# FEATHER KERATIN SYNTHESIS

A thesis submitted by

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To Kath,
for her occasional help and encouragement,

and to Andrew and Ben,
although they were of no help whatsoever.

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## SUMMARY

1. Keratin proteins from adult and embryonic feathers and leg scales of the domestic fowl were characterized as their S-carboxymethyl derivatives. The use of various physico-chemical techniques for the routine analysis of such preparations was investigated. Three groups of proteins were apparent, designated the α-, β- and γ-proteins. The major (β) group of proteins appeared to consist of a heterogeneous family of related keratin proteins. The β-proteins from different tissues were shown to be different, demonstrating that in the different cell lines which originate from embryonic epidermis, different sets of genes for keratin proteins are expressed.

2. N-terminal sequence studies showed that the N-termini of embryonic feather keratin proteins were acetylated. A series of N-terminal peptides of increasing length was prepared from embryonic feather proteins and the homologous N-terminal amino-acid sequences Ac-Ser-SCMC-$\begin{matrix}\text{Phe}\\\text{Tyr}\end{matrix}$-Asn were established, demonstrating that feather keratins are a family of homologous proteins.

3. Quantitative polyacrylamide gel electrophoretic techniques were applied to the study of the kinetics of keratin synthesis during development of the embryonic feather. The major keratin proteins were detectable by 12 days and attained a maximal rate of synthesis after 13 days. The proteins were

synthesized in a coordinated fashion, in contradiction to the two-phase synthesis proposed by earlier workers.

4. Embryonic feathers maintained in culture medium for short periods incorporated $^{14}$C-leucine into proteins. The spectrum of radioactive proteins was dependent on the age of the tissue. The rate of keratin synthesis was low in 12 day tissue and maximal in 14 day tissue, in agreement with the results from quantitative gel electrophoresis.

5. Electron-microscopic autoradiography indicated that individual cells lose the ability to synthesize DNA before keratin becomes detectable but retain the ability to synthesize RNA after keratin becomes detectable.

6. The major protein products of a cell-free system from 14-day feathers (developed in this laboratory by G.A. Partington) were shown to be complete keratin molecules by several criteria. Approximately 50% of the protein molecules which were labelled in the system were shown to have been initiated in the system. These newly initiated molecules were acetylated in the system. Acetyl-CoA was able to act as the acetyl donor. Some evidence was obtained indicating the presence of a mechanism capable of acetylating chemically de-acetylated keratin molecules, suggesting that acetylation occurs at the post-translational level.

## 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:

DAVID J. KEMP

## ACKNOWLEDGEMENTS

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I am also indebted to Drs. R.D.B. Fraser and E. Suzucki of the C.S.I.R.O., Division of Protein Chemistry, Melbourne, for assistance with least-squares analysis, Dr. J.C. Wallace of this Department and Mr. T. Nettelbeck of the Department of Psychology, University of Adelaide for assistance with statistical procedures, and Mr. I.J. O'Donnell of the C.S.I.R.O., Division of Protein Chemistry, Melbourne, for his communication of unpublished results.

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## ABBREVIATIONS

The following abbreviations are used in this thesis. Each abbreviation used is defined in the text once before general use.

| | |
|---|---|
| SCMC | S-carboxymethyl-cysteine |
| MW | molecular weight |
| PAGE | polyacrylamide gel electrophoresis |
| HVPE | high voltage paper electrophoresis |
| PMA | pyridine/N-ethyl morpholine/acetate buffer |
| NEMA | N-ethylmorpholine/acetate buffer |
| PCA | pyrrolidine carboxylic acid |
| TCA | trichloroacetic acid |
| dansyl- or DNS- | 1-dimethylaminonapthalene -5-sulphonyl chloride |
| EGF | epidermal growth factor |

# CHAPTER 1

# GENERAL INTRODUCTION

## A. INTRODUCTORY COMMENTS

During the course of development of the embryonic chick, embryonic feathers first grow and then fill with the intracellular fibrous protein complex, keratin. The general aim of the work described in this thesis was to proceed towards the eventual understanding of the molecular mechanisms involved in the synthesis and the control of the synthesis of the keratin proteins.

In the ensuing discussion, background information relevant to the study of keratin synthesis in general and necessary for the evaluation of the present work is presented.

## B. FEATHER STRUCTURE AND DEVELOPMENT

### 1. *Structure of Embryonic and Adult Feathers.*

#### (a) *Embryonic Feathers.*

Embryonic feathers, otherwise known as the juvenile, down or primary feathers, or *neosoptiles*, form the covering of the newly hatched chick. The morphology of embryonic feathers has been described in many publications (see, for example, Watterson, 1942; Romanoff, 1960; Rawles, 1972), and is illustrated in Figure 1.1. They consist principally of a group of from 10 to 15 barbs, attached to a short calamus at the base. The barbs contain a central core of medulla cells, surrounded by flattened cortical cells. On the basal two-thirds of its length, each barb bears 2 rows of barbules, composed of strings of single,

FIGURE 1.1.

Structure of the embryonic chick feather.

FIGURE 1.1.a

Feather from newly hatched chick, after discarding sheath.

FIGURE 1.1.b

Diagram, illustrating details of the structure of barbs and barbules.

B: barbs

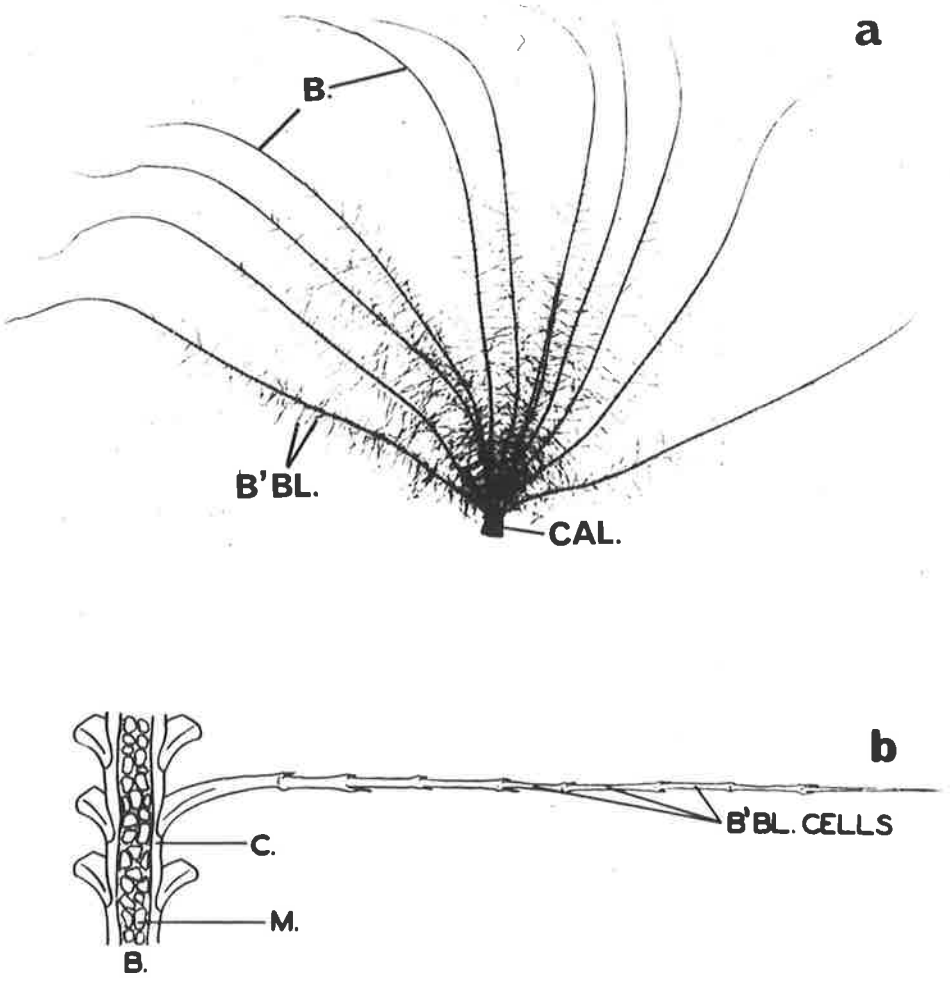
B.BL: barbules

CAL: calamus

C: cortex

M: medulla

(From Watterson, 1942).


a
B.
B'BL.
CAL.
b
B'BL. CELLS
C.
M.
B.

essentially cylindrical, barbule cells, attached end-to-end.

All these cells are dead, dehydrated and filled with keratin.

*(b) Adult Feathers.*

Adult feathers, otherwise known as permanent or definitive feathers, or *teleoptiles*, can be divided into three classes; the contour feathers (*pennae*), the down feathers (*plumae*) and the hair-like (*filoplumae*) (see Voitkevich, 1966). The present work is concerned only with the *pennae*, which henceforth will be referred to as adult feathers. The morphology of the adult feather is shown in Figure 1.2. The feather consists of a shaft, the lower, hollow, region of which is termed the calamus. Above the calamus, the shaft is termed the rachis and is filled with a "pithy" medulla. The rachis bears two rows of barbs, to which are attached barbules. The cells of all these distinct tissues are dead and filled with keratin.

## 2. *Feather Keratins*

*(a) Keratins.*

Keratins are the fibrous intracellular proteins of high cystine content produced in certain epithelial cells of higher vertebrates. The subject has been recently reviewed in some detail by Fraser *et al.* (1972). Keratins are classified into four groups on the basis of their X-ray diffraction patterns as α-, β-, feather or amorphous. The four groups differ in the molecular structure of the

FIGURE 1.2.

Structure of the adult feather.

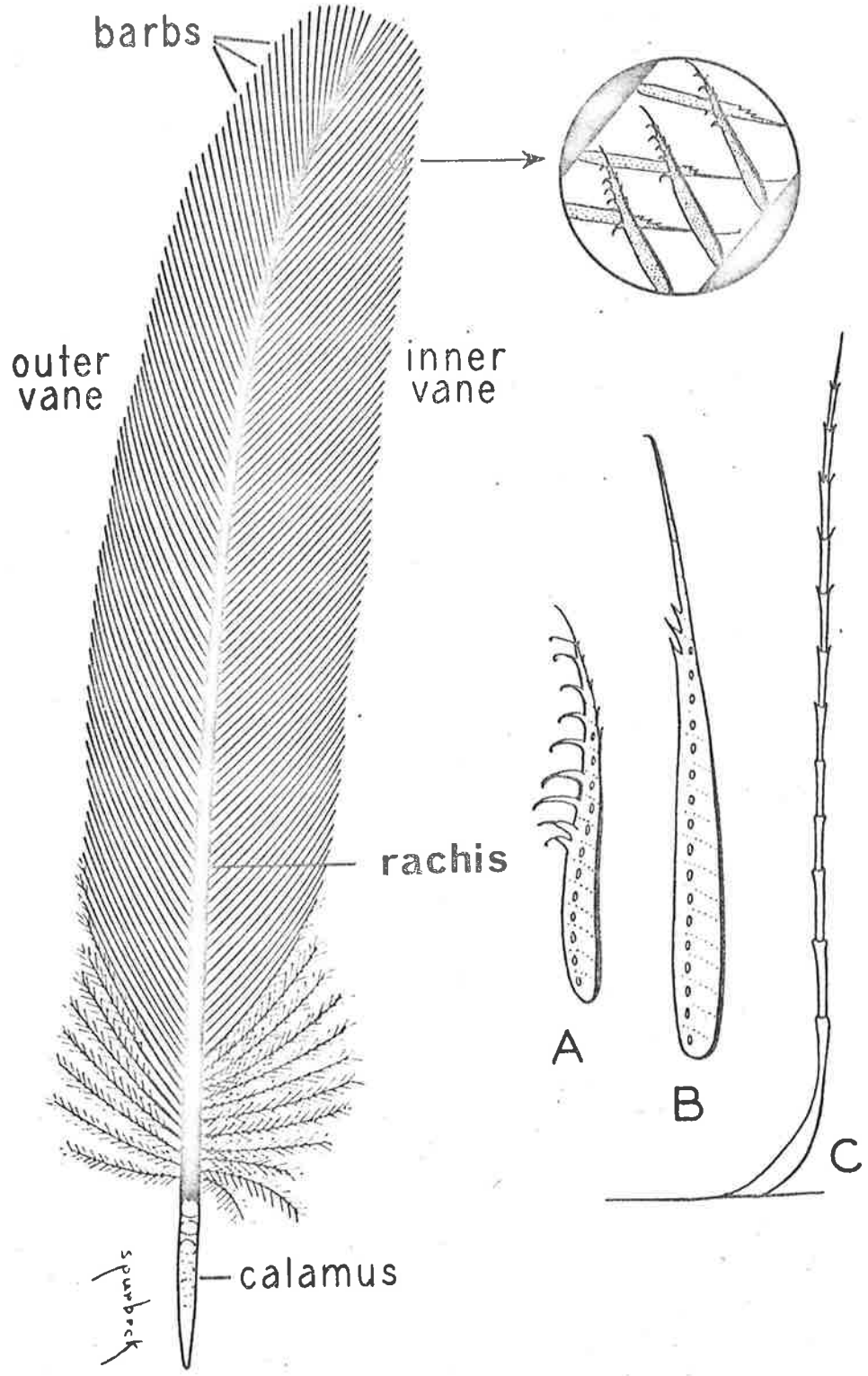
INSET. Detail showing barbules branching off the barbs.

A. Anterior barbule, bearing hooklets (hamuli).

B. Posterior barbule.

C. Downy barbule, from the lower, fluffy region of the feather.

(From Rawles, 1965)


barbs
outer vane
inner vane
rachis
calamus
A
B
C

component proteins.

*(b) Molecular Structure of Feather Keratin.*

Electron-microscopic studies of feather keratin have revealed a filamentous structure (Filshie and Rogers, 1962; Rogers and Filshie, 1963). The filaments are approximately 30 - 35 Å in diameter and of indeterminate length.

X-ray diffraction data, originally reported by Marwick (1931), have led to the postulation of several models for the molecular structure of feather keratin (see Fraser *et al.*, 1972). In the most recent of these (Fraser *et al.*, 1971) the unit of structureis proposed to be a double-helical array of twisted antiparallel pleated sheat, containing four polypeptide chain segments, each with eight residues. It was further suggested that individual feather keratin polypeptide chains may be looped back upon themselves to form the pleated-sheat units.

*(c) Feather Keratin Proteins.*

Methods for solubilization of feather keratin proteins have relied on scission of the disulphide bonds which presumably cross-link the proteins in the native state (Goddard and Michaelis, 1934; Jones and Mecham, 1943; Ward *et al.*, 1946; Woodin, 1954; Rougvie, 1954). Harrap and Woods (1964a,b; 1967) and Woods (1971), in the most detailed studies published to date on the nature of feather keratin proteins prepared stable, soluble feather keratin derivatives by reduction and S-carboxymethylation. The proteins were

extracted in high yield from feathers from various species, and also from the different morphological parts of the feathers. The component proteins all had monomer molecular weights in the vicinity of 10,000 daltons, in good agreement with earlier results (Woodin, 1954). However, they were shown to be heterogeneous by electrophoretic and chromatographic criteria.

Fractionation of the proteins by acid or ethanol precipitation did not result in fractions of differing sulphur content, in contrast to the results obtained with α-keratins (Goddard and Michaelis, 1934; see Fraser *et al.*, 1972). The fractions obtained were similar in composition, being rich in glycine, serine, proline and S-carboxymethyl-cysteine, but deficient in methionine, lysine and histidine, in agreement with the results of Schroeder and Kay (1955). The compositions of proteins from different species or feather parts, although different, showed similar characteristics.

The feather keratin proteins were capable of aggregating to form fibrils (Filshie *et al.*, 1964) which appear to be related in structure to the native proteins (Burke, 1969). Native-type filaments could be regenerated from feather keratin solubilized by oxidation (Rougvie, 1954).

The N-terminal sequences of the proteins from goose feather calamus and rachis were determined by O'Donnell (1971). The N-termini were found to be acetylated, in agreement with earlier studies (Woodin, 1956; Harrap and Woods, 1964a), and the sequence Ac-Ser-SCMC-Tyr was common

to all the preparations studied. Some di- and tripeptide sequences were determined by Schroeder *et al.* (1957) and the distribution of proline residues was studied by Busch (1970). In recent studies (I.J. O'Donnell; personal communication) the complete amino acid sequence of a major feather keratin protein from emu rachis has been determined. The S-carboxymethyl-cysteine residues were concentrated in the N- and C-terminal portions of the molecule whereas the central region of the polypeptide chain was relatively free of these residues, but contained a hydrophobic region with properties suggesting that it corresponded to the β-portion of the molecule postulated by Fraser *et al.* (1971).

In α-keratins, two major fractions are present, namely the low-sulphur and the high-sulphur proteins, which are thought to constitute the microfibrillar and crosslinking-matrix moieties of the keratin structure respectively (Crewther *et al.*, 1965; Rogers, 1969; Fraser *et al.*, 1972). In contrast, the results from protein chemistry, electron-microscopy and X-ray diffraction indicate that feather keratin does not possess such a two-phase structure.

### 3. *Development of the Embryonic Chick Feather*

The development of the embryonic chick feather has been described in detail at the light-microscope level (Davies, 1889; Strong, 1902; Watterson, 1942; Wessells, 1965) and at the electron-microscope level (Kischer, 1963; 1968; Kallman *et al.*, 1967; Matulionis, 1970). Reviews on the development of both adult and embryonic feathers are given

by Romanoff (1960), Lillie (1965) and Voitkevich (1966). The data below are taken mainly from Watterson (1942) and Matulionis (1970).

Gross aspects of the development are summarized in Figures 1.3 and 1.4. At five days of incubation the skin of the chick consists of a layer of cuboidal epithelial cells, overlain by flat peridermal cells and underlain by mesoderm. Soon after, the mesodermal cells condense into localized regions of high cell concentration, and above each of these regions the germinative epithelial layer begins to proliferate. Extensive evidence (to be discussed below) indicates that the dermis plays an important instructive role in the proliferation of the epithelial cells. By about eight days the feather primordia are clearly visible as a series of epidermal thickenings, in clearly defined tracts (placode stage). The primordia grow rapidly outward, forming cylindrical epidermal "humps" with mesodermal cores by 10 days.

Early on the eleventh day, the rapidly dividing epidermal cells in the feather cylinder become organized into a series of discrete sectors, the barb ridges (Fig. 1.5). From this time, the presence of several different cell-types, all derived from the epidermal cells, becomes apparent (Figs. 1.5, 1.6a). By day 12, the embryonic feather consists of a series of barb ridges in a cylindrical arrangement, each containing barb and barbule cells. The cylinder of barb ridges is surrounded externally by the sheath cells and by barb-vane-ridge cells, and internally by a layer of supportive

FIGURE 1.3.

Embryonic Development of the Chick, During the Period of Feather Growth.

Magnification: 10-13 Days, X1.5

14-15 Days, X0.9.

(From Lillie, 1965).

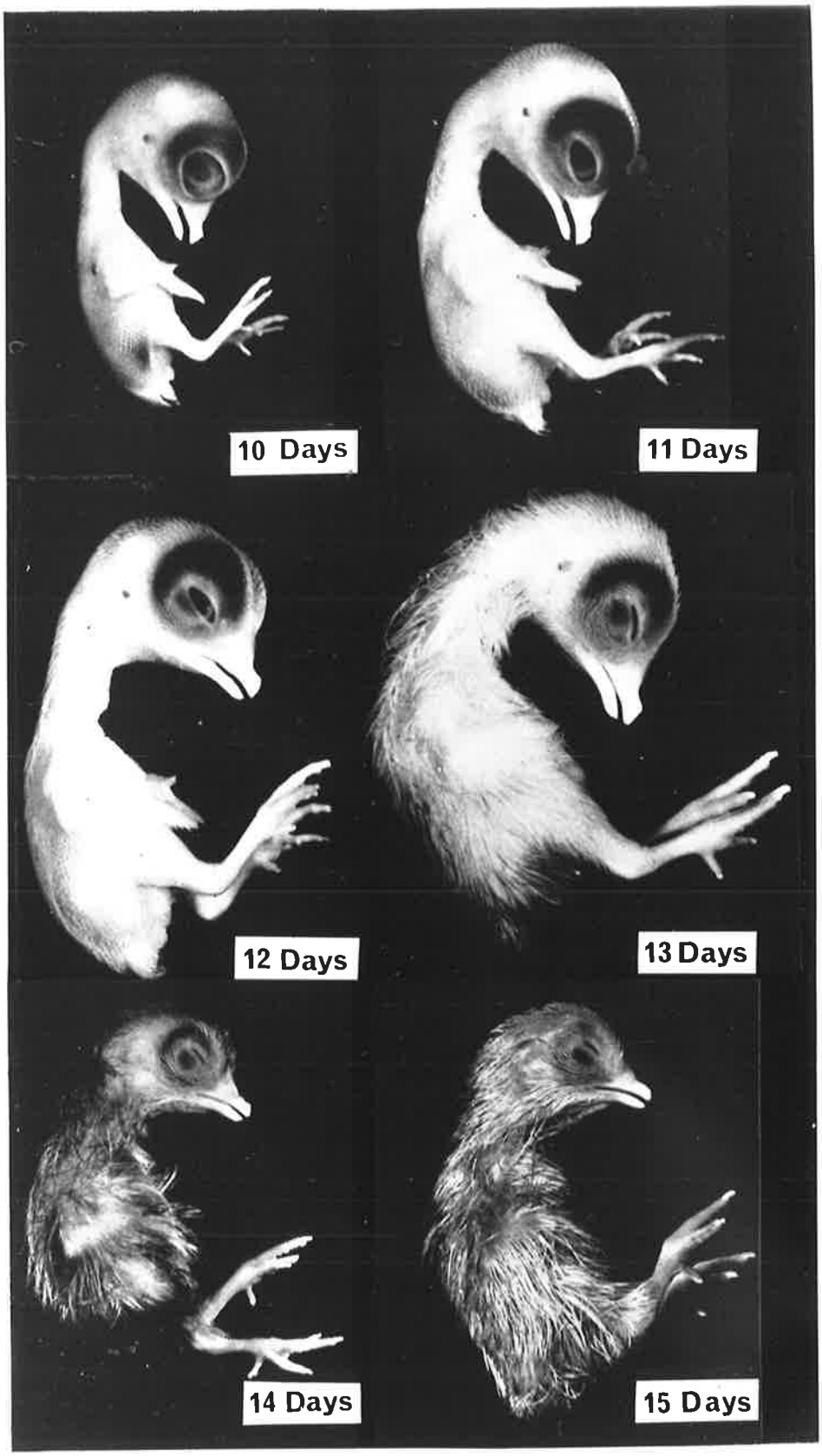
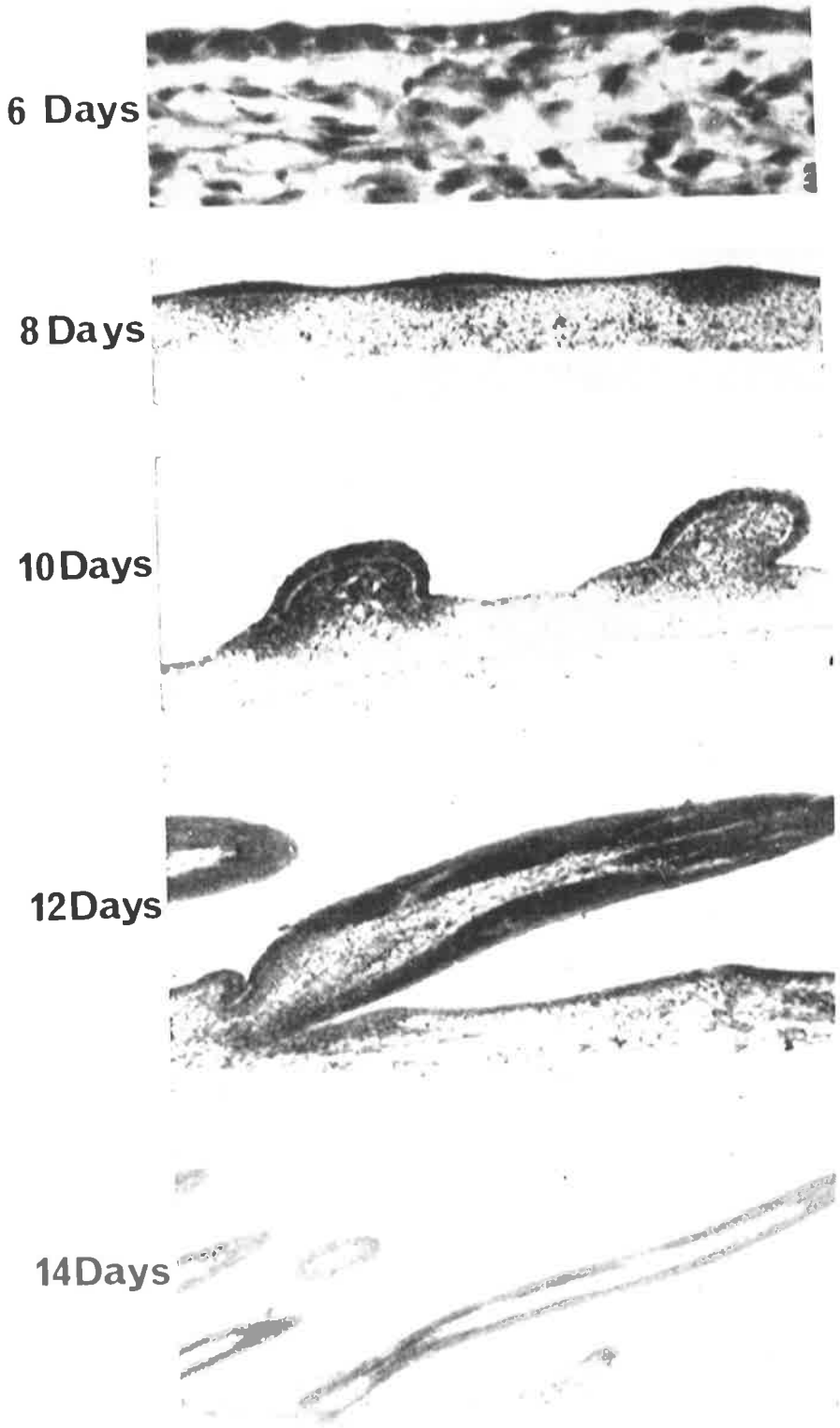

10 Days
11 Days
12 Days
13 Days
14 Days
15 Days

FIGURE 1.4

Histological sections of embryonic feathers during development.

(From Garber, 1968).


6 Days
8 Days
10 Days
12 Days
14 Days

cylinder cells. The barb and barbule cells account for most of the volume of the tissue. A few pulp cells, of mesodermal origin, and capillary vessels occupy the central region of the cylinder.

After 12 days, the feathers rapidly elongate (Fig. 1.6b). The elongation is primarily due to cell elongation and movement, as very little further cell division takes place. An apico-basal gradient of differentiation becomes evident. Keratin fibrils can be detected by electron-microscopy first in the sheath cells at the tip, and then at lower regions of the sheath. The same pattern then occurs in the barb and barbule cells. During the next few days, keratin synthesis proceeds rapidly. The sheath, barb and barbule cells fill with masses of keratin, while the barb-vane-ridge cells and cylinder cells atrophy and the pulp is retracted. After about 17 to 18 days, development is essentially complete.

At 21 days when the chick hatches, the feathers dry out, the sheaths split open and are discarded as the fluffy down feathers open up. The central remnant of atrophied cylinder and pulp cells, initially attached to the basal ring of cells constituting the calamus, is soon discarded.

### 4. *Keratin Synthesis in the Embryonic Feather*

Biochemical studies on keratin synthesis during development of the embryonic feather are limited to those of Bell and his co-workers (Bell, 1964a,b; Bell and Thathachari, 1963; Malt and Bell, 1965; Ben-Or and Bell, 1965; Bell and

FIGURE 1.5

Cellular organization in the embryonic feather during development.

A. Low magnification. B and C, higher magnification. Arrows indicate the areas magnified.

E: Epidermal cells. P: - Peridermal cells.

B.R: - Barb ridges.

P.p: - Pulp cells. Pp.C: - Pulp cavity.

N: - Nucleus. Nl: - Nucleolus.

B: - Basal lamina. BVRC: - Barb-vane-ridge cells.

Sh: - Sheath cells.

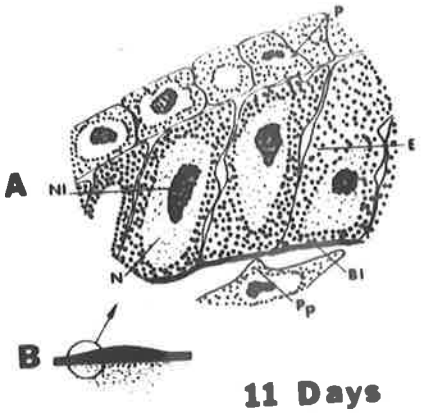
Bl.V: - Blood vessel. B.Bl: - Barbule.

B: - Barb. C: - cylinder cells.

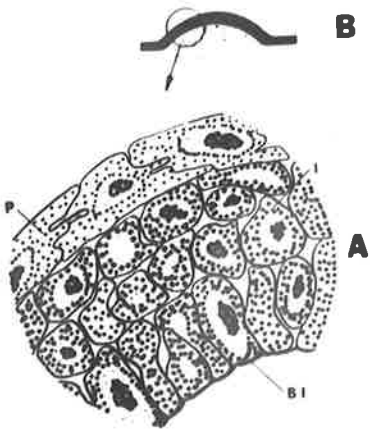
Bc: - Barb-cortical cells. Bm: - Barb medulla cells.

(From Matulionis, 1970).

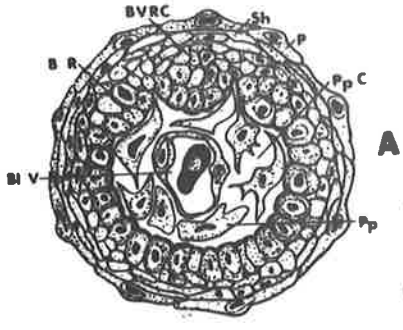
8 Days



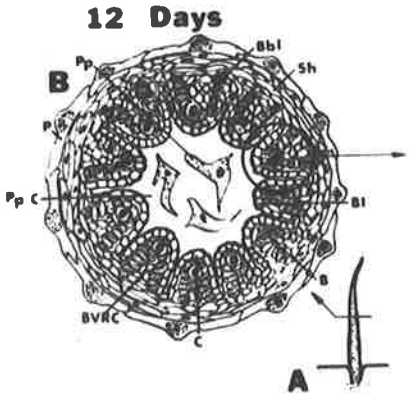

10 Days

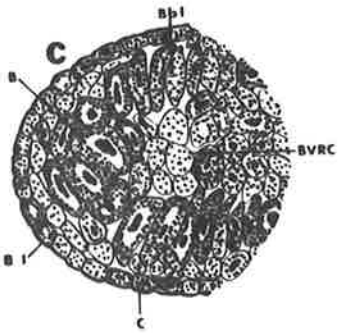



11 Days

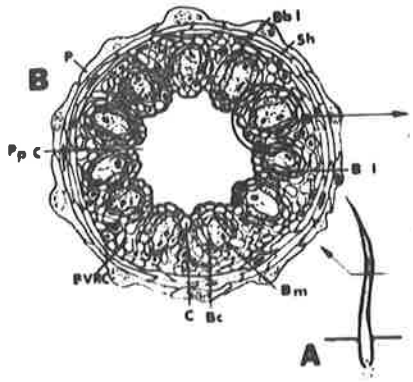



12 Days




14 Days



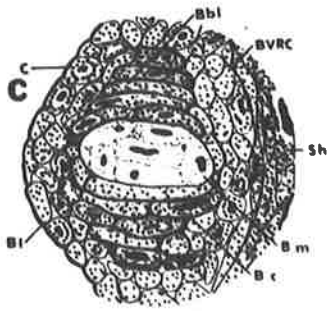



FIGURE 1.6a

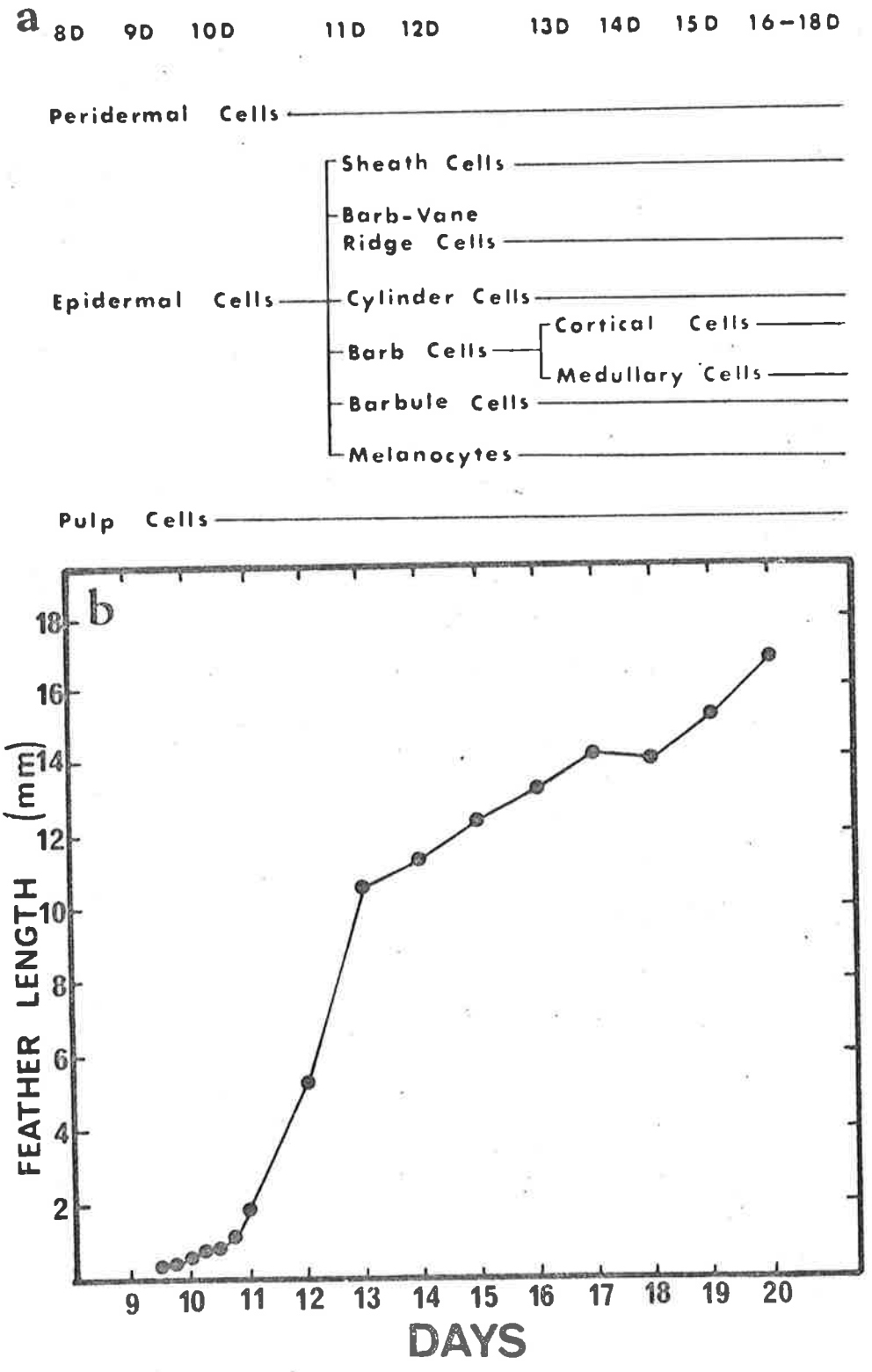
Differentiation of cell-types in the embryonic feather during development.

(From Matulionis, 1970).

FIGURE 1.6b

Embryonic feather elongation.

(Re-drawn from data of Watterson, 1942).


a
8D
9D
10D
11D
12D
13D
14D
15D
16–18D
Peridermal Cells
Epidermal Cells
Sheath Cells
Barb-Vane Ridge Cells
Cylinder Cells
Barb Cells
Cortical Cells
Medullary Cells
Barbule Cells
Melanocytes
Pulp Cells
b
FEATHER LENGTH (mm)
2
4
6
8
10
12
14
16
18
9
10
11
12
13
14
15
16
17
18
19
20
DAYS

Merrill, 1967). In these earlier studies, the keratin proteins were not definitively identified. From the results of gross techniques, such as X-ray diffraction studies on whole feathers (Bell and Thathachari, 1963) and determination of the sulphur content of unfractionated protein extracts (Malt and Bell, 1965) it was concluded that " ... a new protein rich in sulphur is being made for the first time late on the thirteenth day of development", that " ... a sulphur poor fibrillar protein of low-molecular weight is the chief product of the differentiated feather cell early in development ..." and that " ... the sulphur poor polymeric fibrillar molecules are brought into register by the cross-linking and stabilization of the sulphur rich proteins ..." (Malt and Bell, 1965). These conclusions are not consistent with the more recent evidence on the structure of feather keratin discussed above, as there is no basis at present for postulating the existence of high-sulphur and low-sulphur protein groups in feather keratin. Furthermore, the results are not consistent with those of Matulionis (1970), who was able to detect keratin fibrils in the more advanced cells of 12 day feathers.

Quantitative data on the rates of synthesis of defined keratin proteins during embryonic development, a pre-requisite for meaningful studies on the control of keratin synthesis, are therefore lacking.

Other biochemical aspects of feather development which have been studied are included in the following sections.

## C. THE GROWTH AND DEVELOPMENT OF KERATINOCYTES

Although some aspects of the development of feathers have been the subject of extensive experimentation, there are other areas where studies have been extremely limited. It is therefore more profitable to consider not only the feather system but also the data available for other keratinizing systems.

In the following sections, the effects of dermis and other factors on the development and organization of keratinocytes will be discussed.[a]

### 1. *Dermo-epidermal Interactions*

#### (a) *The Specifying Action of Dermis*.

Interactions between the dermis and epidermis have been demonstrated most clearly in embryonic chick skin. Embryonic skin can be separated into its component dermis and epidermis. Dermis and epidermis from the same area or from different areas may be recombined and cultured either *in vitro* or on the chorioallantoic membrane of a host egg (Sengel, 1957, 1971; Rawles, 1963, 1965; Wessells, 1962, 1965). Results from heterotypic combinations show, for example, that dermis from the middorsum of 5 to 8½ day embryos induces feather formation when combined with 9 to 12 day tarsometatarsal epidermis (which would normally form

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[a]The following discussion, in slightly modified form, was contributed to Fraser *et al.* (1972), Chapter 8.

scales). The experiments of Rawles (1963, 1965) also demonstrate that it is only at certain developmental stages that the dermis can exert this "inductive" effect, and that the epidermis can respond to it. A similar situation exists with the dermis and epidermis of the embryonic beak and spur (Rawles, 1963).

Maintenance of epidermal specificity in adult mammals and birds also appears to be determined by the dermis. For example, Billingham and Silvers (1968) showed that in heterotypic recombinations of dermis and epidermis from the sole of foot, ear and trunk of adult guinea pigs, the nature of the dermis always determined the resulting nature of the epidermis in the graft. Earlier experiments had been interpreted as suggesting that in the adult the epidermis was the site of specificity. For example, Wang (1943) implanted adult feather dermal papillae into foreign sites and found that breast feathers developed when saddle papillae were placed in breast follicles. However, Cohen (1964) reinterpreted such experiments to suggest that the local dermis was affecting the transplanted dermal papillae, and that dermis was in fact the site of specificity. More recent experiments (Cohen, 1969) have supported this conclusion.

*(b) The Response of Epidermis.*

It is apparent that epidermis has some organizational capacity of its own. For example, in heterotypic recombinations of chick middorsal epidermis

and tarsometatarsal dermis of suitable age, tarsometatarsal dermis can take part in feather formation. Similarly, Oliver (1966, 1967) has shown that the lower (epidermal) surface of a vibrissa follicle has the capacity to organize a new dermal papilla, whereas the upper two thirds of the follicle cannot elicit such *de novo* papilla formation.

Goetinck (1966) studied a "scaleless" mutant of the chick, and demonstrated that the mutant phenotype was caused by the inability of the epidermis to respond to scale dermis.

The ability of epidermis to respond to different mesenchymal stimuli was further demonstrated by McLoughlin (1961a,b). Recombinations of chick epidermis from 5-day embryonic chick limb buds with mesenchyme from proventriculus, gizzard and heart of the same age resulted in each case in the formation of epithelial structures representative of the type of mesenchyme with which the epidermis was in contact.

### (c) *The Nature of the Interactions Between Dermis and Epidermis.*

Epithelio-mesenchymal interactions are common to morphogenetic systems with epithelial and mesenchymal components (Fleischmajer and Billingham, 1968) but the mechanism of these interactions is not clearly understood (Wessells, 1968). From the fact that the particular type of mesenchyme specifies the type of epithelium, it has been postulated that a specific "inducer" molecule is

passed from the mesenchyme to the epithelium. Experiments in which isolated epidermis was cultured *in vitro* (McLoughlin, 1961a; Wessells, 1962; Dodson, 1963) indicate that dermis was required for mitosis, spreading and orientation of the epidermis. Dodson (1963) showed that frozen-thawed (killed) dermis and collagen gels could maintain to some extent the orientation and mitosis of epidermis, indicating that the role of dermis was that of a support. However, heat killed dermis was a poor substrate. Wessells (1962) showed that isolated epidermis would grow even if a Millipore filter was interposed between the epidermis and dermis and the whole assembly cultured *in vitro*. This experiment demonstrates that dermis and epidermis can interact over a considerable extracellular distance, Wessells (1964) found that a particulate fraction of chick embryo extract or a similar preparation from the dermis alone could support basal cell orientation and mitosis. This fraction has been partially characterized and shown to be non-dialyzable, heat labile and sensitive to proteolytic enzymes.

Wessells (1968) suggests that the data do not require the postulation of a specific chemical inducer. Such factors as changes in mesenchymal cell density and changes in extracellular substances of the mesenchyme that mediate environmental exchanges between epithelial cells could conceivably be responsible for the apparent mesenchymal specificity. It is also evident that although this specificity might explain the particular developmental

fate of a group of epidermal cells, the mesenchyme itself must derive these special characteristics from some other source.

The evidence does not indicate whether the factors required for basal cell mitosis and orientation are identical to those required for specification of the particular differentiation fate the cell will undergo. However, it seems likely that they are different. McLoughlin (1968) has shown that mesenchyme is required for mitosis and basal cell orientation but the particular type of mesenchyme specifies the developmental fate of the epidermis. The factors supporting mitosis and orientation may be relatively trivial, for example nutritional requirements, or a suitable attachment surface. However, the factors operating to select the path of differentiation are likely to be of a more fundamental nature. The protein hormone erythropoietin, which stimulates red blood cell development (Goldwasser, 1966) is an example of a factor with similar properties to the postulated "inducers".

### *(d) The Mechanism of Response of the Epidermis*

Besides considerations of the nature of the interactions between dermis and epidermis, it is pertinent to consider what precisely is being controlled in the epidermis. Early embryonic epidermis consists of a population of apparently identical cells and there are several possible mechanisms which could result in the morphologically and chemically different cells derived

from them. Firstly, the embryonic cells could each be equal and multipotential. A specific dermal stimulus, for example for feather formation, could then select the gene set determining the type of mature cell into which the embryonic cell would differentiate. A second possibility is that different types of cells, such as prospective skin cells and prospective feather cells, are present in the embryonic epidermis and that, the dermal stimulus simply induces certain of these to proliferate. The first of these hypotheses is the more tenable as various experiments indicate the "developmental plasticity" of embryonic epidermis, as evidenced for example by the effects of gizzard and proventricular mesenchyme and the effects of vitamin A on the development of the epidermis, discussed elsewhere in this chapter. The fact that epidermal cells in the presence of vitamin A can apparently differentiate in two directions at once (to be discussed) is particularly convincing on this point.

A third possibility is that the dermal influence controls mitosis, and that selection of the path of differentiation is a secondary effect of interactions among the growing epidermal cell population. Wessells (1967) pointed out that there are two major responses to the dermal influence, namely mitosis and differentiation, but to date it has not been possible to obtain differentiation in the absence of mitosis. Thus the third hypothesis cannot be ruled out. However, it would seem more likely that mitosis is a required step in the path of differentiation.

Evidence has been accumulated in many other systems (Stockdale and Topper, 1966; Holtzer, 1970) to suggest that this is generally true.

In addition to selecting the gene set required for a particular epidermal cell type, the dermal influence must control tissue morphology, and as a prerequisite, epidermal cell division. This was most clearly demonstrated by Dhouailly (1967) who found that chick epidermis, in the presence of duck dermis, grew feathers morphologically similar to duck feathers. The information directing cell division in the chick epidermal cells could only have come from the duck dermal cells, irrespective of its mode of transmission.

The question of whether the same genes for keratin proteins are expressed in all epidermal derivatives has been raised by Crounse (1965) and Wessells (1967). Crounse (1965) suggested that human soft and hard keratin contained a common keratin protein, but the available evidence does not support this view (Fraser *et al.*, 1972). The path of differentiation chosen for a particular group of epidermal cells, apparently specified by the dermis may therefore also specify the particular set of genes for keratin which are to be expressed. There appears to be no direct evidence concerning the time of development at which this choice is made, or its molecular mechanism.

## 2. *Other Factors Affecting Keratinocyte Development*

### *(a) Hormones.*

Besides the effects attributable to dermis, several other factors are known to influence the growth and development of epidermis and its derivatives. Extensive studies have been made on the effects of hormones on epidermal cells, in particular with respect to hair growth as this is readily measured. The dependency of hair growth on adrenal, gonadal, thyroid and pituitary hormone levels has been discussed in detail by Mohn (1958) and Ferguson *et al.* (1965) and the stimulation of wool growth by thyroxine after removal of the thyroid (thyroidectomy) and the suppression of wool growth by administration of adrenal corticosteroids or by removal of the pituitary (hypophysectomy) strongly suggest that hair growth is under hormonal control. The influence of hormones on feather growth has been discussed by Voitkevich (1966). The inhibition of embryonic feather development by hypophysectomy or thyroidectomy indicate a similar control in this case.

Hormonal effects have also been shown to occur *in vitro*. For example, hydrocortisone hastens keratinization in skin explants from chick and rat embryos (Fell, 1962; Weissmann and Fell, 1962), oestrogens induce the keratinization of vaginal epithelium *in vitro* (Biggers *et al.*, 1956; Kimura *et al.*, 1967), and thyroxine accelerates keratinization of dissociated chick skin cells grown as rotating aggregates (Kitano and Kuroda, 1967). Prostaglandins

have a similar effect on embryonic chick skin in culture to that of the epidermal growth factor (see below; Kischer and Furlong, 1967). In one of the few attempts to investigate the mechanism of hormonal action on keratinocytes at a molecular level, Yatvin (1966a) demonstrated that the formation of polyribosomes associated with keratin synthesis in the developing feather was prevented by hypophysectomy. Administration of pituitary gland extract allowed normal development and the concomitant restoration of a normal polysomal profile (Yatvin, 1966b).

*(b) Vitamin A*

Mori (1922) demonstrated that vitamin A deficiency in animals causes certain secretory epithelia to undergo a squamous metaplasia and to keratinize. Lasnitzki (1962) demonstrated this phenomenon *in vitro*, using a chemically defined medium. Hicks (1968) found that during keratinization of the rat bladder epithelium induced by vitamin A deficiency, the cells become cytologically indistinguishable from those of epidermis, including the formation of cytoplasmic filaments and keratohyalin granules, but no stratum corneum was formed.

Fell and Mellanby (1953) demonstrated a second effect of vitamin A. When skin from 7-day chick embryos was cultuvated in the presence of an excess of vitamin A (10-15 I.U. vitamin A/ml culture medium) keratinization was completely inhibited, and more dramatically, the epidermis underwent a complete mucous metaplasia and occasionally formed tracts of actively beating ciliated cells. Typical

goblet cells, secreting mucin were found. Fell (1957) showed that the highly developed metatarsal epidermis of 12- to 18-day chick embryos underwent a similar metaplasia and Fitton-Jackson and Fell (1963) showed that the post-mitotic cell population was the one affected. More highly developed cells were apparently too advanced on their normal path of differentiation to be transformed. When the excess of vitamin A was removed, cells which had undergone the mucous metaplasia were unchanged, but the new generation of postmitotic cells reverted to keratin production. However some of these cells, during the recovery period, contained both secretory globules and keratin filaments. Similar observations have been made in a mammalian system (Hardy, 1967).

Fell (1964b) has suggested the effects of vitamin A may be connected with its known action on lysosomal membranes. However, the extensive nature of the meta-plastic changes may indicate a more fundamental mechanism, acting at the level of the nucleus. It should also be pointed out that the modulation of epithelial cells between the secretory and keratinizing states appears to be a very general phenomenon. Secretory epithelia can be induced to undergo keratinization by a variety of stimuli other than vitamin A. Examples include the effect of carbon dioxide deficiency on chorionic ectoderm (Moscona, 1961) and the effect of oestrogen on vaginal epithelium (Biggers *et al.*, 1956; Takasugi, 1963). Conversely, squamous keratinizing epithelia can be induced to form secretory epithelia by association with heterotypic mesenchyme

(McLoughlin, 1961a,b). Such findings, and the general requirement for mitosis in the expression of these changes, suggest that the sets of gene loci determining whether a cell enters the path to keratinization or secretion of mucin are selected during or after mitosis in the germinatal layer. The finding that during the recovery from an excess of vitamin A some cells can produce both keratin and mucin clearly demonstrates that expression of normally alternative sets of genes concomitantly is possible in the one cell. Such a situation contrasts with other differentiating systems in that protein products that are characteristic of two distinct cell types are not found in one cell type. For example, so far as it is understood at the present time reticulocytes that produce hemoglobin do not synthesize myosin nor do myogenic cells synthesize hemoglobin. Therefore in the case of keratinocytes that are capable of producing both keratin and mucin under the influence of vitamin A, it can be concluded that the necessary sets of genes must be in a state readily available for activation by the appropriate stimulus.

*(c) Epidermal Chalone.*

The studies of Bullough (1967) suggests that epidermal mitotic activity may be controlled at least in part by a diffusible inhibitor of mitosis. Injury to the epidermis leads to increased mitotic activity in the basal cells, and there is an initial over-production of epidermal cells in the area. Bullough and Lawrence (1964) postulated

that as a result of wounding, an antimitotic factor ("chalone") is reduced below its normal concentration, allowing mitosis to take place. It was suggested that the epidermal chalone is produced by differentiating cells and acts on basal cells, determining their rate of division. Bullough (1967) has developed this concept in detail.

Bullough also has found that hair follicles do not respond to the chalone, and pointed out that such epidermal appendages as hairs and feathers would not be expected to be under feedback regulation in this way. These organs do not maintain a constant relationship between the rate of cell division and tissue volume, as is the case in epidermis. Consequently, cell division in adult hair and feather follicles is thought to be under the control of systemic or local factors. In each case the role of dermis, systemic hormones, and specific inhibitors and stimulators of epidermal mitosis must be understood before a complete scheme of control of cell division can be proposed.

*(d) Epidermal Growth Factor.*

Cohen (1962) found that an extract from the submaxillary gland of the male mouse when injected into newborn mice and rats elicited precocious eyelid separation and tooth eruption. This appeared to result from a stimulated epidermal development and keratinization (Cohen and Elliott, 1963). The active principle has been isolated and shown to be a protein which acts

directly on epidermis in organ culture, and stimulates the mitotic rate in basal cells. The stimulation can only occur if the epidermis is in contact with a suitable substrate (Cohen, 1965).

This epidermal growth factor (EGF) stimulated protein and RNA synthesis in epidermal organ cultures and caused an apparent increase in the number of polyribosomes present. A cell-free protein synthesizing system was found to be more active if the epidermal tissue had been pretreated with EGF (Hoober and Cohen 1967a,b; Cohen and Stastny, 1968). These authors suggested that the ability of ribosomes to bind messenger-RNA is enhanced in cells treated with EGF. However, it is difficult to distinguish between cause and effect in such circumstances, and the primary response to EGF cannot be clearly defined.

It should also be pointed out that it is not known whether EGF or similar factors play any role in the normal control of epidermal growth. It is interesting to note in this regard that a similar factor stimulating nerve growth (NGF) has also been isolated from the submaxillary gland of the male mouse. An antiserum prepared against this factor, when injected into newborn mice, inhibits development of the sympathetic nervous system, indicating a genuine role for NGF in nerve development (Levi-Montalcini, 1964). By analogy, it would appear likely that the EGF may play an important role in the control of epidermal growth. The protein has in fact some biological properties which would be predicted for the postulated dermal "inducer" molecule.

*(e) Nutritonal Factors.*

The formation of keratin is subject to variation according to the nutritional status of the animal. An outstanding example of this has been the finding that the rate of wool growth in sheep and the cystine content of the wool was markedly increased when the diet was supplemented with cysteine and sulphur-containing amino acids and proteins by abomasal infusion (Reis and Schinkel, 1963). Analysis of the keratin produced in these circumstances has revealed that the cystine-rich supplement gives rise to the synthesis of a high-sulphur protein with a very high cystine content. Further, it appears that there is an increase in the proportion of high-sulphur proteins synthesized in the follicle (Gillespie, 1965; Gillespie and Reis, 1966). It is not known how this increase of sulphur-containing substrate available to the follicle influences the protein-synthesizing machinery to produce more of one kind of protein, but there is clearly some kind of control mechanism operating.

## D. KERATIN BIOSYNTHESIS

### *1. Control of the Onset of Keratin Synthesis.*

The preceding sections have discussed various physiological influences on the morphogenesis of epidermal tissues, and have also briefly considered the effects of these agents on keratinization. The following sections will consider more closely the relationship of events such as mitosis and nucleic acid synthesis to the onset of synthesis

of keratin proteins in these cells.

