction size was 3.0 ml. Samples (0.1 ml) were removed for ninhydrin analysis after alkaline hydrolysis as described in Methods. The column was calibrated with standard S-carboxymethyl proteins of known molecular weight. The peaks CNBr-1 and CNBr-2 refer to the tubes that were pooled for characterisation of their contents.

CALIBRATION PROTOCOL

Protein	Molecular weight (daltons)	V_e (fraction number)
Ovalbumin	45 500	30.5 (V_o)
Chymotrypsinogen A	25 500	33.5
Apomyoglobin	17 200	42.5
Lysozyme	14 800	45.0
Feather keratin	10 500	51.0
Insulin B	3 450	73.5
Insulin A	2 550	81.5 (V_t)

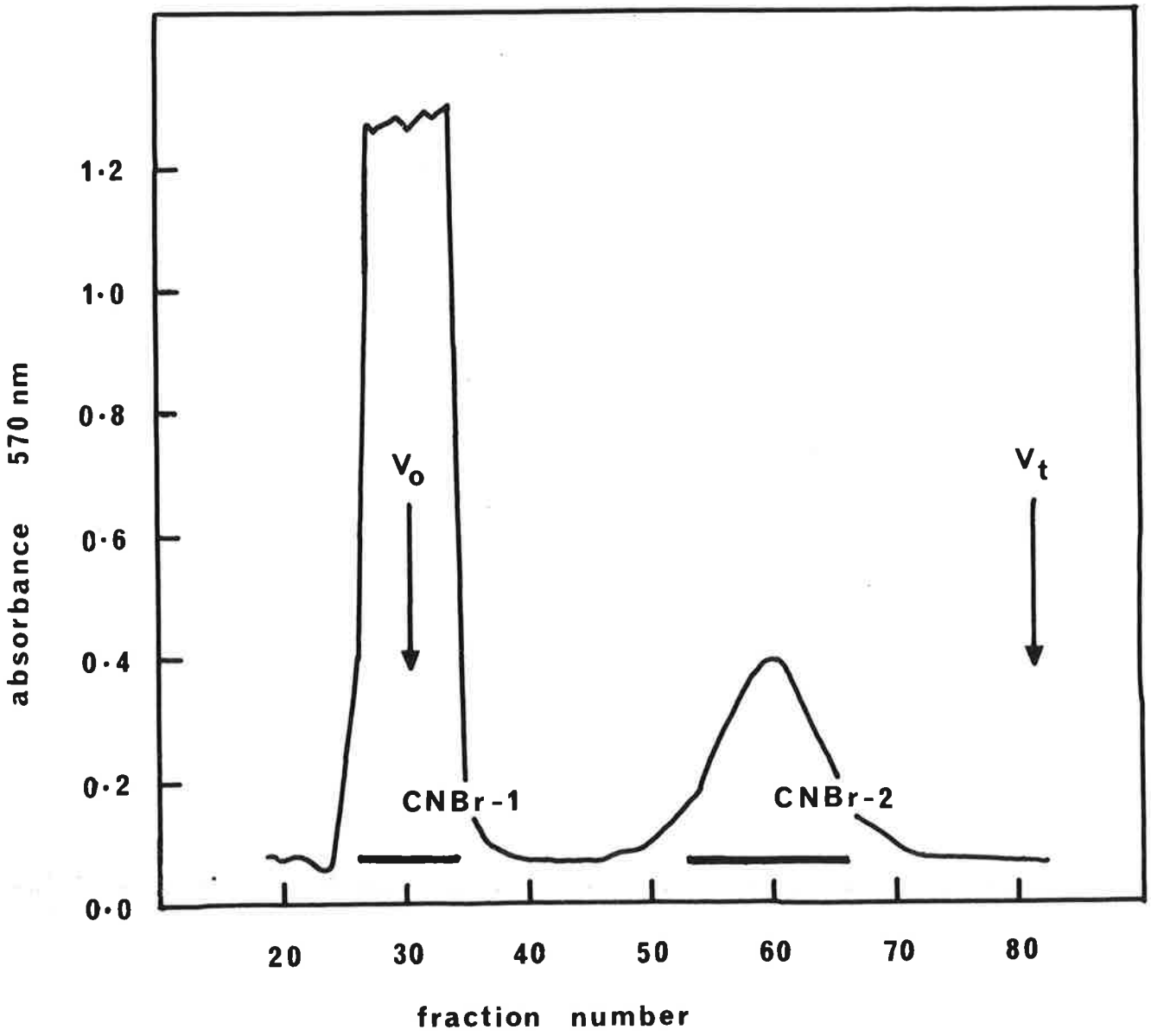

absorbance 570 nm
1·2
1·0
0·8
0·6
0·4
0·2
0·0
V_o
V_t
CNBr-1
CNBr-2
20
30
40
50
60
70
80
fraction number

TABLE 4.2

AMINO ACID COMPOSITION OF THE CYANOGEN BROMIDE FRAGMENTS OF COMPONENT H-III

The values are expressed as residues per 100 residues *(first column)* and are the averages of analyses on two different samples. The possible numbers of residues *(second column)* are also given, assuming; H-III, methionine = 1.00; CNBr-1, histidine = 2.0; CNBr-2, homoserine = 1.0. Amounts indicated by "trace" were present in < 0.05 residues percent.

Amino acid	H-III[a]		CNBr-1		CNBr-2	
SCMcysteine	5.70	23	4.15	14	14.35	9
Aspartic acid	9.25	37	9.10	31	8.15	5
Threonine	4.90	20	4.75	16	6.40	4
Serine	8.00	32	6.85	23	13.95	9
Homoserine	0.00		trace		1.65	1
Glutamic acid	21.75	87	24.95	83	6.65	4
Proline	3.55	14	2.45	8	9.40	6
Glycine	4.70	15	2.80	9	9.55	6
Alanine	5.80	23	6.05	20	4.95	3
Valine	4.75	19	5.05	17	3.25	2
Half-cystine	0.05		trace		trace	
Methionine	0.25	1	trace		trace	
Isoleucine	3.50	14	3.85	13	1.70	1
Leucine	11.30	46	13.95	43	5.05	3
Tyrosine	3.15	13	3.25	11	3.70	2
Phenylalanine	1.75	7	1.15	4	4.80	3
Lysine	2.75	11	2.90	10	1.80	1
Histidine	0.80	3	0.60	2	1.55	1
Arginine	7.85	31	8.65	29	3.50	2
Total number of residues	about 396		about 333			62

[a] These values are from Table 3.7.

larger CNBr-1 fragment did not contain any detectable homoserine, it was concluded that this fragment contained the carboxyl-terminus of H-III. In addition, CNBr-2 was unreactive with dansyl-chloride, whereas CNBr-1 yielded principally DNS-glux.

To characterise the CNBr-2 fragment further, 35 mg (about 5 μmoles) of it were digested with trypsin and pronase and N-acetylserine was recovered in a yield of 0.73 moles/mole of CNBr-2. No N-acetylserine was recovered from similar experiments on CNBr-1 (yields < 0.05 moles/mole). These results firmly established CNBr-2 as the amino-terminal fragment.

Samples of both fragments were examined by polyacrylamide gel electrophoresis. CNBr-1 migrated slightly faster than H-III, from which it was derived, but appeared as a single band. CNBr-2 migrated rapidly to the anode as a single diffuse band.

Thompson and O'Donnell (1967) and O'Donnell (1969) demonstrated that similar cyanogen fragments from Merino wool "component 8" were heterogeneous and could be fractionated into several peaks by gradient elution on DEAE-cellulose using urea buffers. When similar experiments were performed on the fragments CNBr-1 and CNBr-2 isolated here, each chromatographed as a single symmetrical peak which suggested little, if any, heterogeneity of the fragments.

(3) Characterisation of the tryptic peptides of H-III-CNBr-2

75 mg (about 11 μmoles) of CNBr-2 were digested with trypsin as described in Methods and the products were chromatographed on a column of Sephadex G-25 using 50 % aqueous formic acid as eluant (Fig. 4.4). The amino acid analyses of the peaks T_1, T_2 and T_3 obtained are given in Table 4.3. The probable numbers of residues in each peak are given, assuming CNBr-2 contained 62 amino acid residues.

Peak T_3 contained about equal amounts of homoserine, glutamic acid and threonine and did not contain a basic amino acid. Reaction of it with

FIGURE 4.4

CHROMATOGRAPHY OF THE TRYPTIC PEPTIDES OF H-III-CNBr-2 ON SEPHADEX G-25

The tryptic peptides of H-III-CNBr-2 were prepared as described in the text and fractionated on a 65 x 1.2 cm column of Sephadex G-25 in 50 % aqueous formic acid. The flow rate was 10 ml/h and fraction size 1.25 ml. Samples (0.2 ml) were removed for ninhydrin analysis after alkaline hydrolysis as described in Methods. The column was calibrated with standard S-carboxymethyl proteins of known molecular weight. The bars T_1, T_2 and T_3 refer to the tubes that were pooled for characterisation of their contents.

CALIBRATION PROTOCOL

Protein	Molecular weight (daltons)	V_e (fraction number)
Feather keratin	10 500	23.0 (V_o)
Insulin B	3 450	24.0
Lysozyme peptide 2	3 000	26.5
Insulin A	2 550	28.0
Lysozyme peptide 3	1 300	34.0
$K_3Fe(CN)_6$		47.5 (V_t)

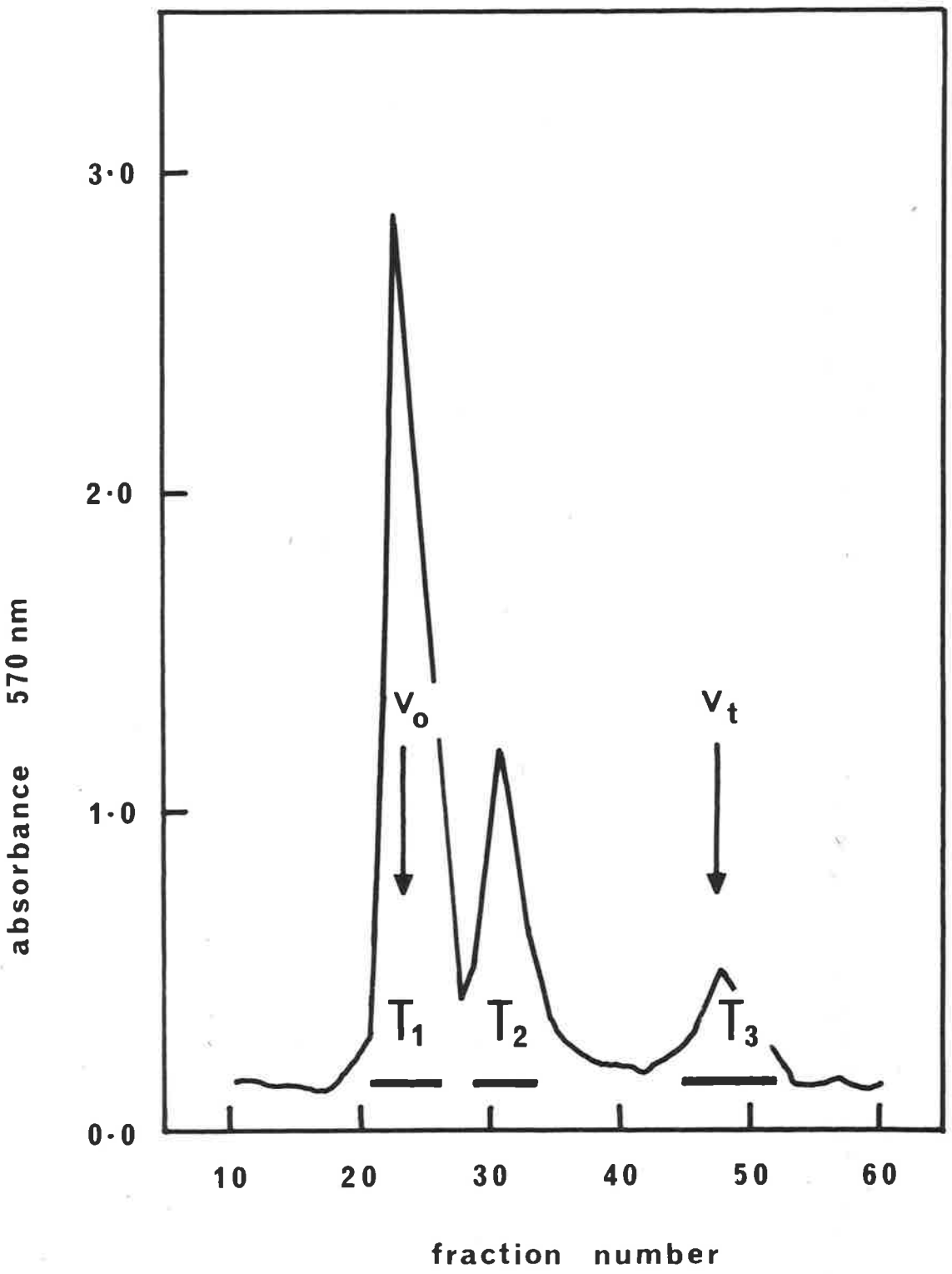

3·0
2·0
1·0
0·0
absorbance 570 nm
V_o
V_t
T_1
T_2
T_3
10
20
30
40
50
60
fraction number

TABLE 4.3

AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES DERIVED FROM H-III-CNBr-2

The values are expressed as residues per 100 residues *(first column)* and are the averages of the analyses on two different samples. The probable numbers of residues *(second column)* are also given, assuming; CNBr-2, homoserine = 1.00; T_1, lysine = 1.0; T_2, arginine = 1.0; T_3, homoserine = 1.0. Amounts indicated by "trace" were present in < 0.05 residues percent.

Amino acid	CNBr-2[a]		T_1		T_2		T_3	
SCMcysteine	14.35	9	14.15	7	16.10	2	4.80	
Aspartic acid	8.15	5	9.10	4	9.05	1	2.15	
Threonine	6.40	4	6.70	3	0.95		21.50	1
Serine	13.95	9	15.10	7	14.60	2	3.20	
Homoserine	1.65	1	0.15				22.20	1
Glutamic acid	6.65	4	6.85	3	1.05		24.95	1
Proline	9.40	6	10.75	5	8.15	1	2.45	
Glycine	9.55	6	11.35	5	7.90	1	4.80	
Alanine	4.95	3	6.40	3	0.65		2.35	
Valine	3.25	2	3.95	2	0.75		2.20	
Half-cystine	trace							
Methionine	trace		trace					
Isoleucine	1.70	1	2.25	1	0.85		0.90	
Leucine	5.05	3	2.05	1	16.00	2	2.70	
Tyrosine	3.70	2	3.30	1.6	3.30	0.4	0.55	
Phenylalanine	4.80	3	0.90	0.4	12.30	1.6	0.25	
Lysine	1.80	1	2.10	1	0.75		3.35	
Histidine	1.55	1	1.85	1	0.60		0.40	
Arginine	3.50	2	2.20	1	8.00	1	0.65	
Total number of residues		62		47		12		3

[a] These values are from Table 4.2.

dansyl-chloride indicated the presence of DNS-glux (as well as traces of DNS-leu). These data suggested T_3 was a single tripeptide which originated from the carboxyl-terminal end of CNBr-2, and its probable sequence was glux-thr-homoser.

Peak T_2 contained one arginine residue per 12 residues. After calibration of the Sephadex G-25 column, the molecular weight of T_2 was found to be about 1500 daltons which suggested it was a single peptide. The peptide was unreactive with dansyl-chloride which suggested a blocked amino-terminus. Accordingly, 7.5 mg (about 5 μmoles) of peptide were digested with pronase by established procedures and N-acetylserine was recovered in a yield of 0.60 moles/mole of T_2. Thus T_2 was probably the amino-terminal tryptic peptide of H-III-CNBr-2.

Peak T_1 contained one arginine and one lysine residue per 47 amino acid residues. Its molecular weight was > 3500 daltons. A 5 mg sample was reacted with carboxypeptidase B and the released amino acid(s) was characterised by dansylation. Only ε-DNS-lysine and ε-α-diDNS-lysine were recovered indicating that lysine was a single carboxyl-terminal amino acid. Therefore, T_1 was probably a single large peptide. It was likely the arginine residue was rendered stable to cleavage by trypsin, possibly by an adjacent acidic amino acid and (or) a proline residue (O'Donnell, 1969). A 10 mg sample of T_1 was digested extensively with trypsin using a 1 : 5 enzyme : peptide ratio for 16 h at 37°. The products were chromatographed on the Sephadex G-25 column and no material of molecular weight < 3500 daltons was recovered (< 2 % of total), indicating the arginyl peptide bond was very resistant to tryptic digestion. Reaction of T_1 with dansyl chloride gave DNS-valine (faint spot) as well as traces of ε-DNS-lysine, O-DNS-tyrosine and DNS-glycine. Valine may therefore be the amino-termial residue of this peptide.

The order of the tryptic peptides of H-III-CNBr-2 was therefore

T_2 - T_1 - T_3. The known details of the primary structure of H-III are summarised in Fig. 4.5.

Peptides T_1 and T_2 contained clearly non-integral amounts of tyrosine and phenylalanine, suggesting that some molecules in each peptide may have contained either tyrosine or phenylalanine where these residues appeared in the sequence. These "replacements" may have arisen from the heterogeneity of H-III which itself is composed of two sub-components as described earlier. Other less-obvious cases of amino acid replacements in the sequence may be present.

D DISCUSSION

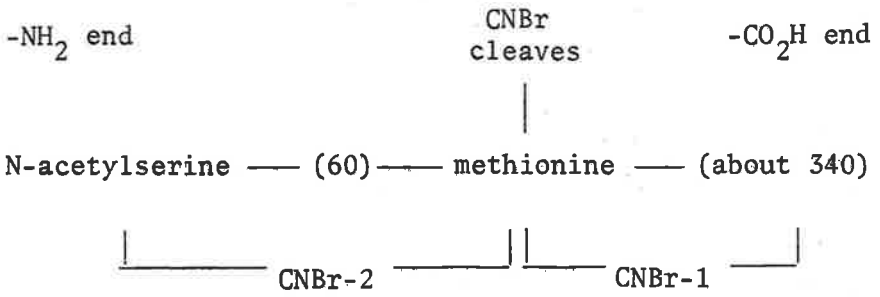
The present studies have shown that both the LoS and HiS proteins have N-acetyl amino-terminal residues. Blocked amino-terminal groups may be a common feature of structural and fibrous proteins as they have been found widely in a number of different proteins. For examples; N-acetylmethionine in Turnip Yellow Mosaic Virus coat protein (Harris and Hindley, 1961); N-acetylserine in Tobacco Mosaic Virus coat protein (Narita, 1958); N-acetylserine in rabbit myosin (Offer, 1965); N-acetylaspartic acid in rabbit muscle actin (Alving and Laki, 1965; Gaetjens and Bárány, 1966); N-acetylthreonine in bovine fibrinogen (and the fibrinopeptide B) (Folk and Gladner, 1960); and other keratins; N-acetylserine in goose feather (O'Donnell, 1971) and chicken feather (Kemp, 1971, personal communication).

The LoS protein component H-III comprised a family of at least two very similar proteins as shown by polyacrylamide gel electrophoresis (Fig. 3.7), amino acid analyses of the tryptic peptides derived from CNBr-2 (Table 4.3) and the near-quantitative yields of the CNBr-1 and CNBr-2 moieties of H-III and of the tryptic peptides of CNBr-2. Indeed, the differences in the chemistry of the polypeptide chains of H-III could be accounted for simply by occasional single amino acid replacements as shown

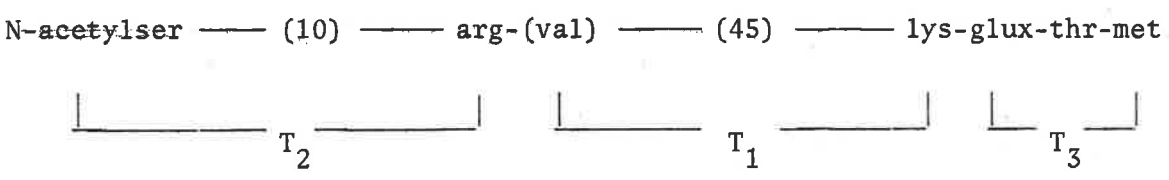
FIGURE 4.5

KNOWN DETAILS OF SEQUENCE OF COMPONENT H-III

INTACT H-III



H-III-CNBr-2



for the wool LoS "component 8" (O'Donnell, 1969).

It is of interest to compare the amino acid composition of the CNBr-2 fragment to that of H-III itself and CNBr-1. CNBr-2 comprised the amino-terminal 62 amino acid residues or about 16 % of the total protein. In contrast to H-III itself, CNBr-2 contained a much higher proportion of SCMcysteine, serine, proline and glycine and a much lower proportion of most acidic, aliphatic and basic amino acids. Indeed, the amino acid composition of CNBr-2 was rather similar to that of the HiS proteins (compare Tables 3.11 and 4.2). Therefore, CNBr-2 probably had very little helix content. A similar cyanogen bromide fragment of about 60 residues was characterised in wool "component 8" (Thompson and O'Donnell, 1967; O'Donnell, 1969) and, interestingly, its amino acid composition was similar to the wool HiS proteins, and, furthermore, to that of the non-helical "tails" isolated by Crewther and Harrap (1967). On the basis of these comparisons, it is predicted that component H-III had a similar structure to the wool LoS proteins in that there are regions of low and high helical content along the length of the molecules of H-III. Moreover, it is likely that there are other regions in H-III-CNBr-1 of low structural order where many of the remaining SCMcysteine, serine, proline and glycine amino acid residues are concentrated as suggested for the wool proteins (Thompson and O'Donnell, 1967; Crewther and Harrap, 1967).

The observations of this and the previous chapter have shown that all properties of the LoS and HiS proteins obtained from both hair and hair follicle origin are identical as far as can be experimentally determined. Therefore, it is reasonable to propose that the amino acid sequence of the corresponding hair and hair follicle proteins is the same. This important conclusion forms the basis of the interpretations of the *in vitro* protein synthesis studies that will be reported in Chapters Seven and Eight of this thesis.

The similarities in amino acid composition of the H-III-CNBr-2 fragment and the HiS proteins introduces the possibility of unusual mechanisms of synthesis of the LoS proteins. Possible mechanims of generation of sequence heterogeneity of the proteins were discussed in Chapter One, and in addition to these, other mechanisms are also applicable. For example, during polypeptide synthesis or assembly of the microfibrils, a cysteine-rich or HiS - like polypeptide might become attached to a cysteine-poor helical peptide to form a single LoS protein chain. This implies the presence of subunits, but these have not been found in the present work. Nevertheless, the possible involvement of subunits in synthesis of the LoS proteins can be tested experimentally using a cell-free protein synthesis system.

CHAPTER FIVE

ELECTRON MICROSCOPIC OBSERVATIONS ON THE SYNTHESIS OF HIGH SULPHUR KERATIN PROTEINS *IN SITU*

A INTRODUCTION

The observations to be reported in this chapter were made during electron microscope studies on sections of guinea pig hair follicles. Densely-stained granules of rectangular or cylindrical shape were observed associated with keratin filaments in the developing cortical cells of the hair follicle. From the morphological evidence to be presented, the reasonable hypothesis is advanced that these granules contain high sulphur keratin (HiS) proteins. If this assertion is correct, it is of importance in relation to the temporal synthesis and mechanism of synthesis of the HiS proteins in the hair follicle and this is discussed.

B METHODS

(a) PREPARATION OF HAIR FOLLICLE CORTICAL CELLS

During the isolation of inner root sheath cells from hair follicles by enzymic digestion as described by Steinert *et al.* (1971), it was seen that numerous cortical cells were also released. This procedure was therefore applied directly to the isolation of cortical cells.

Freshly-depilated guinea pig hair follicles were placed in the "filtration apparatus" of design similar to that of Kawiak *et al.* (1965) and suspended in a buffer of 10 mM tris-HCl (pH 7.4) and 10 mM KCl containing 0.1 % (w/v) trypsin (Sigma, type III, 2-times crystallised). The follicles were stirred for 15 min at 23°. The harvested cells were collected and washed in buffer by centrifugation and suspended in 4 ml of 10 % (w/v) sucrose in the buffer. This suspension was layered onto a 25 ml linear sucrose density gradient of 50 - 70 % sucrose in buffer in a 33 ml cellulose-nitrate tube and centrifuged at 20 000 rev./min for 30 min in a Spinco SW25.1 rotor. Three distinct bands of material were recovered: the 10 - 50 % sucrose interface contained cytoplasmic debris; a diffuse band in the upper

half of the gradient at sucrose concentrations of 50 - 55 % contained inner root sheath cells; and a third diffuse band near the bottom of the gradient at sucrose concentrations of 60 - 65 % contained principally cortical cells. This latter fraction was removed and washed by centrifugation at 500 x *g* in buffer to remove sucrose. About 100 - 200 mg of cortical cells could be prepared by this procedure from 1 g of follicle tissue.

C RESULTS AND OBSERVATIONS

(a) *EXAMINATION OF CROSS-SECTIONS OF GUINEA PIG HAIR FOLLICLES*

(1) Longitudinal sections

In Fig. 5.1 is shown a longitudinal cross-section through a guinea pig hair follicle near the mid keratogenous zone (level 2) (*see* Fig. 1.3) of the follicle. Deposits of densely-stained material appear closely associated with the keratin microfibrils of the cortical cells. These "cortical granules" are about 300 - 500 Å wide by about 1000 - 1500 Å long and their long axes are oriented along the microfibrils. Their appearance coincides precisely with the first appearance of the droplets of protein in the neighbouring cuticle cells (Fig. 5.1).

In Fig. 5.2 is shown a montage of longitudinal cross-sections from different hair follicles showing the association of the granules with the microfibrils at higher magnification. Three clearly different situations exist. In small clusters of filaments the granules appear as circular (or spherical) deposits and are attached to the microfibrils on only one surface *(1)*. In other fields the granules are approximately rectangular-shaped when bounded on both sides by microfibrils *(2)*. In larger clusters of fibrils the granules are smaller, less distinct and bound on all sides by fibrous material *(3)*. In all cases the granules appear devoid of any recognisable ultrastructure.

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PROTEIN BIOSYNTHESIS IN CELL-FREE SYSTEMS PREPARED FROM HAIR FOLLICLE TISSUE OF GUINEA PIGS

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SUMMARY

1. Polyribosomes have been prepared from the hair follicle tissue of young albino guinea pigs and the yields of especially the larger polyribosomes greatly exceeded those isolated from the same source in earlier studies.

2. These polyribosomes were also highly active in cell-free protein synthesis, being about 700 times more active than those prepared previously.

3. The ribonuclease content of the tissue homogenate preparations from young animals was found to be markedly lower than that obtained from older animals and it was concluded that this lower ribonuclease content was responsible for the improved findings in this work.

4. The properties of the protein synthesis systems established from the guinea pig hair follicle polyribosomes have been investigated using standard techniques. During amino acid incorporation, the polyribosomes degraded by an orderly process of run-off of ribosomes from the mRNA followed by release of the nascent protein chains. Experiments suggested that considerable reinitiation of protein chains occurred during incubation. These and other properties of the cell-free protein synthesis systems are similar to those of other animal systems and thus it is concluded that they are typically of the eukaryote cell-type.

INTRODUCTION

The hair follicle is a complex organ which synthesises several unique structural proteins that are deposited intracellularly. The largest group of proteins are the keratins that comprise a major portion of the hair fibre. Thus hair follicle tissue is well suited to a study of the biosynthesis of structural proteins and their subsequent organisation into a highly ordered and functional form. Whereas the mechanism of protein biosynthesis in some animal systems which synthesise principally only one or a small group of proteins is now well established (for example, globin synthesis in reticulocytes[1], crystallin synthesis in lens tissue[2] and myosin synthesis in chicken embryo muscle[3]), comparatively little is known of these mechanisms in hair follicle tissue.

Abbreviation: PEP, phosphoenolpyruvate.

A number of preliminary studies have been conducted. Polyribosomes are present in large numbers in the developing hair follicle cells[4–6]; they have been isolated from the tissue and their ability to direct protein synthesis *in vitro* has been demonstrated[5,7,8]. However, the yields of polyribosomes that were isolated in these studies and their ability to incorporate amino acids into protein in cell-free systems were low, which are factors that have severely handicapped attempts to assess their role in the biosynthesis of hair follicle proteins *in vivo*.

In the present work polyribosomes have been prepared from guinea pig hair follicle tissue in much higher yields which have an activity in cell-free protein synthesis several hundred times greater than those reported previously. This has afforded an opportunity for detailed studies on the properties of these polyribosomes and of the mechanism of protein synthesis *in vitro*. Recently, methods have been described for the isolation of polyribosomes from a related tissue, wool follicles of the sheep, which are similar to those described below[9].

MATERIALS

Radioactive amino acids, [4,5-$^3H_2$]leucine of specific activities 58.1 C/mmole and 2.0 C/mmole and uniformly ^{14}C-labelled phenylalanine of specieficactivity 455 C/mole were obtained from Schwarz BioResearch. $^{32}PO_4^{3-}$ (carrier-free) was obtained from the Australian Atomic Energy Commission. Dithiothreitol, puromycin dihydrochloride (grade II), chloramphenicol, cycloheximide, disodium ATP (grade II), disodium GTP (grade II-S), pyruvate kinase (rabbit skeletal, type II-A, in 2.1 M $(NH_4)_2SO_4$), ribonuclease A (bovine pancreas, type II-A) and deoxyribonuclease (bovine pancreas, ribonuclease-free) were obtained from Sigma Chemical Co. Ribonuclease-free sucrose and trypsin (minimal chymotrypsin content) were obtained from Mann Research Laboratories. Phosphoenolpyruvate (PEP) was prepared as the dipotassium salt[10].

METHODS

Source of tissue

Albino guinea pigs of both sexes were used throughout this work as a source of hair follicle tissue. Animals less than three weeks of age were used (or, more generally, of weight less than 150 g). Hair follicles were exposed by the wax-sheet procedure[5] in the cold (2°) and harvested with animal clippers. All subsequent procedures were conducted at 0–2° or as specified otherwise. Glassware and all equipment for all procedures was sterilised before use to minimise ribonuclease contamination.

Preparation of whole tissue homogenates containing polyribosomes

Follicles were suspended (10 %, w/v) in Buffer A consisting of 0.25 M KCl–20 mM Tris–HCl (pH 7.6)–5 mM $MgCl_2$–1 mM dithiothreitol–5 % sucrose. Homogenisation was performed in a Dounce homogeniser using a loose-fitting pestle (clearance about 0.2 mm) and 15–20 complete strokes. The extent of homogenisation necessary varied with the age of the animal used in the experiment: in very young

animals, the hair follicles were thinner and more easily disrupted, whereas the larger follicles of slightly older animals were more difficult to homogenise. In general, the extent of homogenisation was increased by two strokes from 15 strokes for each 25 g of animal body weight over 90 g (which is the approximate birth weight). The homogenates were filtered through nylon gauze (pore size about 0.05 mm) to remove hair and other debris and then centrifuged at 12 000 $\times g$ for 10 min. This 12 000 $\times g$ supernatant which contained the hair follicle polyribosomes was used as a basis of all experiments and will be referred to throughout this work as the whole tissue homogenate.

Analysis of polyribosome preparations by sucrose density centrifugation

Linear sucrose density gradients (15–40 %, 28 ml) were prepared in Buffer A, loaded with 1.0 ml of sample and centrifuged for 3 h at 22 500 rev./min in a Spinco SW25.1 rotor. The gradients were fractionated from the bottom and the effluent was continuously monitored at 260 nm by an Optica spectrophotometer with flow cell and recorder attachments. Polyribosomes could also be prepared by pelleting through sucrose layers at higher rotor speeds. The most satisfactory method was by centrifugation through 1 M sucrose in Buffer A layered over 2 M sucrose in Buffer A at 225 000 $\times g$ for 2.5 h. Single ribosomes and aggregates of two ribosomes did not sediment under these conditions. Polyribosomes prepared by this method could be resuspended easily and only slight breakage of the larger particles occurred on handling.

Yields of polyribosomes

These were determined spectrophotometrically at 260 nm by assuming that a 1.0 mg/ml suspension has an optical density of 11.8 units/cm (ref. 11).

Cell-free protein synthesis in the whole tissue homogenates

These experiments were performed on the whole tissue homogenate preparations described above. To 0.7 ml of the homogenate was added, to give a final volume of 1.0 ml, 5 μmoles of PEP, 1.0 μmole of ATP, 0.25 μmole of GTP, 20 μg of pyruvate kinase (and 4 μmoles of $(NH_4)_2SO_4$), 5 nmoles of each amino acid excepting leucine or phenylalanine, whichever was used as the labelled amino acid, and either 1 μC (17 pmoles) of [4,5-^{3}H]leucine or 0.5 μC (1.1 nmoles) of uniformly ^{14}C-labelled phenylalanine. The final buffer composition was 0.25 M KCl–20 mM Tris–HCl (pH 7.6)–5 mM $MgCl_2$–1 mM dithiothreitol–5 % sucrose. This was incubated at 37° for times varying up to 60 min. Samples (0.1 ml) were removed into 1.0 ml of ice-cold water and the reactions were terminated with 1.0 ml of 10 % trichloroacetic acid.

Cell-free protein synthesis in the reconstituted system

Polyribosomes that had been pelleted through sucrose layers to remove supernatant factors were resuspended in Buffer B consisting of 0.125 M KCl– 20 mM Tris–HCl (pH 7.6)–7.5 mM $MgCl_2$–1 mM dithiothreitol– 10 % glycerol. A supernatant fraction active in cell-free protein synthesis was obtained by centrifuging the whole tissue homogenate at 125 000 $\times g$ for 1.5 h. The upper quarter and lower quarter of the clear supernatant were discarded and the middle half was retained and dialysed against Buffer B with two changes of 100 vol. The *in vitro* system contained,

in a final volume of 0.6 ml, approx. 150 μg of polyribosomes, 0.2 ml of the dialysed supernatant fraction, 30 μg of deacylated yeast tRNA[12], 2.1 μmoles of PEP, 0.6 μmole of ATP, 0.15 μmole of GTP, 12 μg of pyruvate kinase (and 2.4 μmoles of $(NH_4)_2SO_4$) and 3.0 nmoles of each amino acid excepting leucine and 2.0 μC (1.0 nmole) of [4,5-^{3}H] leucine. The final composition of the buffer was 0.125 M KCl–20 mM Tris–HCl (pH 7.6)–6.5 mM $MgCl_2$–1 mM dithiothreitol–10 % glycerol. The reaction was incubated at 37°. Samples (0.1 ml) of the reaction were removed and terminated as before.

Ribonuclease assay

An *Escherichia coli* B strain was grown in an overnight incubation in a low phosphate medium containing $^{32}PO_4^{3-}$. The nucleic acids were extracted with phenol by established procedures and the labelled DNA was removed by digestion with ribonuclease-free deoxyribonuclease. The labelled *E. coli* RNA was mixed with RNA prepared from baker's yeast[13] to give a specific activity of 450 000 counts/min per mg. Assays contained 1.0 ml of 0.1 M Tris–HCl (pH 7.6), 1.0 ml of 0.05 % yeast–*E. coli* RNA mixture and 1.0 ml of sample containing ribonuclease and were incubated at 37° for 25 min. The reaction was terminated by addition of 1 mg of yeast carrier-RNA and 1.0 ml of ice-cold 20 % trichloroacetic acid. The total trichloroacetic acid precipitable material remaining after incubation was collected onto a glass fibre circle for counting. Sigma ribonuclease type II-A was used as a standard. The rate of conversion of the radioactively-labelled RNA to acid soluble material was linear between 0–100 ng of this ribonuclease.

Measurement of radioactivity

Sucrose density gradients containing radioactive material were collected into vials on fractionation, mixed with the scintillation fluid of BRAY[14] and counted in a Packard Scintillation Spectrometer. Samples from *in vitro* assays that had been treated with trichloroacetic acid to terminate the reaction were centrifuged at 500 × g for 10 min to sediment insoluble material. The pellets were dissolved in 0.1 M NaOH to hydrolyse all labelled amino acyl-tRNA, reprecipitated with trichloroacetic acid and the precipitates were collected onto glass fibre circles for counting by standard procedures. Scintillation fluid containing 0.3 % 2,5-diphenyloxazole and 0.03 % 1,4-bis-(5-phenyloxazolyl)-benzene in toluene was used.

RESULTS

Characterisation and properties of polyribosomes

In Fig. 1 is shown a typical sucrose density gradient profile of polyribosomes obtained from guinea pig hair follicle tissue. The profile is similar to that obtained by WILKINSON[9] for wool follicle tissue but different from that obtained by CLARKE AND ROGERS[7] and FREEDBERG[8] for guinea pig hair follicle tissue, especially in the yield of the large polyribosomes. This profile may be divided into four groups: a major ribosome peak (A) comprising about 50 % of the total ribosomal particles; a group of polyribosomes of 2–8 ribosomal units (B); an unresolved group of larger polyribosomes sedimenting as a broad band (C); and a fourth group comprising a shoulder on the former group, near the bottom of the gradient (D). If different extents of homogenisation were used, yields of the larger polyribosomes were reduced.

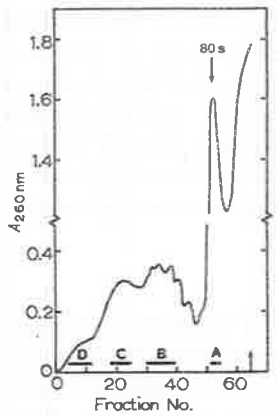

Fig. 1. Sucrose density gradient profile of polyribosomes obtained from guinea pig hair follicle tissue. Whole tissue homogenates were prepared as described in METHODS, and a 1.0 ml sample was centrifuged on a 28 ml 15–40 % sucrose density gradient for 3 h at 22 500 rev./min in Spinco SW25.1 rotor. The gradient was then fractionated as described in METHODS. Sedimentation is towards the left. The vertical arrow represents the last fraction. The Bars A, B, C and D refer to the groups of polyribosomes of increasing size described in the text.

The composition of buffer A used routinely for the preparation of tissue homogenates was determined (Table I). Optimum yields of polyribosomes were clearly recorded at 0.25 M KCl–20 mM Tris–HCl (pH 7.6)– 5 mM $MgCl_2$–1 mM dithiothreitol. Of interest was the marked increase in yield of polyribosomes (about 100 %) when

TABLE I

DETERMINATION OF THE OPTIMUM COMPOSITION OF BUFFER A

The buffer used for each experiment was based on Buffer A. For each experiment, one component was changed to one of the values shown whilst the concentrations of the other components remained as in Buffer A. Hair follicle whole tissue homogenates were then prepared in the modified buffer as described in METHODS and centrifuged through sucrose layers. The pellet containing the polyribosomes was resuspended in unmodified buffer A, clarified by centrifugation at 12 000 $\times g$ and the optical density of the supernatant determined. The individual yields are expressed as mg of polyribosomes per g of follicle tissue and are the mean of two experiments.

Component of Buffer A varied	*Concentration of component used in the different experiments (mM)*	*Yield*
KCl	50	0.3
	100	1.2
	250	2.6
	500	2.0
$MgCl_2$	0	0.2
	5	2.3
	10	1.8
Dithiothreitol	0	1.1
	1	2.7
No dithiothreitol but 2-mercaptoethanol	6	1.4
pH	7.2	1.4
	7.6	2.5
	8.0	0.9

1 mM dithiothreitol was used in place of the more commonly used 6 mM 2-mercaptoethanol. The sulphydryl-rich prekeratin proteins that are solubilised during homogenisation (unpublished, but see refs. 5, 15) became aggregated when buffers not containing dithiothreitol were used for homogenisation. Similarly, extensive aggregation occurred when the KCl concentration was reduced below 0.1 M.

The wax-sheet procedure for the preparation of hair follicles has been criticised[8] because it involves the application of a hot (60°) wax mixture to the skin of the animal prior to exposure of the follicles. Although the skin of the animal is precooled before treatment with wax it was thought that the elevated temperature might lead to degradation of cellular components[8]. To check this, follicles were prepared at different wax temperatures varying from 80 to 45°. Homogenates were prepared and examined by sucrose density gradient centrifugation. The yield of follicles per unit area of skin was reduced at the lower temperatures but there was no significant change in the polyribosome profiles at all temperatures examined, which suggested that the polyribosomal population in the cells of the tissue was not changed during isolation.

Samples from the sucrose density gradients of the C and D groups described were examined in an electron microscope (Siemens Elmiskop I) after negative staining with 1 % uranyl acetate. Group C contained particles of 12–18 ribosomal units and in group D most of the particles had 25–35 ribosomal units (Fig. 2). The polyri-

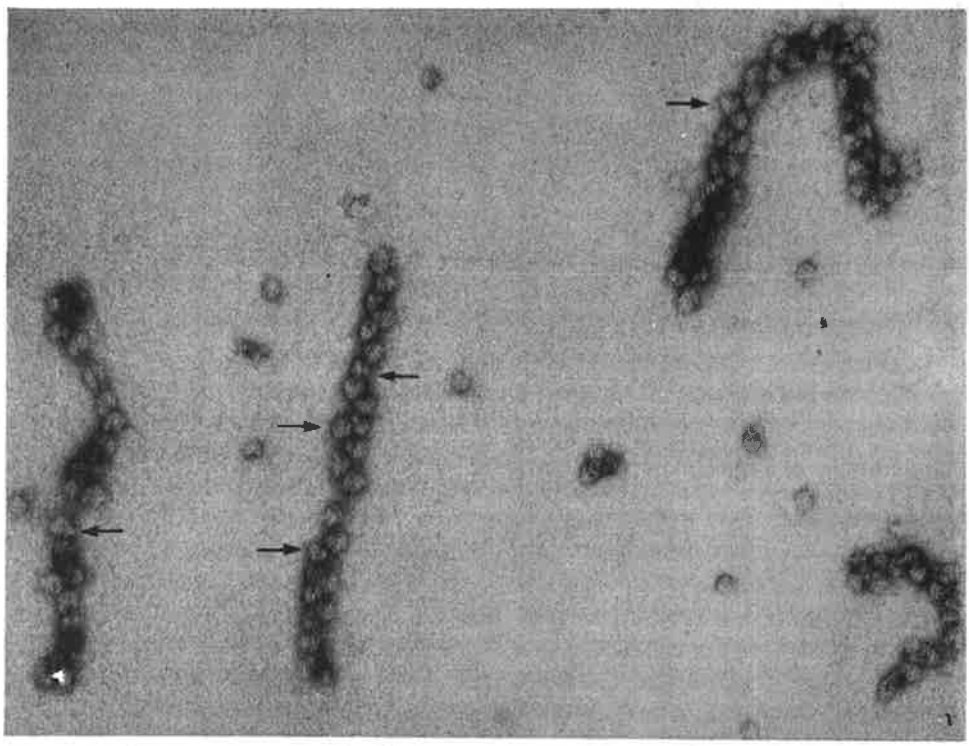

Fig. 2. An electron micrograph of polyribosomes obtained from guinea pig hair follicle tissue. The sample shown was taken from Fraction 8 (and hence from Group D) of a sucrose density gradient and negatively-stained with 1 % uranyl acetate. Magnification × 100 000. In places (arrowed), the subunits of individual ribosomes can be seen, and also, a thin strand about 10 Å in diameter (presumably mRNA) can be seen joining adjacent ribosomes along the polyribosomes.

bosomes of these groups appeared as long chains in the form of extended coils. Aspects of the ultrastructure of individual ribosomes of the polyribosomes are visible in places (arrowed).

To check that the polyribosomes of the C and D groups were not random aggregates of smaller particles, the C and D polyribosomes from three gradients were recentrifuged separately on sucrose density gradients (Fig. 3). Some losses due to

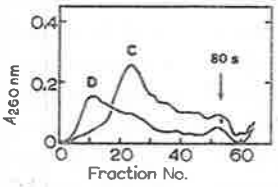

Fig. 3. Recentrifugation of the larger hair follicle polyribosomes. Polyribosomes of the C and D groups from three sucrose density gradients were pooled and sedimented by centrifugation through 2 M sucrose in Buffer A at 225 000 × *g* for 2.5 h. The pellets were carefully resuspended in Buffer A, centrifuged at 12 000 × *g* for 10 min to remove any material not resuspended and the resulting supernatants were layered separately onto 15–40 % sucrose gradients for recentrifugation as in Fig. 1.

mechanical breakage occurred on handling but the larger polyribosomes clearly resedimented to their original positions on the gradient.

Incubation of the hair follicle whole tissue homogenates with ribonuclease (1.0 μg/ml) at 2° for 10 min led to the complete degradation of the polyribosomes to single ribosomes whereas incubations with deoxyribonuclease (10 μg/ml) and trypsin (10 μg/ml) had no significant effect. These observations suggested that the polyribosomes were held together by single-stranded RNA, presumably by messenger RNA.

Ribonuclease activity in follicle tissue homogenates

In earlier studies[5,7,8], it had been shown that high levels of endogenous or contaminating ribonuclease were present in the hair follicle tissue homogenates. In

TABLE II

RIBONUCLEASE ACTIVITY IN HAIR FOLLICLE WHOLE TISSUE HOMOGENATES

Hair follicles were prepared from two animals of a litter of the ages specified. Whole tissue homogenates in Buffer A were prepared as described in METHODS and assayed for ribonuclease activity. The percentage of actively growing hair follicles was determined by plucking fibres from the dorsal region of the animals to be used and examined microscopically. All data shown are the mean values of the two animals used.

Animal age (days)	*Body weight (g)*	*Percentage of active follicles in skin*	*Ribonuclease activity (ng/ml of homogenate)*
1	75	98	3
6	95	95	5
12	110	90	6
16	140	92	6
33	260	67	26
47	310	58	73
about 70	390	49	121
about 98	450	42	118

view of the improved polyribosome profiles prepared in the present work the ribonuclease activity was estimated in homogenates of hair follicles isolated from animals of varying ages (Table II). The level of ribonuclease activity in homogenates from young animals was much lower than that from older animals. The level of about 120 ng/ml of ribonuclease activity using adult animals is similar to the value of 80 ng/ml found by FREEDBERG[8].