The mechanism by which the onset of keratin synthesis (cytodifferentiation) is controlled may be to some degree unrelated to the control of morphogenesis. This situation is most clearly illustrated in the case of the developing embryonic feather. Dermal-epidermal interactions resulting infeather formation begin at about 5 days, as described earlier. However, keratin synthesis does not begin until about 12-13 days, as determined by birefringence, and X-ray diffraction (Bell and Thathachari, 1963) and by electron microscopy (Matulionis, 1970). By this stage, the embryonic feather has almost completed its morphological development. Hypophysectomy of the embryo at about 36 hr does not prevent feather morphogenesis, but does prevent the subsequent synthesis of keratin, as determined by examination of the characteristic polyribosome profiles (Yatvin, 1966a). This evidence suggests strongly that the influences controlling the onset of keratin synthesis are different from those controlling morphogenesis. Presumably however, many events take place at earlier times which are obligatory for the subsequent synthesis of keratins. For example, events in the nucleus at earlier stages may alter the state of repression or activation of certain genes, allowing later stimuli to act on these. The situation is probably similar in other keratinocytes. Keratin is not detectable in the basal cells of epidermis or bulb cells of the hair follicle and the cells synthesizing keratin are therefore physically and temporally separated from the

dividing cell population, thus providing the opportunity for the action of controlling influences at this stage to act on subsequent development.

2. *DNA Synthesis and Mitosis*

It was pointed out earlier that mitosis may be a crucial step in the transition from proliferation to keratinization and evidence has accumulated to suggest that these states of the cell may be mutually exclusive. Autoradiographic studies (Bernstein, 1964) have shown that DNA synthesis is restricted to the basal layers of adult mammalian epidermis. Likewise, Downes *et al.* (1966a) have shown that in wool follicles $^{3}$H-thymidine incorporation is restricted to the basal layers. Mitosis in the wool follicle has been studied in detail by Short *et al.* (1965), Fraser (1965), and Epstein and Maibach (1969).

Primitive chick epidermis can incorporate $^{3}$H-thymidine and undergo mitosis at all levels, but later, as keratin synthesis begins, mitosis is restricted to the basal layer (Wessells, 1963).

DNA is apparently degraded and removed as the cells mature. Autoradiographic studies have shown that $^{3}$H-thymidine disappeared from nuclei during keratinization (Fukuyama and Bernstein, 1961) and Downes *et al.* (1966a) showed that most of the labelled thymidine incorporated into wool follicles is removed within 3 days.

The only strictly biochemical study of DNA synthesis in keratinocytes so far reported is that of

Kischer and Furlong (1967). They examined DNA polymerase and DNAse activity during the development of embryonic chick skin and down feathers and found that DNA polymerase activity was low at early stages, reached maxima at the stages of feather germ outgrowth and of elongation, and dropped abruptly by the onset of keratinization. Nuclear DNAse activity, in contrast, rose slowly to a maximum by the onset of keratinization.

It would appear from these results that keratin synthesis cannot begin until the cells have lost the capacity to synthesize DNA and divide. The effect of vitamin A on gene expression in postmitotic cell populations and the observation that normal differentiation *in vitro* has not been obtained in the absence of mitosis strongly suggest that mitosis is a required step at a crucial stage as a cell enters the path towards synthesis of keratin.

### 3. *RNA Synthesis*

Wilkinson (1970b) investigated the RNA species present in wool follicles using sucrose density-gradient centrifugation. As in other tissues the sedimentation coefficients of the major species were 28, 18 and 4-5S. A heavier species, apparently derived from the nucleus, was also found. Labelling with $^{3}$H-uridine for 15, 30 and 60 minutes resulted in an initial appearance of label in RNA greater than 28S. By 60 minutes, most was in 28S and 18S RNA, suggesting that the heavy species were ribosomal RNA (r-RNA) precursors. The kinetics of entry of $^{3}$H-uridine

into polyribosomes of wool follicles (Wilkinson, 1970a) also showed that r-RNA was the first species to be labelled.

Bell and Merrill (1967) studied the movement of newly synthesized RNA through various pools of cytoplasmic particulates in the developing chick feather. They found that after short pulses of labelled uridine, the labelled RNA was found first in particles sedimenting in the 40-60S region and then in the 85-90S region of sucrose gradients, and later entered polyribosomes. They postulated that the 40-60S particles were involved in RNA transport from the nucleus, as proposed by Belitsina *et al.* (1964).

4. *Stability of the Keratin mRNA*

In many cells committed to the synthesis of one or a few specific proteins, the synthesis is directed at some stage by stable mRNA. Such is the case for example, during development of the reticulcoyte (Wilt, 1965) and the lens (Stewart and Papaconstantinou, 1967). It would appear that a similar situation obtains in the case of keratin synthesis. The stability of mRNA in developing chick skin and feathers has been studied (Bell, 1964; Bell *et al.*, 1965; Scott and Bell, 1964, 1966; Humphreys *et al.*, 1964, 1966; Bell and Merrill, 1967). Skin and feathers from 7- to 13-day chick embryos were incubated *in vitro* for 24 hours with Actinomycin D (ACT-D). At 7 and 9 days, amino acid incorporation was abolished by ACT-D, but by 13 days, about 60 percent of amino acid incorporation was stable to

ACT-D treatment. As keratin synthesis is a major synthetic activity by 13 days, it was concluded that keratin is synthesized on stable m-RNA. Bell and Merrill (1967) showed by autoradiography that protein synthesis in the 12-day keratinizing sheath cells was resistant to an 18 hours incubation with ACT-D.

Stable m-RNA also appears to be present in the developing epidermis. Flamm *et al.* (1966) found that topical application of ACT-D to mouse skin inhibited RNA synthesis but not protein synthesis.

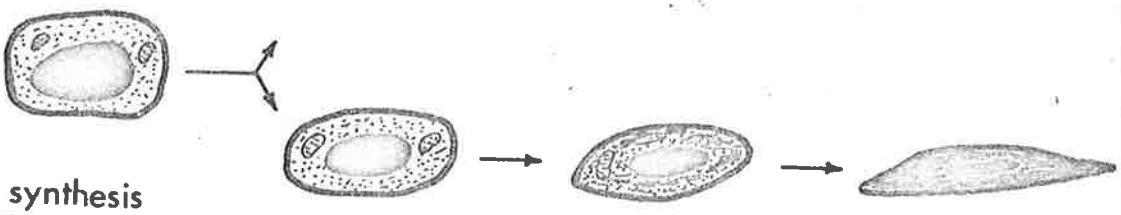
Evidence for the occurrence of stable m-RNA in keratinocytes producing mammalian hard keratin has been obtained by Wilkinson (1970b) who reported that ACT-D did not affect polyribosome profiles from wool follicle tissue for times up to 4 hours.

### 5. *The Pathway of Cytodifferentiation*

The observations on mitosis, DNA synthesis and RNA synthesis, discussed in the previous sections, suggest a general scheme for the cytodifferentiation of keratinocytes (Fig. 1.7). After (or as) the cell undergoes a critical mitosis, some factor operates to switch one or both daughter cells in the direction of keratin production. After this critical mitosis the cell can no longer synthesize DNA or divide. Synthesis of stable keratin mRNA begins, followed by the synthesis of the keratin proteins, possibly after a lag period during which the mRNA is accumulated and stored. As the keratinocyte approaches maturity, synthesis of other

FIGURE 1.7

Generalized diagrams of the life span of a typical keratinocyte.



DNA synthesis

mitosis

RNA synthesis

keratin mRNA synthesis

non-keratin protein synthesis

keratin synthesis

keratin stabilization

cellular proteins and RNA stops and degradation of the nucleus ensues. Stabilization of keratin begins, its synthesis dwindles, and the cell finally dies, filled with keratin.

Although these are clearly the general features of the pathway of cytodifferentiation, the precise mechanism by which any single step is controlled is not understood; also, the relative timing of the events appears to vary in different types of keratinocyte. Of particular interest is the question of whether newly synthesized keratin mRNA is immediately translated, or initially stored and subsequently activated at the level of translation. It should be emphasized, however, that since the synthesis of keratin mRNA is an absolute requirement for subsequent keratin synthesis, the fundamental control of the onset of keratin synthesis occurs at the level of transcription of DNA into RNA.

### 6. *Kinetics of Keratin Synthesis*

So far, keratin synthesis has been discussed as if a single protein was involved but mammalian hard keratin is a complex mixture of proteins and several lines of evidence suggest that synthesis of the component proteins is not concurrent. This implies a temporal compartmentalization in the synthesis of different proteins in the maturing keratinocyte. The sulphur content of the keratin proteins increases with the age of the keratinocyte and one possible explanation is that synthesis of the low-sulphur proteins

precedes that of the high-sulphur proteins (Rudall, 1956). Some support for this view was obtained from electron microscope studies of developing hair (Mercer, 1961; Rogers, 1964) in which the synthesis of microfibrils appeared to precede the deposition of the intermicrofibrillar matrix.

Autoradiographic methods have been employed in studies of the kinetics of synthesis of the keratin proteins of hair and wool (Harkness and Bern, 1957; Ryder, 1958; Sims, 1964; Downes *et al.*, 1963; 1966b; Fraser, 1969b) but no conclusive evidence for a two-stage process has been obtained. If the synthesis of low-sulphur proteins precedes that of high-sulphur proteins this should be detectable by comparing the $^{35}S$ specific activity of the two types of protein at various levels in the follicle after administration of $^{35}S$-cystine. Downes *et al.* (1963) interpreted their findings as supporting a two-stage process, but the results of later experiments (Downes *et al.*, 1966b) were not in accord with a complete compartmentalization of synthesis. One possibility considered was that the synthesis of the two types of protein might occur concurrently but peak at different stages of maturation of the keratinocyte. Strong support for concurrent synthesis of high- and low-sulphur proteins was obtained by Fraser (1969a,b) who isolated both types of proteins from active keratinocytes at all levels of the follicle. The concentration of low-sulphur protein increased steadily during maturation but the concentration of high-sulphur protein

increased sharply during the latter stages of maturation.

Malt and Bell (1965) had suggested a two-stage synthesis of feather keratin similar to that discussed for hair keratins, but the evidence was not definitive.

### 7. *Initiation of Keratin Synthesis*

The synthesis of most proteins in higher cells is now believed to be initiated by a methionine residue, donated by Met-$tRNA_F$, which is subsequently removed from the protein, in most cases while the growing polypeptide is still attached to the ribosome (Jackson and Hunter, 1970; Wilson and Dintzis, 1970; Yoshida *et al.*, 1970; Wigle and Dixon, 1970; Ghosh and Ghosh, 1971; Rho and Bebusk, 1971; Hunter and Jackson, 1971). It has been suggested that acetyl-aminoacyl-tRNAs may act as initiators of N-acetyl proteins (Narita, 1962) and evidence that this occurs has been presented in the cases of ovalbumin (Narita *et al.*, 1968, 1969), and histones (Liew *et al.*, 1970). Since keratin proteins are N-acetylated (O'Donnell and Thompson, 1968; Harrap and Woods, 1964b; O'Donnell, 1971; Haylett and Swart, 1969) it is attractive to postulate that acetyl-aminoacyl-tRNAs function in the initiation of keratin synthesis. Acetyl-aminoacyl-tRNAs could then play a role in the control of keratin synthesis, acting at the level of initiation.

Evidence for control mechanisms operating at the level of initiation of protein synthesis has been obtained in both prokaryotic systems (for example, Steitz, Dube

and Rudland, 1970) and in eukaryotic systems (for example, Heywood, 1970a,b). However, a role for acetyl-aminoacyl-tRNAs in such translational control mechanisms has not been demonstrated.

In recent studies however, extreme doubt has been cast on the possible involvement of acetyl-aminoacyl-tRNAs in initiation by the demonstration that Met-$tRNA_F$ acts as the initiator of crystallins, which are N-acetyl proteins (Berns *et al.*, 1972; Straus *et al.*, 1971). In such cases, the acetylation must result from a post-translational mechanism.

The mechanism of acetylation of keratin proteins has not previously been investigated.

### 8. *Assembly of Keratin Subunits*

Most keratins have a well-defined molecular architecture, and it is of interest to consider how the newly-synthesized protein chains become organized into the final structure. In the case of mammalian hard keratin several lines of evidence suggest that the microfibril is formed by the orderly aggregation of low-sulphur proteins, although it has not so far been possible to establish this by *in vitro* experiments.

The high-sulphur proteins do not appear to be important for the formation of the filaments destined to become microfibrils as the filaments first make their appearance at levels in the follicle where the synthesis of high-sulphur proteins is minimal. Evidence from kinetic

studies, discussed earlier, suggests that the synthesis of high-sulphur protein reaches a maximum at a late stage in the maturation of the keratinocyte and it is difficult to escape the conclusion that the high-sulphur proteins must infiltrate existing bundles of microfibrils in the manner envisaged by Mercer (1961).

Some direct information on the assembly of keratin structures has been provided by autoradiographic studies at the electron microscopic level. Labelled amino acids are rapidly incorporated into the developing fibrils in the hair follicle (Nakai, 1964; Rogers, 1969) suggesting that the newly synthesized keratin proteins spend very little free time in the cytoplasm.

Rogers (1969) found that $^{3}$H-uridine incorporated into the guinea pig hair follicle became associated with growing keratin fibrils within 2 hours. The location of the uridine label suggests that the synthetic apparatus for keratin proteins is localized in the immediate vicinity of the keratin fibrils (Rogers, 1969).

### 9. *Post-synthetic Modification of Keratins*

The most obvious post-synthetic chemical modification of keratins is their stabilization by the formation of disulphide linkages. Despite extensive histochemical studies, no significant information on the nature of this process has been obtained. By analogy with the disulphide-sulphydryl exchange enzyme known in liver microsomes (Goldberger *et al.*, 1963)it is to be expected that

the process is catalysed by a specific enzyme.

Comparisons between the compositions of individual proteins of the hair follicle and of mature hair have revealed little, if any, difference (Frater, 1966; Downes *et al.*, 1966b; Fraser, 1969a; Steinert, 1972).

### *10. Keratin Synthesis In Vitro*

Earlier attempts to isolate polyribosomes from hair follicles (Rogers and Clarke, 1965a,b) and epidermis (Baden and Cohen, 1965; Priestly and Speakman, 1966; Freedberg *et al.*, 1967), met with limited success. More recently Wilkinson (1970a), using better isolation procedures, has obtained excellent polyribosomes from wool follicles and Steinert and Rogers (1971a) have obtained similar polyribosomes from guinea pig follicles.

Polyribosomes from the developing chick feather have also been studied. Until about day 13 of development, very few polyribosomes are detectable. By days 14 to 15, when keratin synthesis is well advanced, the profiles show a decided shift, polyribosoms with 4 to 6 ribosomes predominanting (Humphreys *et al.*, 1964, 1966; Scott and Bell, 1964, 1966; Bell *et al.*, 1965; Yatvin, 1966a,b; Humphreys and Bell, 1967; G.A. Partington, personal communication), and corresponding to the size expected (Heywood *et al.*, 1967; Heywood and Rich, 1968) for those synthesizing the proteins offeather keratin (molecular weight ∿10,000). The earlier studies were confused by the presence of artifactual ribosomal tetramers, induced by

chilling the tissue (Byers, 1966, 1971; Morimoto *et al.*, 1972a,b).

Steinert and Rogers (1971a,b) studied the ability of guinea pig hair follicle polyribosomes to incorporate amino acids into acid-insoluble material, and demonstrated that the major products of the cell-free system were the low-sulphur α-keratin proteins. Similar results have been obtained by Wilkinson (1971).

## E. AIMS OF THE PROJECT

In the preceding discussion, it was pointed out that the embryonic feather first grows and then commences the synthesis of keratin. The system is therefore unique among keratinizing systems in that tissue is readily available at different stages of maturation, and therefore presents an opportunity to study control processes operating during the onset of keratin synthesis. However, the keratin proteins of the embryonic feather had not been characterized and the course of keratin synthesis had not been described in quantitative terms.

The aims of the project were therefore:

1. To identify the keratin proteins of the embryonic chick feather, using as a basis the studies of Harrap and Woods (1964a,b; 1967) on reduced and S-carboxymethylated feather keratin proteins, and to develop suitable analytical procedures for the routine identifidation of the keratin proteins in protein extracts from feathers at different stages of development.

2. To use such procedures to obtain a quantitative description of the course of keratin synthesis during development of the embryonic feather.

3. To develop a suitable experimental system for the study of keratin synthesis and

4. to utilize the results of studies on the above aspects in the study of the control of keratin synthesis at the molecular level.

# CHAPTER 2

# MATERIALS AND GENERAL METHODS

## A. MATERIALS

### *1. Tissue*

In all experiments reported in this thesis, feathers used were from the domestic fowl, *Gallus gallus*. Adult white-leghorn fowls, strain Para 3, a pure-bred strain, were obtained from the Parafield Poultry Station of the Department of Agriculture, Parafield, South Australia. Adult birds were approximately one year old.

Fertilized eggs, a mixture of strains Para 1, 2 and 3, were obtained from the same source. The eggs were stored at 10° for no longer than 7 days, and incubated at 37.8°, 54% humidity in a forced-draught incubator (Saunders Products Pty. Ltd., Adelaide, South Australia) for the required time.

Feathers from eggs which had been incubated a total of, for example, 14 days were designated "14-day feathers".

### *2. Enzymes and Proteins*

*Albumin:* Bovine serum, fraction V. Sigma Chemical Co., St. Louis, U.S.A.

*Carboxypeptidase A:* Hog pancreas, crystallized. Sigma.

*Carboxypeptidase B:* Hog pancreas, in 0.1 M NaCl solution. Sigma.

*α-Chymotrypsin:* Bovine pancreas. Three times crystallized.

Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

*Elastase:* Hog pancreas. Type 1, Twice crystallized. Sigma.

*Insulin:* Crystalline beef. Commonwealth Serum Laboratories, Melbourne, Australia.

Samples of reduced and S-carboxymethylated-A and B chains prepared from this material were gifts from Dr. P.M. Steinert of this Department.

*α-Melanocyte Stimulating Hormone:* Gift from CIBA Chemical Company.

*Pronase:* B grade. Calbiochem, Los Angeles, California, U.S.A.

*Trypsin:* Bovine pancreas, trypsin TPCK. Worthington.

3. *Radioactive Compounds.*

*Acetyl-1[$^{14}C$]-Coenzyme A:* 59 or 57 mC/mmole.

*Algal hydrolysate:* [$^{3}$H]Reconstituted protein hydrolysate (Schwarz Mixture), Lot No. WR-2042. Schwarz Bioresearch, Orangeburg, N.Y., U.S.A.

*L-[$^{14}C$]-Leucine (G):* 316 mC/mmole. Schwarz.

*L-Methyl[$^{3}H$]-Methionine:* 2.6 C/mmole. Schwarz.

*L-[$^{3}H$]-Serine (G):* 1.2 C/mmole. Schwarz.

*Methyl-[$^{3}H$]-Thymidine:* 100 mC/mmole. Radiochemical Centre, Amersham, England.

*5-[$^{3}H$]-Uridine:* 31 C/mmole. Amersham.

4. *Fine Chemicals for Specific Procedures.*

(a) *Extraction, Reduction and Carboxymethylation of Proteins.*

*Ethanolamine:* B.D.H. Ltd., Poole, England.

*Iodoacetic acid:* Sigma. This was recrystallized from petroleum ether (BP 60-80) before use, and stored in the dark at room temperature.

*β-mercaptoethanol:* Sigma. This was distilled under reduced pressure before use, and stored at 2-4°.

*Tris:* Trizma base grade. Sigma.

*Urea:* Reagents puro, Carlo Erbo, Milan, Italy.

*(b) Polyacrylamide Gel Electrophoresis.*

*Acrylamide:* Eastman Organic Chemicals, New York, U.S.A.

*Ammonium Persulphate:* By-Products and Chemicals Pty. Ltd., Sydney, N.S.W., Australia.

*Coomassie Brilliant Blue R-250:* Mann Research Laboratories.

*Ethylenediacrylate:* Borden Chemical Co., Philadelphia, Penn, U.S.A.

*Glycine:* BDH.

*N,N'-methylenebisacrylamide:* Eastman.

*Riboflavin:* B.D.H.

*N,N,N',N'-tetramethylethylenediamine:* Eastman.

*(c) Measurement of Radioactivity.*

*1,4-bis-(5-phenyloxazolyl)-benzene:* Packard Instruments Co. Inc., La Grange, U.S.A.

*2,5-Diphenyloxazole:* Scintillation grade. Packard.

*Glass-fibre filters:* Whatman GF/C. W & R Balston Ltd., England.

*Shellsol A:* Shell Chemicals, Australia.

*Toluene:* Analytical reagent grade. B.D.H.

(d) *Column Chromatography.*

*DEAE-cellulose:* Whatman De-11.

*Dowex-1:* AG 1-XS, minus 400 Mesh, Bio-Rad Laboratories, Richmond, California, U.S.A.

*Dowex-2:* AG 2-X8, 100-200 Mesh, Bio-Rad.

*Dowex-50:* AG 50W-X2, 100-200 Mesh, Bio-Rad.

*Sephadex:* Grades G-100 fine, G-50 fine, G-25 fine and G-10. Pharmacia, Uppsala, Sweden.

(e) *Antibiotics.*

*Actinomycin-D:* Gift from Merck, Sharpe and Dohme Ltd.

*Amicetin:* Gift from Dr. J. Mercer, of this Department, University of Adelaide.

*Cycloheximide:* Sigma.

*Gougerotin:* Gift from Dr. J. Mercer.

*Penicillin G:* Sodium salt. Evans Medical Pty. Ltd., Melbourne, Australia.

*Sparsomycin:* Gift from Dr. A.R. Stanley, National Institutes of Health, Bethesda, Md., U.S.A.

*Streptomycin Sulphate:* Drug Houses of Australia.

5. *Miscellaneous Chemicals*

*Amino acids:* Mann Research Laboratories.

*N-acetyl-glycine:* B.D.H.

*N-acetyl-serine:* Gift from Mr. I.D. Walker of this Department.

*Dansyl amino acids:* Mann.

*Dansyl chloride:* Mann.

*Dithiothreitol:* Sigma.

*N-ethylmorpholine:* Eastman. This was distilled under reduced pressure before use and stored under nitrogen at 2-4°.

*Ninhydrin:* Pierce Chemicals.

6. *Miscellaneous Materials.*

*Cellulose thin-layers:* Chromatogram Sheet 6064. Eastman.

*Chromatography paper:* Whatman 3MM.

*Dialysis Tubing:* Visking. B.D.H. The tubing was boiled in 1% w/v $NaHCO_3$ before use (Thompson and O'Donnell, 1965).

*Graduated centrifuge tubes:* Type 8081. Corning Glass Works, New York, U.S.A.

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

## B. GENERAL METHODS

Only the most general techniques that were used routinely throughout this work are described in the present Chapter. All other procedures are described in the appropriate chapters.

### 1. *Amino-Acid Analysis*

Protein samples were hydrolysed in 6 N HCl *in vacuo* at 110° for 20 hours, unless otherwise stated. A small crystal of phenol was always present to prevent modification of tyrosine (Sanger and Thompson, 1963). HCl

was removed by rotary evaporation. Hydrolsates from protein samples (generally of about 200 µg) were analysed by the procedure of Piez and Morris (1960) using a Beckman 120C analyser modified as described by Harding (1971). Hydrolysates from blocked peptides were usually analysed using only the acidics/neutrals column of the Beckman 120C analyser in instances when thepresence of basic amino acids was not anticipated.

2. *Determination of Radioactivity*

Samples dried on glass-fibre filters were usually counted in a toluene-based scintillation fluid containing 0.35% w/v 2,5-diphenyloxazole and 0.035% w/v 1,4-bis-(5-phenyloxazolyl)-benzene. Occasionally, for economy, "Shellsol-A" was substituted for toluene, although this resulted in decreased efficiency. A Packard Tricarb Liquid Scintillation Spectrometer was used for all radio-activity determinations. Results are generally expressed as the actual counts per minute observed in the aliquot, after subtraction of the background value. The observed counts per minute were generally recorded over 10 or 20 minute periods.

3. *Densitometry of Polyacrylamide Gels.*

Gels were immersed in 10% TCA (Chrambach *et al.*, 1967) and scanned in a "Densicord" recording electrophoresis densitometer (Photovolt Corp., New York, U.S.A.) equipped with an "Integraph" automatic integrator, model 49

(Photovolt Corp.). The white light source and red filter (no. 610 ) were used, with the sensitivity setting on D1 and a scanning speed: chart speed ratio of 1:2. Relative areas under the peaks were determined from the automatic integrator, after subtraction of the background, determined on a suitable area of each gel.

## C. NOMENCLATURE

Most of the studies reported in this thesis were conducted on S-carboxymethylated proteins. To avoid tedium, the prefix SCM- has not been generally used. Thus the terms "feather keratins" "feather proteins" etc., when referring to *extracted* proteins, are defined for the purposes of this thesis as being the S-carboxymethyl derivatives of these proteins, unless otherwise stated. This definition obviously does not apply to any reference to these proteins in their native state.

# CHAPTER 3

# CHARACTERIZATION OF THE KERATIN PROTEINS FROM ADULT AND EMBRYONIC FEATHERS AND SCALES

## A. INTRODUCTION

The present chapter describes studies on the reduced and carboxymethylated proteins extracted from various fully keratinized tissues of the newly-hatched chicken and adult fowl.

The aim of this work was to characterize the proteins of the embryonic chicken feather, in order to develop an assay system for the study of keratin synthesis during development of the embryonic feather. The approach chosen was based on the observations of Harrap and Woods (1964a,b; 1967) who demonstrated that S-carboxymethyl-proteins from the adult feather were amenable to characterization, and the observations of Rogers and co-workers (Rogers and Clarke, 1965b; Kemp and Rogers, 1970; Steinert, 1972) who demonstrated that characterization of S-carboxymethyl-proteins from guinea pig hair provided an approach to studies on the biosynthesis of hair keratins.

During the course of studies on the proteins of various feather tissues, it became apparent that there were differences between them, as observed by Harrap and Woods (1964a). It was therefore of particular interest to determine whether these differences originated from the presence of dissimilar polypeptide chains in the distinct feather tissues that develop from embryonic epidermis, or from varying amounts of the same polypeptide chains. The results presented firmly establish that the keratin proteins synthesized in certain distinct feather tissues are in fact different.

## B. METHODS

### 1. *Preparation of Tissues*

Adult wing feathers were dissected with scissors into their four morphological parts, namely rachis, calamus, barbs and medulla (Chapter 1.A.1.b). Embryonic body feathers were plucked by hand. Scales were removed from the anterior tarsometatarsal region with the aid of forceps. Feather sheaths were collected from a tray in which several chickens had hatched and discarded their sheaths. Contaminating matter was removed from the sheaths by swirling them in acetone in a petri dish. The sheaths accumulated around the outer edge of the dish during this process, and were collected with a wide-mouthed pasteur pipette.

All tissues were washed successively in 0.1% w/v Tween 80 detergent, water, ethanol and acetone, and dried in a stream of warm air. Rachis, calamus and adult scales were cut into pieces approximately 3 mm x 5 mm with scissors, to facilitate their subsequent solubilization. Protein was extracted by reduction and carboxymethylation.

### 2. *Preparation of Reduced and S-carboxymethylated Protein Extracts*

A procedure for the preparation of reduced and S-carboxymethylated feather keratin proteins was developed, based on the results of Harrap and Woods (1964a) and O'Donnell and Thompson (1964). The procedure satisfied

the requirement for the rapid and routine extraction of protein from multiple samples of tissue. It gave high yeilds with essentially complete carboxymethylation and with minimal risk of protein degradation or chemical modification.

Tissue samples were placed in 25 ml conical "Quick-fit" flasks, and incubated at 37° for 3 hr. with a solution containing 8.0 M urea, 0.1 M β-mercaptoethanol, and 0.5 M ethanolamine/HCl, pH 10.5 ("reducing solution") on a gyro-tatory shaker (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.). A ratio of 10 ml of "reducing solution" per 100 mg of tissue sample was generally used. The tissue was homogenized after one and three hr. of incubation using a hand-held Potter-Elvejhem homogenizer. For each 10 ml of "reducing solution" used, 6 ml of a solution containing 3.0 M Tris/HCl at pH 8.0 was added in order to lower the pH of the reaction mixture to pH 8.7 - 8.8. Carboxy-methylation was effected by adding 2.0 ml of a 30% w/v solution of iodoacetic acid that was 0.3 M with respect to Tris and had been neutralized and adjusted to pH 8.2 by the addition of 5.0 M KOH. The pH of the reaction mixture at this stage was 8.6 - 8.7. The carboxymethylation reaction was allowed to proceed for 20min at room temperature by which time the mixture was negative to the nitroprusside test (Feigl, 1947). β-mercaptoethanol (0.3 ml) was then added, and the reaction mixture was incubated at 37° for 30 min on a gyrorotary shaker. A further 2 ml of the iodoacetate solution was then added

and the mixture was again allowed to stand for 20 min at room temperature, in order to effect a second cycle of reduction and carboxymethylation. β -mercaptoethanol (0.4 ml) was added to destroy excess iodoacetate, and insoluble debris was removed by centrifugation (36,000 g, 1 hr., 25°). The supernatant was dialysed exhaustively, first against monodistilled water and then against glass-distilled water. The dialysate was then freeze-dried.

### 3. *Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) at pH 9.5 using 10% polyacrylamide gels was performed as described by Canal Industrial Corp., Bethesda, Md., with urea at a final concentration of 5 M in the gels and loading buffer.

PAGE at pH 7.5 was performed as described by Williams and Reisfeld (1964), using the orthophosphoric acid buffer system. The gels contained urea at a final concentration of 5 M. Protein samples were loaded in the high pH gel loading buffer.

PAGE at pH 2.7 in 2.5 M urea was performed as described by Panyim and Chalkely (1969). Protein bands were stained with Coomassie Brilliant Blue R-250 in 10% TCA (Chambrach *et al.*, 1967) and recorded with the Photovolt Densitometer.

### 4. *Preparative Polyacrylamide Gel Electrophoresis*

Protein fractions were prepared from the adult

feather rachic proteins by PAGE at pH 9.5 as described above, using a large scale (1 litre gel capacity) cooled slab-electrophoresis apparatus, essentially similar to that described by Raymond (1964). After a run the gel slab was placed in 10% TCA to precipitate the proteins bands. The regions of gel containing visible precipitated protein bands were cut out of the slab with a razor blade and homogenized briefly, using a Lourdes homogenizer, in 20 volumes of a solution containing 4 M urea, 0.005 M EDTA, and 1 M Tris/HCl, pH 9.0. The homogenized gel was extracted with at least 4 changes of this solution for a minimum of 24 hr. Gel particles were removed by filtration and centrifugation and the extracted protein was recovered by freeze-drying or rotary-evaporating the extract after dialysis against glass distilled water. Three successive electrophoretic fractionations were required to obtain a sufficient degree of purity. Yields were approximately 25% for each cycle.

5. *Isoelectric Focussing*

Gel isoelectric focussing on the extracted proteins was performed by the technique of Wrigley (1968), using pH 3-5 ampholytes (LKB Produkter, Stockholm) with or without 2 M urea.

6. *Peptide Mapping*

Protein samples were digested at 10 mg/ml concentration in 0.2 M N-ethylmorpholine acetate buffer,

pH 8.3 (NEMA) for 2 hr. at 25° using trypsin treated with N-tosylphenylalanine-chloromethyl ketone at 1% w/w enzyme/substrate ratio. The peptides were subjected to electrophoresis in pyridine acetate buffer, pH 4.7 , for 1 hr. at 1000V on cellulose TLC plates.

### 7. *Immunological Techniques*

Antisera to S-carboxymethyl-keratins from whole adult feathers were prepared in rabbits and the γ-globulin fraction obtained from these sera using methods employed for guinea pig hair follicle S-carboxymethyl-proteins (Kemp and Rogers, 1970). Immunodiffusion was performed by the method of Ouchterlony (1962) using 1% agar gels in 0.01 M sodium phosphate buffer, pH 7.1.

### 8. *Aggregation*

Protein samples (10 mg) were dissolved in 1 ml of NEMA and incubated at 37° for 24 hr. They were then examined by electron microscopy, using negative staining and stored at 2-4° to allow gel formation.

## C. RESULTS

### 1. *Preparation of Reduced S-carboxymethyl Proteins*

Initially, feather proteins were extracted by the procedure of Harrap and Woods (1964a). Yields and PAGE patterns similar to those reported by Harrap and Woods (1964a, 1967) were obtained for rachis proteins.

However, lengthy extractions were required and furthermore, the use of thioglycollic acid as a reducing agent can lead to chemical modification of proteins by contaminating thio-esters (White, 1960). Therefore, a rapid method for protein extraction involving β-mercaptoethanol as used by O'Donnell and Thompson (1964) for the extraction of wool proteins was investigated. This method usually gave identical results to the procedure of Harrap and Woods (1964a) but ocassionally, for unknown reasons, the pH of the extraction mixture rose to 12.0 - 12.5, and it was found that a series of additional bands of low mobility resulted, as determined by PAGE at pH 9.5. In order to overcome the problem, 0.5 M ethanolamine was used to buffer the solution at pH 10.5. Ethanolamine was chosen as the buffer because such a compound would be expected to react with any cyanate present in the concentrated urea reaction mixture, thereby protecting the proteins against carbamylation.

All three extraction procedures yielded identical patterns for rachis and barb proteins, as determined by PAGE at pH 9.5. Yields obtained using the β-mercaptoethanol-urea-ethanolamine-HCl procedure are shown in Table 3.1. The spectral properties of all the preparations indicated that they were not detectably contaminated with nucleic acids.

TABLE 3.1.

YIELDS OF SOLUBLE PROTEIN FROM THE VARIOUS TISSUES.

Yields are shown as dry weights of the products as percentages of the dry weight of the starting material.

| Tissue | Yield |
|---|---|
| Rachis[a] | 84 |
| Calamus[a] | 81 |
| Barbs[a] | 82 |
| Medulla[b] | 90 |
| Embryonic Feather[c] | 76 |
| Adult Scales[a] | 76 |
| Embryonic Scales[a] | 73 |

[a]Average of 2 independent experiments.

[b]1 Experiment only.

[c]Average of 4 independent experiments.

## 2. *Properties of Reduced S-carboxymethyl Proteins from Adult and Embryonic Feathers and Scales*

### *(a) Solubility of the Proteins in Water.*

During the dialysis step of the preparation, a fine, opalescent suspension appeared in the extracts from embryonic feathers and from adult and embryonic scales, but not in the extracts from adult feather components. This water-insoluble material was not removed prior to freeze-drying unless otherwise stated, as the aim of the work reported in this Chapter was to characterize all the proteins present in such extracts. Some properties of the water-insoluble material are described in Chapter 3.C.4.

### *(b) Polyacrylamide Gel Electrophoresis at pH 9.5.*

In preliminary studies, the use of gels of 7.5, 10 and 15% acrylamide concentration was investigated in the presence or absence of 5 M urea. Similar band patterns were obtained in all cases but 10% gels were the most satisfactory. The presence of urea prevented occasional "streaking" presumably due to aggregation. Coomassie Blue stain appeared to be more sensitive than Amido Black, although both gave qualitatively identical patterns.

Typical patterns obtained by PAGE at pH 9.5 are shown in Figure 3.1. In each case, considerable fractionation was obtained. Three groups of bands were apparent. The fastest moving ($\alpha$) group of these, designated bands $\alpha 1$- $\alpha 4$ occurred in small amounts, predominantly in barbs, medulla and embryonic feather. The second ($\beta$) group, of intermediate

FIGURE 3.1.

PAGE at pH 9.5 of proteins from adult and embryonic scales and feathers. 10% acrylamide gels containing 5 M urea, 8 cm long were used. 50µg protein/gel was loaded. Electrophoresis was performed at 2 mA/gel until the bromophenol blue marker reached the end of the gel. Bands were stained with Coomassie Blue. The division of bands into groups α, β and γ and the nomenclature for all bands is shown. α1 - γ4 refer to the feather proteins. βS1 - γS4 refer to the scale proteins.

R: - rachis proteins

C: - calamus proteins

B: - barbs proteins

M: - medulla proteins

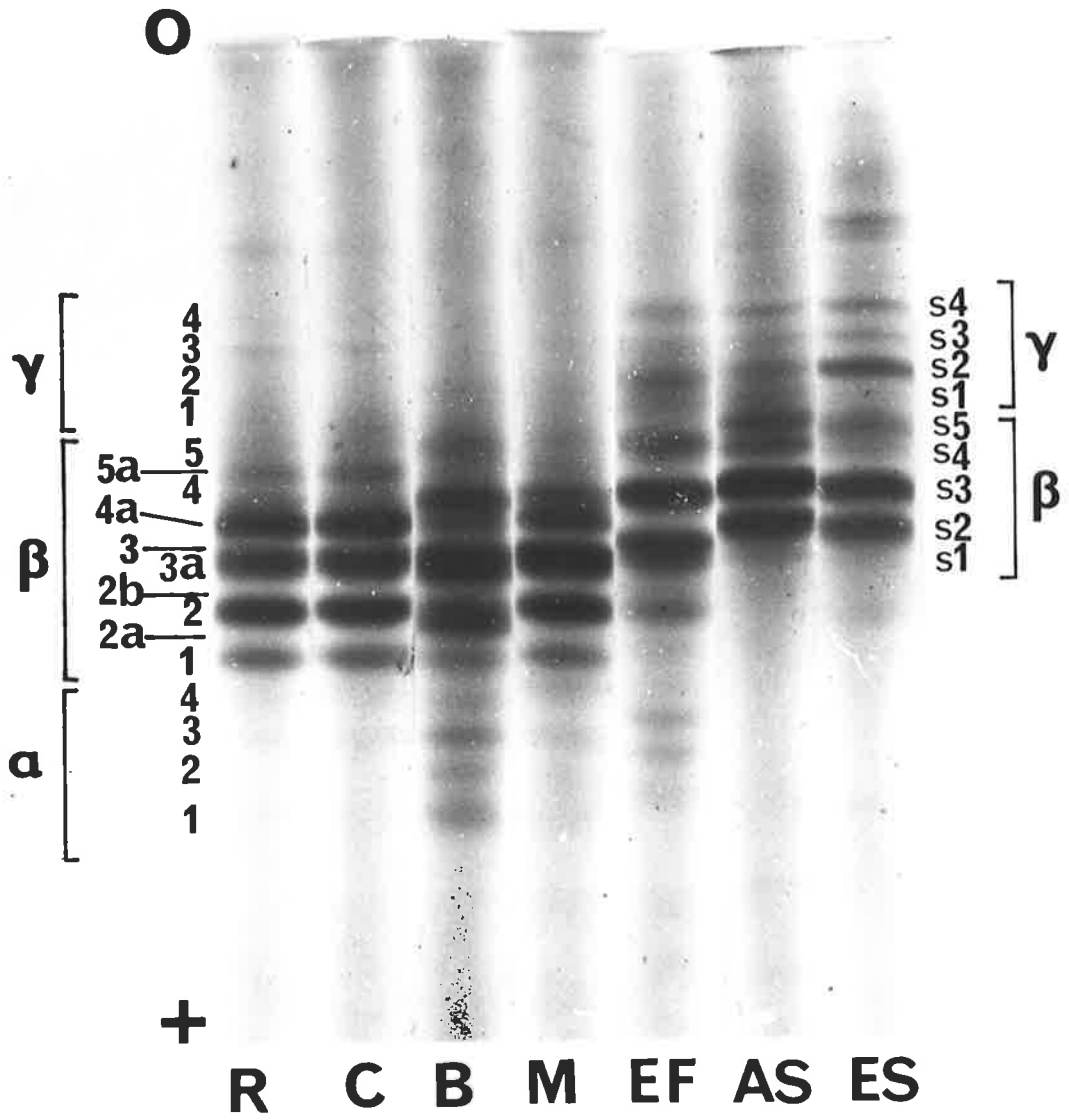
EF: - embryonic feather proteins

AS: - adult scale proteins

ES: - embryonic scale proteins

O: - origin

\+ - anode


O
γ
β
α
4
3
2
1
5a
4a
3
2b
2a
5
4
3a
2
1
4
3
2
1
+
s4
s3
s2
s1
s5
s4
s3
s2
s1
γ
β
R
C
B
M
EF
AS
ES

mobility, designated bands β1- β5 contained the major proteins of each tissue although the distribution of these varied greatly from tissue to tissue. The third group (γ), that are the slowest moving bands and designed γ1- γ4, occurred, in embryonic feather and in both adult and embryonic scales but were virtually absent from adult feather. These γ-bands varied in amount relative to the β-bands in different preparations.

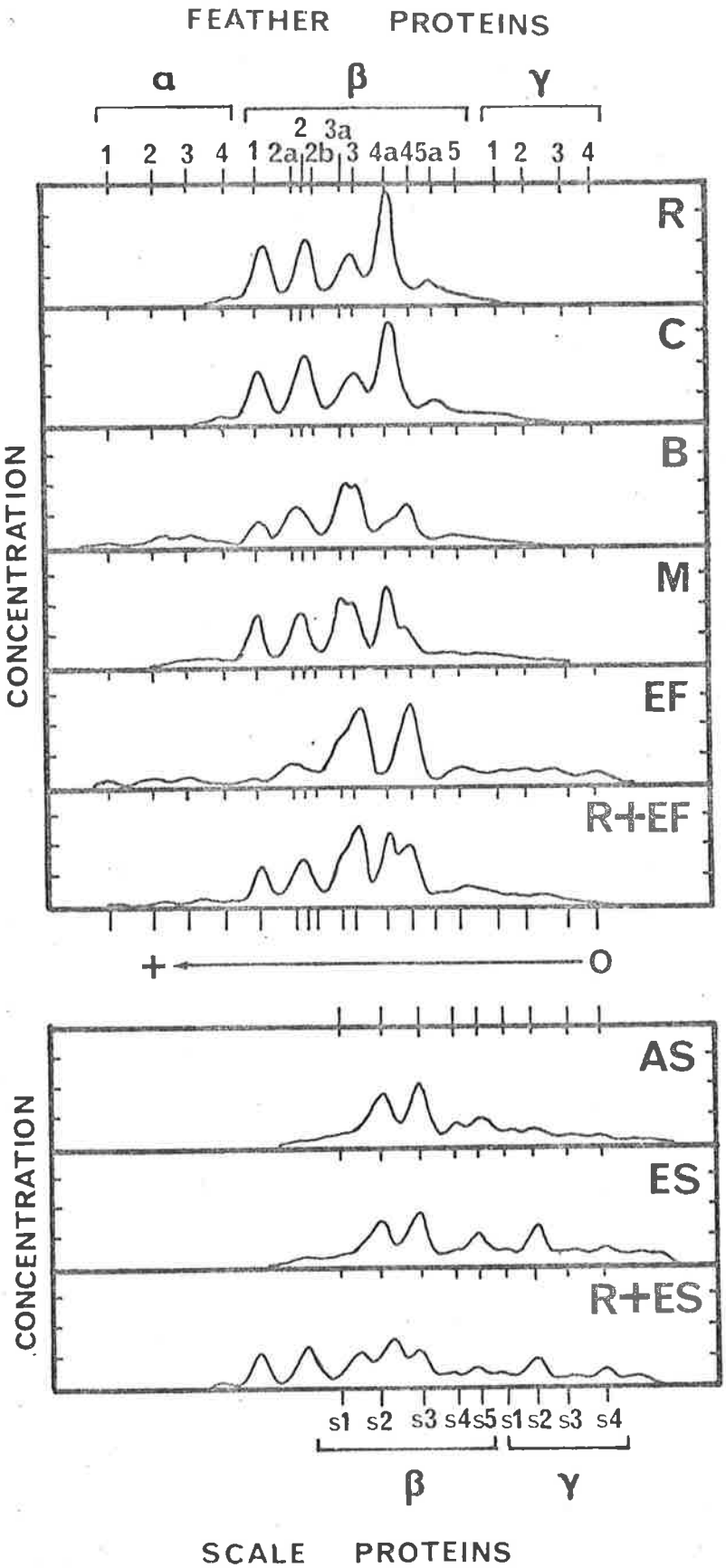
The differences between the β-group of bands of each tissue are demonstrated more clearly by densitometer tracings of the gels (Figure 3.2). Bands β1- β4 of rachis and calamus appeared as symmetrical peaks, but bands β3 and β4 of barbs and medulla quite clearly each contained at least two components, designated β3 and β3a, β4 and β4a respectively. Embryonic feather gave a distinctly different pattern. However, co-electrophoresis of embryonic feather proteins with rachis proteins (Figure 3.2, R + EF) demonstrated that the major proteins of embryonic feather were in bands β3 and β4.

Adult and embryonic scale proteins appeared identical to each other. Co-electrophoresis of embryonic scale proteins with adult rachis proteins (Figure 3.2, R + ES) and embryonic scale proteins with embryonic feather proteins (not shown) demonstrated that the major bands of the β-region of feather proteins have different mobilities to those of scale proteins.

The identity or non-identity of mobility of the

FIGURE 3.2.

Densitometer traces of the protein-containing regions of the high-pH gels shown in Figure 3.1.
R, C etc.: as in Figure 1. R + EF: co-electrophoresis of rachis proteins (25 µg) with embryonic feather proteins (25 µg).
R + ES: co-electrophoresis of rachis proteins (25 µg) with embryonic scale proteins (25 µg).
The direction of migration is indicated by the arrow. Concentration is in arbitrary units and refers to the concentrations of Coomassie Blue dye bound.


FEATHER PROTEINS
α
β
γ
1 2 3 4 1 2a 2b 3 2 3a 4a 4a5a 5 1 2 3 4
R
C
B
M
EF
R+EF
CONCENTRATION
+
O
AS
ES
R+ES
CONCENTRATION
s1 s2 s3 s4 s5 s1 s2 s3 s4
β
γ
SCALE PROTEINS

protein bands in preparations from the various tissues, as deduced above from comparison of gels and co-electrophoresis, was confirmed by PAGE at pH 9.5 using an analytical slab-gel apparatus in which the preparations were fractionated as a series of adjacent, overlapping sets of bands.

*(c) Polyacrylamide Gel Electrophoresis at pH 7.5.*

Fractionation of the feather and scale proteins by PAGE at pH 7.5 (Figure 3.3) resulted in patterns similar to those obtained by PAGE at pH 9.5, particularly in the case of the α- and β-proteins. The γ-proteins however, gave different patterns. Subsequent studies (by I.D. Walker) have confirmed the identity of the α- and β-proteins, but the relationship between particular γ-protein bands on pH 9.5 and pH 7.5 gels is not clear.

*(d) Polyacrylamide Gel Electrophoresis at pH 2.7.*

Using the 8 cm gel system at pH 2.7 (Figure 3.4) a maximum of eight major bands (A - H) was observed in the preparations. In addition, a band of much greater mobility (the "fast" band) was present in embryonic feather and as a trace in embryonic scales, but not in the other tissues (Figure 3.4a). It was subsequently found that on 20 cm gels (Figs. 3.5 and 3.6) most of these bands could be further resolved into additional ones designed A1 - H2. The major protein bands of embryonic feather (A1 - E) were also observed in barbs and medulla whereas only traces were present in rachis and calamus. The groups of bands

FIGURE 3.3

PAGE at pH 7.5 of proteins from adult and embryonic scales and feathers. 7.5% acrylamide gels containing 5 M urea, 8 cm long were used. 100 µg protein/gel was loaded.

All other details as for Figure 3.1.

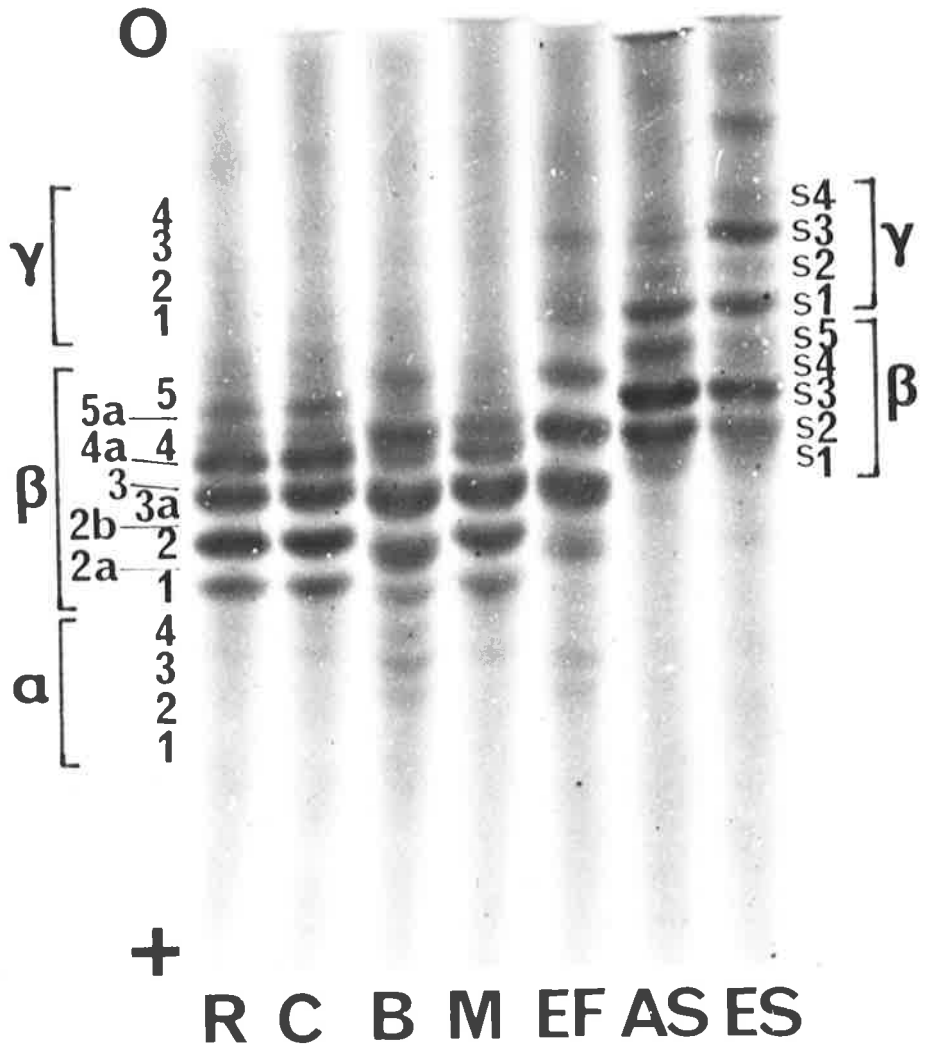

O
γ
4
3
2
1
β
5a
5
4a
4
3
3a
2b
2
2a
1
α
4
3
2
1
+
s4
s3
s2
s1
γ
s5
s4
s3
s2
s1
β
R C B M EF AS ES

FIGURE 3.4.

PAGE at pH 2.7 of proteins from adult and embryonic feathers and scales. 50 µg protein/gel was loaded. 15% gels containing 2.5 M urea, 8 cm long were used. Electrophoresis was performed at 2 mA/gel for (a) 2 hr; (b) 4 hr. Bands were stained with Coomassie Blue.

R, C etc.: - as in Figure 3.1.

- : - cathode

Feather protein bands and scale protein bands are designated A - H and SA - SE respectively. The "fast" component of embryonic feather can be seen in Figure 3.4a. In Figure 3.4b, the difference in mobility of feather and scale proteins is clear.

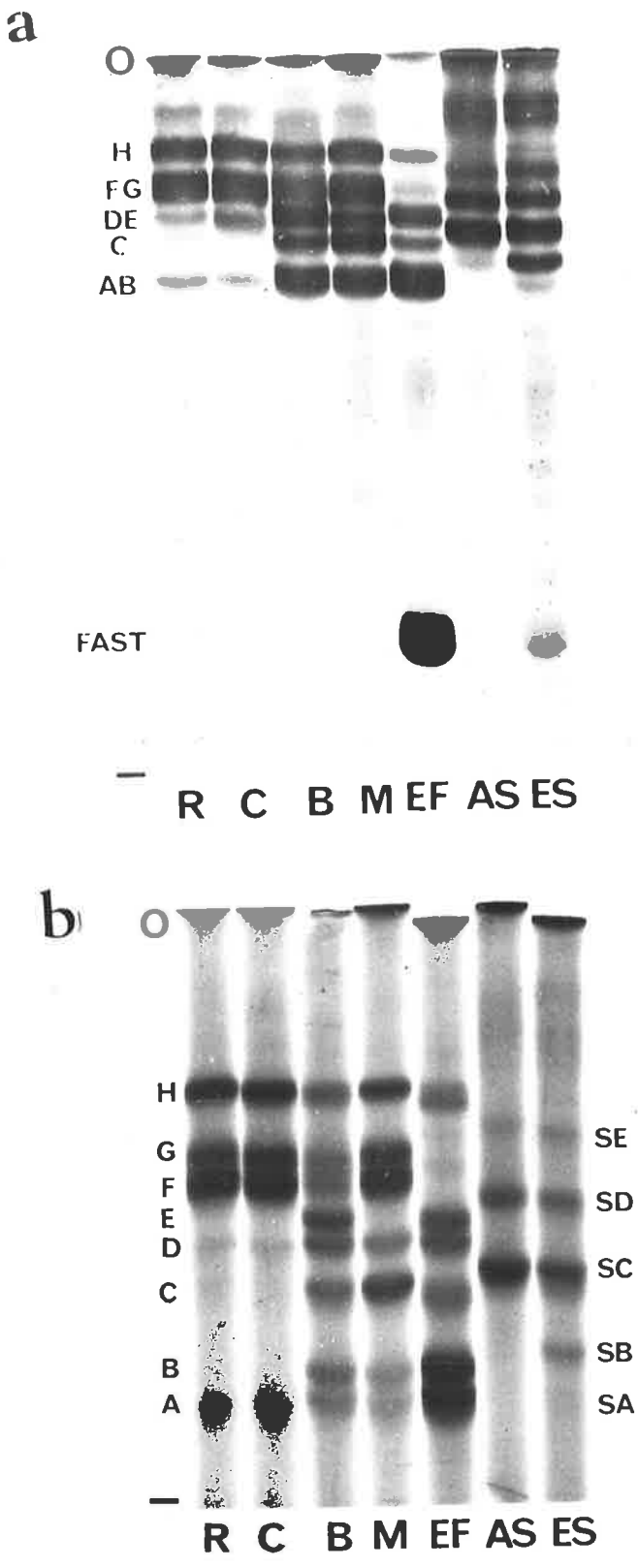

a
O
H
FG
DE
C
AB
FAST
R C B M EF AS ES
b
O
H
G
F
E
D
C
B
A
SE
SD
SC
SB
SA
R C B M EF AS ES

FIGURE 3.5.