Cell-free protein synthesis in the whole tissue homogenate system

The time course for the incorporation of tritiated leucine into trichloroacetic acid insoluble material by the whole tissue homogenates was linear for the first 15 min but then reached a plateau level. The properties of this system are illustrated in Table III. The results were reproducible over several experiments although there

TABLE III

PROPERTIES OF THE WHOLE TISSUE HOMOGENATE CELL-FREE PROTEIN SYNTHESIS SYSTEM

Details of the complete whole tissue homogenate reaction are given in METHODS. Reactions were incubated for 60 min. The labelled amino acids were *leucine and **phenylalanine.

Experiment	*Counts/min*	*% of complete*
*Complete	21 720	100
−GTP	13 800	64
−ATP regeneration	14 820	68
−ATP and ATP regeneration	5 460	25
−Amino acids	11 880	55
+Puromycin, $5 \cdot 10^{-5}$ M	8 340	38
$2 \cdot 10^{-4}$ M	1 680	8
+Chloramphenicol, $5 \cdot 10^{-5}$ M	21 540	99
$4 \cdot 10^{-4}$ M	21 000	97
+Cycloheximide, $5 \cdot 10^{-6}$ M	15 420	71
$2 \cdot 10^{-5}$ M	6 180	28
$3 \cdot 10^{-3}$ M	720	3.3
+Ribonuclease, 10 μg	480	2.2
+Polyuridylic acid, 20 μg	20 400	94
200 μg	14 340	66
**Complete	7 800	100
+Polyuridylic acid, 20 μg	11 700	150
200 μg	62 880	806

was some variability in the dependence for added amino acids between different experiments. This most probably reflected variations in the pool sizes of free leucine or leucyl-tRNA in the follicle tissue homogenates of different guinea pigs.

Puromycin inhibited incorporation substantially and at $2 \cdot 10^{-4}$ M inhibition was almost complete. Similarly, cycloheximide at $3 \cdot 10^{-3}$ M completely inhibited incorporation and at $5 \cdot 10^{-6}$ M incorporation was still inhibited by 29 %. Chloramphenicol at $4 \cdot 10^{-4}$ M had little or no effect on amino acid incorporation. Since chloramphenicol at this concentration specifically inhibits protein synthesis by prokaryote cell-type (or mitochondrial) polyribosomes[16], and since cycloheximide specifically inhibits protein synthesis by eukaryote cell-type polyribosomes[17–19], it can therefore be assumed that bacterial contamination in this system was minimal and that cytoplasmic hair follicle polyribosomes were responsible for all incorporation

observed. Polyuridylic acid in high concentration inhibited the incorporation of leucine in this system by 34 %, suggesting that there was competition between endogenous messenger RNA and added polyuridylic acid for the attachment of ribosomes. When phenylalanine was used as the labelled amino acid it was seen that the hair follicle polyribosomes were directed by polyuridylic acid to synthesise polyphenylalanine.

Cycloheximide is reported to have dual inhibitory action on protein synthesis in eukaryote tissue[17–19]: at high concentrations it inhibits translation, but at very low concentrations it inhibits reinitiation. Thus it is possible that about 30–35 % of the total amino acid incorporation that occurred in this system was due to the elongation of protein chains newly initiated *in vitro*. This is supported by the observation on the effects of high concentrations of polyuridylic acid on leucine incorporation.

The effects of these manipulations on the structural integrity of the polyribosomes was investigated using sucrose gradient centrifugation. During incubation of the whole tissue homogenates with added ATP, an ATP regeneration source and unlabelled amino acids, the polyribosomes were degraded, the largest being degraded most rapidly (Fig. 4). Incubation with a low concentration of added ribonuclease (0.1 μg/ml) also resulted in degradation of the polyribosomes (Fig. 4).

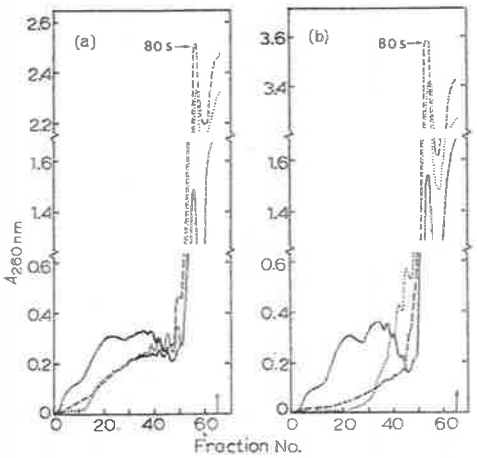

Fig. 4. The effect of incubation on the structural integrity of hair follicle polyribosomes. Whole tissue homogenate preparations were incubated as described in METHODS but with unlabelled amino acids (5 nmoles of each) for (a) 5 min and (b) for 10 min in the absence (. . .) or presence (- - -) of 0.1 μg/ml of added ribonuclease. Incubations were terminated by chilling and examined by sucrose density gradient centrifugation as in Fig. 1. A control homogenate sample (———) which had been held at 0° was centrifuged simultaneously.

In order to determine whether the degradation of the polyribosomes that occurred on incubation was due to protein synthesis or to the action of the ribonuclease present in the whole tissue homogenates, these experiments were repeated on tissue homogenates containing hair follicle polyribosomes that had been labelled *in vivo* with tritiated leucine (Fig. 5). In the absence of added ribonuclease the polyribosomes were degraded to ribosomes during incubation (Fig. 5a) and the labelled nascent

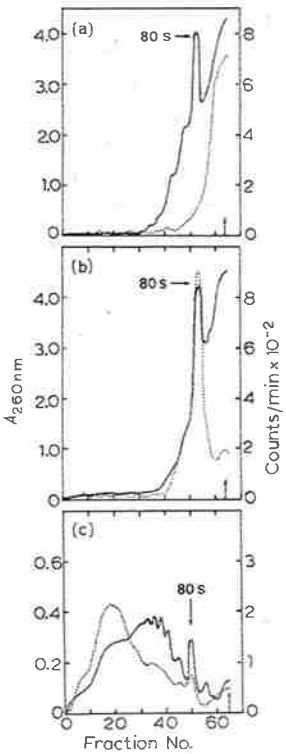

Fig. 5. The effect of incubation on the structural integrity of hair follicle polyribosomes bearing labelled nascent protein chains. Hair follicle polyribosomes were labelled *in vivo* by intracardial injection of [4,5-$^3H_2$]leucine (specific activity 58.1 C/mmole, 300 μC/100 g of animal body weight) into two guinea pigs. Trial experiments indicated that a pulse time of 10 min labelled the polyribosomes most satisfactorily, by about 50 000 disint./min per mg. The labelled polyribosomes were prepared free of the highly labelled supernatant fraction by centrifugation through sucrose layers. A 125 000 $\times g$ supernatant fraction was prepared from a third unlabelled animal. This preparation was *not* dialysed before use. Approx. 0.7 mg samples of the labelled polyribosomes were then incubated at 37° with unlabelled amino acids (5 nmoles of each), 1 mM ATP and an ATP regeneration source (see METHODS) for 10 min in 1.0 ml of the unlabelled supernatant fraction in the absence (a) or presence (b) of 0.1 μg/ml of added ribonuclease. The incubations were terminated by chilling and examined by sucrose density gradient centrifugation as in Fig. 1. A control sample of labelled polyribosomes suspended in buffer A which had been held at 0° was centrifuged simultaneously (c). The gradients were fractionated and prepared for counting as described in METHODS. ———, absorbance at 260_{nm}; . . ., counts/min.

protein chains were released into the supernatant: almost all of the label on the gradient appeared above the ribosome peak. However, in the presence of added ribonuclease, the labelled polyribosomes were degraded to labelled ribosomes (Fig. 5b): the labelled nascent protein chains were not released from the ribosomes. Since in the absence of added ribonuclease very little radioactivity remained associated with the ribosomes (Fig. 5a), it is concluded that the amount of ribonuclease activity present in the homogenates (about 5 ng/ml) was not sufficient to cause significant degradation of the polyribosomes *per se* during incubation. Low concentrations of ribonuclease can hydrolyse single-stranded (and in this case messenger) RNA but do not significantly effect attachment of tRNA species bearing nascent protein chains to ribosomes[20].

This experiment therefore suggested that, during amino acid incorporation in hair follicle tissue homogenates, the polyribosomes degraded by an orderly process of run-off of ribosomes from the messenger due to protein synthesis, coupled with the release of the protein chains, as in other eukaryote cell-type systems[1,21,22].

The effects of the antibiotics puromycin and cycloheximide on the structural integrity of the hair follicle polyribosomes during incubation were also investigated using similar techniques. $2 \cdot 10^{-4}$ M puromycin protected the polyribosomes from degradation (Fig. 6a) but at $2 \cdot 10^{-5}$ M significant breakdown occurred. Similarly, $3 \cdot 10^{-3}$ M cycloheximide (Fig. 6b) protected the structure of the polyribosomes, but

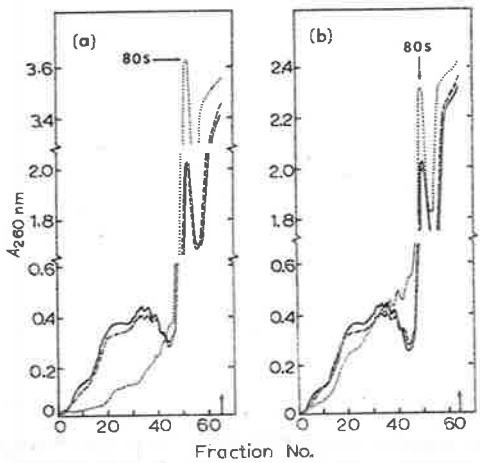

Fig. 6. The effect of incubation with puromycin and cycloheximide on the structural integrity of hair follicle polyribosomes. Whole tissue homogenate preparations were incubated at 37° for 5 min as described in METHODS but with unlabelled amino acids (5 nmoles of each) and with (a) puromycin at $2 \cdot 10^{-5}$ M (. . .) and $2 \cdot 10^{-4}$ M (- - -) or (b) cycloheximide at $5 \cdot 10^{-4}$ M (. . .) and $3 \cdot 10^{-3}$ M (- - -). The reactions were terminated by chilling and examined by centrifugation on sucrose density gradients as in Fig. 1. A control homogenate sample (———) which had been held at 0° was centrifuged simultaneously in each case.

at $5 \cdot 10^{-4}$ M breakdown occurred. When polyribosomes labelled *in vivo* with tritiated leucine were incubated with $2 \cdot 10^{-4}$ M puromycin, the nascent protein chains were released (Fig. 7a): almost all of the label on the gradient appeared above the ribosome peak. The size of the polyribosomes was also maintained, as before (Fig. 6a). This observation is not entirely consistent with the studies of WILLIAMSON AND SCHWEET[23]. These workers showed that in the presence of energy and amino acids and $2 \cdot 10^{-4}$ M puromycin, degradation of reticulocyte polyribosomes occurred *in vitro*, and that nascent protein chains were released as the peptidyl-puromycin derivatives. Further, they postulated that the degradation of the polyribosomes was due to randon reinitiation of peptide bond formation along the messenger. Hence, as the hair follicle polyribosomes used in the present work did not degrade during incubation with $2 \cdot 10^{-4}$ M puromycin, it is possible that this reinitiation process did not occur. The peptidylpuromycin or puromycin molecules themselves may competitively inhibit the ribosomal binding site such that random reinitiation of peptide bond formation along the messenger RNA is prevented. At lower concentrations

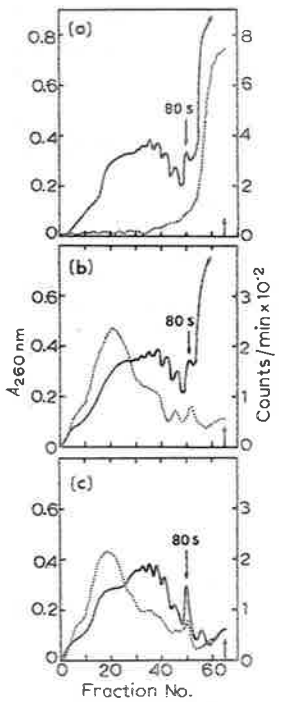

Fig. 7. The effect of incubation with puromycin and cycloheximide on the structural integrity of hair follicle polyribosomes bearing labelled nascent protein chains. Hair follicle polyribosomes were labelled *in vivo* and prepared as described in Fig. 5. Samples were incubated at 37° for 10 min in 1.0 ml of an unlabelled supernatant fraction as in Fig. 5 in the presence of either $2 \cdot 10^{-4}$ M puromycin (a) or $3 \cdot 10^{-3}$ M cycloheximide (b). The incubations were terminated by chilling and examined by sucrose density gradient centrifugation as in Fig. 1. A control sample of labelled polyribosomes was centrifuged simultaneously (c). Other details are as in Fig. 5. ———, absorbance; . . . ., counts/min.

of puromycin (Fig. 6a) degradation of the hair follicle polyribosomes proceeded, presumably by release of the nascent protein chains and random reinitiation of peptide bond formation by the same mechanism as that postulated by WILLIAMSON AND SCHWEET[23].

When incubated with cycloheximide (Fig. 7b) the size of the labelled polyribosomes was maintained and the label on the gradient remained distributed as in the control sample of labelled polyribosomes (Fig. 7c); the nascent protein chains were not released by this antibiotic. The concentration of cycloheximide required for this inhibition was high, presumably due to the presence of 1 mM dithiothreitol in the incubation system[19]. The use of high levels of cycloheximide therefore provides a convenient method for the rapid termination of protein synthesis *in vitro* whilst the structural integrity of the polyribosomes is maintained.

Cell-free protein synthesis in the reconstituted system

Optimum conditions for the incorporation of labelled leucine into trichloroacetic acid precipitable material were determined. The reconstituted system was most active at 0.125 M KCl–20 mM Tris–HCl (pH 7.6)–6.5 mM $MgCl_2$–1 mM dithio-

threitol. Addition of deacylated yeast tRNA (50 μg/ml) was found to stimulate incorporation slightly. The time course for amino acid incorporation was linear for the first 20 min and reached a plateau level at 40 min.

The properties of the reconstituted system are given in Table IV. These results were reproducible over several experiments. Incorporation was totally dependent

TABLE IV

PROPERTIES OF THE RECONSTITUTED CELL-FREE PROTEIN SYNTHESIS SYSTEM

Details of the complete reconstituted system are given in METHODS. Each reaction in these experiments contained 154 μg of polyribosomes from a single preparation and was incubated for 60 min.

Experiment	*Counts/min*	*% of complete*
Complete	22 880	100
−GTP	17 480	76
−ATP regeneration	11 930	52
−ATP and ATP regeneration	4 100	18
−Amino acids	9 100	40
−Yeast tRNA	19 220	84
−Polyribosomes	790	3.4
−Supernatant fraction	260	1.1
—Dithiothreitol	10 270	45
—Dithiothreitol + 2-mercaptoethanol, $6 \cdot 10^{-3}$ M	14 620	64
+Puromycin, $5 \cdot 10^{-5}$ M	2 420	11
$2 \cdot 10^{-4}$ M	640	2.8
+Chloramphenicol, $1 \cdot 10^{-4}$ M	21 930	96
$4 \cdot 10^{-4}$ M	21 320	93
+Cycloheximide, $5 \cdot 10^{-6}$ M	15 790	69
$5 \cdot 10^{-4}$ M	6 380	28
$3 \cdot 10^{-3}$ M	950	4.2
+Ribonuclease, 10 μg	460	2.0
+Polyuridylic acid, 20 μg	21 400	94
200 μg	14 640	64

upon added polyribosomes, supernatant factors and ATP and partially dependent on added amino acids, reducing agents and yeast tRNA. In the absence of dithiothreitol considerable precipitation of the sulphydryl-rich prekeratin proteins[5,15] occurred during incubation, even if 6 mM 2-mercaptoethanol was added in its place. Antibiotics and ribonuclease affected the activity in the manner expected of eukaryote cell-type polyribosomes. Inhibition by high concentrations of cycloheximide and low concentrations of polyuridylic acid suggested that reinitiation occurred in the reconstituted *in vitro* system also.

Attempts were made to increase the dependence of the reconstituted system on added amino acids by modification of the supernatant fraction. More extensive dialysis of it was not effective; nor was passage of the supernatant through columns of Sephadex G-50 (medium) equilibrated in Buffer B. It is concluded that much of the endogenous tRNA was charged with amino acids.

The effect of the temperature of the wax used for the preparation of hair follicles from the guinea pig skin on the incorporation ability of the follicle polyribosomes was investigated. Follicles were prepared as described at different wax temperatures varying from 80 to 45°. Polyribosomes were then prepared from each of the

temperature samples and assayed in the *in vitro* protein synthesis system. No significant variation in incorporation was observed between the different temperature samples which suggested that the hot wax sheet procedure for the preparation of hair follicles did not alter the properties of the isolated polyribosomes.

The rate of release from the polyribosomes of protein chains synthesised during incubation was investigated (Fig. 8). About 55 % of the total trichloroacetic acid

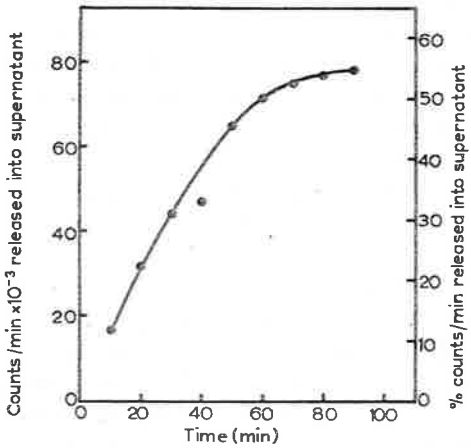

Fig. 8. Estimation of the rate of release of protein chains from the polyribosomes. The composition of the reconstituted cell-free incubation system used is given in METHODS. Samples were removed from an incubation at various times and terminated by the addition of cycloheximide to a final concentration of $3 \cdot 10^{-3}$ M and chilled. The samples were centrifuged at 125 000 × *g* for 1.5 h to pellet the ribosomal material. The pellet and supernatant were then assayed separately for radioactivity. The curve shows the amount of radioactivity that was released from the polyribosomes during the course of the incubation and the percentage of the total trichloroacetic acid precipitable counts incorporated that were released into the supernatant.

precipitable material incorporated during incubation was released from the polyribosomes. Thus it is concluded that the polyribosomes steadily released completed protein chains at a substantial rate as amino acid incorporation proceeded.

The activity of the *in vitro* reconstituted system was estimated as follows. In an incubation mixture containing 150 μg of polyribosomes and 2 μC (1000 pmoles) of tritiated leucine, about 22 000 counts/min or about 65 000 disint./min (15 pmoles) were incorporated. Since the leucine content of the total hair follicle proteins was about 10 % (unpublished observations), and if it can be assumed that all follicle proteins were represented in the polyribosomal population, then the *in vitro* system incorporated about 1000 pmoles of amino acids per mg of polyribosomes. In comparison, when similar calculations were applied to previous experiments[7], the polyribosomes incorporated only about 1.4 pmoles of amino acids per mg of polyribosomes. Further calculations in the present work show that each ribosome incorporated an average of about two amino acid residues. This degree of incorporation is lower than that expected from the evidence presented earlier of degradation of polyribosomes by run-off of ribosomes from a messenger during amino acid incorporation. This apparent anomaly could be explained by the presence in the dialysed supernatant of endogenous aminoacyl-tRNA, as already mentioned.

DISCUSSION

The yields of especially the larger polyribosomes isolated in the present work clearly exceeded those of earlier experiments[5,7,8]. The guinea pigs used here were much younger than those used previously. The number of actively growing hair follicles in animals less than three weeks of age is about 90–95 % whereas this number is reduced to less than 50 % in animals older than two months. Accordingly, higher yields of polyribosomes have been found. Observations in this work have demonstrated that greater yields of polyribosomes are obtained when buffers of high ionic strengths are used. Similar observations have been made in wool follicle tissue[9] and chick embryo muscle tissue[24]. Of interest also is the increased yield of polyribosomes using buffers containing dithiothreitol instead of the more commonly-used 2-mercaptoethanol. While it is appreciated that dithiothreitol is a superior reducing agent[25] and that hair follicle tissue contains large amounts of sulphydryl-rich proteins[5,15], the precise reason for the higher yields of polyribosomes is not clear.

The most significant reason for greater yields of the larger polyribosomes in the present work is the much lower level of ribonuclease activity in the tissue homogenates. Hair follicle tissue homogenates prepared from young animals contained only trace amounts of ribonuclease activity (about 5 ng/ml) whereas in homogenates prepared from older animals, of about the same age as those used in previous studies, the ribonuclease content was much greater (about 120 ng/ml). The reason why higher levels of ribonuclease activity in the hair follicle tissue of older animals is found is not clear. In another study (R. M. CLARKE AND G. E. ROGERS, unpublished) a substrate-film technique on skin slices containing hair follicles from adult guinea pigs was employed to locate the source of the high ribonuclease activity found in tissue homogenates. It was shown that the sebaceous gland adjoining the hair follicle canal and the epidermis surrounding the hair follicle itself contained large amounts of an acid ribonuclease. (See also FREEDBERG *et al.*[26].)

Although large polyribosomes from hair follicle tissue have been isolated here, about 50 % of the total ribosomal particles produced were present as single ribosomes. This finding cannot be accounted for by shearing of the polyribosomes during homogenisation because the observation was made (not shown) that when polyribosomes labelled *in vivo* were separated on sucrose density gradients, the small polyribosomes and single ribosomes were only slightly labelled. FAN AND PENMAN[27] have shown that in dividing eukaryote cells, about 75 % of the total cytoplasmic ribosomes are present as single ribosomes. In the present study, many of the cells that were disrupted during homogenisation would have originated from the lowest regions of the follicle and these cells are known to be actively dividing. Thus the high proportion of single ribosomes in the homogenates might have had their origin in this manner.

Studies in a number of animal tissues which synthesise only one or a small group of proteins, such as reticulocytes[1], lens[2,28] and muscle[3,24] suggest that there is a relationship between the size of the polypeptide chain and the size of the polyribosome that directs its synthesis; this relationship is of the order of 30 amino acids (or 3000–4000 daltons) per ribosome. If this relationship is used to postulate the sizes of the protein chains that hair follicle polyribosomes synthesise, they amount to, group B, 10 000–30 000; Group C, 40 000–50 000; Group D, 90 000–120 000. By comparison, the keratin proteins of guinea pig hair can be fractionated into two groups, one with

a range of molecular weight 10 000–30 000 (the "high-sulphur" or matrix component) and the other, of molecular weight 43 000 (the "low-sulphur" or microfibrillar component). Both of these types of proteins can be readily isolated from hair follicle tissue (refs. 15, 29 and P. M. STEINERT AND G. E. ROGERS, unpublished). It is not possible to speculate on the role of the Group D polyribosomes in protein synthesis in the hair follicle.

In the present work detailed studies were made on the properties of the hair follicle polyribosomes and of the amino acid incorporation systems established with them. Using a simple tissue homogenate the hair follicle polyribosomes incorporated tritiated leucine into trichloroacetic acid precipitable material at a substantially greater rate than observed previously[5,7,8]. Amino acid incorporation in both whole tissue homogenates and reconstituted systems proceeded at a linear rate for about 20 min. It is likely that considerable polypeptide chain elongation took place during this time as shown by the steady decrease in polyribosome size and the steady release of protein chains from the polyribosomes during incubation. It was estimated that the reconstituted system established in the present work was about 700 times more active than that established previously[7]. In addition, it is likely that considerable polypeptide chain reinitiation occurred during incubation, as shown by the inhibitory effects on leucine incorporation of low concentrations of cycloheximide and high concentrations of polyuridylic acid.

Further studies on the biosynthesis of the individual hair follicle protein species *in vitro* and *in vivo* should be possible as the systems developed in the present work were highly active in cell-free protein synthesis.

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Biochim. Biophys. Acta, 232 (1971) 556–572

FIGURE 5.1

LONGITUDINAL CROSS-SECTION THROUGH A GUINEA PIG HAIR FOLLICLE

The section was made near the mid keratogenous region (level 2) (*see* Fig. 1.3) of the hair follicle. The specimen was prepared as described in General Methods (*see* page 42) and stained on the grid for 5 min with 1 % (w/v) $KMnO_4$.

Numerous densely-stained "cortical granules" are associated with the clusters of keratin microfibrils.

Magification: x 25 000.
Cu = cuticle; Co = cortex; n = nucleus; r = ribosomes; v = vesicle; m = mitochondrion.


m
v
Co
r
v
m
n
r
Co
Cu

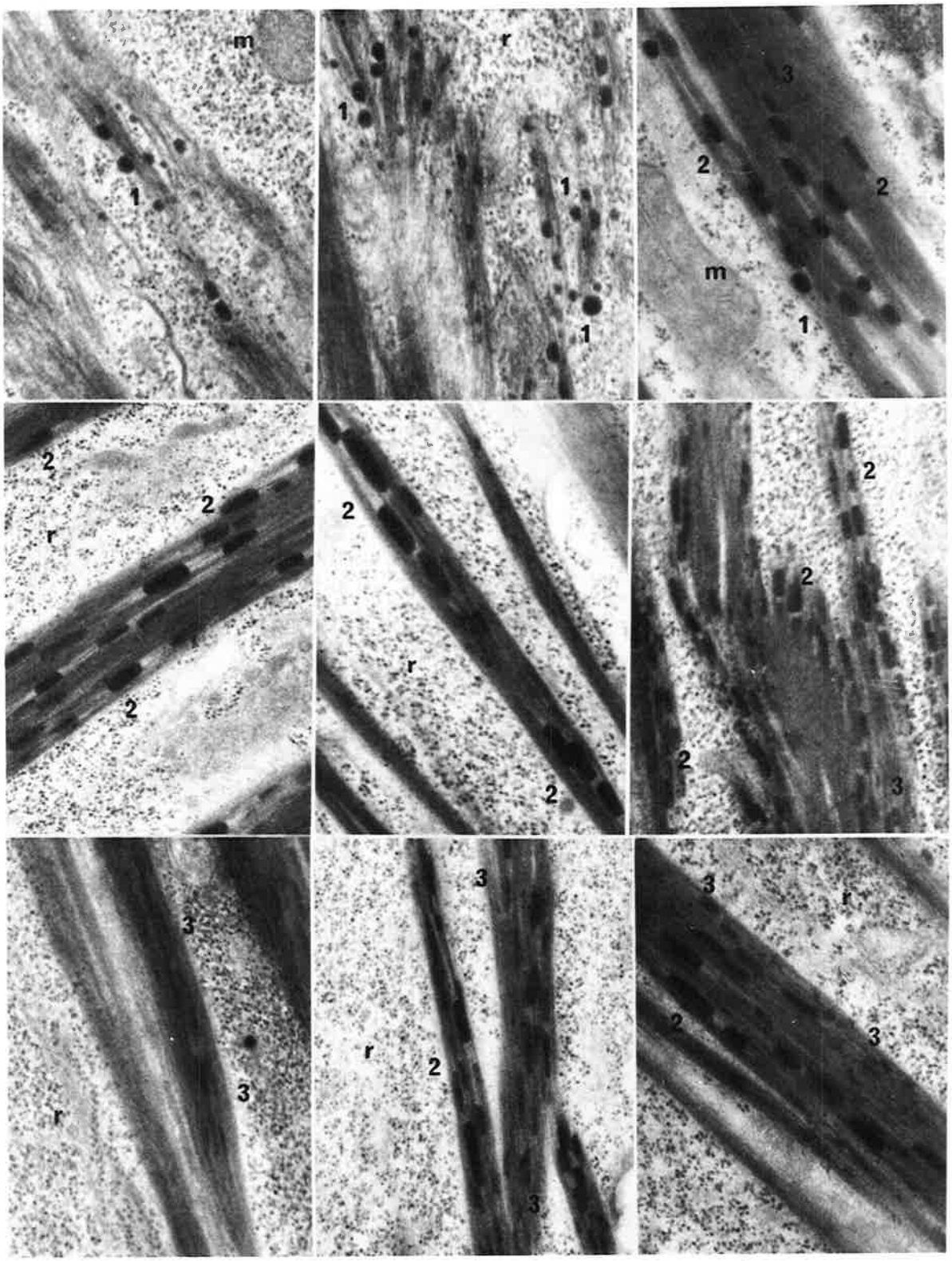
FIGURE 5.2

MONTAGE OF LONGITUDINAL CROSS-SECTIONS THROUGH SEVERAL DIFFERENT GUINEA PIG HAIR FOLLICLES

All specimens were prepared as described in Fig. 5.1.

The cortical granules appear as in Fig. 5.1. Areas labelled *1*, *2* and *3* refer to the different states of association of the cortical granules with the microfibrils as described in the text.

Magnification in each field is: x 40 000.
r = ribosomes; m = mitochondrion.


m
1
r
1
1
1
3
2
2
m
1
2
2
r
2
2
2
r
2
2
2
2
2
3
3
r
3
r
2
3
3
r
2
3
3
r
2
3

The cortical granules are present only in one or two cell-lengths (50 - 100 μm) of the follicle: they are absent from cells either below or above the specific level at which the cuticle proteins first become apparent. Moreover, the cortical granules are present in all guinea pig hair follicles examined.

(2) Transverse sections

In Fig. 5.3 is shown a transverse cross-section through a guinea pig hair follicle at the level at which the granules appear in longitudinal section. Numerous densely-stained areas of roughly circular shape appear associated with the microfibrils and it is likely that these are cortical granules as shown in Figs. 5.1 and 5.2. Droplets of protein are also present in the neighbouring cuticle cells (Fig. 5.3). In Fig. 5.4 is a montage of transverse cross-sections showing association of the granules with the microfibrils at higher magnification. Again, three distinct situations exist. In some areas *(1)* there are regions of low electron density between microfibrils where matrix material appears to be absent. These microfibrils appear poorly-oriented in the plane of the section. An intermediate stage is evident *(2)* in which granules are located amongst groups of poorly-oriented microfibrils and only the peripheral microfibrils are ordered. Elsewhere densely-stained deposits appear amongst microfibrils *(3)* in which the microfibrils appear oriented in the plane of the section as in fully-hardened keratin. These different observations may reflect different stages of association of the material of the cortical granules with microfibrils.

In lower levels of the hair follicle the microfibrils are poorly-oriented in the plane of the section and there is no evidence of densely-stained material between the microfibrils (Fig. 5.5a and stage *1* of Fig. 5.4). On the other hand, at levels above the region where the granules are first

FIGURE 5.3

TRANSVERSE CROSS-SECTION THROUGH A GUINEA PIG HAIR FOLLICLE

The section was made near the mid keratogenous region (level 2) of the hair follicle. The sample was prepared as described in Fig. 5.1 except that it was stained with 1 % (w/v) uranyl acetate in 0.1 M veronal buffer (pH 7.5) for 30 min before embedding in "Araldite". The section was further stained on the grid for 5 min with 1 % (w/v) $KMnO_4$.

The densely-stained regions are assumed to be the cortical granules.

Magnification: x120 000.
Cu = cuticle; Co = cortex; m = mitochondrion; cm = cell membrane; r = ribosomes; mt = microtubule spindle.

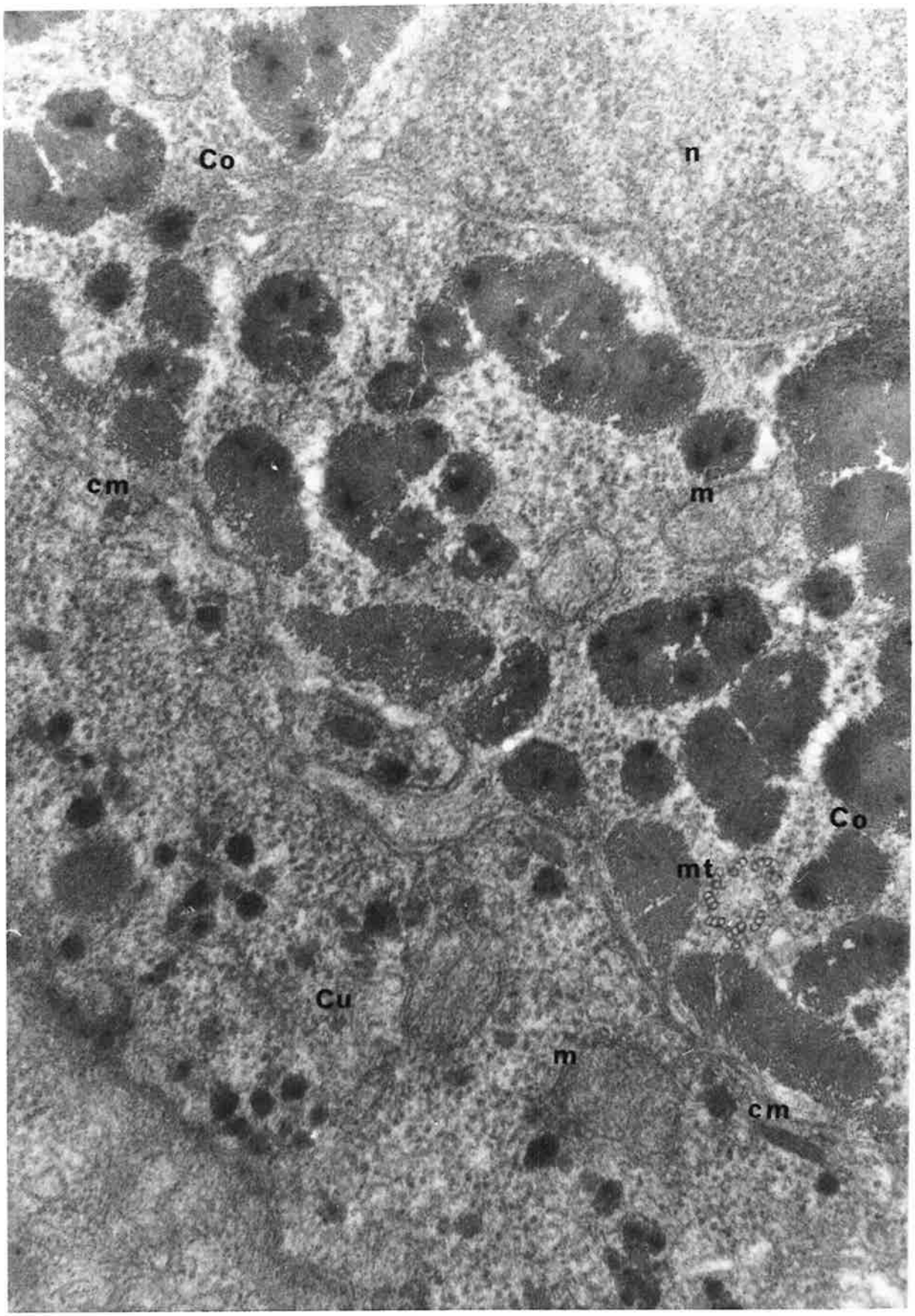

Co
n
cm
m
Co
mt
Cu
m
cm

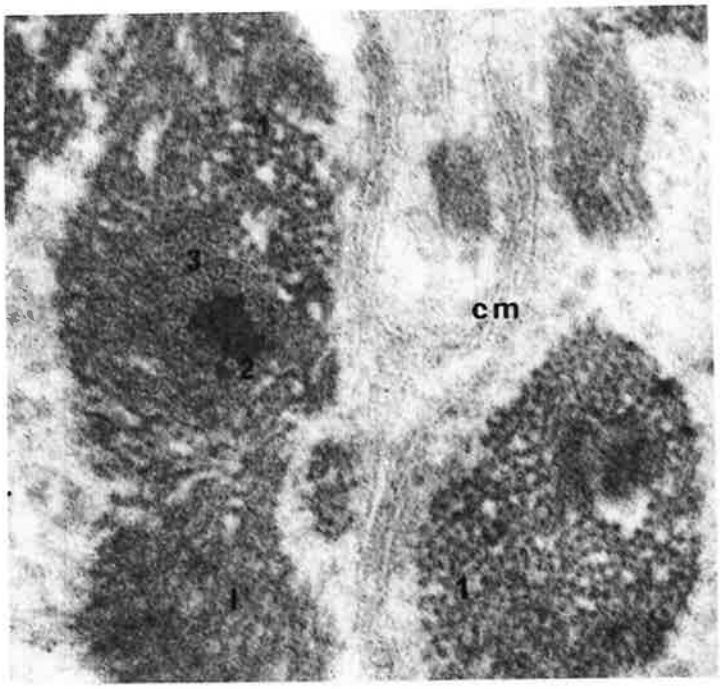
FIGURE 5.4

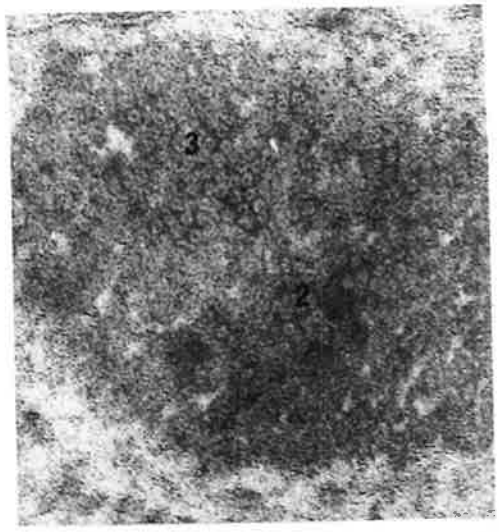
MONTAGE OF TRANSVERSE CROSS-SECTIONS THROUGH SEVERAL DIFFERENT GUINEA PIG HAIR FOLLICLES

All specimens were prepared as described in Fig. 5.3.

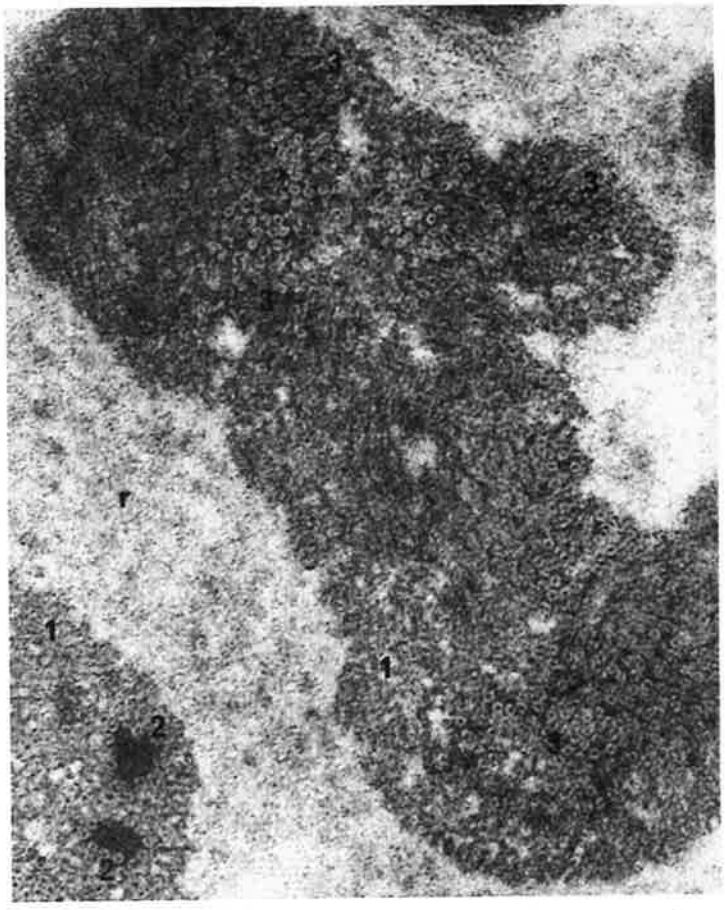
The cortical granules appear as in Fig. 5.3. The areas labelled *1*, *2* and *3* refer to the different states of association of the cortical granules with the microfibrils as described in the text.

Magnification in each field is: x 200 000.
cm = cell membrane; r = ribosomes.


3
cm
2
1
1


3
2


1
2
3


3
3
r
1
2
1
2

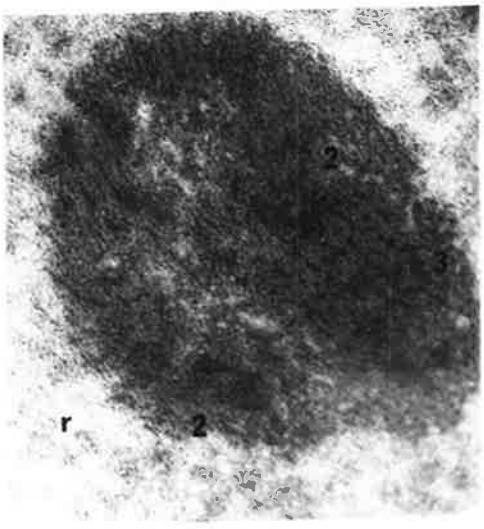

2
3
r
2

FIGURE 5.5

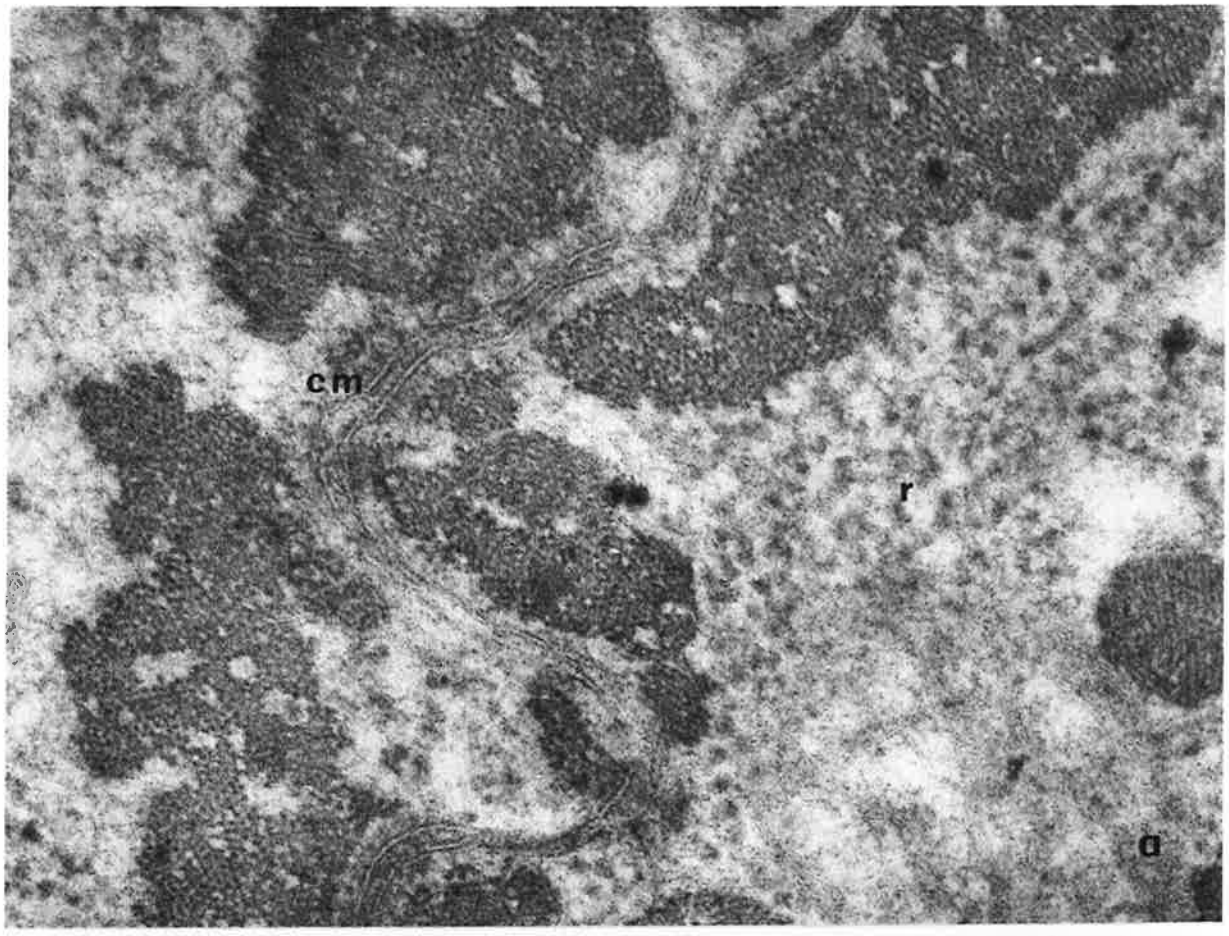
TRANSVERSE CROSS-SECTIONS THROUGH A GUINEA PIG HAIR FOLLICLE AT DIFFERENT LEVELS IN THE FOLLICLE

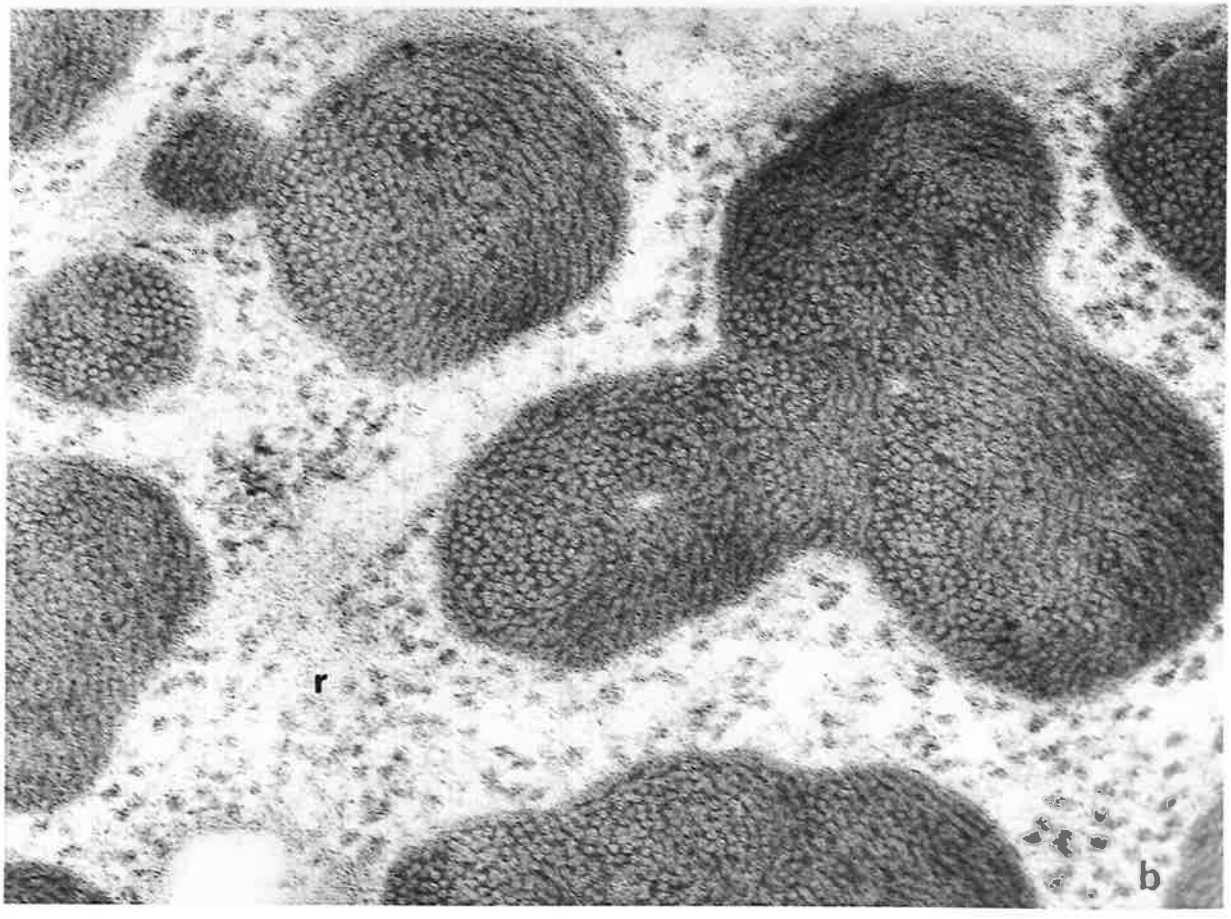
The sections were prepared as described in Fig. 5.3.