PAGE at pH 2.7 of proteins from adult and embryonic feathers. 100 µg protein/gel was loaded. 15% gels containing 2.5 M urea, 20 cm long were used. Electrophoresis was performed at 500 volts for 24 hr. Bands were stained with Coomassie Blue.

R, C, etc. : - as in Figure 3.1.

- : - cathode.

Bands are designated A1 - H2.

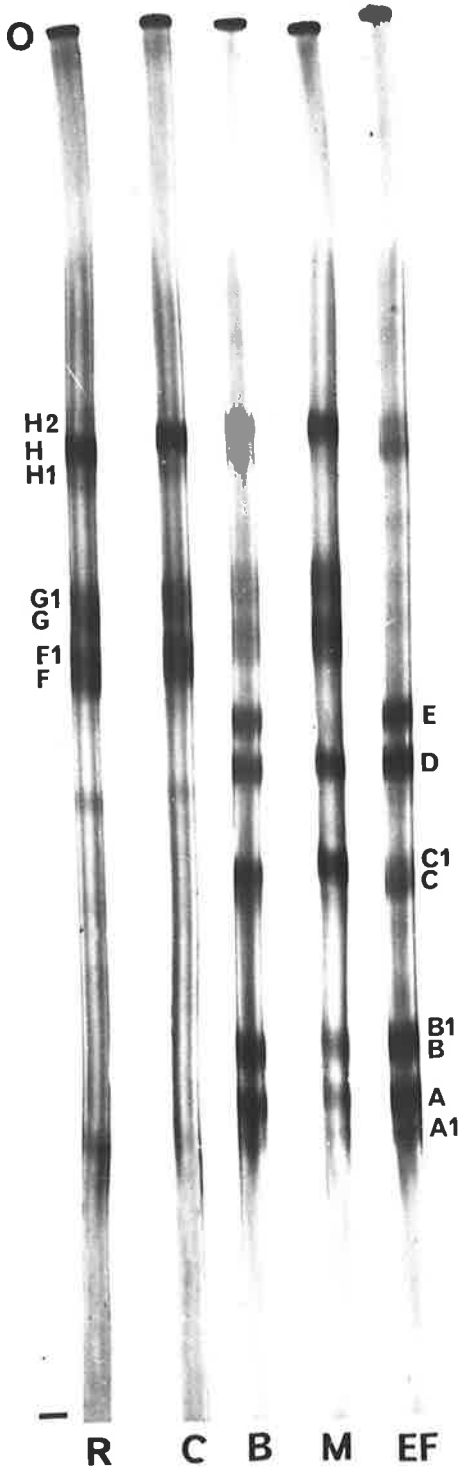
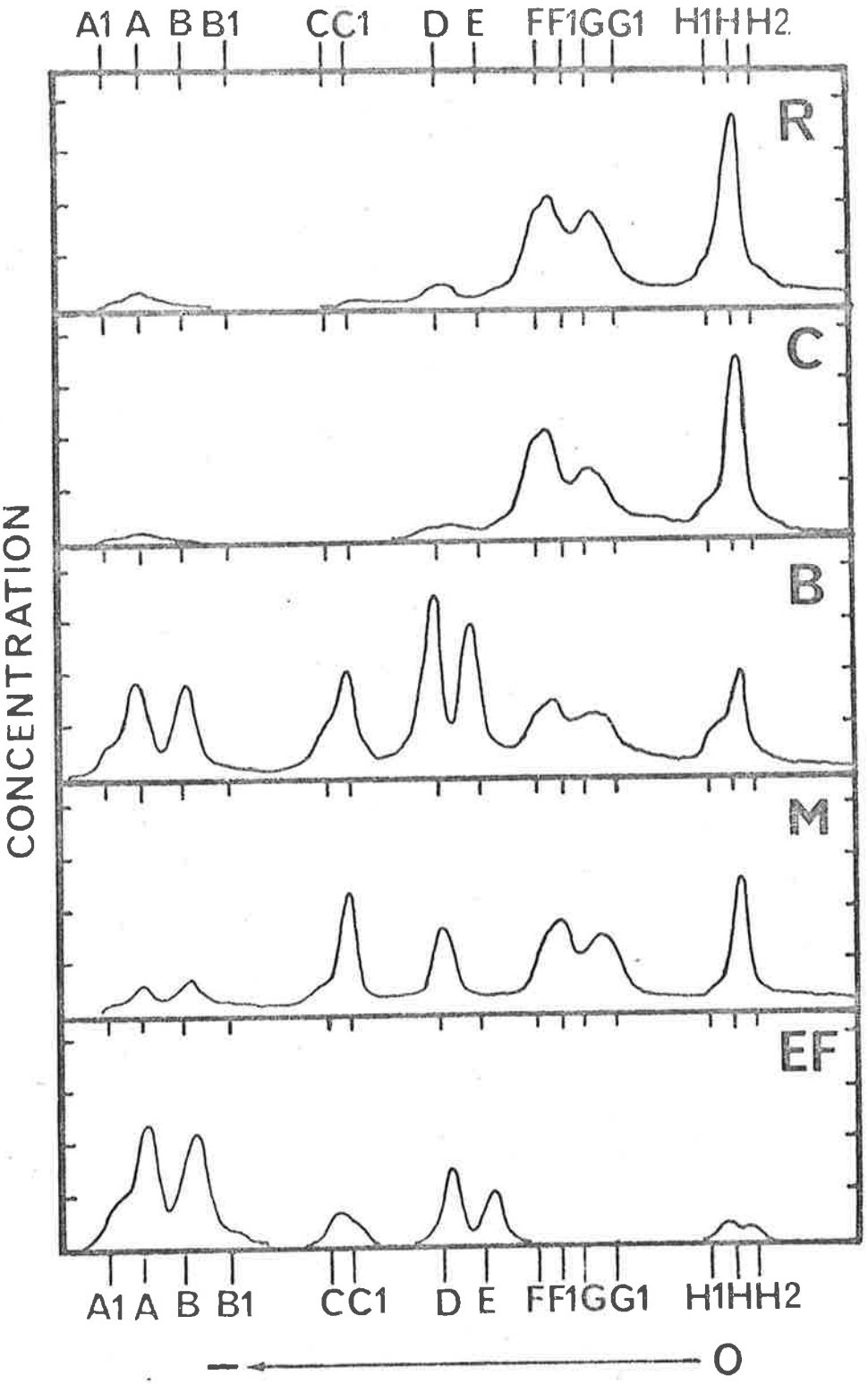

O
H2
H
H1
G1
G
F1
F
E
D
C1
C
B1
B
A
A1
R
C
B
M
EF

FIGURE 3.6.

Densitometer traces of the low-pH gels shown in Figure 3.5. Details as in Figure 3.2. Feather protein bands A1 - H2 are indicated. It is clear from densitometry that in the F - G region, particularly in the case of calamus, there are minor components at the leading and trailing edges. However, for simplicity, these will not be discussed further.


A1 A B B1 C C1 D E F F1 G G1 H1 H H2
R
C
B
M
EF
CONCENTRATION
A1 A B B1 C C1 D E F F1 G G1 H1 H H2
O

designated F and G, derived from proteins of rachis, calamus, barbs and medulla of adult feather were absent in embryonic feather although there were traces of similar bands in this region. Band E of embryonic feather was not detectable in rachis, calamus or medulla.

The protein bands of adult and embryonic scales were qualitatively identical to each other (Figure 3.4) but they differed in their relative amounts. All the bands of scale (SA - SE) had different mobilities from those of feathers.

*(e) Isoelectric Focussing.*

Preliminary experiments using wide range ampholytes (pH 3-10) indicated that all detectable proteins in the various preparations had isoelectric points in the region of pH 3-4. In further experiments therefore, ampholytes in the range of pH 3-5 were used. The number and pattern of bands were found to be dependent on the time the current was applied although reproducible at a given time (Frater, 1970). The clearest pattern was obtained at 5 hr. (Figure 3.7).

It can be seen that the different parts of adult feather have some protein bands in common and that some of these are present in embryonic feather. However, in the high pH region some of the protein components are completely absent from rachis and calamus. Isoelectric focussing of embryonic feather proteins demonstrated that this tissue has a large proportion of protein components that have isolectric points that are in the higher region of the pH

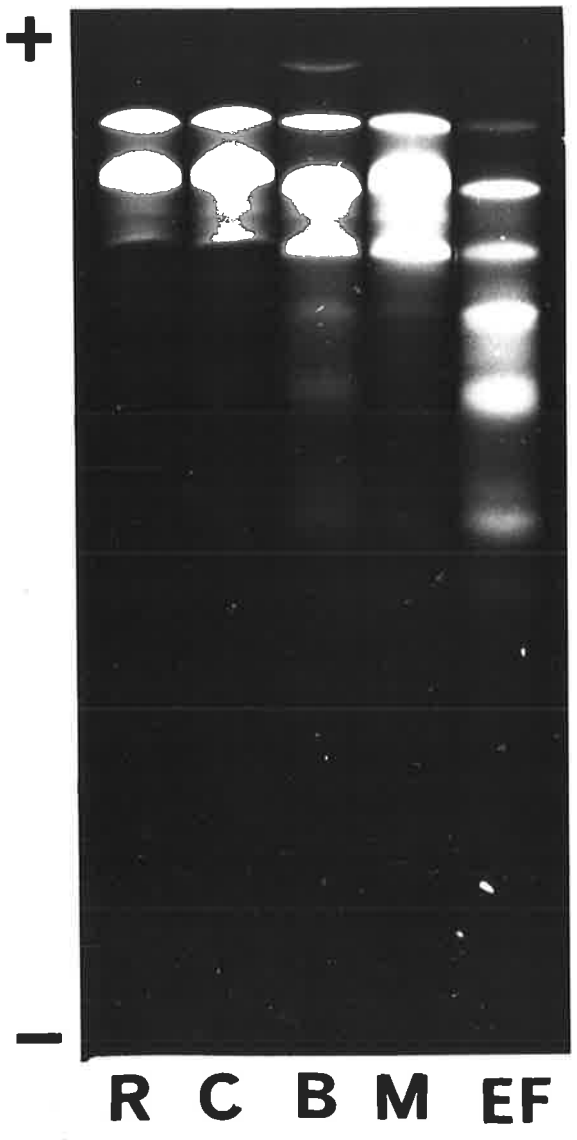
FIGURE 3.7.

Isoelectric focussing in polyacrylamide gels, in the absence of urea, for 5 hr, using pH 3 - 5 ampholytes. 50 µg protein/gel was loaded. Bands were precipitated with 10% TCA and photographed unstained, using darkfield illumination.

R, C etc.-: - as in Figure 3.1.

\+ : - anode (low pH) end.

\- : - cathode (high pH) end.


+
−
R C B M EF

range 3-4. This result is consistent with the observations made by PAGE at pH 2.7 and by amino acid analysis.

The proteins of scales did not focus under these conditions, but precipitated in the gels throughout their length. The addition of 2 M urea to the gels prevented this precipitation but the pH gradient did not extend below pH 3.45 after a 5 hr. run. The resulting patterns for both feathers and scales were not reproducible and did not allow any firm conclusions to be drawn.

*(f) Peptide Mapping.*

One-dimensional electrophoresis (Figure 3.8) of the tryptic peptides prepared from the proteins of each tissue gave results consistent with the above observations. Barbs, medulla and embryonic feather gave patterns that were similar and were more complex than those of rachis and calamus. Adult and embryonic scales were again similar to each other, but contained some peptides not present in the feather preparations. The same results were obtained when the peptides were fractionated by paper electrophoresis at pH 4.7, 3.7 and 6.5 in pyridine-acetate buffers.

*(g) Immunodiffusion.*

Preliminary experiments indicated that the precipitin line patterns obtained were dependent upon antigen concentration. Double-diffusion on the various preparations against anti-feather-protein serum is shown in Figure 3.9, at three different antigen concentrations. Rachis and

FIGURE 3.8.

Electrophoresis on cellulose thin-layers of tryptic peptides from feather and scale proteins. Approximately 500 µg of each preparation was loaded. Conditions were as given in Methods. Stained with ninhydrin.

R, C etc.: - as in Figure 3.1.

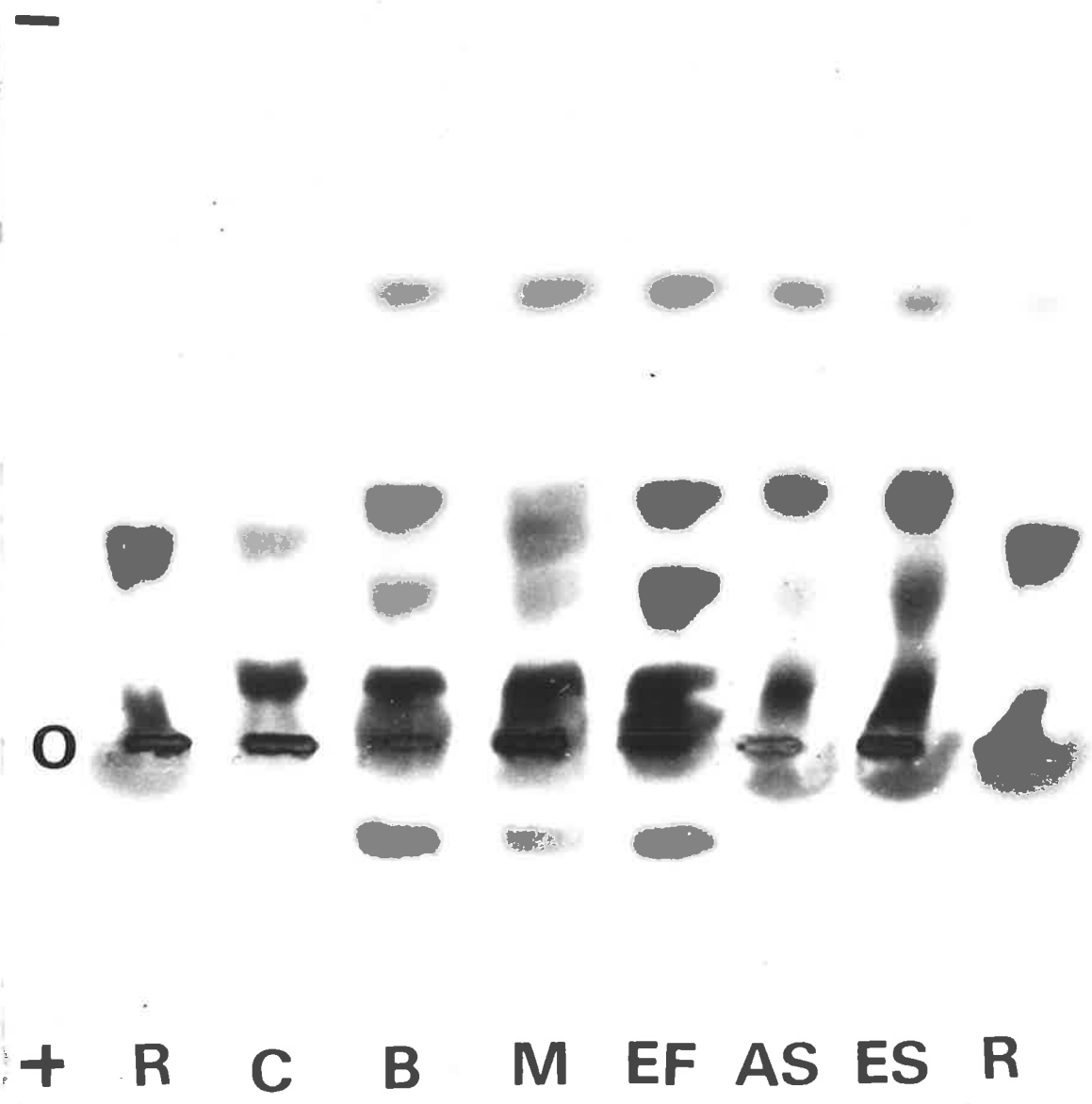

−
O
+ R C B M EF AS ES R

Figure 3.9.

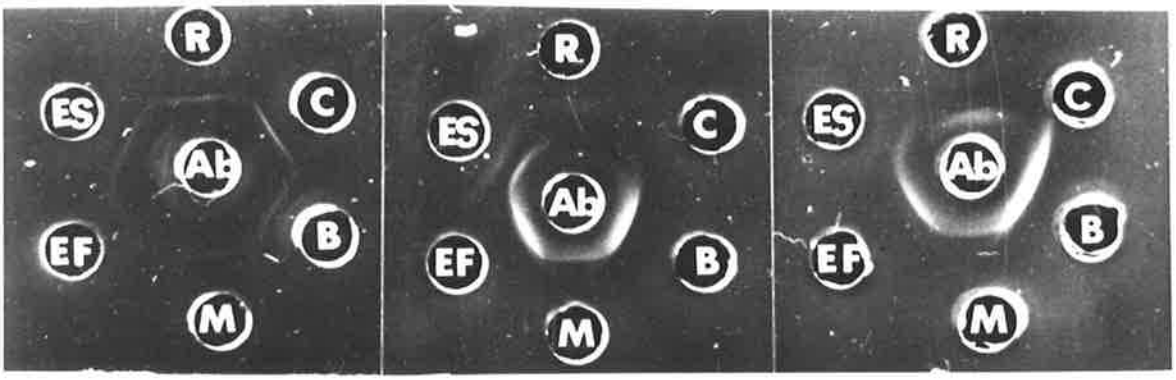
Immunodiffusion of proteins from adult and embryonic feathers and scales against rabbit antifeather SCM-proteins serum. Protein (antigen) concentration:

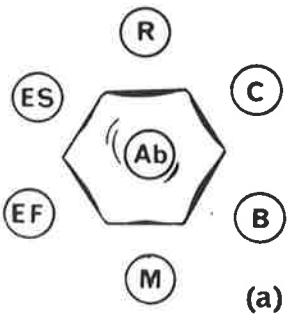
(a) 10 mg/ml; (b) 1.25 mg/ml; (c) 0.31 mg/ml

R, C, etc.: - as in Figure 3.1.

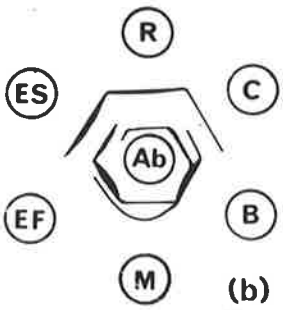
Ab: - Antiserum.

The unstained gels were photographed using darkfield illumination.

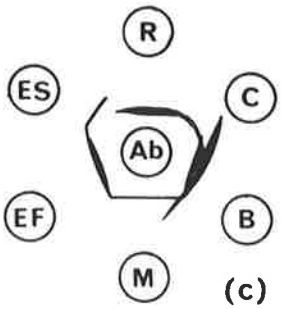



(a)




(b)




(c)

calamus were immunologically identical at all antigen concentrations tested. At the lower concentrations, all other feather preparations contained at least one additional antigen (precipitin line) not present in rachis and calamus. Scale proteins contained at least two antigens common to feather.

These reactions were not prevented by the incorporation of 0.025 M S-carboxymethyl-cysteine (a potential hapten inhibitor) into the agar, indicating that the reactions were not determined by the S-carboxymethyl groups. Also, no reaction was obtained with S-carboxymethyl-proteins from guinea pig hair, further indicating that hapten effects were not involved.

*(h) Aggregation.*

Harrap and Woods (1967) found that S-carboxymethyl-keratin preparations from adult rachis aggregated into a gel if allowed to stand for some time in borate buffer and that long fibrils were formed during this process (Filshie *et al.*, 1964). Consequently, samples of theproteins from adult feather rachis, calamus, barbs and medulla, from embryonic feather and scales and from adult scales were tested for their ability to aggregate. All formed apparently identical fibrils within 24 hr as determined by electron microscopy (Figure 3.10) and all eventually gelled.

*(i) Amino Acid Compositions.*

Amino acid compositions of the proteins from each tissue are shown in Table 3.2. The results for the adult feather components agree well with those of Harrap and Woods

FIGURE 3.10.

Electron-micrograph of fibrils prepared from feather and scale proteins. Negatively stained with uranyl-acetate. Fibrils of similar diameter are evident in each preparation. The length of the fibrils varies in the different preparations. The length increases with increasing times of incubation.

R, B etc.: - as in Figure 3.1.

Magnification: X60,000.

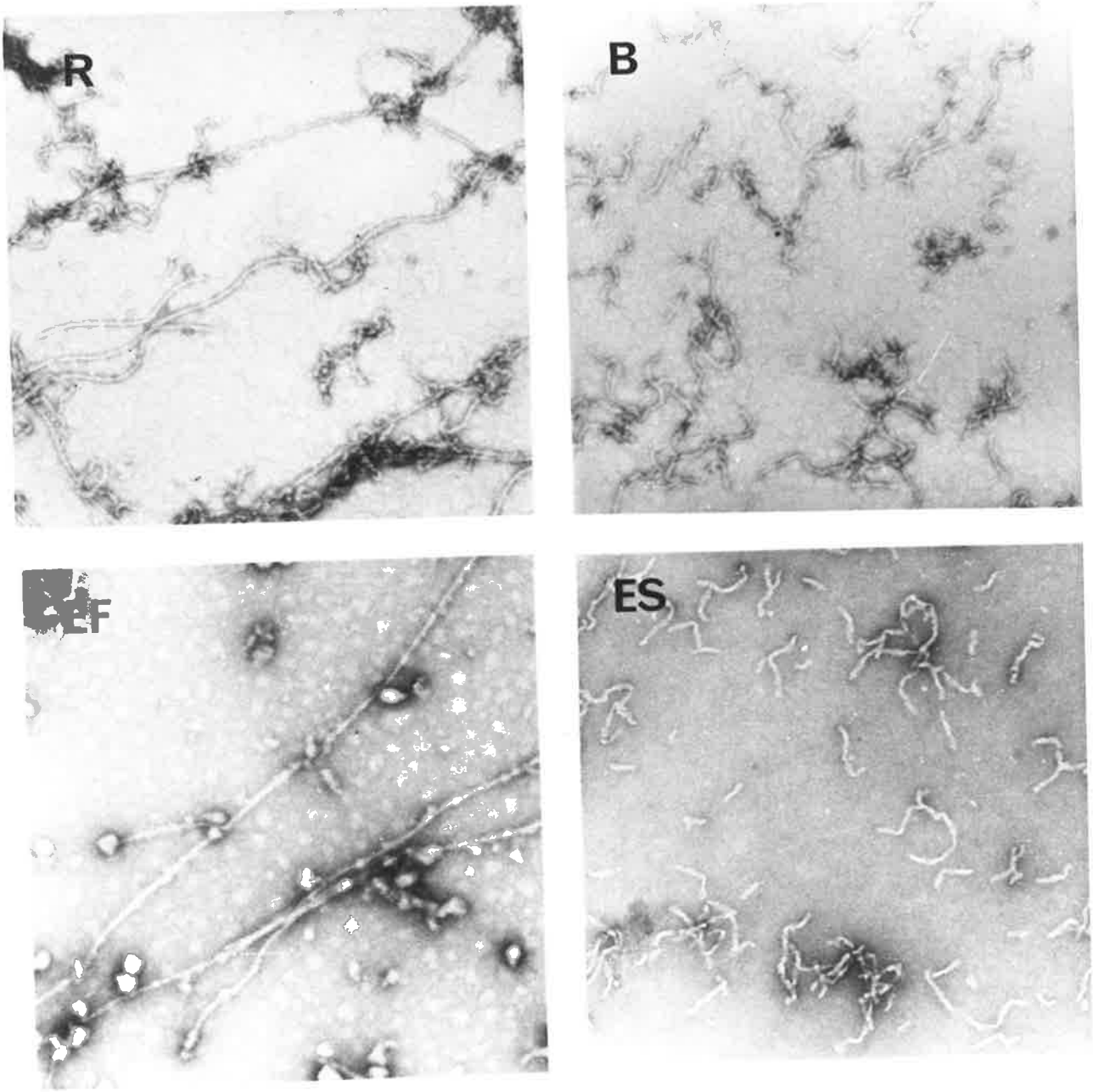

R
B
EF
ES

TABLE 3.2. AMINO ACID COMPOSITIONS OF THE UNFRACTIONATED KERATIN PROTEINS FROM VARIOUS TISSUES (Moles/100 moles).

| Tissue | Rachis[a] | Calamus[b] | Barbs[b] | Medulla[b] | Embryonic[b] Feather | Adult[b] Scale | Embryonic[b] Scale |
|---|---|---|---|---|---|---|---|
| SCM-Cysteine | 8.0 | 7.8 | 7.7 | 7.9 | 8.1 | 5.4 | 5.8 |
| Aspartic acid | 5.7 | 5.5 | 5.7 | 5.9 | 6.0 | 4.2 | 4.2 |
| Threonine | 4.1 | 4.2 | 5.2 | 4.1 | 4.0 | 3.9 | 3.7 |
| Serine | 14.7 | 13.4 | 14.9 | 13.9 | 12.0 | 9.4 | 9.5 |
| Glutamic acid | 6.8 | 7.2 | 8.2 | 7.8 | 7.6 | 5.8 | 5.8 |
| Proline | 11.7 | 9.8 | 10.7 | 9.7 | 11.3 | 6.9 | 7.7 |
| Glycine | 13.6 | 14.8 | 11.8 | 13.6 | 12.5 | 26.3 | 25.8 |
| Alanine | 8.5 | 9.1 | 5.4 | 7.3 | 4.5 | 6.5 | 7.9 |
| Half-cystine | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| Valine | 8.0 | 7.9 | 8.2 | 7.7 | 8.4 | 6.5 | 6.4 |
| Methionine | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.7 | 0.6 |
| Isoleucine | 2.9 | 3.2 | 4.4 | 3.6 | 4.6 | 2.7 | 2.6 |
| Leucine | 7.8 | 8.4 | 7.1 | 8.1 | 7.5 | 6.3 | 5.8 |
| Tyrosine | 1.3 | 1.6 | 1.6 | 1.5 | 2.5 | 6.6 | 6.2 |
| Phenylalanine | 3.1 | 3.2 | 3.7 | 3.8 | 4.2 | 3.0 | 2.6 |
| Lysine | 0.2 | 0.2 | 0.2 | 0.5 | 0.3 | 0.9 | 1.0 |
| Histidine | 0.0 | 0.1 | 0.1 | 0.2 | 1.0 | 0.7 | 0.6 |
| Arginine | 3.6 | 3.7 | 4.7 | 4.4 | 5.5 | 4.3 | 3.9 |

[a]Average of 3 analyses, on 20, 44 and 72 hr hydrolysates.

[b]One analysis, on 20 hr hydrolysate.

(1964a). The composition of embryonic feather proteins was similar to that of adult. Adult and embryonic scale proteins, although almost identical to each other, were significantly different from feather proteins, particularly in their high content of glycine (approximately 26%). The high glycine content is similar to that of the feather-type keratin proteins from goanna claw (Fraser *et al.*, 1972). The S-carboxymethyl-cysteine, serine, methionine and tyrosine contents were also significantly different to those of the featherproteins.

### 3. *Isolation of Rachis Protein Fractions by Preparative Polyacrylamide Gel Electrophoresis*

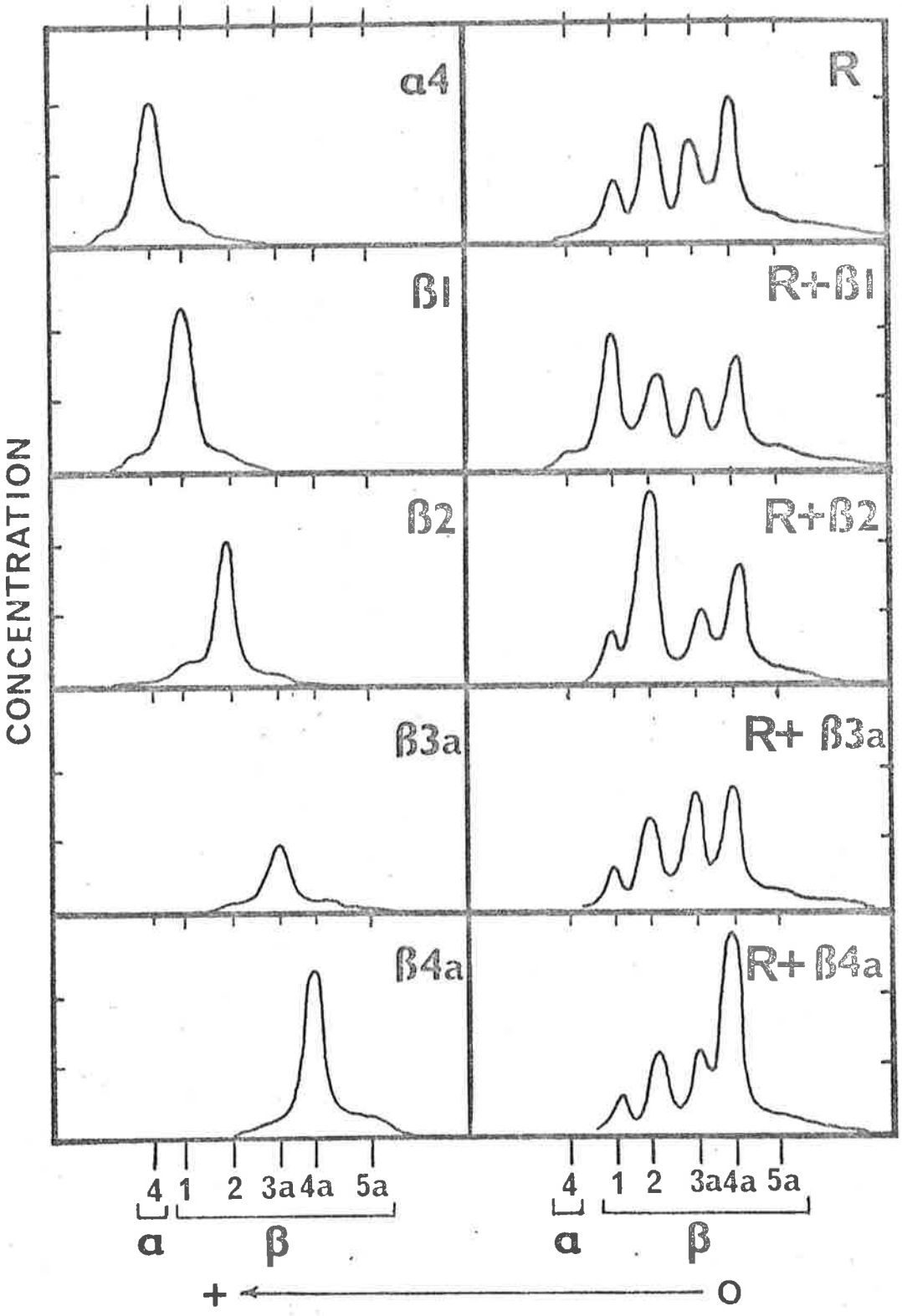
Preparative PAGE at pH 9.5 was used for the isolation of feather-keratin protein fractions during the initial stages of this project. At that time, no useful chromatographic procedures had been developed for fractionation of the feather proteins and it was believed that each tissue simply contained quantitative variations in the amount of several different keratin proteins that were common to them all.

Purified samples of bands α4, β1, β2, β3a and β4a from rachis were prepared. Densitometer traces of these fractions on analytical gels at pH 9.5 are shown in Figure 3.11. Identity of the fractions was established by co-electrophoresis with unfractionated rachis proteins in each case (Figure 3.11), and also by analytical slab-gel electrophoresis. Three unsuccessful attempts were made

FIGURE 3.11.

Densitometer traces of bands purified by high-pH PAGE. Details as in Figure 3.2.

R + β1 etc. : -co-electrophoresis of rachis and band β1, etc.


CONCENTRATION
α4
R
ß1
R+ß1
ß2
R+ß2
ß3a
R+ ß3a
ß4a
R+ ß4a
4 1 2 3a 4a 5a
α β
4 1 2 3a 4a 5a
α β
+
o

to run these fractions on low-pH gels. Most of the proteins did not enter the gel and the remainder formed several bands, none of which had the same mobility as any band band in the control gels. Most probably some solubilised polyacrylamide contaminating the preparations interacted with the proteins at the low pH, causing the anomalous behaviour (Weiner *et al.*, 1972).

Amino-acid compositions of the fractions are shown in Table 3.3. Control experiments showed that the presence of solubilised polyacrylamide in the rachis fractions did not affect the analyses providing hydrolysis was carried out in the presence of phenol, to protect tyrosine. S-carboxymethyl-cysteine was destroyed to a varying extent. Each purified fraction was rich in serine, glycine and proline but contained little methionine, lysine or histidine. Their compositions, which are typical of feather keratin, indicate the existence of a family of closely-related protein species.

4. *Nature of the "Water-Insoluble" Proteins of Embryonic Feather*

(a) *Identification of the γ-Proteins and the "Fast Band"*.

The suspended material which formed during dialysis of embryonic feather proteins (Chapter 3.C.2(a)) was removed by centrifugation at 36,000 g for 1 hr. PAGE of the total embryonic feather proteins, of embryonic feather proteins from which this material had been removed

TABLE 3.3. AMINO ACID COMPOSITIONS OF FEATHER PROTEIN FRACTIONS (moles/100 moles)

| Major band Isolated | α4[a] | β1[b] | β2[a] | β3a[a] | β4a[b] |
|---|---|---|---|---|---|
| SCM-Cysteine | 6.7 | 6.6 | 5.0 | 3.6 | 5.5 |
| Aspartic acid | 6.0 | 6.5 | 6.6 | 5.3 | 5.0 |
| Threonine | 4.2 | 3.9 | 4.2 | 4.5 | 4.7 |
| Serine | 14.9 | 14.7 | 15.6 | 12.7 | 14.2 |
| Glutamic acid | 9.0 | 8.9 | 8.8 | 6.8 | 5.4 |
| Proline | 10.2 | 10.0 | 10.2 | 9.4 | 10.9 |
| Glycine | 13.2 | 13.0 | 12.7 | 17.5 | 15.9 |
| Alanine | 8.1 | 7.9 | 8.0 | 13.8 | 10.9 |
| Half-cystine | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Valine | 8.3 | 9.3 | 9.2 | 6.7 | 7.2 |
| Methionine | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Isoleucine | 3.4 | 3.2 | 3.9 | 3.1 | 3.2 |
| Leucine | 6.7 | 6.5 | 6.8 | 8.4 | 10.4 |
| Tyrosine | 1.3 | 1.4 | 1.1 | 1.3 | 1.2 |
| Phenylalanine | 4.1 | 4.2 | 4.1 | 3.0 | 2.3 |
| Lysine | 0.3 | 0.0 | 0.0 | 1.0 | 0.0 |
| Histidine | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 |
| Arginine | 3.7 | 4.0 | 3.7 | 2.7 | 3.0 |

[a]One analysis on 20 hr hydrolysate.

[b]Two analyses, on 20 and 72 hr hydrolysates.

("embryonic feather water-soluble proteins" and of the water-insoluble material is shown in Figure 3.12. It was clear (Figure 3.12a) that the γ-proteins were concentrated in the water-insoluble fraction. On pH 2.7 gels (Figure 3.12b) the "fast" band was concentrated in the water-insoluble fraction. It was not possible to conclude that the γ-proteins exclusively constitute the "fast" band, however, as both gel systems revealed that the fractionation of β- from γ-proteins was not complete.

The electron-microscopic appearance of the water-insoluble material after resuspension in urea and dialysis against water until the opalescence returned is shown in Figure 3.13. The particles obtained appear identical to those obtained from total embryonic feather proteins. It is therefore likely that the water-insoluble fraction, at least in part, does not possess the ability to fibrillate. In contrast to the β-proteins, this material exists as rounded, apparently amorphous aggregates, both in water and in NEMA.

### (b) *Isolation of γ-Proteins by DEAE-Cellulose Chromatography*.

In the course of other studies, a series of partially purified fractions containing γ-proteins was obtained by DEAE-cellulose chromatography. The procedure used was that of I.D. Walker (personal communication), except that the water-insoluble material that contains the γ-components was not removed by centrifugation prior to the

FIGURE 3.12

PAGE of soluble and insoluble fractions from embryonic feather proteins.

FIGURE 3.12a.

PAGE at pH 9.5. Conditions as in Figure 3.1.

FIGURE 3.12b.

PAGE at pH 2.7. Conditions as in Figure 3.4a.

EF: - unfractionated embryonic feather proteins.

EFS: - embryonic feather supernatant proteins.

EFI: - embryonic feather water-insoluble proteins.

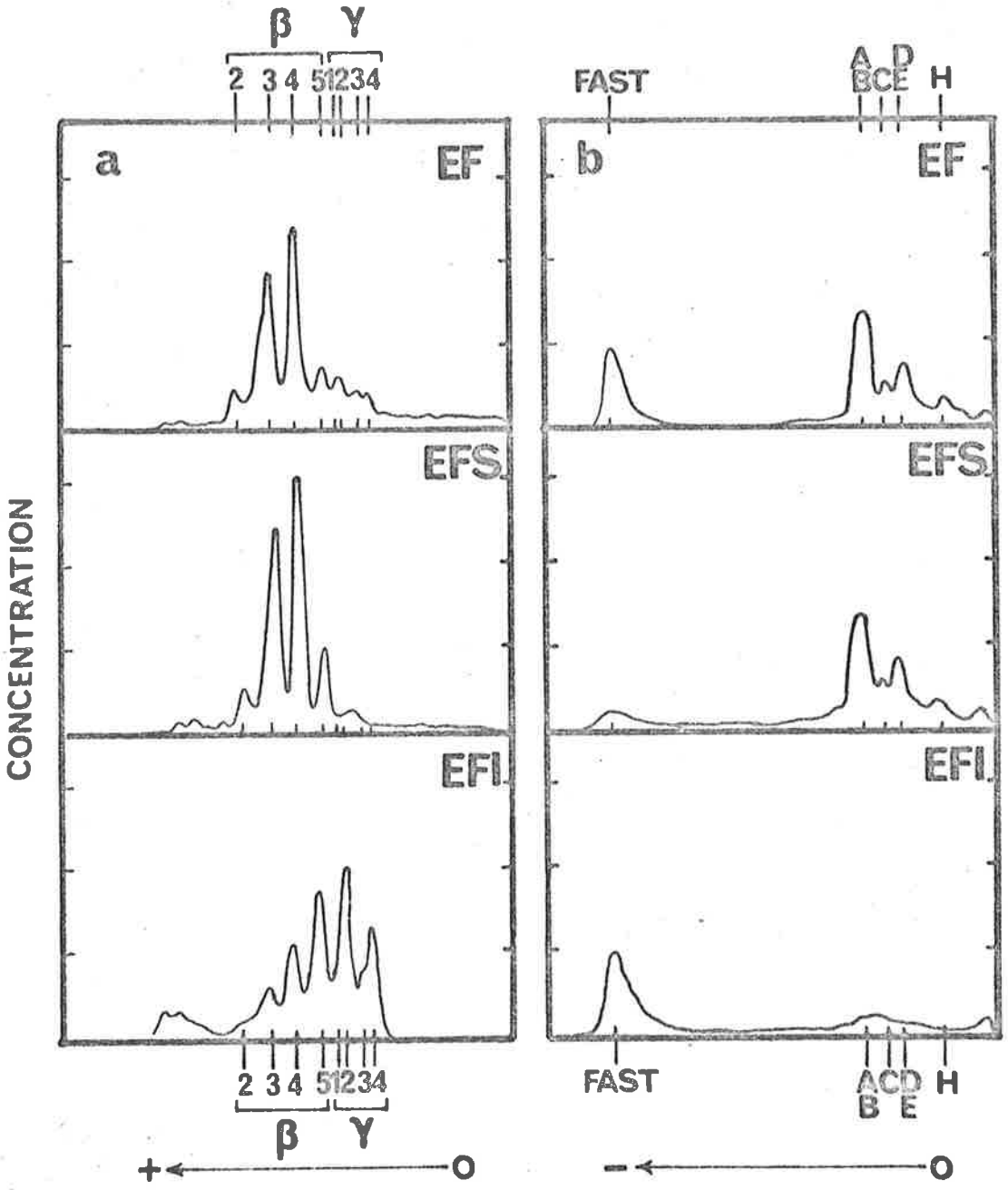

β γ
2 3 4 5 1 2 3 4
a
EF
EFS
EFI
CONCENTRATION
2 3 4 5 1 2 3 4
β γ
+
O
FAST
A B C D E H
b
EF
EFS
EFI
FAST
A C D H
B E
-
O

FIGURE 3.13

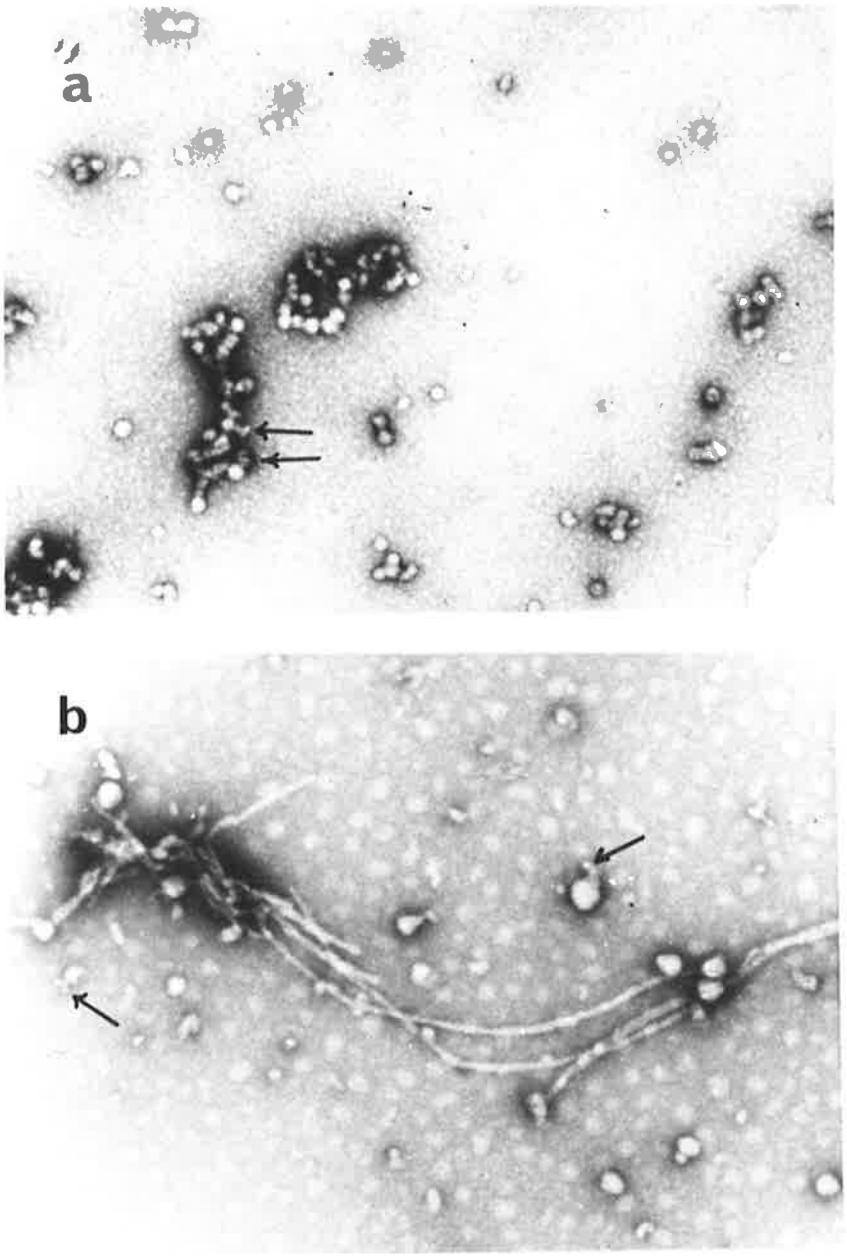
Electron-micrographs of particles obtained

(a) from the "water-insoluble" fraction of embryonic feather proteins;

(b) for comparison, the particles obtained from total embryonic feather proteins, as in Figure 3.10.

Negatively stained with uranyl acetate. The rounded particles appear to consist of smaller particles (arrows).

Magnification: X60,000.


a
b

chromatographic fractionation in the presence of 8 M urea. The profile obtained (Figure 3.14) was similar to that for water-soluble proteins obtained by Walker but contained in addition the peaks designated 1 and 2. These, in addition to peak 3, contained predominantly γ-proteins, as determined by PAGE at pH 7.5 (Fig. 3.15). The amino acid compositions of fractions 1-3 are shown in Table 3.4. In fractions 1 and 2, the contents of aromatic and basic amino acids, particularly tyrosine and histidine, were significantly higher than those of the other feather proteins. Fraction 3 was of similar composition to the other feather proteins however, as found by Walker (personal communication).

### 5. *Embryonic Feather-Sheath Proteins*

Proteins were prepared from embryonic feather sheaths, and characterized by PAGE at pH 9.5 and at pH 7.5 (Figure 3.16). One major band was obtained, running in the region of the γ-proteins, as well as a minor band of lower mobility. The pattern was totally unlike that of the other feather tissues investigated. This result was not unexpected, in that the feather sheath appears from earlier evidence to be different from the rachis and calamus of feather, in that it gives a different X-ray diffraction pattern and contains α-keratin-type structure (Rudall, 1947). Furthermore, the amino acid composition of the sheath protein preparation (Table 3.5) was distinctly different to those of the proteins from adult or embryonic feathers or scales, particularly with regard to the contents of S-carboxymethyl

FIGURE 3.14.

Isolation of γ-protein fractions by chromatography on DEAE-cellulose. Embryonic feather proteins (1 gm) were fractionated on a column (100 cm x 2.5 cm) of DEAE-cellulose, using a gradient of total volume 1 litre, from 0 to 2.5 M in potassium chloride. The buffer used to equilibrate the column and for the gradient consisted of 8.0 M urea, 0.001 M EDTA and 0.01 M Tris/HCl, pH 7.0. Flow rate was 96 ml/hr, and 10.5 ml fractions were collected. Fractions were pooled as shown by the bars. γ-proteins were present in fractions 1-3. Other fractions were used for the N-terminal sequence experiments described in Chapter 4.

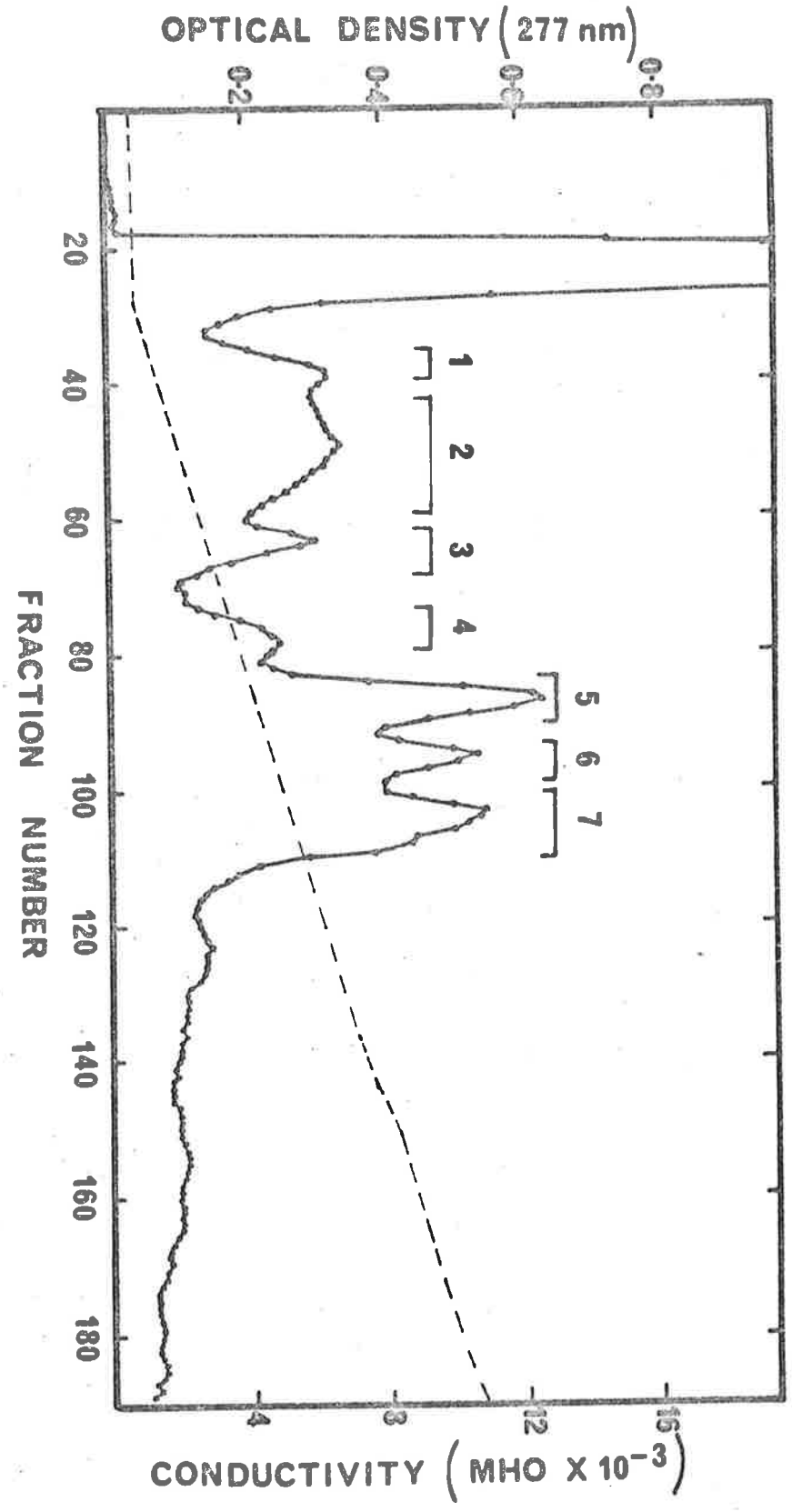

OPTICAL DENSITY (277 nm)
0·2
0·4
0·6
0·8
20
40
60
80
100
120
140
160
180
FRACTION NUMBER
1
2
3
4
5
6
7
4
8
12
16
CONDUCTIVITY (MHO X 10⁻³)

FIGURE 3.15.

PAGE at pH 7.5 of γ-protein fractions purified by DEAE-cellulose chromatography. Conditions as in Figure 3.3. Approximately 50 µg/gel was loaded.

EF: - unfractionated embryonic feather proteins.

1,2,3: - γ-protein fractions, from Figure 3.14.

EF + 1 etc.: - co-electrophoresis of EF and fraction 1, etc.