(a) Cross-section at a level about 100 μm below where the cortical granules were first detected. The microfibrils are not surrounded by a dense layer of stain and appear poorly oriented in the plane of the section.

(b) Cross-section at a level about 100 μm above where the cortical granules were first detected. The microfibrils are well oriented in the plane of the section and have dense deposits of stain about their peripheries as in fully-hardened keratin.

Magnification: x 120 000.
cm = cell membrane; r = ribosomes.


cm
r
a


r
b

seen, the microfibrils within each macrofibril appear well-oriented in the plane of the section and have densely-stained peripheries as in fully-hardened keratin (Fig. 5.5b). At this level there are no clearly discernible cortical granules.

(b) ATTEMPTED ISOLATION OF CORTICAL GRANULES

These studies were undertaken in order to determine the nature of the cortical granules by direct isolation and characterisation.

(1) Preliminary studies

Hair follicle tissue homogenates were prepared in a buffer consisting of 10 mM tris-HCl (pH 7.6) and 10 mM KCl using a Dounce homogeniser (Kontes Glass, clearance about 0.07 mm) and examined in the electron microscope by negative-staining. Although numerous other cytoplasmic particles were present in these homogenates, no structures identifiable as cortical granules were found. It therefore seemed more profitable to extend these studies to preparations of cortical cells prepared as described in Methods.

(2) Studies on cortical cells

Material was released from the cortical cells by homogenisation of a suspension (about 100 mg/ml) in the 10 mM tris-HCl (pH 7.6) and 10 mM KCl buffer in a Dounce homogeniser (Kontes Glass, clearance about 0.07 mm). Such homogenates contained fibrous material (presumably keratin microfibrils) to which were frequently attached rectangular-shaped bodies (Fig. 5.6). These had dimensions similar to the granules seen in section and it seemed likely that they were indeed cortical granules.

Variations in the disruption techniques, such as homogenisation in the presence of detergents, 0.1 % (w/v) trypsin and ultrasonication, failed to release the granules from the fibrous material. Indeed, the two were

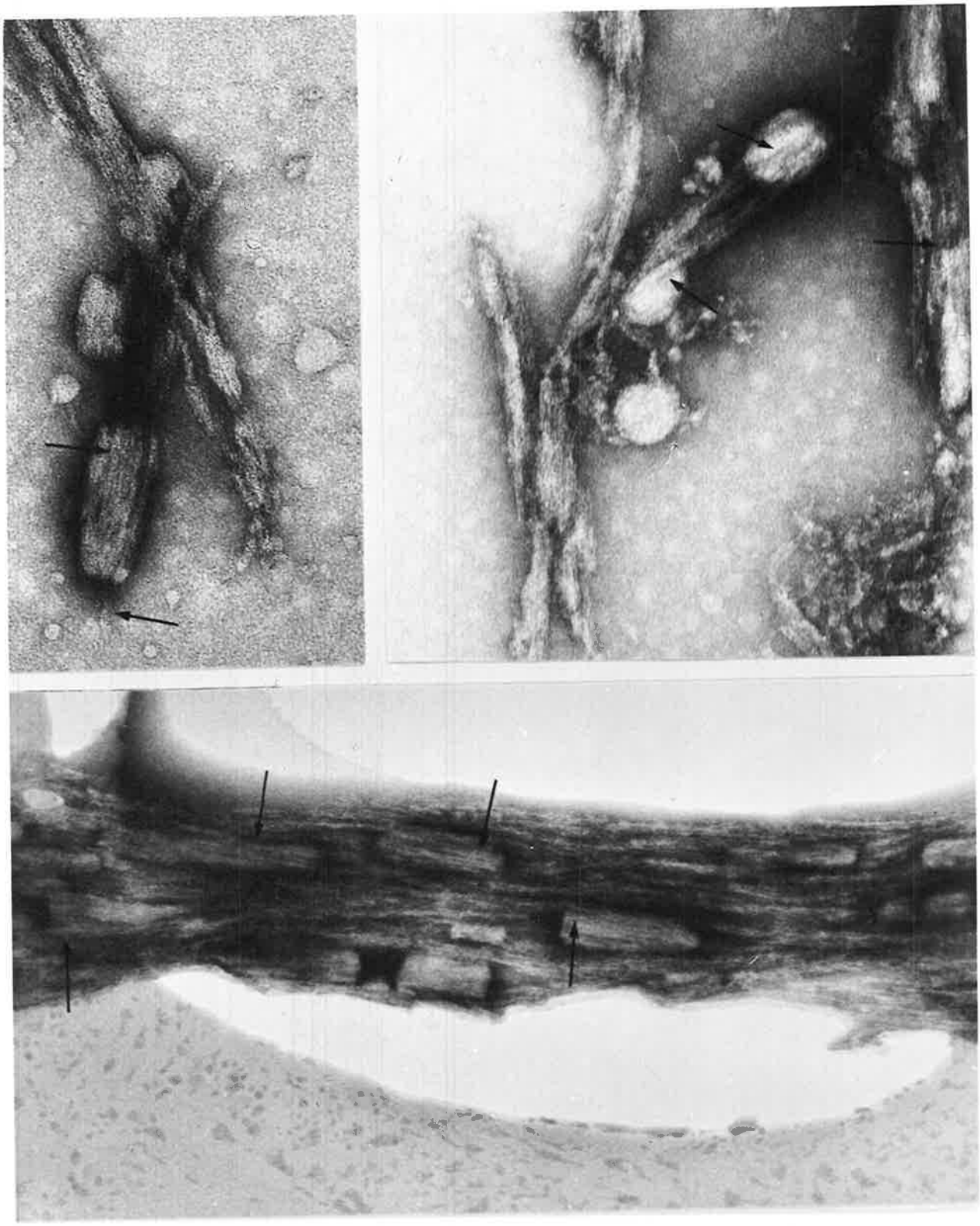
FIGURE 5.6

CORTICAL GRANULES IN HOMOGENATE PREPARATIONS OF CORTICAL CELLS

The homogenates were prepared from the cortical cells as described in the text. The specimens were stained with 1 % (w/v) uranyl acetate.

The cortical granules appear as rectangular-shaped bodies and have grooves along their surfaces and in places filaments are attached at their edges (arrowed).

Magnification: x 200 000.



associated with one another; filaments about 40 - 50 Å were seen attached to the granules and grooves about 50 Å wide were apparent on them (Fig. 5.6).

It was not possible to obtain any further information on the chemistry or structure of the granules as the quantity prepared by these procedures was very low and so chemical analysis of them was not possible.

(c) OCCURRENCE OF CORTICAL GRANULES

Hair follicles from a number of different animals were examined in the electron microscope for the presence of the cortical granules after sectioning (Table 5.1). Information on the contents of matrix (HiS) proteins in the hairs produced by these animals and the amount of cystine in the proteins is also given. This is listed in order to test an hypothesis that there might be a relationship between the presence of the cortical granules and the matrix and cystine contents of the proteins of the hairs of these animals.

The cortical granules were present only in the guinea pig and rabbit follicles, and in support of the hypothesis mentioned, the hair fibres of these animals contained high levels of matrix proteins and cystine. In contrast, however, poodle hair and "sulphur-enriched" wool also contained high levels of cystine but the granules were not present in the follicles of these specimens. It would therefore appear that the hypothesis stated above is untenable.

D DISCUSSION

The cortical granules described here are structures that have not been described previously in cross-sections of guinea pig hair follicles (but see Swift, 1968; see below). Their presence suggests that they may be important in development in the guinea pig hair follicle.

The working hypothesis of this chapter is that the cortical

TABLE 5.1

OCCURRENCE OF CORTICAL GRANULES

Hair follicles from the different animal species listed were prepared for electron microscopy as described in General Methods (*see* page 42). Data on the matrix protein and SCMcysteine content of matrix proteins from hairs of these animals was obtained from Gillespie and Inglis (1965), except as noted. These values represent typical values of the different types of hairs; the actual values for the animals from which the follicles were obtained in this work were not determined.

Source of hair follicles	Cortical granules	Matrix proteins: % of kerateine proteins	SCMcysteine content of matrix proteins (g of N per 100 g of total N)
Poodle [a]	No	50	21.7
Guinea pig	Yes	34	21.7
Rabbit	Yes	29	21.7
Human	No	38	19.4
Merino sheep (control diet)	No	22	15.9
Merino sheep [b] (on high methionine diet)	No	34	18.6

[a] Values from Gillespie (1971, personal communication). These values are "extrapolated" values based on a sulphur content of 5.9 - 6.1 %. Direct estimates were not possible since only about 30 % of the poodle hair could be solubilised.

[b] These follicles were obtained from Dr. R.M. Clarke of the C.S.I.R.O. Division of Animal Physiology, Prospect, N.S.W., Australia.

granules contain HiS proteins. This notion arose from electron microscope observations in both longitudinal and transverse cross-sections. There appeared to be different states of association of the cortical granules with the microfibrils and these states may represent different *stages* of association. Thus, at the earliest stages, the cortical granules appear as circular (or spherical) bodies attached to one surface of a small cluster of microfibrils. As more fibrils are added the cortical granules adopt an elongated rod shape, and eventually disappear into progressively smaller particles as the fibril clusters enlarge. This is to say that as the microfibrils are added to a cluster, the amorphous granule material becomes dispersed among the fibrils.

Some experimental evidence was obtained which supports the hypothesis. In transverse sections the material of the granules associates with the microfibrils in a manner reminiscent of the structure of fully-hardened keratin. Each microfibril in the vicinity of a cortical granule is aligned in the plane of the section and has a layer of densely-stained material about its periphery. The matrix proteins of fully-hardened keratin and the cortical granule material stain in an analagous fashion with OsO_4 and $KMnO_4$. The close structural association of the cortical granules with microfibrils seen *in situ* was also observed in homogenate preparations. The granules frequently had filaments or grooves on their surfaces. These granule-filament complexes were resistant to mild exposures of proteolytic enzymes which is analagous to the known resistance of hardened keratin to proteolytic enzyme degradation.

Some indirect information on the chemical nature of the granules has appeared (Swift, 1968). Using a histological stain sensitive to cystine applicable at the electron microscope level, he reported the presence of "rectangular-shaped deposits of cystine in the cortex" of guinea pig hair

follicles "associated with the filament bundles". It is possible these deposits were the structures seen here.

However, it was not possible to obtain any direct chemical information on the composition of the cortical granules. It is emphasised that such information is essential before the present hypothesis that the cortical granules contain HiS proteins can be accepted. Moreover, the granules were found only in albino guinea pig and rabbit hair follicles and there was no obvious correlation between their presence in hair follicles from different animals and the matrix or cystine content of the hair fibres from the different animals. It is possible that the granules are structures unique to animals of the *rodenta* class. More extensive studies are necessary to establish this.

Notwithstanding these comments, some further points are worthy of consideration if it is assumed that these cortical granules contain HiS proteins. Firstly, their presence indicates the stage when the HiS proteins first appear in hair follicle and it is apparent that this stage is well after the first appearance of the microfibrillar (LoS) proteins. This fact reaffirms the observations that were made earlier by other workers (for example, Rogers, 1964) on the appearance of HiS proteins during follicle development. As described in Chapter One (*see* page 27) some autoradiography studies have indicated that synthesis of the keratin mRNA species occurs very early in the follicle (Sims, 1967; Rogers, 1970, unpublished studies). Therefore, since HiS proteins do not appear for a considerable period of time after the synthesis of their mRNA, restriction of the free expression of mRNA for the HiS proteins may occur. This regulation may operate at some post-transcriptional steps of protein synthesis as summarised in Chapter One. This matter will be considered further in later chapters of this thesis.

Secondly, it appears that there are two distinct stages in the

synthesis of the proteins. Initially, there is a rapid burst of synthesis of protein which is deposited onto the microfibrils in the form of the regular-shaped granules. This deposit of amorphous proteins is dispersed during further addition of microfibrils. After this, it is proposed that further synthesis of matrix proteins is maintained at a rate parallel with the rate of further microfibrillar synthesis.

It is of interest that the cortical granules were first detected at the same level at which the dense cystine-rich droplets of protein appeared in the neighbouring cuticle cells of the developing hair follicle. That there may be some significance in the simultaneous appearance of these two entities is supported by some general similarities in properties of the proteins of the hair cuticle and matrix (that is; low molecular weight, high cystine content and amorphous structure.

The possible involvement of the cortical granules in development in the hair follicle of the guinea pig is reminiscent of the appearance and involvement of keratohyalin granules in the stratum granulosum layer of developing mammalian epidermis. These granules appear suddenly and rapidly become enmeshed with keratin fibrils (in the transitional stage of T cells of Brody (1960) and finally disappear altogether in the hardened layers (the stratum corneum). These keratin filaments are embedded in a densely-stained amorphous matrix analagous to the situation in hair and it has been postulated that the keratohyalin granules are precursors of this matrix protein (for example, Brody, 1959 and 1964; Odland, 1964) although an alternative suggestion has been made (Mercer, 1961). This assertion has gained considerable support from the recent isolation and characterisation of the keratohyalin granules from rat skin (Matoltsy and Matoltsy, 1970) in which it was shown that the protein was similar to the matrix protein of hair keratin. Thus, in view of the morphological similarities between the keratohyalin

granules and the cortical granules described in this work, it would seem reasonable, *a priori*, that the cortical granules are in fact deposits of matrix proteins.

CHAPTER SIX

ISOLATION AND CHARACTERISATION OF POLYRIBOSOMES FROM GUINEA PIG HAIR FOLLICLE TISSUE

A INTRODUCTION

In this chapter is described the isolation and characterisation of polyribosomes from guinea pig hair follicle tissue. Such a study is an important prerequisite to studies on protein synthesis *in vitro*.

A number of studies have shown that polyribosomes are present in large numbers in the developing hair follicle cells (for example, Rogers and Clarke, 1965; Freedberg, 1970; Clarke and Rogers, 1970b). However, the yields of polyribosomes that were isolated in these studies were very low with the predominant species being single ribosomes and polyribosomes with only two or three ribosomes. The presence of high levels of ribonuclease were believed to be responsible for these low yields (Freedberg, 1970; Clarke and Rogers, 1970b).

In the present chapter it will be shown that hair follicle polyribosomes can be isolated in much greater yields than in the previous studies by using very young animals as the source of tissue and buffers of high ionic strength for the preparation of the tissue homogenates.

In a more recent study (Wilkinson, 1970b), methods were described for the isolation of polyribosomes from a related tissue, wool follicles of the sheep. The yields and properties of these polyribosomes were very similar to those described in the present work.

B METHODS

(a) RIBONUCLEASE ASSAY

A sensitive ribonuclease assay procedure was developed to estimate the ribonuclease activity in homogenates of hair follicle tissue.

An *Escherichia coli* B strain was grown in an overnight incubation in a low phosphate medium containing $^{32}PO_4^{3-}$ (100 μc/ml). The nucleic acids

were extracted with phenol by established procedures and the labelled DNA was removed by digestion with ribonuclease-free deoxyribonuclease. The labelled *E. coli* RNA was mixed with RNA prepared from baker's yeast (Crestfield *et al.*, 1955) to give a specific activity of 450 000 counts/min/mg. Assays contained 1.0 ml of 0.1 M tris-HCl (pH 7.6), 1.0 ml of 0.05 % (w/v) yeast-*E. coli* RNA mixture and 1.0 ml of sample containing ribonuclease and were incubated at 37^{o} for 25 min. The reaction was terminated by addition of 1 mg of carrier yeast RNA and 1.0 ml of very cold (< -15^{o}) 20 % (w/v) trichloroacetic acid (TCA). The total TCA precipitable material remaining after incubation was collected onto a glass fibre circle for counting. Sigma type II-A ribonuclease was used as a standard. The rate of conversion of the radioactively-labelled RNA to acid soluble material was linear between 0 - 100 ng of this ribonuclease. Ribonuclease activities as low as 2 ng could be estimated by this procedure.

(b) ISOLATION OF HAIR FOLLICLE POLYRIBOSOMES

Homogenates of guinea pig hair follicles were prepared as described below, filtered through nylon gauze (pore size about 0.5 mm), centrifuged at 12 000 x *g* for 10 min to pellet the cell debris and then analysed by sucrose density gradient centrifugation. All procedures were carried out at 2^{o}.

Hair follicle tissue prepared from very young animals (age less than one week, weight about 100 g) was suspended (10 % , w/v) in buffer A consisting of 0.25 M KCl, 20 mM tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol and 5 % (w/v) sucrose (Heywood *et al.*, 1967). Two methods of homogenisation were investigated; the first, using a motor-driven teflon plunger in a glass vessel with clearance about 0.3 mm (Potter-Elvehjem); and the second, using a glass-glass hand-operated vessel with clearance about 0.2 mm (Dounce). The first method led to extensive frothing and was

difficult to control as analyses of homogenates prepared in the same manner on sucrose density gradients gave inconsistent profiles of polyribosomes. In contrast, the second method could be more readily controlled as homogenates prepared in the same manner gave consistent and more satisfactory profiles of polyribosomes. Therefore, this method was used routinely.

Trial experiments indicated that the degree of homogenisation required to obtain good yields of larger polyribosomes varied with the extent of homogenisation employed. If more than 20 strokes were used, the yield of the larger polyribosomes was less than that obtained when about 15 strokes were used, which suggested breakage by shearing. Similarly, if only 5 strokes were used, the yield of polyribosomes was reduced. Therefore, about 15 strokes were routinely used. The ratio of single ribosomes to polyribosomes of 6 ribosomes was always 4 : 1 (*see* Fig. 6.1). Furthermore, the extent of homogenisation necessary varied with the age of the animals used in the experiment: the slightly larger follicles of older animals (up to 3 weeks of age, weight about 150 g) required more disruption. In general, the extent of homogenisation was increased by 2 strokes from 15 strokes for each 25 g of animal body weight over 90 g.

C RESULTS

(a) ISOLATION OF POLYRIBOSOMES

(1) Standard polyribosome profile

A typical standard polyribosome profile prepared from guinea pig hair follicle tissue is shown in Fig. 6.1. This profile is similar to that obtained by Wilkinson (1970b) for wool follicle tissue, but greatly different from the profiles obtained by Clarke and Rogers (1970b) and Freedberg (1970) for guinea pig hair follicle tissue, especially in the yield of the larger polyribosomes. This profile may be divided into four groups: a major ribosome peak (A) comprising about 50 % of the total ribosomal particles; a

FIGURE 6.1

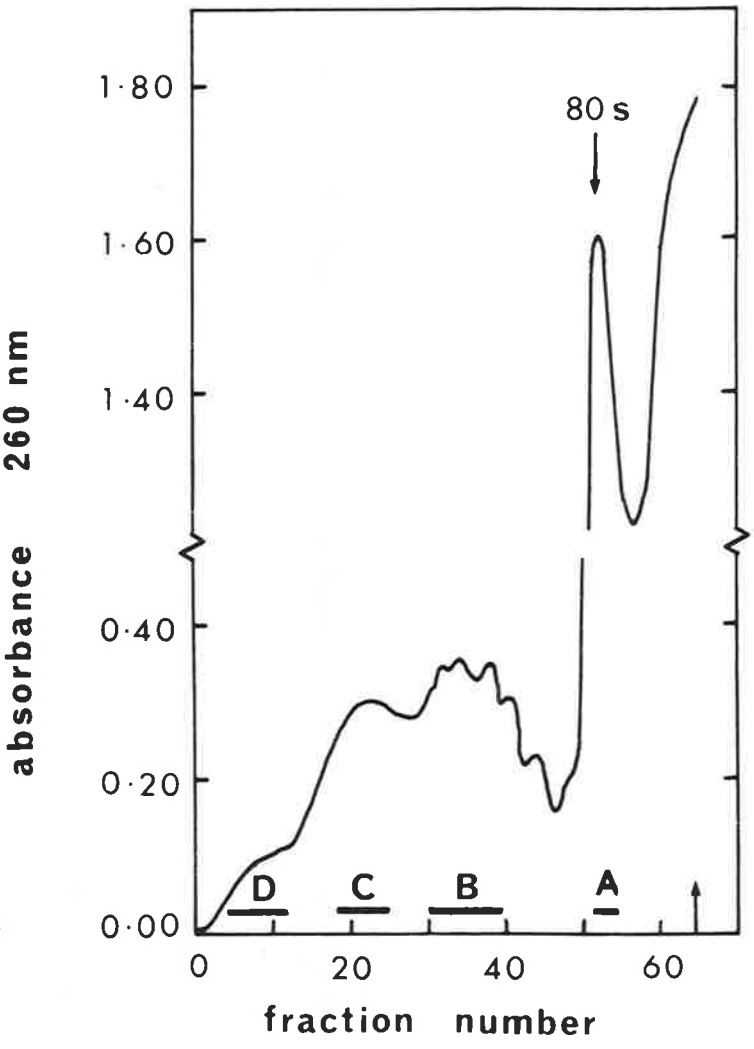
SUCROSE DENSITY GRADIENT CENTRIFUGATION OF POLYRIBOSOMES OBTAINED FROM GUINEA PIG HAIR FOLLICLE TISSUE

STANDARD SUCROSE DENSITY GRADIENT

The homogenate was prepared as described in the text, filtered though nylon gauze (pore size about 0.5 mm) and centrifuged at 12 000 x *g* for 10 min to pellet the cell debris.

A 1.0 ml homogenate sample was centrifuged on a 28 ml linear 15 - 40 % sucrose density gradient at 2° and 22 500 rev./min in a Spinco SW25.1 rotor. The gradient was then fractionated from the bottom and the effluent was continuously monitored at 260 nm by an Optica spectrophotometer with flow cell and recorder attachments. Sedimentation is towards the left. The vertical arrow represents the top of the gradient. 80*S* indicates the peak of single ribosomes.

The bar A refers to the group of single ribosomes and the bars B, C and D refer to the groups of polyribosomes of increasing size described in the text.


absorbance 260 nm
1·80
1·60
1·40
0·40
0·20
0·00
80 s
D
C
B
A
0
20
40
60
fraction number

group of polyribosomes of 2 - 9 ribosomal units (B) as seen by the oscillations on the profile; an unresolved group of larger polyribosomes sedimenting as a broad band (C); and a fourth group comprising a shoulder on the former group near the bottom of the gradient (D).

(2) Determination of the optimum composition of buffer A

The composition of buffer A used routinely for the preparation of tissue homogenates was checked with a view to increasing the yields of polyribosomes (Table 6.1). The buffer used initially was similar to that used by Heywood *et al.* (1967) for the characterisation of embryonic chicken muscle polyribosomes and Wilkinson (1970b) for the characterisation of wool follicle polyribosomes. Optimum yields of polyribosomes were clearly recorded at 0.25 M KCl, 5 mM $MgCl_2$, pH 7.6 and 1 mM dithiothreitol. Of interest was the marked increase in yield of polyribosomes (near 100 %) when 1 mM dithiothreitol was used as a reducing agent in place of the more commonly used 6 mM 2-mercaptoethanol. The sulphydryl-rich prekeratin proteins that are solubilised during homogenisation became aggregated when buffers not containing dithiothreitol were used for homogenisation. Similarly, extensive aggregation occurred when the KCl concentration was reduced below 0.1 M. Therefore, this buffer was used unchanged and used routinely for the isolation of polyribosomes from hair follicle tissue. The homogenates prepared with this buffer and in the manner described will be referred to throughout this work as the "whole tissue homogenates".

The wax-sheet procedure for the preparation of hair follicles has been criticised (Wilkinson, 1970b) because it involves the application of a hot (60°) wax mixture to the skin of the animal prior to exposure of the follicles. Although the skin is precooled before treatment with wax it was thought that the elevated temperature might lead to degradation of cellular components (Wilkinson, 1970b). To check this, follicles were prepared at

TABLE 6.1

DETERMINATION OF THE OPTIMUM COMPOSITION OF BUFFER A

The buffer used for each experiment was based on buffer A. For each experiment, one component was changed to one of the values shown whilst the concentrations of the other components remained as in buffer A. Hair follicle tissue homogenates were then prepared in the modified buffer as described in Methods and the polyribosomes pelleted through sucrose layers as described in Fig. 6.3b. The pellets containing the polyribosomes were resuspended in *unmodified* buffer A, clarified by centrifugation at 12 000 x *g* and the absorbance of the solution determined at 260 nm. The individual yields are expressed as mg of polyribosomes per g of follicle tissue and are the mean of two experiments.

Component of buffer A varied	Concentration of component used in the different experiments (mM)	Yield
KCl	50	0.3
	100	1.2
	250	2.6
	500	2.0
$MgCl_2$	0	0.2
	5	2.3
	10	1.8
Dithiothreitol	0	1.1
	1	2.7
No dithiothreitol but 2-mercaptoethanol	6	1.4
pH	7.2	1.4
	7.6	2.5
	8.0	0.9

five different wax temperatures, 80°, 70°, 60° (normal), 53° and 45°. Homogenates were prepared and examined by sucrose density gradient centrifugation. The yield of follicles per unit area of skin was reduced at the two lower temperatures but there was no significant change in either the polyribosome profiles or the yields of polyribosomes per g of follicles at all temperatures. This suggested that there was no significant change in the polyribosomal population in the cells of the tissue during the isolation procedure and hence no cellular damage was induced by the hot wax.

(3) Ribonuclease activity in the hair follicle tissue homogenates

In earlier studies (Freedberg, 1970; Clarke and Rogers, 1970b) it had been shown that high levels of endogenous or contaminating ribonuclease were present in the hair follicle tissue homogenates. In view of the improved polyribosome profiles prepared in this work, the ribonuclease activity in homogenates of hair follicles of animals of varying ages was estimated (Table 6.2). The level of ribonuclease activity in homogenates from very young animals was much lower than that obtained from older animals; the level rises rapidly from about 5 ng/ml in very young animals to about 120 ng/ml in near-adult and adult animals. Interestingly, near-adult animals were used in the previous experiments of the other workers. The level of about 120 ng/ml is similar to the value of 80 ng/ml cited by Freedberg (1970).

(b) PROPERTIES OF THE HAIR FOLLICLE POLYRIBOSOMES

(1) Structure

Samples from the sucrose density gradients of the groups A - D described in Fig. 6.1 were examined in the electron microscope after negative-staining with uranyl acetate (Fig. 6.2). Group A contained single ribosomes and ribosomal subunits (Fig. 6.2a). Group B contained polyribosomes of 2 - 9

TABLE 6.2

RIBONUCLEASE ACTIVITY IN HAIR FOLLICLE TISSUE HOMOGENATES

Hair follicles were prepared from two animals of a litter of the ages specified. Whole tissue homogenates in buffer A were prepared as described in the text and assayed for ribonuclease activity. The percentage of actively growing hair follicles was determined by plucking fibres from the dorsal region of the animals to be used and examined microscopically. All data shown are the mean values of the two animals used.

Animal age (days)	Body weight (g)	Percentage of active follicles in skin	Ribonuclease activity (ng/ml of homogenate)
1	75	98	3
6	95	95	5
12	110	90	6
16	140	92	6
33	260	67	26
47	310	58	73
about 70	390	49	121
about 98	450	42	118

FIGURE 6.2

ELECTRON MICROSCOPY OF THE HAIR FOLLICLE POLYRIBOSOMES

The samples were taken from a standard sucrose density gradient and negatively-stained with 1 % uranyl acetate.

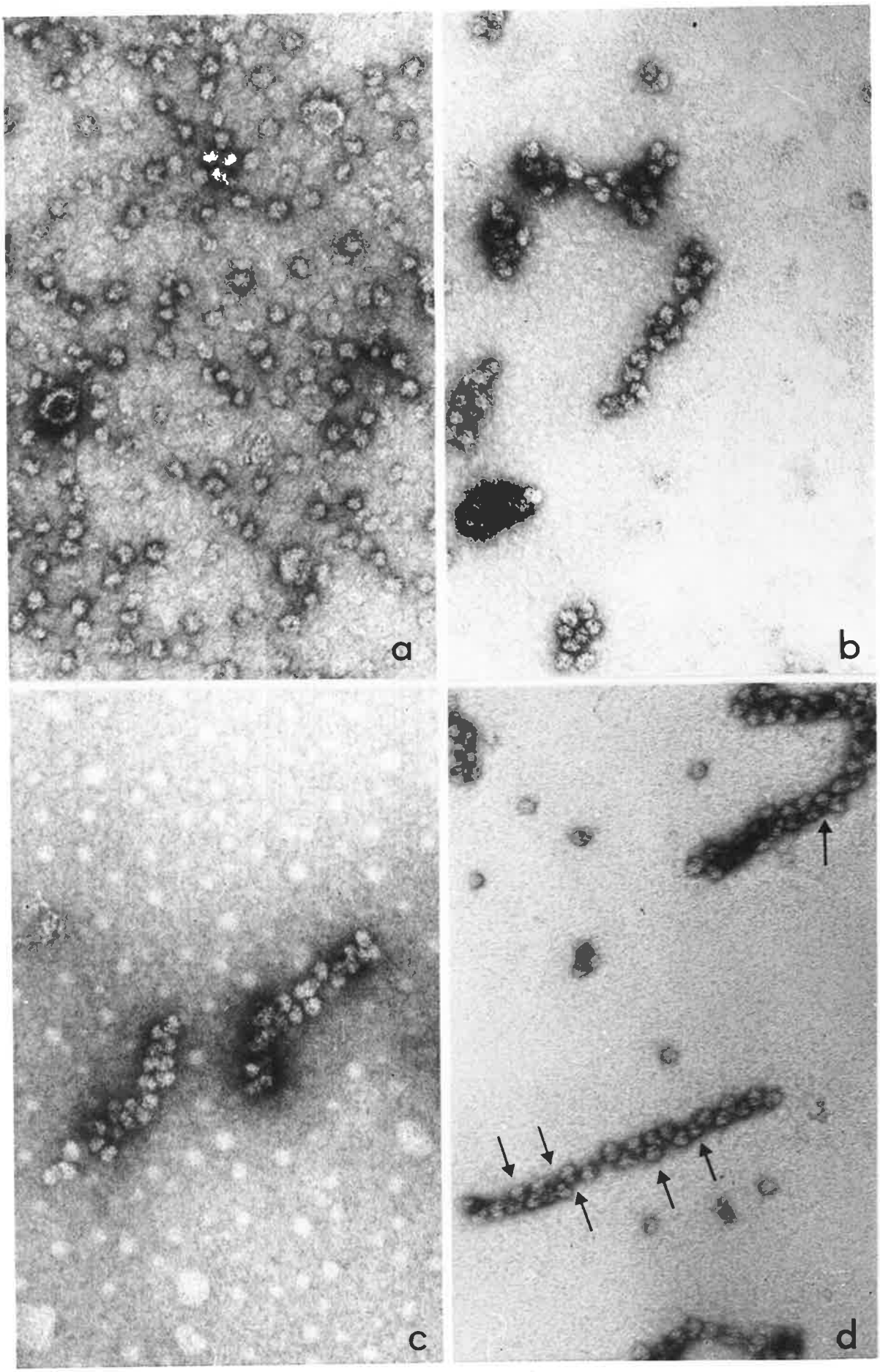
The magnification in each sample is: x 100 000.

(a) Sample from tube 53 (group A). This sample contains mostly single ribosomes and ribosomal subunits.

(b) Sample from tube 38 (group B). This sample contains several polyribosomes of sizes varying from 2 to about 9 ribosomal units.

(c) Sample from tube 18 (group C). This sample contains two polyribosomes of size about 15 to 18 ribosomal units. The ribosomes on these polyribosomes appear to be arranged in a helical manner.

(d) Sample from tube 8 (group D). This sample contains two polyribosomes of size about 25 to 35 ribosomal units. In places (arrowed) the subunits of individual ribosomes can be seen and also a thin strand about 10 Å in diameter (presumably mRNA) can be seen adjoining adjacent ribosomes along the polyribosome.


a
b
c
d

ribosomal units (Fig. 6.2b). Group C contained particles of 12 - 18 ribosomal units (Fig. 6.2c) and in group D most of the particles had 25 - 35 ribosomal units (Fig. 6.2d). The polyribosomes of the larger groups appeared as long chains in the form of extended coils. Aspects of the ultrastructure of individual ribosomes of the polyribosomes are visible in places (arrowed).

To check that the polyribosomes of the C and D groups were not random aggregates of smaller particles, the C and D polyribosomes of three gradients were pooled, pelleted at high speed and recentrifuged separately on gradients (Fig. 6.3a). Only slight losses due to mechanical breakage occurred on handling. The large polyribosomes clearly resedimented to their original positions on the gradients. In a similar experiment, the polyribosomes present in a whole tissue homogenate were pelleted by centrifugation through sucrose layers (Fig. 6.3b). Again only slight breakage of the large polyribosomes occurred but the single ribosomes did not pellet. This procedure therefore provided a convenient method for the preparation of polyribosomes from a whole tissue homogenate free of the single ribosomes and supernatant proteins.

(2) Effect of enzymes on hair follicle polyribosomes

Incubation of the hair follicle whole tissue homogenates with ribonuclease (1.0 µg/ml) at 2^{o} for 10 min led to the complete degradation of polyribosomes to single ribosomes (Fig. 6.4) whereas incubation with ribonuclease-free deoxyribonuclease (10 µg/ml) and trypsin (10 µg/ml) had no significant effect (Fig. 6.4). These observations suggested that the polyribosomes were held together by single-stranded RNA, presumably mRNA.

(3) Labelling of hair follicle polyribosomes in vivo

The purpose of these experiments was to investigate whether the nascent protein chains that are presumably present on the polyribosomes could be

FIGURE 6.3

RECENTRIFUGATION OF HAIR FOLLICLE POLYRIBOSOMES

(a) NATURE OF THE C AND D GROUP POLYRIBOSOMES

Polyribosomes from the C and D groups from three sucrose density gradients were pooled and sedimented by centrifugation at 225 000 x *g* for 2.5 h. The pellets were carefully resuspended in buffer A, clarified by centrifugation at 12 000 x *g* for 10 min to remove any material not resuspended and the resulting supernatants were layered separately onto sucrose density gradients for recentrifugation as in Fig. 6.1.

(b) PREPARATION OF POLYRIBOSOME PELLETS

Polyribosomes present in a whole tissue homogenate were sedimented by centrifugation through 1 M sucrose in buffer A layered over 2 M sucrose in buffer A at 225 000 x *g* for 2.5 h. The pellet was resuspended in buffer A and clarified by centrifugation at 12 000 x *g* for 10 min. The concentration of polyribosomes was adjusted to 0.25 mg/ml (assuming a 1.0 mg/ml suspension of polyribosomes has an absorbance of 11.8 units/cm (Rich, 1967)). A 1.0 ml sample was then centrifuged on a sucrose density gradient as in Fig. 6.1.

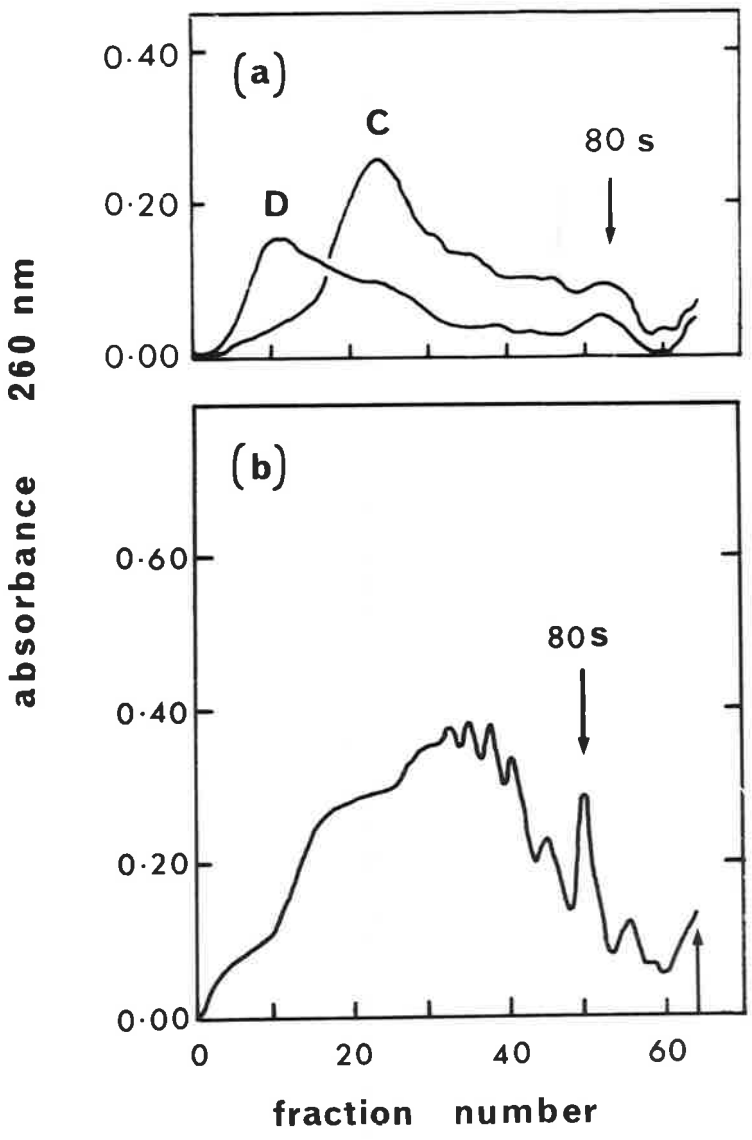
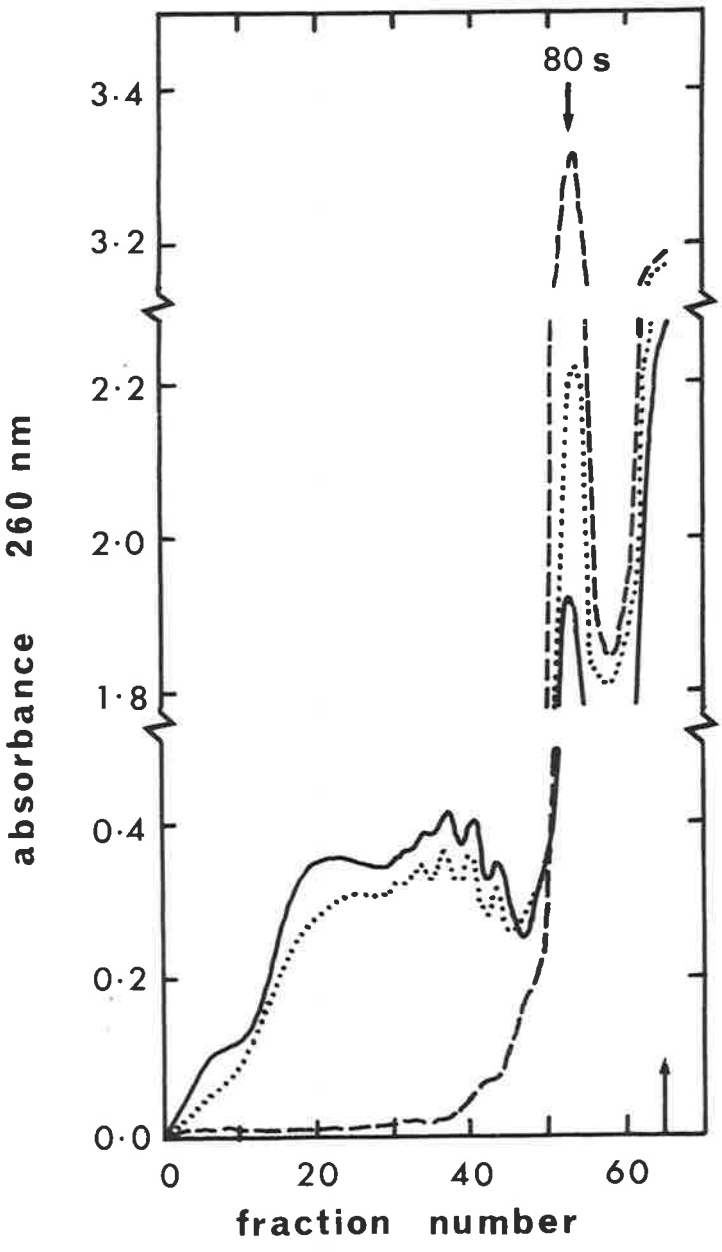

(a)
C
D
80 s
0·40
0·20
0·00
(b)
80s
0·60
0·40
0·20
0·00
0
20
40
60
absorbance 260 nm
fraction number

FIGURE 6.4

EFFECT OF ENZYMES ON HAIR FOLLICLE POLYRIBOSOMES

Three 1.0 ml samples of a whole tissue homogenate preparation were incubated separately for 10 min at 2^{o} with; ribonuclease (1.0 µg/ml, -----); ribonuclease-free deoxyribonuclease (10 µg/ml, ———); and trypsin (TPCK-trypsin) (10 µg/ml, ·····). Each sample was then centrifuged on a sucrose density gradient as in Fig. 6.1.


80 s
3·4
3·2
2·2
2·0
1·8
0·4
0·2
0·0
absorbance 260 nm
0
20
40
60
fraction number

labelled, since such labelled polyribosomes would be useful for investigations on the mechanism of protein synthesis *in vitro*. Three guinea pigs were injected intracardially with tritiated leucine of high specific activity and whole tissue homogenates were prepared at 5, 10 and 30 min after injection. The polyribosomes of each homogenate were then characterised (Fig. 6.5). At each time, radioactivity appeared all over the gradient, but at the shorter times, the larger polyribosomes were more highly labelled, especially at 10 min. At 30 min, however, the polyribosomes were labelled to a lesser degree, suggesting dilution of the isotope by endogenous leucine. In each case the single ribosomes were only slightly labelled which suggested that there had been little breakage of the polyribosomes during isolation, although material smaller than 80*S* ("supernatant") was highly labelled, as expected. The C and D group polyribosomes were always more highly labelled. This may simply reflect the leucine content of the nascent protein chains on these groups of polyribosomes.

Samples of the whole tissue homogenates from each time sample were pelleted through sucrose layers to remove the highly labelled supernatant and Fig. 6.5d shows the distribution of radioactivity over the polyribosomes obtained from the 10 min sample. The radioactivity profile was nearly identical to that shown in Fig. 6.5b. The specific activity of the pelleted polyribosomes was also determined and the values obtained were; 5 min incorporation, 10 800 counts/min/mg; 10 min incorporation, 17 500 counts/min/mg; 30 min incorporation, 6 800 counts/min/mg.

D DISCUSSION

The yields of especially the larger polyribosomes isolated in the present work clearly exceeded those of earlier experiments (Freedberg, 1970; Clarke and Rogers, 1970b). The principal reason for the greater yields of the larger polyribosomes was the much lower level of ribonuclease activity in

FIGURE 6.5

LABELLING OF HAIR FOLLICLE POLYRIBOSOMES WITH RADIOACTIVE LEUCINE *IN VIVO*

Three guinea pigs were injected intracardially with {4,5-^{3}H}leucine (specific activity, 58.1 C/mmole, 100 μc per 100 g of animal body weight). One animal was killed at 5 min, another at 10 min and the third at 30 min after injection. Whole tissue homogenates were then prepared.

1.0 ml samples of each homogenate were centrifuged separately on sucrose density gradients as in Fig. 6.1.

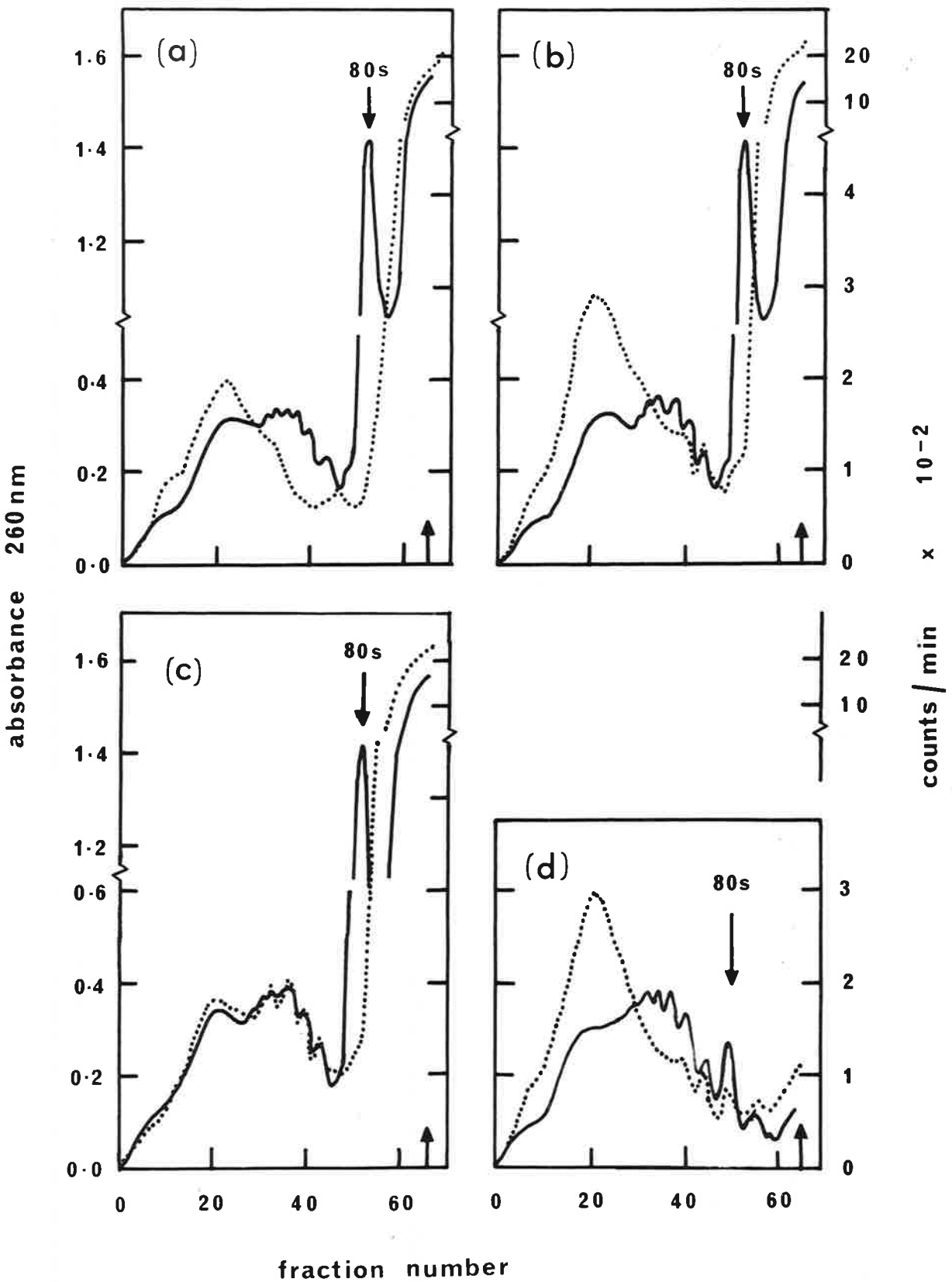
(a) 5 min sample.

(b) 10 min sample.

(c) 30 min sample.

(d) A sample of the whole tissue homogenate from the 10 min labelled animal was centrifuged through sucrose layers to pellet the polyribosomes as described in Fig. 6.3b. Approximately 0.25 mg of the polyribosomes were suspended in 1.0 ml of buffer A and analysed on a sucrose density gradient as in Fig. 6.1.

After centrifugation the gradients were fractionated directly into vials and mixed with the scintillation fluid of Bray (1960) for measurement of radioactivity.


(a)
80s
(b)
80s
(c)
80s
(d)
80s
absorbance 260 nm
counts / min
x 10−2
fraction number
1·6
1·4
1·2
0·4
0·2
0·0
0·6
20
10
4
3
2
1
0
0
20
40
60

tissue homogenates. Hair follicle tissue homogenates prepared from young animals contained only trace amounts of ribonuclease activity (about 5 ng/ml whereas in homogenates prepared from older animals, of about the same age as those used in the previous studies, the ribonuclease activity was much greater (about 120 ng/ml). The reason why higher levels of ribonuclease activity in the hair follicle tissue of older animals is found is not clear. In earlier studies of Clarke and Rogers (unpublished) a substrate-film technique on skin slices containing hair follicles from adult animals was employed to locate the source of the ribonuclease activity. It was shown that the sebaceous gland adjoining the hair follicle canal and the epidermis surrounding the hair follicle itself contained large amounts of an acid ribonuclease (see also, Freedberg *et al.*, 1967).

Several other reasons for the higher yields of polyribosomes isolated in this study are apparent. The guinea pigs used here were much younger than those used previously. The number of hair follicles that are actively growing in animals less than three weeks of age is 90 - 95 % of the total population whereas this number is reduced to less than 50 % in animals older than two months. Accordingly, higher yields of polyribosomes were found. Greater yields were also obtained when buffers of high ionic strength were used. Similar observations have been made in wool follicle tissue (Wilkinson, 1970b) and chick embryo muscle tissue (Heywood *et al.*, 1968). Of interest was the increased yield of polyribosomes using buffers containing dithiothreitol instead of the more commonly used 2-mercapto-ethanol. While it is appreciated that dithiothreitol is a superior reducing agent (Cleland, 1964) and that hair follicle tissue contains large amounts of sulphydryl-rich proteins, the precise reason for the higher yields of polyribosomes is not clear.

Although large polyribosomes have been isolated from hair

follicle tissue in this study, about 50 % of the total ribosomal particles isolated were single ribosomes. This finding cannot be accounted for by shearing of the polyribosomes during homogenisation because the observation was made (Fig. 6.5) that when polyribosomes labelled *in vivo* were separated on sucrose density gradients, the single ribosomes were only slightly labelled. Fan and Penman (1970) have shown that in dividing eukaryote cells, about 75 % of the total cytoplasmic ribosomes are present as single ribosomes. In the present study, many of the cells that were disrupted during homogenisation would have originated from the lowest regions of the follicle and these cells are known to be actively dividing and not synthesising structural proteins. Thus the high proportion of single ribosomes in homogenates might have had their origin in this manner.

From the studies reported in a number of tissues which synthesise only one or a small number of proteins, such as reticulocytes (Lamfrom and Knopf, 1964), lens (Schoenmakers *et al.*, 1967; Spector and Travis, 1966) and muscle (Heywood *et al.*, 1967) , it was suggested that there is a relationship between the size of the polypeptide chain and the size of the polyribosome that directs its synthesis (Heywood *et al.*, 1967; Heywood and Rich, 1968); this relationship is of the order of 30 amino acid residues (or 3000 - 4000 daltons) per ribosome. If this relationship is used to postulate the sizes of the protein chains that hair follicle polyribosomes synthesise, they amount to; group B, 10 000 - 30 000 daltons; group C, 40 000 - 50 000 daltons; and group D, 90 000 - 120 000 daltons. By comparison, the keratin proteins of guinea pig hair and hair follicle tissue can be fractionated into two groups, one with a range of molecular weight 10 - 28 000 daltons (the HiS or matrix component) and the other, of molecular weight 45 000 daltons (the LoS or microfibrillar component) (*see* Chapter Three). Accordingly, the group B and C polyribosomes isolated

in this work may be responsible for the synthesis of the HiS and LoS proteins, respectively. It is not possible to speculate on the role of the group D polyribosomes.

The largest polyribosomes isolated here appeared ordered, possibly helical structures of 2 or 3 ribosomes per turn when seen in negatively-stained preparations in the electron microscope. Other reports on the ordered arrangement of polyribosomes isolated from other sources have been made (for examples, Heywood *et al.*, 1967; Spector and Travis, 1966; dePetris, 1970). However, interpretations on the structures of the isolated polyribosomes should be made with caution since artifacts could arise by dehydration during preparation of the samples for electron microscopy (Shelton and Kuff, 1966). Nevertheless, the structure of these negatively-stained polyribosomes is consistent with the ordered and occassionally helical arrays of polyribosomes seen *in situ* in hair follicle tissue (Orwin, 1969; *see* Figs. 5.1 and 5.2). The possible involvement of these structurally arranged polyribosomes in control of protein synthesis has been mentioned (*see* Chapter One, page 13).

CHAPTER SEVEN

MECHANISM OF PROTEIN SYNTHESIS IN CELL-FREE SYSTEMS PREPARED FROM GUINEA PIG HAIR FOLLICLE TISSUE

A INTRODUCTION

In this chapter studies on the mechanism of protein synthesis in cell-free systems containing hair follicle polyribosomes will be described. In the previous chapter it was shown that polyribosomes could be isolated from hair follicle tissue in much greater yields than in previous studies and that a principal reason for this improvement was the much lower level of ribonuclease activity in the tissue homogenates. Not supprisingly therefore, it will be shown in this chapter that the activity of cell-free protein synthesis directed by these polyribosomes is much greater than that reported in the previous studies of Freedberg (1970) and Clarke and Rogers (1970b). This has afforded an opportunity for a detailed study of the mechanism of protein synthesis *in vitro*.

B METHODS

(a) COMPOSITION OF THE WHOLE TISSUE HOMOGENATE CELL-FREE PROTEIN SYNTHESIS SYSTEM

These experiments were performed on the whole tissue homogenate preparations prepared as described in the previous chapter (*see* pages 88 and 89) and the system contained 0.7 ml of homogenate in a final volume of 1.0 ml. The composition of the buffer was identical to that of buffer A. The labelled amino acid was either high specific activity {4,5-^{3}H}leucine (58.1 C/mmole) (1 µc, 17 pmoles) or uniformly ^{14}C-labelled phenylalanine (455 C/mole) (0.5 µc, 1.1 nmoles). In addition, the complete system contained 0.25 µmole of GTP, 1.0 µmole of ATP, an ATP regenerating system of 5 µmoles of phosphoenolpyruvate and 20 µg of pyruvate kinase (and 4 µmoles of $(NH_4)_2SO_4$) and 5 nmoles of each of 19 unlabelled amino acids, excluding either leucine or phenylalanine, whichever was used as the labelled amino acid. This reaction mixture was incubated at 37^{o} for times varying up to 60 min.

Samples were removed into 1.0 ml of ice-cold water and the reactions were terminated with 1.0 ml of 10 % (w/v) trichloroacetic acid (TCA), followed by centrifugation at 1500 x *g* for 10 min to sediment the insoluble material. The pellets were dissolved in 0.5 ml of 0.1 N NaOH to hydrolyse all amino acyl-tRNA, reprecipitated with 1.0 ml of 10 % TCA and the precipitates were collected onto glass fibre circles by filtration in a Millipore apparatus. The filtrates were washed with 10 % TCA (2 x 5 ml), acetone - 1 % (v/v) HCl (1 x 5 ml) and ether (1 x 5 ml) and the circles were dried in an oven at 110^{o} for 15 min. Toluene-based scintillation fluid was used for measurement of radioactivity.

(b) COMPOSITION OF THE RECONSTITUTED CELL-FREE PROTEIN SYNTHESIS SYSTEM

The buffer B initially used for this system was similar to that of Heywood *et al.* (1967) and contained 0.15 M KCl, 20 mM tris-HCl (pH 7.6), 7.5 mM $MgCl_2$, 1 mM dithiothreitol and 10 % (w/v) glycerol. The system contained, per 4.0 ml, 1.0 mg of polyribosomes that had been pelleted through sucrose layers to remove supernatant proteins (*see* Fig. 6.3b), and 2.0 ml of a dialysed "supernatant fraction". This was prepared from a whole tissue homogenate by centrifugation at 125 000 x *g* for 1.5 h. The middle half of the resultant supernatant was removed and dialysed against buffer B with 2 changes of 100 volumes. In initial studies the *in vitro* system also contained per 4.0 ml, 1.0 µmole of GTP, 4 µmoles of ATP, an ATP generating source of 20 µmoles of phosphoenolpyruvate and 80 µg of pyruvate kinase (and 16 µmoles of $(NH_4)_2SO_4$), 15 µc (7.5 nmoles) of {4,5-^{3}H}leucine and 20 nmoles of each of the other 19 unlabelled amino acids. This reaction mixture was incubated at 37^{o} for times varying up to 90 min. Samples were removed and the reactions were terminated as described above.

In later studies using this system (*see* Fig. 7.6) the final phosphoenolpyruvate and $MgCl_2$ concentrations were reduced to 3.5 mM and

6.5 mM, respectively. For maximal incorporation, the KCl concentration was also reduced to 0.125 M. Deacylated yeast tRNA (50 µg/ml) prepared by the method of Holley (1967) was also added (*see* Table 7.3).

C RESULTS

(a) CELL-FREE PROTEIN SYNTHESIS IN THE WHOLE TISSUE HOMOGENATE SYSTEM

(1) Properties of the in vitro system

The time course of the incorporation of tritiated leucine into TCA insoluble material was linear for the first 15 min but reached a plateau value by 30 min.

The properties of the system are shown in Table 7.1. These results were reproducible over several experiments although there was some variability in the dependence for added amino acids between different experiments. This partial degree of dependence and the variability of it most probably reflected variations in the pool sizes of free leucine or leucyl-tRNA in the follicle tissue homogenates of different guinea pigs. In addition, the system was only partially dependent on added GTP, ATP and ATP regeneration suggesting there is a substantial pool of these in the tissue homogenates.

The effects of various antibiotics was also investigated. Puromycin inhibited incorporation substantially and at $2 \cdot 10^{-4}$ M, inhibition was almost complete. Cycloheximide at $3 \cdot 10^{-3}$ M completely inhibited incorporation and even at $5 \cdot 10^{-6}$ M the incorporation was inhibited by 29 %. Chloramphenicol at $4 \cdot 10^{-4}$ M had little or no effect on amino acid incorporation. Since chloramphenicol at this concentration specifically inhibits cell-free protein synthesis by prokaryote cell-type polyribosomes (von Ehrenstein and Lipmann, 1961), and since cycloheximide specifically inhibits cell-free protein synthesis by eukaryote cell-type polyribosomes

TABLE 7.1

PROPERTIES OF THE WHOLE TISSUE HOMOGENATE CELL-FREE PROTEIN SYNTHESIS SYSTEM

The details of the complete whole tissue homogenate cell-free system are described in Methods. All reactions contained the same homogenate preparation and were incubated for 60 min.

Experiment	Counts/min	% of complete
Complete [a]	21 720	100
- GTP	13 800	64
- ATP regeneration	14 280	68
- ATP and ATP regeneration	5 460	25
- Amino acids	11 800	55
+ Puromycin, $5 \cdot 10^{-5}$ M	8 340	38
$2 \cdot 10^{-4}$ M	1 680	7.8
+ Chloramphenicol, $5 \cdot 10^{-5}$ M	21 540	99
$4 \cdot 10^{-4}$ M	21 000	97
+ Cycloheximide, $5 \cdot 10^{-6}$ M	15 420	71
$2 \cdot 10^{-5}$ M	6 180	28
$3 \cdot 10^{-3}$ M	720	3.3
+ NaF, $2 \cdot 10^{-4}$ M	19 320	89
$2 \cdot 10^{-2}$ M	15 640	72
+ Ribonuclease, 10 µg	480	2.2
+ Polyuridylic acid, 20 µg	20 400	94
200 µg	14 340	66
Complete [b]	7 800	100
+ Polyuridylic acid, 20 µg	11 700	150
200 µg	62 880	806

The labelled amino acids were: [a] leucine; [b] phenylalanine.

(Wettstein *et al.*, 1964; Lin *et al.*, 1966; Baliga *et al.*, 1969), it can therefore be assumed that bacterial contamination in this system was minimal and that cytoplasmic hair follicle polyribosomes were responsible for all incorporation observed.

Cycloheximide is reported to have a dual inhibitory action on cell-free protein synthesis in eukaryote systems (Wettstein *et al.*, 1964; Lin *et al.*, 1966; Baliga *et al.*, 1969): at high concentration it inhibits translation and at low concentrations it inhibits initiation. Thus it is possible that about 30 - 35 % of the total amino acid incorporation that occurred in this system was due to the elongation of protein chains newly initiated *in vitro*. This was supported by the inhibitory action of high concentrations of polyuridylic acid on leucine incorporation in this system by 34 % (Table 7.1) suggesting that there was competition between endogenous mRNA and added artificial messenger for the attachment of ribosomes. In addition, NaF which also inhibits initiation, inhibited protein synthesis here by 28 %. When phenylalanine was used as the labelled amino acid, it is seen that the hair follicle polyribosomes were directed by the polyuridylic acid to synthesise polyphenylalanine. Ribonuclease completely inhibited protein synthesis as expected.

In order to investigate further the poor dependence of this system for added amino acids, an estimate of the level of the endogenous amino acids in the whole tissue homogenates was made (Table 7.2). The concentration of most amino acids in the tissue homogeantes was of the order of 10^{-3} - 10^{-4} M and since the concentration of tissue in the homogenates was 10 % (w/v), the concentration of the amino acids *in vivo* was of the order of 10^{-2} - 10^{-3} M. The amino acid concentration of most amino acids was generally similar between the different samples, but there were major variations in the levels of serine, proline and cystine. These differences may reflect variations in the dietary or nutritional status of the different

TABLE 7.2

LEVEL OF ENDOGENOUS AMINO ACIDS IN WHOLE TISSUE HOMOGENATES

Guinea pig hair follicle tissue homogenates were prepared and 1.0 ml samples were deproteinised with 1 % picric acid (Stein and Moore, 1954). After centrifugation to remove precipitated protein, the picric acid was removed from the supernatant on a 5 x 0.5 cm column of Dowex AG 2W-X8 in 0.02 N HCl. The eluate was collected, dried by rotary film evaporation and a sample analysed. Although there were several other ninhydrin-positive components, only the common amino acids were considered. The values for three different whole tissue homogenates are given and are expressed as nmoles/ml of homogenate

Amino acid	Sample number		
	1	2	3
Aspartic acid	415	330	340
Threonine	405	375	490
Serine	775	405	600
Glutamic acid	1105	1005	1055
Proline	445	220	305
Glycine	970	1155	875
Alanine	540	485	440
Half-cystine	90	30	55
Valine	185	135	175
Methionine	45	55	35
Isoleucine	85	80	55
Leucine	230	285	215
Tyrosine	75	55	55
Phenylalanine	20	25	25
Lysine	150	140	125
Histidine	125	90	95
Arginine	270	300	255

animals. It was considered that a brief dialysis of the whole tissue homogenates against buffer A before incubation should increase the dependence of the system on the added amino acids and thus increase incorporation. However, experiments showed that after a 30 min dialysis, after which time the level of endogenous amino acids should have been reduced at least ten-fold, the degree of incorporation was decreased by about two-fold, suggesting that the removal of some other essential components had occurred.

(2) Effect of incubation on the structural integrity of the hair follicle polyribosomes

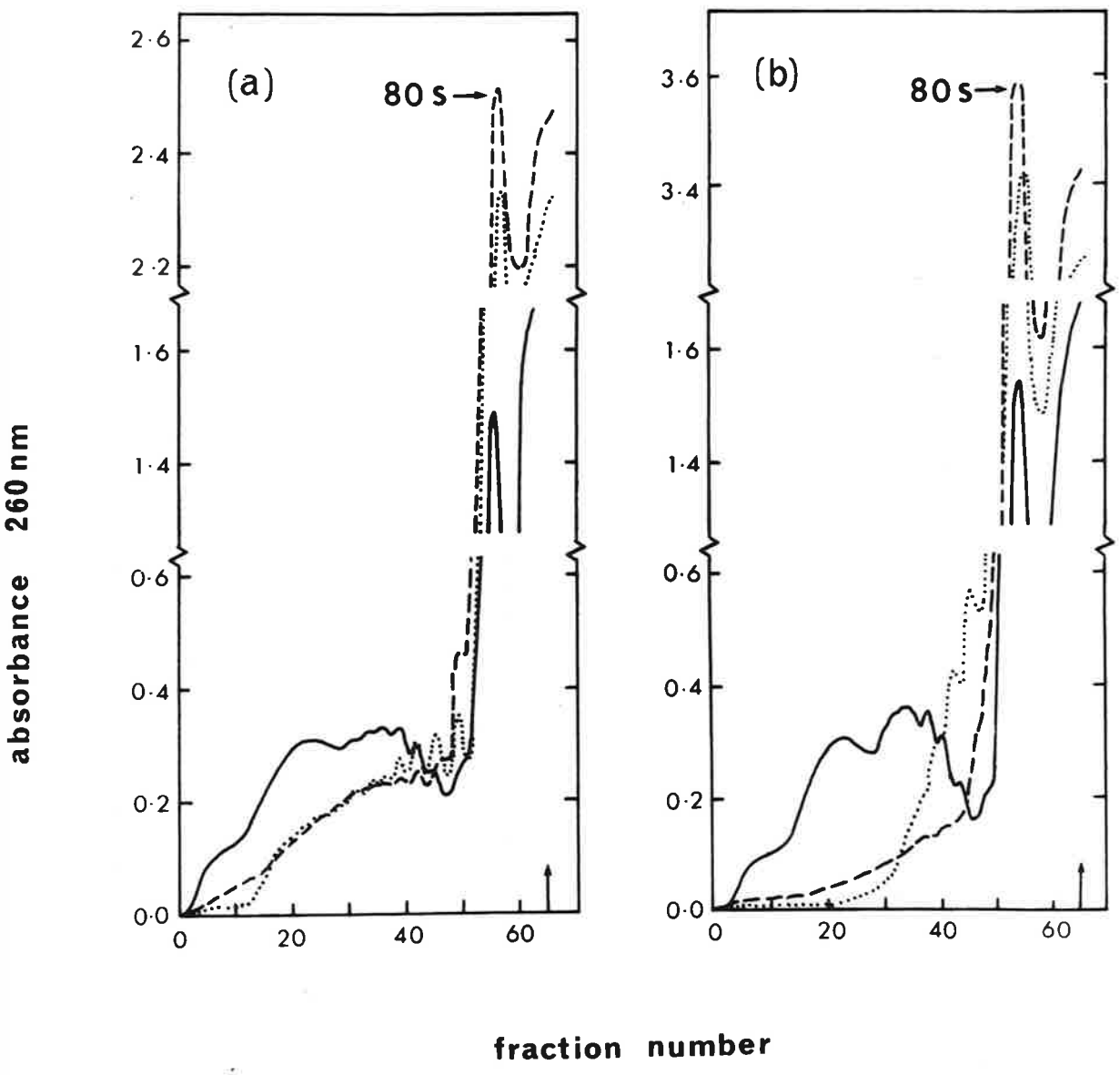
During incubation of the whole tissue homogenates with added ATP, an ATP regeneration source and unlabelled amino acids, the polyribosomes were degraded, the largest being degraded most rapidly (Fig. 7.1). Incubation with a low concentration of added ribonuclease (0.1 µg/ml) also resulted in degradation of the polyribosomes (Fig. 7.1).

In order to determine whether the degradation of the polyribosomes was due to protein synthesis or to the action of the ribonuclease present in the whole tissue homogenates, these experiments were repeated on tissue homogenates containing hair follicle polyribosomes that had been labelled *in vivo* with tritiated leucine (Fig. 7.2). In the absence of added ribonuclease the polyribosomes were degraded to ribosomes during incubation (Fig. 7.2a) and the labelled nascent protein chains were released into the supernatant: almost all of the label on the gradient appeared above the ribosome peak. However, in the presence of added ribonuclease, the labelled polyribosomes were degraded to labelled ribsomes (Fig. 7.2b): the labelled nascent protein chains were not released from the ribosomes. Since in the absence of added ribonuclease very little radioactivity remained associated with the ribosomes (Fig. 7.2a), it is concluded that the amount of ribonuclease activity present in the homogenates (about 5 ng/ml) was not

FIGURE 7.1

THE EFFECT OF INCUBATION ON THE STRUCTURAL INTEGRITY OF HAIR FOLLICLE POLYRIBOSOMES

Whole tissue homogenate preparations were incubated as described in Methods but with unlabelled amino acids (5 nmoles of each) for *(a)* 5 min and *(b)* 10 min in the *absence* (-----) or *presence* (∘∘∘∘∘) of 0.1 µg/ml of added ribonuclease. Incubations were terminated by chilling and examined by sucrose density gradient centrifugation as in Fig. 6.1. A control homogenate sample (———) which had been held at 0^{o} was centrifuged simultaneously.


(a)
80 s
(b)
80 s
absorbance 260 nm
fraction number
2·6
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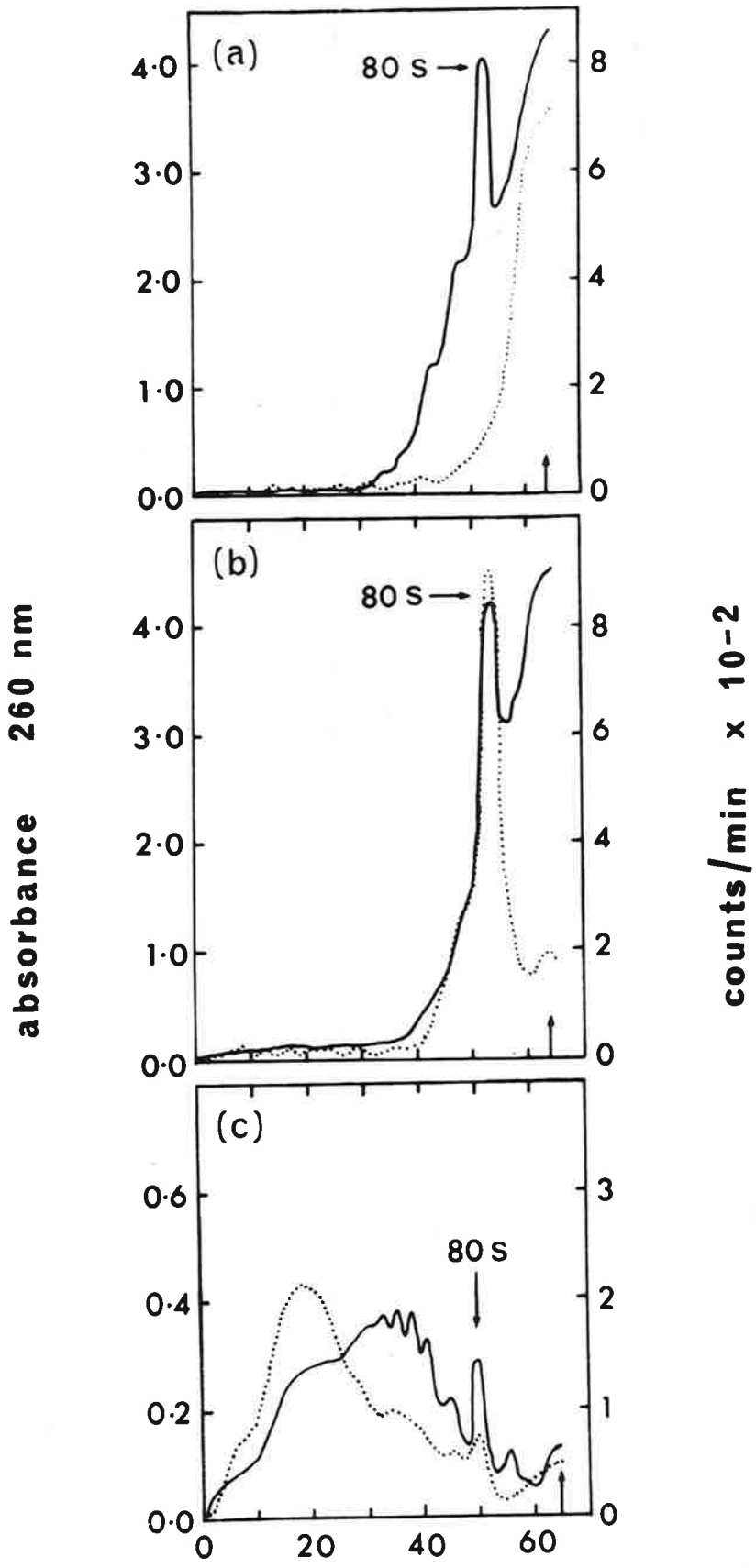
3·6
3·4
0
20
40
60

FIGURE 7.2

THE EFFECT OF INCUBATION ON THE STRUCTURAL INTEGRITY OF HAIR FOLLICLE POLYRIBOSOMES BEARING LABELLED NASCENT PROTEIN CHAINS

Hair follicle polyribosomes were labelled *in vivo* with tritiated leucine and prepared free of the highly labelled supernatant as described in Fig. 6.5d. A 125 000 x *g* supernatant fraction was prepared from another unlabelled animal. The preparation was *not* dialysed before use. Approximately 0.25 mg samples of the labelled polyribosomes were then incubated at 37^{o} with unlabelled amino acids (5 nmoles of each) as described in Methods for 10 min in 1.0 ml of the unlabelled supernatant fraction in the *absence (a)* or *presence (b)* of 0.1 µg/ml of added ribonuclease. The incubations were terminated by chilling and examined by sucrose density gradient centrifugation as in Fig. 6.1. A control sample of labelled polyribosomes suspended in buffer A which had been held at 0^{o} was centrifuged simultaneously *(c)*. ——— , absorbance at 260 nm; ----- , counts/min. The gradients were fractionated and prepared for measurement of radioactivity by addition of the scintillation fluid of Bray (1960).


(a)
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0·6
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0·2
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8
6
4
2
0
3
2
1
0
0
20
40
60
absorbance 260 nm
counts/min x 10-2
fraction number

sufficient to cause significant degradation of the polyribosomes *per se* during incubation. It is important to mention that low concentrations of ribonuclease can hydrolyse single-stranded (and in this case messenger) RNA but do not significantly affect attachment of tRNA species bearing nascent protein chains to ribosomes (Pestka, 1968).

This experiment therefore suggested that during amino acid incorporation in hair follicle tissue homogenates, the polyribosomes degraded by an orderly process of run-off of ribosomes from the messenger due to protein synthesis, coupled with the release of the protein chains, as in other eukaryote systems (for example, Lamfrom and Knoff, 1964; Goodman and Rich, 1963; Hardesty *et al.*, 1963).

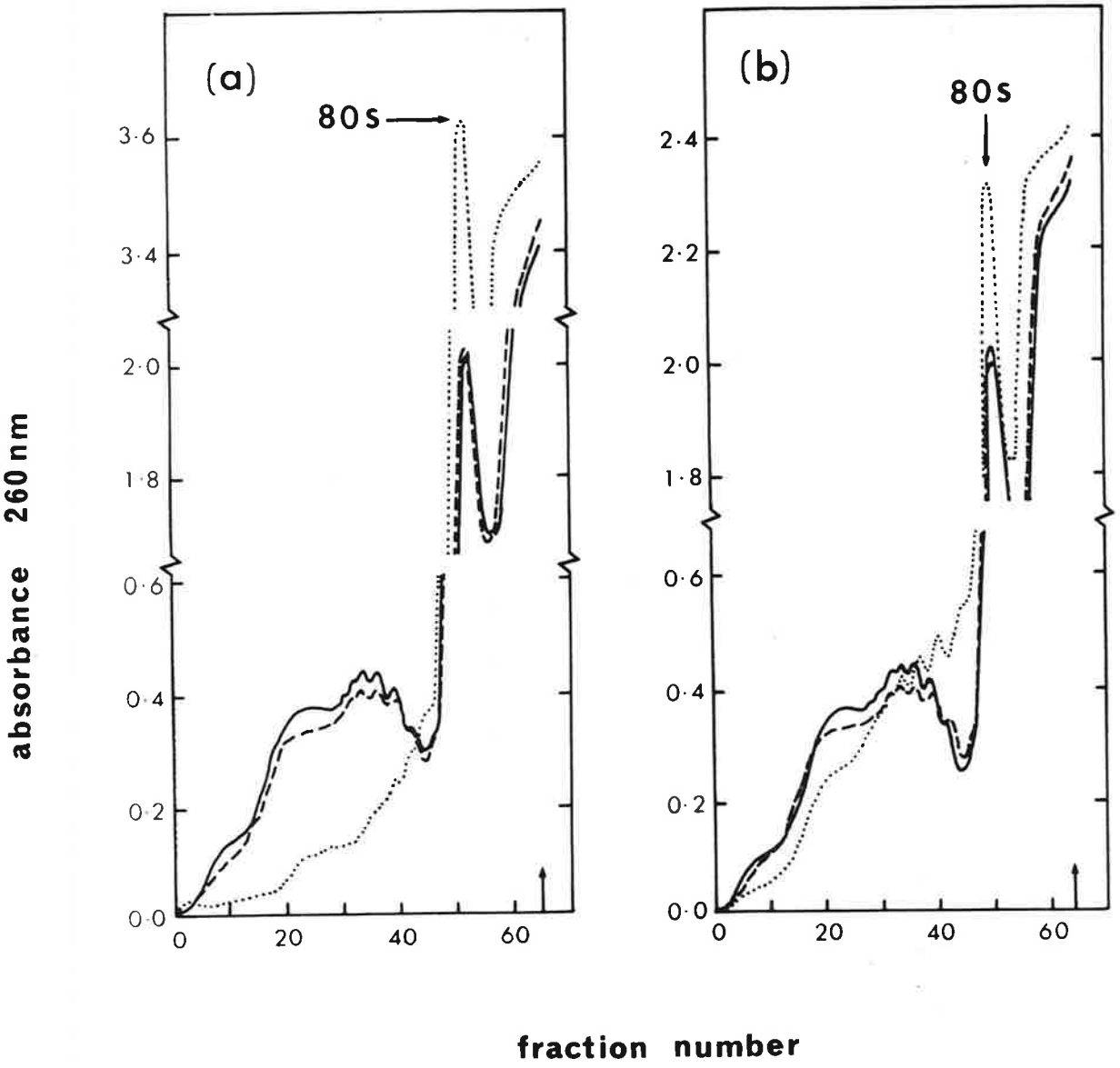
(3) Effect of incubation with antibiotics on the structural integrity of hair follicle polyribosomes

The effects of the antibiotics puromycin and cycloheximide on the structural integrity of the hair follicle polyribosomes during incubation was investigated using similar techniques. $2 \cdot 10^{-4}$ M puromycin protected the polyribosomes from degradation (Fig. 7.3a) but at $2 \cdot 10^{-5}$ M significant breakdown occurred. Similarly, $3 \cdot 10^{-3}$ M cycloheximide (Fig. 7.3b) protected the structure of the polyribosomes, but at $5 \cdot 10^{-4}$ M breakdown occurred. When polyribosomes labelled *in vivo* with tritiated leucine were incubated with $2 \cdot 10^{-4}$ M puromycin, the nascent protein chains were released (Fig. 7.4a): almost all of the label on the gradient appeared above the ribosome peak. The size of the polyribosomes was also maintained as before (Fig. 7.3a). This observation was not entirely consistent with the studies of Williamson and Schweet (1965). These workers showed that in the presence of ATP and amino acids and $2 \cdot 10^{-4}$ M puromycin, degradation of reticulocyte polyribosomes occurred *in vitro*, and that nascent protein chains were released as the peptidyl-puromycin derivatives. Further, they

FIGURE 7.3

THE EFFECT OF INCUBATION WITH PUROMYCIN AND CYCLOHEXIMIDE ON THE STRUCTURAL INTEGRITY OF HAIR FOLLICLE POLYRIBOSOMES

Whole tissue homogenate preparations were incubated at 37^{o} for 5 min as described in Methods but with unlabelled amino acids (5 nmoles of each) with *(a)* puromycin at $2 \cdot 10^{-4}$ M (-----) and $2 \cdot 10^{-5}$ M (·····) or cycloheximide at $3 \cdot 10^{-3}$ M (-----) and $5 \cdot 10^{-4}$ M (·····) *(b)*. The reactions were terminated by chilling and examined by centrifugation on sucrose density gradients as in Fig. 6.1. A control sample of homogenate (———) which had been held at 0^{o} was centrifuged simultaneously in each case.


(a)
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(b)
80s
absorbance 260 nm
fraction number
0·0
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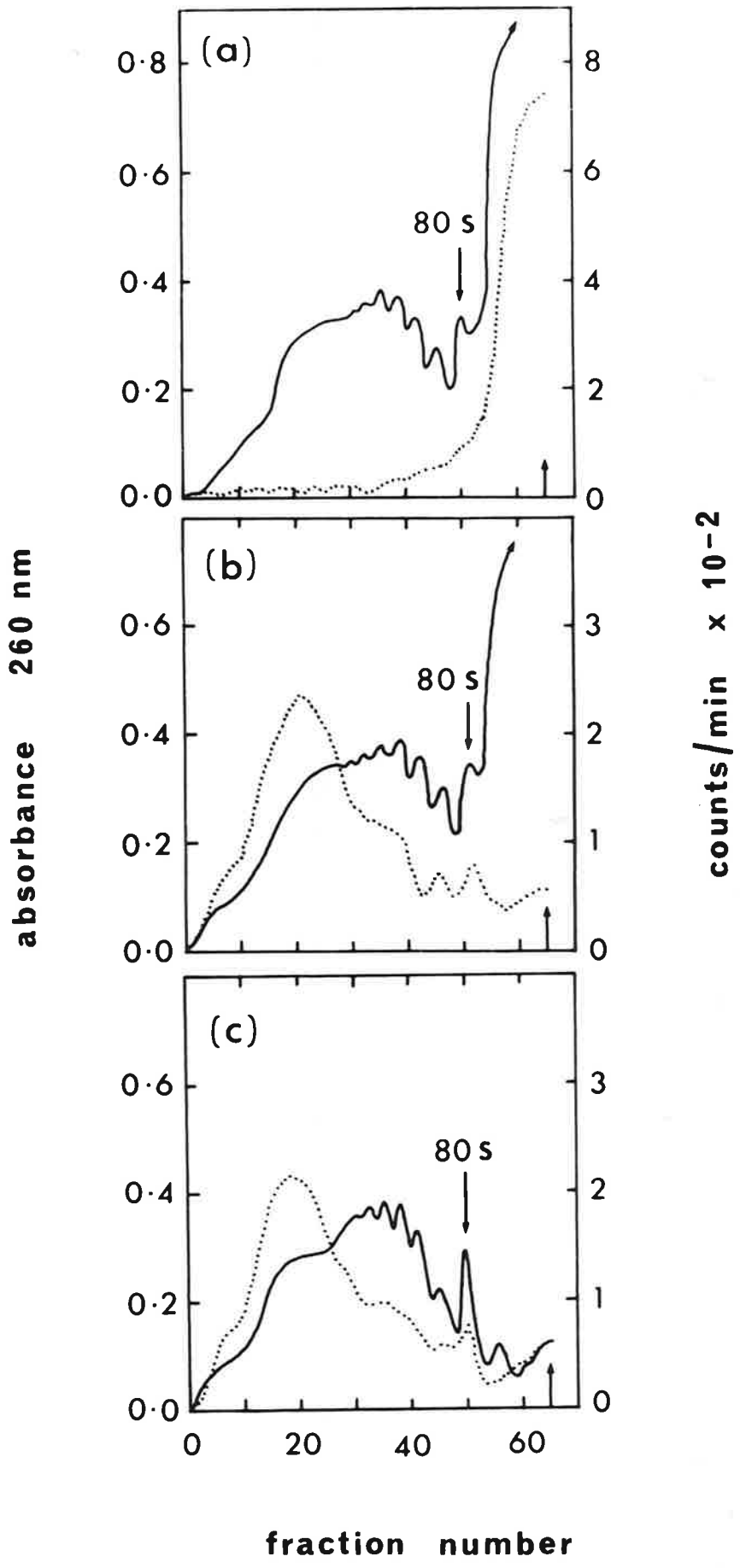
2·0
3·4
3·6
2·2
2·4
0
20
40
60

FIGURE 7.4

THE EFFECT OF INCUBATION WITH PUROMYCIN AND CYCLOHEXIMIDE ON THE STRUCTURAL INTEGRITY OF HAIR FOLLICLE POLYRIBOSOMES BEARING LABELLED NASCENT PROTEIN CHAINS

Hair follicle polyribosomes were labelled *in vivo* and prepared as described in Figs. 6.5d and 7.2. Samples were incubated at 37^{o} for 10 min in 1.0 ml of an unlabelled supernatant fraction as in Fig. 7.2 in the presence of either *(a)* $2 \cdot 10^{-4}$ M puromycin or *(b)* $3 \cdot 10^{-3}$ M cycloheximide. Other details are as in Fig. 7.2. ——— , absorbance at 260 nm; ----- , counts/min. A control sample of labelled polyribosomes suspended in buffer A which had been held at 0^{o} was centrifuged simultaneously *(c)*.


(a)
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0
20
40
60
absorbance 260 nm
counts/min x 10-2
fraction number

postulated that the degradation of the polyribosomes was due to random reinitiation of peptide bond formation along the messenger. Since the hair follicle polyribosomes used in the present work did not degrade during incubation with $2 \cdot 10^{-4}$ M puromycin, it is possible that this reinitiation process did not occur. The peptidyl-puromycin or puromycin molecules themselves may have competitively inhibited the ribosomal binding site such that random reinitiation of peptide bond formation along the mRNA was prevented. At lower concentrations of puromycin (Fig. 7.3a) degradation of the hair follicle polyribosomes proceeded, presumably by release of nascent protein chains and random reinitiation of peptide bond formation by the same mechanism as that postulated by Williamson and Schweet (1965).

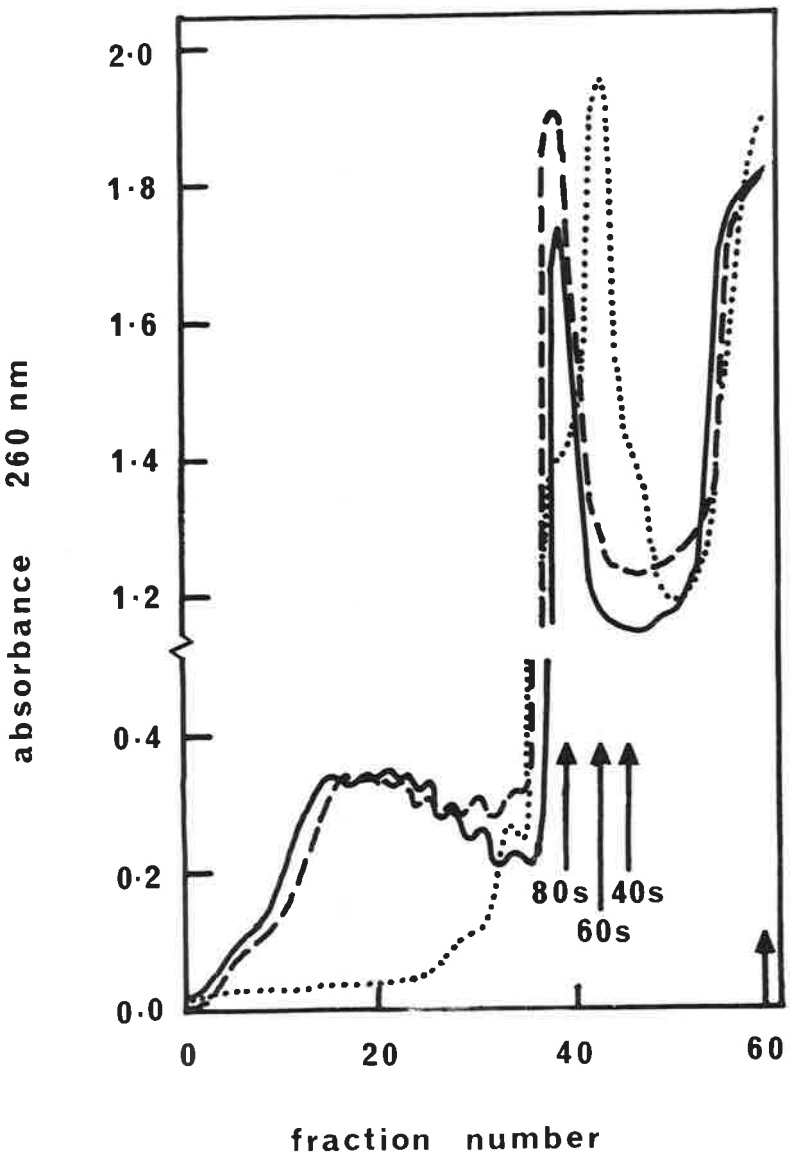
When incubated with cycloheximide (Fig. 7.4b) the size of the labelled polyribosomes was maintained and the label on the gradient remained distributed as in the control sample of labelled polyribosomes (Fig. 7.4c): the nascent protein chains were not released by this antibiotic. The concentration of cycloheximide required for this inhibition was high, presumably due to the presence of 1 mM dithiothreitol in the incubation system (Baliga *et al.*, 1969). The use of high levels of cycloheximide therefore provided a convenient method for the rapid termination of protein synthesis *in vitro* whilst the structural integrity of the polyribosomes was maintained.

The stability of the hair follicle polyribosomes that had been incubated with high concentrations of puromycin or cycloheximide was investigated on sucrose density gradients prepared in a high salt buffer (Fig. 7.5). Puromycin-treated polyribosomes were almost completely degraded to single ribosomes and ribosomal subunits whereas the structure of the cycloheximide-treated polyribosomes was essentially unchanged. These observations were in accord with those of Zylber and Penman (1970) on HeLa

FIGURE 7.5

THE EFFECT OF HIGH SALT ON THE STRUCTURAL INTEGRITY OF HAIR FOLLICLE POLYRIBOSOMES INCUBATED WITH PUROMYCIN OR CYCLOHEXIMIDE

Whole tissue homogenate preparations were incubated at 37^{o} for 10 min as described in Methods but with unlabelled amino acids (5 nmoles of each) and with $2 \cdot 10^{-4}$ M puromycin (·····) or $3 \cdot 10^{-3}$ M cycloheximide (-----). The reactions were terminated by chilling. The KCl and $MgCl_2$ concentrations were increased to 0.50 M and 50 mM, resepctively, by addition of the solid reagents (Zylber and Penman, 1970). The samples were then examined by centrifugation on linear 15 - 60 % sucrose density gradients prepared in a *modified* buffer A in which the KCl and $MgCl_2$ concentrations had been increased to 0.50 M and 50 mM, respectively (Zylber and Penman, 1970), as in Fig. 6.1, but were centrifuged for 6 h. A control sample of homogenate (———) which had been held at 0^{o} but was also treated with high salt, was centrifuged on a high salt gradient simultaneously.