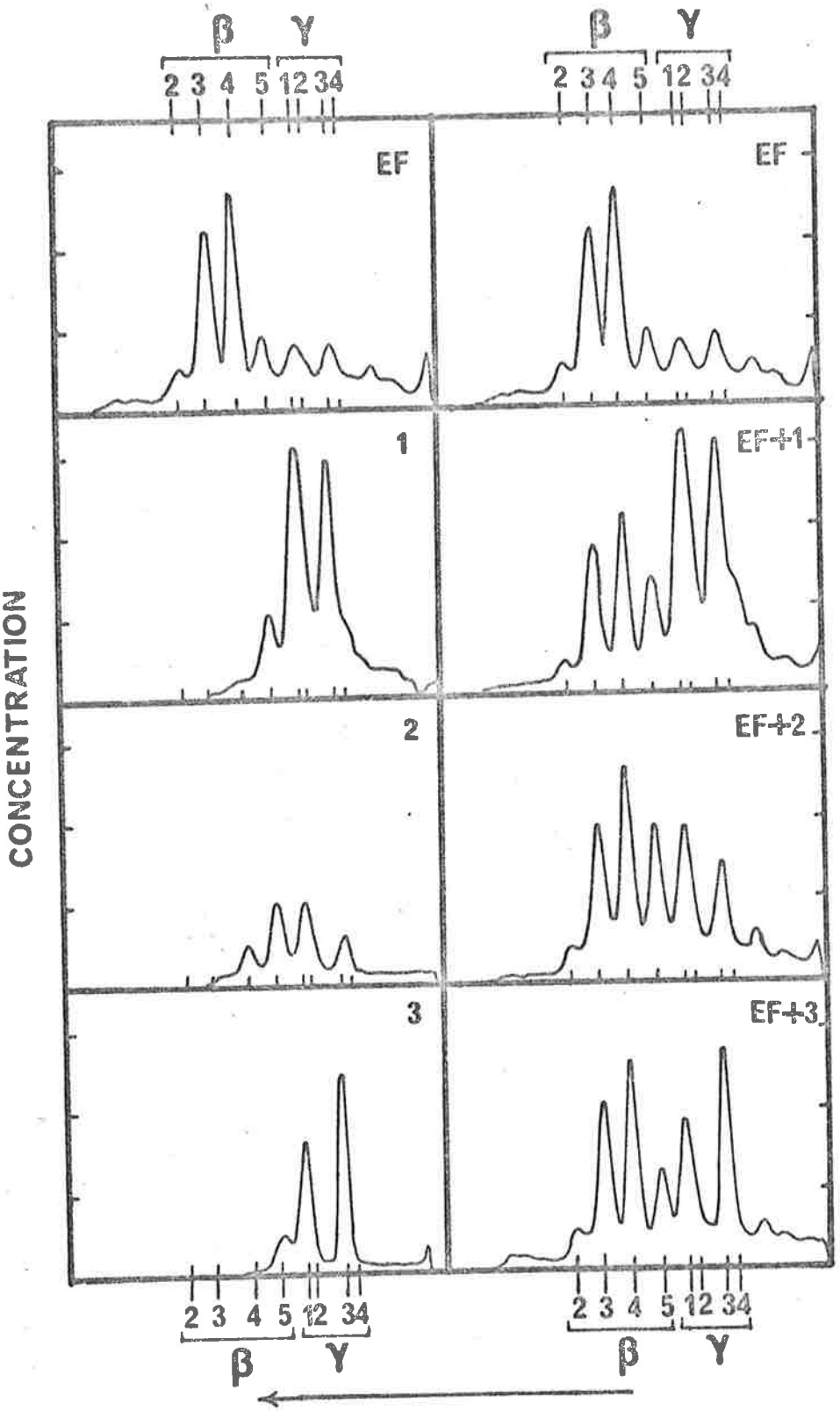

β γ
2 3 4 5 12 34
EF
EF+1
EF+2
EF+3
1
2
3
CONCENTRATION
β γ
2 3 4 5 12 34

TABLE 3.4. AMINO ACID COMPOSITIONS OF γ-PROTEIN FRACTIONS FROM DEAE-CELLULOSE CHROMATOGRAPHY (moles/100 moles)

| Fraction | 1[a] | 2[a] | 3[a] | EF[b] |
|---|---|---|---|---|
| SCM-Cysteine | 2.6 | 3.4 | 5.1 | 8.1 |
| Aspartic acid | 9.2 | 7.8 | 5.4 | 6.0 |
| Threonine | 1.8 | 2.7 | 5.5 | 4.0 |
| Serine | 9.1 | 11.1 | 16.5 | 12.0 |
| Glutamic acid | 5.7 | 6.1 | 7.4 | 7.6 |
| Proline | 4.7 | 7.7 | 8.9 | 11.3 |
| Glycine | 15.7 | 15.2 | 16.6 | 12.5 |
| Alanine | 1.7 | 3.0 | 4.0 | 4.5 |
| Half-Cystine | - | - | - | - |
| Valine | 2.9 | 4.8 | 6.4 | 8.4 |
| Methione | trace | - | - | - |
| Isoleucine | 1.8 | 3.2 | 4.3 | 4.6 |
| Leucine | 7.0 | 7.3 | 7.0 | 7.5 |
| Tyrosine | 10.1 | 7.9 | 4.4 | 2.5 |
| Phenylalanine | 7.4 | 6.2 | 3.0 | 4.2 |
| Lysine | 1.1 | 1.2 | 0.4 | 0.3 |
| Histidine | 9.2 | 6.2 | 0.6 | 1.0 |
| Arginine | 6.8 | 6.2 | 4.2 | 5.5 |

[a]One analysis, on 20 hr hydrolysate

[b]Unfractionated embryonic feather proteins (from Table 3.2).

FIGURE 3.16.

PAGE of embryonic feather and feather sheath proteins. 100 µg/gel was loaded.

a. PAGE at pH 7.5. Conditions as in Figure 3.3.

b. PAGE at pH 9.5. Conditions as in Figure 3.1.

EF: Embryonic feather proteins.

Sh: - sheath proteins.

Sh + EF: - co-electrophoresis of sheath and embryonic feather proteins.


a
b
O
O
+
+
SH
SH + EF
EF
SH
SH + EF
EF

TABLE 3.5. AMINO ACID COMPOSITION OF FEATHER SHEATH PROTEINS (moles/100 moles)

| Sample | Sheath[a] | EF[b] | α-Keratin[c] |
|---|---|---|---|
| SCM-Cysteine | 3.52 | 8.1 | 6.8 |
| Aspartic acid | 9.34 | 6.0 | 8.1 |
| Threonine | 5.21 | 4.0 | 4.4 |
| Serine | 8.11 | 12.0 | 7.3 |
| Glutamic acid | 9.76 | 7.6 | 14.1 |
| Proline | 4.80 | 11.3 | 4.2 |
| Glycine | 9.40 | 12.5 | 8.8 |
| Alanine | 7.94 | 4.5 | 6.4 |
| Half-Cystine | - | - | - |
| Valine | 7.35 | 8.4 | 5.9 |
| Methionine | - | - | 0.6 |
| Isoleucine | 5.05 | 4.6 | 3.7 |
| Leucine | 8.41 | 7.5 | 10.3 |
| Tyrosine | 3.32 | 2.5 | 4.3 |
| Phenylalanine | 4.03 | 4.2 | 3.0 |
| Lysine | 5.91 | 0.3 | 4.1 |
| Histidine | 2.33 | 1.0 | 0.7 |
| Arginine | 5.52 | 5.5 | 7.3 |

[a]One analysis, on 20 hr hydrolysate

[b]Unfractionated embryonic feather proteins (from Table 3.2).

[c]SCMK-A from wool (from Thompson and O'Donnell, 1962).

cysteine, proline and lysine. The composition was similar to the SCMK-A fraction of α-keratins (Thompson and O'Donnell, 1962), particularly with regard to the contents of serine, proline and glycine.

## D. DISCUSSION

### *1. Heterogeneity of Feather Proteins*

This work was aimed at providing an analytical basis for the study of specific gene action in the development of keratinizing tissues in the chicken. Quantitative densitometry of the extracted S-carboxymethyl-proteins on polyacrylamide gels (Figures 2 and 5) provides a suitable technique for measuring the mobility and the amount of each band. However, the question of whether each band resolved by polyacrylamide gels contains one gene product or more is obviously important. The heterogeneity of keratin proteins from other keratinizing tissues (see Fraser *et al.*, 1972) suggests the need for caution in such interpretation.

The possibility that any of the bands are aggregates can be ruled out because the same protein species occur in the various feather tissues in different amounts. The major bands from rachis proteins obtained by PAGE at pH 9.5, were isolated, and these re-ran as single bands on gels at pH 9.5, further ruling out the possibility that some bands are aggregates. Moreover, amino acid analyses of the unfractionated proteins from each tissues

exclude the possibility that any of the major bands are artefacts due to incomplete reduction or carboxymethylation.

The results from isoelectric focussing, peptide maps and immunodiffusion are entirely consistent with the results from PAGE but do not, however, allow any further conclusions as to the number of polypetpide chain species present. In further studies in this laboratory (I.D. Walker, personal communication) the number of polypeptide chain species has been investigated by a combination of chromatographic isolation and PAGE. It has been found that most of the major bands observed by PAGE contain more than one polypeptide chain.

2. *Differences in the Proteins of Feathers and Scales*

The results from PAGE demonstrate that the major β-group of protein species of scales are different from those of feathers. Besides the developmental significance this observation serves as an excellent experimental control to show that all the proteins are in fact tissue-specific. They are not, for example, ribosomal proteins or other cellular proteins which might be expected to be common to each tissue.

3. *Microheterogeneity and Relationship to Keratin Structure*

*(a) β-Proteins.*

The results from PAGE and isoelectric focussing, the immunological cross-reactions between feather and scale

proteins and the ability of these proteins to fibrillate and gel *in vitro*, suggest that they consist of a family of closely-related protein chains. Moreover, the similarity in molecular weight (Harrap and Woods, 1964b; Jeffrey, 1970) and amino acid composition (present results) of all the β-protein species suggests that they have all arisen by duplications and subsequent mutational divergence from a common ancestral gene. This mode of origin is generally accepted for the diversity of hemoglobin chains and other protein families (Zuckerkandl and Pauling, 1965). The N-terminal sequence studies described in Chapter 4 support this concept.

Various models have been proposed for the structure of feather keratin based on X-ray diffraction (Fraser *et al.*, 1971) and electron microscopic evidence (Rogers and Filshie, 1963). It is likely that each type of β-protein chain is a structurally equivalent monomer in the keratin quaternary structure and the filament unit (microfibril) of avian keratins could be built from any one of them.

*(b) α- and γ-Proteins.*

These two groups of proteins were present in relatively minor amounts in various tissues. Although they are tissue-specific (Figures 3.1 - 3.3) in that, for example, the γ-proteins do not occur in adult feather, the question arises as to whether or not they are keratins. Band α4 from rachis was isolated and shown to have an amino-

acid composition typical of feather keratin (Table 3.3). The γ-proteins, however, appear to be distinctly different to the β-proteins in that they are relatively insoluble in water and those present in DEAE-cellulose fractions 1 and 2 have dissimilar amino acid compositions. It would appear likely that they do not form fibrils. Subsequent studies (see Chapter 4) have indicated that they do not possess the N-terminal sequence common to the major β-proteins. The γ-proteins are not essential for feather keratin structure as they do not occur in significant amounts in adult feathers.

It is also of particular importance to note that the amount of γ-proteins, relative to β-proteins, varied in different preparations. Several factors could have contributed to the observed variation. Such factors could include the efficiency of the extraction procedure, the length of time between hatching of the chicks and collection of the feathers, and the stringency of the washing procedures. If the γ-proteins represent proteins contaminating the feathers, such variables could greatly influence their subsequent yields. It is difficult, however, to see how (apparently) identical proteins could occur as extrinsic contaminants of tissues in such diverse environments as the embryonic feather and adult scales.

These results suggest that the γ-proteins are not keratins. Their nature is not obvious, however. It is possible that they are a distinct class of keratin proteins from cylinder cells or barb-vane-ridge cells. These cells

are discarded with the central pulp remnant after hatching, but are possible contaminants of the feathers. X-ray diffraction studies (Rudall, 1947) have indicated that cylinder cells contain α-keratin, suggesting that the proteins of these cells would be different to the other feather proteins. It is pertinent to note that proteins of unknown function (the high-glycine-high-tyrosine proteins) with somewhat similar properties occur in extracts from hair and other α-keratins (Gillespie, 1960; Zahn and Biela, 1968a,b; Gillespie and Darskus, 1971).

### 4. *Embryonic Feather Sheath Proteins*

During embryonic feather development, the sheath commences keratin synthesis before the barbs or barbules (Bell and Thathachari, 1963; Matulionis, 1970). It was therefore of some importance to identify the sheath proteins before examining the kinetics of keratin synthesis (Chapter 5). The results show that the sheath possesses a spectrum of proteins totally unlike that of the other tissues investigated. This observation is consistent with X-ray diffraction studies (Rudall, 1947) which indicate that the sheath has an α-keratin type structure.

### 5. *Significance in Relation to Development*

Embryonic feathers, feather sheaths, and scales have been shown to contain some different species of protein chains. Thus, different structural genes are expressed in the epidermal-derived cells as a direct or indirect result

of dermal "inductive" influences. The expression of these genes represents the dominant synthetic activity of the cells after a defined temporal point in the development of each tissue.

Studies on recombination of dermis and epidermis from various regions have clearly shown that feather and scale morphogenesis from epidermal cells is determined to some extent at least by dermis by as yet poorly-understood processes (Chapter 1). The studies of Dhouailly (1967) have demonstrated that the morphology of feathers and hence control of cell proliferation, is controlled by the nature of the dermis. Cytological studies (see Chapter 1) have shown the presence of different cell types in embryonic feathers, for example, barbule, barb - cortex and barb - medulla and sheath cells. The studies of Kato (1969) have shown that at least one of the cell types (scale cells) can be induced by scale dermis to differentiate from chorionic epithelial cells which in the normal state do not differentiate in such a manner.

The present study shows that in the differentiation of morphologically-distinct derivatives of the embryonic epidermis such as feathers, feather sheaths and scales, distinct groups of protein chains are produced. These observations lead to the conclusion that there exists a major set of structural genes for all of the different protein chains of feather keratin and that the particular set of these genes that is expressed in the cells of feather is entirely different from the one expressed in scale cells. Similarly,

the group of proteins (bands A - E) synthesized by barb and barbule cells which are the predominant cells in embryonic feathers (Bell and Thathachari, 1963) is distinct from the proteins (bands F - H) synthesized by the rachis cells of the adult feather. Here again, one concludes that the gene set selected for expression is distinct in two different populations of cells.

It is seen from an examination of the variety of protein chains present in the different parts of adult feather, the rachis, calamus, barbs and the medulla (Figure 5) that within experimental error the amounts of the proteins represented by bands F, G and H remain constant relative to one another. The proteins of bands A and B from each of these tissues also show a similar relationship. In contrast, no constant relationship exists between the proteins of bands A and H or those of bands C, D and E. It is possible that each group of proteins showing this "coordinated" behaviour reflects the presence of a unique population of cells within the tissue, exclusively synthesizing the proteins of the group in a coordinately controlled manner. The apparent lack of coordination between the groups of proteins would therefore result from the presence of varying numbers of each of the discrete cell types from tissue to tissue, each type having its distinct set of active genes.

# CHAPTER 4

# N-TERMINAL SEQUENCES OF EMBRYONIC FEATHER PROTEINS

## A. INTRODUCTION

The studies reported in the present chapter were undertaken as a pre-requisite for the studies on initiation of keratin synthesis described in Chapter 6.

Harrap and Woods (1964b) showed that the N-termini of feather keratins from adult fowl rachis were acetylated. O'Donnell (1971) utilized the procedure of Narita (1958) for the selective isolation of N-blocked peptides and found that the proteins from adult goose calamus and rachis all had the same N-terminal sequence, namely Ac-Ser-SCMC-Tyr.

The preparation of a series of N-terminal peptides of increasing size from embryonic chick feather proteins is described in this chapter. The procedures have furnished results which establish that the N-termini of embryonic feather keratin proteins are acetylated, and provide routine techniques for the isolation of N-terminal peptides, suitable for studies on keratin biosynthesis (Chapter 6). In addition, firm support is provided for the hypothesis (Chapter 3) that the various keratin proteins constitute a homologous family, which presumably have arisen by gene duplication and subsequent mutational divergence.

## B. METHODS

Blocked peptides were isolated by fractionation of proteolytic digests on Dowex-50, in a manner similar to that originally described by Narita (1958) and subsequently used for feather proteins by O'Donnell (1971).

*1. Proteolytic Digestions*

*(a) Pronase Procedure.*

The method of Harding and Rogers (1971), which has been shown to degrade many proteins to their component amino acids was adapted as described. Embryonic feather proteins were dissolved in NEMA (10 mg/ml, w/v) and incubated at 37° with the following enzymes in succession:

trypsin, 1% w/w, 1 hr.

chymotrypsin, 1%, w/w, 16 hr.

pronase, 10%, w/w, 24 hr.

pronase, 5%, w/w, 24 hr.

pronase, 5%, w/w, 24 hr.

A small crystal of thymol was always added, to inhibit the growth of bacteria.

*(b) Other Enzymes.*

Proteins were dissolved in NEMA as above, and incubated at 37° with the required enzyme at the stated concentrations for the following times:

| | |
|---|---|
| chymotrypsin: | 2% w/w, 24 hr. |
| elastase: | 2% w/w, 4 hr. |
| trypsin: | 2% w/w, 4 hr. |
| carboxypeptidase-A: | 5%, w/w, 24 hr. |
| carboxypeptidase-B: | 2%, w/w, 4 hr. |

In the case of a combined digestion with chymotrypsin and carboxypeptidase-A the proteins were first incubated with chymotrypsin as described above, boiled for 5 min., and then incubated with carboxypeptidase-A. The mixture was

not boiled between incubations with trypsin and carboxypeptidase-B.

2. *Deproteinization of Proteolytic Digests*

Pronase and chymotrypsin/carboxypeptidase-A digests were deproteinized by picric acid precipitation and the picric acid was subsequently removed by Dowex-2 chromatography as described by Stein and Moore (1954).

3. *Isolation of Blocked Peptides by Dowex-50 Chromatography*

Enzymic digests were fractionated by chromatography on Dowex AG 50W-X8, 100-200 mesh ($H^+$ form), in $H_2O$ (Narita, 1958; O'Donnell, 1971). A column of about 30 ml bed size was used for 100 mg of starting material. Samples which had previously been deproteinized and recovered in 0.02 N HCl were concentrated to about 5 ml by rotary evaporation prior to loading on the column. Samples which had not been deproteinized and were in NEMA were adjusted to pH 5.2 - 5.4 by the addition of acetic acid prior to loading. Blocked peptides were eluted from the column with 40 ml of $H_2O$, and the eluates were taken to dryness by rotary evaporation.

4. *Chromatography on Sephadex G-10*

Samples were applied to a column (80 cm x 2.5 cm) of Sephadex G-10, equilibrated in 0.02 N HCl, and eluted with the same solvent at a flow rate of 20 ml/hr. The

effluent was monitored manually by the ninhydrin reaction (Yemm and Cocking, 1955) after alkaline hydrolysis (Moore and Stein, 1954).

5. *Chromatography on Dowex-1*

A procedure for the fractionation of blocked or acidic amino acids and peptides by chromatography on Dowex-1 was developed, based on the results of Offer (1965) and Alving and Laki (1966). Samples were applied in $H_2O$ to a 65 cm x 0.6 cm column of Dowex AG1 W - X8, -400 mesh, in the formate form, and eluted at 35° at a flow rate of 30 ml/hr. with a gradient of increasing formic acid concentration. The gradient finally adopted after studies with model compounds is shown in Table 4.1. The effluent was monitored with the Technicon Peptide Analyser, essentially as described by Catravas (1964). Fractions were collected at 5 or 10 minute intervals.

6. *Chromatography on Sephadex G-50*

Samples were applied to a column (61 cm x 1.4 cm) of Sephadex G-50 fine, equilibrated in pyridine/morpholine/acetic acid buffer, pH 9.0 (PMA, Schroeder *et al.*, 1962) and eluted with the same buffer, at a flow rate of 10 ml/hour. Fractions were collected every 12 minutes. The effluent was monitored manually by the ninhydrin reaction after alkaline hydrolysis.

TABLE 4.1. COMPOSITION OF THE GRADIENT FOR DOWEX-1 CHROMATOGRAPHY.

[Each chamber contained 30 ml of formic acid.]

| Autograd Chamber No. | 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 |

7. *High Voltage Paper Electrophoresis*

Samples were fractionated by high voltage paper electrophoresis (HVPE) in pyridine/acetate buffer, pH 6.5, as described by Offord (1966) using an apparatus similar to that described by Michl (1951).

Amino acids and unblocked peptides were detected with ninhydrin (0.1% w/v in ethanol). Blocked peptides were detected by the chlorination/starch/potassium iodide procedure of Rydon and Smith, 1957); this procedure, however, was found to be unreliable and insensitive for N-acetyl amino acids and dipeptides, as observed by others (Offer, 1965; Alving and Laki, 1966; Mak and Waley, 1968). Peptides containing tyrosine were detected with α-nitroso-β-napthol/nitric acid (Acher and Crocker, 1952). Peptides containing arginine were detected with the phenanthrenequinone reagent as described by Easly *et al.* (1969).

Peptides were eluted from the paper for preparative purposes by the technique of Edstrom (1968). The paper segments were generally washed four times with 1-2 ml of NEMA.

8. *N-terminal analysis* by the Dansyl chloride procedure was carried out as described by Gros and Labouesse (1969). DNS-amino acids were identified by two-dimensional chromatography on polyamide layers as described by Woods and Wang (1967).

## C. RESULTS

### 1. *Isolation of the N-Terminal Dipeptide*

#### (a) *Isolation by the Pronase Procedure.*

Preliminary studies using the DNS technique indicated that the N-termini of the feather proteins were blocked. Blocked peptides were therefore prepared by Dowex-50 chromatography of a deproteinized pronase digest of unfractionated embryonic feather proteins (600 mg).

##### (i) *Fractionation of the Dowex-50 eluate.*

The Dowex-50 eluate was fractionated by chromatography on Sephadex G-10 (Figure 4.1). Four peaks, G-10.1 - G-10.4 resulted. Acetic acid was detected by gas chromatography of an acid hydrolysate of peak G-10.3, but not of the other peaks (I.D. Walker, personal communication).

Each of the four Sephadex G-10 peaks was fractionated by chromatography on Dowex-1 (Figure 4.2). In each case, one major peak was obtained (D-1.1 - D-1.4). In subsequent experiments, the Sephadex G-10 step was omitted, and the same 4 peaks were obtained by Dowex-1 chromatography of the Dowex-50 eluate (Figure 4.2.f). The amino acid compositions of the Dowex-1 fractions are shown in Table 4.2.

##### (ii) *Nature of the fractions isolated by Sephadex G-10 and Dowex-1 Chromatography.*

The composition of peak D-1.1 indicates that it was probably a mixture of contaminating peptides.

FIGURE 4.1.

Fractionation of the Dowex-50 eluate from a pronase digest of unfractionated embryonic feather proteins (600 mg) on Sephadex G-10. Procedures were as described in the text. Fractions under peaks 1 - 4 were pooled as indicated by the bars.

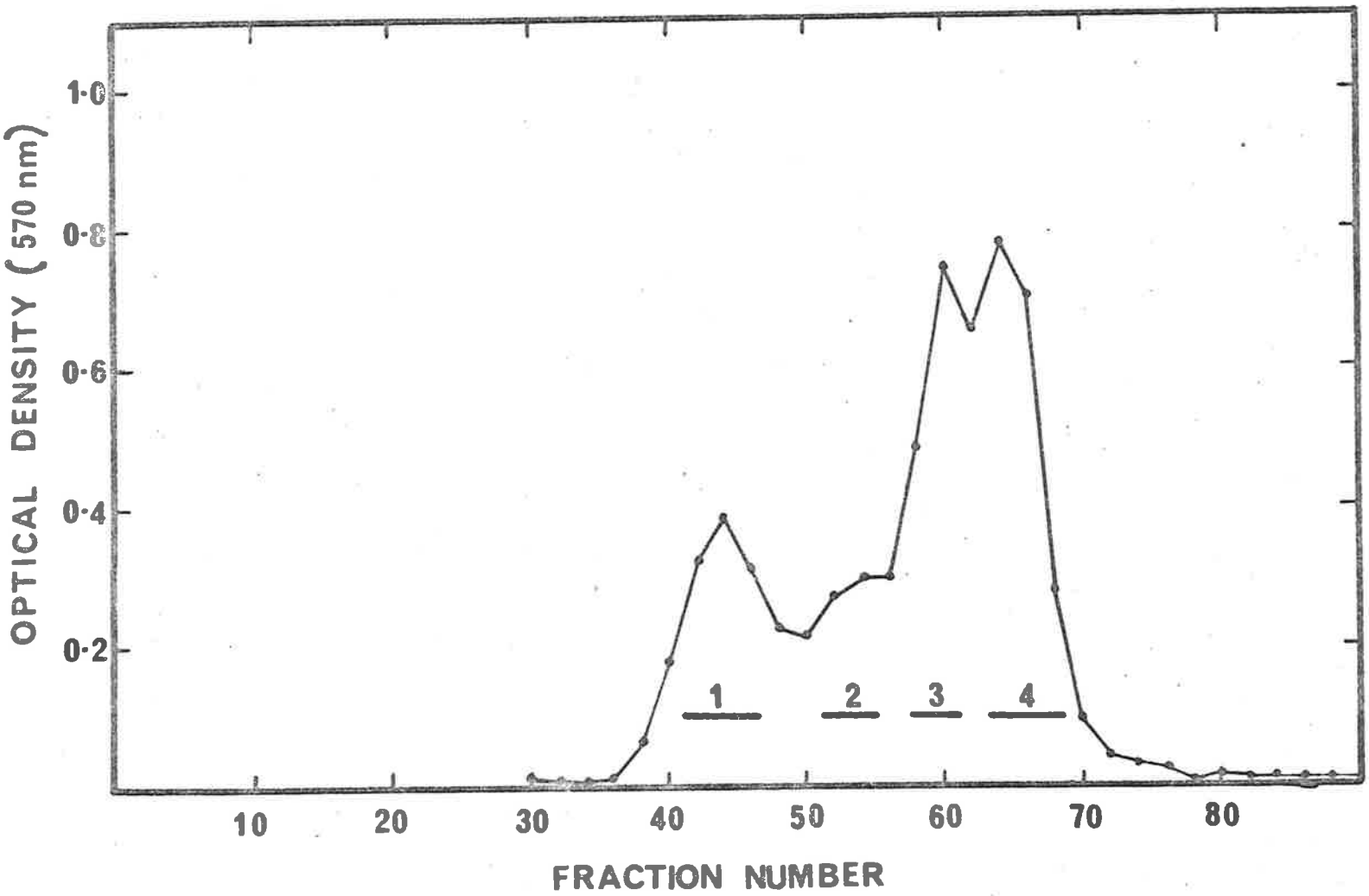

OPTICAL DENSITY (570 nm)
1·0
0·8
0·6
0·4
0·2
1
2
3
4
10
20
30
40
50
60
70
80
FRACTION NUMBER

FIGURE 4.2.

Chromatography of pronase peptide fractions on Dowex-1. Procedures were as described in the text.

a. Chromatography of model compounds. 100 - 250 µg of each compound denoted was chromatographed, in separate runs.

b - e. Chromatography of Sephadex G-10 fractions 1-4, from Figure 4.1.

f. Chromatography of unfractionated Dowex-50 eluate from a pronase digest of embryonic feather proteins (100 mg).

Dowex-1 fractions 1-4 are indicated.

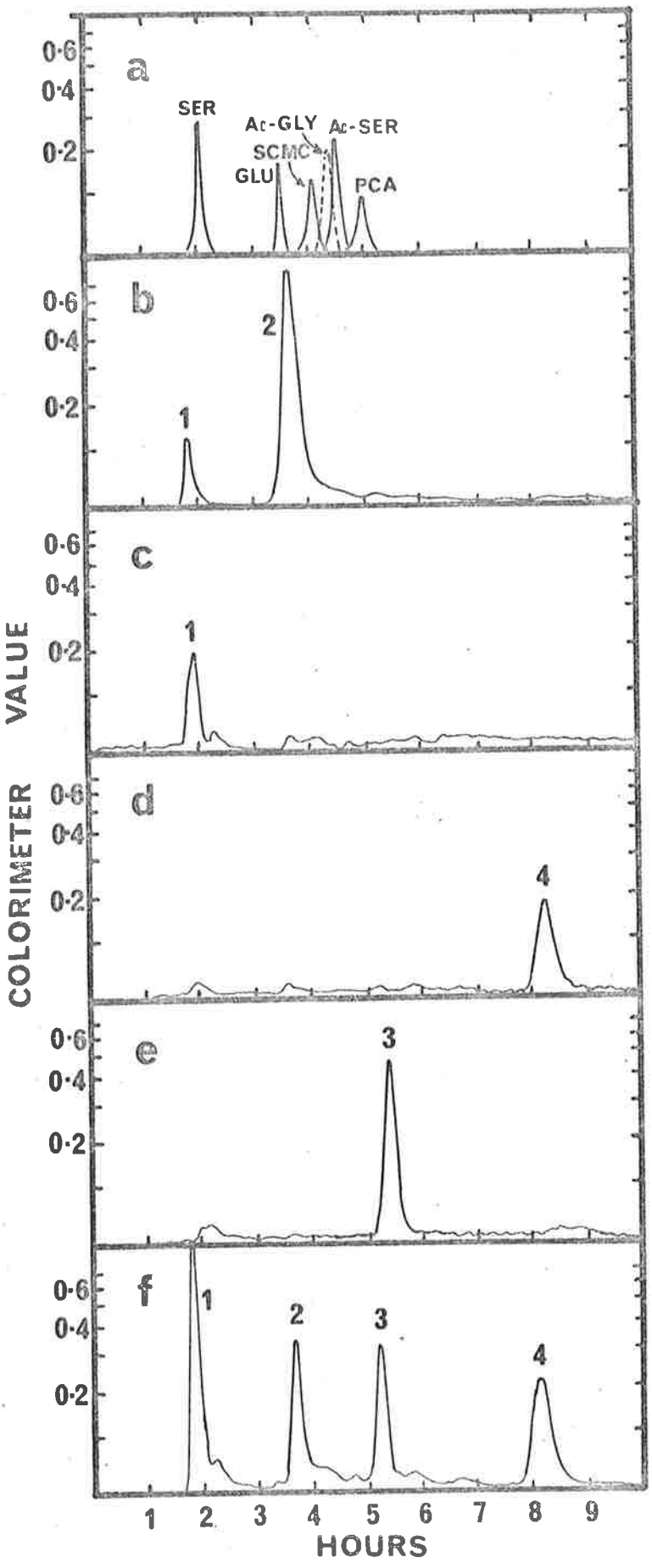

a
SER
Ac-GLY
Ac-SER
SCMC
GLU
PCA
b
1
2
c
1
d
4
e
3
f
1
2
3
4
0·6
0·4
0·2
COLORIMETER VALUE
1 2 3 4 5 6 7 8 9
HOURS

TABLE 4.2. AMINO-ACID COMPOSITIONS OF FRACTIONS FROM DOWEX-1 CHROMATOGRAPHY.

Analyses were performed using the long column of a Beckman 120C amino acid analyser. Values are expressed as residue ratios, relative to the predominant amino-acid. Amounts indicated by "trace" were present in <0.05 residues.

| Fraction | 1 | 2 | 3 | 4 | 4[a] |
|---|---|---|---|---|---|
| SCM-Cysteine | - | 0.05 | - | 0.70 | 0.86 |
| Aspartic acid | 0.09 | 0.08 | trace | 0.05 | trace |
| Threonine | 0.08 | trace | - | - | - |
| Serine | 0.69 | 0.68 | trace | 1.00 | 1.00 |
| Glutamic acid | 0.30 | 0.77 | 1.00 | 0.05 | trace |
| Proline | 1.00 | 1.00 | - | - | - |
| Glycine | 0.49 | 0.08 | trace | 0.07 | - |
| Alanine | 0.13 | trace | - | trace | - |
| Half-Cystine | - | - | - | - | - |
| Valine | 0.17 | 0.05 | - | 0.21 | - |
| Methionine | - | - | - | - | - |
| Isoleucine | 0.06 | - | - | trace | - |
| Leucine | 0.17 | trace | - | trace | - |
| Tyrosine | - | - | - | - | - |
| Phenylalanine | - | - | - | trace | - |

[a]Prepared by Sephadex G-10 chromatography, followed by Dowex-1 chromatography.

The position of elution from Dowex-1 (indicating 1 net negative charge) and composition of peak D-1.2 suggests that it contained a peptide of structure PCA-(ser,pro). Peak D-1.3 was PCA, as it contained only glutamic acid after hydrolysis and co-eluted with an authentic sample of PCA on Dowex-1.

The composition and elution position of peak D-1.4, together with the presence of acetate in G-10.3, indicated that it contained a peptide of structure Ac-(ser,SCMC). Accordingly, a sample of this material, which had been purified by Sephadex G-10 chromatography followed by Dowex-1 chromatography, was subjected to partial acid hydrolysis (2 N HCl in 50% aqueous ethanol, 1 hr., 110°; Greenstein and Winitz, 1961, p.1243) to remove the acetate group. After taking to dryness, the partial-acid-hydrolysate was re-chromatographed on Dowex-1. One retarded peak was obtained, eluting between glutamic acid and SCMC, of composition 0.7 SCMC: 1.0 serine. N-terminal analysis of this fraction by the DNS procedure gave DNS-serine and an unknown spot, possibly DNS-Ser-SCMC.

It was concluded that the structure of peptide G-10.3 - D-1.4 was Ac-Ser-SCMC, and that the peptide was the N-terminal peptide of at least some of the embryonic feather proteins. Overall yield after allowing for sampling was approximately 0.2 moles/mole.

*(b) Isolation by the Chymotrypsin/Carboxypeptidase A Procedure*

As the yield of the N-terminal dipeptide was

low in the experiments described above and the sequence was identical to that of other feather keratins (O'Donnell, 1971), the use of chymotryptic digestion followed by carboxypeptidase-A as a preparative procedure was investigated. This combination of enzymes would be expected to give the N-terminal dipeptide in high yield if the N-terminal sequence was Ac-Ser-SCMC-Tyr (O'Donnell, 1971). A digest was prepared and the Dowex-50 eluate was fractionated by chromatography on Dowex-1 (Figure 4.3). The only major retarded product obtained eluted in the same position as Ac-Ser-SCMC derived from pronase digests and was of composition 0.9 SCMC : 1.0 serine. The material which was not retarded was presumed to be contaminants.

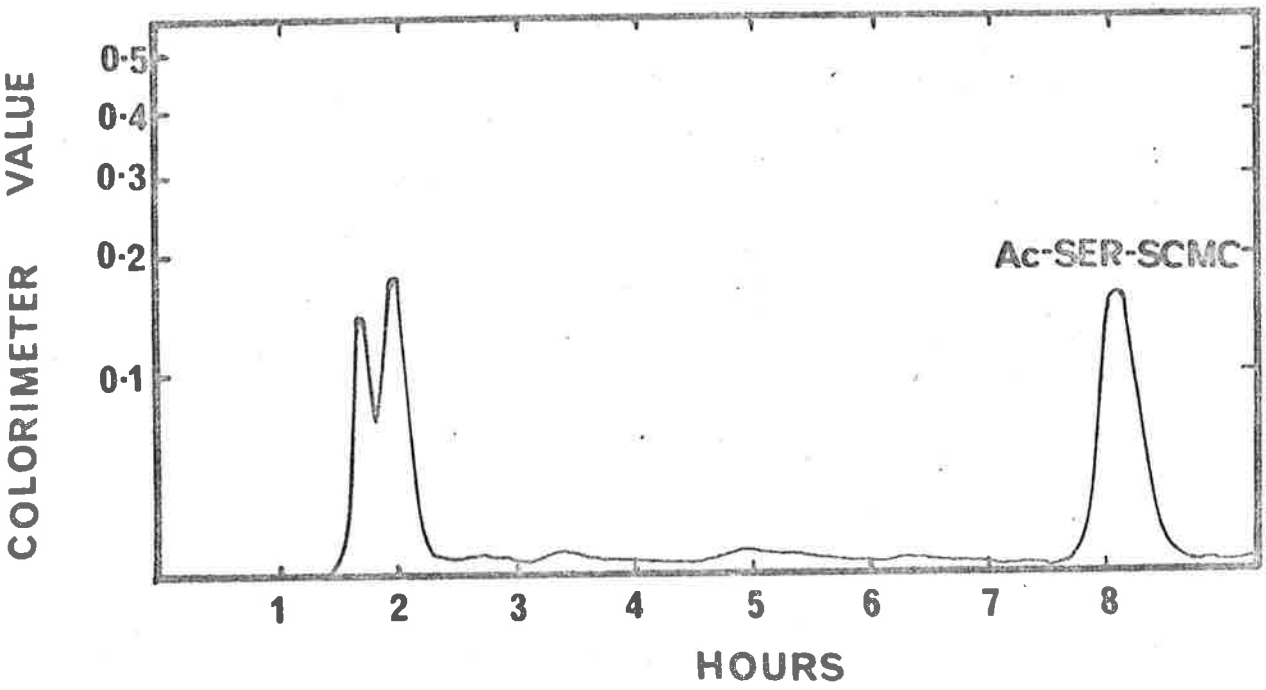
A sample of the peptide (approx. 2 µmoles) was subjected to HVPE at pH 6.5. A single ninhydrin negative, chlorination positive spot of m = 1.12 was obtained, in good agreement with the mobility of Ac-Ser-SCMC predicted from molecular weight and charge (Offord, 1966). Yield was 0.22 moles/mole.

### (c) *Isolation of the N-Terminal Dipeptide from Purified Fractions of Embryonic Feather Proteins*

A series of embryonic feather protein fractions was prepared by DEAE-cellulose chromatography as described by I.D. Walker (personal communication; see Figure 3.14). The N-terminal dipeptide was prepared from samples (10 mg) of DEAE-cellulose fractions 3-7 (Figure 3.14) by the chymotrypsin/carboxypeptidase A procedure and purified by

FIGURE 4.3.

Chromatography of the Dowex-50 eluate from a chymotrypsin/carboxypeptidase-A digest of embryonic feather proteins (50 mg) on Dowex-1. Procedures were as described in the text.


COLORIMETER VALUE
0·5
0·4
0·3
0·2
0·1
Ac-SER-SCMC
1
2
3
4
5
6
7
8
HOURS

Dowex-1 chromatography. The nature of the fractions tested and yield of Ac-Ser-SCMC from each of these fractions is shown in Table 4.3.

It is apparent that Ac-Ser-SCMC was the major N-terminal dipeptide of the β-proteins in fractions 4-7, as yields ranged from 0.45 - 0.59 moles/mole. The yield from fraction 3 (containing predominantly γ-proteins) was much lower (0.13 moles/mole) and probably could be accounted for by contamination of this fraction by β-proteins. The yield from unfractionated feather proteins was also lower than that from any of the purified β-protein fractions.

2. *Isolation of N-Terminal Tripeptides from Unfractionated and Fractionated Embryonic Feather Proteins*

As the β-protein fractions had the same N-terminal dipeptide, it was of interest to investigate whether this homology extended to the third residue. Samples (50 mg) of unfractionated feather proteins and some of the purified protein fractions (as described in Table 4.3) were digested with chymotrypsin, followed by Dowex-50 fractionation and then HVPE at pH 6.5. In each case, a ninhydrin negative, chlorination positive, α-nitroso-β-napthol positive band, of mobility 0.84 - 0.91 was obtained. The amino acid compositions and yields of these peptides after purification by HVPE at pH 6.5 are shown in Table 4.4.

Paper chromatography in pyridine/iso-amylalcohol/water (30:30:35; Baglioni, 1961) of a sample of the peptides

TABLE 4.3. YIELDS OF N-TERMINAL DIPEPTIDES FROM EMBRYONIC FEATHER PROTEIN FRACTIONS.

The yield from Fraction 4 was determined by quantitating the amount of serine in the dipeptide obtained, after purification of the dipeptide by Dowex-1 chromatography.

Yields from Fraction 3 and from Fractions 5-7 were determined from the areas of the peptide analyser chromatogram peaks, relative to that from Fraction 4. In all cases, a molecular weight of 10,000 was assumed.

| Sample | Major Component Present[a] | Yield (moles/mole) |
|---|---|---|
| Unfractionated | | 0.33 |
| DEAE-Fraction 3 | Band γ1 + γ3 | 0.13 |
| " 4 | " β5 | 0.49 |
| " 5 | " β4 | 0.59 |
| " 6 | " β3 | 0.45 |
| " 7 | " β3a | 0.46 |

[a]Determined by PAGE at pH 7.5 on samples of the DEAE-cellulose fractions. Each purified component gave 1 major band at this pH, but has been shown to consist of more than 1 polypeptide chain (I.D. Walker; personal communication).

TABLE 4.4. COMPOSITION AND YIELDS OF N-TERMINAL TRIPEPTIDES FROM EMBRYONIC FEATHER PROTEINS.

Analyses were performed using the long column of a Beckman 120C amino acid analyser. Values are expressed as residue ratios, relative to serine. Amounts indicated by "trace" were present in <0.05 residues. One analysis only was performed on all samples except fraction 7 (2 analyses).

| Sample | Unfractionated[a] | Unfractionated[b] | Fraction 5[a] | Fraction 6[a] | Fraction 7[a] |
|---|---|---|---|---|---|
| SCM-Cysteine | 0.91 | 0.92 | 0.88 | 1.04 | 0.96 |
| Aspartic acid | 0.06 | 0.09 | 0.06 | trace | trace |
| Threonine | trace | - | trace | - | - |
| Serine | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Glutamic acid | 0.12 | 0.06 | 0.10 | trace | trace |
| Proline | - | - | - | - | - |
| Glycine | 0.09 | 0.14 | 0.10 | trace | 0.07 |
| Alanine | trace | trace | trace | - | - |
| Half-Cystine | - | - | - | - | - |
| Valine | trace | trace | - | - | - |
| Methionine | - | - | - | - | - |
| Isoleucine | trace | trace | trace | trace | - |
| Leucine | 0.06 | trace | 0.06 | trace | trace |
| Tyrosine | 0.36 | 0.16 | 0.24 | 0.33 | 0.81 |
| Phenylalanine | 0.66 | 0.63 | 0.74 | 0.80 | 0.29 |
| YIELD[c] | 0.51 | n.d. | 0.40 | 0.46 | 0.28 |

[a] After purification by HVPE at pH 6.5.

[b] After purification by HVPE at pH 6.5, followed by chromatography in pyridine/isoamyl alcohol/water.

[c] Moles/mole, assuming a molecular weight of 10,000.

from unfractionated embryonic feather proteins, after purification by HVPE, gave one ninhydrin-negative band, Rf 0.37. The amino acid composition of this material is shown in Table 4.4.

The results demonstrated that there are at least two homologous N-terminal tripeptides of embryonic feather keratin proteins, namely Ac-Ser-SCMC-Phe and Ac-Ser-SCMC-Tyr. The relative amounts of these two species varied greatly in the different purified protein fractions (Table 4.4).

### 3. *Isolation of N-Terminal Peptides After Digestion with Elastase*

Samples (50 mg) of unfractionated embryonic feather proteins were digested with elastase, followed by Dowex-50 fractionation and then HVPE at pH 6.5. A broad ninhydrin-negative band, m = 0.75 was obtained. Paper chromatography of this material in pyridine/iso-amyl alcohol/water gave two ninhydrin-negative bands, designated E1 (Rf 0.29) and E2 (Rf 0.17). The amino acid compositions of these fractions are shown in Table 4.5.

It would appear from these results that E1 is the N-terminal tetrapeptide, of structure Ac-Ser-SCMC-$\{^{\text{Phe}}_{\text{Tyr}}\}$-Asn. In contrast to the N-terminal tri- and tetrapeptides, the total phenylalanine plus tyrosine content of fraction E2 did not approximate the SCMC and serine content. It would therefore appear possible that fraction E2 contains a mixture of homologous pentapeptides of general structure

TABLE 4.5. AMINO-ACID COMPOSITIONS OF N-TERMINAL ELASTASE PEPTIDES.

Analyses were performed using the long column of a Beckman 120C amino acid analyser. Values are expressed as residues ratios, relative to serine. Amounts indicated by "trace" were present in <0.05 residues.

| Sample | E1[a] | E2[b] |
|---|---|---|
| SCM-Cysteine | 0.75 | 0.91 |
| Aspartic acid | 0.71 | 0.72 |
| Threonine | - | - |
| Serine | 1.00 | 1.00 |
| Glutamic acid | 0.17 | 0.47 |
| Proline | - | 0.40 |
| Glycine | 0.18 | 0.28 |
| Alanine | 0.07 | 0.16 |
| Half-Cystine | - | - |
| Valine | - | trace |
| Methionine | - | - |
| Isoleucine | trace | trace |
| Leucine | - | 0.39 |
| Tyrosine | 0.19 | 0.35 |
| Phenylalanine | 0.55 | 0.22 |

[a]Average of 2 analyses.

[b]Average of 3 analyses.

$$\text{Ac-Ser-SCMC-}\{\overset{\text{Phe}}{\underset{\text{X}}{\text{Tyr}}}\}\text{-Asn-}\{\overset{\text{Y}}{\underset{\text{Z}}{}}\}$$

where X, Y, Z are glutamic acid (or gluamine), proline, or leucine.

4. *Isolation of N-Terminal Tryptic Peptides*

*(a) Trypsin/Carboxypeptidase B Procedure.*

Embryonic feather proteins (50 mg) were digested with trypsin, followed by carboxypeptidase-B. The digest was fractionated by Dowex-50 chromatography and then by HVPE at pH 6.5. One major acidic ninhydrin-negative band, designated Tc, was obtained. The amino acid composition of this material is shown in Table 4.6.

*(b) Sephadex G-50 Procedure.*

*(i) Identification of Sephadex G-50 fractions containing N-terminal tryptic peptides.*

Embryonic feather proteins (100 mg) were digested with trypsin and freeze-dried. The peptides were taken up in 1.0 ml of 50% aqueous pyridine and fractionated on Sephadex G-50 in PMA. The effluent was monitored by the ninhydrin reaction after alkaline hydrolysis. Aliquots (0.1 ml) from alternate fractions were dried on paper and stained to detect tyrosine. A typical profile is shown in Figure 4.4. Three major peaks of ninhydrin-positive material were obtained, G-50.1 - G-50.3. Peaks G-50.2 and G-50.3 contained tyrosine.

TABLE 4.6. AMINO-ACID COMPOSITIONS OF N-TERMINAL TRYPTIC PEPTIDES.

Values are expressed as residue ratios, relative to arginine, serine or SCMC.

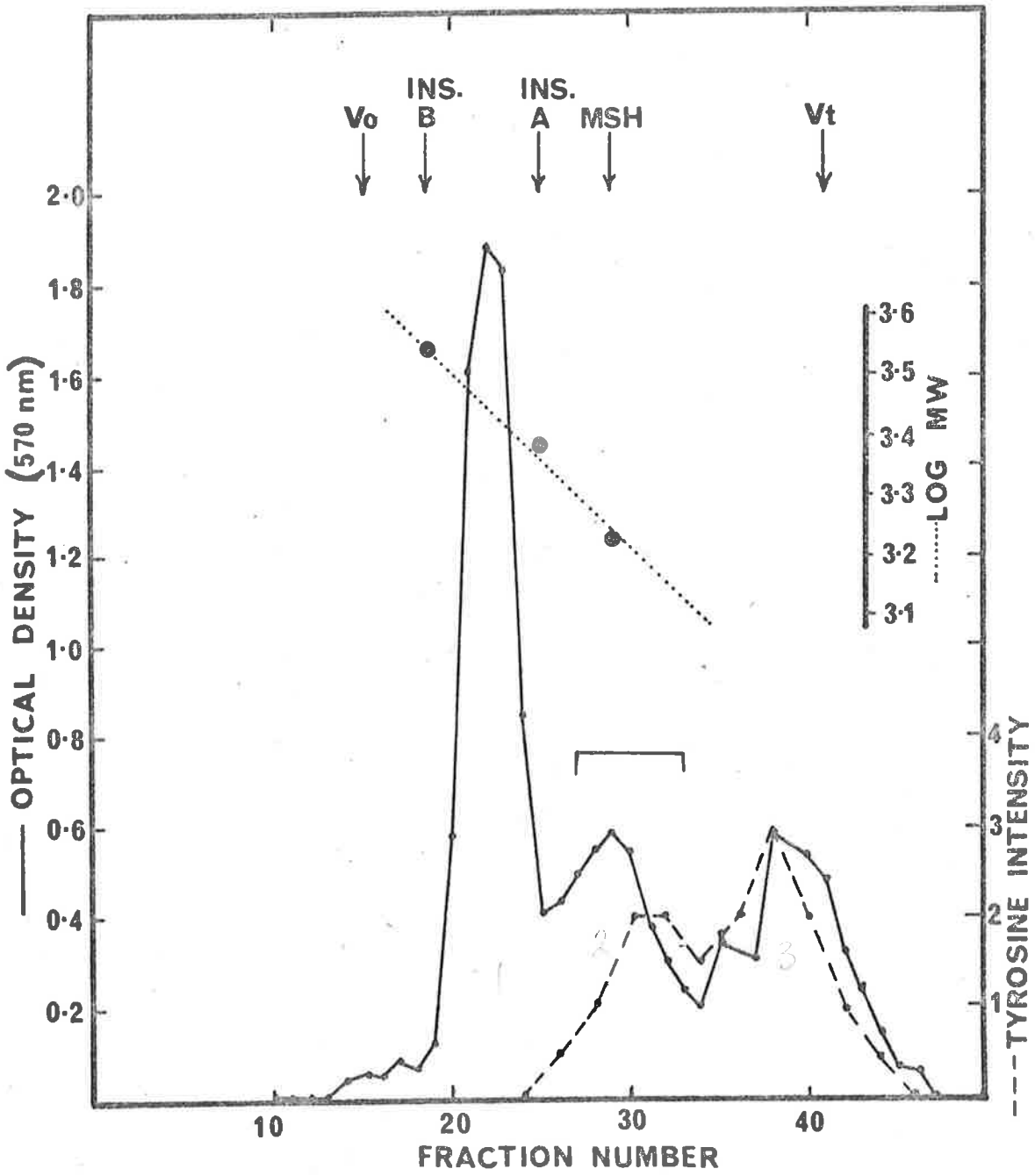
| Sample | Tc[a] | Tc[b] | T1[a] | T1[c] | T2[d] |
|---|---|---|---|---|---|
| SCM-Cysteine | 2.00 | 2.00 | 1.97 | 2.00 | 1.79 |
| Aspartic acid | 0.85 | 0.89 | 1.52 | 1.19 | 1.26 |
| Threonine | 0.44 | 0.46 | 0.57 | 0.44 | 0.61 |
| Serine | 1.23 | 1.25 | 1.06 | 1.05 | 1.00 |
| Glutamic acid | 0.96 | 1.09 | 0.60 | 0.61 | 1.12 |
| Proline | 1.54 | 1.64 | 2.28 | 1.96 | 2.51 |
| Glycine | 0.46 | 0.47 | 0.74 | 0.51 | 0.84 |
| Alanine | 0.43 | 0.40 | 0.63 | 0.49 | 0.67 |
| Half-Cystine | - | - | - | - | - |
| Valine | 0.36 | 0.63 | 0.58 | 0.43 | 0.46 |
| Methionine | - | - | - | - | - |
| Isoleucine | 0.06 | 0.12 | - | 0.02 | 0.19 |
| Leucine | 0.74 | 0.81 | 0.94 | 0.95 | 1.24 |
| Tyrosine | 0.26 | 0.28 | 0.10 | 0.26 | 0.20 |
| Phenylalanine | 0.22 | 0.27 | 0.39 | 0.36 | 0.29 |
| Lysine | - | - | - | - | 0.08 |
| Histidine | - | - | - | - | - |
| Arginine | - | - | 1.00 | 1.00 | 0.87 |

[a]One analysis; [b]One analysis; some preparation, after repurification by chromatography on Dowex-50.
[c]Average of 2 analyses, on sample prepared independently.
[d]Average of 2 analyses.

FIGURE 4.4.

Fractionation of a tryptic digest of embryonic featherproteins on Sephadex G-50. Procedures were as described in the text.

Fractions pooled are indicated by the bars. The elution volumes of standards of known molecular weight are indicated by arrows. "Tyrosine Intensity" refers to visual inspection of spot-tests with α-nitroso-β-napthol.


Vo
INS. B
INS. A
MSH
Vt
OPTICAL DENSITY (570 nm)
LOG MW
TYROSINE INTENSITY
FRACTION NUMBER
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
3·6
3·5
3·4
3·3
3·2
3·1
4
3
2
1
10
20
30
40
1
2
3

Accordingly, fractions across peaks G-50.2 and G-50.3 were tested for the presence of the tyrosine-containing N-terminal tryptic peptide(s) in the following manner. Aliquots (0.4 ml) from tubes 26-42 were treated with chymotrypsin (0.2 ml of a 1 mg/ml solution in NEMA, 37°, 24 hr) and then taken to dryness. The chymotryptic digests were subjected to HVPE at pH 6.5, and stained for the presence of tyrosine. Fractions 26-34 contained a tyrosine-positive acidic peptide of m = 0.85. All fractions tested contained other tyrosine positive material in addition.

In one experiment, it was found that the N-terminal tripeptide could be prepared by chymotryptic digestion, Dowex-50 chromatography and pH 6.5 HVPE of the material in G-50.2. The amino acid composition of this material was similar to that of the N-terminal tripeptides prepared from unfractionated embryonic feather proteins.

It was concluded that peak G-50.2 contained N-terminal tryptic peptides, as chymotryptic digestion of this peak produced N-terminal chymotryptic peptides.

*(ii) Isolation of N-terminal tryptic peptides.*

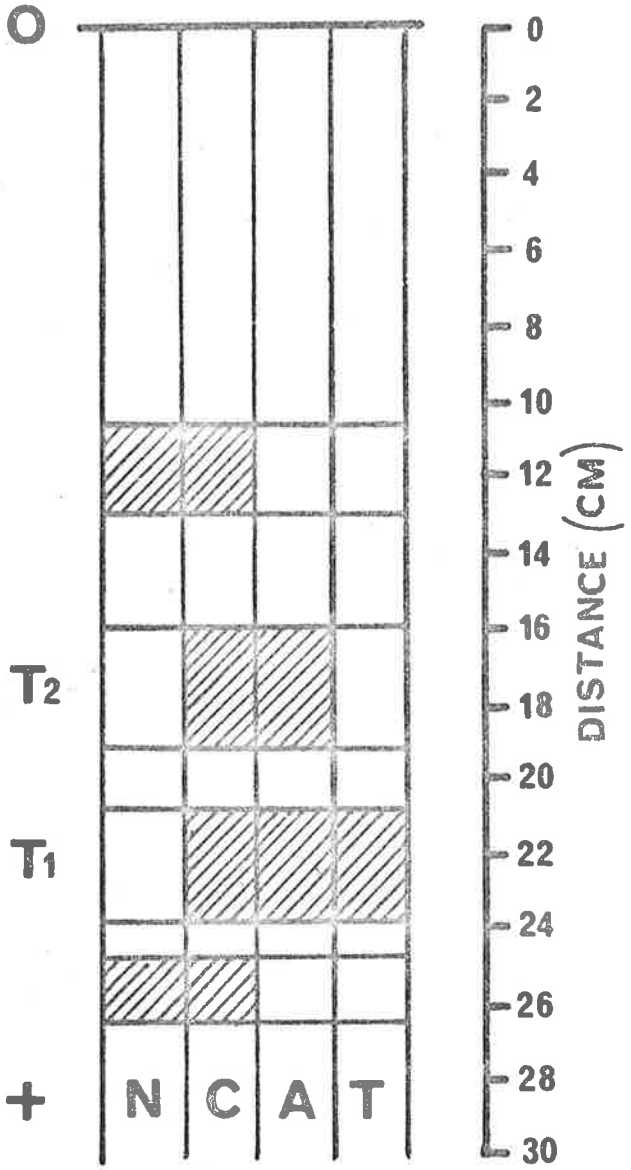
The fractions from peak G-50.2 were pooled as shown in Figure 4.4, freeze-dried, loaded as a wide strip on paper and subjected to HVPE at pH 6.5. Guide strips were then cut from the paper and stained to detect peptides. The fractionation and staining properties of the acidic peptides obtained are indicated diagrammatically in Figure 4.5. The staining properties of the peptides

FIGURE 4.5.

HVPE at pH 6.5 of acidic tryptic peptides from Sephadex G-50 peak 2. Electrophoresis was for 1 hour at 3000 V. After electrophoresis, test strips were stained to reveal material positive to the ninhydrin reaction (N), the α-nitroso-β-napthol reaction for tyrosine (T), the phenanthrenequinone reaction for arginine (A), or the chlorination/ starch/KI reaction (C), as described in the text.

O : origin

+ : anode


O
T2
T1
+
N
C
A
T
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
DISTANCE (CM)

designated T1 and T2 were consistent with them being N-terminal tryptic peptides.

The amino acid composition of peptides T1 and T2 (Table 4.6) also were consistent with them being N-terminal tryptic peptides, and were similar both to each other and to the peptide(s) obtained by the trypsin carboxypeptidase-B procedure.

*(iii) Molecular weight of N-terminal tryptic peptides.*

The Sephadex G-50 column was calibrated with blue dextran, S-carboxymethyl-insulin A and B chains, α-melanocyte-stimulating-hormone, and potassium ferricyanide (Figure 4.4). Peak G-50.2 eluted in a similar volume to α-melanocyte-stimulating-hormone (13 residues, MW 1648).

## D. DISCUSSION

The results presented in this chapter clearly establish that the N-termini of embryonic feather keratin proteins are acetylated, as is the case for all keratin proteins studied to date (Chapter 1). The N-terminal sequence information obtained is summarized and interpreted in Table 4.7.

The N-terminal sequences Ac-Ser-SCMC-$\{^{\text{Phe}}_{\text{Tyr}}\}$-Asn were established definitively by the compositions of the peptides isolated after treating the proteins separately

TABLE 4.7. SUMMARY AND INTERPRETATION OF N-TERMINAL SEQUENCE INFORMATION OBTAINED IN THE PRESENT WORK.

| | |
|---|---|
| PRONASE: | Ac-Ser-SCMC $\xrightarrow{H^+}$ Ser-SCMC $\xrightarrow[H^+]{\text{DNS-Cl}}$ DNS-Ser |
| CHYMOTRYPSIN/ CARBOXYPEPTIDASE A: | Ac-Ser-SCMC |
| CHYMOTRYPSIN: | Ac-Ser-SCMC-{Phe, Tyr} |
| ELASTASE 1: | Ac-Ser-SCMC-{Phe, Tyr}-Asn |
| ELASTASE 2:[a] | Ac-Ser-SCMC-{Phe, Tyr, X}-Asn-{Y, Z} |
| TRYPSIN/ CARBOXYPEPTIDASE B:[a] | Ac-Ser-SCMC-{Phe, Tyr, X}-Asn-(Pro,SCMC,Leu,Pro,Glx,{Thr, Ala, Gly, Val} ) |
| TRYPSIN 1:[a] | Ac-Ser-SCMC-{Phe, Tyr, X}-Asn-(Pro,SCMC,Leu,Pro,Glx,{Thr, Ala, Gly, Val} ) _____Arg |
| TRYPSIN 2:[b] | Ac-Ser-SCMC-{Phe, Tyr, X}-Asn-(Pro,SCMC,Leu,Pro,Glx,Gly,{Thr, Ala, Pro, Val}) _____Arg |
| EMU RACHIS:[b] | Ac-Ser-SCMC-Tyr -Asn- Pro-SCMC-Leu-Pro _____Arg |

[a]The interpretation shown is based on the N-terminal tetrapeptide sequence established in the present work and the amino acid compositions of the peptides. The amino acids not accounted for by the N-terminal tetrapeptides are aligned to maximise homology with the known N-terminal sequence of emu rachis proteins.

[b]I.J. O'Donnell; personal communication.

with pronase, chymotrypsin, chymotrypsin plus carboxypeptidase-A and elastase. The presence of a third residue at position 3 is suggested by the composition of the elastase peptide E2. If this is so, it is perhaps surprising that additional N-terminal chymotryptic peptides were not obtained; however, lack of efficient cleavage sites, extensive heterogeneity, and the lack of sensitivity of the chlorination procedure can be invoked to explain their absence. Furthermore, the low yields of the N-terminal di- and tri-peptides (Tables 4.3 and 4.4) are in accord with (an) additional amino acid(s), not susceptible to chymotrypsin, at position 3.

The structures of the N-terminal tryptic peptides (Table 4.7) are somewhat more speculative. Nevertheless, certain conclusions can be drawn. Of the residues not accounted for by the N-terminal tetrapeptides, some (proline, leucine, SCMC, glutamic acid) tend to be present in near molar amounts in each of the three different products obtained. It is noteworthy that, except for the presence of glutamic acid (or glutamine), these can be written as a sequence identical to that of emu feather keratin (I.J. O'Donnell; personal communication). In each of the three products, other residues (e.g., threonine, alanine, valine) were present in significant but not molar amounts. It would therefore appear probable that the N-terminal tryptic peptides from embryonic feather keratin are homologous with those from emu rachis keratin, but contain an additional 3-4 amino acids. Amino acid substitutions occur at position 3 in the different polypeptides, and presumably,

at several other positions.

The molecular weight of the Sephadex G-50 fraction S-50.2, containing the N-terminal tryptic peptides, was estimated at 1650 daltons or approximately 13 residues (Figure 4.4), a value in good agreement with the amino acid compositions of peptides Tc, T1 and T2.

The total amounts of tyrosine plus phenylalanine were significantly less than molar in each tryptic peptide obtained, in agreement with elastase peptide(s) E2. Excellent recoveries of tyrosine plus phenylalanine were obtained from the amino acid analyser for the N-terminal chymotryptic peptides and elastase peptide(s) E1, indicating that technical problems in the determination of these amino acids were not responsible for their low values in the other peptides. The data therefore require that at least three amino acids (Tyr, Phe, X) can occur at position 3 in the sequences of different feather keratin polypeptide chains. Furthermore, since at least three residues apparently occur in position 3 in each of the distinct tryptic peptides T1 and T2, a minimum of six different N-terminal tryptic peptides is deduced.

The above data, although not definitively establishing more than the N-terminal tetrapeptide sequence, establish that there are more than two N-terminal sequences present in embryonic feather proteins. Thus the presence of different sequences cannot be entirely accounted for by allelic variation, and there must be more than one gene for embryonic feather keratin proteins per haploid chromosome set.

The evidence for a series of homologous N-terminal peptides in embryonic feather keratin proteins provides support for the concept (Chapter 3) that the heterogeneity of feather keratin proteins results from gene duplication and subsequent mutational divergence (see, for example, Zukerkandl and Pauling, 1965). Sequence studies on the high-sulphur keratin proteins of wool have indicated such duplications, both of genes and of regions within genes, with subsequent mutational divergence (Gillespie *et al.*, 1968; Haylett and Lindley, 1968; Lindley *et al.*, 1971; Haylett and Swart, 1969; Ellerman, 1971).

The results obtained in the present Chapter do not support an alternative hypothesis, namely that the heterogeneity of keratin proteins results from their post-translational modification. Since the proteins have acetylated N-termini, the presence of the acetyl group may in fact be the result of a post-translational modification. On the other hand, the acetyl group may function in the initiation of keratin synthesis (see Chapters 1 and 6). The results obtained in the present Chapter provide the basis for studies on the initiation of keratin synthesis *in vitro* and the possible role of acetyl-ser-tRNA in the mechanism of initiation, described in Chapter 6.

## E. APPENDIX 1.

## FIBRILLATION OF A TRYPTIC PEPTIDE FRACTION FROM EMBRYONIC FEATHER PROTEINS

After fractionation of embryonic feather tryptic peptides on Sephadex G-50 (Figure 4.4), it was observed that the contents of tubes containing peak G-50.1 formed a gel upon standing. After storage for 1 day at room temperature followed by 2 days at 2-4°, tube 22 (Figure 4.4) could in fact be inverted without loss of the contents. Electron-microscopic examination of the material from peak G-50.1 revealed the presence of fibrils, apparently identical to those formed from intact feather keratin (Figure 4.6).

The amino acid composition ofthis material is shown in Table 4.8. The fibrillating peptide fraction was enriched in serine and hydrophobic amino acids compared with feather keratin, and depleted in S-carboxymethyl-cysteine.

The molecular weight of the fraction was estimated at 3856 daltons by amino acid analysis (assuming 1 mole arginine/mole) and at 2820 daltons by Sephadex G-50 chromatography. This rather large discrepancy in molecular weights might result from a variety of reasons. Firstly, the peak could also contain C-terminal peptides, resulting in a low value for arginine. Secondly, the peak could be a mixture of homologous internal peptides which interact in some manner with the Sephadex, resulting in an anomalous apparent molecular weight (see, for example, Milne and Wells, 1970). A third, more interesting, explanation is

FIGURE 4.6.

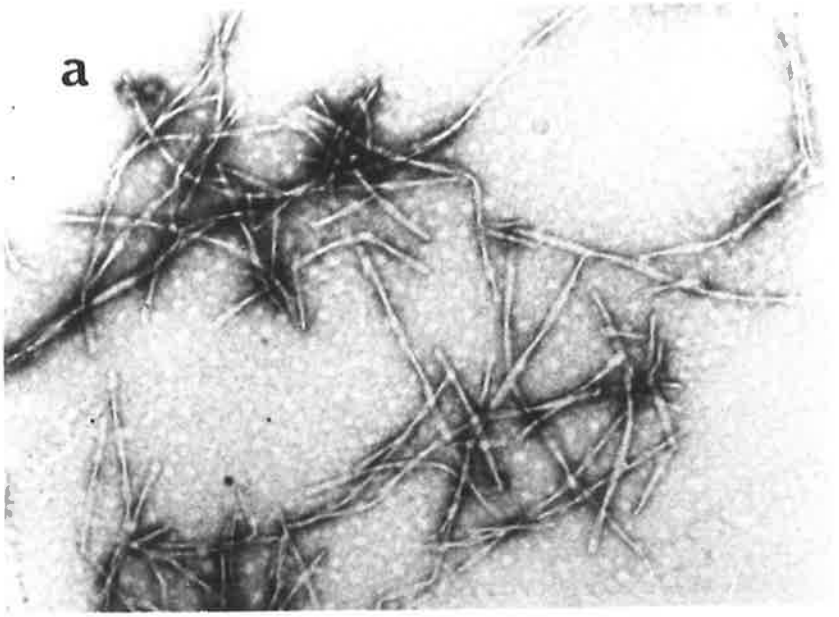
Electron-micrographs of fibrils obtained,

(a) from fraction 22 of Figure 4.4.

(b) for comparison, fibrils obtained from embryonic feather proteins, as in Figure 3.10.

Negatively stained with uranyl acetate.

Magnification: X60,000.


a

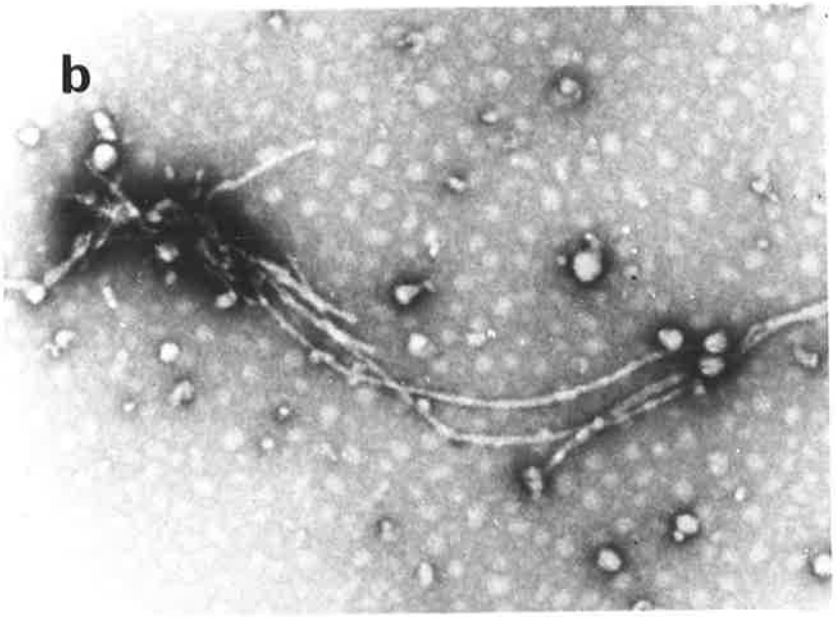

b

TABLE 4.8. AMINO-ACID COMPOSITION OF THE FIBRILLATING TRYPTIC PEPTIDE FRACTION FROM SEPHADEX G-50 CHROMATOGRAPHY.

Values are expressed as residue ratios, relative to arginine.

| | |
|---|---|
| SCM-Cysteine | 0.62 |
| Aspartic acid | 1.33 |
| Threonine | 1.94 |
| Serine | 7.83 |
| Glutamic acid | 3.05 |
| Proline | 4.13 |
| Glycine | 5.81 |
| Alanine | 2.10 |
| Half-Cystine | - |
| Valine | 4.25 |
| Methionine | - |
| Isoleucine | 2.85 |
| Leucine | 2.83 |
| Tyrosine | trace |
| Phenylalanine | 1.46 |
| Lysine | - |
| Histidine | - |
| Arginine | 1.00 |

that fraction G-50.1 consists of a family of homologous internal tryptic peptides and that each molecule existed as a folded compacted structure during the course of chromatography, resulting in a low apparent molecular weight value.

These results can be interpreted in terms of the structure proposed for native feather keratin (Fraser *et al.*, 1971). It has been shown that fibrils from S-carboxymethyl-keratin proteins contain the cross-β conformation (Burke, 1969) and suggested that this represents packing of the pleated sheet units side-to-side, instead of end-to-end as in the native state (Fraser *et al.*, 1972). The present results would therefore provide some chemical evidence in support of the postulates that

(a) the whole molecule is not necessary for formation of the pleated sheet units;

(b) the cystine residues are concentrated in the non-β portion of the molecule, and

(c) the pleated sheet portion of the molecule is not at the N terminus.

Further, if the discrepancy in molecular weight values obtained *does* result from folding of each molecule, into a pleated sheet unit, then

(d) the pleated sheet portion of the molecule is situated more or less centrally in the molecule, as the

tryptic peptides containing this sequence are internal peptides; and

(e) the pleated sheet units are formed by multiple folding of a single polypeptide chain.

Similar observations of fibrillating tryptic peptides have been made with emu rachis (I.J. O'Donnell; personal communication) and fowl calamus (I.D. Walker; personal communication). In the case of emu rachis, the fibrillating peptide was shown to be an internal peptide, and the cystine residues were found to be concentrated in the N- and C-terminal regions of the molecule.

# CHAPTER FIVE

# KERATIN SYNTHESIS DURING DEVELOPMENT OF THE EMBRYONIC FEATHER

## A. INTRODUCTION

The present chapter describes quantitative studies on keratin synthesis during development of the embryonic chick feather.

Previous studies on keratin synthesis in intact cells have been hindered by one or more of three major problems. In the hair follicle system for example, although the keratin proteins have been characterized, the tissue does not actively incorporate labelled amino acids during culture, and no satisfactory procedure for obtaining living tissue at different stages of development has been reported (see, for example, Clarke, 1967; Fraser, 1969b; Steinert, 1972). Although cultured mouse skin will incorporate labelled amino acids into protein, the keratin proteins are not well characterized and tissue has not been obtained at different developmental stages (see, for example, Sugawara and Bernstein, 1971). Studies on the developing embryonic chick feather were previously hampered by the lack of a suitable assay system for the keratin proteins, as these had not been definitely identified (Bell and Thathachari, 1963; Malt and Bell, 1965; Ben Or and Bell, 1965).

In the work described below, the results presented in Chapter 3 have been applied to the quantitative description of keratin synthesis during development of the embryonic feather. Results obtained by quantitative PAGE have been compared with the results of radioisotope incorporation and of electron-microscopic studies. Electron-

microscopic autoradiography has been used to investigate the temporal relationship of the onset of keratin synthesis with respect to nucleic acid synthesis within individual cells.

## B. METHODS

### 1. *Preparation of Embryonic Feathers*

Embryos were carefully removed from the eggs and rinsed twice with Hanks' solution. The feathers were removed using jewellers' forceps, and placed in Hanks' solution. Wing feathers were not collected, as these develop differently to body feathers (Maderson, 1942). When measurement of the packed volumes of feathers was required, the feathers were placed in graduated glass centrifuge tubes in Hanks' solution and centrifuged at 4500 g for 10 min., using an MSE Super Minor Centrifuge. The packed volumes were measured immediately after centrifugation. The volumes of feathers used ranged from 0.1 - 0.5 ml in different experiments. All operations were performed at room temperature (approximately 25°).

### 2. *Extraction of Feather Proteins*

After the determination of packed volumes, proteins were extracted from the feathers by reduction and carboxymethylation as described in Chapter 3. Younger feathers were lysed upon contact with the urea/β-mercaptoethanol solution, which became extremely viscous, presumably due to the presence of nucleic acids. Both these effects

decreased markedly with increase in developmental age.

During dialysis, varying amounts of a stringy, white precipitate formed. This was particularly marked in extracts from younger tissue, and increased with increasing times of dialysis. Removal of the precipitate by centrifugation resulted in the selective depletion of several of the bands of intermediate mobility on pH 9.5 polyacrylamide gels (see Results). The precipitate was therefore evenly dispersed by homogenization immediately before sampling for the determination of protein content, unless stated otherwise. The homogenized dialysates were made up to a known volume with water, and the protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Values were expressed as mg protein per ml of packed feather tissue. The preparations were freeze-dried, and taken up at a protein concentration of 10 mg/ml in 4 M urea.

The extracts, particularly from the younger tissue, were contaminated with nucleic acids, as determined by UV spectra. No attempt was made to remove the nucleic acids as these do not interfere appreciably with the Lowry reaction (Lowry *et al.*, 1951) and do not stain with Coomassie Brilliant Blue (Dahlberg *et al.*, 1969).

### 3. *Quantitative Polyacrylamide Gel Electrophoresis*

Samples containing known amounts of protein were fractionated by PAGE at pH 9.5, 7.5 or 2.7 as described in Chapter 3. In any one experiment, identical amounts of total

protein from each embryonic feather extract were applied to the gels; usually the amount was either 50 or 100 µg of protein. A standard curve was obtained in each experiment by running a set of gels loaded with known amounts of total 21-day embryonic feather proteins. After the run, protein in the gels was precipitated in 10% TCA for 30 min. and then stained for 1-2 days in 0.05% Coomassie Brilliant Blue in 10% TCA (Chrambach *et al.*, 1967). The gels were then rinsed in 60% ethanol to remove precipitated dye and allowed to stand overnight in 10% TCA in the dark. The band patterns were recorded by densitometry. The areas under appropriate peaks of the densitometer traces were calculated, using the automatic integrator.

As the peaks were not completely resolved by densitometry after PAGE at pH 9.5 and pH 7.5, the number of integrator units between the lowest point of successive "troughs" in the densitometer traces was considered to represent the area of the peak between them. Although this procedure would presumably result in the overestimation of some peaks andunderestimation of others, the similarity in profiles of the keratin-containing regions of the gels at days 13-15 should ensure that such weighting of peaks be relatively constant at each age, thereby providing a valid means of comparison.

In the case of PAGE at pH 2.7, the bands in groups A + B and D + E were not resolved sufficiently for their individual quantitation by the above procedure, and as a consequence, they were quantitated as these groups. The

curve shapes and relative areas of bands A and B were then determined by least squares analysis of the densitometer traces (R.D.B. Fraser and E. Suzucki; personal communication).

The amount of protein in each band was determined from the standard curve, assuming equal dye binding by the proteins in each band. This assumption appears valid for the different feather keratin proteins (I.D. Walker; personal communication).

4. *Incorporation of $^{14}$C-Leucine by Feather Tissue*

12-15-day feathers were collected and washed in Hanks' solution as described above. They were then incubated for 2 hr. at 37° in Hanks' solution, containing amino acids at the concentrations used in Charity Waymouth's medium, supplemented with serine (75 mg/litre), proline (50 mg/litre), alanine (50 mg/litre) and glutamine (350 mg/litre). $^{12}$C-Leucine was omitted, and 5.0 µCi/ml of $^{14}$C-leucine (sp. act. 316 mC/mmole) was added. The medium contained chloramphenicol (10 µg/ml) and penicillin (10 µg/ml). Incubation was terminated by removing the incubation medium and adding 1.0 ml of cold Hanks' solution containing 0.1% leucine. The packed volumes of the feathers were then determined, and protein extracted as described above.

5. *Polyacrylamide Gel-Electrophoresis of Radioactive Proteins*

Samples containing known amounts of $^{14}$C-protein were subjected to PAGE at pH 7.5 as described in Chapter 3, except

that ethylene diacrylate was used as the cross-linking agent in the gels to facilitate the subsequent determination of radioactivity (Cain and Pitney, 1968). It should be pointed out that initial attempts were made to use the pH 9.5 system in this manner but the results were poorly reproducible because the gels were unstable. This finding contrasts with the results of Cain and Pitney (1968). Ethylene diacrylate gels were stable at pH 7.5. After electrophoresis, the gels were stained and analysed by densitometry as described above. The gels were then sliced into 1 mm sequents, using a razor-blade type gel slicer (Mickle Engineering Co., Gomshall, Surrey, England). Each slice was placed in a glass vial (0.8 cm x 4.5 cm) and solubilized by treatment with 1.0 M ammonium hydroxide (0.4 ml; 60° for 2 hours, or overnight at room temperature). The solution was absorbed onto a glass fibre filter and dried at 110° for the determination of radioactivity.

### 6. *Incorporation of $^{3}$H-Thymidine and $^{3}$H-Uridine by 12-day Feathers*

In order to prevent morphological damage which might interfere with subsequent electron-microscopy, feathers were carefully teased from 12-day embryos still attached to small pieces of skin, using jeweller's forceps. As much as possible of the attached skin was then removed from each feather, taking care not to grasp the feathers themselves with the forceps during these operations. The feathers were then incubated for 30 min. at 37° in Charity Waymouth's

medium containing 50 µCi/ml of $^{3}$H-thymidine (sp. act. 100 mC/mmole) or $^{3}$H-uridine (sp. act. 31 C/mmole). The medium was then drained off, and the feathers were washed quickly twice with cold medium containing unlabelled uridine or thymidine (1 mg/ml) and prepared for electron-microscopic autoradiography. In control experiments, ACT-D was present during the incubation with $^{3}$H-uridine or the feathers were incubated in medium containing ACT-D for 18 hours after the incorporation. ACT-D was used at a concentration of 50 µg/ml (Bell and Merrill, 1967).

7. *Electron Microscopy*

Samples were fixed for 1 hr. in a solution containing 2% v/v glutaraldehyde and 0.1 M sodium cacodylate, pH 7.2, and then washed in a solution containing 0.18 M sucrose in 0.1 M sodium cacodylate for 30 min. Post-fixation was in a solution containing 2% w/v osmic acid in 0.1 M sodium cacodylate for 30 min., followed by a wash in 1% w/v uranyl acetate, and dehydration through graded acetones. The samples were then embedded in araldite. Sections were cut on an LKB microtome, using glass knives. The sections were picked up on carbon coated grids, and stained for 15 min in lead citrate. The sections were examined in a Siemens Elmiskop I at 80 kV, using a 50 µ objective aperture.

*8. Electron-microscopic Autoradiography*

After incubation with $^{3}$H-thymidine or $^{3}$H-uridine, the feathers were fixed and stained as described above, except that the glutaraldehyde fixative contained 1 mg/ml unlabelled thymidine or uridine. After staining, the sections were coated with a thin carbon film and covered with a film of Ilford L4 photographic emulsion. The sections were exposed for 60 days at 4°, and then developed in Microdol (Caro, 1964) or fine grain developer (Paweletz, 1967).

## C. RESULTS

*1. Changes in Total Protein Content During Feather Development*

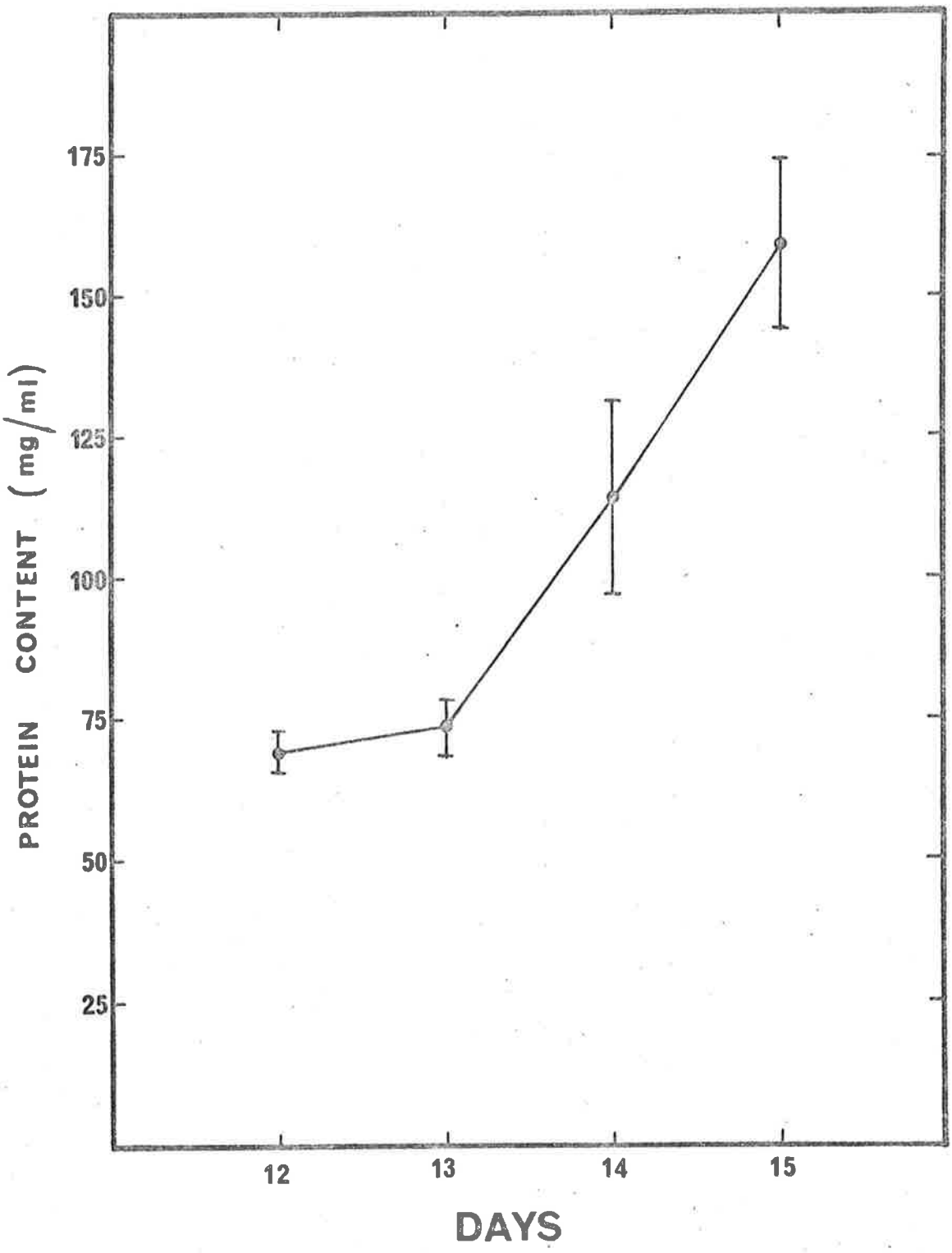
Total protein contents of feathers at various developmental ages are shown in Fig. 5.1. Statistical analysis of the data (see caption to Figure 5.1) demonstrated that significant increases in protein content occurred between days 13 and 14 and between days 14 and 15. By day 15 the protein content was over double that at day 13, indicating a dramatic change in rate of protein synthesis at about 13 days.

In individual experiments, values consistently higher or lower than the mean for protein content at each age were observed. Whether this effect resulted from the extraction procedure or from differences in batches of embryos is not

FIGURE 5.1.

Total Protein Content of Feathers During Development.

Total protein content per ml of packed feathers was determined as described in Methods. Results shown are the mean of 5 independent experiments, plus and minus the standard errors of the means. In two of these experiments, the tissue had previously been incubated for 2 hours with $^{14}$C-leucine, as described in Methods. Analysis of variance showed a significant effect arising from feathers of different ages ($p = 0.0002$) but although there was some scatter between the independent experiments, the effect was just above the critical $\alpha$-level of 0.05 ($p = 0.0538$). The Newman-Keuls A-Posteriori Test Between Means (Winer, 1962) showed no significant difference ($\alpha = 0.05$) between all but one of the 10 possible pairs of experiments. There was no significant difference between the protein content at days 12 and 13 ($p > 0.05$), but significant differences between the protein content at days 13 and 14 ($0.01 < p < 0.05$) and days 14 and 15 ($p < 0.01$).


175
150
125
100
75
50
25
PROTEIN CONTENT (mg/ml)
12
13
14
15
DAYS

known. However, in every experiment, the observed protein content increased between days 13 and 14 and between days 14 and 15.

The scatter in experimental points at days 14 and 15 presumably also reflects to some extent the individual variation in development of different embryos. At these ages, as the protein content is rapidly increasing, variations in individual development over a total time of about 12 hours, spread across the mean, would account for much of the observed scatter. Such variation in developmental age would be expected (Lillie, 1965).

### 2. *Determination of Keratin Content During Feather Development by Quantitative Polyacrylamide Gel Electrophoresis*

Aliquots of the protein preparations were fractionated by polyacrylamide gel electrophoresis, stained and analysed by quantitative densitometry as described in the Methods section.

#### *(a) Polyacrylamide Gel Electrophoresis at pH 9.5*

Marked differences in patterns were observed between protein preparations depending on whether the precipitate formed during dialysis was removed by centrifugation or not. The γ-proteins were removed to a varying extent by this step, as was found for the 21-day embryonic feather proteins (Chapter 3.C.4.a). In order to avoid removing some of the slower moving β-components as well (Chapter 3.C.4.a)

centrifugation at this stage was omitted in subsequent studies.

In the 11- and 12-day feather extracts, protein bands corresponding in mobility to the major feather keratins (bands β2 -β5) were present only as traces (Figure 5.2). These bands rapidly increased in quantity after day 12, and had already become the most abundant protein species in the feathers by day 15.

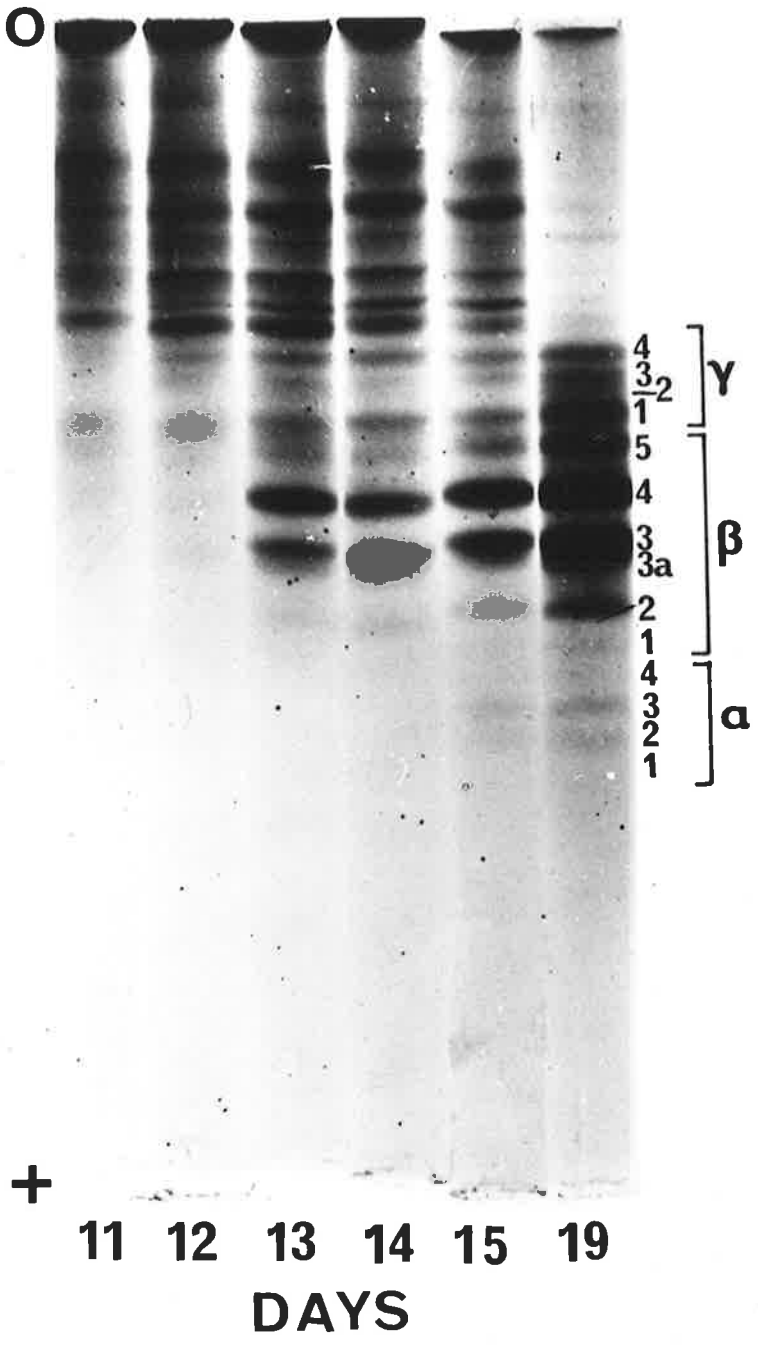
The α-proteins appeared to follow a similar course during development to the β-proteins, but were not detectable as early, presumably because of their low amount. The γ-proteins followed a different pattern during development. These were readily detectable at 11-12 days, and did not undergo a similar rapid increase in amount after day 12.

The precise pattern of protein bands in the γ-region varied somewhat in different experiments. However, their pattern was very similar from gel to gel in each individual experiment, and the same general conclusions could always be drawn, namely that the γ-proteins were present at 11-12 days and did not undergo the same rapid increase as the proteins after day 12. No such variation ever occurred in the cases of the α- and β-proteins in different experiments. It would appear that resolution of the γ-proteins is particularly sensitive to slight variations in the electrophoretic conditions. It is also possible that the insolubility in water of the γ-proteins led to selective and variable losses of some components during manipulation.

FIGURE 5.2.

PAGE at pH 9.5 of Proteins from Feathers During Development.

Samples (100 µg/gel) of the reduced and carboxymethylated protein preparations from feathers at 11-19 days of development were subjected to polyacrylamide gel electrophoresis at pH 9.5 and stained with Coomassie Blue as described in Methods. O: origin; + : anode. Bands α1 - γ4 are indicated.


O
4
3
2
1
γ
5
4
3
3a
2
1
β
4
3
2
1
α
+
11 12 13 14 15 19
DAYS

*(b) Polyacrylamide Gel Electrophoresis at pH 7.5.*

The patterns of keratin bands obtained by PAGE at pH 7.5 (Figure 5.3) were essentially identical to those obtained by PAGE at pH 9.5 (Figure 5.2), in that the β-bands rapidly increased in amount after day 12. The pattern in the γ-region was somewhat different, however. The value of this system lay in its applicability to the fractionation of radioactive proteins (see below).

*(c) Polyacrylamide Gel Electrophoresis at pH 2.7.*

Fractionation of proteins from 11-19 day feathers by PAGE at pH 2.7 is shown in Figures 5.4 and 5.5.

After electrophoresis for six hours in this system (Figure 5.4) the major keratin bands (A-H) were not as well resolved from the background of non-keratin proteins as in the high pH system. It was clear, however, that there was a rapid increase in amount of each keratin band after day 12.

After electrophoresis for shorter times (Figure 5.5), it was observed that the "fast" component was not present in extracts from 11-15-day feathers but was present in the extract from 19-day feathers.

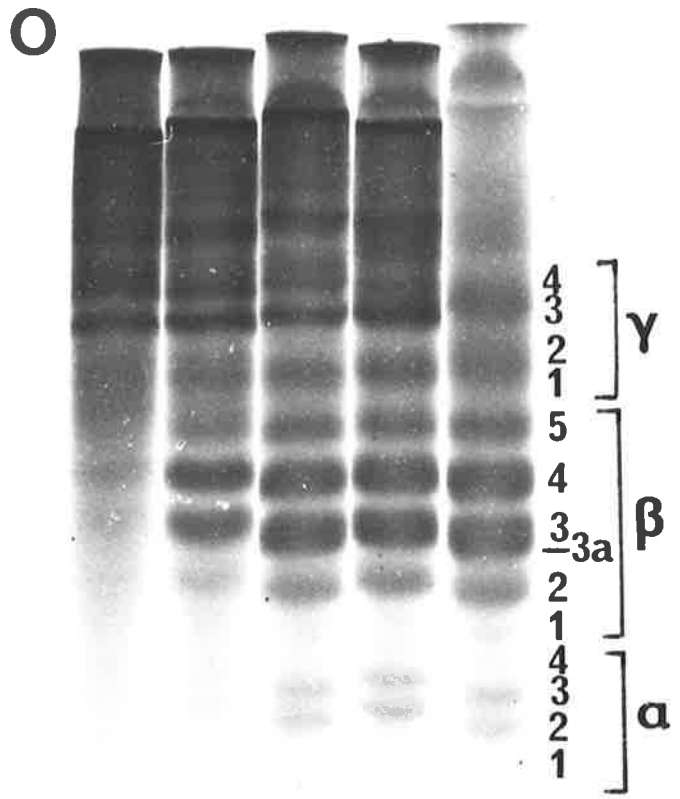
The time course of synthesis of the "fast" component therefore contrasts with the time courses of synthesis of both the γ-proteins, as detected by PAGE at pH 9.5 and pH 7.5 and the major keratin proteins, as detected by all PAGE systems.

FIGURE 5.3.

PAGE at pH 7.5 of Proteins from Feathers During Development.

Samples (100 µg/gel) of the reduced and carboxymethylated protein preparations from feathers at 12-15 days of development were subjected to polyacrylamide gel electrophoresis at pH 7.5 and stained as described in Methods.

O: origin; + : anode. Bands α1 - γ4 are indicated.




+

12 13 14 15 21

DAYS

FIGURE 5.4.

PAGE at pH 2.7 of Proteins from Feathers During Development.

Samples (50 µg/gel) of the reduced and carboxymethylated protein preparations from feathers at 12-15 days of development were subjected to polyacrylamide gel electrophoresis at pH 2.7 for 6 hours at 2 mA/gel and stained as described in Methods. For comparison the pattern obtained from 21-day feather protein is shown. O: origin; - : cathode.

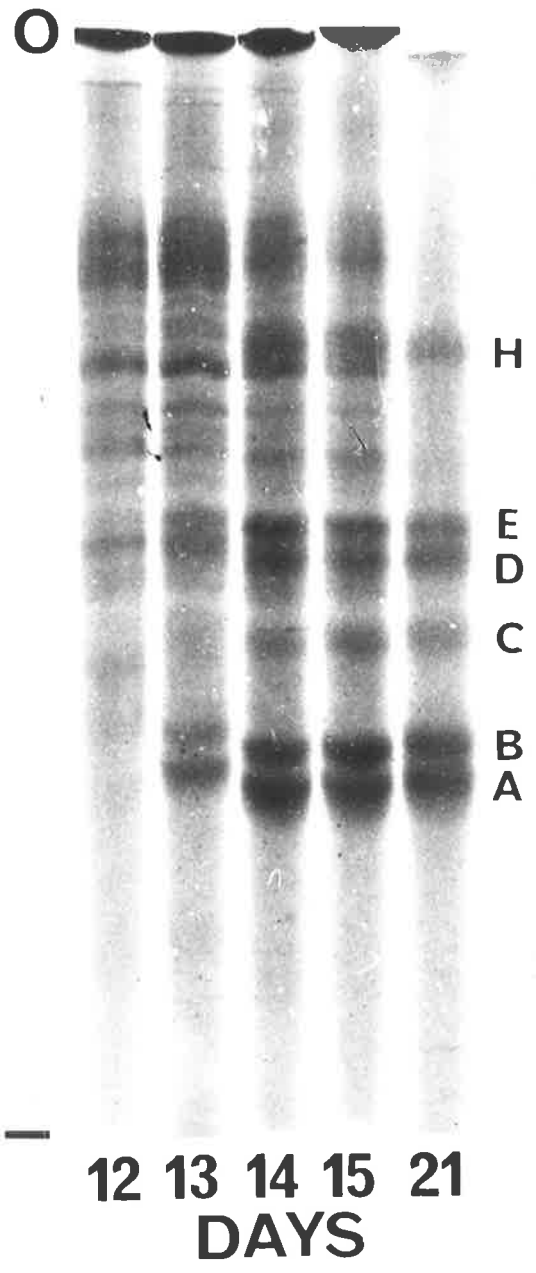
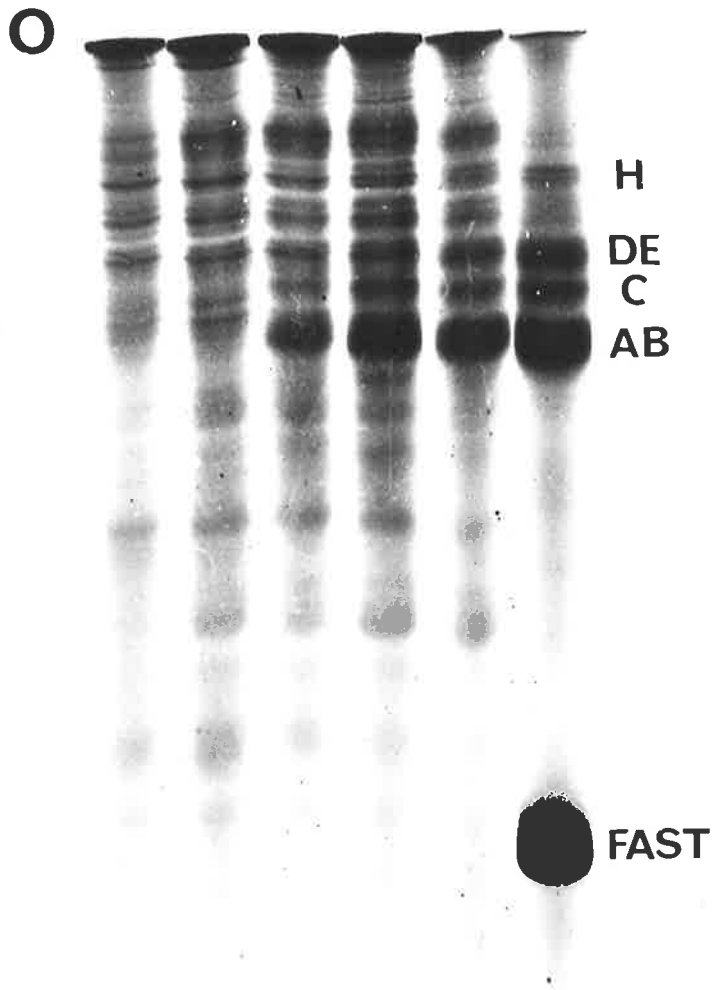

O
H
E
D
C
B
A
12 13 14 15 21
DAYS

FIGURE 5.5.

PAGE at pH 2.7 of Proteins from Feathers During Development.

Samples (50 µg/gel) of the reduced and carboxymethylated protein preparations from feathers at 11-19 days of development were subjected to PAGE at pH 2.7 for 2.75 hours at 2 mA/gel and stained as described in Methods. O: origin; - : cathode. Bands A-H and the "fast" band are indicated.




– 11 12 13 14 15 19

DAYS

*(d) Quantitation of Protein Bands on Polyacrylamide Gels.*

Standard curves were prepared by densitometry of gels upon which known amounts of 21-day embryonic feather proteins had been fractionated, as described in Methods. The total integrator values for all bands on these gels were plotted against protein loaded for each gel system (Figure 5.6). In each system, a linear relationship over a usable range was observed. The accuracy of these values was least in the case of PAGE at pH 2.7, presumably because it was necessary to load samples of less than 0.02 ml quantitatively in this system to obtain maximal resolution (Panyim and Chalkley, 1969).

Artefacts in quantitative densitometry of polyacrylamide gels can arise, in that the effects of protein spreading in bands of different mobility can lead to erroneous values (Krushi and Narayan, 1968). For the present purposes, howeve, such effects would not be expected to alter the general conclusions since a similar profile of keratin bands was observed at each embryonic age.

The amounts of the major keratin bands resolved in each of the three PAGE systems per ml of packed 12-15-day feathers are shown in Figures 5.7 - 5.9. Similar results were obtained in all three systems. An increase in amount of each band occurred between days 12 and 13, followed by a more rapid increase during each of the next two days. Furthermore, the amounts of each band relative to the other remained approximately constant at each age.

FIGURE 5.6.

Standard Curves for Quantitative PAGE of Embryonic Feather Keratin Proteins.

Samples (12.5 µg-100 µg) of reduced and carboxymethylated 21-day embryonic feather proteins were fractionated by PAGE, stained and analysed by densitometry. The total amount of Coomassie Brilliant Blue dye bound to protein in each gel, after subtraction of background stain, was determined using the automatic integrator. The amount of protein applied to the gels was determined by the method of Lowry *et al.* (1951). Values are in arbitrary units, and are dependent on the time of staining and the sensitivity settings of the densitometer and integrator. Any such values therefore apply only to a particular run.

FIGURE 5.6a.

Standard Curve for Proteins After Fractionation by PAGE at pH 9.5.

FIGURE 5.6b.

Standard Curve for Proteins After Fractionation by PAGE at pH 7.5.

FIGURE 5.6c.

Standard Curve for Proteins After Fractionation by PAGE at pH 2.7.

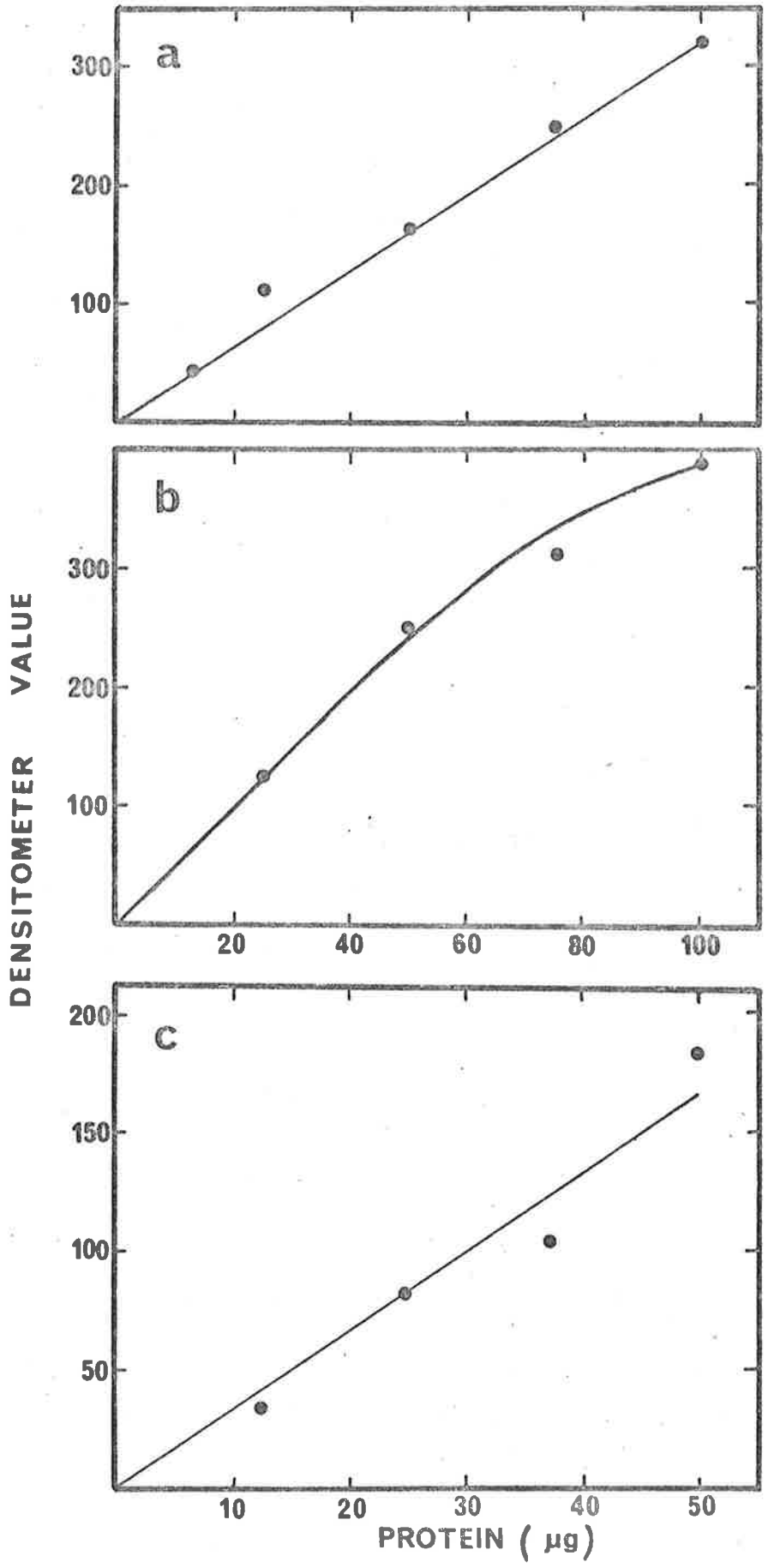

a
b
c
300
200
100
300
200
100
200
150
100
50
20
40
60
80
100
10
20
30
40
50
DENSITOMETER VALUE
PROTEIN ( µg)

FIGURE 5.7.

Changes in Keratin Content During Feather Development, as Determined by PAGE at pH 9.5.

Values shown for bands β2 - β5 are the average of 2 independent experiments, as in Figure 5.2a. Gels were run in duplicate in each experiment. Values are expressed as mg of protein in each resolvable band per ml of packed feather tissue.

●——● : Band β4

○——○ : Bands β3a + β3

■——■ : Band β5

□——□ : Band β2.

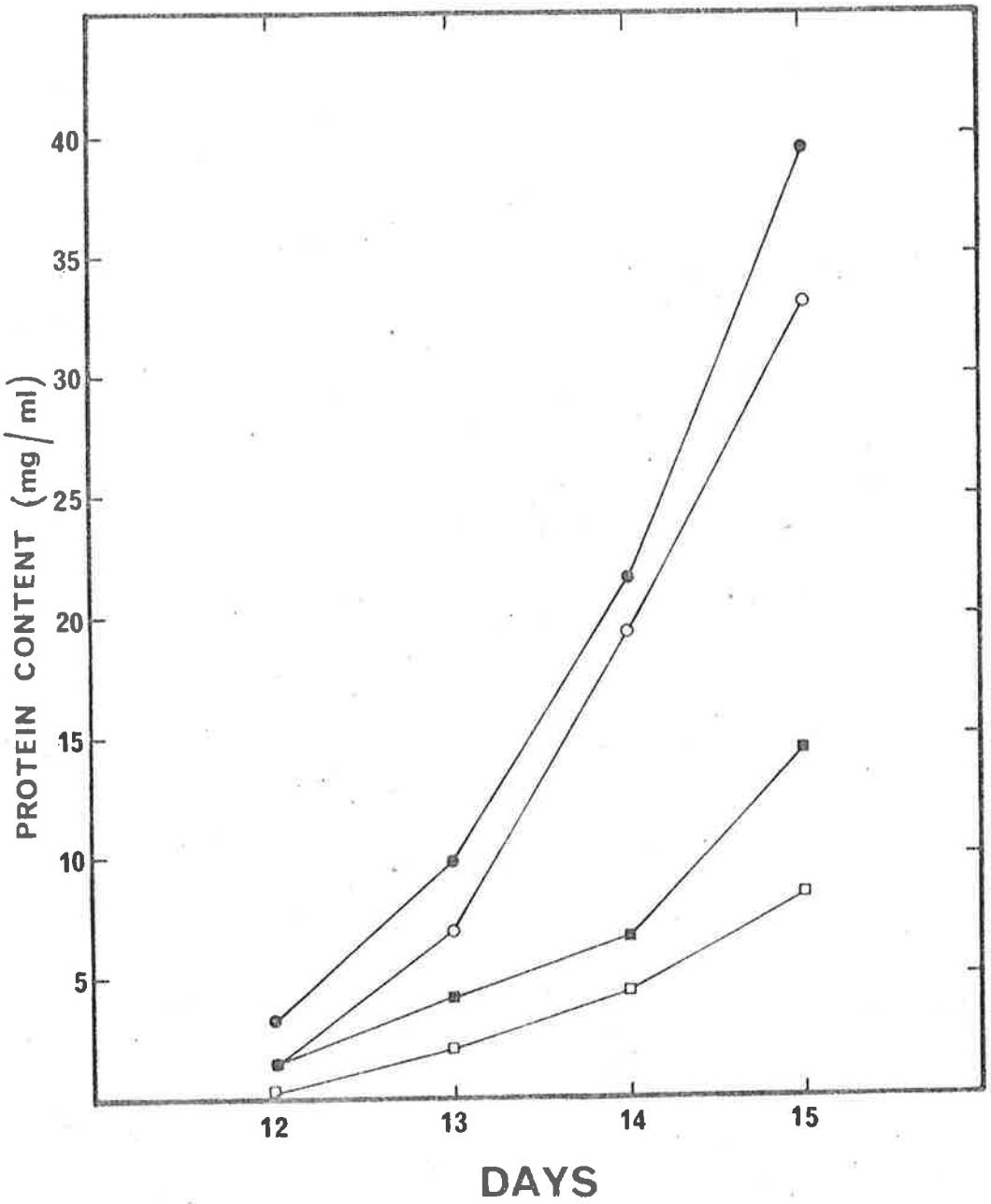

PROTEIN CONTENT (mg / ml)
40
35
30
25
20
15
10
5
12
13
14
15
DAYS

FIGURE 5.8.