2·0
1·8
1·6
1·4
1·2
0·4
0·2
0·0
absorbance 260 nm
0
20
40
60
fraction number
80s
40s
60s

cells. These authors inferred that the presence of the peptidyl-tRNA species was important for maintainence of polyribosome or ribosome structure in the conditions of high ionic strength. Thus untreated polyribosomes or polyribosomes that had been treated with cycloheximide were unaffected by the high salt concentration. On the other hand, treatment of polyribosomes with puromycin and high salt provides a convenient method for the preparation of ribosomal subunits (*see* Fig. 9.1).

(b) CELL-FREE PROTEIN SYNTHESIS IN THE RECONSTITUTED SYSTEM

(1) Composition of the in vitro system

The composition of the buffer B used was investigated with a view to increasing the incorporation of leucine into acid insoluble material. The concentration of $MgCl_2$ for optimal activity varied with the concentration of phosphoenolpyruvate (Fig. 7.6). It appeared that the phosphoenolpyruvate complexed with $MgCl_2$, thereby removing it from the reaction. Optimal activity was obtained at 3.5 mM phosphoenolpyruvate and 6.5 mM $MgCl_2$. Similar experiments showed that the system was more active at 0.125 M KCl. The composition of buffer B and of the composition of the reaction system was therefore changed accordingly.

(2) Properties of the in vitro system

The time course of the incorporation of tritiated leucine into TCA precipitable material was linear for the first 20 min and reached a plateau value by 40 min.

The properties of the reconstituted system are shown in Table 7.3. These results were reproducible over several experiments. Incorporation was totally dependent on added polyribosomes, supernatant fraction and ATP and partially dependent on added amino acids, reducing agents and yeast tRNA. In the absence of dithiothreitol considerable

FIGURE 7.6

DETERMINATION OF THE OPTIMUM $MgCl_2$ AND PHOSPHOENOLPYRUVATE (PEP) CONCENTRATIONS IN THE RECONSTITUTED CELL-FREE SYSTEM

The composition of the reconstituted cell-free system used was as described in Methods except that the $MgCl_2$ concentration was varied as shown over three different phosphoenolpyruvate concentrations. A single batch of polyribosomes and supernatant were used in the experiment. Each reaction sample contained 77 µg of polyribosomes. The samples were incubated for 60 min and the total TCA insoluble counts incorporated were determined. The $MgCl_2$ concentration was varied at; O - O , 0 mM PEP; ● - ● , 3.5 mM PEP; ▲ - ▲ , 5.0 mM PEP.

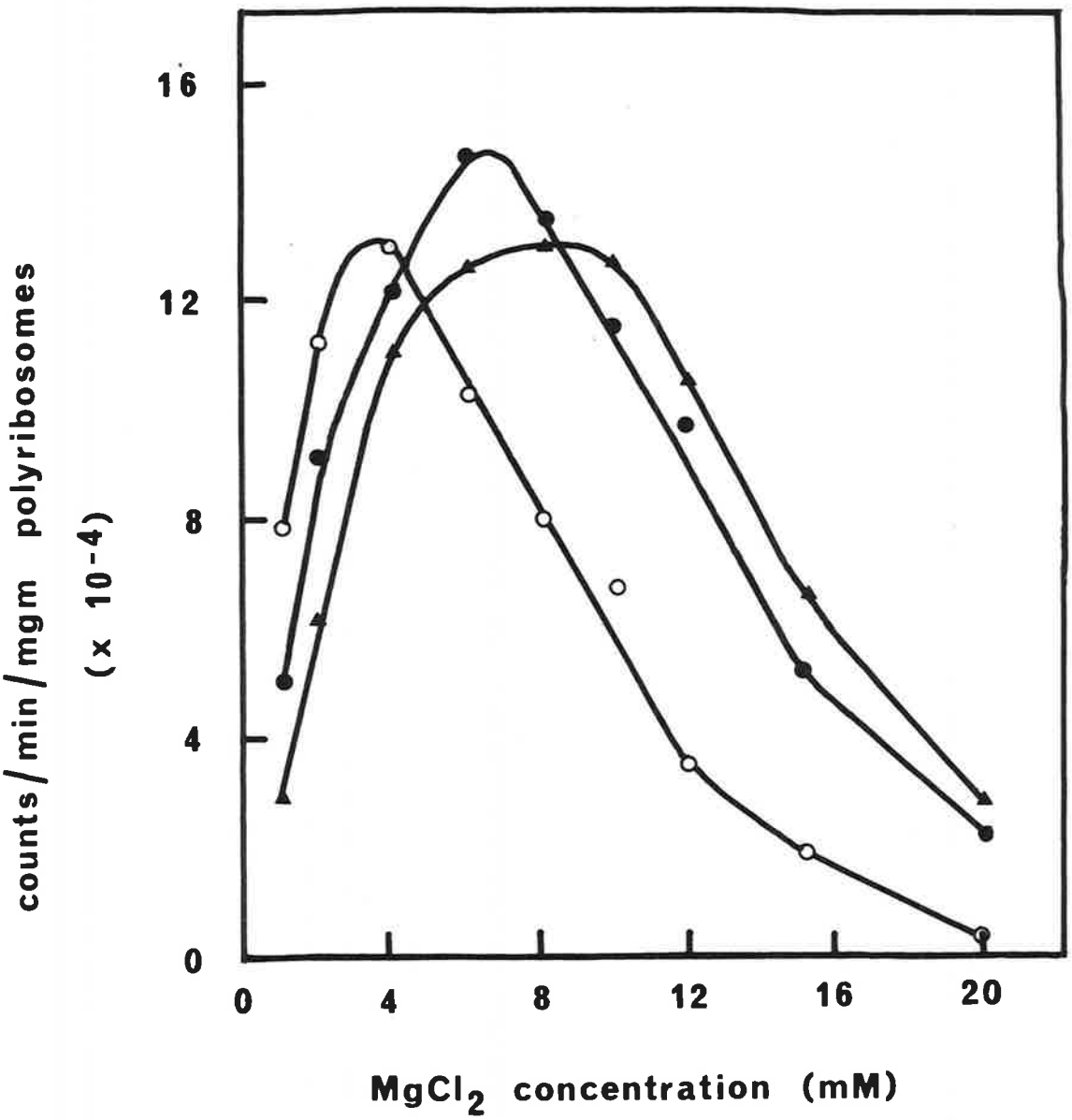

16
12
8
4
0
0
4
8
12
16
20
counts/min/mgm polyribosomes
(x 10-4)
MgCl2 concentration (mM)

TABLE 7.3

PROPERTIES OF THE RECONSTITUTED CELL-FREE PROTEIN SYNTHESIS SYSTEM

Details of the complete reconstituted system are given in the Methods. Each reaction in these experiments contained 154 µg of polyribosomes from a single preparation and was incubated for 60 min.

Experiment	Counts/min	% of complete
Complete	22 800	100
- GTP	17 480	76
- ATP regeneration	11 930	52
- ATP and ATP regeneration	4 100	18
- Amino acids	9 100	40
- Yeast tRNA	19 220	84
- Polyribosomes	790	3.4
- Supernatant fraction	260	1.1
- Dithiothreitol	10 270	45
- Dithiothreitol + 2-mercaptoethanol, $6 \cdot 10^{-3}$ M	14 620	64
+ Puromycin, $5 \cdot 10^{-5}$ M	2 420	11
$2 \cdot 10^{-4}$ M	640	2.8
+ Chloramphenicol, $1 \cdot 10^{-4}$ M	21 930	96
$4 \cdot 10^{-4}$ M	21 320	93
+ Cycloheximide, $5 \cdot 10^{-6}$ M	15 790	69
$5 \cdot 10^{-4}$ M	6 380	28
$3 \cdot 10^{-3}$ M	960	4.2
+ NaF, $5 \cdot 10^{-3}$ M	19 220	84
$2 \cdot 10^{-2}$ M	16 020	70
+ Ribonuclease, 10 µg	460	2.0
+ Polyuridylic acid, 20 µg	21 400	94
200 µg	14 640	64

precipitation of the sulphydryl-rich keratin proteins occurred during incubation, even if 6 mM 2-mercaptoethanol was added in its place. Attempts were made to increase the dependence of the reconstituted system on added amino acids by modification of the supernatant fraction. More extensive dialysis of it was not effective; nor was passage of it through columns of Sephadex G-25 (medium) equilibrated in buffer B. A "pH 5 enzyme" fraction was prepared (Hoagland and Askonas, 1963). The sulphydryl-rich keratin proteins present in the supernatant also precipitated near pH 5 and were difficult to redissolve. The pH 5 preparation used inhibited incorporation by about five-fold. It was concluded that there was a significant concentration of leucyl-tRNA in the supernatant fraction.

Antibiotics and other inhibitors of protein synthesis affected the activity in essentially the same manner as observed in the whole tissue homogenate cell-free system. Inhibition by cycloheximide, NaF and high concentrations of polyuridylic acid suggested that initiation occurred in this system also.

The effect of the temperature of the wax used for the preparation of hair follicles from guinea pig skin on the incorporating ability of the follicle polyribosomes was investigated. Follicles were prepared as described at five different wax temperatures; 80^{o}, 72^{o}, 60^{o} (normal), 52^{o} and 44^{o}. Polyribosomes were then prepared from the follicles obtained from each temperature sample and assayed in the *in vitro* protein synthesis system. No significant variation in incorporation was observed between the different temperature samples which suggested that the hot wax procedure for the preparation of hair follicles did not alter the properties of the isolated polyribosomes.

(3) Activity of the in vitro system

The activity can be estimated as follows using the data given

in Table 7.3. In an incubation mixture containing 150 µg of polyribosomes and 2 µc (1000 pmoles) of tritiated leucine, about 22 000 counts/min or about 65 000 distintegrations/min (15 pmoles) were incorporated. Since the leucine content of the total hair follicle proteins was about 10 % (*see* Table 3.1), and if it can be assumed that all follicle proteins were represented in the polyribosomal population, then the *in vitro* system incorporated about 1000 pmoles of amino acids per mg of polyribosomes. In comparison, when similar calculations were applied to previous experiments of Clarke and Rogers (1970b), their polyribosomes incorporated only about 1.4 pmoles of amino acids per mg of polyribosomes.

These observations can be extended to calculate that each ribosome incorporated an average of about two amino acid residues. This degree of incorporation is lower than that expected from the evidence presented earlier of degradation of polyribosomes by run-off of ribosomes from a messenger during amino acid incorporation. This apparent anomaly was most likely due to the presence in the dialysed supernatant fraction of leucyl-tRNA which effectively lowered the specific activity of the leucine in the incubation system.

(4) Activity of different size classes of hair follicle polyribosomes

Polyribosomes from the size classes B, C and D and single ribosomes from group A (*see* Fig. 6.1) were assayed for protein synthesis activity in the reconstituted system (Table 7.4). The larger polyribosomes were more active than than the total mixture, and group B polyribosomes were less active. These differences may indicate the differences in leucine contents of the proteins that are synthesised by the different size groups of polyribosomes. Single ribosomes were much less active, as expected.

TABLE 7.4

ACTIVITY OF DIFFERENT SIZE GROUPS OF HAIR FOLLICLE POLYRIBOSOMES

Polyribosomes of the size groups B, C and D and single ribosomes from group A (*see* Fig. 6.1) were prepared from three standard sucrose density gradients by pelleting through 2 M sucrose (1 M sucrose for ribosomes) in buffer A by centrifugation at 225 000 x *g* for 2.5 h. The pellets were resuspended in buffer B, clarified by centrifugation at 12 000 x *g* for 10 min and assayed in the reconstituted cell-free protein synthesis system. Each assay reaction contained 100 - 200 µg of the polyribosomes (or ribosomes). The degree of incorporation is expressed as counts/min/mg of polyribosomes (or ribosomes) and the values given are the averages of four different experiments.

Polyribosome Size Group	Number of Ribosomes	counts/min/mg
A	1	9 400
B	2 - 9	98 700
C	12 - 20	173 300
D	25 - 35	163 700

(5) Release of protein chains from the hair follicle polyribosomes during incubation

The rate of release from the hair follicle polyribosomes of protein chains synthesised during incubation was investigated (Fig. 7.7). About 55 % of the total TCA precipitable material incorporated during incubation was released from the polyribosomes. Thus it is concluded that the polyribosomes steadily released completed protein chains at a substantial rate as amino acid incorporated proceeded.

D DISCUSSION

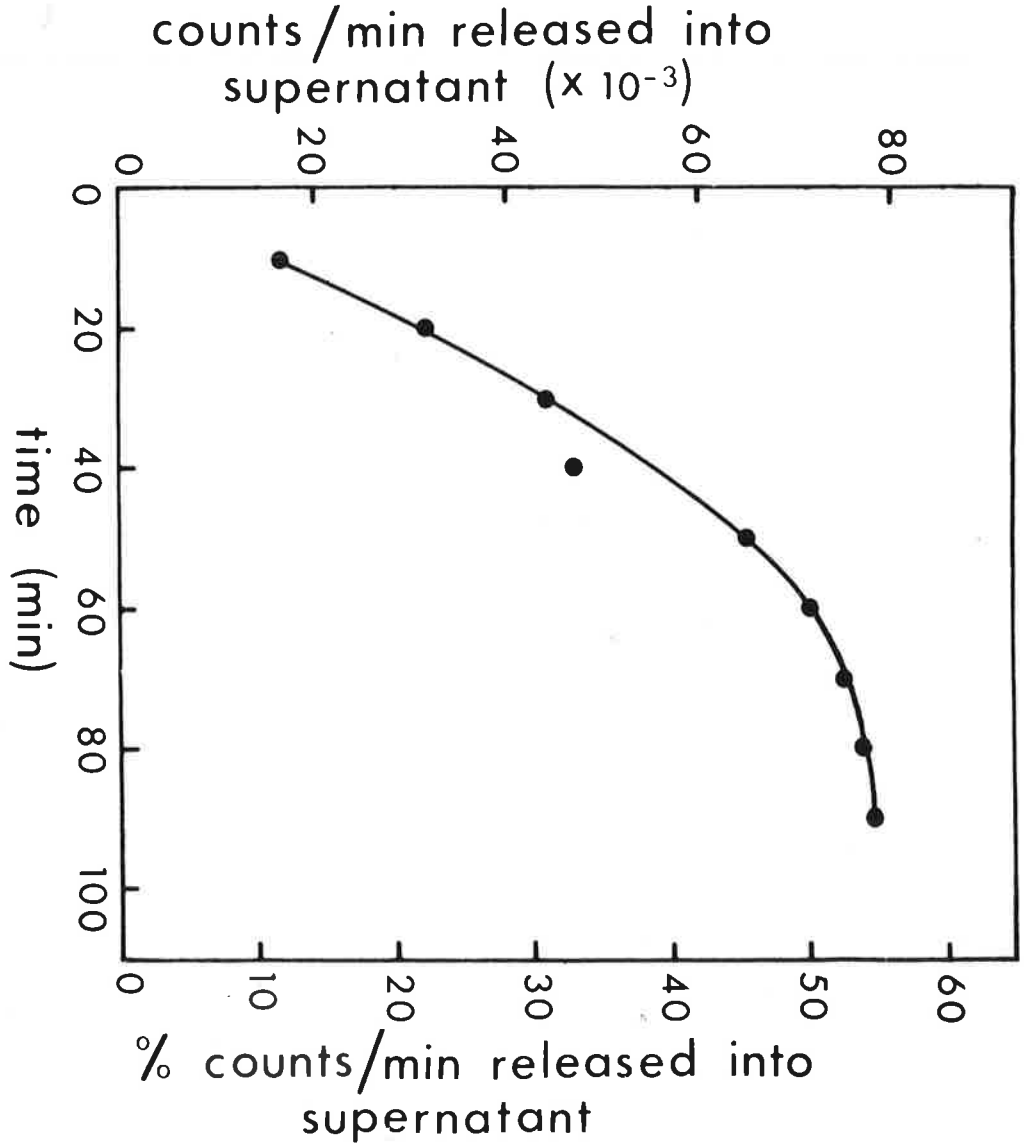
The hair follicle polyribosomes that have been isolated in this work directed the incorporation of amino acids into acid precipitable material at a substantially greater rate than those prepared previously (Freedberg, 1970; Clarke and Rogers, 1970b). Indeed, the reconstituted system established here was nearly 1000 times more active than a similar system established by Clarke and Rogers (1970b). This was most likely due to the lower levels of ribonuclease activity and the consequent higher yields of the larger polyribosomes present in the tissue homogenates. However, the activity of the whole tissue homogenate system was variable owing to the variations in the levels of endogenous amino acids in the homogenates. Attempts to reduce this variability and increase incorporation were largely unsuccessful. For this reason, a reconstituted system was developed and it was found that this was less variable, more dependent on added amino acids and about as active in amino acid incorporation.

Amino acid incorporation in all cell-free systems proceeded at a linear rate for 15 - 20 min. It is likely that considerable polypeptide chain elongation took place in this time as shown by the steady decrease of polyribosome size and the steady release of protein chains from the polyribosomes during incubation. The nature of these released protein

FIGURE 7.7

RATE OF RELEASE OF PROTEIN CHAINS FROM HAIR FOLLICLE POLYRIBOSOMES DURING INCUBATION

The composition of the reconstituted system used is given in Methods. Samples were removed from an incubation at various times and terminated by the addition of cycloheximide to a final concentration of $3 \cdot 10^{-3}$ M and chilled. The samples were centrifuged at 125 000 x *g* for 1.5 h to pellet the ribosomal material. The pellet and supernatant were then assayed separately for radioactivity. The curve shows the amount of radioactivity that was released from the polyribosomes during the course of the incubation and the percentage of the total acid precipitable counts incorporated that were released into the supernatant.


counts/min released into supernatant (x 10⁻³)
0
20
40
60
80
time (min)
0
20
40
60
80
100
0
10
20
30
40
50
60
% counts/min released into supernatant

chains will be described in the next chapter.

From the behaviour of these polyribosomes on incubation in the absence and presence of various antibiotics and other inhibitors of protein synthesis, it is concluded that the mechanism of protein synthesis directed by the hair follicle polyribosomes is very similar to that directed by polyribosomes obtained from other eukaryote cell-types.

An important aspect of this work was the finding that polypeptide chain initiation may have occurred during incubation, as shown by the inhibitor studies. Clearly, the present evidence is insufficient, and the most satisfactory demonstration of initiation *in vitro* would involve the complete synthesis of a protein from its amino-terminal residue, *in vitro*. Such studies using the cell-free protein synthesis systems developed in the present chapter will be described in the next two chapters.

CHAPTER EIGHT

THE *IN VITRO* SYNTHESIS OF THE GUINEA PIG HAIR FOLLICLE PROTEINS

A INTRODUCTION

The experiments described in the previous chapter demonstrated that guinea pig hair follicle polyribosomes directed the incorporation of amino acids into high molecular weight polypeptide material in cell-free systems at a high rate. It was therefore of considerable interest to investigate the nature of the polypeptides that were labelled *in vitro*.

It was also demonstrated in Chapter Seven that about 50 % of the total acid-insoluble radioactivity was released from the ribosomes during incubation. In this chapter studies on the nature of these released polypeptides are described and it is shown that they are in fact completed protein molecules which are identical to the native hair follicle proteins.

B METHODS

(a) PREPARATION OF THE RADIOACTIVELY-LABELLED PROTEIN FRACTIONS

The composition of the reconstituted cell-free protein synthesis system used in these studies was exactly as described in Methods of Chapter Seven (*see* page 97) except that variations to the concentration and specific activity of the radioactive amino acid precursors were made as required (*see* later experiments).

The procedure for the isolation of the keratin proteins from the guinea pig hair follicle tissue described in Chapter Three involved extraction with a buffer containing 8 M urea and 0.1 M 2-mercaptoethanol followed by alkylation of the sulphydryl-rich proteins with an excess of iodoacetate (*see* Chapter Three, page 41). This extracted the hair follicle (F) proteins as their stable S-carboxymethyl kerateine (SCMK) derivatives. Accordingly, the cell-free incubations were terminated by addition of cycloheximide to a final concentration of 3 mM and then centrifuged at 125 000 x *g* for 1.5 h to pellet the ribosomal material. The supernatant was made to 8 M urea, 0.1 M 2-mercaptoethanol and 0.1 M tris-HCl (pH 9.0)

and mixed for 30 min to ensure complete reduction of all sulphydryl groups. Alkylation with iodoacetate was performed as described in Chapter Three and the solution was finally dialysed exhaustively against a buffer of 10 mM tris-HCl (pH 7.6) and 1 mM EDTA. The dialysed radioactive proteins were mixed with an additional quantity of native guinea pig F-SCMK proteins. The amount was varied between different experiments as required.

The different groups of hair follicle proteins were then prepared and purified from the pH 4.4 soluble (F-SCMK-B) and insoluble (F-SCMK-A) protein fractions as described in Chapter Three (*see* Table 3.2).

C RESULTS

(a) FEASIBILITY EXPERIMENTS

In order to characterise the proteins synthesised during cell-free incubation it was necessary to increase their specific activity to the highest possible level. Therefore, the following experiments were performed to examine possible methods of achieving this using several different radioactive amino acids.

In Fig. 8.1a it is seen that at least 7 - 8 nmoles of amino acids are required per mg of polyribosomes for optimal incorporation, irrespective of the nature of the amino acid. In addition, the degree of incorporation was directly proportional to the specific activity of the radioactive amino acid used in the incubation (Fig. 8.1b), above specific activities of about 1 μc/nmole. The non-linearity at the low specific activities was possibly due to the presence in the supernatant fraction of endogenous amino acids or amino acyl-tRNA species as mentioned ealier.

The data of Fig. 8.1a also demonstrate that the degree of incorporation of a particular amino acid was proportional to the content of that amino acid in the total hair follicle proteins extracted by the urea

FIGURE 8.1

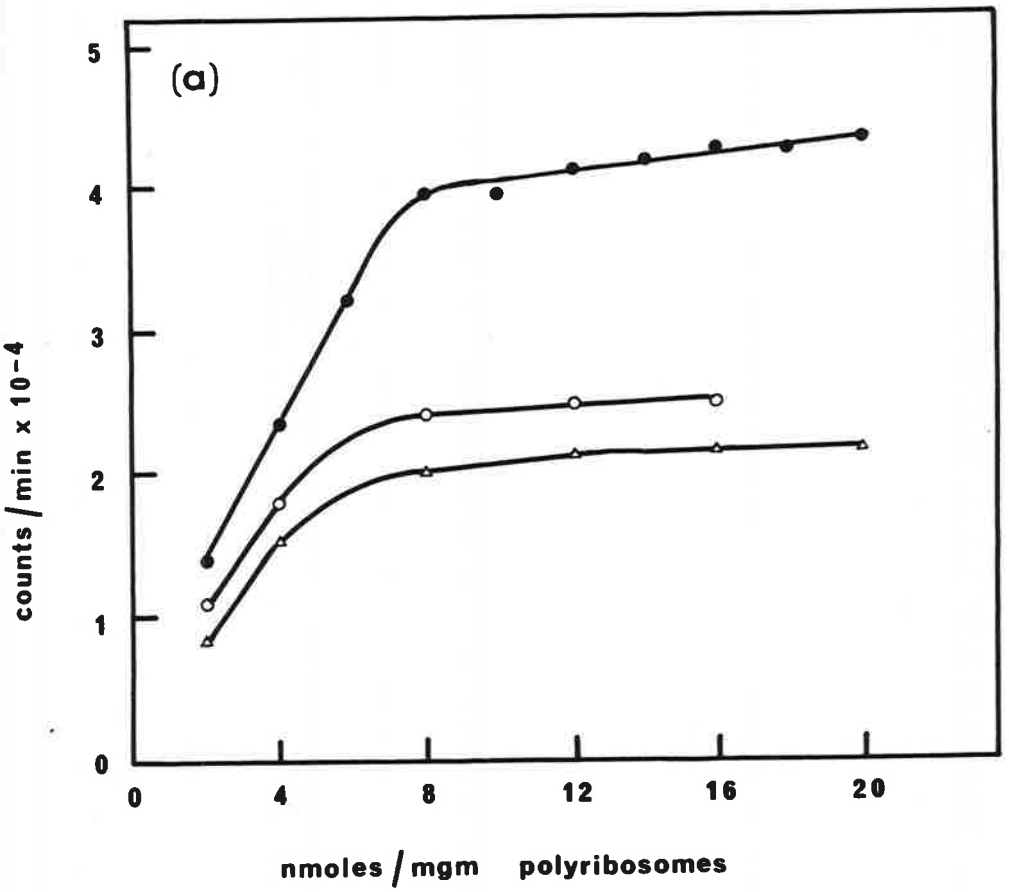
IN VITRO PROTEIN SYNTHESIS FEASIBILITY EXPERIMENTS

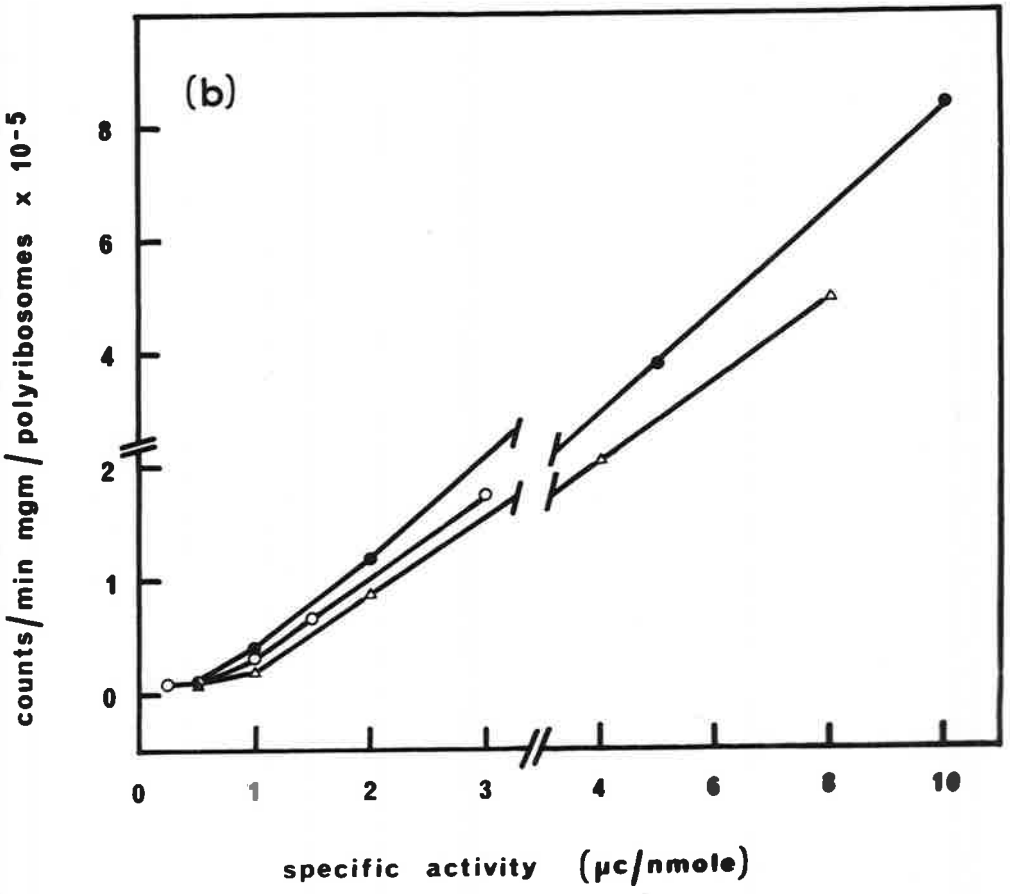
(a) DETERMINATION OF THE OPTIMAL CONCENTRATION OF RADIOACTIVE AMINO ACIDS

The composition of the reconstituted cell-free protein synthesis system used was as described in Chapter Seven (*see* page 97) except that the amount of radioactive amino acid was varied between 2 - 20 nmoles/mg of polyribosomes. The specific activity of the radioactive amino acid was maintained at 1 μc/nmole. Each sample was incubated for 60 min and a portion was withdrawn for measurement of incorporated acid-insoluble radioactivity. The radioactive amino acids used were; ● - ● , L-{4,5-^{3}H}leucine; O - O , L-{2-^{3}H}serine; and Δ - Δ , L-{2-^{3}H}alanine.

(b) THE EFFECT OF INCREASING RADIOACTIVE AMINO ACID SPECIFIC ACTIVITY ON INCORPORATION

The composition of the reconstituted cell-free protein synthesis system used was as above except that the specific activity of the radioactive amino acids was varied between the range of 0.25 - 10.0 μc/nmole. The concentration of the radioactive amino acid was maintained at 10 nmoles/mg of polyribosomes. Each sample was incubated for 60 min and a portion was withdrawn for measurement of incorporated acid-insoluble radioactivity. The radioactive amino acids were; ● - ● , L-{4,5-^{3}H}leucine; O - O , L-{2-^{3}H}serine; Δ - Δ , L{2-^{3}H}alanine.


(a)
counts / min x 10-4
0
1
2
3
4
5
0
4
8
12
16
20
nmoles / mgm polyribosomes


(b)
counts / min mgm / polyribosomes x 10-5
0
1
2
4
6
8
0
1
2
3
4
6
8
10
specific activity (μc/nmole)

buffer. For example, at a specific activity of 3 μc/nmole, about 240 000 counts/min of leucine were incorporated into the proteins (Fig. 8.1b) in which the leucine content is about 10 moles percent (*see* Table 3.1) (that is, about 24 000 counts/min per percent of leucine). The values respectively for serine were 180 000 counts/min and 8 moles percent (that is, about 22 000 counts/min per percent of serine) and for alanine, 120 000 counts/min and 5 moles percent (that is, about 24 000 counts/min per percent of alanine). It was therefore possible to predict the approximate degree of incorporation of an amino acid or mixture of amino acids under known conditions of amino acid specific activity and concentration.

Predictions on the incorporation into a particular group of hair follicle proteins were possible if it was further assumed that incorporation was proportional to the content of that group of proteins in the total hair follicle extract. For example, since the F-group-2 (LoS) proteins comprise about 70 % of the total hair follicle proteins (*see* Table 3.4) and have a leucine content of about 10 moles percent (*see* Table 3.3), then if 400 000 counts/min of leucine were incorporated in an incubation (Fig. 8.1b) it could be predicted that about 140 000 counts/min would be incorporated into the LoS proteins. In the same incubation, about 5000 counts/min would be incorporated into the F-group-4 (HiS) proteins (the content of HiS proteins is about 10 % of the total proteins and the leucine content is near 2.5 moles percent).

Preliminary studies using radioactive serine and leucine showed that these predictions were in fact accurate. Such predictions greatly facilitated the design of experiments and made possible the detailed studies reported here.

(b) CHARACTERISATION OF THE LoS PROTEINS SYNTHESISED IN VITRO

The experiments of Chapter Three demonstrated that the F-group-2

(LoS) proteins had a molecular weight of 43 - 48 000 daltons and when separated on polyacrylamide gels appeared as four distinct bands. Other experiments have shown that these proteins are also antigenic (Frater, 1968 and 1969; Kemp and Rogers, 1970). These properties formed the basis for the characterisation of the LoS proteins that were synthesised in the cell-free systems of this work.

(1) Chromatography on Sephadex G-200

A cell-free incubation using radioactive leucine was performed and when the labelled F-SCMK-A protein fraction was chromatographed on Sephadex G-200 the elution profile shown in Fig. 8.2a was obtained. These proteins had a marked propensity for aggregation both with themselves and other highly charged protein molecules (*see* Chapter Three), even in the presence of 8 M urea. These aggregates and the other lower molecular weight protein species (F-group-3 proteins) were removed from the LoS proteins by repeated re-chromatography on Sephadex (*see* Fig. 3.6) and after one recycle the elution profile of Fig. 8.2b was obtained. The radioactivity profile co-chromatographed precisely with the absorbance profile suggesting that the labelled proteins were completed polypeptide chains of the same size as the native proteins.

(2) Polyacrylamide gel electrophoresis (PAGE)

A sample of the labelled proteins prepared as described in Fig. 8.2b was subjected to PAGE as described previously (*see* Fig.3.1). Determination of the absorbance of the stain as well as the amount of radioactivity in each gel slice provided unambiguous comparison of the two parameters. As shown in Fig. 8.3 the radioactivity co-electrophoresed precisely with the main protein bands which suggested that the labelled proteins were completed protein chains with the same molecular charge as the native proteins.

FIGURE 8.2

CHROMATOGRAPHY OF THE LABELLED F-SCMK-A PROTEINS SYNTHESISED *IN VITRO* ON SEPHADEX G-200

A standard reconstituted cell-free protein synthesis system was employed using L-{4,5-^{3}H}leucine. The details were; specific activity, 10 µc/nmole; 7.5 nmoles of leucine/mg of polyribosomes; 2.5 mg of polyribosomes. The labelled F-SCMK-A protein fraction was prepared as described in Methods with the expected total yield of 750 000 counts/min (about 25 mg).

A 165 x 1.6 cm column of Sephadex G-200 column in urea buffer (*see* Fig. 3.2) was loaded with a 3 ml sample containing 15 mg (450 000 counts/min) of the labelled F-SCMK-A protein fraction. The absorbance of the 3.0 ml fractions was measured at 276 nm and a portion of each fraction was mixed with 1 mg of carrier unlabelled guinea pig F-SCMK protein and treated with 10 % TCA. Acid-insoluble material was collected onto glass fibre circles for measurement of radioactivity. ——— , absorbance; ..... , radioactivity.

(a) Chromatography of the labelled F-SCMK-A protein fraction. The bar represents the tubes that were pooled, dialysed exhaustively against buffer and concentrated by ultrafiltration (*see* page 47) for re-chromatography in *(b)*. In *(b)* approximately 210 000 counts/min (about 7 mg) were applied.

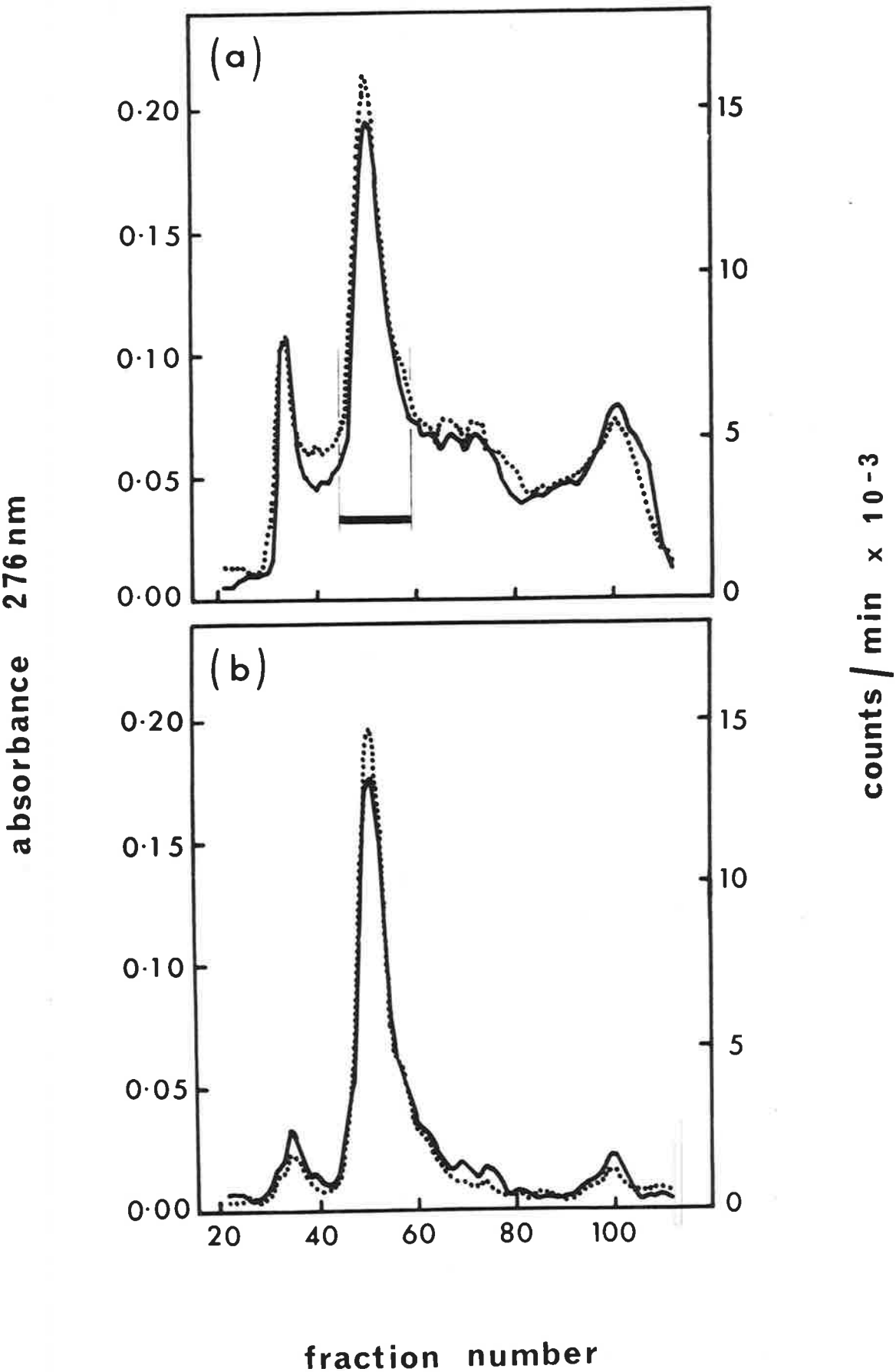
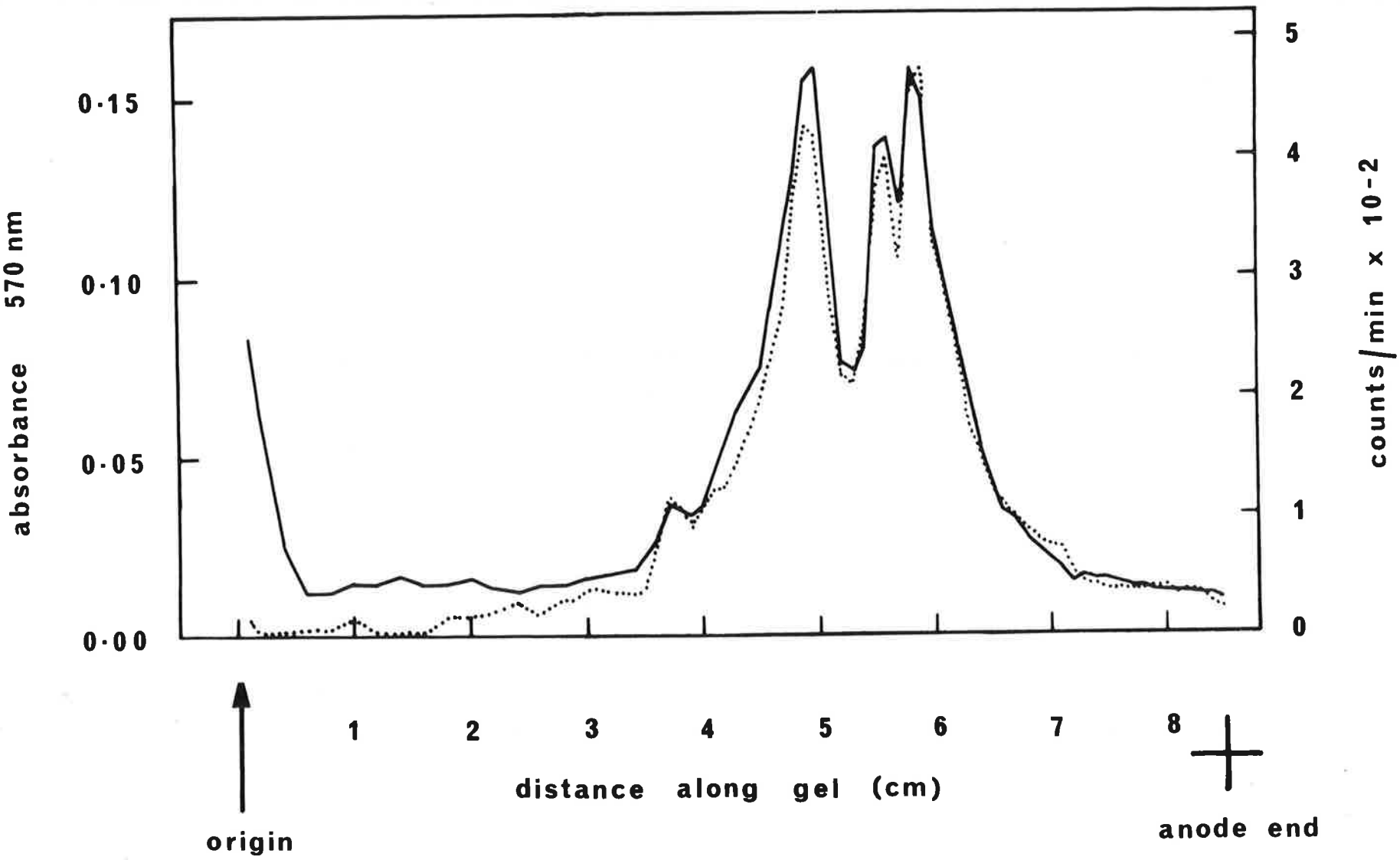

(a)
(b)
0·20
0·15
0·10
0·05
0·00
15
10
5
0
20
40
60
80
100
absorbance 276 nm
counts / min x 10-3
fraction number

FIGURE 8.3

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF THE LABELLED LoS PROTEINS

The polyacrylamide gel at pH 9 in the presence of 5 M urea was prepared as described in General Methods (*see* page 41) except that the usual cross-linking reagent (N,N'-methylene-bisacrylamide) was replaced by N,N'-diallyltartardiamide (Anker 1970).

A 0.3 mg (9000 counts/min) sample of the labelled protein obtained from Fig. 8.2b was electrophoresed on the gel at 3 mA for 4 h. The gel was then stained with 0.05 % coomassie brilliant blue in 10 % TCA for 2 days. Excess stain was removed with 50 % aqueous ethanol and the gel was frozen and sliced into 1.0 mm lengths. Each slice was dissolved in 2 % (w/v) periodic acid (Anker, 1970) (2.0 ml) in 30 min at 23^{o} and the absorbance of the stain determined at 570 nm. The solution was mixed with 1 mg of carrier unlabelled F-SCMK protein, treated with 10 % TCA and acid-insoluble material was collected onto glass fibre circles for measurement of radioactivity. ——— , absorbance; ····· , radioactivity.


absorbance 570 nm
0·15
0·10
0·05
0·00
counts/min x 10-2
5
4
3
2
1
0
1
2
3
4
5
6
7
8
distance along gel (cm)
origin
anode end

This experiment (together with the previous purification step) was performed routinely on each preparation of labelled proteins and in all cases similar results were obtained.

(3) Immunoprecipitation experiments

Experiments on the reaction of the purified LoS proteins with various antisera are demonstrated in Fig. 8.4. The labelled proteins reacted chiefly with the F-SCMK-A antiserum (Fig. 8.4a). A low degree of cross-reaction of the S-carboxymethyl feather keratin protein antiserum was evident, but this may have been due to cross-reaction with the S-carboxymethyl groups on the two different types of keratin proteins (Frater, 1968; Kemp and Rogers, 1970). In addition, only native unlabelled guinea pig LoS protein competed with the labelled protein for reaction with the F-SCMK-A antiserum (Fig. 8.4b). Neither S-carboxymethyl-ovalbumin (which had a similar molecular weight and charge to the LoS proteins) nor chicken S-carboxymethyl feather keratin protein competed significantly with the antiserum.

(4) Characterisation of the amino-terminal tryptic peptides

In another experiment hair follicle proteins were labelled with tritiated algal hydrolysate. Component F-III was prepared and purified as described in Chapter Three and the tryptic peptides of the amino-terminal cyanogen bromide fragment CNBr-2 were characterised (Fig. 8.5) by the procedures developed and described in Chapter Four. The peptides T_1 and T_2 were clearly labelled. The tripeptide T_3 was only slightly labelled presumably since the threonine residue was the only amino acid common to the tripeptide and radioactive amino acid mixture, and its specific activity was low. The amino-terminal tryptic peptide T_2 contained 900 counts/min of radioactivity which was consistent with the occurrence of *de novo* protein synthesis.

This experiment showed that the chemical properties of the

FIGURE 8.4

REACTION OF THE LABELLED PROTEINS SYNTHESISED *IN VITRO* WITH ANTISERA

The samples of labelled proteins used in these experiments were obtained from the experiment of Fig. 8.2b.

(a) PRECIPITATION

Samples contained 0.25 ml of normal rabbit serum (▲-▲), or sera prepared against the native guinea pigF-SCMK-A proteins (●-●) or chicken S-carboxymethyl feather keratin protein (■-■) and increasing amounts of the labelled F-SCMK-A protein purified as described in Fig. 8.2b in 1.0 ml of 0.14 M NaCl - 10 mM phosphate buffer (pH 7.1). The samples were incubated at 37° for 2 h and then 2° for 18 h. Precipitated material was collected by centrifugation and filtered onto glass fibre circles for measurement of radioactivity.

(b) PRECIPITATION WITH COMPETITION

Samples contained 20 µg (600 counts/min) of the purified labelled F-SCMK-A protein in 1.0 ml of the NaCl - phosphate buffer used in *(a)* and varying amounts of one of the competitor proteins, S-carboxymethyl-ovalbumin (▲-▲), chicken S-carboxymethyl feather keratin protein (■-■) and native guinea pig F-SCMK-A protein that had been purified on Sephadex G-200 (●-●). Precipitated material that formed on incubation was collected for measurement of radioactivity as in *(a)*.