Changes in Keratin Content During Feather Development, as Determined by PAGE at pH 7.5.

FIGURE 5.8a.

Values shown for bands β2 - β5 are the average of two independent experiments, as in Figure 5.3. Gels were run in duplicate in each experiment. Values are expressed as mg of protein in each resolvable band per ml of packed feather tissue.

●——● : Band β4

○——○ : Band β3a + β3

■——■ : Band β5

□——□ : Band β2

FIGURE 5.8b.

Values Shown for Bands α1 - α4 are from one experiment only, as in Figure 5.4, but the gels were overloaded (500 μg/gel) to allow densitometric quantitation of the α-bands.

●——● : Bands α2 and α3

○——○ : Band α4

■——■ : Band α1

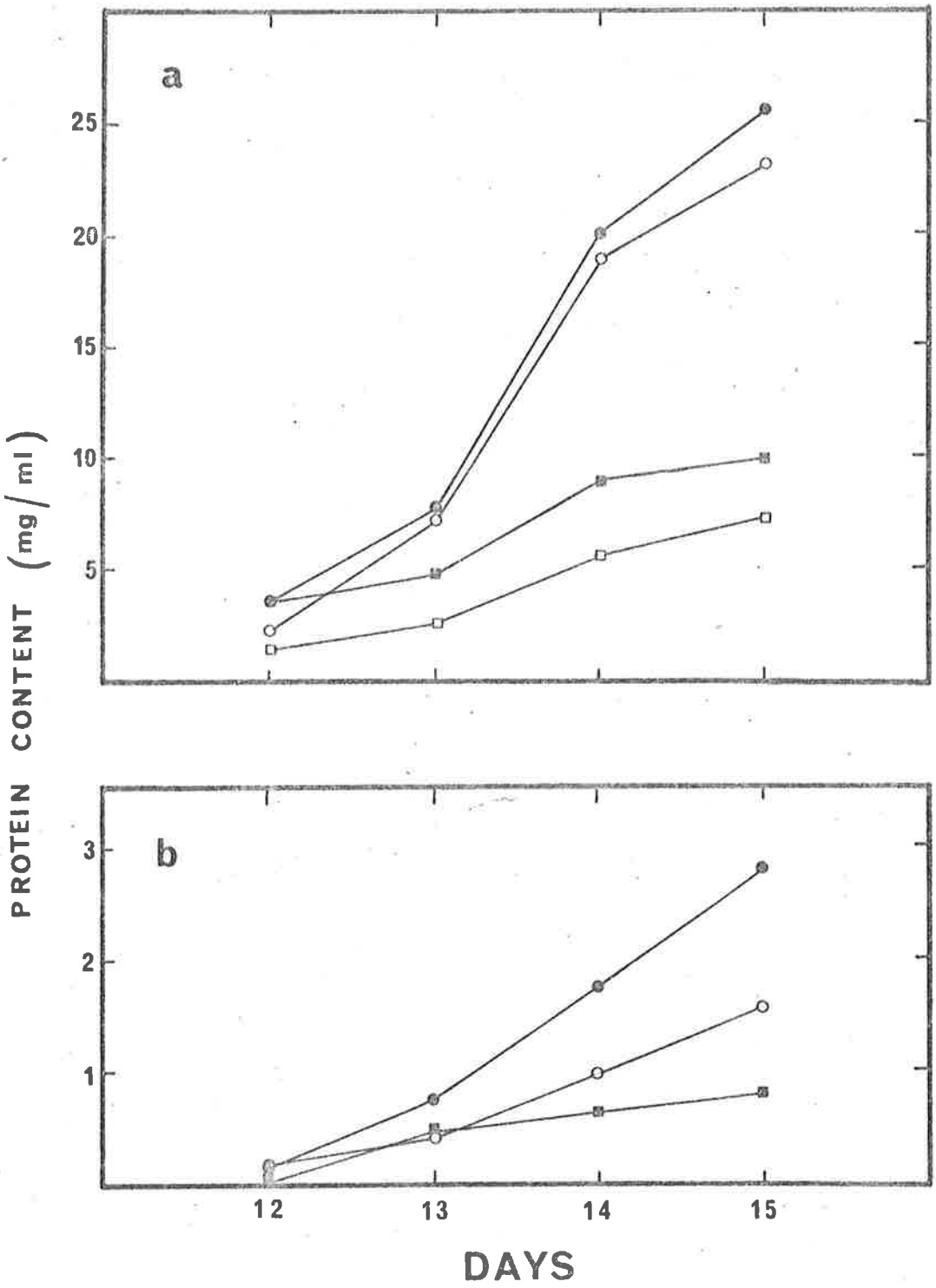
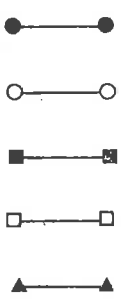
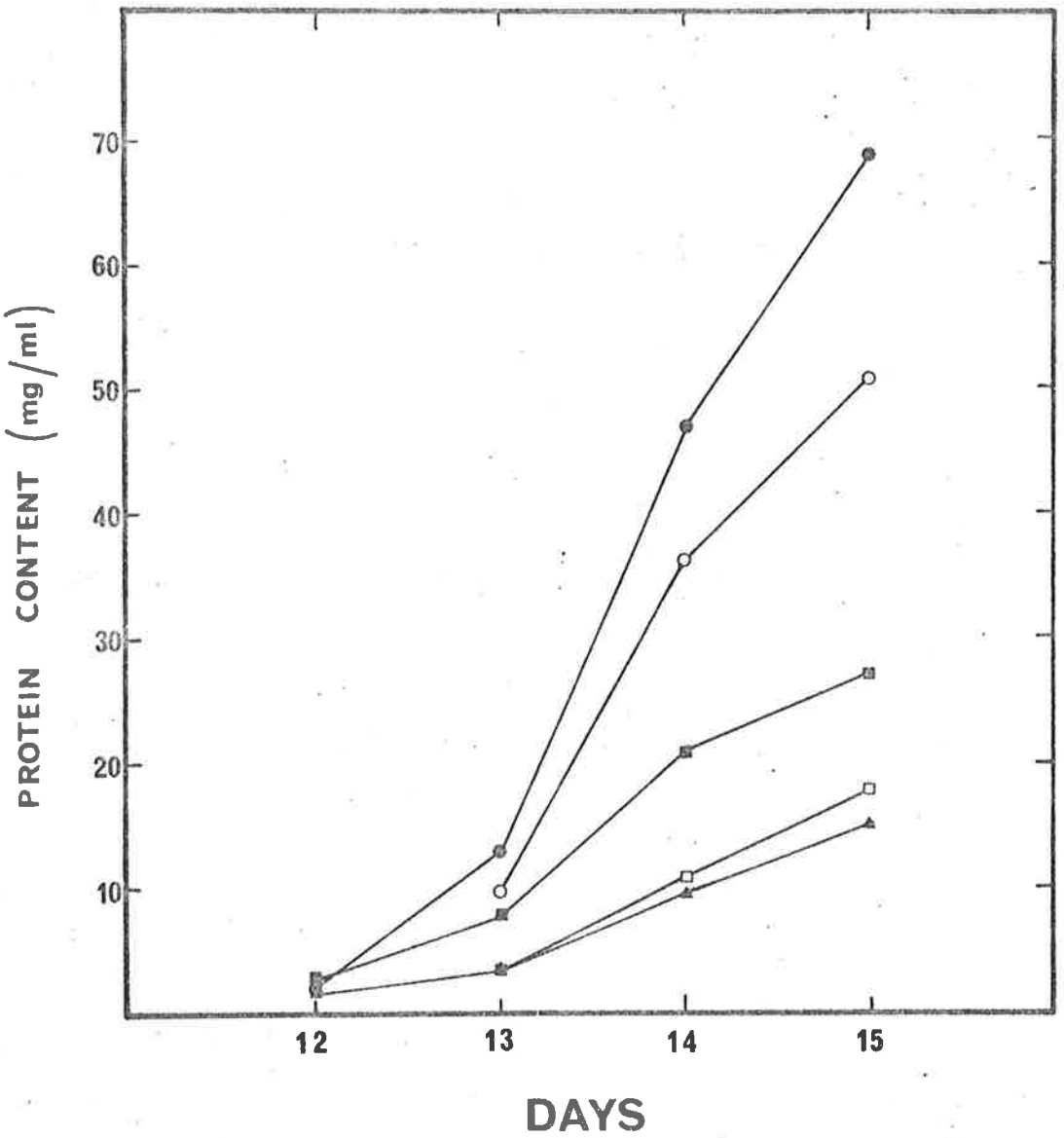

a
b
25
20
15
10
5
3
2
1
12
13
14
15
DAYS
PROTEIN CONTENT (mg/ml)

FIGURE 5.9.

Changes in Keratin Content During Feather Development, as Determined by PAGE at pH 2.7.

Values for bands A + B, C, and D + E are the average of 2 independent experiments, as in Figure 5.4. The values for bands A and B were determined by least-squares analysis. Gels were run in duplicate in each experiment. Values are expressed as mg of protein in each band (or group of bands) per ml of packed feather tissue.




PROTEIN CONTENT (mg/ml)
70
60
50
40
30
20
10
12
13
14
15
DAYS

Examination of photographs of pH 9.5 and pH 7.5 gels (Figures 5.2 and 5.3) reveals a background smear of stained material, predominantly of lower mobility than band β-4, which decreased as both developmental age and band mobility increased. The relatively high values obtained at days 12 and 13 for band β-5 would appear to derive from this background. It is also evident that all values obtained at day 12 would include some contribution from background staining. Quantitation of the γ-bands was not possible, as the background was too high in the region.

The results suggest a coordinated synthesis of the keratin proteins, as opposed to the two-phase model of Malt and Bell (1965). The rate of synthesis of each resolvable component became maximal after 13 days of development.

### (e) *Changes in Total Keratin Content During Feather Development*

Estimations of total keratin content were made (Figure 5.10) by summing the values for each keratin band resolved by PAGE at pH 9.5, 7.5 or 2.7 (Figures 5.7 - 5.9) of the proteins in preparations from feathers of each age. The values obtained from the three different gel systems were in good agreement. Furthermore, the increase in total protein content between days 13 and 14 and between days 14 and 15 could be accounted for to a large extent by the increase in keratin content during these periods, indicating that keratins were the major proteins synthesized.

FIGURE 5.10.

Changes in Total Keratin Content Durng Feather Development.

The values shown were obtained by summing the values for keratin bands at each age in Figures 5.7, 5.8 or 5.9.

●——● : Total protein content (from Figure 5.1).

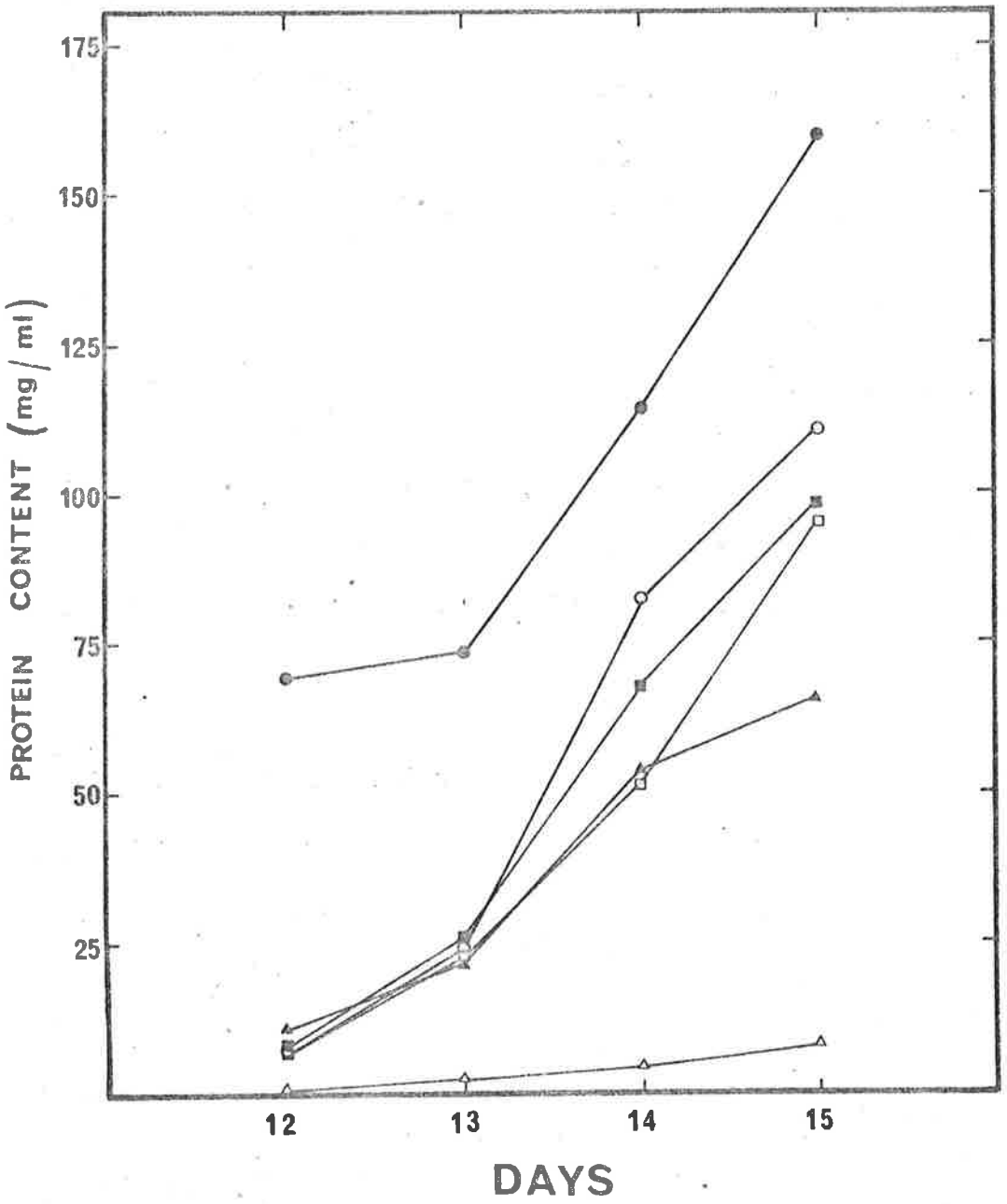
□——□ : Total content of bands β2 - β5, determined by PAGE at pH 9.5

▲——▲ : Total content of bands β2 - β5, determined by PAGE at pH 7.5.

○——○ : Total content of bands A - E, determined by PAGE at pH 2.7.

△——△ : α-band content (from Figure 5.8b).

■——■ : Total keratin content. The mean of the values obtained for the sum of bands β1 - β5, as determined by PAGE at pH 9.5 and pH 7.5, and bands A - E as determined by PAGE at pH 2.7 was determined. The value obtained for the α-bands (Figure 5.8b) was then added to the mean. As the α-bands are thought to account for the H bands at pH 2.7 (I.D. Walker; personal communication), this procedure should give the total value for all keratin components except any which run in the γ-region of pH 9.5 and 7.5 gels.


175
150
125
100
75
50
25
PROTEIN CONTENT (mg / ml)
12
13
14
15
DAYS

### 3. *Determination of the Rates of Keratin Synthesis During Feather Development by Incorporation of $^{14}$C-Leucine*

Studies on the incorporation of $^{14}$C-leucine into acid precipitable material using 14-day feathers demonstrated a linear rate of incorporation over a 2-hour period (Figure 5.11), indicating that the tissue had remained viable. Plucked feathers from 12-15 day embryos were therefore incubated with $^{14}$C-leucine for 2 hours, and the radioactive proteins extracted. The total incorporation per ml of packed feathers at each age (Table 5.1) varied somewhat in the different experiments.

Samples of the radioactive proteins were fractionated by PAGE at pH 7.5. Densitometer tracings and radioactivity profiles of such gels are shown in Fig. 5.12. The profiles were very similar in the different experiments. In particular, very little radioactivity was associated with the keratin region of the gel at day 12, whereas by days 14 and 15, keratin proteins were the predominant radioactive species.

The rate of keratin synthesis at each age was estimated by summing the radioactivity of gel slices in each band of the keratin containing region of each gel (Fig. 5.13). As estimated in this manner, keratin synthesis in cultured feather tissue accounted for only 7% of the total protein synthesis in 12-day feathers and had increased to greater than 25% of total protein synthesis in 14- and 15-day feathers (Figure 5.13a). This increase could be accounted for by an increase in the rate of keratin synthesis

FIGURE 5.11.

Incorporation of $^{14}$C-Leucine into TCA-Precipitable Material by 14-Day Feathers During Culture.

Approximately 1 ml of 14-day feathers were incubated in Hanks' solution (10 ml) containing amino acids and antibiotics, as described in Methods. The medium contained 0.1 μCi/ml of $^{14}$C-L-leucine (sp.act. 316 mC /mmole). Samples containing feathers plus medium were taken at intervals, using a wide-mouthed pasteur pipette. Incorporation was stopped by adding cold Hanks' solution containing 0.1% $^{12}$C-L-leucine. Feathers were collected by brief centrifugation, and dissolved in 2 ml of reducing solution (Chapter 3), over a period of 1 hr at 37°, with occasional vortex-mixing. Water (2 ml) was then added, and the mixture was centrifuged (36,000 g, 1 hr) to remove insoluble material. Aliquots of the supernatant were treated with 10 volumes of 10% TCA for 1 hr at 0°. Precipitated protein was collected by centrifugation and taken up in 1.0 ml of 0.1 M NaOH. Aliquots of this solution were taken for the determination of radioactivity and protein content. Values shown are the average of duplicate incubations.

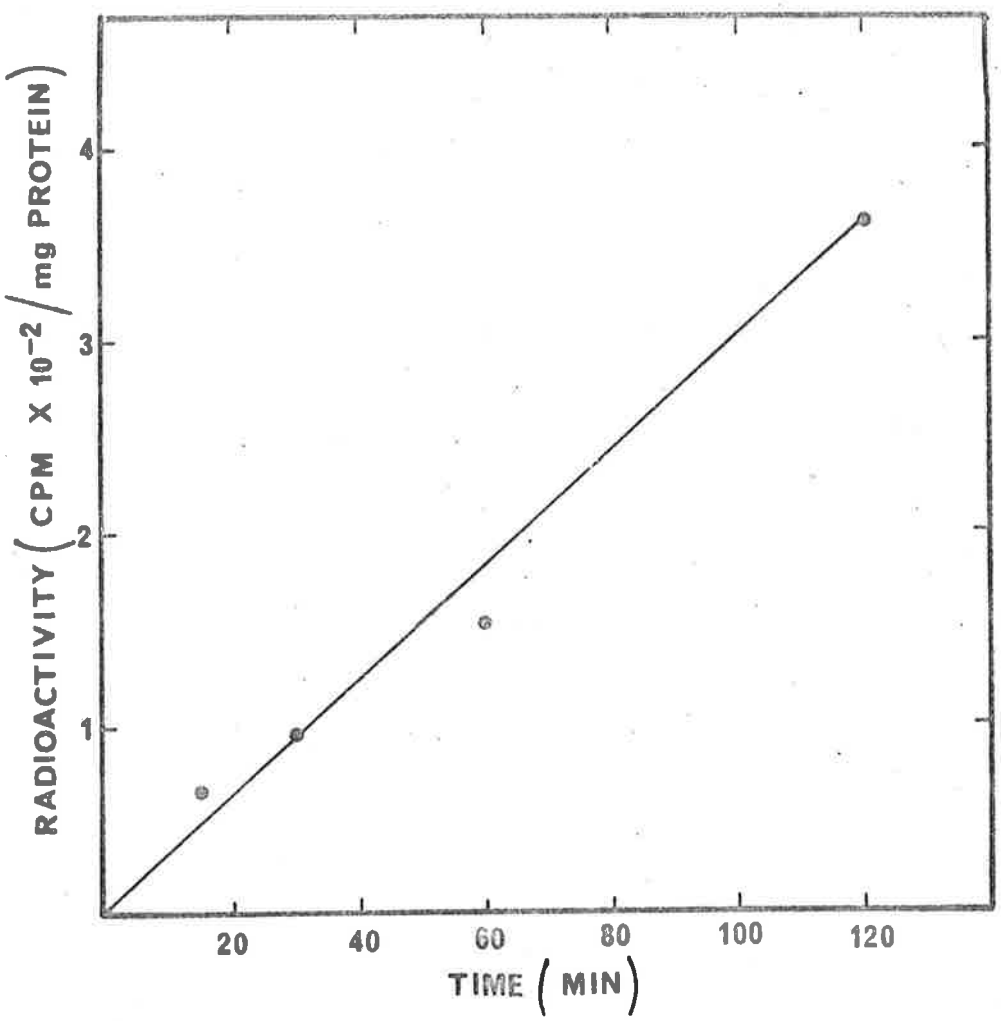

RADIOACTIVITY ( CPM X $10^{-2}$ / mg PROTEIN )
1
2
3
4
20
40
60
80
100
120
TIME ( MIN )

TABLE 5.1. INCORPORATION OF $^{14}$C-LEUCINE/ML OF FEATHERS IN CULTURE AT DAYS 12-15.

Feathers of each age were incubated with $^{14}$C-L-leucine. The tissue volumes, protein content and radioactivity were determined as described in Methods. Values are expressed as cpm/ml tissue/hr x $10^{-5}$.

| Day | 12 | 13 | 14 | 15 |
|---|---|---|---|---|
| Expt. 1 | 5.8 | 3.9 | 5.2 | 6.2 |
| Expt. 2 | 8.9 | 7.7 | 6.9 | 3.3 |
| Average | 7.3 | 5.8 | 6.0 | 4.7 |

FIGURE 5.12

PAGE at pH 7.5 of Proteins Labelled with $^{14}$C-Leucine.

Samples (400 µg/gel) of reduced and carboxymethylated proteins from feathers at 12-15 days of development, which had been labelled for 2 hr with $^{14}$C-leucine, were subjected to polyacrylamide gel electrophoresis at pH 7.4 as described in the Methods section. The gels were crosslinked with ethylenediacrylate (Cain and Pitney, 1968). The results shown are from experiment 1 of Table 5.1.

Bands β2 - β5 are indicated.

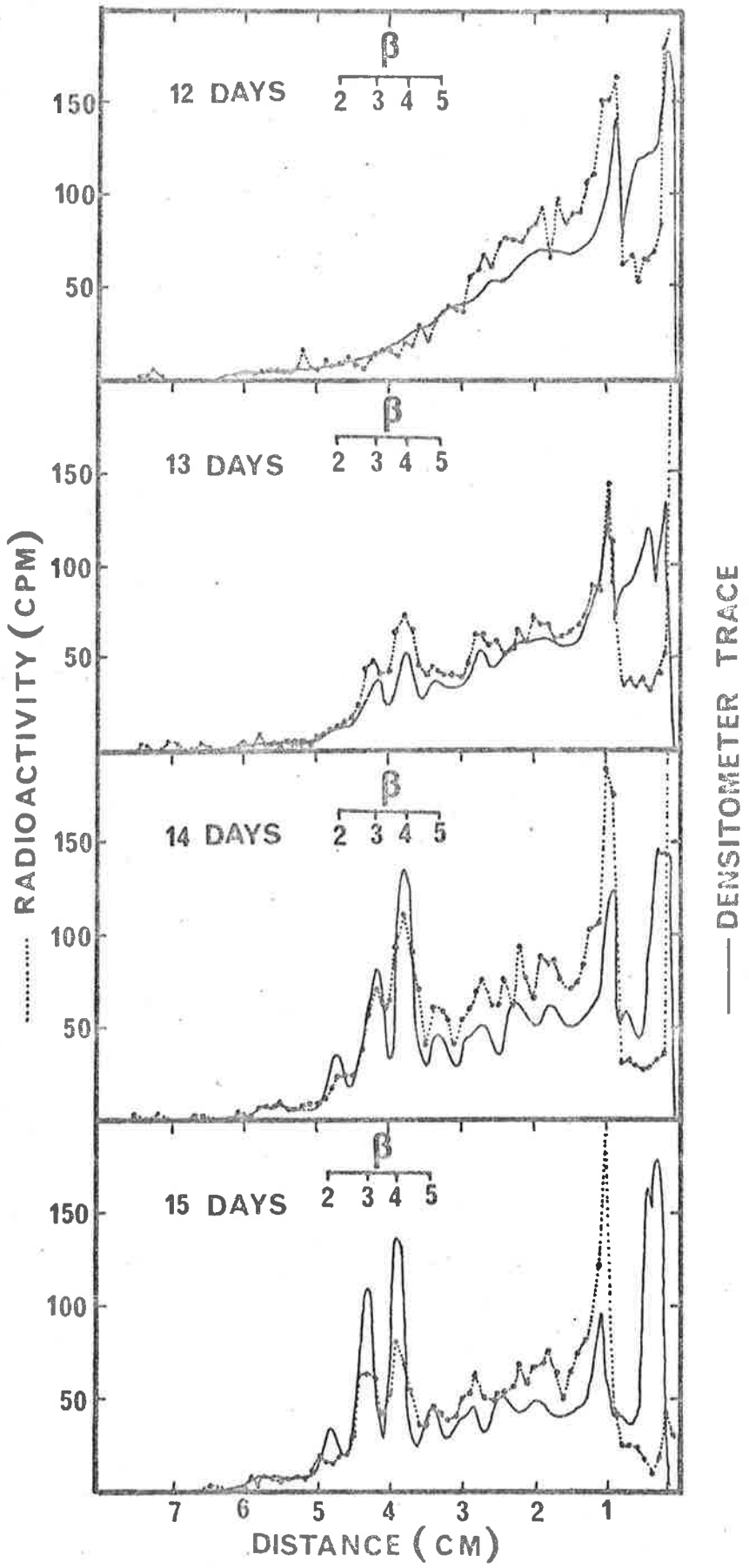

12 DAYS
13 DAYS
14 DAYS
15 DAYS
β
2 3 4 5
150
100
50
RADIOACTIVITY (CPM)
DENSITOMETER TRACE
7 6 5 4 3 2 1
DISTANCE (CM)

FIGURE 5.13

Determination of the Rates of Keratin Synthesis During Feather Development by Incorporation of $^{14}$C-Leucine.

Values were obtained by summing the radioactivity of gel slices (Figure 5.12) corresponding to the relevant keratin bands, and are the average of 2 independent experiments.

FIGURE 5.13a.

Incorporation of $^{14}$C-leucine into keratin bands β2 - β5 at days 12-15, as per cent of total incorporation.

FIGURE 5.13b.

Total incorporation of $^{14}$C-leucine into keratin bands β2 - β5 at days 12-15, per ml of packed feathers, per hr.

FIGURE 5.13c.

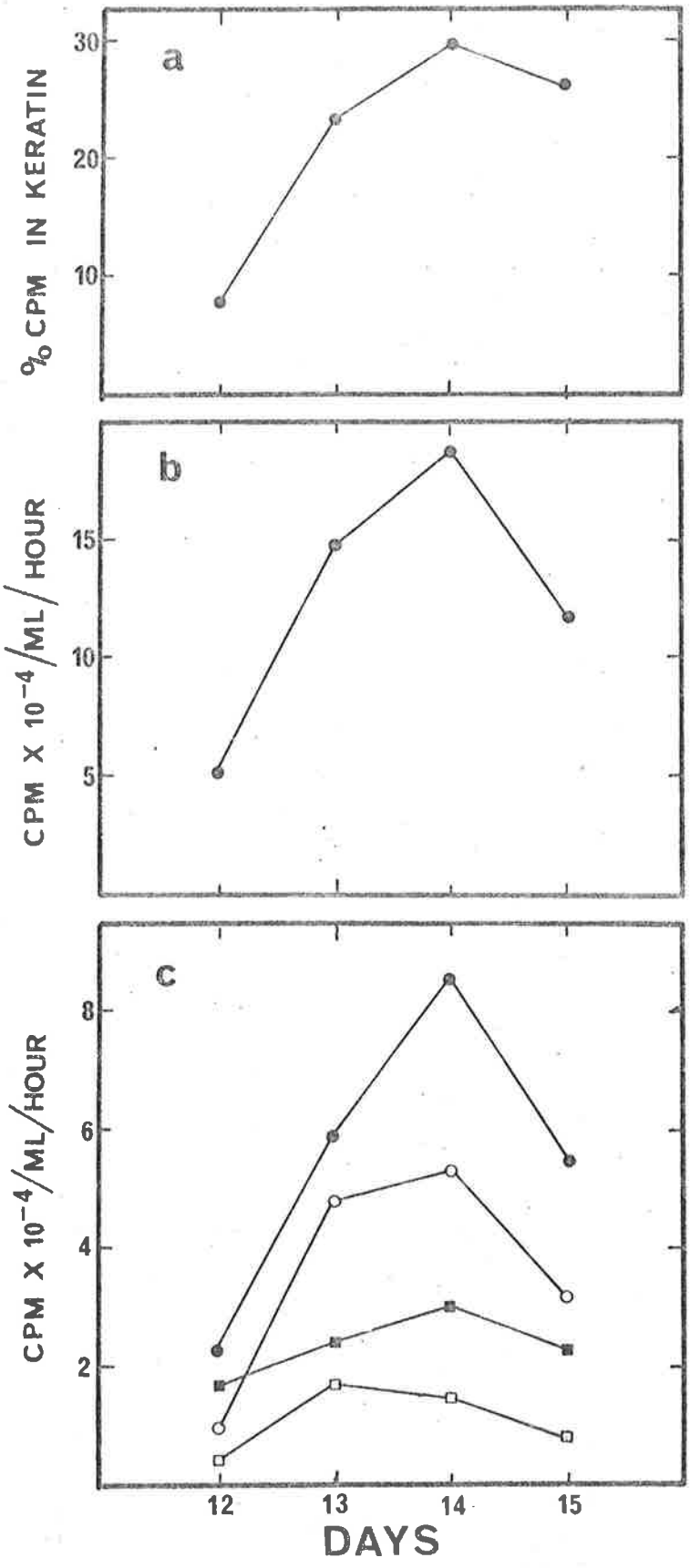
Relative rates of incorporation of $^{14}$C-leucine into keratin bands β2 - β5 at days 12-15, per ml of packed feathers, per hr.

●——● : Band β5

○——○ : Bands β3a + β3

■——■ : Band β5

□——□ : Band β2


a
% CPM IN KERATIN
30
20
10
b
CPM X $10^{-4}$/ML/HOUR
15
10
5
c
CPM X $10^{-4}$/ML/HOUR
8
6
4
2
12
13
14
15
DAYS

in tissue at these ages (Fig. 5.13b). Furthermore, the relative rates of synthesis of keratin proteins in each polyacrylamide gel band remained approximately constant in tissue at each age (Figure 5.13c) in agreement with the results obtained by quantitative densitometry (Figures 5.7 - 5.9).

4. *Electron Microscopic Observations on Keratin Synthesis During Feather Development*

(a) *Occurrence of Keratin Fibrils in 12-15 Day Feather Cells.*

Electron micrographs of transverse sections of feathers at various ages of development are shown in Figures 5.14 - 5.15. Relatively few keratin fibrils were detectable in 12-day feathers, except in the sheath cells, in agreement with the results of Matulionis (1970). The fibrils then rapidly increased in abundance with age until by 15 days, fibrils accounted for the majority of the cytoplasmic area. The quantitative observations on synthesis of feather keratin proteins described above therefore correlate well with electron-microscopic visualization of keratin fibrils during development of the feather.

(b) *$^{3}$H-Thymidine Autoradiography.*

12-day feathers were incubated with $^{3}$H-thymidine for 30 min. and subjected to electron microscopic autoradiography as described in the Methods section. The autoradiographs were then examined for the presence of labelled cells which

FIGURES 5.14 - 5.15.

Electron-micrographs of Feathers at 12-15 Days.

Transverse sections through barb ridges, about mid-way along the feathers. At 12 days, the nuclear volume is large relative to that of the cytoplasm. No keratin is detectable in the cytoplasm, although traces of keratin can be found nearer the tips of the feathers. At 13 days, keratin fibrils can be seen in the ribosomal cytoplasm. The keratin fibrils increase in size and abundance during the next two days, and occupy a large portion of the cytoplasm by 15 days. The nuclei at this latter stage are small relative to the cytoplasm, and contain condensed chromatin.

Keratin fibrils are indicated by arrows.

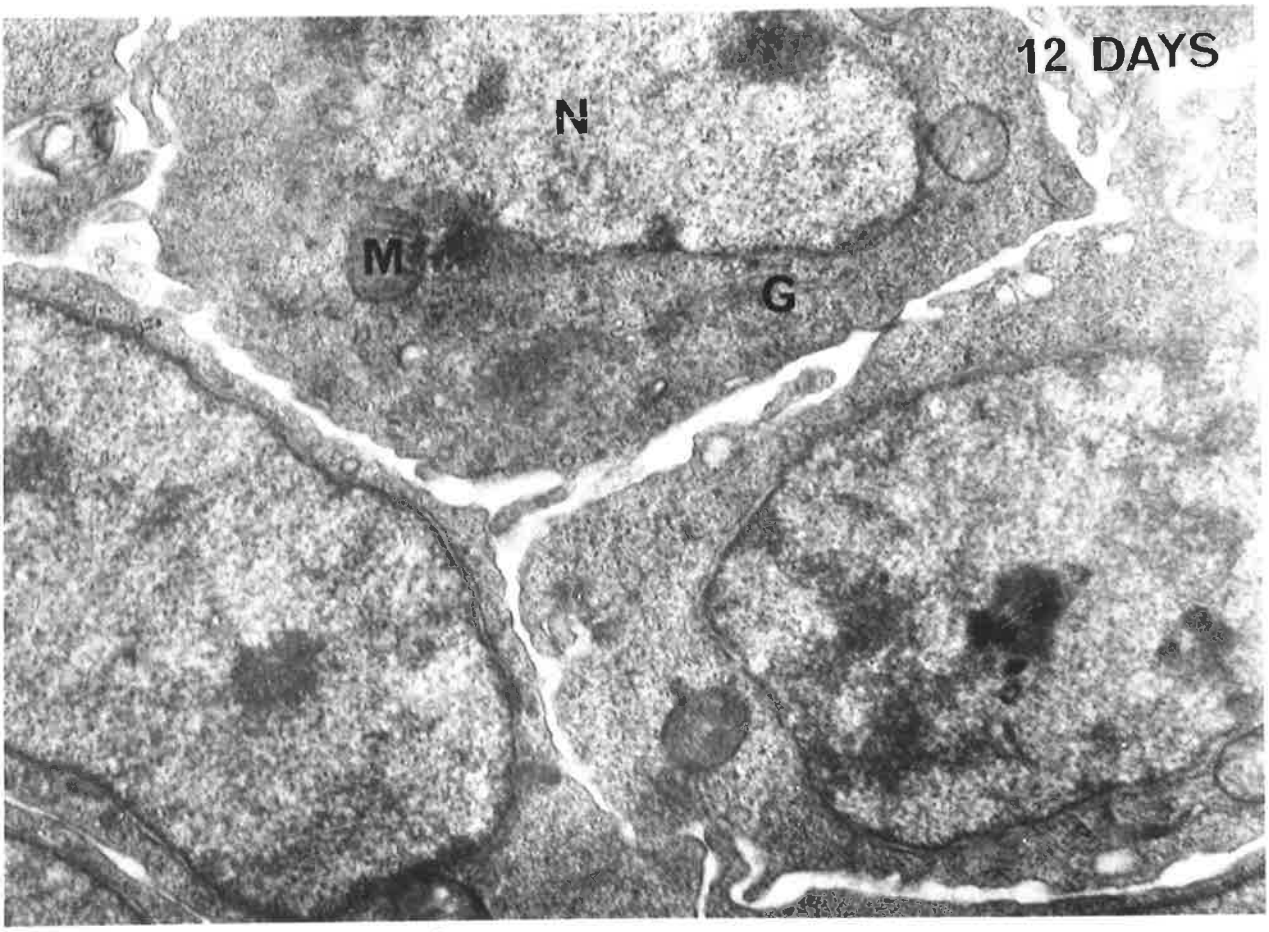
N: Nuclei

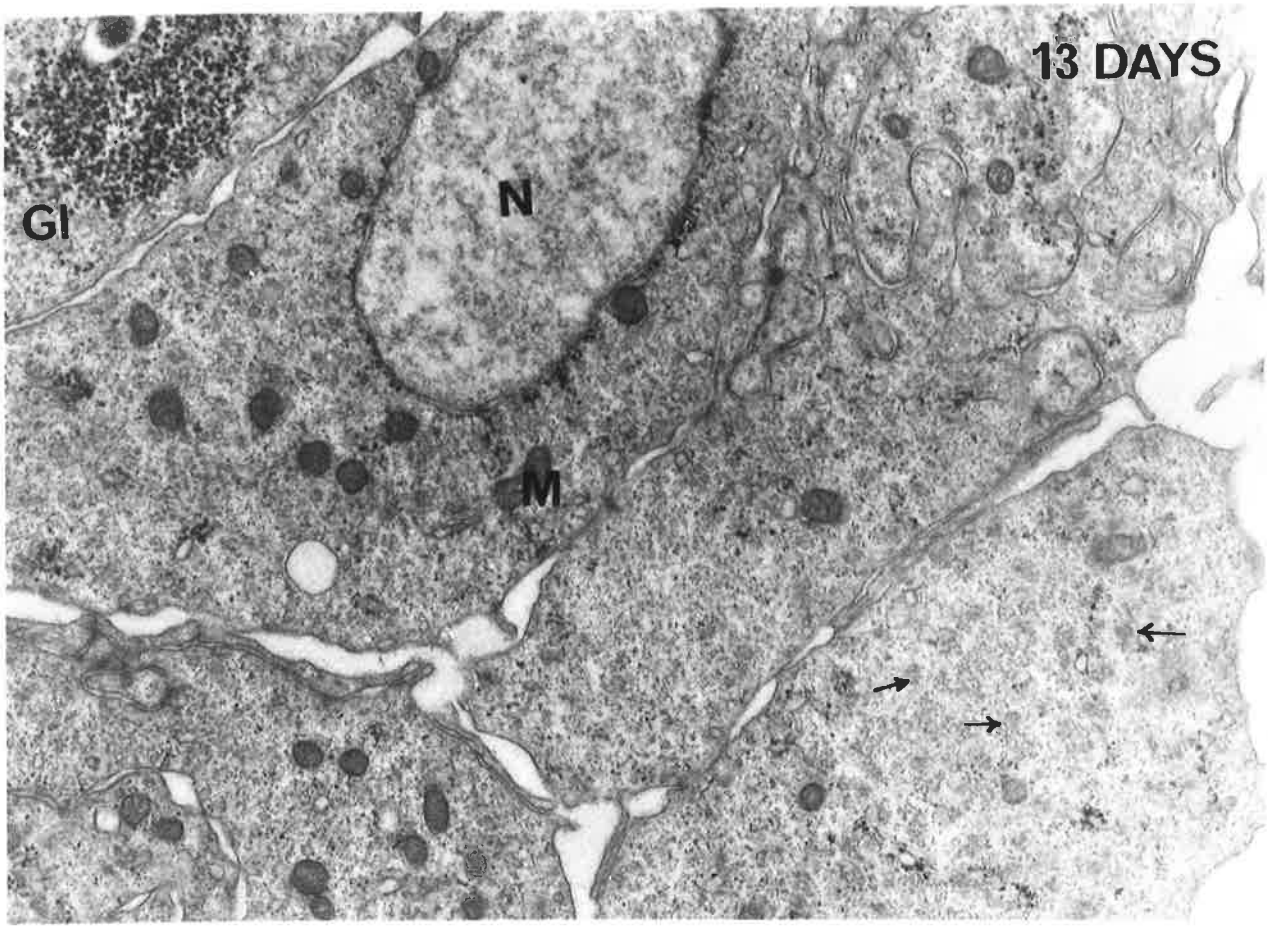
M: Mitochondria

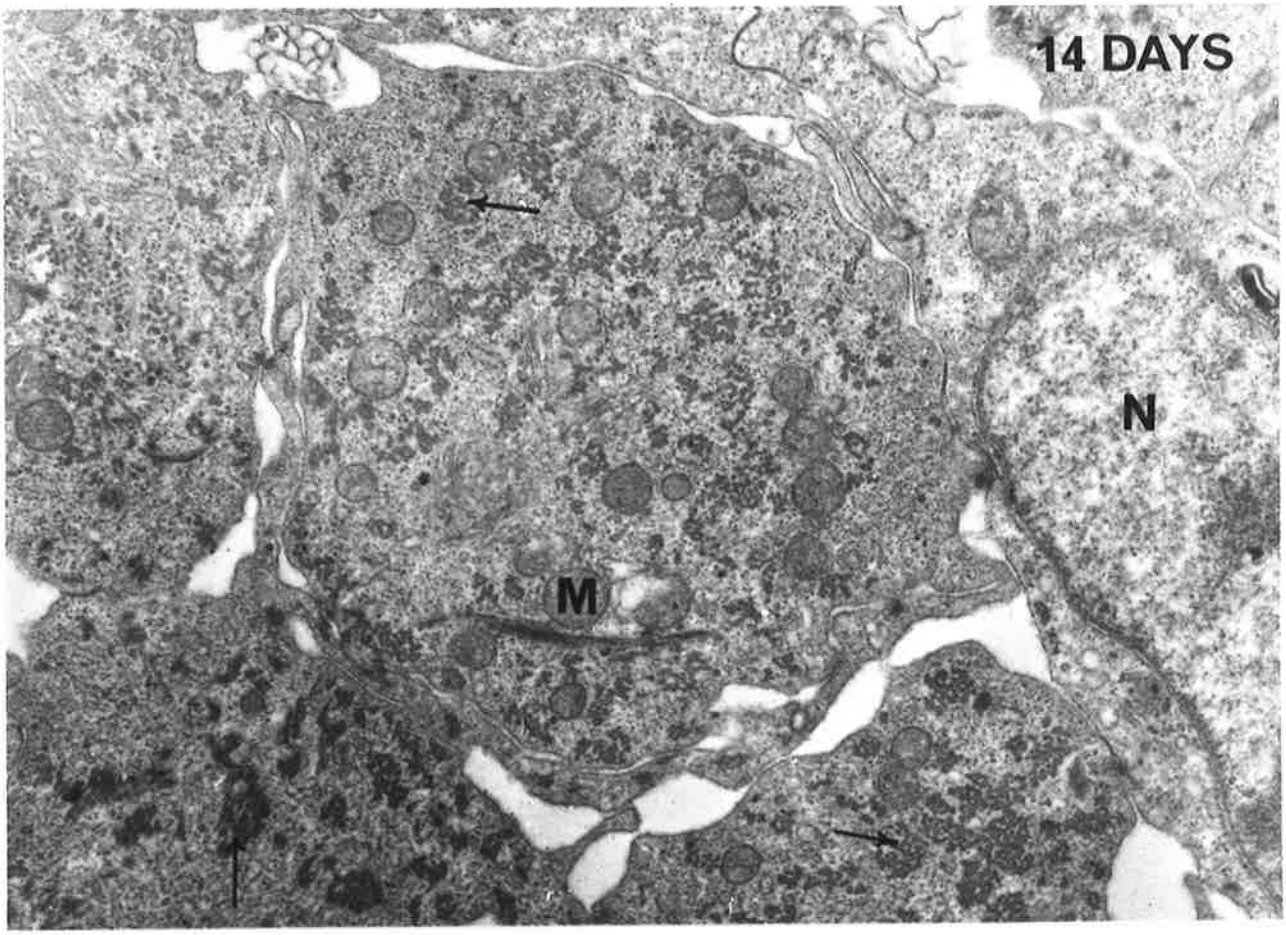
G: Golgi region

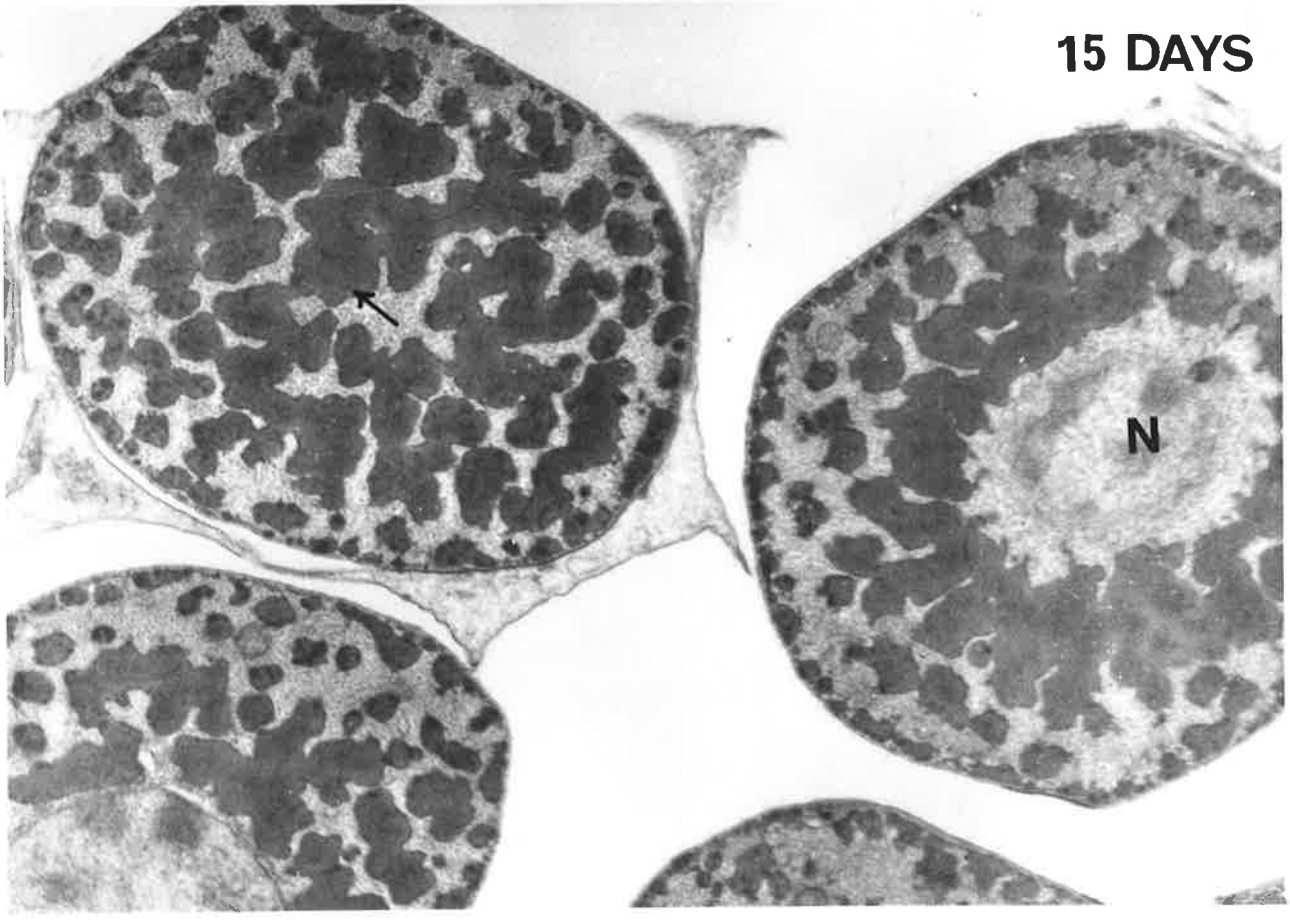
GI: Glycogen

Magnification: X10,000.


12 DAYS
N
M
G


13 DAYS
GI
N
M


14 DAYS
N
M


15 DAYS
N

contained detectable keratin fibrils.

In feathers labelled with $^{3}$H-thymidine, silver grains were localized over the nuclei of cells in the basal region. Sheath cell nuclei were not labelled. In contrast, keratin fibrils were present inthe barb and barbule cells only at the tip of the feather, and in sheath cells from the tip almost down to the base. Areas from the basal region containing barb and barbule cells with labelled nuclei were photographed and examined in detail for the presence of keratin fibrils (Figure 5.16). No cell was found which had incorporated thymidine and also contained keratin fibrils. The cells examined in such a manner are described by Table 5.2. Although a larger sample of cells would appear desirable, technical problems were prohibitive at the required magnification.

It was concluded that keratin fibrils were not detectable in cells which had incorporated thymidine within 30 min. of their subsequent preparation.

*(c) $^{3}$H-Uridine Autoradiography.*

In contrast to the results from $^{3}$H-thymidine autoradiography, cells were found which had actively incorporated $^{3}$H-uridine and also contained keratin fibrils (Figure 5-17). This result was true whether or not the feathers were incubated for 18 hours in the presence of ACT-D after the incorporation. The incorporation of $^{3}$H-uridine was reduced by 70% by the presence of ACT-D during the incubation as determined by autoradiography

FIGURE 5.16.

Electron-microscopic Autoradiograph of a 12-Day Feather Labelled with $^{3}$H-Thymidine.

Label is restricted to the nuclei of barb and barbule cells, which do not contain detectable keratin fibrils. In contrast the sheath cells, which are not labelled, contain keratin fibrils.

Keratin fibrils are indicated by arrows.

BR: Barb ridge

SH: Sheath

P: Periderm cell.

N: Nucleus.

LN: Labelled nucleus.

Magnification: X5,000.

Inset: High magnification view of area enclosed by rectangle. Keratin fibrils can be seen against the background of ribosomal cytoplasm.

Magnification: X50,000.

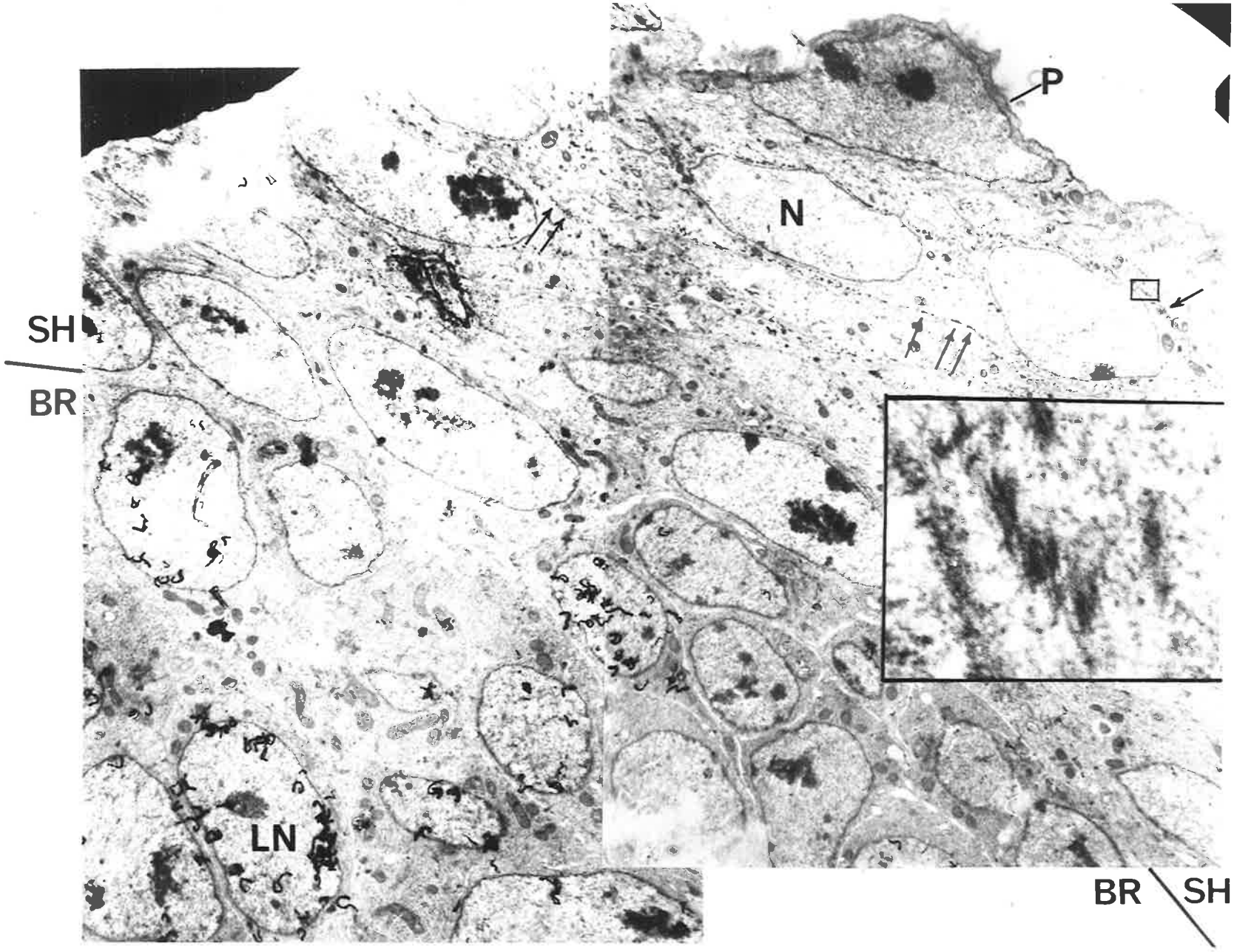

P
N
SH
BR
LN
BR
SH

TABLE 5.2. DISTRIBUTION OF $^{3}$H-THYMIDINE-LABELLED NUCLEI AMONG 12-DAY FEATHER CELLS EXAMINED IN DETAIL BY ELECTRON MICROSCOPY

| | Barb Cells[a] | Sheath Cells |
|---|---|---|
| Total Cells[b] | 152 | 43 |
| Cells with labelled[c] Nucleus | 61 | 0 |
| Cells containing detectable keratin . | 0 | 27 |
| Average number of grains/labelled nucleus | 30 | - |

[a]Includes the precursors of all cell types of the barb ridges (Chapter 1).

[b]17 Electron microscope fields were photographed at a magnification of 3000 X and printed at an optical magnification of 3 X.