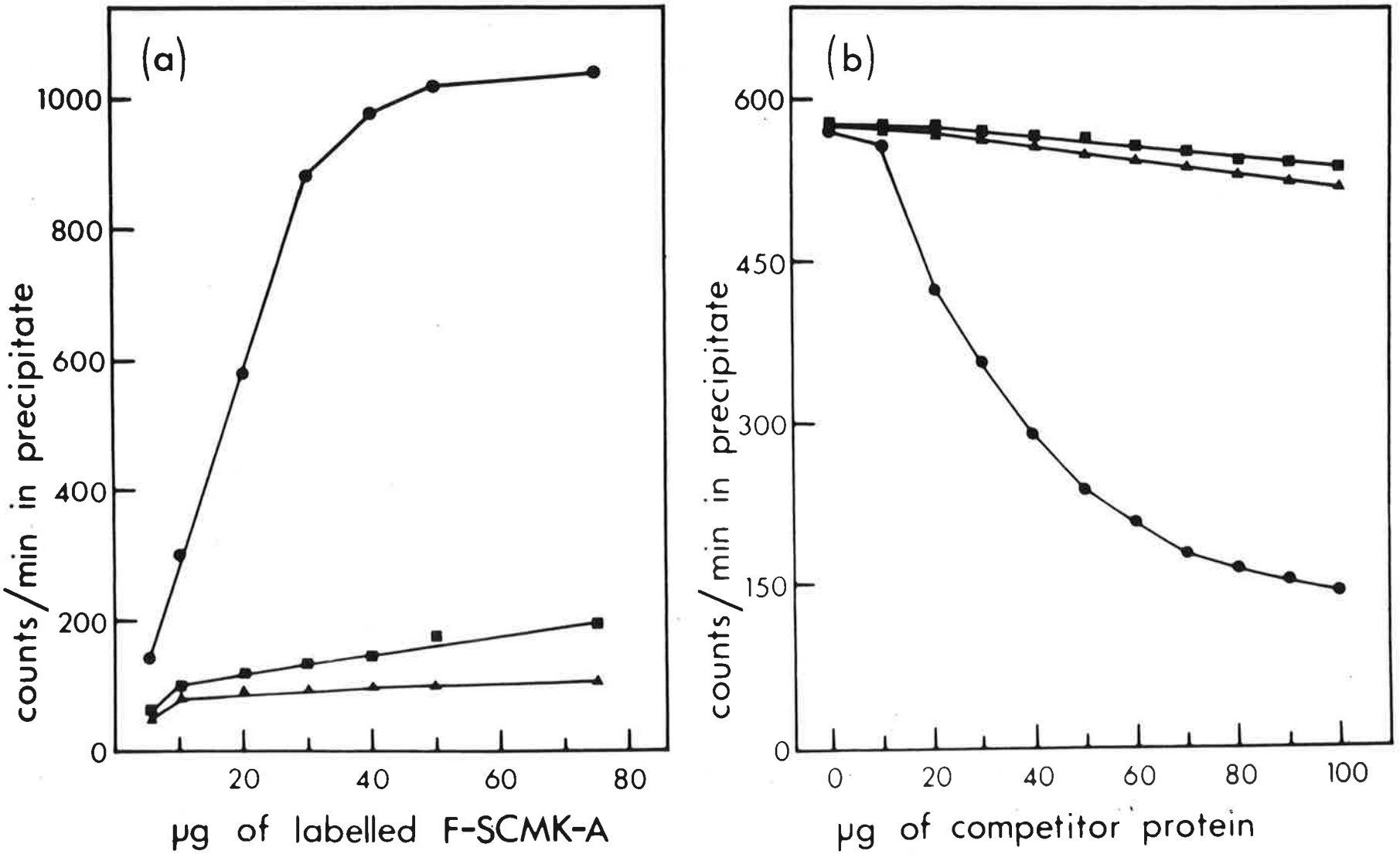
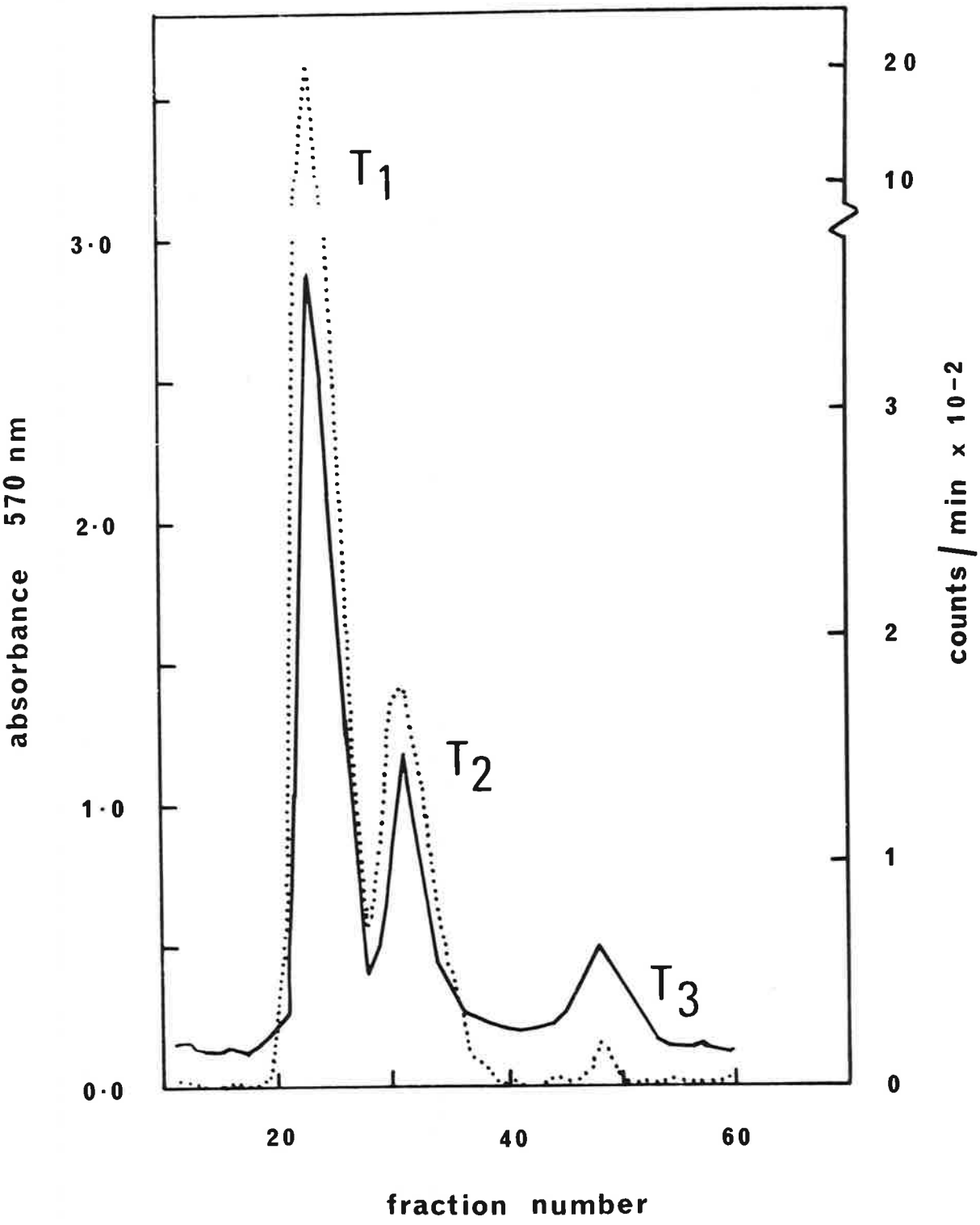

(a)
1000
800
600
400
200
0
counts/min in precipitate
20
40
60
80
µg of labelled F-SCMK-A
(b)
600
450
300
150
0
counts/min in precipitate
0
20
40
60
80
100
µg of competitor protein

FIGURE 8.5

CHROMATOGRAPHY OF THE TRYPTIC PEPTIDES DERIVED FROM LABELLED F-III-CNBr-2 PROTEIN SYNTHESISED *IN VITRO* ON SEPHADEX G-25

A standard cell-free protein synthesis system was employed and the radioactive label was tritiated reconstituted algal hydrolysate which contained 14 different radioactive amino acids. The details of the incubation were; 6.8 mg of polyribosomes; 100 μc of label per mg of polyribosomes; and where necessary, the concentration of each amino acid was adjusted to 10 nmoles/mg of polyribosomes. (Due to the low specific activities of some radioactive amino acids, their concentration was unavoidably higher than this). The labelled LoS proteins were then prepared as described in the preceeding experiments with a total yield of $3.2 \cdot 10^6$ counts/min (about 1.4 g of protein). From this, 600 000 counts/min (about 310 mg) of labelled component F-III were prepared by chromatography on DEAE-cellulose as described in Fig. 3.5. After purification (*see* Fig. 3.6), the CNBr-2 fragment was prepared (*see* Fig. 4.3) (9450 counts/min; about 45 mg) and was digested with trypsin as described in Chapter Four (*see* page 66). The labelled tryptic peptides were chromatographed on a column of Sephadex G-25 using the same conditions as in Fig. 4.4. A 0.2 ml sample of each 1.25 ml fraction was removed for ninhydrin analysis after alkaline hydrolysis (Fig. 4.4) and the remainder of each fraction was evaporated to dryness in a glass scintillation counting bottle at 110°. The solid material which remained was dissolved in water (2.5 ml) and the scintillation fluid of Bray (1960) was added (10 ml) for the measurement of radioactivity. ——— , absorbance at 570 nm; ····· , radioactivity.


absorbance 570 nm
3·0
2·0
1·0
0·0
20
40
60
fraction number
20
10
3
2
1
0
counts / min x 10-2
T1
T2
T3

labelled component F-III synthesised *in vitro* were closely similar or identical to those of the native protein component synthesised *in vivo*.

(c) CHARACTERISATION OF THE HiS PROTEINS SYNTHESISED IN VITRO

In Chapter Three it was demonstrated that the F-group-4 (HiS) proteins consisted of a complex group of many components. However, the proteins were arbitrarily classified into four subgroups on the basis of their molecular weights, separation on polyacrylamide gels and SCMcysteine contents. The HiS proteins synthesised *in vitro* were characterised using these properties.

(1) Chromatography on Sephadex

The elution profile of the F-SCMK-B protein fraction labelled *in vitro* using tritiated leucine on Sephadex G-200 in urea buffer is shown in Fig. 8.6a. The tubes which contained the HiS proteins were pooled as shown and re-chromatographed on Sephadex G-100 using formic acid (Fig. 8.6b). In both cases, the radioactive proteins did not co-chromatograph precisely with the native proteins but they did have the same molecular weights. Two explanations for the differences in the profiles were apparent. Firstly, it was established that the leucine content of the HiS proteins varied between the subgroups (*see* Table 3.11); the lower molecular weight proteins had about twice as much leucine as the high molecular weight proteins. Thus it might be expected the former proteins would be more labelled. Secondly, the different types of HiS proteins might not have been synthesised in the same amounts. Further experiments were designed to investigate these possibilities.

(2) PAGE

Proteins labelled *in vitro* using radioactive serine were purified by the chromatographic procedure described in Fig. 8.6a and a sample

FIGURE 8.6

CHROMATOGRAPHY OF THE LABELLED F-SCMK-B PROTEIN FRACTION SYNTHESISED *IN VITRO* ON SEPHADEX

The labelled F-SCMK-B protein fraction used in this experiment was derived from a reconstituted cell-free protein synthesis experiment using L-{4,5-^{3}H}leucine identical to that described in Fig. 8.2 except that 7.3 mg of polyribosomes were used and the expected yield of 98 000 counts/min (about 34 mg) was obtained.

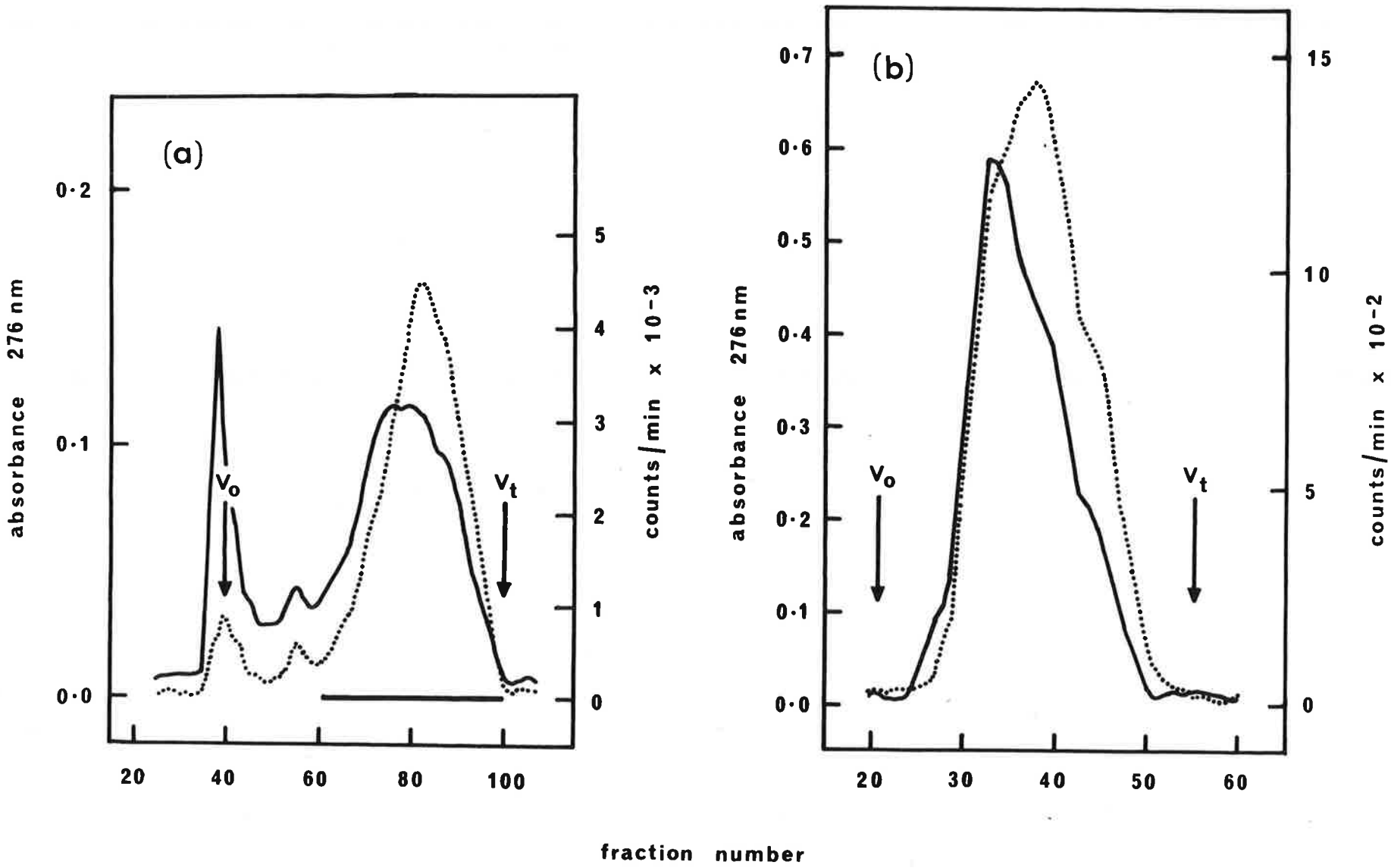
(a) CHROMATOGRAPHY ON SEPHADEX G-200

The details of the chromatography experiment and measurement of radioactivity were identical to those used in Fig. 8.2. The bar represents the tubes that were pooled, dialysed exhaustively against buffer and concentrated by rotary film evaporation (*see* page 47) for re-chromatography in *(b)*.

(b) CHROMATOGRAPHY ON SEPHADEX G-100

The details of this chromatography experiment were identical to those used in Fig. 3.9. Approximately 25 mg (70 000 counts/min) of protein were applied. A 0.5 ml sample of each fraction was dried at 110^{o} onto glass fibre circles in glass vials and the toluene-based scintillation fluid was added for the measurement of radioactivity.

In both *(a)* and *(b)*; ——— , absorbance at 276nm; ····· , radioactivity.


(a)
absorbance 276 nm
counts/min x 10-3
Vo
Vt
0·0
0·1
0·2
0
1
2
3
4
5
20
40
60
80
100
(b)
absorbance 276nm
counts/min x 10-2
Vo
Vt
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0
5
10
15
20
30
40
50
60
fraction number

was electrophoresed on a polyacrylamide gel (Fig. 8.7). The radioactivity co-electrophoresed precisely with the densitometer trace which suggested that the labelled proteins were completed protein molecules with the same molecular charges as the native proteins. However, the faster migrating and lower molecular weight protein species were more labelled that the slower migrating and higher molecular weight proteins (Fig. 8.7). Since the serine content of the different subgroups of HiS proteins was approximately constant (*see* Table 3.11) these results suggested that the lower molecular weight proteins were synthesised to a greater extent in the cell-free experiments.

Similar results were obtained when other batches of labelled HiS proteins were examined, indicating that these differences were reproducible.

(3) Chromatography on DEAE-cellulose

A batch of low specific activity labelled HiS proteins was prepared using radioactive leucine and chromatographed on DEAE-cellulose at pH 4.5 (Fig. 8.8). Each of the four subgroups of proteins (*see* Fig. 3.10) was labelled but as before there were marked differences in the degrees of labelling of each subgroup. In order to characterise this further, tubes were pooled as shown to determine the relative specific activities of each subgroup (Table 8.1). It is clear that after consideration of the variable leucine content of each subgroup, the proteins of lowest molecular weight and lowest SCMcysteine content were synthesised in the greatest amount during cell-free protein synthesis.

(d) SYNTHESIS OF OTHER HAIR FOLLICLE PROTEINS IN VITRO

Apart from the LoS and HiS proteins, the hair follicle synthesises several other types of structural proteins, in addition to other cytoplasmic proteins. The other structural proteins are the group-3 and the

FIGURE 8.7

POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE LABELLED HiS PROTEINS

A standard reconsituted cell-free protein synthesis system was employed using L-{2-^{3}H}serine as the radioactive amino acid. The conditions were; 4.3 mg of polyribosomes; specific activity, 3 μc/nmole; 8 nmoles of serine per mg of polyribosomes. The labelled F-SCMK-B protein fraction was prepared and the HiS (F-group-4) proteins were obtained with a total yield of 39 000 counts/min (about 2 mg).

A 0.2 mg (about 4000 counts/min) sample of this protein was electrophoresed on a gel containing the cross-linking reagent N,N'-diallyltartardiamide at 3 mA for 3 h. The gel was stained, traced with a densitometer, sliced into 1.0 mm pieces, dissolved and prepared for the measurement of radioactivity as in Fig. 8.3. ——— , densitometer trace; ····· , radioactivity.

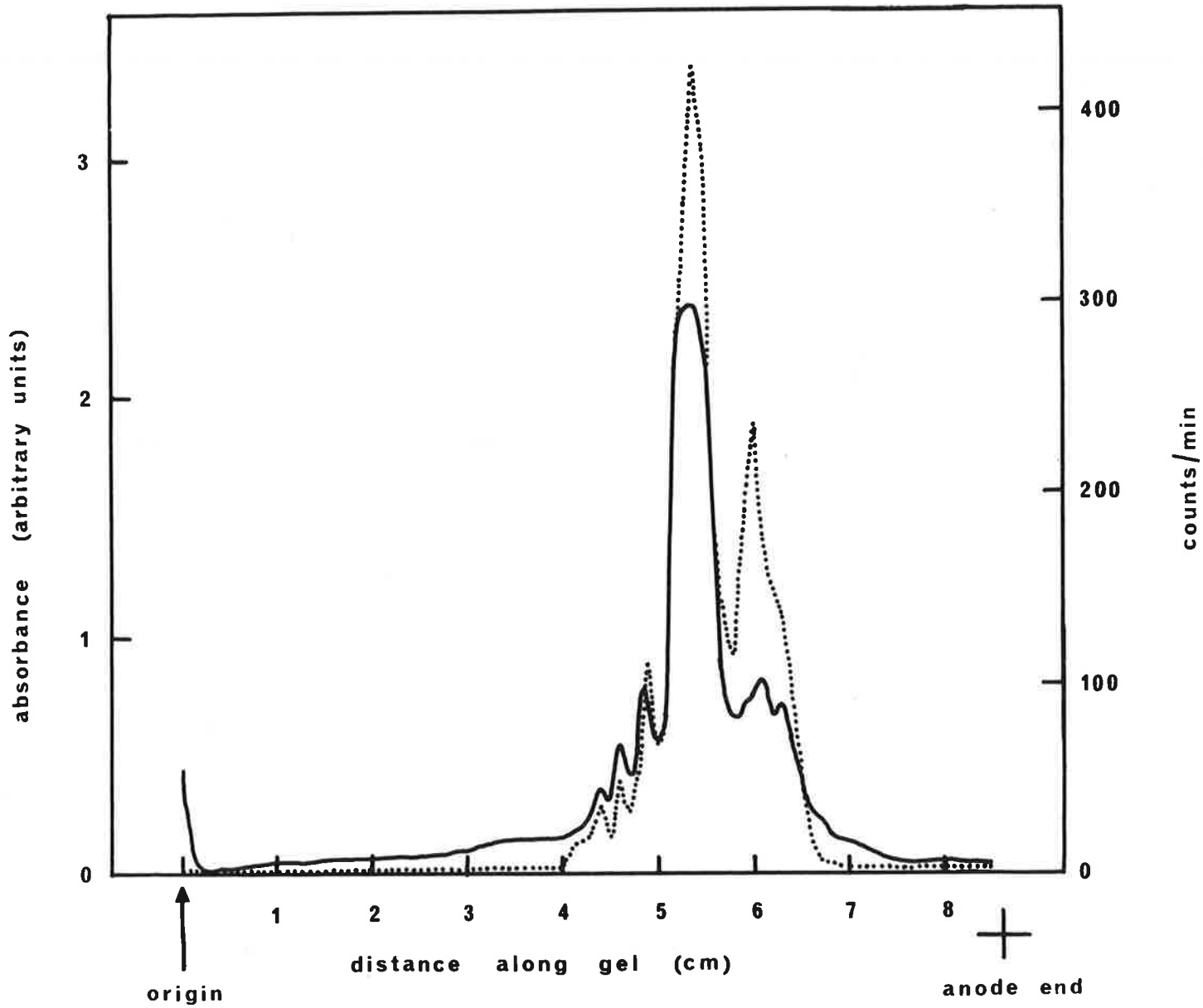

absorbance (arbitrary units)
counts/min
0
1
2
3
100
200
300
400
1
2
3
4
5
6
7
8
distance along gel (cm)
origin
anode end

FIGURE 8.8

CHROMATOGRAPHY OF THE LABELLED HiS PROTEINS SYNTHESISED *IN VITRO* ON DEAE-CELLULOSE

The labelled HiS proteins used in this experiment were derived from a reconstituted cell-free protein synthesis experiment using {4,5-^{3}H}leucine identical to that described in Fig. 8.2 except that 4.8 mg of polyribosomes were used and the expected yield of 65 000 counts/min (about 48 mg) was obtained. This labelled protein was applied to a 15 x 1.6 cm column of DEAE-cellulose in acetate buffer (pH 4.5) and chromatographed as described in Fig. 3.10. A 0.5 ml sample of each 3.0 ml fraction was diluted with water, mixed with 0.5 mg of carrier F-SCMK protein, treated with 10 % TCA and the acid-insoluble material was collected onto glass fibre circles for the measurement of radioactivity. ——— , absorbance at 276 nm; ····· , radioactivity.

The bars marked 1 - 4 inclusive refer to the tubes that were pooled for characterisation of their contents (*see* Table 8.1).

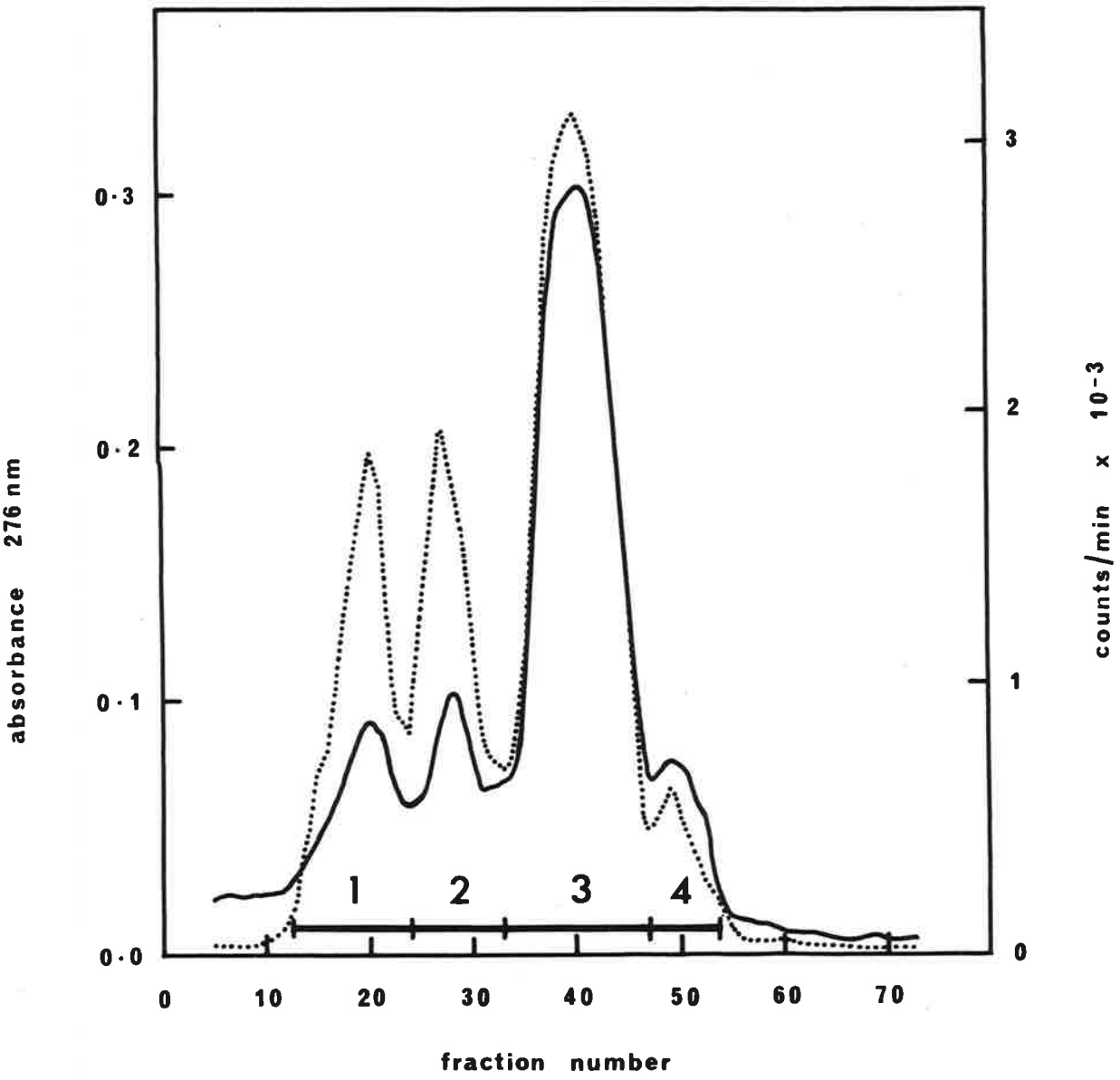

absorbance 276 nm
0·3
0·2
0·1
0·0
counts/min x 10⁻³
3
2
1
0
1
2
3
4
0
10
20
30
40
50
60
70
fraction number

TABLE 8.1

COMPARATIVE SPECIFIC ACTIVITIES OF THE SUBGROUPS OF HiS PROTEINS SYNTHESISED *IN VITRO*

The protein samples were obtained from the experiment described in Fig. 8.8. The tubes containing the peaks 1 - 4 inclusive were pooled as shown in Fig. 8.8, dialysed exhaustively against water and freeze-dried. The dried samples were weighed and reconstituted in water (5.0 ml). A 1.0 ml sample was removed, precipitated with 10 % TCA and collected onto a glass fibre circle for the measurement of radioactivity. The amino acid composition data shown was obtained from Table 3.11.

Subgroup number	4	3	2	1
Approximate molecular weight (daltons)	28 000	22 000	14 000	10 000
SCMcysteine content (moles percent)	27.2	24.2	20.8	19.0
Leucine content (moles percent)	1.8	2.7	3.0	3.5
Counts/min/mg of protein	420	1180	1620	2460
Specific activity: (counts/min/mg/percent of leucine x 10^{-2})	2.3	4.4	5.4	7.0

group-1 proteins (*see* Chapter Three). As shown in Fig. 8.2a the low molecular weight F-group-3 proteins were labelled suggesting that they were synthesised *in vitro*. The F-group-1A proteins of Fig. 8.2a probably arose by aggregation of the LoS (F-group-2) proteins. The small but significant amount of label associated with the F-group-1B proteins (Fig. 8.6a) could be interpreted as indicating that the medulla and inner root sheath proteins were also synthesised *in vitro*. However, no further experiments were performed on either group.

D DISCUSSION

The experiments reported in the present work have demonstrated that a reconstituted *in vitro* protein synthesis system derived from the guinea pig hair follicle tissue will synthesise completed keratin protein molecules that appear identical to the native proteins synthesised *in vivo*. Some evidence was also adduced which suggested that the other major types of hair follicle proteins were synthesised *in vitro*. Several criteria based on different properties of the proteins, namely, molecular size, molecular charge and antigenic properties are provided as evidence for this assertion. Another criterion of identity, coincidence of radioactivity with peptides derived from the labelled LoS proteins, was also demonstrated.

These experiments therefore infer that all hair follicle proteins and particularly the keratin proteins are synthesised *in vivo* by the classical ribosomal-dependent mechanism established in many other eukaryote organisms. The present work is not compatible with the previous suggestions (*see* Chapter One) that some hair follicle proteins might be synthesised by non-ribosomal-dependent mechanisms, such as enrichment with cysteine or cysteine-rich peptides (Gillespie, 1965). Indeed, the present experiments provide definitive evidence against the existence of unusual mechanisms of protein synthesis in hair follicle development.

Of considerable interest in these experiments were the observations on the rates of synthesis of the different components within each group of keratin proteins. Each LoS protein component was approximately equally labelled (Fig. 8.3) and since the leucine content of each component was similar (7 - 8 moles percent), it is concluded that these proteins were synthesised in a co-ordinate fashion during cell-free protein synthesis. However, a different situation was apparent for the HiS proteins. The proteins of lowest molecular weight and SCMcysteine content were synthesised in much greater amounts *in vitro* which indicated that synthesis of all HiS proteins was not co-ordinated.

Since each keratin protein could be synthesised *in vitro* it is clear that the HiS proteins of higher molecular weight and SCMcysteine content must be synthesised by the ribosomal-dependent mechanism in larger amounts *in vivo* during the terminal stages of follicle development. The tissue homogenates used for the isolation of the protein synthesis components did not contain particles from the highest region of the follicle where terminal development occurs. Therefore, variations in the synthesis of the HiS proteins in the cell-free system could be due to the paucity of some component required for synthesis of the proteins of higher molecular weight and SCMcysteine content. These components could be messenger RNA, initiation factors or modulating tRNA species.

A similar disproportionate relationship between the different subgroups of HiS proteins was observed in the earlier studies on the characterisation of the hair and hair follicle proteins (*see* Chapter Three). Therefore, it is also possible that the types of proteins that were synthesised *in vitro* reflected the spectrum of proteins that were synthesised in the hair follicle up to the level which was disrupted during homogenisation.

The achievement of cell-free protein synthesis of at least the keratin proteins of the hair follicle should now facilitate more extensive and detailed studies on the mechanism of control of the synthesis of these proteins. Preliminary studies on the mechanism of initiation of the hair follicle proteins will be described in the next chapter.

It was shown in Chapter Seven that 30 % of the total acid-insoluble radioactivity incorporated during cell-free protein synthesis was due to elongation of chains newly-initiated *in vitro*. While it is clear from the present experiments that pre-existing nascent chains must have been completed *in vitro*, and apart from the experiment of Fig. 8.5, it is not known whether newly-initiated protein chains were also completed *in vitro*. Experiments designed to investigate this are described in the next chapter.

CHAPTER NINE

INITIATION OF THE SYNTHESIS OF THE GUINEA PIG HAIR FOLLICLE KERATIN PROTEINS *IN VITRO*

A INTRODUCTION

As a beginning to the study of the control of protein synthesis in guinea pig hair follicle tissue, the mechanism of initiation has been investigated. This seemed the most profitable aspect to study for several reasons. Firstly, observations reported earlier in this thesis indicated that initiation very likely occurs *in vitro*. Such evidence arose from studies using specific inhibitors of initiation (*see* Tables 7.1 and 7.3) and from the detection of label in the amino-terminal tryptic peptide of component F-III synthesised *in vitro* (*see* Fig. 8.5). Secondly, the suggestion has been made that the mechanism of initiation might be different for the LoS and HiS proteins and different from the general cytoplasmic proteins (Fraser *et al.*, 1972). Thirdly, the observations of Chapter Five suggested that there was a definite point in follicle development at which HiS protein synthesis was initiated. One possible mode by which a switch-on could be mediated is the appearance of a HiS protein - specific factor operative at a post-transcriptional level.

The first part of the work of the present chapter was designed to establish that initiation of the keratin proteins does in fact occur *in vitro*. In a second part, the mechanism of initiation was investigated using ribosomal subunits obtained from guinea pig hair follicle tissue.

B METHODS

(a) PREPARATION OF LABELLED RIBOSOMAL SUBUNITS FROM HAIR FOLLICLE TISSUE

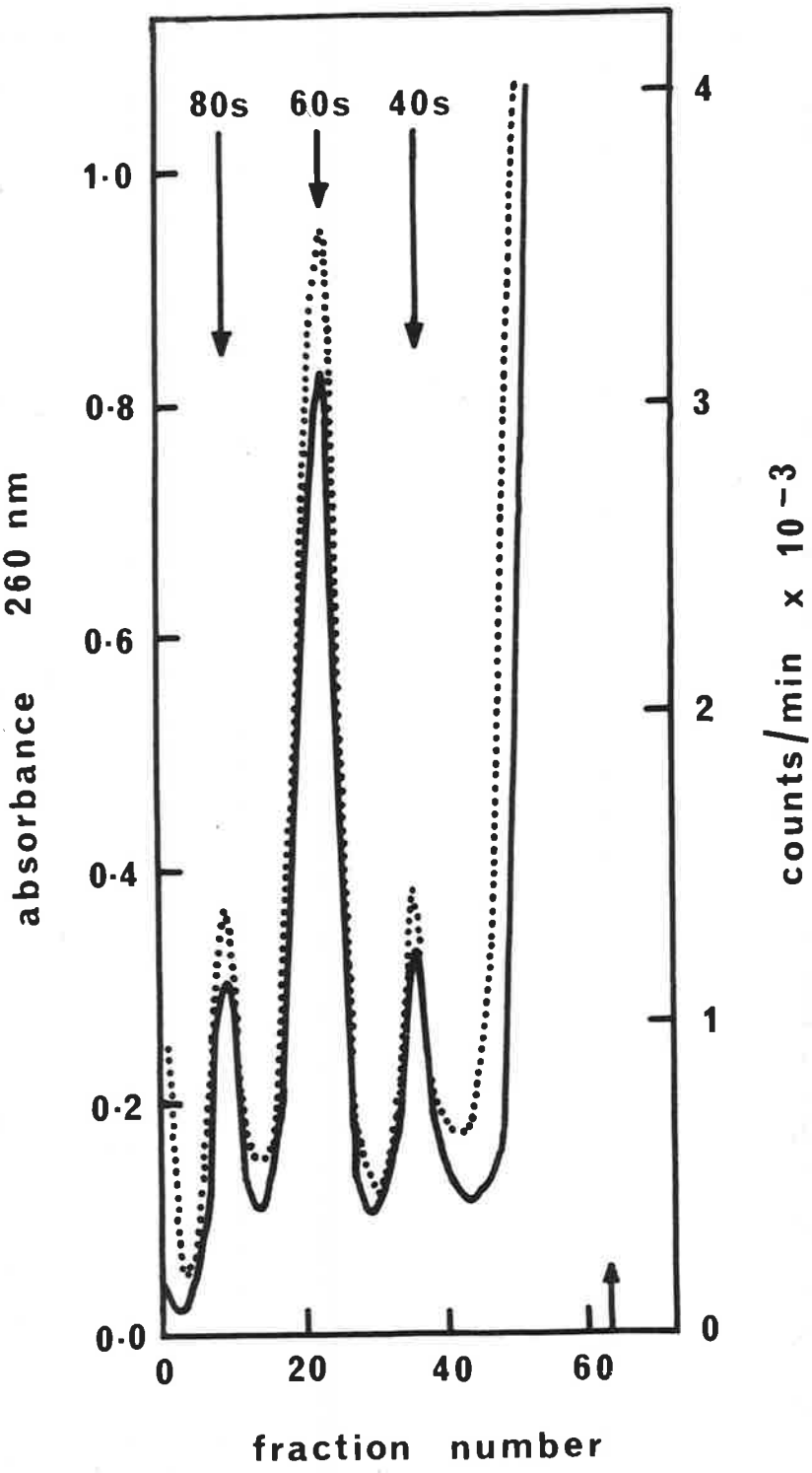
Radioactively-labelled guinea pig hair follicle ribosomal subunits were prepared by the method described in Fig. 9.1 using methods adapted from Zylber and Penman (1970) and the experiment shown in Fig. 7.5. The ribosomal subunits were separated from one another and the single ribosomes and supernatant proteins and possessed a specific activity of

FIGURE 9.1

PREPARATION OF LABELLED RIBOSOMAL SUBUNITS FROM HAIR FOLLICLE POLYRIBOSOMES

Two guinea pigs were injected intraperitoneally with {5-^{3}H}uridine (specific activity, 25 C/mmole, 100 µc per 100 g of animal body weight) and pulsed for 16 h. Whole tissue homogenates were prepared and incubated with 5 . 10^{-4} M puromycin at 37° for 5 min. The incubations were chilled and the KCl and $MgCl_2$ concentrations were increased to 0.50 M and 50 mM, respectively (*see* Fig. 7.5). These preparations were then pooled and centrifuged on 56 ml linear 15 - 30 % sucrose density gradients prepared in *modified* buffer A in which the KCl and $MgCl_2$ concentrations had been increased to 0.50 M and 50 mM, respectively (*see* General Methods, page 43) at 2° and 23 000 rev./min in an MSE swinging bucket rotor for 16 h. After centrifugation, the gradients were fractionated and one was prepared for the measurement of radioactivity by addition of the scintillation fluid of Bray (1960) (*see* Fig. 6.5). ——— , absorbance at 260 nm; ····· , counts/min.

The contents of the fractions corresponding to the 40*S* and 60*S* ribosomal subunits of the other two gradients were pelleted by centrifugation at 225 000 x *g* for 2.5 h. The pellets were resuspended in *unmodified* buffer A using a Dounce homogeniser (with a loose-fitting pestle) by five complete strokes. The concentration of subunits was adjusted spectrophotometrically (*see* Fig. 6.3b) to about 0.5 mg/ml.


80s
60s
40s
1·0
0·8
0·6
0·4
0·2
0·0
absorbance 260 nm
4
3
2
1
0
counts/min x 10⁻³
0
20
40
60
fraction number

165 000 counts/min/mg. The yield of ribosomal subunits was almost quantitative in that about 90 % of the polyribosomes were degraded to the subunits. It is likely that most of the single ribosomes that were recovered in the experiment had not been actively involved in protein synthesis *in vivo* as these were not dissociated to subunits by the puromycin-high salt treatment (Zylber and Penman, 1970; *see* page 103).

C RESULTS

(a) DE NOVO SYNTHESIS OF KERATIN PROTEINS IN VITRO

As discussed in Chapter Seven (*see* page 107), one definitive method of establishing initiation of polypeptide synthesis *in vitro* would be the demonstration of *de novo* protein synthesis. This could be achieved by detection of radioactive label in the amino-terminal amino acid residue of the proteins synthesised *in vitro*.

Therefore, two cell-free protein synthesis experiments were performed by the procedures described in Chapter Eight using radioactive serine and alanine for characterisation of the LoS and HiS proteins, respectively. After the incubations, the labelled F-group-2 and F-group-4 protein fractions were prepared and purified by the established procedures and the amino-terminal N-acetyl amino acids were isolated after enzyme digestion of the proteins. The products were chromatographed on the peptide analyzer (Fig. 9.2). It can be seen that both N-acetylserine (Fig. 9.2a) and N-acetylalanine (Fig. 9.2b) were labelled which suggested that initiation had indeed occurred. However, it was also mentioned that peptides of the composition PCA-ser and PCA-(ser, pro) which arose during enzymic digestion of the H-group-2 protein component H-III eluted from the analyzer column in the same region as the N-acetylserine (*see* Chapter Four, page 69) and it was therefore possible that the radioactivity which co-eluted with N-acetylserine may have been due to one or more of these peptides from the radioactive

FIGURE 9.2

CHARACTERISATION OF THE AMINO-TERMINAL N-ACETYL AMINO ACIDS LABELLED *IN VITRO*

The details of the *in vitro* synthesis experiments were as described in Chapter Eight.

(a) CHARACTERISATION OF THE LoS (F-group-2) PROTEINS

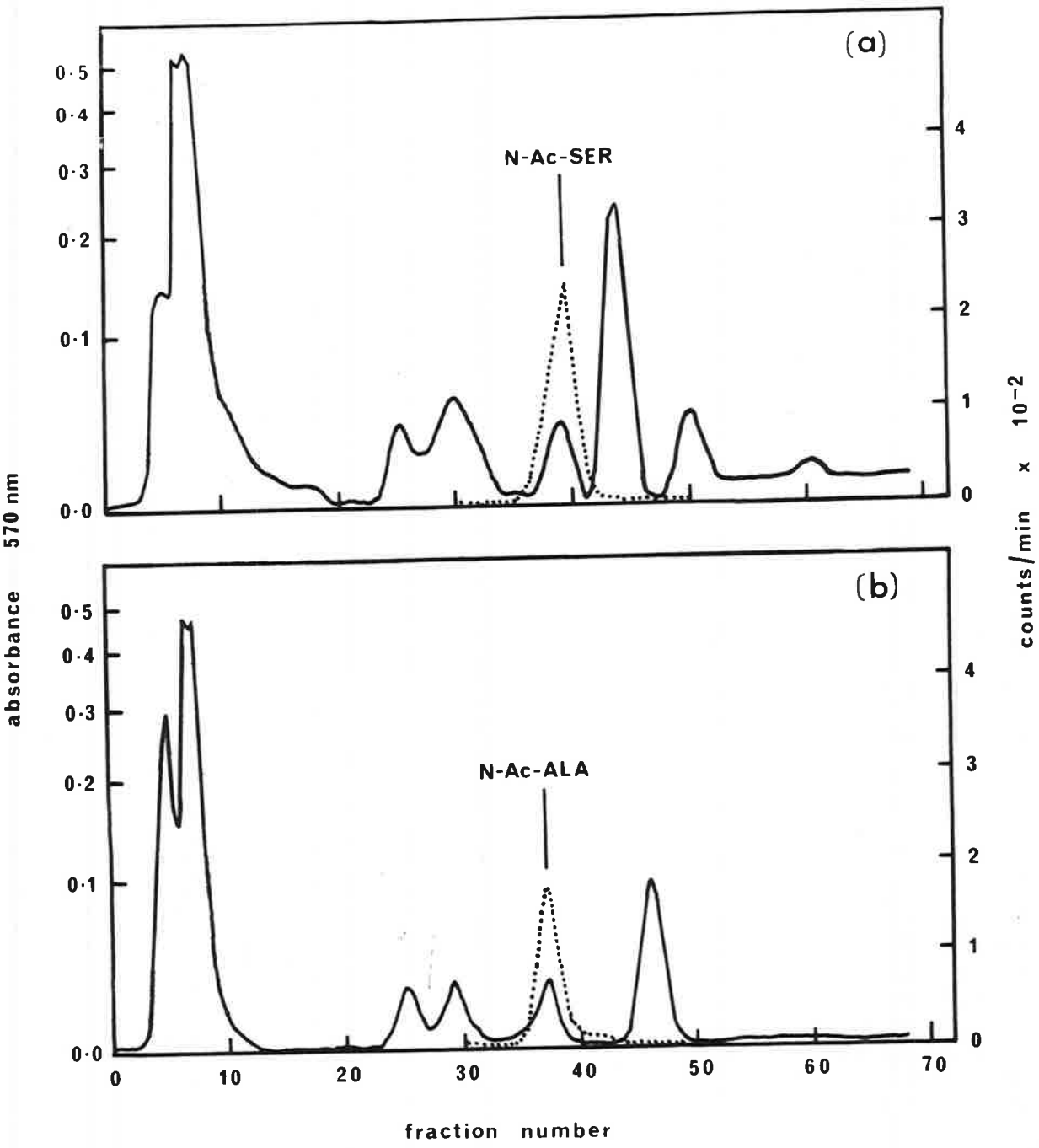
The details of the incorporation were; L-{2-^{3}H}serine; specific activity 3 µc/nmole; 8 nmoles of serine per mg of polyribosomes; 7.2 mg of polyribosomes.

(b) CHARACTERISATION OF THE HiS (F-group-4) PROTEINS

The details of the incubation were; L-{2-^{3}H}alanine; specific activity, 8 µc/nmole; 7.5 nmoles of alanine per mg of polyribosomes; 6.3 mg of polyribosomes.

After incubation, the F-group-2 and F-group-4 protein fractions were prepared from *(a)* and *(b)*, respectively. The N-acetyl species were prepared as described in Chapter Four (*see* page 66) and were then chromatographed on the peptide analyzer column (*see* Fig. 4.1). Aliquots of the fractions were removed for quantitation of the N-acetyl amino acids by amino acid analysis after acid hydrolysis and the remainder was dried onto glass fibre circles in glass vials at 110°. Measurement of radioactivity was done after addition of the toluene-based fluid. ———, absorbance at 570 nm; ····· , radioactivity.

In *(a)* the glass fibre circles of fractions 36 - 42 which contained the peak of N-acetylserine were retained for re-isolation of it. The circles were firstly washed in toluene to remove the scintillation reagents and then in 2 M formic acid to disperse the circles and dissolve the compound. After removal of the glass fibres by centrifugation, the formic acid was dried by rotary film evaporation and the solid residue was redissolved in water for further characterisation.


(a)
N-Ac-SER
(b)
N-Ac-ALA
absorbance 570 nm
counts/min x 10^{-2}
fraction number
0·0
0·1
0·2
0·3
0·4
0·5
0
1
2
3
4
0
10
20
30
40
50
60
70

F-LoS proteins. To check this, the N-acetylserine isolated in Fig. 9.2a was further characterised by high voltage paper electrophoresis (Fig. 9.3). Clearly, only N-acetylserine was labelled. The radioactivity which co-electrophoresed with serine probably arose by degradation of N-acetylserine during the preparation of the sample for the experiment. Accordingly, it can be claimed that the amino-terminal residue, N-acetylserine, of the LoS proteins was labelled. The same experiment on the labelled N-acetylalanine isolated in Fig. 9.2b was not done. The likelihood of the recovery of PCA-ala sequences in the HiS proteins was low since the amino acids glutamic acid and alanine were present in these proteins in much lower amounts than were glutamic acid and serine in the LoS proteins.

Estimations on the degree of initiation of the LoS and HiS proteins are possible from these experiments. 425 000 counts/min of labelled LoS proteins were used and if all the chains present had been made *de novo*, then they would be uniformly labelled with serine. This means that there would be 14 000 counts/min per serine residue (there are about 32 residues of serine per mole; *see* Table 3.3). In the experiment of Fig. 9.2a, N-acetylserine was recovered in a yield of 0.46 moles/mole of protein and 915 counts/min; that is, 2000 counts/min for the amino terminal serine residue or about 0.14 of the uniform value. Hence 14 % of all completed protein molecules present in the labelled protein fraction had been synthesised *de novo* in the cell-free system. A value of 16 % was obtained in another identical experiment. In the case of the HiS proteins, 75 000 counts/min were used which is equivalent to 12 500 counts/min per alanine residue (there are about 6 alanine residues per mole) if the protein was uniformly labelled with alanine. In the experiment of Fig. 9.2b, N-acetylalanine was recovered in yields of 0.53 moles/mole of protein (and N-acetylalanine-SCMcysteine, 0.08 moles/mole)

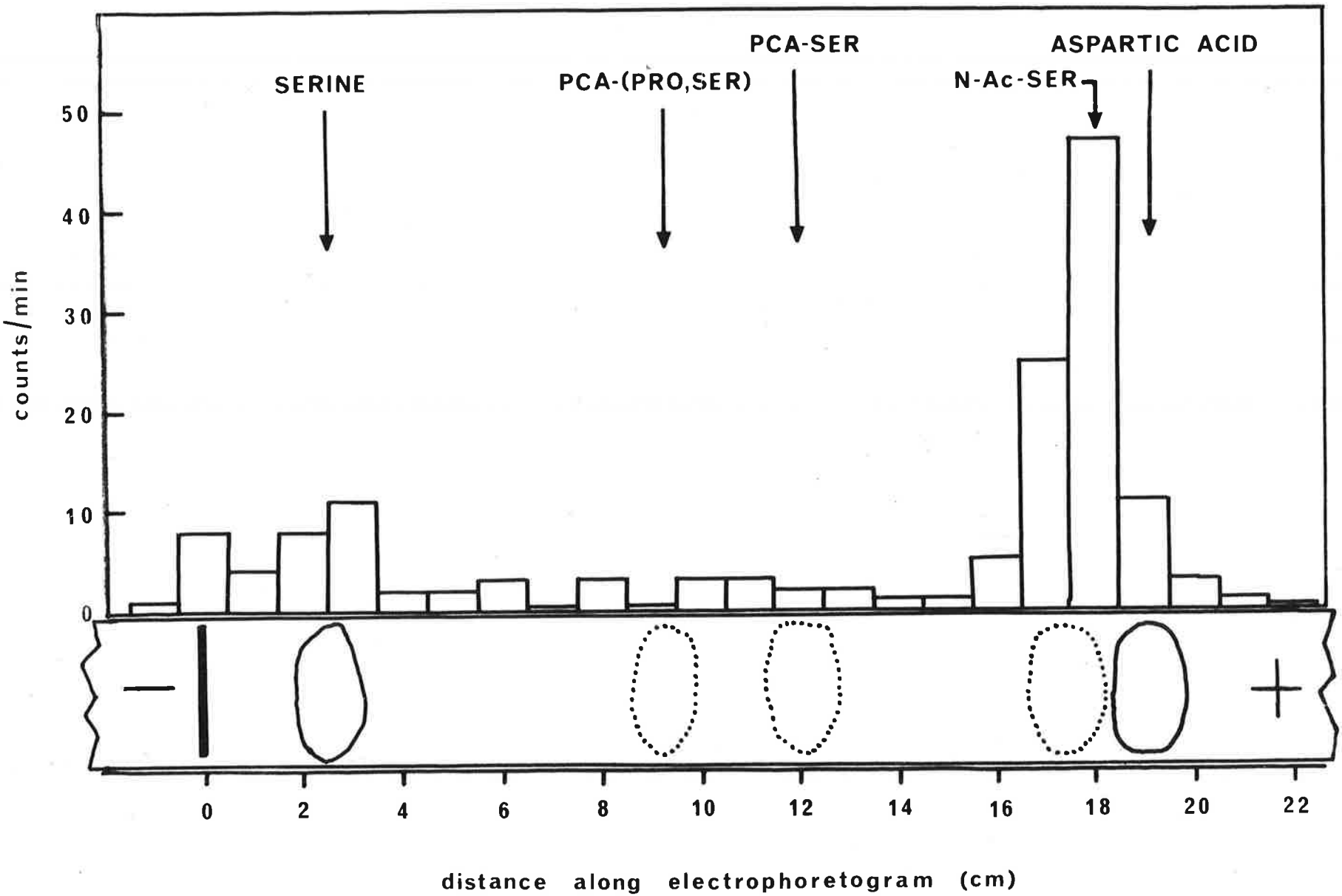
FIGURE 9.3

CHARACTERISATION OF THE N-ACETYLSERINE OBTAINED FROM THE *IN VITRO* SYNTHESIS OF THE LoS PROTEINS BY HIGH VOLTAGE PAPER ELECTROPHORESIS

The sample of N-acetylserine for characterisation was prepared as described in Fig. 9.2. This sample, together with marker compounds of serine and aspartic acid were then electrophoresed for 1 h at 2000 V at pH 6.5 using a buffer system and apparatus similar to that of Michl (1951).

After detection of the amino acids with ninhydrin, the electrophoretogram was cut into 1.0 cm lengths. Each piece was washed with 2 M formic acid (5 ml) for 1 h in a glass scintillation counting bottle to elute the amino acid and peptide material. The paper was removed and the formic acid was evaporated to dryness at 110°. The solid material which remained was redissolved in water (1.0 ml) and the scintillation fluid of Bray (1960) was added (4.0 ml) for the measurement of radioactivity.

The approximate electrophoretic mobilities of N-acetylserine, PCA-ser and PCA-(ser, pro) relative to aspartic acid were calculated on the basis of their molecular weights and charges at pH 6.5 from the data of Offord (1966) and are indicated by the dotted spots.


SERINE
PCA-(PRO,SER)
PCA-SER
N-Ac-SER
ASPARTIC ACID
counts/min
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
distance along electrophoretogram (cm)

and 142 counts/min. Therefore, using the same calculations as before it is seen that only about 2 - 3 % of the HiS protein chains were completely synthesised *in vitro*.

(b) INVESTIGATION OF THE MECHANISM OF INITIATION USING RIBOSOMAL SUBUNITS

As was described in Chapter One, initiation in eukaryote cell-type systems, like prokaryote cell-types, is believed to involve firstly attachment of a 40*S* ribosomal subunit to the mRNA followed by association of an initiating amino acyl-tRNA species and lastly by attachment of the 60*S* ribosomal subunit. Therefore, if initiation does occur in cell-free systems established from hair follicle tissue, it should be possible to demonstrate the attachment of hair follicle ribosomal subunits to polyribosomes after a short incubation.

Samples of the labelled 40*S* ribosomal subunits were added to a cell-free protein synthesis system at various times during the incubation and incubated for 2 min (Fig. 9.4). When added at zero time or after 5 min (Figs. 9.4a and 9.4b, respectively), significant amounts became associated with predominantly the largest polyribosomes, but when added at 10 min (Fig. 9.4c), however, no significant association occurred. The attachment of the 40*S* ribosomal subunits at the earliest times of incubation was consistent with the occurrence of initiation. These observations were in accord with studies in other eukaryote cell-type systems such as HeLa cells (Baglioni *et al.*, 1969) and reticulocytes (Howard *et al.*, 1970) where it was shown that initiation occurred only during the first few minutes of incubation. When labelled 40*S* ribosomal subunts were incubated with polyribosomes and 2 . 10^{-2} M NaF, they did not become associated with the ribosomes or polyribosomes (Fig. 9.4e) indicating that attachment had been inhibited. Likewise, when incubated in the absence of ribosomes (that is, in the absence of mRNA) (Fig. 9.4f), the 40*S* subunits did not associate to

FIGURE 9.4

INCUBATION OF HAIR FOLLICLE POLYRIBOSOMES WITH LABELLED 40*S* RIBOSOMAL SUBUNITS

The incubation system contained 250 µg of polyribosomes that had been pelleted through sucrose layers, suspended in 1.0 ml of a supernatant fraction prepared from a whole tissue homogenate by centrifugation at 125 000 x *g* for 1.5 h. This was *not* dialysed before use. In addition, the system contained 50 µg of unlabelled 60*S* ribosomal subunits (also prepared as described in Fig. 9.1), unlabelled amino acids (5 nmoles of each), 1 mM ATP and an ATP regenerating source of 5 mM phosphoenolpyruvate and 20 µg of pyruvate kinase and was incubated at 37°. 51 µg (8300 counts/min) samples of the labelled 40*S* ribosomal subunits were added at various times during the incubation and the incubations were continued for a further 2 min. After this time the reactions were chilled and layered on 28 ml linear 15 - 60 % sucrose density gradients and centrifuged as in Fig. 7.5. After centrifugation, the gradients were fractionated and prepared for counting as described in Fig. 6.5. ——— , absorbance at 260 nm; ····· , counts/min.

(a) The labelled subunits were added at zero time.

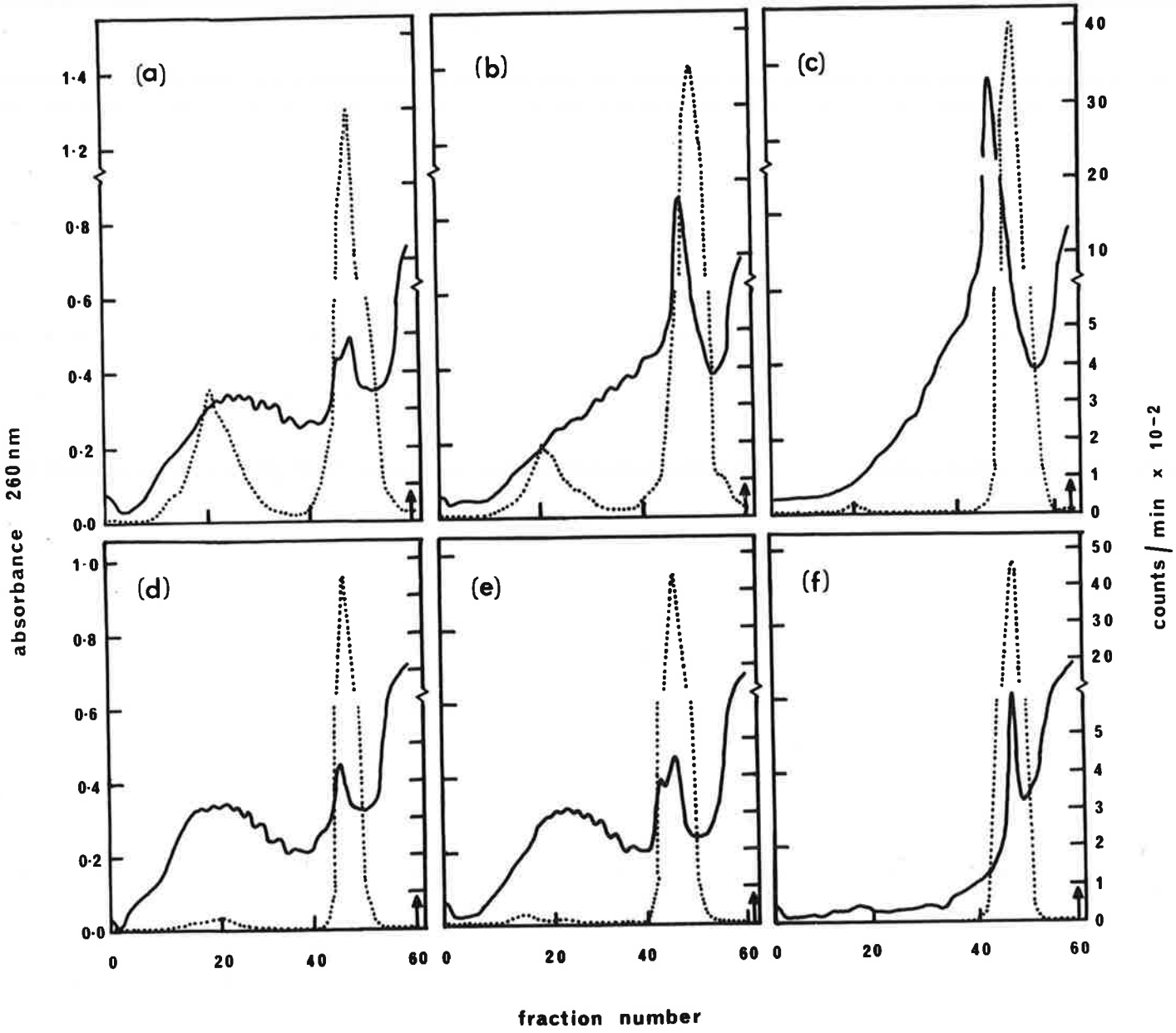
(b) The labelled subunits were added at 5 min.

(c) The labelled subunits were added at 10 min.

(d) The labelled subunits were added at zero time but the mixture was incubated at 0°.

(e) The labelled subunits were added at zero time and the incubation also contained 20 mM NaF.

(f) The incubations contained 100 µg of unlabelled 40*S* subunits (also prepared as described in Fig. 9.1) in place of the polyribosomes. The labelled subunits were added at zero time.


(a)
(b)
(c)
(d)
(e)
(f)
absorbance 260 nm
counts / min x 10-2
fraction number
0·0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
0
1
2
3
4
5
10
20
30
40
50
0
20
40
60

form larger particles.

To characterise this association further, experiments using labelled 60*S* ribosomal subunits were performed (Fig. 9.5). The 60*S* ribosomal subunits also attached to the polyribosomes after a 2 min incubation (Fig. 9.5a) suggesting the formation of complete ribosomes since the attachment of the 60*S* ribosomal subunits is considered to be the last step of initiation prior to the formation of the first peptide bond. On extended incubation, the label appeared in progressively smaller polyribosomes (Fig. 9.5b) and eventually principally in dissociated 60*S* subunits (Fig. 9.5c), which was suggestive of movement of the newly-associated ribosomes along the messenger. Thus it is likely the ribosomal subunits associated to form functional ribosomes capable of messenger translation and presumably synthesis of protein chains. $3 \cdot 10^{-3}$ M cycloheximide added 2 min after incubation with the labelled ribosomal subunits inhibited this translation process (Fig. 9.5e). $2 \cdot 10^{-2}$ M NaF added 2 min after the incubation began inhibited further initiation, but the newly-initiated ribosomes translated the messenger and appeared in small polyribosomes after a further 5 min (Fig. 9.5f).

These experiments therefore suggested that a significant degree of polypeptide initiation occurred during cell-free protein synthesis in hair follicle tissue homogenates. However, this initiation occurred predominantly on the group C polyribosomes which were postulated to be responsible for the synthesis of the LoS proteins (*see* page 94); little initiation occurred on the group B polyribosomes which presumably synthesise the HiS proteins.

D DISCUSSION

Further calculations on the activity of the cell-free systems are possible using the data of Figs. 9.4 and 9.5. From the known

FIGURE 9.5

INCUBATION OF HAIR FOLLICLE POLYRIBOSOMES WITH LABELLED 60*S* RIBOSOMAL SUBUNITS

The incubation system used in these experiments was similar to that used in Fig. 9.4 except that 50 µg of unlabelled 40*S* ribosomal subunits were added and 54 µg (8700 counts/min) samples of the labelled 60*S* subunits were added at the beginning of the incubation and were incubated for various times. Other details are as in Fig. 9.4. ——— , absorbance at 260 nm; ····· , counts/min.

(a) Incubated for 2 min.

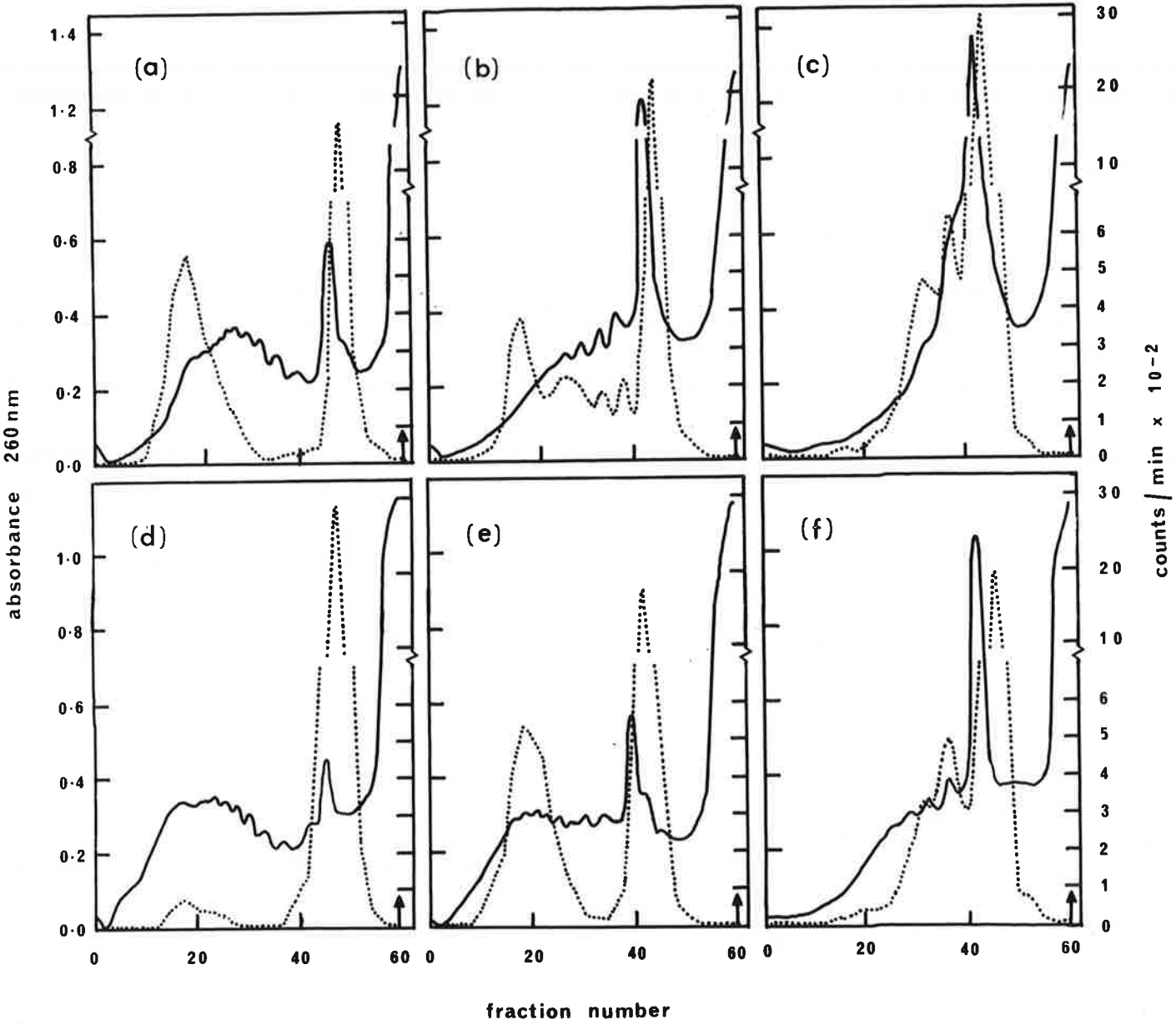
(b) Incubated for 7 min.

(c) Incubated for 12 min.

(d) Incubated at 0^{o} for 12 min.

(e) Incubated for 2 min at which time cycloheximide was added to a final concentration of $3 \cdot 10^{-3}$ M, and the incubation was continued for a further 10 min.

(f) Incubated for 2 min at which time NaF was added to a final concentration of $2 \cdot 10^{-2}$ M and the incubation was continued for a further 5 min.


(a)
(b)
(c)
(d)
(e)
(f)
absorbance 260 nm
counts / min x 10-2
fraction number
0
20
40
60
0·0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1
2
3
4
5
6
10
20
30

specific activity of the labelled ribosomal subunits and measurement of the the amount of radioactivity due to them that associated with the larger polyribosomes during the first 2 min of incubation (Figs. 9.4a and 9.5a), it is possible to calculate that approximately 14 µg of 40*S* (Fig. 9.4a) or 32 µg of 60*S* (Fig. 9.5a) ribosomal subunits attached in this time. The approximate molecular weights of the 40*S* and 60*S* ribosomal subunits are $1 \cdot 10^6$ and $2 \cdot 10^6$ daltons, respectively (Watson, 1970). Therefore, about $9 - 10 \cdot 10^{12}$ of each ribosomal subunit became attached and presumably formed functional ribosomes within the first 2 min of incubation. Similar calculations at the other times of incubation (Figs. 9.4b and 9.4c) respectively show that about $3 \cdot 10^{12}$ and $0.5 \cdot 10^{12}$ ribosomal subunits attached [a]. If it can be assumed that this decreasing rate of attachment follows an exponential decay pattern, then during the first 10 min of incubation, by which time initiation had essentially stopped (Fig. 9.4c), about $25 \cdot 10^{12}$ ribosomal subunits *in toto* had attached [a]. 250 µg of polyribosomes contain about $50 \cdot 10^{12}$ ribosomes (assuming a molecular weight value of $3 \cdot 10^6$ for an 80*S* ribosome; Watson, 1970). The polyribosomes used in these experiments varied in size from 2 ribosomes to about 20 ribosomes and the average size was probably near 10 ribosomes. Therefore, the 250 µg of polyribosomes used contained about $5 \cdot 10^{12}$ polyribosomes. From this it is seen that about 5 ribosomal subunits (or 5 functional ribosomes) became attached to or initiated to each polyribosome. These calculations show that the average number of translation cycles [b] that could have occurred on the polyribosomes with an average of 10 ribosomes in this system was about 0.5 - 1.5. This figure is likely to be closer to

---

[a] These values are likely to be underestimated as unlabelled ribosomal subunits would exist free in the incubation system by 5 and 10 min, thereby reducing the specific activity of the added labelled ribosomal subunits.

[b] A translation cycle of 1.0 is defined as the traverse of a single ribosome along the entire length of the mRNA, from initiation to termination (Kaempfer and Meselson, 1969).

1.5 since after 10 min of incubation, most of the attached subunits had dissociated from the polyribosomes (Fig. 9.5c). These calculations would therefore predict that a substantial proportion of the completed protein chains synthesised in the cell-free system were synthesised *de novo* (that is; up to one third of the total). Indeed, the values for the LoS proteins approached this order.

The method used for the quantitative estimate of initiation was not strictly accurate since only *completed* protein chains were considered. It is likely that more protein chains in fact initiated *in vitro* but were not completed and not released from the ribosomes during incubation. A more precise estimate of the degree of initiation was not possible.

Experiments showed that there was a disproportionate affinity of the ribosomal subunits for attachment to the group C polyribosomes that have been postulated to direct the synthesis of the LoS proteins (*see* page 94). This observation was unexpected and is at variance with a number of studies in other eukaryote systems (for example, reticulocytes, Howard *et al.*, 1970; and muscle, Kabat and Rich, 1969), where it was shown that *functional* ribosomal subunits rapidly equilibrated with polyribosomes of various sizes so that the relative specific activities were equal. A possible reason for this unexpected result is that some of the ribosomal subunits were not functional. However, it is considered that functional ribosomal subunits were indeed involved in the cell-free reactions used in the present experiments for the following reason. The ribosomal subunits were prepared from labelled polyribosomes that had been labelled *in vivo* with uridine for 16 h after which time label would have been equilibrated throughout the follicle. Furthermore, the experiments described in the previous chapter have clearly shown that polyribosomes which synthesised both the LoS and HiS proteins *in vitro* were present in the total polyribosome

population. Therefore subunits which had been actively involved in the synthesis of the keratin proteins *in vivo* would have been utilised in the *in vitro* experiments.

In view of the foregoing discussion, two explanations of the results obtained in this chapter are possible. The first is that the ribosomal subunits did not initiate to any significant extent with the group B polyribosomes. Since it was postulated (*see* page 94) that the group B polyribosomes direct the synthesis of the HiS proteins, this implies that the mechanism of initiation of the HiS proteins was minimally active *in vitro* and therefore possibly different from that of the LoS proteins. The alternative explanation is that the ribosomal subunits cycled only slowly through the group B polyribosomes during protein synthesis *in vitro*. This implies that the rate of translation of the HiS proteins was lower than that for the LoS proteins *in vitro*. It is also possible that both explanations apply.

Comparatively simple molecular mechanisms for both of these possibilities exist and are readily amenable to experimentation. These and their relevance to the possible control of keratin protein synthesis at post-transcriptional levels will be discussed in the next chapter of this thesis.

CHAPTER TEN

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

In highlighting the salient achievements of the work described in this thesis and discussing their significance, it is pertinent to recall those major problems to which the work was directed. These were: (a) The nature of the prekeratin proteins. Earlier studies on wool proteins had shown that there were significant differences between the keratin proteins of the presumptive cortical cells and the mature wool fibre. (b) The mechanism(s) of synthesis of the keratin proteins. That the classical ribosomal-dependent mechanism is operative in hair (and wool) follicle cells was demonstrated several years ago (*see* Chapter One, page 27), but factors such as the effect of nutrition on HiS protein synthesis and the heterogeneity of the HiS proteins led Gillespie (1965) to suggest the existence of a non-ribosomal-dependent mechanism of synthesis of some of the proteins. (c) The nature of the origin of the heterogeneity of the keratin proteins.

Obviously, these problems are inter-related and the implications of many of the findings on them will now be discussed.

Firstly, there is the question of the comparison of the hair follicle with the hair keratin proteins. All the keratin proteins of the hair follicle were present in the mature hair fibre itself and their properties from both sources appeared to be identical. Earlier work had shown that the HiS proteins (that is, proteins soluble at pH 4.4) from wool follicle tissue have a lower molecular weight and lower SCMcysteine content that the HiS proteins of the wool fibre (Rogers, 1959b; Downes *et al.*, 1966a; Fraser, 1969) and this was observed in the present work. It was shown in this work that about one third of the guinea pig hair follicle proteins soluble at pH 4.4 were clearly different from the HiS protein class. Furthermore, the present studies showed that there are major quantitative

differences because HiS proteins of hair of highest molecular weight and SCMcysteine content are present in much smaller amounts in the hair follicle extracts. Therefore, the overall lower molecular weight and SCMcysteine content of the follicle proteins compared with those of hair is due to the quantitative differences in the HiS proteins themselves and the presence of the "non-HiS" proteins but is not due to differences in the properties of the proteins.

One concludes that there are no post-synthetic modifications of the proteins before they become consolidated in the hair fibre. Since the proteins of the hair follicle are discrete entities indistinguishable from those of the keratinised hair, the suggestion of Gillespie (1965) of addition of amino acids or peptides to precursor proteins during biosynhtesis would appear to be untenable.

The second aspect thoroughly investigated in the present work was the ribosomal-dependent mechanism of protein synthesis. *Inter alia,* these studies concluded that the mechanism of protein synthesis was very similar to that established for other eukaryote systems. More specifically, during incubation in the cell-free systems established from hair follicle tissue, the polyribosomes released completed protein chains that appeared identical to the native proteins synthesised *in vivo*. All amino acid incorporation and therefore *in vitro* protein synthesis was inhibited by the addition of the established inhibitors of protein synthesis or by the ommission of components such as polyribosomes or a supernatant fraction.

From these observations it can be reasonably deduced that the LoS and HiS proteins are synthesised only by the classical ribosomal-dependent mechanism *in vivo*.

It therefore follows that the heterogeneity of the proteins does not arise from unusual mechanisms of protein synthesis. It is more likely

that a very large number of genes exist for the LoS and HiS proteins and that other intracellular effects regulate the expression of these genes. The multiplicity of genes might have arisen by extensive duplication of ancestral genes followed by subsequent mutations, deletions and insertions by various chromosomal events. Indeed, some evidence for the occurrence of these processes has appeared recently from the amino acid sequence analyses of Lindley *et al.* (1971), Haylett *et al.* (1971) and Ellerman (1971). For example, Ellerman (1971) has shown that a HiS protein from wool contains a triple repeat of a decapeptide sequence and this strongly suggests that duplication of at least a part of an ancestral gene has occurred.

Accordingly, the other problems of (1) the quantitative differences in the rates of synthesis of the LoS and HiS proteins and in the subgroups of HiS proteins themselves; (2) the temporal sequence of synthesis of the LoS and HiS proteins; and (3) the dietary regulatory effects, could be adequately accounted for by regulation of gene expression at transcriptional or post-transcriptional levels by the processes encountered in other eukaryote systems and summarised in Chapter One.

The observations of Chapter Five on the cortical granules suggested that synthesis of HiS proteins begins well after LoS protein synthesis begins. Evidence was cited earlier (*see* Chapter One, page 27) that mRNA synthesis occurs at a very early stage of development in the keratogenous zone of the hair follicle. Because of the long delay in appearance of the HiS proteins, this implies that restriction of the expression of the mRNA for the HiS proteins occurs and this in turn suggests regulation of protein synthesis at a post-transcriptional level.

The development of the highly active cell-free protein synthesis systems in this work afforded an opportunity to investigate possible post-transcriptional levels of control.

The experiments described in Chapter Nine which measured the degree of *de novo* protein synthesis and rates of ribosomal - polyribosomal recycling showed marked differences between the HiS and LoS proteins. These observations were interpreted in two ways: (1) the mechanism of initiation of the HiS proteins is different from that of the LoS proteins; or (2) the rate of translation of the mRNA for the HiS proteins is less than that for the mRNA for the LoS proteins *in vitro*. Ilan and Ilan (1971) showed that different initiation factors appear at different stages of insect pupation, and in the hair follicle, HiS proteins may be synthesised only after the appearance of specific initiation factors which are different from those of the LoS proteins. Alternatively, a different initiating amino acyl-tRNA species may appear as has been suggested by Rogers and Kemp (in Fraser *et al.*, 1972). Thus a different initiation mechanism for the LoS and HiS proteins is teleologically attractive since it would provide a means by which the temporal synthesis of the proteins could be regulated.

The most likely mechanism by which differences in the rates of translation of the LoS and HiS proteins could be effected is by the limitation of a component necessary for translation of the mRNA. Since the HiS proteins contain extra-ordinarily high levels of cysteine (up to 27 moles percent), regulation of the level of cysteinyl-tRNA offers an immediate possibility. If the pool of cysteinyl-tRNA or its rate of formation is rate-limiting *in vitro* then not only would the rate of synthesis of the HiS proteins be lower than that of the LoS proteins, but also the HiS proteins of lower cysteine content would be synthesised *in vitro* in comparatively larger amounts that the proteins of higher cysteine content. This model is also applicable to the *in vivo* situation. In dietary conditions where the intracellular level of cysteine is high (and therefore cysteinyl-tRNA is high), the HiS proteins of highest cysteine content could be synthesised in greater amounts relative to the proteins of

lower cysteine content. The reverse would apply in dietary conditions where the intracellular cysteine level is very low.

Both of these models are readily amenable to experimentation. In order to investigate the former, initiation factors could be prepared from group B and group C polyribosomes and used in the cell-free systems to establish whether the factors are different. In the second model, cysteinyl-tRNA prepared from hair follicle tissue could be added to cell-free systems to establish whether the HiS proteins of higher cysteine content can be produced in relatively larger amounts.

These experiments are now entirely feasible as a consequence of the developmental work on the cell-free protein synthesis systems reported in this thesis. Moreover, these and other similar systems could be usefully applied to further studies on the macromolecular events involved in keratin biosynthesis.

APPENDIX A

PUBLICATIONS

PAPERS PUBLISHED [a]

(a) DESCRIBING STUDIES NOT INCLUDED IN THIS THESIS

1. The Characterisation of Protein-Bound Citrulline
   (with H.W.J. Harding and G.E. Rogers)
   Biochim. Biophys. Acta, 175 (1969) 1.

2. The Isolation of Non-Keratin Protein Filaments from Inner Root Sheath Cells of the Hair Follicle
   (with P.Y. Dyer and G.E. Rogers)
   J. Invest. Dermatol., 56 (1971) 49.

(b) DESCRIBING STUDIES INCLUDED IN THIS THESIS

1. Protein Biosynthesis in Cell-free Systems Prepared from Hair Follicle Tissue of Guinea Pigs
   (with G.E. Rogers)
   Biochim. Biophys. Acta, 232 (1971a) 556.

2. The Synthesis of Hair Keratin Proteins *In Vitro*
   (with G.E. Rogers)
   Biochim. Biophys. Acta, 238 (1971b) 150.

PAPERS PRESENTED AT MEETINGS

1. The Isolation and Characterisation of Protein-Bound L-Citrulline
   (with G.E. Rogers)
   Proc. Aust. Biochem. Soc., 1 (1968) 123.

2. The Isolation of a Fibrous Non-Keratin Protein from Hair Follicle Cells
   (with G.E. Rogers and P.Y. Dyer)
   Proc. Aust. Biochem. Soc., 3 (1970) 55.

3. The Synthesis of Hair Keratin Proteins *In Vitro*
   (with G.E. Rogers)
   Proc. Aust. Biochem. Soc., 4 (1971) 64.

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[a] Reprints are enclosed at the back of this thesis

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THE SYNTHESIS OF HAIR KERATIN PROTEINS *IN VITRO*

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SUMMARY

The "low-sulphur" keratin proteins that comprise the major fibrous protein of hair have been synthesised in a reconstituted cell-free system containing polyribosomes and supernatant factors isolated from guinea pig hair follicle tissue. The evidence for this is precise co-chromatography on Sephadex and co-electrophoresis on acrylamide gels of the radioactively labelled proteins synthesised *in vitro* with the native proteins extracted from the hair follicle. Further evidence is that the labelled proteins precipitated with antisera prepared against the native hair follicle "low-sulphur" keratin proteins.

INTRODUCTION

The hair follicle synthesises several unique structural proteins that are deposited intracellularly. The largest group of proteins which constitute the major protein of hair are the keratins. This group is composed of two different types of proteins, the "low-sulphur" or microfibrillar component and the "high-sulphur" or matrix component, which together form the structurally ordered keratin complex in the hair fibre. Therefore hair follicle tissue is well suited to a study of the biosynthesis of the structural proteins and their subsequent organisation into an ordered and functional form.

Several preliminary studies have confirmed the presence of polyribosomes in hair follicle tissue[1–3], which on isolation, incorporated amino acids in cell-free systems into protein[2,4,5]. However, these studies were limited by the high levels of ribonuclease activity in the tissue homogenates[4,5]. Most recently, techniques involving very young animals for the source of tissue and buffers of high ionic strength for the preparation of homogenates containing polyribosomes have been used in which the level of ribonuclease activity was very low[6]. The polyribosomes present in these tissue homogenates incorporated amino acids into protein at a much greater rate than those of earlier experiments, and during amino acid incorporation, the polyribosomes degraded by an orderly process by run-off of the ribosomes from the messenger followed by release of the nascent protein chains. About 55 % of the total acid-insoluble radioactivity incorporated during incubation was released from the ribosomes[6]. It is shown in the present work that the radioactively labelled protein molecules that are released are identical to the native hair follicle proteins. The "low-sulphur" keratin proteins have been used for this demonstration since they are most readily characterised.

MATERIALS AND METHODS

[4,5-$^3H_2$]Leucine of specific activity 53.9 C/mmole was obtained from Schwarz BioResearch. Dithiothreitol, cycloheximide, disodium ATP (Grade II), disodium GTP (Grade II-S) and pyruvate kinase (rabbit skeletal, Type II in 2.1 M $(NH_4)_2SO_4$ were obtained from Sigma Chemical Co. Ribonuclease-free sucrose was obtained from Mann Research Laboratories. Phosphoenolpyruvate was prepared as the dipotassium salt[7].

Preparation of hair follicle tissue components

Hair follicles from albino guinea pigs of body weight less than 150 g (age less than 3 weeks) were prepared as described previously[2,6]. Homogenates were prepared in Buffer A consisting of 0.25 M KCl, 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol and 5 % sucrose using a Dounce homogeniser[6], and centrifuged at 12 000 $\times g$ for 10 min to give the "whole tissue homogenate". During homogenisation, some native sulphydryl-rich keratin proteins of the tissue were solubilised.

Polyribosomes that were present in the whole tissue homogenate were pelleted through 2 M sucrose in Buffer A by centrifugation at 225 000 $\times g$ for 2.5 h and resuspended in Buffer B consisting of 0.125 M KCl, 20 mM Tris–HCl (pH 7.6), 7.5 mM $MgCl_2$, 1 mM dithiothreitol and 10 % glycerol. A supernatant fraction active in cell-free protein synthesis was prepared by centrifugation of the whole tissue homogenate at 125 000 $\times g$ for 1.5 h. The middle half of this supernatant was retained and dialysed against two changes of 100 vol. of Buffer B.

In vitro cell-free protein synthesis

The *in vitro* system contained, in a volume of 4.0 ml of Buffer B, 1 mg of polyribosomes, 2 ml of the dialysed supernatant fraction, 1 mM ATP, 0.25 mM GTP, 3.5 mM phosphoenolpyruvate, 80 μg of pyruvate kinase (and 16 μmoles of $(NH_4)_2SO_4$), 200 μg of deacylated yeast tRNA[8], 20 nmoles of each amino acid excepting leucine and 75 μC (8 nmoles) of [4,5-$^3H_2$]leucine and was incubated at 37° for 90 min. The reaction was terminated by addition of cycloheximide to a final concentration of 3 mM (see ref. 6), chilled and centrifuged at 125 000 $\times g$ for 1.5 h to pellet the ribosomal material. The supernatant containing the labelled proteins was retained.

Preparation of the radioactively labelled "low-sulphur" keratin protein fraction

The procedure for the isolation of the keratin proteins from guinea pig hair follicle tissue involved extraction with buffers containing 8 M urea and 0.1 M 2-mercaptoethanol, followed by alkylation of the sulphydryl-rich keratin proteins with an excess of iodoacetate[9–11]. This extracted the hair follicle (F) proteins as their stable *S*-carboxymethyl kerateine (SCMK) derivatives. Accordingly, the supernatant obtained from the cell-free incubation containing the labelled proteins synthesised *in vitro* and the native sulphydryl-rich keratin proteins was made to 8 M urea, 0.1 M 2-mercaptoethanol and 0.1 M Tris–HCl (pH 9.0) and mixed for 30 min to ensure complete reduction of all sulphydryl groups. Iodoacetic acid dissolved in 3 M Tris–HCl (pH 9.0) (200 mg/ml) was added to a final concentration of 0.15 M and mixed

for a further 30 min to effect complete alkylation of the sulphydryl groups, and the solution was finally dialysed exhaustively against water. These dialysed proteins were mixed with an additional 25 mg of native guinea pig F-SCMK proteins. The pH was lowered with 0.2 M acetate buffer (pH 4.4) and the fraction (A) insoluble at pH 4.4 was collected. This labelled F-SCMK-A fraction (about 20 mg) possessed a specific activity of 30 counts/min per μg.

Sephadex chromatography

Sephadex G-200 (medium) was washed in dry ether to remove fines[12], equilibrated in water for 1 week and then in buffer of 8 M urea, 0.2 M KCl, 50 mM Tris–HCl (pH 7.6) and 1 mM EDTA.

Acrylamide gel electrophoresis

Electrophoresis[13] was conducted on 7.5 % acrylamide gels at pH 9 in the presence of 5 M urea but the usual cross-linking reagent (*N,N'*-methylenebisacrylamide) was replaced by *N,N'*-diallyltartardiamide[14].

Preparation of rabbit antisera

Antisera to chicken *S*-carboxymethyl feather keratin and the native guinea pig F-SCMK-A proteins were prepared as described elsewhere[11].

RESULTS

The F-SCMK-A protein fraction contains about 70 % of the total hair follicle proteins and comprises the main "low-sulphur" keratin proteins that become the microfibrillar moeity of the hair fibre. These proteins have a molecular weight of about 43 000 and when separated on acrylamide gels appear as 3 distinct bands[9]. The proteins are also antigenic[11]. These properties of the proteins formed the basis of the characterisation of the proteins synthesised in the cell-free system in this work.

Chromatography on Sephadex G-200

When the labelled F-SCMK-A proteins were chromatographed on Sephadex G-200, the elution profile shown in Fig. 1a was obtained. The keratin proteins have a marked propensity for aggregation both with themselves and with other highly charged protein molecules[9,15], even in the presence of strong dissociating reagents. These aggregates and other contaminating low molecular weight proteins can be removed from the main 43 000 molecular weight protein species by repeated rechromatography on Sephadex[9,15], and after one recycle the elution profile of Fig. 1b was obtained in which most contamination had been removed. The radioactivity profile co-chromatographed precisely with the optical density profile. If incompleted labelled protein molecules were present, they would be expected to have a lower molecular weight than the native proteins.

Acrylamide gel electrophoresis

Samples of the labelled protein purified as shown in Fig. 1b were subjected to electrophoresis on acrylamide gels. Determination of the optical density of the stain

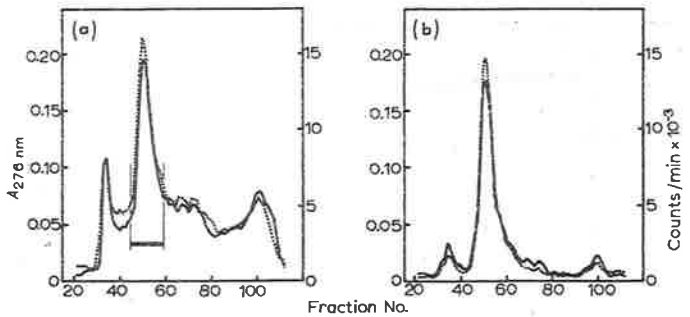

Fig. 1. Chromatography of labelled proteins synthesised *in vitro* on Sephadex G-200. A column 165 cm × 1.6 cm was prepared and loaded with a 3-ml sample containing 15 mg (450 000 counts/min) of labelled F-SCMK-A protein. The absorbance of the 3.0-ml fractions was measured at 276 nm and a portion of each fraction was mixed with 1 mg of carrier guinea pig F-SCMK protein and treated with 10 % trichloroacetic acid. Acid insoluble material was collected onto glass fibre circles for counting. ———, absorbance; · · ·, radioactivity. a. Chromatography of the labelled F-SCMK-A protein. The bar represents the tubes that were pooled for re-chromatography in b.

as well as the counts/min of radioactivity in each gel slice provided unambiguous comparison of the two parameters. As shown in Fig. 2, radioactivity co-electrophoresed precisely with the main protein bands. If incompleted labelled protein molecules were present they would be expected to migrate more rapidly than the native proteins.

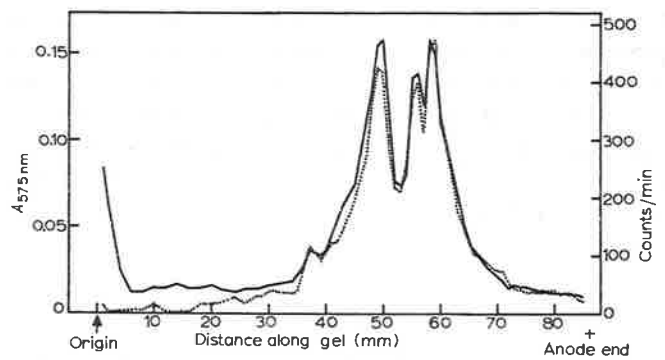

Fig. 2. Acrylamide gel electrophoresis of labelled proteins synthesised *in vitro*. A 0.3-mg (9000 counts/min) sample of the labelled protein obtained from Fig. 1b was electrophoresed on the gel at 3 mA for 4 h. The gel was then stained with 0.1 % coomassie brilliant blue in 10 % trichloroacetic acid for 2 days. Excess stain was removed with 50 % aq. ethanol and the gel was frozen and sliced into 1.0-mm lengths. Each slice was dissolved in 2 % periodic acid[14] (2.0 ml) in 30 min at 23° and the absorbance of the stain determined at 575 nm. The solution was mixed with 1 mg of carrier F-SCMK protein, treated with 10 % trichloroacetic acid and acid insoluble material was collected onto glass fibre circles for counting. ———, absorbance; · · ·, radioactivity.