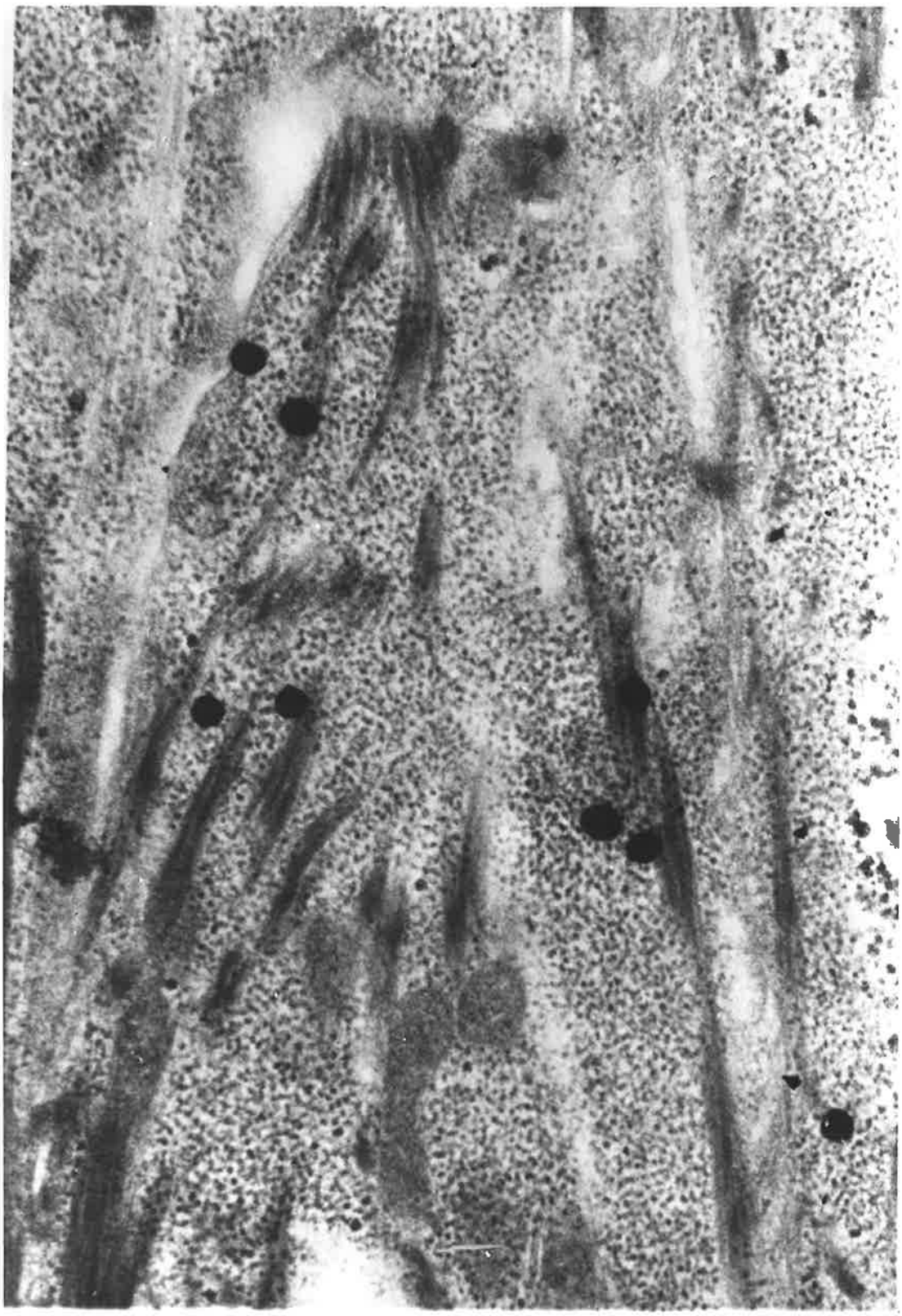
[c]Background (no. of grains over unlabelled cells) was 1.0 silver grains/cell. Cells with 2 or less silver grains/nucleus were counted as unlabelled cells.

FIGURE 5.17.

Electron-microscopic Autoradiograph of a 12-Day Feather Labelled with $^{3}$H-Uridine.

Keratin fibrils and label are evident within the one cell.

Magnification: X48,000.



at the light-microscope level, indicating that the $^{3}$H-uridine had been incorporated into RNA. It was concluded that cells containing keratin fibrils are capable of RNA synthesis. The nature of this RNA was not investigated.

It was observed that a large percentage of the silver grains were localized in the immediate vicinity of keratin fibrils. In an attempt to quantitate this observation, the number of silver grains with their centres directly over keratin fibrils and the total number of silver grains was determined. Considerations on the nature of the process of autoradiography (Rogers, 1967) suggest that grains considered to be associated with keratin would result from disintegrations within 1000 Å of the keratin fibrils. The relative area of keratin fibrils to the total area of keratin-free cytoplasm was estimated by cutting out the "fibrils" from prints and weighing them. As the section thickness (approx. 500 Å) approximated the thickness of the fibrils, this procedure would be expected to give a reasonable estimate of the relative volumes of keratin and cytoplasm.

The results are shown in Table 5.3. The relative number of grains per unit area of keratin was much greater than the number of grains per unit area of the remainder of the cytoplasm, whether or not the tissue had been "chased" with ACT-D. A t-test performed on this data, assuming each electron-microscope plate to be an independent observation, indicated that the association of silver grains with fibrils was statistically significant (Table 5.4).

These results suggest that newly-synthesized RNA

TABLE 5.3. DISTRIBUTION OF $^{3}$H-URIDINE LABEL OVER KERATIN FIBRILS AND CYTOPLASM IN 12-DAY FEATHER CELLS

| Field | EXPERIMENT A $^{3}$H-Uridine Pulse | | EXPERIMENT B $^{3}$H-Uridine Pulse/ACT-D Chase | |
|---|---|---|---|---|
| | Grains/unit area of Cytoplasm[a] | Grains/unit area of Keratin | Grains/unit area of Cytoplasm[a] | Grains/unit area of Keratin |
| 1 | 0.52 | 2.50 | 0.68 | 1.68 |
| 2 | 0.32 | 4.29 | 1.57 | 1.80 |
| 3 | 0.50 | 0.00 | 2.53 | 2.70 |
| 4 | 0.79 | 2.73 | 1.46 | 6.54 |
| 5 | 0.69 | 1.54 | 1.95 | 3.08 |
| 6 | 1.11 | 2.22 | 1.61 | 2.86 |
| 7 | 0.79 | 5.83 | 0.48 | 2.50 |
| 8 | 0.42 | 4.00 | 0.87 | 3.66 |
| 9 | 1.02 | 0.83 | 0.87 | 0.00 |
| | $t = 3.24$, $0.001 < p < 0.01$ | | $t = 2.27$, $0.02 < p < 0.05$ | |

[a]The term "cytoplasm" refers to the cytoplasmic area remaining after subtraction of that area occupied by keratin fibrils.

in the cytoplasm of cells of the 12-day feather rapidly becomes associated with keratin fibrils. Similar observations have been made in the guinea pig hair follicle (Rogers, 1969).

## D. DISCUSSION

### *1. Changes in Protein Content and Synthesis*

The techniques described for measuring both the total protein content and the quantity of each keratin protein band resolved on polyacrylamide gels per ml of packed feather tissue were found to provide simple and useful methods for studying the synthesis of keratin proteins during development of the embryonic chick feather. Use of the parameter "packed tissue volume" is not ideal since the results do not necessarily reflect precisely the changes in protein content per cell. However, it is virtually impossible to accurately determine the number of cells in such a tissue sample in any simple, routine manner. DNA content is also not a suitable parameter, both for technical reasons and because it is likely that DNA is degraded and removed during later stages of cytodifferentiation as in other keratinizing tissues (Fukuyama and Bernstein, 1961; Downes *et al.*, 1966a).

The total protein content per unit volume of packed feathers increased markedly between days 13 and 14 and thereafter (Figure 5.1), suggesting a rapid change in the net rate of protein synthesis. That this change

principally involved the synthesis of keratin proteins was demonstrated quantitatively by gel electrophoresis (Figures 5.7 - 5.10), in agreement with histological and X-ray diffraction observations (Bell and Thathachari, 1963), electron microscopic observations (Matulionis, 1970) and chemical and immunological observations (Malt and Bell, 1965; Ben-Or and Bell, 1965).

The incorporation of $^{14}$C-leucine into the keratin proteins during this period of development further demonstrated the change in spectrum of proteins synthesized (Figures 5.12 and 5.13).

The results, in general, were in good agreement with the results from quantitative polyacrylamide gel electrophoresis. However, keratin synthesis accounted for a maximum of only 30% of total protein synthesis, as determined by the radiochemical technique. This result would not appear to be compatible with the observation (Fig. 5.10) that keratin synthesis accounted predominantly for the increase in protein content during development. Several explanations of the discrepancy are possible. For example, the culture conditions may have been sub-optimal for keratin synthesis. Alternatively, for example, the leucine pool-size may be significantly lower in basal cells than in the developmentally more advanced apical cells, or the permeabilities of the cells may vary.

*2. Kinetics of Keratin Synthesis*

Quantitative polyacrylamide gel electrophoresis (Figures 5.7 -5.9) demonstrated that within the present limits of resolution and accuracy, an increase in the amount of each of the predominant keratin protein bands began after 12 days. Furthermore, the relative amounts of these bands remained constant during development. The rates of synthesis of each major resolvable keratin band became maximal after 13 days. The α-proteins appeared to follow a similar course of development to the β-proteins, but were not measurable as early because of their relatively low amount.

In contrast, the γ-proteins were detectable at 11 days, and did not undergo the same increase after day 12. Several lines of evidence suggest, however, that most of the γ-proteins are not keratins (Chapters 3.D.3.b and 4.C.1.c.). It is possible that the γ-proteins are precursors of the β-proteins. If this were so, it would not be predicted that the amount of $^{14}$C-leucine label incorporated into the β-proteins relative to the amount of label incorporated into the γ-proteins would change so dramatically in the feather tissues pulse-labelled with $^{14}$C-leucine for two hours at days 12-15 (Figure 5.12). It is possible that the γ-proteins are nuclear proteins, as their synthesis in feathers at days 11-12 correlates with the time of maximal cell proliferation.

Malt and Bell (1965) postulated a two-phase synthesis of keratin during development of the embryonic chick feather. They suggested that a fibrous protein of

low sulphur content was first synthesized, followed by the synthesis of a sulphur-rich matrix protein. However, these proteins were not definitively isolated. In the case of hair and wool proteins which are known to have this microfibril-matrix structure (see Fraser *et al.*, 1972) the available evidence supports a mode of synthesis in which the low-sulphur fibrous proteins begin to be laid down before the matrix proteins (Downes *et al.*, 1963; 1966; Fraser, 1969a,b). Evidence from electron microscopy (Rogers and Filshie, 1963) and from protein-chemical studies (Harrap and Woods, 1964a,b; Woods, 1971; Chapter 3) indicates that in contrast, no such microfibril-matrix structure exists in feather keratin. Furthermore, X-ray diffraction studies (Fraser *et al.*, 1971; Burke, 1970) support the concept that the 10,500 molecular weight feather proteins constitute the feather microfibrils.

The results from quantitative gel electrophoresis described in the present work do not support a two-phase synthesis of the major feather keratin proteins during development of the embryonic feather. Within the present degree of accuracy the major keratin proteins are present in similar relative amounts at all stages of development, demonstrating a coordinated synthesis of the major keratin proteins. Synthesis of the minor γ-group of proteins, which are thought not to be keratins, begins well before that of the β-proteins, however, and it is probable that the γ-proteins are those thought to be the fibrous sulphur-deficient keratin proteins by Malt and Bell (1965).

Traces of keratin were detected as early as day 11 by PAGE at pH 9.5 and pH 7.5. No evidence yet obtained indicates whether these entirely represent keratin synthesis in a few feathers or cells more advanced in development than the rest of the population, or whether there is in addition a low basal level of keratin synthesis prior to the major onset of keratin synthesis after 13 days. Such a "proto-differentiated state" has been postulated for the case of enzyme synthesis in the developing pancreas by Rutter *et al.* (1967).

### 3. *Electron-Microscopic Observations on Feather Keratin Synthesis*

The electron microscope visualization of keratin fibrils at various stages of feather development confirmed the results of Matulionis (1970) and correlated well with the results from PAGE of the extracted proteins. As the fibrils are large polymers of the keratin subunits detected by PAGE, the observed correlation suggests that there is no large pool of keratin monomers in the feather cells before fibrils can be seen. Consequently, electron-microscopic autoradiography provided a method of investigating the temporal relationship of DNA, RNA and keratin synthesis within individual cells.

Keratin fibrils were not observed in cells which had incorporated $^{3}$H-thymidine immediately before observation, but were detected in cells which had incorporated

$^{3}$H-uridine. Thus it would appear likely that the cells retain the ability to synthesise RNA after the onset of keratin synthesis, but that DNA synthesis and keratin synthesis are mutually exclusive events within the one cell. These conclusions are subject to the obvious limitation of the sensitivity of detection of keratin fibrils. Nevertheless, a similar relationship exists between DNA synthesis (and hence mitosis) and overt cyto-differentiation in certain othersystems, for example, the embryonic chick myoblast (Holtzer, 1970). The results of Kischer and Furlong, 1967) support the conclusion that DNA synthesis ceases by the time the major onset of feather keratin synthesis begins.

$^{3}$H-Uridine label, which apparently was incorporated into RNA, was associated with keratin fibrils after the 30 min. pulse, and was still associated with the fibrils after 18 hours in the presence of ACT-D. Similar observations have been made after incorporation of labelled uridine in the guinea pig hair follicle (Rogers, 1969). It was suggested (Rogers, 1969) that keratin synthesis occurs at the surface of the fibrils and that the newly synthesized proteins aggregate with the pre-existing fibrils while still attached to polyribosomes.

Association of the synthetic machinery with the finished product in this manner suggests the possibility of translational control of protein synthesis (Cline and Bock, 1966). Association of polyribosomes with feather keratin fibrils has recently been observed by electron

microscopy of broken cell preparations (P.Y. Dyer; personal communication). A similar association of polyribosomes has been observed with myosin fibrils (Cedergren and Harary, 1964; Larson *et al.*, 1969). On the other hand, no evidence for such an association has been obtained from studies on isolated polyribosomes from embryonic feathers (G.A. Partington; personal communication).

It is evident from the gel profiles of keratin proteins observed in the present work that feather tissue samples can be obtained at different developmental stages, from that at 11-12 days when keratin synthesis is negligible, to that at later stages (14-15 days) when the predominant activity is synthesis of keratin. The quantitative methods described for the study of keratin synthesis during this transition should therefore be particularly valuable for the analysis of the mechanisms controlling the onset of keratin synthesis.

Furthermore, the ability of cultured feathers to incorporate $^{14}$C-leucine into a spectrum of proteins at least resembling the *in vivo* situation at each age of development should facilitate studies on the control of keratin synthesis. Such a system for the study of keratin synthesis has not previously been available.

# CHAPTER 6

# KERATIN SYNTHESIS IN A CELL-FREE SYSTEM FROM THE EMBRYONIC FEATHER

## A. INTRODUCTION

Synthesis of specific proteins in cell-free preparations (Schweet *et al.*, 1958) has been achieved in a number of systems from cells which are committed to the synthesis of large quantities of a restricted set of proteins. In particular, the synthesis of several fibrous proteins in such systems has been observed. Examples of these include muscle proteins (Heywood and Rich, 1968), collagen, (Lazarides and Lukens, 1971), crystallins (Straus *et al.*, 1971) and recently, α-keratin proteins (Steinert and Rogers, 1971; Wilkinson, 1971). In so-called "lysate" systems, the initiation of protein synthesis has been demonstrated (Lamfrom and Knopf, 1964). Such systems, in which initiation of synthesis of specific proteins occurs, permit the direct study at the molecular level of potential control mechanisms, acting at the level of translation of mRNA (for example, among others, Lockard and Lingrel, 1969; Heywood, 1970; Rouke and Heywood, 1972; Nienhuis *et al.*, 1971; Pemberton *et al.*, 1972).

A lysate system prepared from 14-day embryonic chick feathers has been shown to be highly active in the incorporation of radioactive amino acids into acid-insoluble material (G.A. Partington; personal communication; see Appendix A). It was therefore of interest within the above context to determine whether the system synthesized keratin proteins, and if so, whether initiation of the synthesis of these proteins occurred.

The present chapter presents evidence that the 14-day feather lysate system predominantly synthesizes intact feather keratin proteins, and initiates the synthesis of these proteins. The N-termini of the newly-synthesized keratin proteins were found to be acetylated.

The results of studies aimed at determining aspects of the mechanism of initiation and acetylation of keratin proteins are also reported.

## B. METHODS

### 1. *Incorporation of Radioactive Precursors into Proteins in the Lysate System*

Preparation of the lysates and their incubation with labelled precursors was performed by Mr. G.A. Partington of this Department. A description of the lysate system is therefore given in Appendix A. For each experiment described, variables such as radioactive precursor used, time of incubation and any other relevant conditions are given in the text or in captions to figures.

### 2. *Preparation of Reduced and Carboxymethylated Radioactive Proteins from the Lysate Supernatants*

To each 2 ml of post-ribosomal supernatant from the lysate, 10 ml of urea/β-mercaptoethanol/ethanolamine solution was added, and 2 cycles of reduction and carboxymethylation were performed essentially as described in Chapter 3.B.2. except that the initial reduction step was generally for 1 hour, and the centrifugation was omitted.

*3. Polyacrylamide Gel Electrophoresis*

PAGE at pH 7.5 or pH 2.7, using gels cross-linked with ethylene diacrylate, followed by densitometry, slicing and determination of radioactivity was performed as described in Chapter 5.B.5.

*4. DEAE-Cellulose Chromatography*

Radioactive proteins were fractionated on a column (50 cm x 1.6 cm) of DEAE-cellulose essentially as described in Chapter 3.C.4.b. using a linear gradient of 500 ml total volume. Flow rate was 0.8 ml/min and 6.0 ml fractions were collected. Optical density readings at 277 nm were taken , and aliquots were treated with 10% TCA for the determination of acid-insoluble radioactivity.

*5. Sephadex G-100 Chromatography*

Samples were applied to a column (92 cm x 1.0 cm) of Sephadex G-100 fine, equilibrated with 50% v/v aqueous formic acid (Steinert, 1972). The flow-rate was 5.0 ml/hour and 1.0 ml fractions were collected. Optical densities at 277 nm were determined. Aliquots were absorbed onto glass fibre filters and dried for the determination of radioactivity.

*6. N-terminal Analysis*

N-terminal dipeptides were isolated by either the trypsin/chymotrypsin/pronase or chymotrypsin/carboxypeptidase A procedures, followed by deproteination and Dowex-50

chromatography as described in Chapter 4.B.1-3.

After Dowex-1 chromatography as described in Chapter 4.B.5 aliquots of the fractions were dried on glass-fibre filters for the determination of radioactivity.

Identity of the labelled N-terminal dipeptide was confirmed by HVPE at pH 6.5 (Chapter 4.B.7). After electrophoresis and drying, the paper was cut into 0.5 inch strips, which were placed in scintillation fluid for the determination of radioactivity.

### 7. *Determination of the Specific Activity of $^{3}$H-Serine in the Proteins and N-Terminal Dipeptide*

Samples of the protein and N-terminal dipeptide were hydrolysed in 6 N HCl at 110° for 20 hours *in vacuo*. The hydrolysates were taken to dryness and dissolved in Beckman Automatic Amino Acid Analyzer loading buffer. Aliquots were taken for the determination of serine by amino acid analysis, and aliquots were absorbed onto glass fibre filters and dried for the determination of radioactivity. Results were expressed as cpm/μmole serine

### 8. *Preparation of "De-acetylated SH-keratin"*

Twenty-one day embryonic chick feathers (100 mg) were extracted with urea/β-mercaptoethanol/ethanolamine as described in Chapter 3.B.2, but the proteins in the extract were not carboxymethylated. After centrifugation at 38,000 g for 30 min, the extract was dialysed against four changes of water overnight and freeze-dried.

The freeze-dried protein was taken up in 5 ml of 2 M HCl in 50% v/v ethanol and subjected to partial acid hydrolysis at 100° for 10 min (Greenstein and Winitz, 1961). Water (10 ml) was then added, and the mixture was freeze-dried. The freeze-dried material was stored *in vacuo*.

The above procedure would be expected to produce a mixture containing among many other partial acid hydrolysis products, de-acetylated but otherwise undegraded keratin polypeptide chains, in varying states of oxidation.

### 9. *Preparation of Nascent Polypeptide Chains*

After incubation of lysates with labelled amino acids for the stated times, the ribosomes/polyribosomes were collected by centrifugation, generally at 203,000 g for 90 min. The ribosomal pellet was resuspended at alkaline pH to hydrolyse the peptidyl-tRNA bonds (Bresler *et al.*, 1966). Generally 0.5 ml of a solution containing 10 mM dithiothreitol adjusted to pH 10.2 - 10.4 with N-ethylmorpholine was used for this purpose, and the mixture was incubated for 3 hours at 37° (Yoshida *et al.*, 1970). No difference was observed if Tris/HCl (0.1 M, pH 9.0), or ammonium bicarbonate (1 M, pH 9.7) was substituted for the N-ethylmorpholine solution. After incubation, the mixture was cleared by centrifugation (203,000 g for 90 min).

### 10. *Fractionation of Nascent Chain Preparations on Sephadex G-25*

The supernatant from the alkali-treated ribosomal

preparations was loaded *in toto* on a column of Sephadex G-25 fine (89 cm x 1.0 cm) equilibrated in PMA containing 0.01 M β-mercaptoethanol. The flow rate was 10 ml/hour and 1.0 ml fractions were collected. Aliquots, generally of 0.2 ml from alternate tubes, were absorbed onto glass fibre filters and dried for the determination of radioactivity .

### 11. *Fractionation of Nascent Chain Preparations on Sephadex G-50*

Samples were applied to a column (67 cm x 1.2 cm) of Sephadex G-50 fine, equilibrated with 75% v/v aqueous formic acid. The flow rate was 5 ml/hour and 1.0 ml fractions were collected. Aliquots were absorbed onto glass fibre filters and dried for the determination of radioactivity.

### 12. *Fractionation of Nascent Chain Preparations on Sephadex G-10*

Samples were applied to a column of Sephadex G-10 (76 cm x 1.4 cm), equilibrated with PMA. The flow rate was 20 ml/hour, and 1.0 ml fractions were collected. Aliquots were absorbed onto glass fibre filters for the determination of radioactivity.

### 13. *Fractionation of Nascent Chain Preparations on Dowex-50*

In order to determine whether such preparations had free amino groups, samples were loaded in water onto a column (5 cm x 1 cm) of AG 50W-XB, 100-200 mesh ($H^+$ form) in

$H_2O$ (Narita, 1958), and eluted with water. Eight fractions, each of 2.0 ml were collected, and the column was then washed with 2.0 M pyridine/acetate, pH 6.5 in order to elute any bound peptides. A further eight fractions were collected. Aliquots were absorbed onto glass fibre filters and dried for the determination of radioactivity.

### 14. *High Voltage Paper Electrophoresis at pH 1.9*

Samples were applied to Whatman 3 MM paper and subjected to electrophoresis (2000 V, 1 hour) using the formic acid/acetic acid mixture of Offord (1966).

After electrophoresis and drying, the paper was cut into 0.5 inch strips. Each strip was cut into small pieces, and shaken for 1 hour in 1.0 ml of NEMA. Aliquots were absorbed onto glass fibre filters and dried for the determination of radioactivity.

### 15. *N-terminal Analysis by the Dansyl Chloride Procedure*

Dansylation of nascent chain preparations was carried out as described by Gros and Labouesse (1969). The hydrolysed reaction products were mixed with DNS-methionine and DNS-methionine sulphone and fractionated by HVPE at pH 4.40 (Gray, 1967). After electrophoresis, the DNS-methionine and DNS-methionine sulphone spots were cut out, eluted with pH 4.40 electrophoresis buffer, absorbed onto glass fibre filters and dried for the determination of radioactivity. Control experiments with $^3$H-methionine indicated that the recovery of radioactivity was about 40%.

C. RESULTS

1. *Characterization of the Proteins Synthesized in 14-Day Lysates*

(a) *Polyacrylamide Gel Electrophoresis.*

(i) Polyacrylamide gel electrophoresis at pH 7.5. Aliquots of the radioactive protein samples prepared from lysates labelled with $^{14}$C-leucine, $^{3}$H-serine or $^{14}$C-algal hydrolysate were mixed with embryonic feather proteins and subjected to PAGE at pH 7.5. Densitometer tracings and radioactivity profiles of typical gels are shown in Figure 6.1. Alignment of the profiles was unambiguously established by determining the optical density at 570 nm of the Coomassie Brilliant Blue dye in the gel slices after their solubilization in ammonium hydroxide (Cain and Pitney, 1968). The optical density in the keratin region of such gels was contributed almost entirely by the added carrier protein. It should be noted in Figure 6.1 that the optical density in the low-mobility regions of the gels was contributed by proteins in the samples of lysate. The volumes of the total incorporation mixtures that were applied to the gels varied in different experiments, according to the level of radioactivity.

The mobility of the major peaks of radioactivity coincided precisely with keratin bands β2 - β5, for material labelled with $^{14}$C-leucine, $^{3}$H-serine, or $^{14}$C-algal hydrolysate. Some radioactivity also co-electrophoresed with the α- and γ-proteins. In the case of the preparation labelled with $^{3}$C-serine, the recovery of counts applied to the gel was determined. The high yield obtained (approx. 94%) indicates

FIGURE 6.1.

PAGE at pH 7.5 of Radioactive Proteins Synthesized in the Lysate.

After reduction and carboxymethylation of the supernatant fraction, an aliquot was mixed with 100 µg of 21-day embryonic feather proteins and subjected to PAGE at pH 7.5. Bands β2 - β5 are indicated.

FIGURE 6.1a.

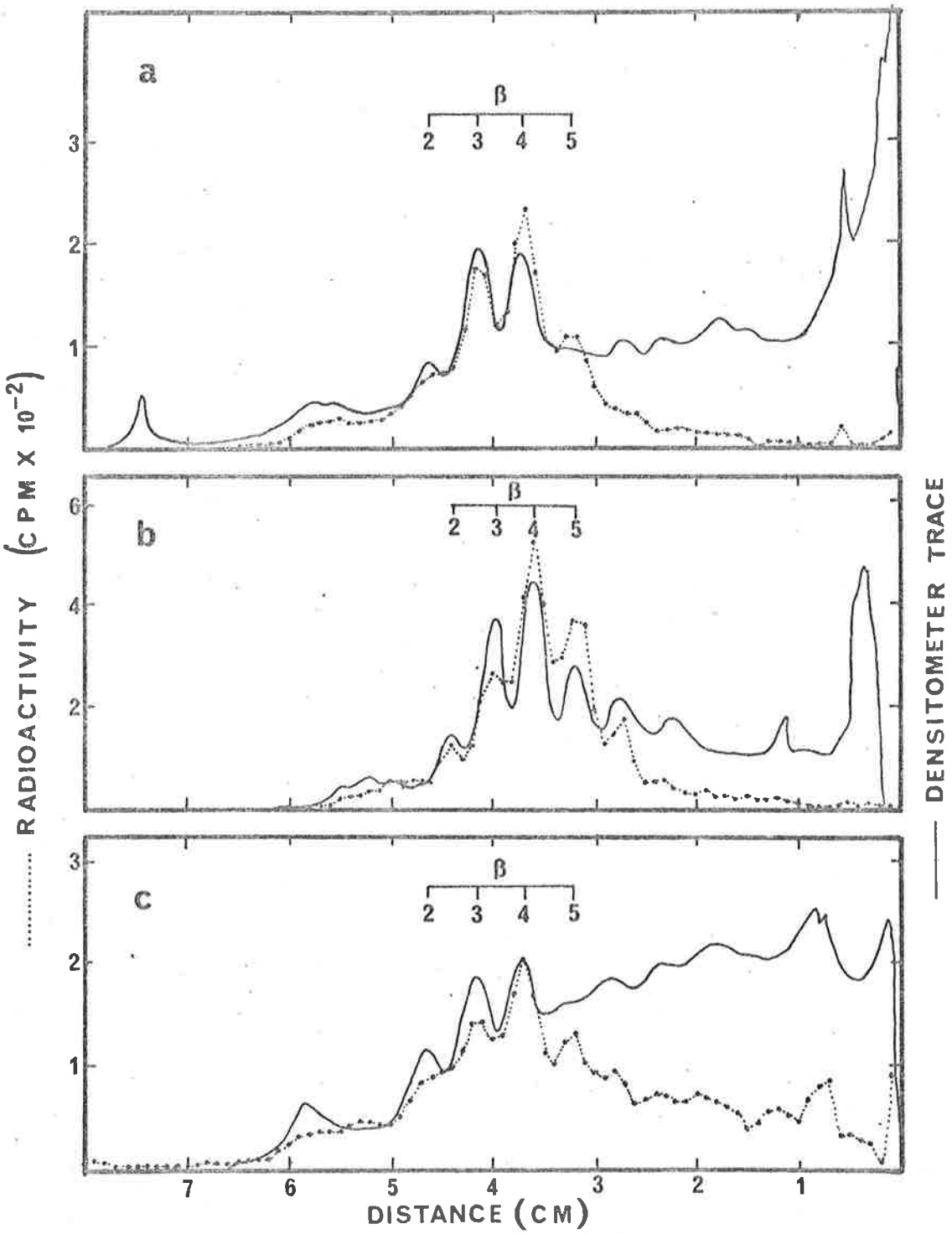
The lysate was incubated for 45 min. with 5 µC of $^{14}$C-leucine, Sp. Act. 313 µC/mMole. One-third of the total was loaded.

FIGURE 6.1b.

The dialyzed lysate was incubated for 1 hr with 50 µC of $^{3}$H-serine, Sp. Act. 1.2 C/mMole. One-fifteenth of the total was loaded.

FIGURE 6.1c.

The lysate was incubated for 1 hr with 10 µC of $^{14}$C-algal hydrolysate. One-third of the total was loaded.


a
b
c
β
2 3 4 5
RADIOACTIVITY (CPM X $10^{-2}$)
DENSITOMETER TRACE
DISTANCE (CM)
7 6 5 4 3 2 1

that all species of proteins into which serine had been incorporated in the lysate were displayed by PAGE at pH 7.5. The results suggest that the major protein products of the lysate were the keratin bands β2 - β5.

(ii) PAGE at pH 2.7. Co-electrophoresis of proteins labelled with $^{14}$C-leucine and embryonic feather proteins on pH 2.7 gels is shown in Figure 6.2. The radioactivity profile again corresponded closely to the densitometer profile, indicating that the major protein products of the cell-free system were keratins of bands A and B.

*(b) DEAE-cellulose Chromatography.*

Chromatography on DEAE-cellulose of proteins labelled with $^{3}$H-serine, to which embryonic feather proteins (100 mg) had been added, is shown in Figure 6.3. The major peak of radioactivity co-chromatographed with the peak of optical density contributed by the major keratin proteins. Lower peaks of radioactivity corresponded to optical density peaks of α- and γ-proteins, in a manner similar to the results obtained by PAGE at pH 7.5. The peak of optical density on the front has been shown to result in part from non-proteinaceous material and in part from traces of keratin components which, for unknown reasons, do not bind to the DEAE-cellulose (I.D. Walker, personal communication). Presumably, the unbound keratin fraction accounts at least in part for the peak of radioactivity on the front, although the possibility that other radioactive proteins were present has not been excluded.

FIGURE 6.2.

PAGE at pH 2.7 of Radioactive Proteins Synthesized in the Lysate.

Details of the procedures are identical to those in Figure 6.1a, except that the fractionation was by PAGE at pH 2.7.

Bands A-E are indicated.

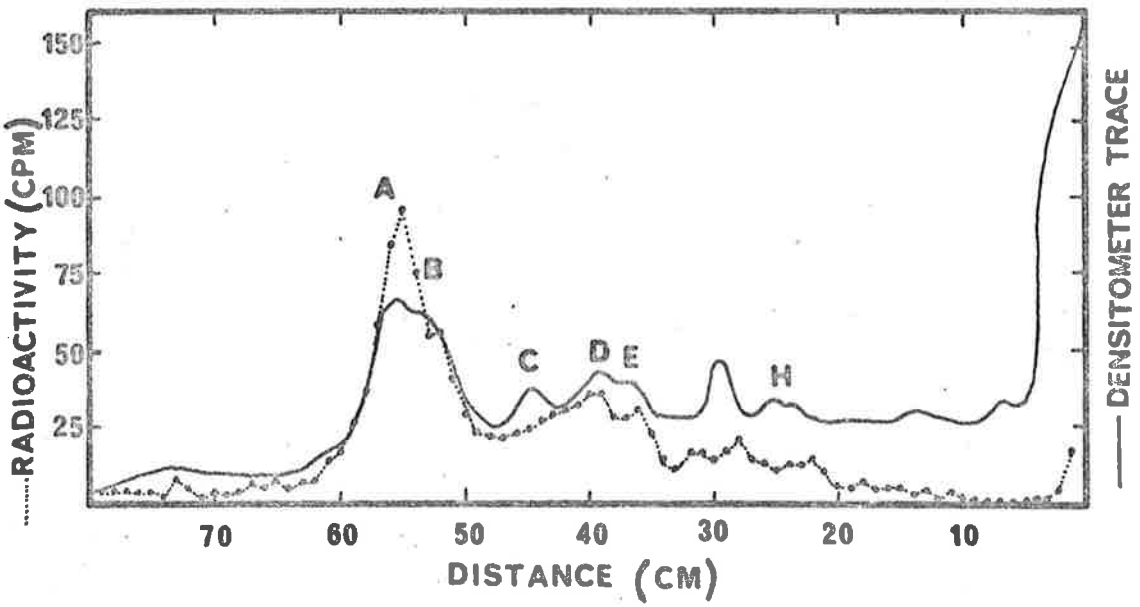
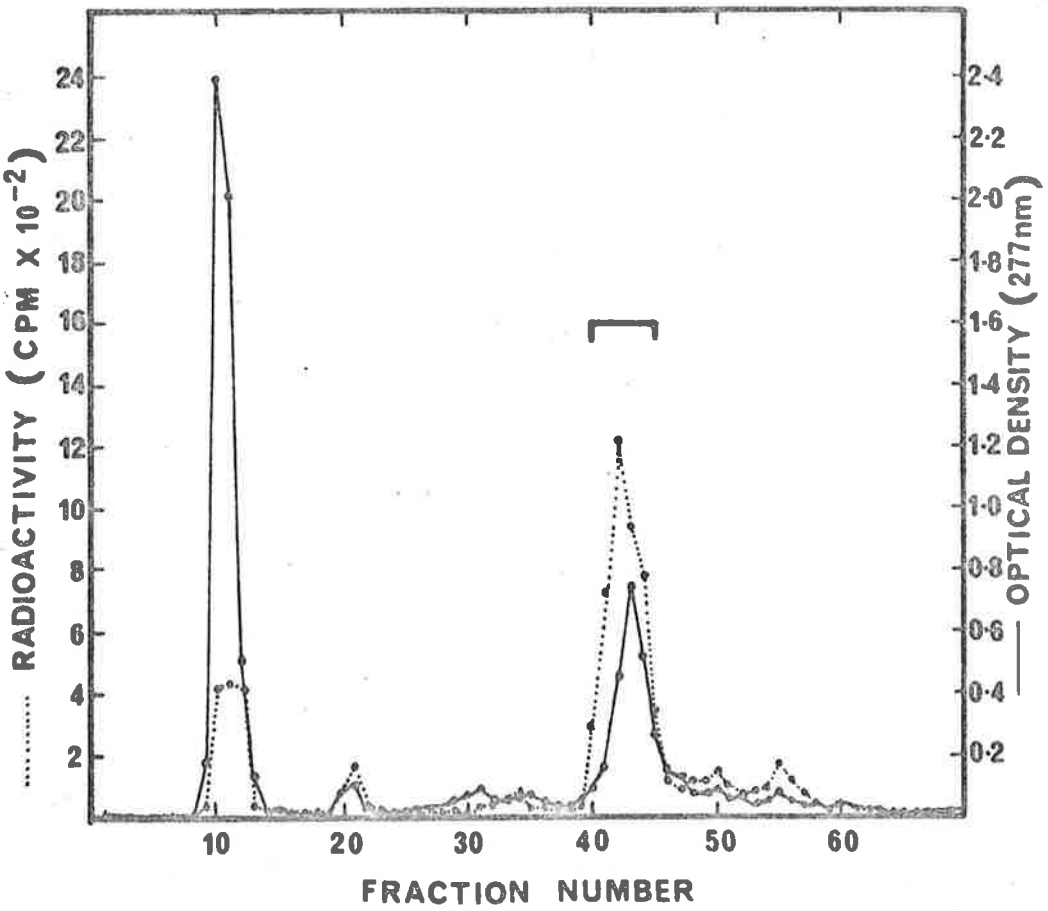

150
125
100
75
50
25
RADIOACTIVITY (CPM)
A
B
C
D
E
H
70
60
50
40
30
20
10
DISTANCE (CM)
DENSITOMETER TRACE

FIGURE 6.3.

Fractionation of Radioactive Proteins Synthesized in the Lysate by DEAE-Cellulose Chromatography.

Dialyzed lysate was incubated for 1 hr with 75 μC of $^{3}$H-serine, Sp.Act. 3 C/mMole. After reduction and carboxymethylation of the supernatant fraction, 100 mg of 21-day embryonic feather proteins were added and the mixture was fractionated by DEAE cellulose chromatography as described in Methods.

Fractions pooled for subsequent experiments are shown by the bar.


RADIOACTIVITY (CPM X $10^{-2}$)
OPTICAL DENSITY (277nm)
FRACTION NUMBER
24
22
20
18
16
14
12
10
8
6
4
2
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
10
20
30
40
50
60

The specific activity across the major keratin peak was not constant. The specific activity in proteins labelled with $^{3}$H-serine of component β5, as determined by PAGE at pH 7.5 (Fig. 6.1b) was greater than the specific activities of β4 or β3. Components β5, β4 and β3 elute in that order from DEAE-cellulose under conditions of higher resolution (I.D. Walker, personal communication; see Fig. 3.14) thus rendering the decrease in specific activity across the keratin peak on DEAE-cellulose compatible with the results from PAGE at pH 7.5.

*(c) Sephadex G-100 Chromatography.*

The major peak fractions from DEAE-cellulose chromatography of proteins labelled with $^{3}$H-serine, pooled as shown by the bar in Figure 6.3, were dialyzed against water and freeze-dried. A sample (5 mg) of this material was then analyzed by chromatography on Sephadex G-100 (Fig. 6.4). The major peak of radioactivity precisely co-chromatographed with the peak of optical density corresponding to the major keratin proteins.

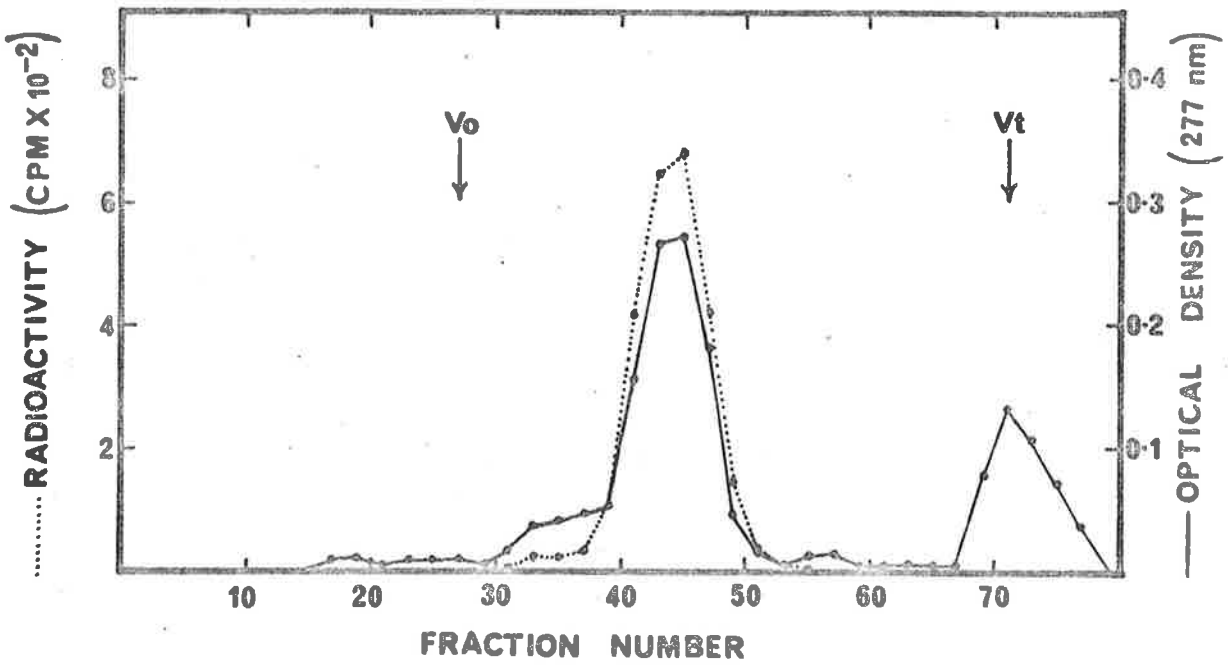
A similar result was obtained when an unfractionated preparation of proteins labelled with $^{14}$C-algal hydrolysate in the lysate system was co-chromatographed with embryonic feather proteins on Sephadex G-100 except that a small peak of higher molecular weight material was also observed.

The results from Sephadex G-100 chromatography provide support for the conclusion that keratin proteins were synthesized in the lysate system. Further, it would appear

FIGURE 6.4.

Fraction of Radioactive Proteins Synthesized in the Lysate by Sephadex G-100 Chromatography.

The sample consisted of 5 mg of the proteins labelled with $^{3}$H-serine and fractionated by DEAE cellulose chromatography as shown in Figure 6.3. 0.5 ml aliquots from the 1.0 ml fractions were taken for the determination of radioactivity.


RADIOACTIVITY (CPM X 10⁻²)
OPTICAL DENSITY (277 nm)
Vo
Vt
8
6
4
2
0·4
0·3
0·2
0·1
10
20
30
40
50
60
70
FRACTION NUMBER

that the preparations were not significantly contaminated with lower molecular weight partially-completed chains.

2. *Comparison of the Proteins Synthesized in 14-Day Lysates with Proteins Synthesized in Lysates From Feathers of Other Ages*

In order to compare the protein products of lysates from 12-, 13-, 14- and 15-day feathers, radioactive proteins were prepared from the post-ribosomal supernatants of lysates of these ages and subjected to PAGE at pH 7.5.

Difficulties were encountered with preparations from tissue younger than 14-days. The ability of these younger preparations to incorporate amino acids into protein was low (G.A. Partington, personal communication) and consequently, large protein samples of low specific activity were applied to the gels. These gels were found to run in an anomalous manner. In one experiment using a 12-day lysate labelled with $^{14}$C-algal hydrolysate, sufficient radioactivity was obtained to overcome this problem. The gel profile is shown in Figure 6.5a compared with a 14-day gel profile (Figure 6.5b). This result, which must be considered preliminary, indicates that the major protein products of the 12-day lysate were non-keratin proteins, with only traces of keratin being synthesized. Such a result would be expected from studies on keratin synthesis *in vivo* (Chapter 5).

In contrast, the 15-day lysate system behaved in a manner similar to that of the 14-day system, in that the major protein products appeared to be keratins (not shown).

FIGURE 6.5.

Comparison of the Proteins Synthesized in 12- and 14-Day Lysates.

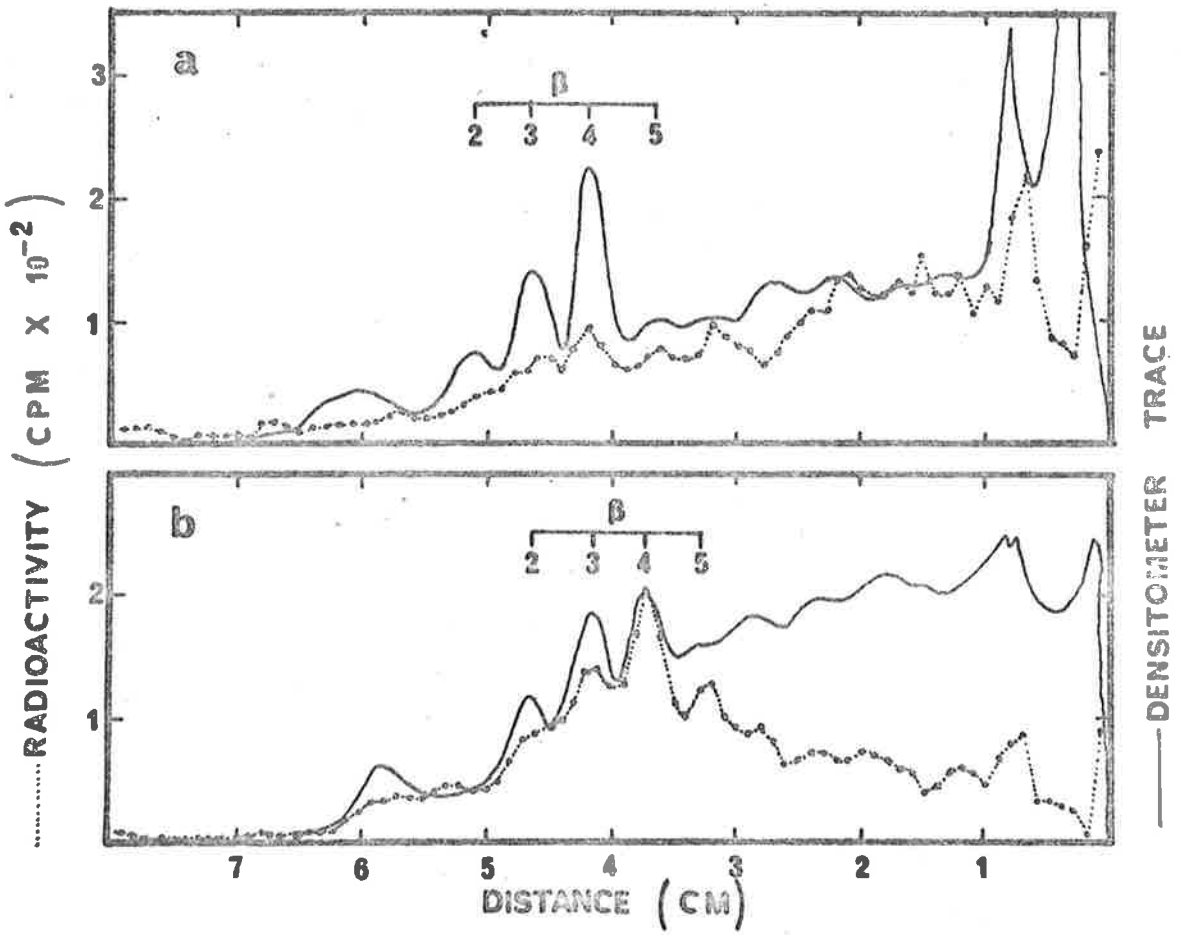
Samples were fractionated by PAGE at pH 7.5, after addition of 21-day embryonic feather proteins (100 µg). Bands β2 - β5 are indicated.

FIGURE 6.5a.

12-day lysate was incubated for 1 hour with 5 µC of algal hydrolysate. One-half of the total was loaded.

FIGURE 6.5b.

14-day lysate, as for Figure 6.1c.


a
b
β
2 3 4 5
3
2
1
RADIOACTIVITY (CPM X $10^{-2}$)
DENSITOMETER TRACE
7 6 5 4 3 2 1
DISTANCE (CM)

### 3. *Isolation of the N-Terminal Dipeptide From the Proteins Synthesized in 14-Day Lysates*

The results described above provide strong evidence that the 14-day feather lysate system synthesized and released keratin proteins. However, the observed synthesis may have involved only completion and release of pre-existing incomplete nascent chains (Bishop *et al.*, 1960), or, in addition, the synthesis and release of chains initiated in the lysate. It would be expected that a lysate system prepared in the present manner (Appendix A) would be capable of initiation of protein synthesis (Lamfrom and Knopf, 1964).

If initiation of the synthesis of feather keratin proteins was occurring in the lysate, it would be expected that after incorporation of $^{3}$H-serine into protein in the lysate the N-terminal serine residue of these proteins should be radioactive. If, in addition, the acetylation mechanism was active in the lysate, it should be possible to isolate Ac-[$^{3}$H]-Ser-SCMC from the proteins, after enzymic digestion.

These predictions were examined in the experiments described below.

#### (a) *Isolation of the N-Terminal Dipeptide by the Pronase Procedure*

Unfractionated proteins, labelled with $^{3}$H-serine in the lysate, were mixed with embryonic feather proteins (50 mg), digested with trypsin, chymotrypsin and pronase, and the N-terminal peptide was purified by Dowex-50 chromatography.

Fractionation of the Dowex-50 eluate by chromatography on Dowex-1 is shown in Figure 6.6a. A major peak of radioactivity co-eluting with Ac-Ser-SCMC was obtained. In addition, two other major peaks were obtained, one corresponding to the peptide PCA-(ser,pro) and another, of unknown nature, eluting after PCA.

To confirm the identity of Ac-$^{3}$H-Ser-SCMC, a sample of this material purified by Dowex-1 chromatography in a similar experiment was subjected to HVPE at pH 6.5 (Figure 6.6b). The peak of radioactivity migrated with the mobility (m = 1.16) expected for Ac-Ser-SCMC (Chapter 4).

*(b) Isolation of the N-Terminal Dipeptide by the Chymotrypsin/Carboxypeptidase Procedure*

The DEAE-cellulose purified featherkeratin proteins, labelled with $^{3}$H-serine (from Figure 6.3) were digested with chymotrypsin followed by carboxypeptidase A and the N-terminal dipeptide was isolated by Dowex-50 chromatography. Fractionation of the Dowex-50 eluate by chromatography on Dowex-1 is shown in Figure 6.7a. One major peak of radioactivity, co-eluting with Ac-Ser-SCMC was obtained. HVPE of this material at pH 6.5 gave one peak, m = 1.12 (Figure 6.7b).

In this experiment, the percentage of chains initiated in the cell-free system was estimated by determining the specific activity of serine in the intact proteins and the specific activity of serine in the N-terminus (Table 6.1). The percentage of total radioactivity in the N-terminus in this

FIGURE 6.6.

Isolation of the N-terminal Dipeptide From Radioactive Proteins Synthesized in the Lysate by the Pronase Procedure.

The preparation of $^{3}$H-serine-labelled protein was that described in Figure 6.1b.

FIGURE 6.6a.

Fractionation of the Dowex-50 eluate by chromatography on Dowex-1. Fractions were collected at 5 minute intervals. 0.2 aliquots were taken for the determination of radioactivity. Other details are given in Methods and the text.

FIGURE 6.6b.

HVPE at pH 6.5 of the N-terminal dipeptide after purification by Dowex-1 Chromatography. The sample was obtained from an experiment similar to that shown in Figure 6.6a. Electrophoresis was for 1 hour at 2500 V. The position of Ile (centre) and Asp (front) markers is shown.

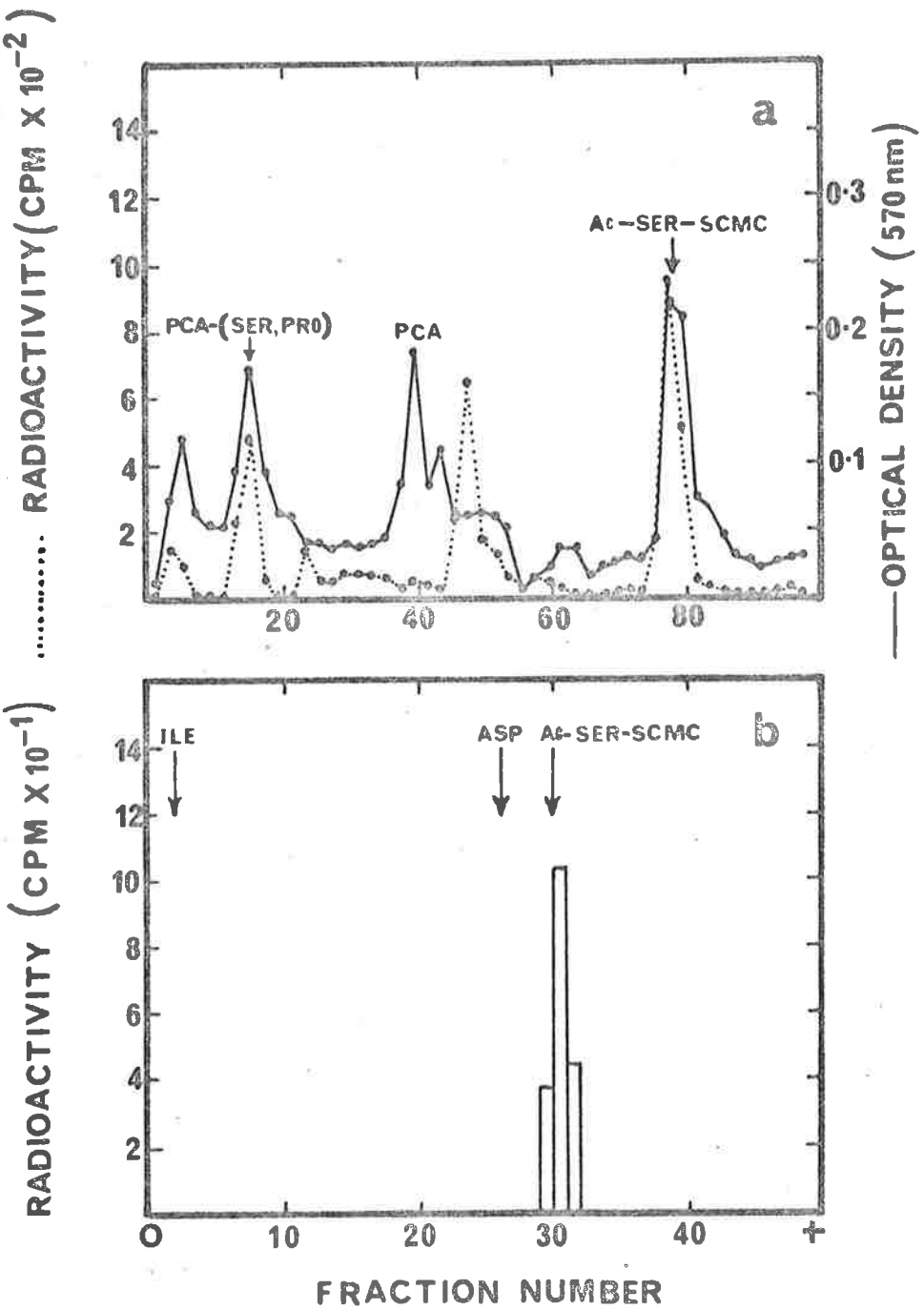

a
b
PCA-(SER,PRO)
PCA
Ac-SER-SCMC
ILE
ASP
Ac-SER-SCMC
RADIOACTIVITY (CPM X 10⁻²)
RADIOACTIVITY (CPM X 10⁻¹)
OPTICAL DENSITY (570 nm)
FRACTION NUMBER

FIGURE 6.7.