Immunoprecipitation experiments

Experiments on the reaction of the column-purified labelled protein with various antisera are demonstrated in Fig. 3. The labelled protein reacted chiefly with the F-SCMK-A antiserum (Fig. 3a). A low degree of cross-reaction of the

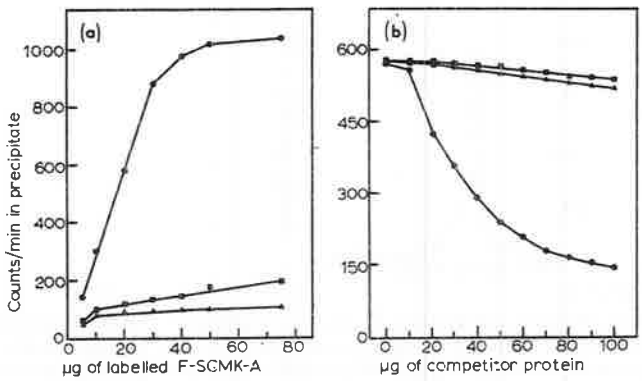

Fig. 3. Reaction of labelled protein synthesised *in vitro* with antisera. a. Precipitation. Samples contained 0.25 ml of normal rabbit serum (▲–▲), or sera prepared against the native guinea pig F-SCMK-A proteins (●–●) or chicken *S*-carboxymethyl feather keratin protein (■–■) and increasing amounts of the labelled F-SCMK-A protein purified as described in Fig. 1b in 1.0 ml of 0.14 M NaCl–10 mM phosphate buffer (pH 7.1). The samples were incubated at 37° for 2 h and then at 2° for 18 h. Precipitated material was collected by centrifugation and filtered onto glass fibre circles for counting. b. Precipitation with competition. Samples contained 20 µg (600 counts/min) of the purified labelled F-SCMK-A protein in 1.0 ml of the NaCl–phosphate buffer used in a and varying amounts of one of the competitor proteins, *S*-carboxymethyl ovalbumin (▲–▲), chicken *S*-carboxymethyl feather keratin protein (■–■) and native guinea pig F-SCMK-A protein that had been purified on Sephadex G-200 (●–●). Precipitated material that formed on incubation was collected for counting as in a.

chicken *S*-carboxymethyl feather keratin protein antiserum was also evident, but this may have been due to cross-reaction with the *S*-carboxymethyl groups on the two different types of keratin proteins[11,16]. In addition, only native unlabelled guinea pig F-SCMK-A protein (which had also been purified on Sephadex G-200) competed with the labelled protein for reaction with the F-SCMK-A antiserum (Fig. 3b). Neither *S*-carboxymethyl ovalbumin (which has a similar molecular weight and charge to the F-SCMK-A proteins) nor chicken *S*-carboxymethyl feather keratin protein competed significantly with the antiserum.

DISCUSSION

The experiments reported in the present work have demonstrated that a reconstituted *in vitro* protein synthesis system derived from guinea pig hair follicle tissue will synthesise completed "low-sulphur" keratin protein molecules that appear identical to the native proteins synthesised *in vivo*. Three criteria based on different properties of the proteins, namely, molecular size, molecular charge and antigenic properties, are provided as evidence for this assertion. Another criterion of identity, coincidence of radioactivity with peptides derived from the labelled proteins, has not been established at this time.

It was shown previously[6] that about 30 % of the total acid-insoluble radioactivity incorporated during cell-free protein synthesis was due to the elongation of chains newly initiated *in vitro*. While it is clear from these experiments that pre-existing nascent protein chains must have been completed *in vitro*, it is not known whether newly initiated chains were also completed *in vitro*.

The guinea pig hair follicle synthesises several other important structural proteins in addition to the "low-sulphur" keratin proteins described above. The "high-sulphur" keratin proteins which become the matrix of the hair fibre are, like the "high-sulphur" proteins of wool[17], very heterogeneous in both molecular size and charge. Nothing is known of the mechanism of synthesis of these proteins or of the origin of their extensive heterogeneity. The other major structural proteins synthesised in the hair follicle are those of the inner root sheath and medulla. Both of these contain the amino acid citrulline[18] and both are extensively cross-linked by the γ-isopeptide link, ε-(γ-glutamyl)lysine[19] but little information is available on their biosynthesis. Therefore, by using an active cell-free protein synthesis system similar to that in the present work which will permit the synthesis of completed protein molecules, it should be possible to investigate aspects of the mechanism of biosynthesis of these various types of proteins of the hair follicle.

ACKNOWLEDGEMENTS

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THE CHARACTERISATION OF PROTEIN-BOUND CITRULLINE

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SUMMARY

1. The amino acid citrulline, generally not found as a constituent of proteins, has been isolated and purified from the medulla protein of the quill of the African porcupine and from the inner root sheath protein of the hair follicle of the guinea pig.

2. The properties of the amino acid isolated from the two sources were found to be identical to those of L-citrulline. The identity of the amino acid has therefore been unequivocally established.

3. The proteins of the medulla and of the inner root sheath isolated as tryptic polypeptides contain, respectively, 760 and 235 μmoles of citrulline per g.

4. Citrulline has been released almost completely from the proteins by digestion with subtilisin. Furthermore, limited amino acid sequences containing citrulline have been determined in purified peptides released by combined tryptic and peptic digestion of the proteins. Earlier indications that citrulline is covalently bound in peptide linkage in the proteins have been confirmed.

5. The possible origins of the citrulline in the proteins are briefly discussed.

INTRODUCTION

Citrulline is widely distributed as a free amino acid in plants and animals but it has not been found as a general constituent of proteins. The occurrence of citrulline in protein and polypeptide material has been reported[1–6], but these reports have either not been fully substantiated or must be considered unlikely in view of the more recent studies on the amino acid composition of many proteins.

An apparently unique occurrence of citrulline in proteins was established when ROGERS[7] isolated inner root sheath material from hair follicles and demonstrated substantial quantities of this amino acid in the cell-proteins of this tissue. Citrulline has also been shown to be present in the protein (or proteins) that occur in the cells of the central canal or medulla of hair fibres of several species[8] and of modified hairs, namely the quills of the porcupine[9].

Abbreviations: DNS, dansyl-group or 1-dimethylamino-5-naphthalenesulfonyl-group; PTH, phenylthiohydantoin.

The inner root sheath and medulla proteins, like keratins, are insoluble in protein solvents but they are dissimilar from keratins in being readily hydrolysed by trypsin and contain only trace amounts of cystine. Several experiments have already indicated[7–12] that the citrulline present in these proteins is covalently bound in high-molecular-weight polypeptides and not merely adsorbed to the proteins. More recently, citrulline has been detected in the protein of the cuticle layer of the hair fibre[13].

Before an amino acid can be accepted as a new constituent of a protein, the evidence for its occurrence should meet certain criteria[14]. In earlier work, insufficient quantities of protein were isolated to permit full characterisation of the citrulline[8–10,15]. It is the purpose of this paper to report the isolation and characterisation of citrulline and the establishment of citrulline residues in amino acid sequences of particular peptides isolated from the cell-proteins of both inner root sheath and medullary cells.

MATERIALS AND METHODS

The source of proteins containing citrulline

A protein or group of proteins containing this amino acid has not been isolated intact from either inner root sheath or medullary tissue nor is it known how many protein species containing it might be present in the cells of these tissues. Consequently, investigations of the chemistry of the proteins concerned were performed on the whole tissues (inner root sheath or medulla). Alternatively, the proteins were quantitatively removed by digestion with trypsin and separated from the cell debris and keratin contamination. This proteolytic procedure reduces the proteins to soluble polypeptides which still contain all of the protein-bound citrulline.

In the present paper where reference is made to 'inner root sheath protein' or 'medulla protein' this does not connote isolation of the 'native' protein or proteins from them.

Isolation of inner root sheaths and medulla tissue

Hair follicles of the guinea pig (*Cavia porcellus*) were exposed by the wax–sheet method[9] and the sheaths removed according to the established procedure[10]. The medulla was dissected from quills of the African porcupine (*Hystrix cristata*) with a sharp scalpel, ground in a Wiley mill to through-40 mesh size and washed with water and organic solvents to remove ninhydrin-positive material and lipid.

Isolation of citrulline

L-Citrulline (Calbiochem) was used as a standard throughout.

Separation of citrulline from protein hydrolysates. The protein contained in the inner root sheath and medulla material was removed as polypeptides by digestion with crystalline trypsin (Mann Research Lab.; minimal chymotrypsin content). Digestion was conducted at 37° in 0.01 M NH_4HCO_3 (pH 8.3) using an enzyme : protein ratio of 1 : 100. Digestion was terminated at 3 h by freeze-drying the supernatant after centrifuging at 5000 × *g*.

The water-soluble tryptic peptides derived from the medulla and inner root sheath preparations were hydrolysed by refluxing in glass-distilled constant-boiling HCl. The hydrolysis time for the isolation of citrulline was 12 h to minimise destruction

of the amino acid. A protein to acid ratio of 1:200 (w/v) was used. Acid was removed by passing the hydrolysate through a short column of Dowex 50 X4 (H^+ form).

Citrulline was separated from the mixture of amino acids according to HIRS, MOORE AND STEIN[16]. The amino acids were adsorbed on a column of Dowex 1 X8 (acetate form). The fractions containing the neutral and basic amino acids were pooled and then applied to a column of Dowex 50 X4 (H^+ form). Separation of the mixture was initiated at 20° with 1.00 M HCl and completed at 30° using a linear acid gradient between 1.00 and 2.50 M HCl. Citrulline was eluted at an acid concentration of about 2.3 M HCl and was recovered from the acid by adsorption on a small column of Dowex 50 X4 (H^+ form) and eluted with 0.5 M pyridine–acetate buffer at pH 5.3.

Amino acids in fractions from the ion-exchange columns were determined by the procedure of YEMM AND COCKING[17]; citrulline was estimated quantitatively according to ARCHIBALD[18] or the modification of MCLEAN, NOVELLO AND GURNEY[19]. Assays of acid hydrolysates of both free and protein-bound citrulline showed a potentiation of colour by approx. 50% but the factor responsible has not been identified. The extent of potentiation was determined from standard amounts of citrulline to which had been added optimal amounts of a 120-h hydrolysate of citrulline in which this amino acid had been completely destroyed.

Purification of citrulline. Contaminating basic amino acids and some salts were removed by passing the crude citrulline through a short column of Dowex 50 X4 (NH_4^+ form) with water. Traces of contaminating salts were removed from the citrulline on a column of Sephadex G-10 using de-ionised water as eluant and an impurity of NH_4^+ detected by nesslerisation was removed by heating at 40° *in vacuo* over P_2O_5 for 2 days. The remaining impurities were removed by the formation of the copper complex[14]. The citrulline was recovered from the copper citrullinate and crystallised by evaporating to dryness over P_2O_5 in a desiccator. Traces of water from the product were removed by heating at 45° *in vacuo* over P_2O_5.

Characterisation of the isolated citrulline

Descending paper chromatography was performed using Whatman No. 1 paper and ascending thin-layer chromatography was conducted on Eastman 'Chromagram' sheets (grade K301R2). In both instances a minimum of five solvent systems[20] were applied. Amino acids were located by spraying with 0.1% (w/v) ninhydrin in ethanol. Ehrlich's reagent, 1% (w/v) *p*-dimethylaminobenzaldehyde in 1 M HCl, was used for the detection of citrulline[21].

Oxidation of the isolated citrulline samples with L-amino acid oxidase was performed according to the method of MALMSTADT AND HADJIIOANNUO[22] using an oxygen-electrode. The reaction mixture contained 1.0 μmole citrulline, 5 μg catalase (Calbiochem, B. grade) and 1 mg L-amino acid oxidase (snake venom; Worthington Chemical Corp.) in 3.40 ml of 0.1 M Tris–HCl buffer at pH 7.4. Reaction was complete in 10 min. The extent of oxidation was used to determine the configuration of the isolated citrulline.

Proteolytic release of citrulline from citrulline-proteins

The medulla and inner root sheath tissue and tryptic peptides derived from them, were hydrolysed with subtilisin (Nutritional Biochemicals Corp.) at an enzyme: protein ratio of 1:100 in 0.01 M ammonium acetate at pH 7.4 for 7 h at 37°. The ci-

trulline released was collected by adsorption on Dowex 50 X8 (H^+ form) and quantitatively determined.

Sequence of citrulline peptides

Citrulline-containing peptides were prepared from the proteins of porcupine quill medulla by the action of crystalline trypsin[8]. The tryptic peptides, precipitated by adjusting the pH to 3.5 ('tryptic core') were further digested with pepsin (Sigma, 5 times crystallised) using an enzyme:protein ratio of 1.5:100 in 5% (v/v) aqueous formic acid at pH 2 for 24 h at 37°. The peptides in this mixture were fractionated on a column of Dowex 50 X2 with a pyridine–formic acid gradient (pH 2.6 to 9.1) and the elution of peptides was followed by ninhydrin estimations on aliquots of each fraction after alkaline hydrolysis[23]. Samples were quantitatively estimated for the presence of citrulline by the method of McLEAN, NOVELLO AND GURNEY[19]. Fractions were pooled and submitted to two-dimensional high-voltage paper electrophoresis at pH 6.5 and 3.7, and those containing a minimal number of peptides and a favourable citrulline content were studied. Peptides were purified by separate steps of one-dimensional paper electrophoresis and paper chromatography; their purity was checked by N-terminal amino acid analysis using the dansyl (DNS) technique of GRAY AND HARTLEY[24].

Amino acid analyses of the peptides were carried out using an automatic analyser. The ornithine that arises from the hydrolysis of the citrulline side-chain during acid hydrolysis was estimated in the analysis and the value was added to the citrulline estimate to give a 'total citrulline' value.

Amino acid sequence analyses were performed using a combination of the DNS end-group method with the stepwise Edman degradative procedure[24] using trifluoroacetic acid for cyclisation[25]. Preliminary studies on authentic citrulline and 1,9-citrulline bradykinin showed that the degradative method could be applied satisfactorily and caused only a minimal breakdown of citrulline. The phenylthiohydantoin (PTH) derivatives of asparagine and glutamine were extracted according to the method of NEDKOV AND GENOV[26] and identified by thin-layer chromatography. The DNS-derivatives of the N-terminal amino acids were identified by one-dimensional thin-layer chromatography on silica gel G layers and polyamide layers[27]. α-DNS-citrulline undergoes partial breakdown to α-DNS-ornithine during the acid hydrolysis so that spots corresponding to both these compounds were seen when citrulline residues were N-terminal.

RESULTS AND DISCUSSION

Amino acid content of the proteins

The destruction of the ureido-group of citrulline occurs under hydrolytic conditions with constant-boiling HCl, yielding ornithine, CO_2 and NH_3. Consequently, in the direct colorimetric determination of the citrulline content of the proteins it is necessary to correct for this breakdown by performing citrulline assays during the course of acid hydrolysis and extrapolate to zero time. The extrapolated values were corrected for the potentiation effect that occurs in the colorimetric analysis of hydrolysates of citrulline. The time course of digestion is shown in Fig. 1.

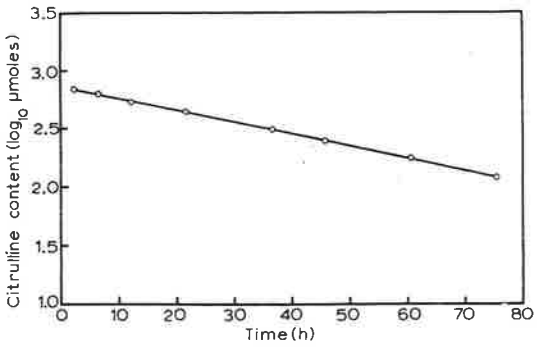

Fig. 1. The rate of hydrolytic destruction of free and bound citrulline. Authentic L-citrulline and the tryptic polypeptides of the medulla and inner root sheath preparations were separately hydrolysed (105°) at a ratio of 1:200 of protein (or equivalent quantity of L-citrulline) and constant-boiling HCl. The citrulline contents were determined at various times. Since the rates of destruction were found to be identical the results are presented as a single plot. Extrapolation to zero time yielded a recovery figure of 99.5% for the L-citrulline and enabled calculation of the citrulline contents of the two proteins.

TABLE I

AMINO ACID COMPOSITION OF THE MEDULLA AND INNER ROOT SHEATH PROTEINS

All values are expressed as μmoles/g of tryptic peptides of the tissue. Arginine, aspartic acid and the amide contents were not determined in the present work; their values presented here are taken from other studies (unpublished).

Amino acid	*Protein source*	
	African porcupine quill: medulla	*Guinea pig: inner root sheath*
Arg	260	265
Ammonia	[945]	[1160]
Asp	460	510
Ala	370	440
Citrulline	760*	235*
Cys	Trace	Trace
Glu	2625	1510
Gly	310	520
His	95	100
Ile	135	250
Leu	710	680
Lys	675	630
Orn	90	30
Met	45	160
Phe	215	220
Pro	165	245
Ser	260	505
Thr	140	225
Tyr	175	170
Val	330	345

* Corrected for citrulline breakdown.

The citrulline contents of tryptic peptides prepared from medulla and inner root sheath material determined by this procedure were respectively 760 and 235 μmoles per g (dry wt.).

The quantitative analyses of all amino acids in the proteins with the exception of arginine and aspartic acid were determined from the column chromatograms and the values are given in Table I. The similarities that are evident between the medulla and inner root sheath analyses indicate a close relationship between the proteins from the two tissues. It can be seen that the values for citrulline are identical with those obtained by the direct assays discussed above.

Isolation and properties of citrulline

Ion-exchange chromatography of acid hydrolysates of the medulla and inner root sheath protein yielded crude fractions of citrulline that required further purification. Citrulline assays showed that the purified citrulline isolated from both tissues was $> 99.5\%$ pure. The elemental analysis of it (Table II) agreed with the calculated values and with analyses performed on authentic citrulline which had been recrystallised from water.

TABLE II

ELEMENTAL COMPOSITION OF ISOLATED CITRULLINE

Analyses were performed by the Australian Microanalytical Service, C.S.I.R.O., Melbourne.

	Element (%)			
	C	*H*	*O*	*N*
Calculated for: $C_6H_{13}N_3O_3$	41.1	7.4	27.5	24.0
Observed values for citrulline from:				
Medulla	41.1	7.4	27.9	23.7
Inner root sheath	40.8	7.4	27.6	24.1
Authentic	40.9	7.6	27.2	23.6

Melting points were determined on isolated and authentic preparations of citrulline (219–220°, decomp.), their crystalline copper complexes (259–260°, decomp.) and DNP-derivatives (139–140°, decomp.). There were no significant differences in the values between the isolated and authentic materials. Furthermore, examination by paper and thin-layer chromatography showed without exception that the citrulline preparations were indistinguishable from authentic citrulline.

The infrared spectra given by the isolated material and authentic citrulline are shown in Fig. 2. No significant difference between preparations was observable.

The values of dextrorotation of citrulline isolated from the medulla and inner root sheaths were identical with the authentic material and they were all increased in the positive direction on the addition of acid, in conformity with the rule for L-amino acids[14]. In addition, the preparations of citrulline were submitted to the action of L-amino acid oxidase and the results showed that the isolated citrulline from both inner root sheath and medulla was of the L-configuration in both instances.

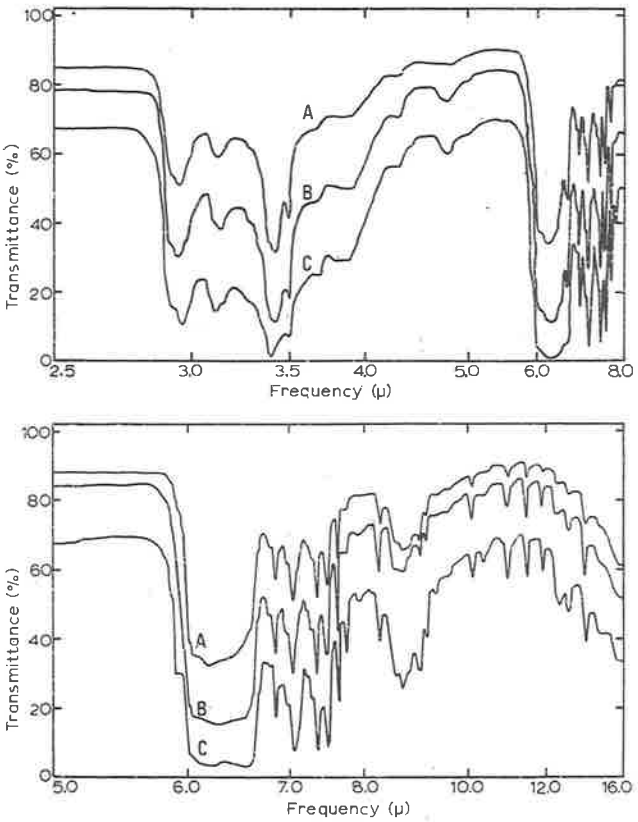

Fig. 2. Infrared spectra of citrulline. (A) authentic L-citrulline, (B) citrulline from medulla and (C) citrulline from the inner root sheath. Nujol mull; Perkin-Elmer "Infracord-237" spectrometer.

The evidence for L-citrulline in peptide linkage

The results of earlier work indicated that citrulline is covalently-bound in the proteins investigated in the present study. Thus vigorous washing techniques with a variety of solvents will not release free citrulline[9] even though the tissues themselves give intense colour reactions for this amino acid with both Ehrlich's reagent[11] and the Fearon reagent[9]. Further, the protein material in the medulla and inner root sheath tissues is remarkably resistant to extraction with most reagents, although readily accessible to proteolytic attack[7–10,12]. Moreover, it was shown that exopeptidases liberate citrulline from tryptic and peptic peptides. In the present work subtilisin was found to release free citrulline almost quantitatively from the tryptic peptides as seen in Fig. 3.

The identification of amino acid sequences containing citrulline in purified peptides (Table III) together with the foregoing evidence, firmly establish that L-citrulline occurs in peptide linkage in high molecular weight polypeptides.

The precise mechanism by which citrulline arises in the proteins is as yet unclear. It could be incorporated *de novo* but it would appear unreasonable to suppose that keratinising tissues should alone possess this capability. A more attractive hypothesis

TABLE III

N-TERMINAL AMINO ACID SEQUENCES

*Peptide**	*Sequence*
I M-TP4/6a	Asp–Cit–Phe–Cit–
I M-TP4/7d	Cit–Cit–Val–Cit–Cit–(Glu, Gln)–Val**
I M-TP6/7b	Leu–Leu–Glu–Cit–Cit–
I M-TP7/1	Phe–Cit–Glx–Glx–
II M-TP5/3b	Leu–Cit–Gln–
II M-TP10/2a	Asp–Cit–Cit–Phe–
1,9-Citrulline bradykinin	Cit–Pro–Pro–

Abbreviation: Cit, citrulline.

* The notation adopted for the peptides refers to the type of digestion used and their order of elution from the initial column chromatograms. The relationships between the peptides and to the primary structure of the protein is not known at present.

** The C-terminal valine was determined from carboxypeptidase A digestion of the peptide. The presence of the Gln residue in this peptide was deduced from the electrophoretic mobility of the DNS-peptide[30].

for which evidence has already been adduced[10] is that citrulline residues are produced by desimidation of arginine residues after their activation and incorporation[7–11,28]. A process of this kind would be unique although precedents for amino acid modification after incorporation into a polypeptide are known, such as the hydroxylation of proline during collagen biosynthesis[29]. The origin of citrulline in the proteins and the relationship of its mode of formation with the process of keratinisation is being investigated.

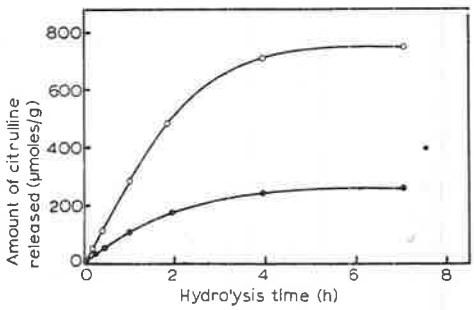

Fig. 3. Release of citrulline from the tryptic peptides of the medulla and inner root sheath proteins by subtilisin. The citrulline released from both proteins was in excess of 95% of the total after 7 h incubation. ○—○, medulla tryptic peptides; ●—●, inner root sheath tryptic peptides.

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THE ISOLATION OF NON-KERATIN PROTEIN FILAMENTS FROM INNER ROOT SHEATH CELLS OF THE HAIR FOLLICLE*

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ABSTRACT

Protein filaments obtained from the cells of the inner root sheath layers of the guinea pig hair follicle have been isolated and characterized. They are hollow tubes approximately 80 Å in diameter and are of indeterminate length. The protein of the filaments is unique in that it contains the amino acid citrulline and its amino acid composition is very similar to that of the total protein obtained from the inner root sheath cells as soluble polypeptides by digestion with crystalline trypsin.

The intracellular filaments of the inner root sheath cells are chemically and structurally quite distinct from the keratin microfibrils that are present in the neighboring cortical cells of the follicle. It is suggested that the filaments play a role in the development of cell shape in the hair follicle in a manner analogous to that which is accepted for microtubules in many types of cells.

The proteins contained in the mature inner root sheath cells of hair follicles and the medullary cells of hair fibres have been shown to contain substantial amounts of the amino acid citrulline (1–7). Since the protein contained in these tissues is highly resistant to dissolution by normal protein solvents (2), chemical studies have been performed on soluble polypeptides derived from the tissues by digestion with crystalline trypsin or pepsin. Limited sequence analyses of polypeptides derived from the proteins of these and a related tissue (the medulla of porcupine quills) indicates that the citrulline is chemically bound in the proteins by peptide linkages (3, 7).

Previous studies have indicated that the protein of the inner root sheath is composed of filaments of the α-type (5, 6), oriented in the cell in the direction of fibre growth, while the protein of the medulla is not frankly filamentous (6). In this paper we show that the protein of the inner root sheath can be isolated in the form of morphologically distinct filaments and describe their properties.

MATERIALS AND METHODS

The hair follicles of male albino guinea pigs aged one to three weeks were exposed by the wax-sheet procedure (6, 8), removed with animal clippers and suspended in a buffer of 10 mM tris-chloride pH 7.4 containing 10 mM KCl.

Isolation of mature inner root sheath tissue. The published method (5, 6) for the preparation of inner root sheaths was unsatisfactory due to the presence of hair and denatured insoluble protein. Consequently, follicles were dispersed by gentle agitation in a buffer of 8 M urea, 10 mM tris-chloride pH 7.4, 25 mM 2-mercaptoethanol at 4° for 5 min. The soluble prekeratin and other cytoplasmic proteins were alkylated at pH 9.0 by addition of solid iodoacetic acid to 50 mM until −SH negative and centrifuged at 38,000 g for 15 min. The pellet containing the mature inner root sheaths, keratinized hair and cellular debris was washed free of the solubilized proteins and urea by centrifugation at 500 g in 10 mM tris-chloride buffer pH 7.4, resuspended in 5 ml of 10% sucrose in this buffer and layered onto a 24 ml discontinuous sucrose gradient in a 30 ml cellulose-nitrate tube. The gradient employed was composed of 6 ml layers of 70, 60, 50 and 20% sucrose in the same buffer and was centrifuged at 22,500 rpm for 30 min. in a Spinco SW25.1 rotor. Only inner root sheaths banded at the 50–60% sucrose interface. The denser hair fibres banded at the 60–70% sucrose interface and the other cytoplasmic debris remained at higher levels on the gradient. The purified sheaths were examined by light microscopy using phase-contrast and polarized-light optics and were observed to be free of other particles. The sheaths were recovered and washed by centrifugation to remove sucrose.

Preparation of filaments. Filaments could be released from inner root sheaths isolated by the procedure described above. The most satisfactory method for release was limited digestion with a 0.1% solution of a proteolytic enzyme in the tris-

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chloride-KCl buffer. Four different enzymes were tested: crystalline trypsin (Sigma, Type III, 2-times crystallized), α-chymotrypsin (Worthington, 3-times crystallized), pronase (Calbiochem, B-grade) and Difco trypsin (Difco Labs., 1:250 preparation). The enzyme solutions were clarified by centrifugation at 50,000 rpm in a Spinco 50.1 rotor for 2 hr immediately before use. Digestions were performed in a "filtration apparatus" similar to that described by Kawiak *et al.* (9) for times varying up to 15 min at 20°. At the completion of the digestion the cell suspensions were chilled to 0° and the cells harvested and washed by centrifugation in the buffer at 500 g to remove enzyme. Filaments were then released from the cells by homogenization in the tris-chloride-KCl buffer in a close-fitting Dounce homogenizer (Kontes glass; clearance approximately 0.07 mm). The homogenates were centrifuged at 4,000 g for 5 min to remove cellular debris. The resultant supernates contained the inner root sheath protein filaments and were retained for further studies.

The same procedure could be applied to intact follicles thus avoiding the pre-isolation of the inner root sheaths. In these circumstances the cells of the follicle bulbs were completely digested within about 10 min; the surrounding inner root sheaths had become detached from the follicles and were disrupted into single cells which could then be readily separated in the filtration apparatus.

Trypsin digestion of inner root sheaths to polypeptides. The standard method in protein chemistry for the enzymic cleavage of proteins to polypeptides is to use purified proteolytic enzymes of known specificity. Accordingly, the procedure for the release of the *total* protein of the inner root sheath tissue was by digestion with a pure, crystalline proteolytic enzyme. As in earlier sequence studies (7), the digestions were performed using crystalline trypsin (Mann, minimal chymotrypsin content) at 37° in 10 mM NH_4HCO_3 (pH 8.3) using an enzyme:tissue ratio of 1:100. The reaction was terminated after 3 hr by freeze-drying the supernate obtained by centrifugation for 5 min at 4,000 g.

Amino acid analysis. Samples for amino acid analysis were hydrolyzed for 28 hr in constant-boiling HCl at 110°, freed of HCl by evaporation and analyzed in a Technicon amino acid analyser. Filament preparations were dialyzed for 4 hr against water and then freeze-dried before hydrolysis.

Electron microscopy. Specimens for electron microscopy were examined in a Siemens Elmiskop I electron microscope. Homogenate preparations were examined after negative-staining with 2% uranyl acetate. Freshly depilated guinea pig hair follicles were fixed in 2% glutaraldehyde, post-fixed in 1% osmic acid, dehydrated in acetone and embedded in araldite by standard procedures. Sections were stained on the grid for 90 min with 1% potassium permanganate.

RESULTS

Fine structure of filaments in situ. A transverse cross-section through the inner root sheath layer of a guinea pig hair follicle is shown in Fig. 1a at a stage when the Henle layer of the sheath has become a hardened rigid structure. Filaments appear as hollow tubes about 80 Å in diameter. Fig. 1b shows a transverse cross-section through the cortical region of the follicle at the same level. Keratin microfibrils approximately 80 Å in diameter can be seen. They are clearly different in their staining properties. Their "cores" and the interfilamentous regions (matrix) are more densely stained than the inner root sheath filaments.

Isolation of filaments. In earlier studies (2) attempts to release the fibrous protein material of the inner root sheath by a variety of techniques were not successful. However, it had been noted that during proteolytic digestion of the inner root sheaths (to release the protein as soluble polypeptides), the sheaths were initially disrupted into single highly-birefringent cells, the fibrous contents of which were removed only after longer digestion. We have found that homogenization of the cells produced during the early stages of this digestion releases the protein filaments.

Homogenization of sheaths in buffer before enzyme treatment did not produce filaments, but instead, large membrane-bound clumps of fibrous material. Moreover, homogenates of intact hair follicles prepared in the tris-chloride buffer did not show the presence of filaments. Of the proteolytic enzymes tested, the method employing digestion with Difco trypsin, for approximately 15 min at 20° on intact follicles or purified inner root sheaths gave the highest yields of filaments per unit weight of starting material.

A typical preparation of filaments negatively-stained by uranyl acetate is shown in Fig. 2a. These filaments were prepared from whole guinea pig hair follicle tissue by the method described. The filaments are approximately 80 Å in width, several microns in length and appear to have a distinct "core" throughout their length (Fig. 2b).

It was determined by electron microscopy using a variety of negative-staining techniques, that such preparations of filaments were almost completely devoid of other cytoplasmic particles

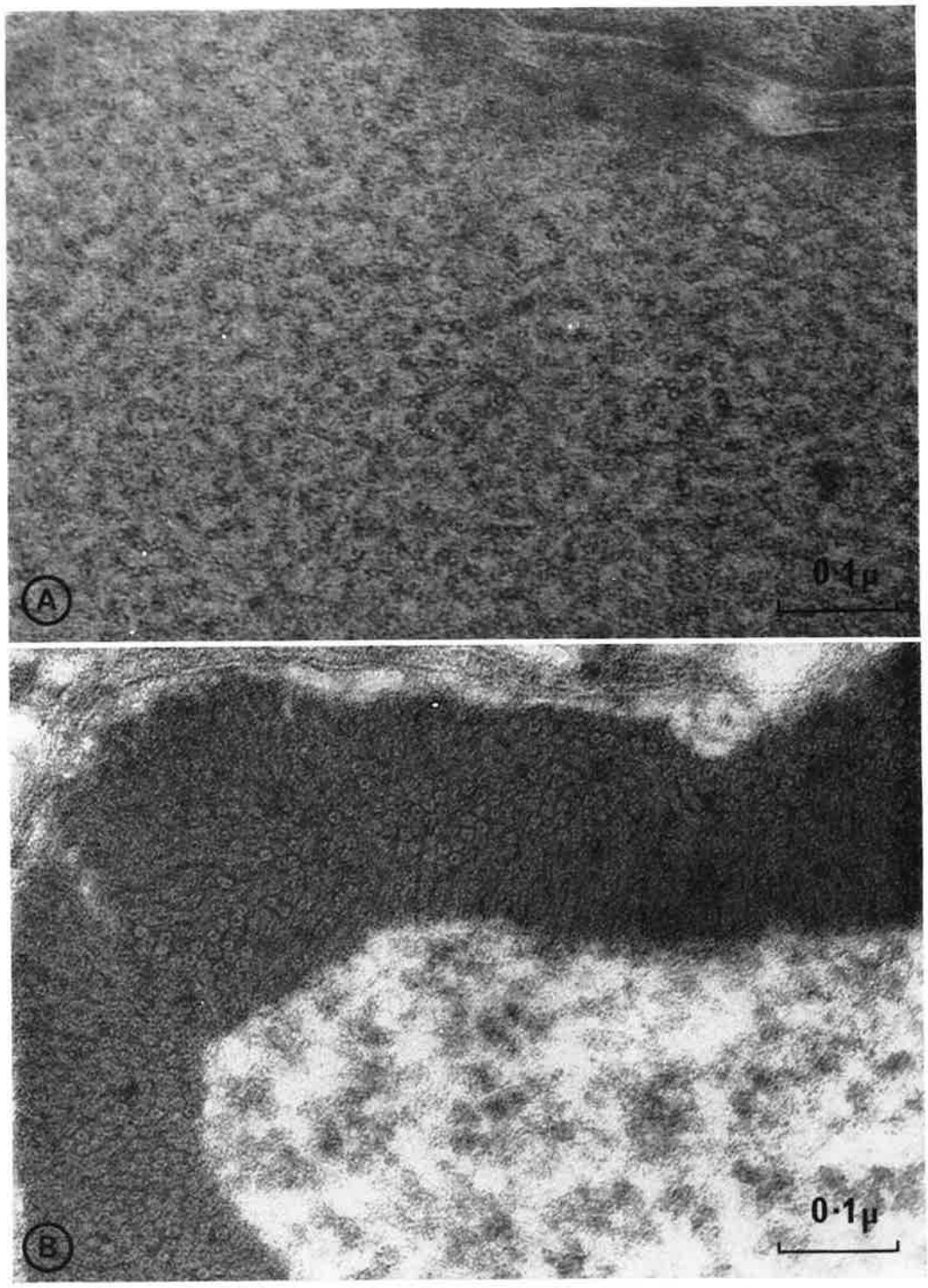

FIG. 1. Transverse cross-section through a guinea pig hair follicle. (a) Cross-section through a cell of the Henle layer of the inner root sheath showing the hollow tubular structure of the filaments. The filaments are approximately 80 A in diameter. The inter-filamentous regions are of very low electron density. (b) Cross-section through the cortical region of the follicle at the same level. Each microfibril is approximately 80 A in diameter and has a densely-stained core and the microfibrils are surrounded by a densely-staining matrix. Specimens prepared as described in Materials and Methods. × 180,000.

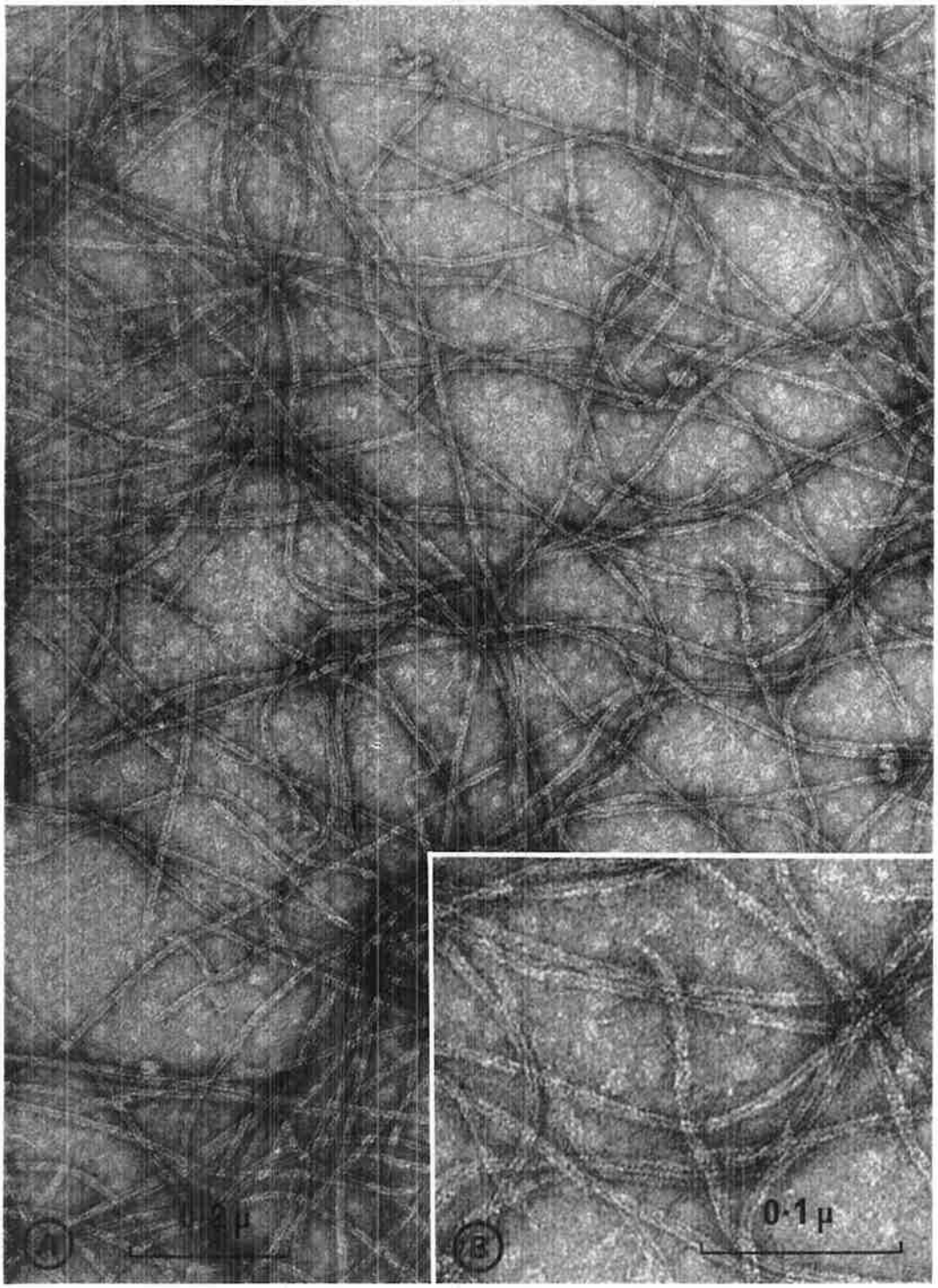

FIG. 2. Inner root sheath filaments prepared from whole guinea pig follicle tissue by digestion with Difco trypsin for 15 min at 20°. The majority of the filaments shown here are about 80 A in diameter and are more than 1 micron long. They show a dense core throughout their length which appears to constitute one third of their diameter. Negatively-stained with 2% uranyl acetate. (a) × 120,000. (b) × 210,000.

and were considered to be sufficiently pure for further studies.

Amino acid analyses. It can be seen from the Table that the amino acid composition of the inner root sheath filament preparations and the low sulfur keratin proteins (H-SCMK-A) extracted from guinea pig hair are completely different (columns 1–3 cf column 4). Comparison of the inner root sheath filament preparations with the low sulfur keratin proteins, instead of the total keratin proteins of hair (H-SCMK), is justified as this fraction is thought to derive from the microfibrillar moiety of the original hair fibre (10). Thus the inner root sheath filaments are chemically as well as structurally unlike those of keratin.

The analyses of the filaments are quite similar to the analyses of the tryptic polypeptides derived from the *total* proteins of the inner root sheath. There are, however, four amino acids which vary significantly; in the filaments the content of citrulline is higher and the contents of tyrosine, phenylalanine and lysine are lower than those in the tryptic peptides of whole inner root sheaths. This suggests the presence in whole inner root sheaths of other protein species that do not contain citrulline and which are absent from the filament preparations.

TABLE

Amino acid analyses of the protein samples

(All values are expressed as moles percent and represent the average of two experiments)

Amino acid	Filaments isolated from inner root sheaths	Tryptic polypeptides derived from inner root sheaths	H-SCMK-A*
SCM-cysteine	0.0	0.0	5.3
Aspartic acid	10.2	9.4	8.7
Threonine	3.2	3.0	4.2
Serine	6.5	5.8	6.4
Glutamic acid	24.0	22.5	16.7
Proline	4.1	3.5	3.5
Citrulline†	4.1	3.2	0.0
Glycine	7.7	7.1	4.4
Alanine	6.6	6.2	6.6
Valine	4.8	4.8	5.8
Half-cystine	0.7	0.7	0.0
Methionine	2.3	2.1	0.4
Isoleucine	3.4	3.2	3.5
Leucine	9.8	9.1	10.0
Tyrosine	1.9	2.7	1.9
Phenylalanine	1.7	3.1	1.5
Ornithine	1.4	1.0	0.0
Lysine	4.3	8.8	3.7
Histidine	1.4	1.4	0.9
Arginine	3.6	3.7	7.0
% Recovery of protein material hydrolyzed as amino acid	74	78	93

* From unpublished studies. The protein was extracted from guinea pig hair (H) by reduction and then alkylation with iodoacetic acid to give the S-carboxymethyl kerateine (SCMK) derivative in which the half-cystine residues have been converted to SCM-cysteine residues. Fraction A is prepared by precipitation at pH 5.0. This kerateine fraction (H-SCMK-A) is lower in SCM-cysteine content than the total hair keratin proteins (H-SCMK) and is believed to derive from the microfibrils of the original hair fibre (10).

† During acid hydrolysis citrulline undergoes partial decomposition to ornithine. The citrulline value given is the sum of the citrulline remaining after 28 hr and the ornithine produced during this time.

DISCUSSION

General properties. It has been recognized for some time from electron microscope studies that the cells of the mature inner root sheath mainly contain oriented filaments about 80 Å in diameter (5, 6). Previous studies on the total proteins derived from the tissue have shown the presence of the amino acid citrulline (1–7). However, it was not known whether the citrulline was located in the filaments or in some other protein present in the inner root sheath cells. Attempts to isolate the fibrous protein or an intact protein species had not been successful. Consequently, previous chemical studies were performed on soluble polypeptides derived from the tissue which was the only known means of removing the protein content from the cells. In contrast, the release of the filaments from the inner root sheath cells as described in the present work has been achieved by employing a short period of proteolytic digestion. The present isolation from the inner root sheath cells of morphologically distinct protein filaments which contain citrulline establishes the existence of this amino acid in the protein of the filaments and not primarily in a non-filamentous protein of these cells.

The constitution of the filaments in terms of the number of protein species that contain citrulline has not been determined. Such a deter-

mination will be difficult since recent work (H. W. J. Harding and G. E. Rogers, unpublished) has shown the presence of γ-peptide (isopeptide) links between adjacent polypeptide chains. These cross-links prevent the separation of the component chains and their dissolution by the usual solvents for proteins.

It has not been possible to establish a criterion of chemical purity for these inner root sheath filaments. Although the possibility of contamination of the filaments by other nonfibrous protein material cannot be overlooked, the amino acid analyses suggest that the extent of this contamination is minimal.

Despite the similar diameters of inner root sheath filaments and keratin microfibrils (approximately 80 Å), they differ from one another in at least two properties. Keratin microfibrils show a different affinity for the permanganate section-stain due to the presence of the sulfur-rich matrix within their cores and between their peripheries (11). Secondly, the amino acid composition of the inner root sheath filaments is entirely different from that of α-keratin. The virtual absence of cystine and the presence of citrulline are the salient features of difference.

Possible functional relationship with microtubules. The primary function of the inner root sheath is considered to be a structural one (12, 13). The cells of the sheath are thought to constrain the growing hair in the follicle and thus contribute a cooperative effect to the elongation of the cortical cells internal to them (12, 13). Further, the outward movement of the inner root sheath relative to the hair is regarded as being the cause of the flattened form of the cuticle cells by establishment of a "shearing" action (12). These morphogenic forces could be expected to be dependent upon the fibrous elements of the inner root sheath cell.

The structural elements that are prominently involved in the acquisition of cell shape during growth and development of many cells are the cytoplasmic microtubules; these structures are becoming well characterized especially in their occurrence as outer fibres of sperm tails (14) and cilia (15) and as microtubules in neurones (16–18). It is of interest that cytoplasmic microtubules are not prominent features of either developing or mature inner root sheath cells (unpublished observations of this laboratory). Thus it is suggested that the filaments of these cells replace microtubules as the determinants of cell shape and structure.

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