Isolation of the N-terminal Dipeptide From Radioactive Proteins Synthesized in the Lysate by the Chymotrypsin/ Carboxypeptidase A Procedure.

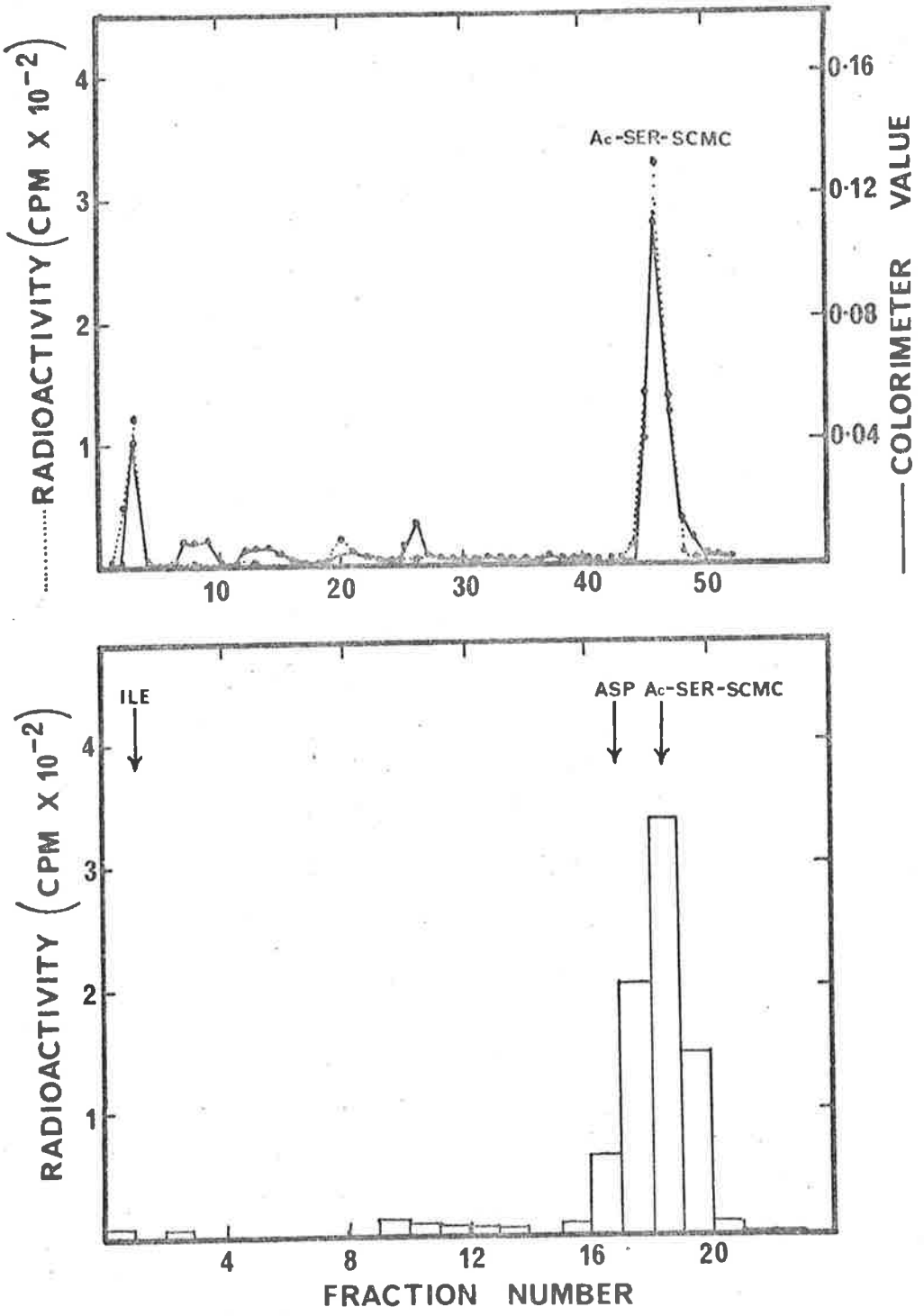
The preparation of $^{3}$H-serine-labelled protein was taken from the fractions indicated by the bar in Figure 6.3.

FIGURE 6.7a.

Fractionation of the Dowex-50 eluate by chromatography on Dowex-1. Fractions were collected at 10 minute intervals. 0.5 ml aliquots were taken for the determination of radioactivity.

FIGURE 6.7b.

HVPE at pH 6.5 of the N-terminal dipeptide after purification by Dowex-1 chromatography. The sample was obtained from the experiment shown in Figure 6.7a. Electrophoresis was for 1 hour at 2500 V.


RADIOACTIVITY (CPM X $10^{-2}$)
COLORIMETER VALUE
Ac-SER-SCMC
0·16
0·12
0·08
0·04
1
2
3
4
10
20
30
40
50
ILE
ASP
Ac-SER-SCMC
RADIOACTIVITY (CPM X $10^{-2}$)
1
2
3
4
4
8
12
16
20
FRACTION NUMBER

experiment and in the experiments in which the pronase procedure was used is also shown in Table 6.1.

The results provide firm evidence that the N-terminal serine residue of the completed keratin proteins synthesized in the lysate had, to a large extent, been incorporated into the proteins in the lysate. Furthermore, the radioactive N-terminal serine was acetylated in the cell-free system.

*(c) Calculation of the Number of Released Chains Per Polysome which were Initiated in the Lysate*

The number of released chains per polysome which had initiated in the lysate was calculated in the following manner.

KNOWN.

(a) Average number of ribosomes/polysome at start = 5 (G.A. Partington, personal communication).

(b) Average serine content of the proteins = 12/mole (Chapter 3).

(c) Specific activities of the N-terminal serine ($S_N$) and total protein-bound serine ($S_T$) (Table 6.1).

ASSUMED.

(e) Random distribution of serine in the proteins.

(f) Random distribution of ribosomes along mRNA.

(g) All polysomes are active.

CALCULATION

From (a), (b), (e) and (f), the average number of serine residues in nascent chains per polysome = 30. To complete and release all these chains, a further 30 serine

TABLE 6.1. PER CENT OF CHAINS LABELLED IN THE SYSTEM THAT WERE INITIATED IN THE SYSTEM.

| Expt. | Calculation of % Initiation from % cpm in N-terminus[a] | Calculation of % Initiation from Specific Activities of N-terminus and Protein |
|---|---|---|
| 1[b] | 30 | n.d. |
| 2[b,c] | 6 | n.d. |
| 3[d] | 51 | 53 |

[a]Assuming that for 100% initiation one-twelfth of total cpm would be in N-terminus.

[b]Pronase procedure for isolation of the N-terminal dipeptide.

[c]Lysate was dialysed for 1.1/2 hr instead of 2.1/2 hr.

[d]Chymotrypsin/Carboxypeptidase-A procedure for isolation of the N-terminal dipeptide.

residues must be added in the lysate. After addition of these 30 residues, the specific activities in released chains can therefore be represented by

$$S_N = \frac{0}{5} \qquad S_T = \frac{30}{60}$$

After 1 newly initiated chain has been synthesized and released, the specific activities can be represented by

$$S_{N1} = \frac{1}{5 + 1} \qquad S_{T1} = \frac{30 + 12}{60 + 12}$$

After n newly initiated chains have been released,

$$S_{Nn} = \frac{1}{5 + n} \qquad S_{Tn} = \frac{30 + 12n}{60 + 12n}$$

$\therefore$ for $\frac{SN}{ST} = 0.67$,

$$\frac{\frac{n}{5 + n}}{\frac{30 + 12n}{60 + 12n}} = 0.67$$

$\therefore$ $n = 5.8$

$\therefore$ in completed, released chains, the number per polysome which initiated in the lysate = 5.8, and the total number completed and released per polysome = 5.8 + 5 = 10.8. The

% of labelled released chains which had initiated in the lysate was therefore

$$\frac{5.8}{10.8} \times 100\% = 53\%.$$

4. *Studies on the Mechanism of N-Acetylation of Keratin Proteins in 14-Day Feather Lysate*

As the foregoing results had indicated that the mechanism for N-terminal acetylation of the keratin proteins was active in the 14-day feather lysate system, some aspects of this mechanism were investigated.

(a) *Characterization of Proteins Labelled by Incubation of the Lysate in the Presence of $^{14}$C-Acetyl-CoA.*

Preliminary experiments indicated that incubation of 14-day feather lysate in the presence of $^{14}$C-acetyl-CoA resulted in the incorporation of radioactivity into acid-precipitable material. Accordingly, proteins were prepared from the supernatants of such lysates, mixed with embryonic feather proteins and fractionated by PAGE at pH 7.5. The densitometer trace and radioactivity profile of such a gel is shown in Figure 6.8.

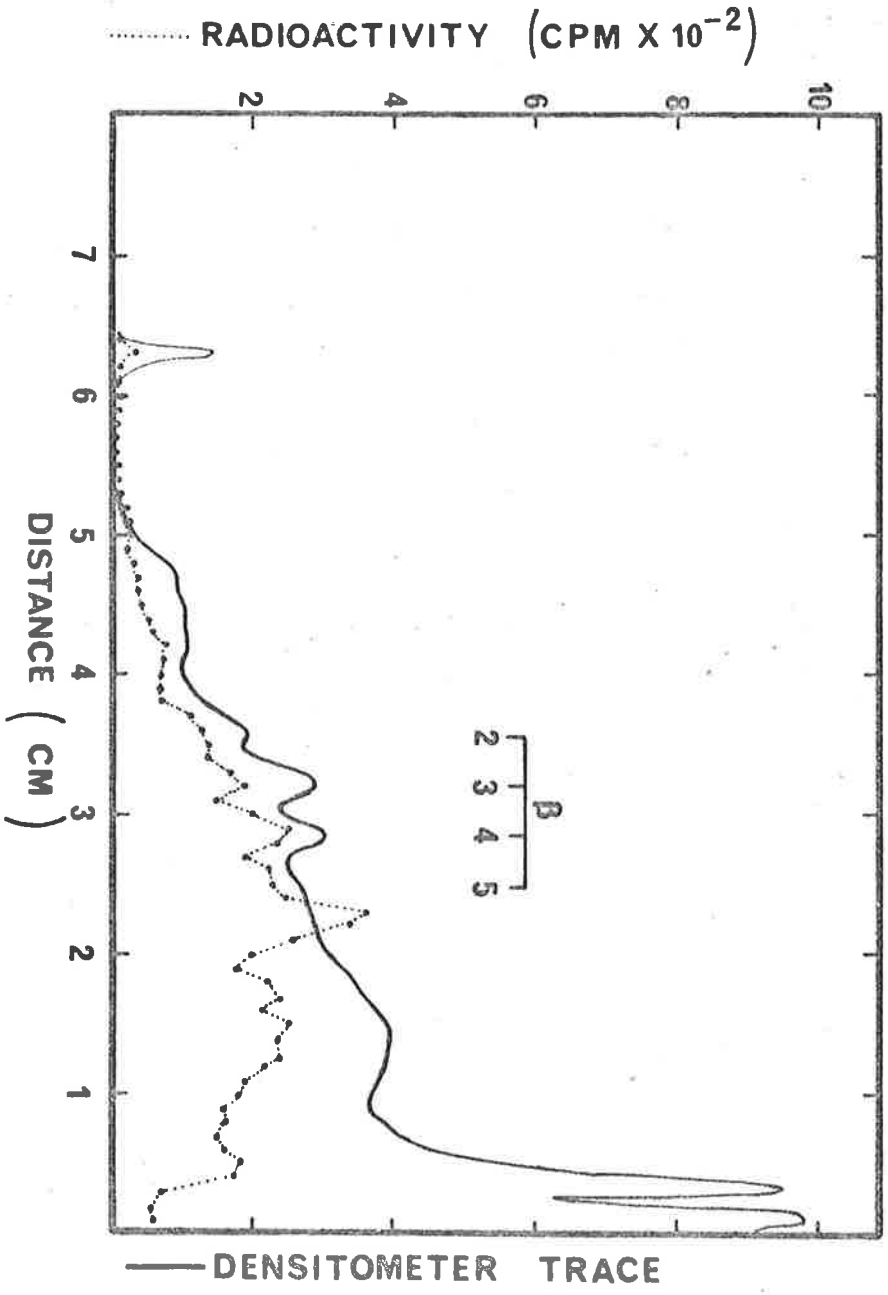
Peaks of radioactivity co-electrophoresed with keratin bands β2 - β5. However, much of the radioactivity was associated with material of lower mobility. This second result was not unexpected, in view of the widespread occurrence

FIGURE 6.8.

PAGE at pH 7.5 of Proteins Labelled in the Lysate in the Presence of $^{14}$C-acetyl-CoA.

The dialyzed lysate was incubated for 1 hour with 5 µC of $^{14}$C-acetyl-CoA, Sp. Act. 59 C/mMole. After reduction and carboxymethylation of the supernatant fraction, one-fifth of the total preparation was mixed with 100 µg of 21-day embryonic featherproteins and subjected to PAGE at pH 7.5.

Bands β2 - β5 are indicated.


RADIOACTIVITY (CPM X $10^{-2}$)
2
4
6
8
10
DISTANCE (CM)
1
2
3
4
5
6
7
β
2 3 4 5
DENSITOMETER TRACE

of post-synthetic acetylation of proteins by acetyl-CoA (Allfrey *et al.*, 1966; Nohara *et al.*, 1968). The results indicate that acetyl-CoA can act as an acetyl donor to feather keratin proteins, in addition to other proteins present in the cell-free system.

*(b) Isolation of the N-Terminal Dipeptide from Proteins Labelled in the Presence of $^{14}$C-Acetyl-CoA.*

Unfractionated SCM-proteins labelled in the presence of $^{14}$C-acetyl-CoA were digested with chymotrypsin followed by carboxypeptidase A and the N-terminal dipeptide was isolated by Dowex-50 chromatography. Fractionation of the Dowex-50 eluate by chromatography on Dowex-1 is shown in Figure 6.9a. One peak of radioactivity, co-eluting with Ac-Ser-SCMC, was obtained. HVPE of this material at pH 6.5 gave one radioactive peak, m = 1.16 (Figure 6.9b).

In order to confirm that the radioactivity was in the acetyl moiety of Ac-Ser-SCMC, aliquots of the material, after purification by Dowex-1 chromatography, were subjected to acid hydrolysis (6 N HCl, 30 min, 100°). In control experiments, water was substituted for HCl. After boiling, 0.2 ml of 5 N acetic acid was added and the mixtures were dried, taken up in 0.5 ml of 5 N acetic acid, absorbed onto glass fibre filters and dried for the determination of radioactivity. This treatment resulted in the loss of 96% of the radioactivity from the samples which had been hydrolysed in 6 N HCl, as compared with the controls. It was concluded that acid

FIGURE 6.9.

Isolation of the N-terminal Dipeptide From Proteins Labelled in the Lysate in the Presence of $^{14}$C-Ac-CoA.

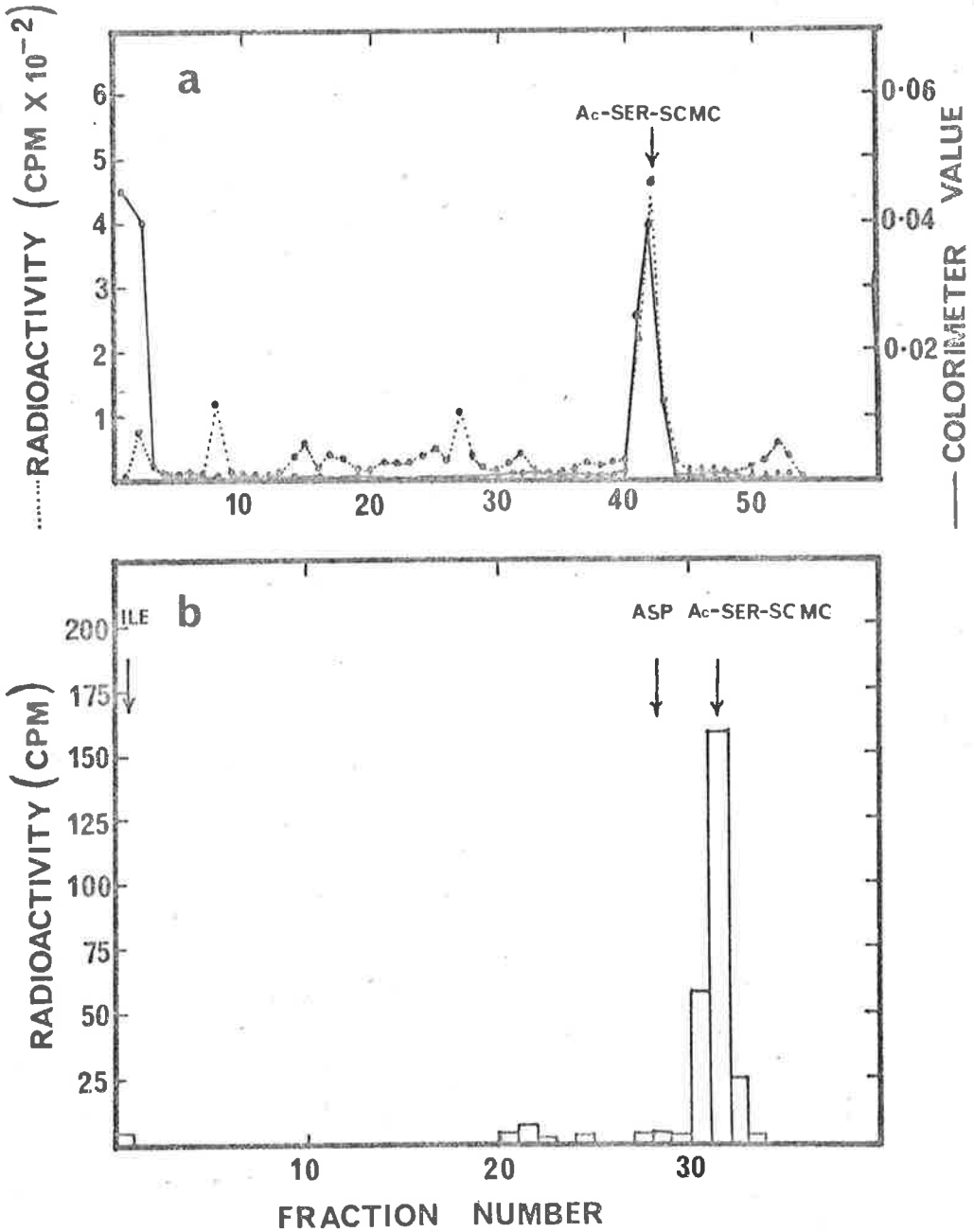
The sample used contained three-fifths of the total preparation described in Figure 6.8.

FIGURE 6.9a.

Fractionation of the Dowex-50 eluate by chromatography on Dowex-1. Fractions were collected at 10 minute intervals, and 1.0 ml aliquots were taken for the determination of radioactivity.

FIGURE 6.9b.

HVPE at pH 6.5 of the N-terminal dipeptide after purification by Dowex 1 chromatography. Electrophoresis was for 30 min at 4,500 V.


a
Ac-SER-SCMC
RADIOACTIVITY (CPM X 10⁻²)
COLORIMETER VALUE
0·06
0·04
0·02
6
5
4
3
2
1
10
20
30
40
50
b
ILE
ASP
Ac-SER-SC MC
RADIOACTIVITY (CPM)
200
175
150
125
100
75
50
25
10
20
30
FRACTION NUMBER

hydrolysis converted the radioactivity into a volatile form, as would be expected if the radioactivity was in the acetyl moiety of Ac-Ser-SCMC.

### (c) *Effects of "de-acetylated" Keratin on Incorporation of $^{14}$C-Acetate into the N-Terminal Dipeptide.*

The above results indicated that the lysate system acetylated newly initiated polypeptide chains and also that acetyl-CoA could act as an acetyl donor to keratin molecules. The effect of adding a partial-acid hydrolysate of feather keratin (in the SH-form) was investigated. If the system contains a protein-acetylase, certain of the products present in such a partial acid hydrolysate could be expected to act as substrates for this enzyme. The experiment was based, in part, on that of Marchis-Mouren and Lippman (1965).

Lysate from 14-day feathers was incubated with $^{14}$C-acetyl-CoA in the presence or absence of "de-acetylated keratin". After the incubations, the total material in each incubation mixture was reduced and carboxymethylated. The effect of "de-acetylated keratin" on the incorporation of radioactivity into acid-insoluble material varied in the individual experiments (Table 6.2).

Fractionation of the carboxymethylation reaction mixture on Sephadex G-25 (Figure 6.10) revealed that "de-acetylated keratin" greatly stimulated the incorporation of radioactivity into material of intermediate molecular weight. This material apparently was not precipitated by TCA (Table

TABLE 6.2. INCORPORATION OF RADIOACTIVITY FROM $^{14}$C-ACETYL-CoA INTO ACID-INSOLUBLE MATERIAL IN THE PRESENCE AND ABSENCE OF "DE-ACETYLATED KERATIN".

Dialysed lysate was incubated with $^{14}$C-acetyl-CoA (2.5 μC, Sp. Act. 59 mC/mMole) for 1 hr, in the presence or absence of "de-acetylated keratin" (3 mg). 5 ml of urea/β-mercapto-ethanol/ethanolamine was then added, and 0.2 ml aliquots were taken and treated with 10% TCA for the determination of radioactivity. Total cpm incorporated are shown.

| Expt. | Control | + "de-acetylated keratin" |
|---|---|---|
| 1 | 40,600 | 53,400 |
| 2 | 32,800 | 19,300 |

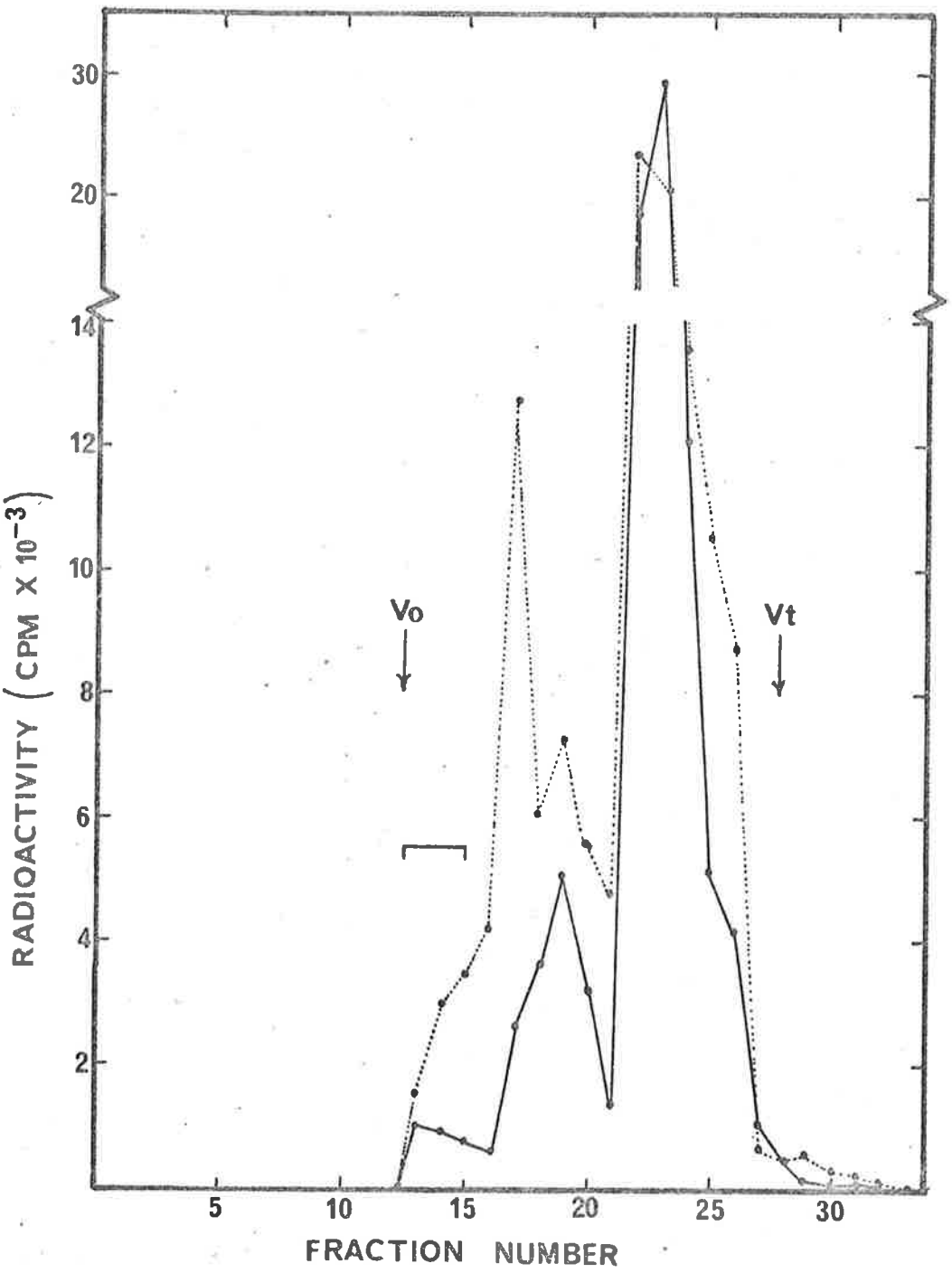
FIGURE 6.10.

Fractionation of Products Labelled by $^{14}$C-Acetyl-CoA in the Lysate in the Presence or Absence of "de-acetylated keratin" on Sephadex G-25.

The material described in Table 6.2 (experiment 1) was fractionated on a column (108 cm x 2 cm) of Sephadex G-25 fine, equilibrated in PMA. Flow rate was 30 ml/hr. Fractions were collected at 10 minute intervals. 0.5 ml aliquots were taken, absorbed onto glass fibre filters and dried for the determination of radioactivity. Fractions pooled for subsequent HVPE are shown by the bar.

——— : control

..... : + de-acetylated keratin.


RADIOACTIVITY (CPM X $10^{-3}$)
30
20
14
12
10
8
6
4
2
Vo
Vt
5
10
15
20
25
30
FRACTION NUMBER

6.2). The fractions shown by the bars in Figure 6.10 were pooled, digested with chymotrypsin and fractionated on Dowex-50. The Dowex-50 eluates were then fractionated by HVPE at pH 6.5 (Figure 6.11). The stimulation of incorporation by "de-acetylated keratin" apparently was due to incorporation into the N-termini of peptides other than Ser-SCMC-Tyr.

In an independent experiment, (experiment 2 in Table 6.2) high molecular weight material was obtained by dialysis of the carboxymethylation reaction mixture instead of Sephadex chromatography, and blocked peptides were isolated by the chymotrypsin/carboxypeptidase-A procedure. Fractionation of the Dowex-50 eluate from the preparation on Dowex-1 is shown in Figure 6.12. Again, "de-acetylated keratin" greatly stimulated incorporation into non-dialysable material, which, after enzymic digestion, resembled blocked amino acids and peptides, suggesting the presence of a protein-acetylase in the lysate .

### 5. *Attempts to Determine the Nature of the Initiator of Keratin Synthesis Using 14-Day Lysates*

The approach chosen involved the use of inhibitors of translation to accumulate nascent chains (Jackson and Hunter, 1970) followed by Sephadex G-25 fractionation of the nascent chains (Yoshida *et al.*, 1970). It was hoped that this approach would allow the unambiguous determination of the N-terminal amino-acid sequence(s) of short nascent chains, at the radiochemical level. In such experiments, detection of the sequence Met-Ser-SCMC-$\{^{\text{Tyr}}_{\text{Phe}}\}$ would argue

FIGURE 6.11.

Fractionation by pH 6.5 HVPE of Chymotryptic Digests of the Products Labelled by $^{14}$C-acetyl-CoA in the Presence or Absence of "de-acetylated keratin".

The material indicated by the bars in Figure 6.10 was digested with chymotrypsin and fractionated by Dowex-50 chromatography. One half of the total material in each Dowex-50 eluate was then subjected to HVPE at pH 6.5 (4000 V, 30 min). The paper was cut into 0.5 inch strips for the determination of radioactivity.

——— : control

..... : + de-acetylated keratin.

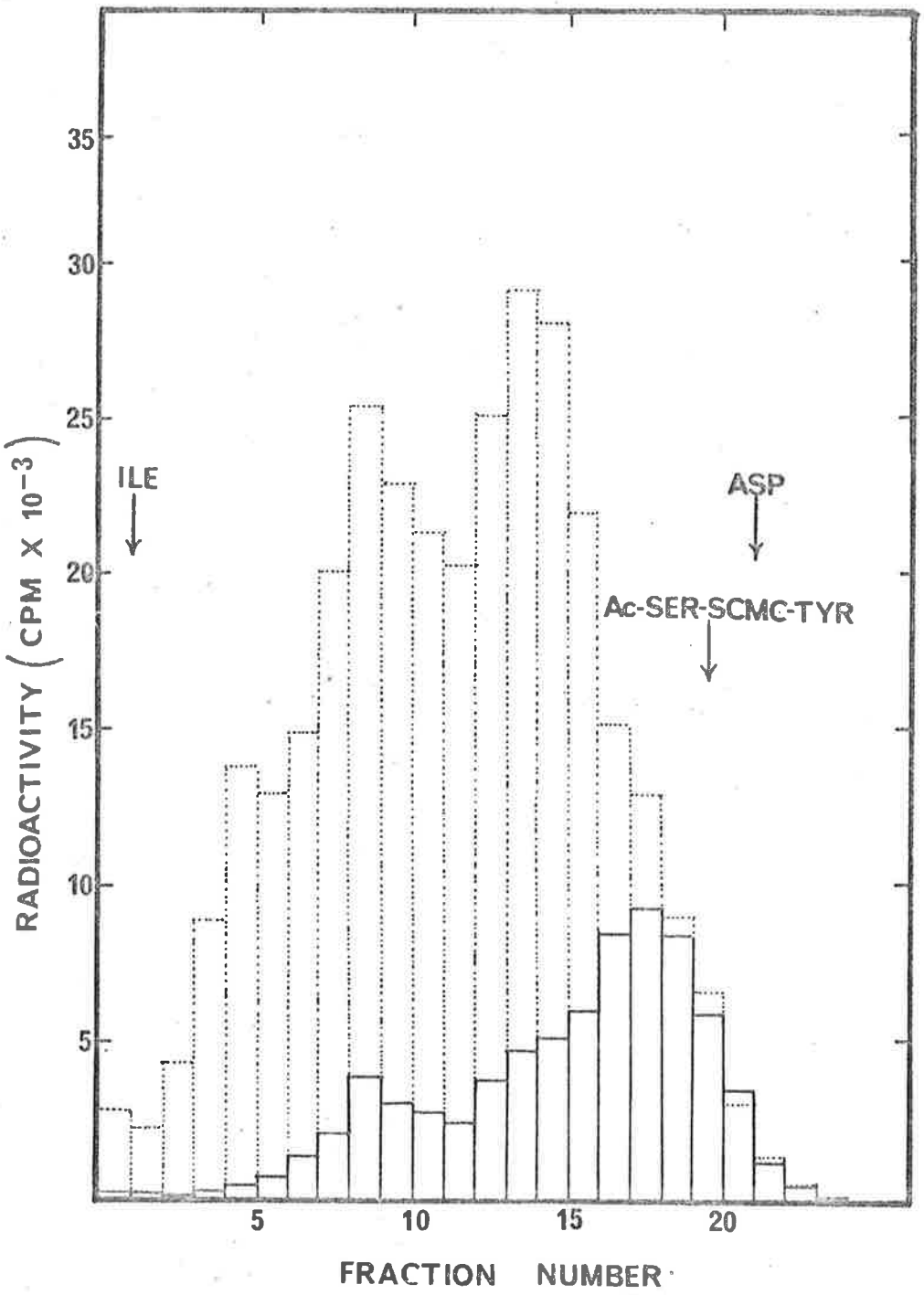

RADIOACTIVITY ( CPM X $10^{-3}$ )
35
30
25
20
15
10
5
ILE
ASP
Ac-SER-SCMC-TYR
5
10
15
20
FRACTION NUMBER

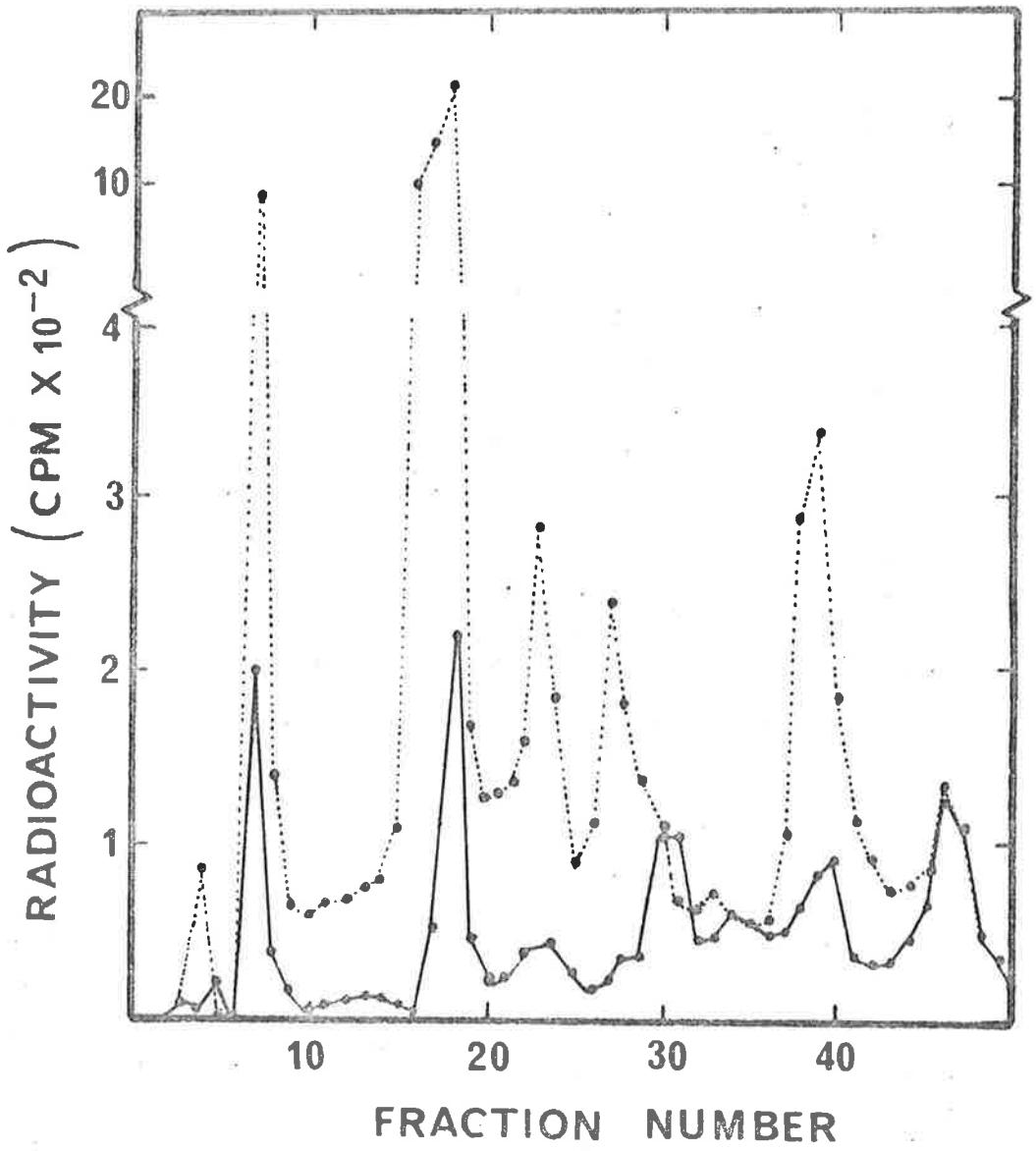
FIGURE 6.12.

Fractionation by Dowex-1 Chromatography of Chymotrypsin/Carboxypeptidase-A Digests of the Products Labelled by $^{14}$C-acetyl-CoA in the Presence or Absence of "de-acetylated keratin".

The material described in Table 6.2 (experiment 2) was digested with chymotrypsin and carboxypeptidase-A and fractionated by Dowex-50 chromatography. The Dowex-50 eluates were then fractionated by Dowex-1 chromatography.

——— : control

..... : + de-acetylated keratin.


RADIOACTIVITY (CPM X 10⁻²)
20
10
4
3
2
1
10
20
30
40
FRACTION NUMBER

strongly for the involvement of methionyl-tRNA in the initiation of keratin synthesis, as is the case in other systems (Jackson and Hunter, 1970; Yoshida *et al.*, 1970; Wilson and Dintzis, 1970; Wigle and Dixon, 1970. Alternatively, identification of short nascent chains with the N-terminal sequence Ac-Ser-SCMC-$\{^{Tyr}_{Phe}\}$ would argue for the involvement of Ac-Seryl-tRNA in the initiation of keratin synthesis, as has been proposed for certain other systems (Narita *et al.*, 1968, 1969; Liew *et al.*, 1970). If Ac-seryl-tRNA was the initiator of keratin synthesis, translational control of keratin synthesis at the level of initiation would be possible (see Chapter 1.D.7).

The N-terminal (initiating) methionine has been shown to be largely removed from nascent chains after they attain the length of about 30 residues (Yoshida *et al.*, 1970; Wilson and Dintzis, 1970). It was therefore imperative for the present purposes that nascent chains of low molecular weight be accumulated in order to test the above hypotheses.

*(a) Incorporation of $^{3}$H-Serine into Nascent Chains in 14-Day Feather Lysates.*

After short-term incubations of lysate with $^{3}$H-serine in the absence of inhibitors of protein synthesis, fractionation of nascent chain preparations on Sephadex G-25 gave profiles typified by that shown in Figure 6.13a. Peaks of radioactivity were obtained that were completely excluded or completely retarded by the Sephadex, but very little material of intermediate size was evident. Similar results

FIGURE 6.13.

Fractionation of Nascent Chain Preparations Labelled with $^{3}$H-Serine by Sephadex G-25 Chromatography.

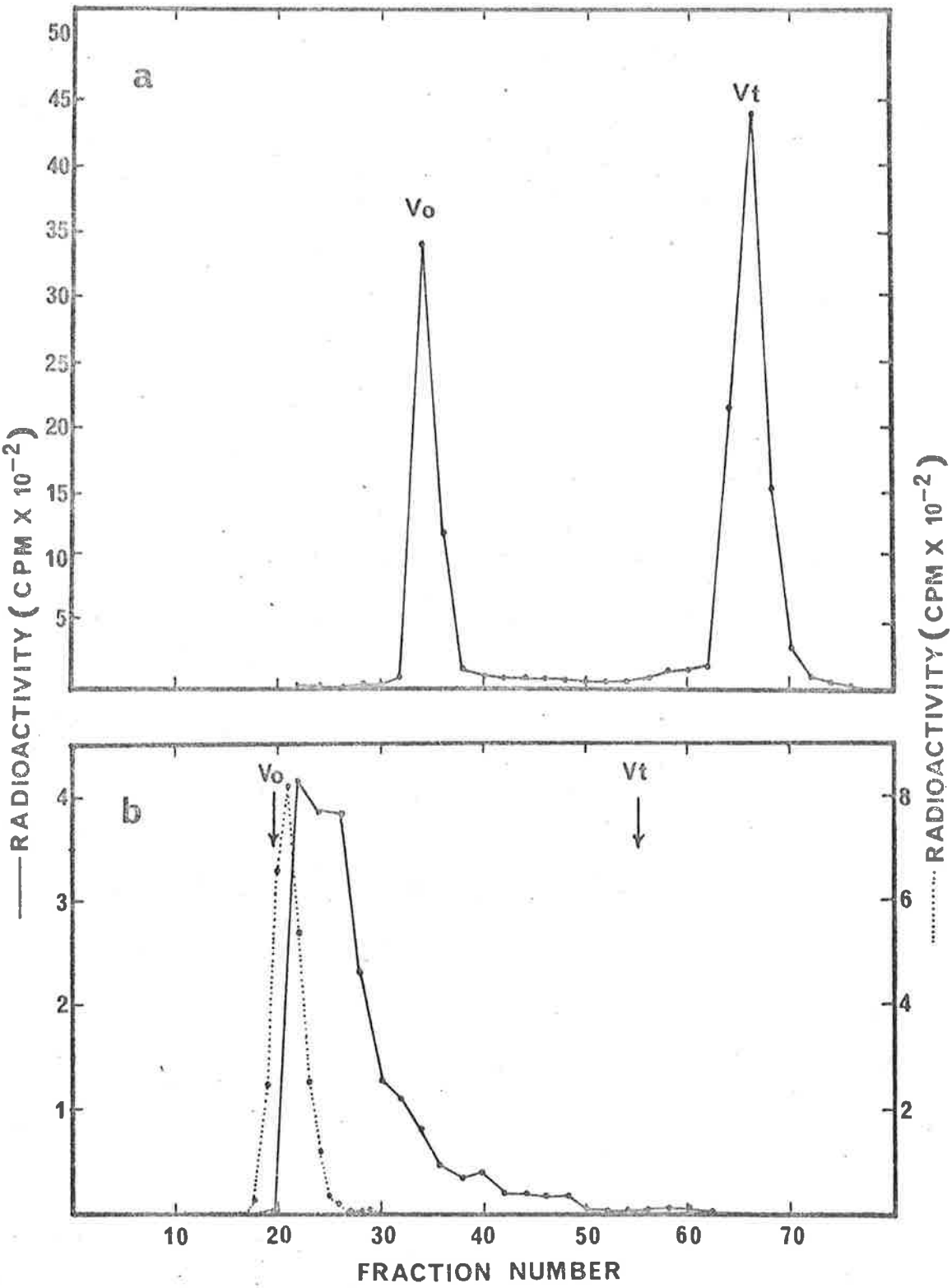
Dialyzed lysate was incubated for 7 min with 50 μC of $^{3}$H-serine, Sp. Act. 1.2 C/mmole. Ribosomal material was recovered by centrifugation, treated at pH 10.4, and the supernatant from this mixture after centrifugation was fractionated by Sephadex G-25 chromatography. 0.2 ml aliquots were taken for the determination of radioactivity.

FIGURE 6.13b.

Fractionation of Nascent Chains on Sephadex G-50.

——— The fractions containing the peak at Vo in Figure 6.13a were pooled, dried down, and reduced and carboxymethylated in a final volume of 0.9 ml. The reaction mixture was then fractionated on Sephadex G-50 as described in Methods. 0.2 ml aliquots were taken for the determination of radioactivity.

..... Fractionation of a sample of protein from the supernatant of a lysate labelled with $^{3}$H-serine for 1 hr, prepared as described in Figure 6.1b.


a
Vo
Vt
b
Vo
Vt
RADIOACTIVITY (CPM X 10⁻²)
RADIOACTIVITY (CPM X 10⁻²)
FRACTION NUMBER

were obtained when $^{14}$C-algal hydrolysate was used as the radioactive precursor.

The material from the peak at Vo in Figure 6.13a was taken to dryness and reduced and carboxymethylated in a final volume of 0.9 ml. The reaction mixture was then fractionated on Sephadex G-50 under dissociating conditions (Figure 6.13b). Comparison of the elution profile with that of protein from the supernatant of a 14-day lysate labelled for 1 hour with $^{3}$H-serine (Figure 6.13b) demonstrated that the average molecular weight of the radioactive material in peak 1 from Figure 6.13a was lower than that of completed feather keratin chains. At least some of the material in this peak therefore appeared to consist of relatively high molecular weight nascent chains.

In attempts to accumulate nascent chains of lower molecular weight, the effects of the antibiotics amicetin, gougerotin and sparsomycin were investigated. At appropriate concentrations, these antibiotics are thought to selectively inhibit translation, without affecting initiation (Jackson and Hunter, 1970; Battaner and Vazquez, 1971), giving rise to the accumulation of short nascent chains on polyribosomes. Results are shown in Figure 6.14. At the concentrations used (see caption to Figure 6.14) the antibiotic amicetin led to the presence of a peak of radioactivity (Peak 1, Figure 6.14a) of low apparent molecular weight. A similar peak was observed when the incubations were carried out in the presence of gougerotin or sparsomycin, but in lower yield (Figure 6.14b,c).

The material from peak 1 (amicetin experiment)

FIGURE 6.14.

Fractionation of Nascent Chain Preparations Labelled with $^{3}$H-Serine in the Prescence of Inhibitors of Translation by Sephadex G-25 Chromatography.

Details were as for Figure 6.13a, except that;

FIGURE 6.14a.

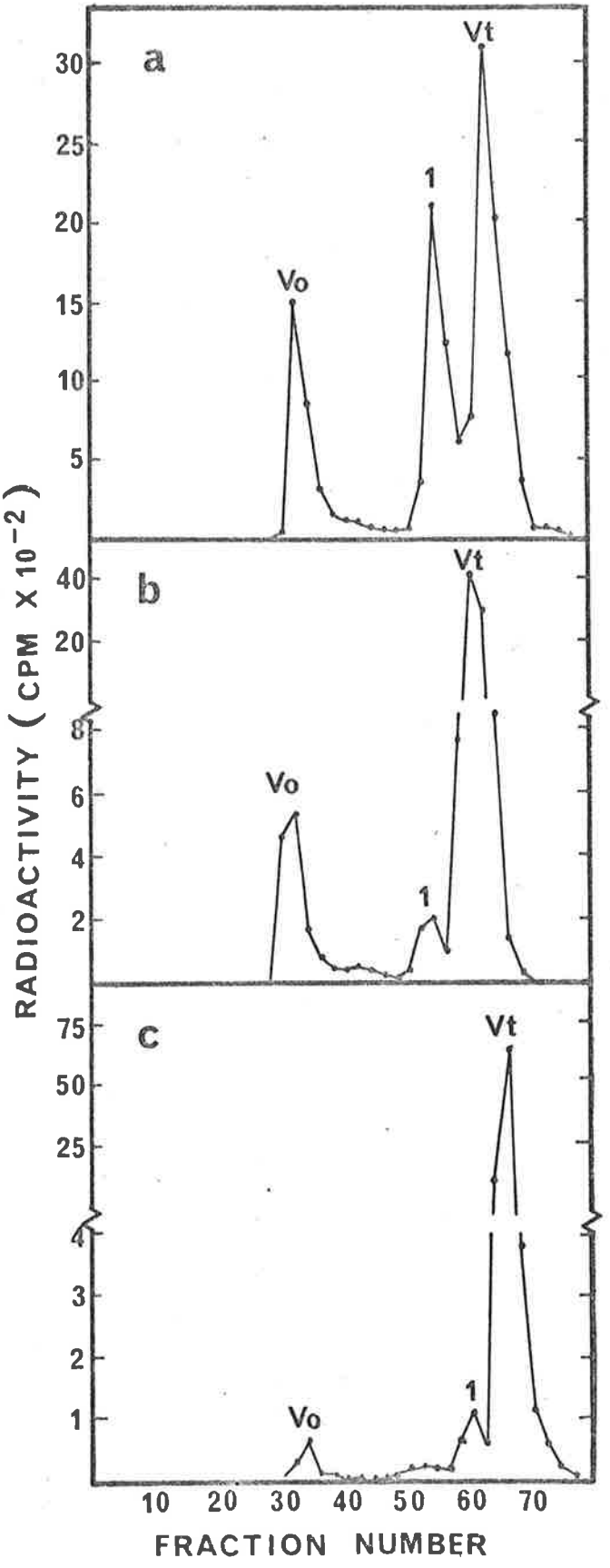
The incubation was for 20 min in the presence of 70 µCi of $^{3}$H-serine, Sp. Act. 1.2 C/mmole, and Amicetin at a concentration of 1 mM.

FIGURE 6.14b.

The incubation was for 20 min in the presence of 70 µCi of $^{3}$H-serine, Sp. Act. 1.2 C/mmole and Gougerotin at a concentration of 10 µM.

FIGURE 6.14c.

The incubation was for 20 min in the presence of 50 µCi of $^{3}$H-serine, Sp. Act. 1.2 C/mmole and Sparsomycin at a concentration of 40 µM.


a
b
c
Vt
Vo
1
RADIOACTIVITY (CPM X 10⁻²)
FRACTION NUMBER
10 20 30 40 50 60 70

was characterized in some detail. The preparation was taken to dryness, reduced and carboxymethylated in a final reaction volume of 0.9 ml, and the reaction mixture was fractionated on Sephadex G-10 to remove reagents. Properties of the radioactive material from peak 1 (Figure 6.14a) are summarized in Table 6.3, The properties suggest that the material was a blocked di- or tripeptide. However, it was neither Ac-Ser-SCMC nor N-formyl-methionyl serine, as shown by its mobility at pH 6.5 and acid stability.

It was considered possible that the material was a diketopiperazine. Dipeptidyl tRNAs could be expected to yield diketopiperazines at alkaline pH (Greenstein and Winitz, 1961). If this were so, however, the observed mobility at pH 6.5 would require that one of the constituent amino acids possessed an acidic side chain (Offord, 1966) and therefore could not be formed from methionyl-seryl-tRNA. Other possibilities are (1) the material is not related to the initiation of keratin synthesis, (2) initiation of keratin synthesis occurs by an unsuspected mechanism, or (3) the material has been chemically modified in the lysate during incubation or during subsequent processing.

The blocking group could not be acetate or formate, as revealed by its stability to acid hydrolysis (Greenstein and Winitz, 1961).

(b) *Incorporation of $^{3}$H-Methionine into Nascent Chains in 14-day Feather Lysates*

The presence of amicetin or sparsomycin during

TABLE 6.3. PROPERTIES OF THE MATERIAL SYNTHESIZED IN FEATHER LYSATE IN THE PRESENCE OF AMICETIN AND $^{3}$H-SERINE.

The material was from peak 1 in Figure 6.14a.

| Procedure | Result |
|---|---|
| 1. Sephadex G-10 chromatography | 1 peak, eluting between $(Leu)_3$ and $^{14}$C-algal hydrolysate. |
| 2. Dowex-50 chromatography | Not bound. |
| 3. HVPE at pH 6.5 | 1 peak, acidic, m = 0.93. |
| 4. Dowex-1 chromatography | 1 peak, eluting after PCA. |
| 5. HVPE at pH 1.9 | Neutral. |
| 6. Partial acid hydrolysis (2 N HCl in 50% EtOH, 30 min, 110°) | >90% stable, as determined by HVPE at pH 6.5 and pH 1.9. |
| 7. Amino acid analysis | Serine was the only radioactive material in the acid-hydrolysate, fractionated on Beckman long column. |

incubation of feather lysate and also of rabbit reticulocyte lysate (Jackson and Hunter, 1970) with $^{3}$H-methionine resulted in highly retarded peaks of radioactivity on Sephadex G-25 (Peaks 1 in Figure 6.15), a result similar to that obtained with $^{3}$H-serine (Figure 6.14). In contrast this peak was not present when $^{3}$H-alanine was used as the label (not shown). As there is no alanine residue near the N-terminus of feather proteins (Chapter 4) this result suggests that the material labelled with serine and methionine was related to the N-termini of keratin proteins.

The material (peaks 1, 2 and 3) prepared in this manner from a rabbit reticulocyte lysate which has been incubated with $^{3}$H-methionine in the presence of sparsomycin was subjected to HVPE at pH 3.5 (Jackson and Hunter, 1970). In contrast to the results of Jackson and Hunter, none of the material had a mobility greater than that of methionine. Furthermore, N-terminal analysis by the Dansyl procedure did not give DNS-methionine.

Properties of the material prepared in this manner in a feather lysate in the presence of sparsomycin are shown in Table 6.4. The properties of the material prepared in the presence of amicetin were similar as far as they were investigated, in that the material did not bind to Dowex-50 and was neutral at pH 1.9, indicating that it was blocked.

In parallel studies on the material at Vt in Figure 6.15b, it was found that this material bound to Dowex-50, indicating that it was not blocked. This material had a mobility identical to methionine, as determined by HVPE at

FIGURE 6.15.

Fractionation of Nascent Chain Preparations Labelled with $^{3}$H-Methionine in the Presence of Inhibitors of Translation by Sephadex G=25 Chromatography.

Details were as for Figure 6.13a, except that incubations were for 20 min, and the incubation mixtures contained $^{3}$H-Methionine (50 µC, Sp. Act. 2.6 C/mmole).

FIGURE 6.15a.

The incubation mixture contained feather lysate, and Amicetin at a concentration of 1 mM.

FIGURE 6.15b.

The incubation mixture contained feather lysate and Sparsomycin at a concentration of 40 µM.

FIGURE 6.15c.

The incubation mixture contained reticulocyte lysate and Amicetin at a concentration of 1 mM.

FIGURE 6.15d.

The incubation mixture contained reticulocyte lysate and Sparsomycin at a concentration of 40 µM.

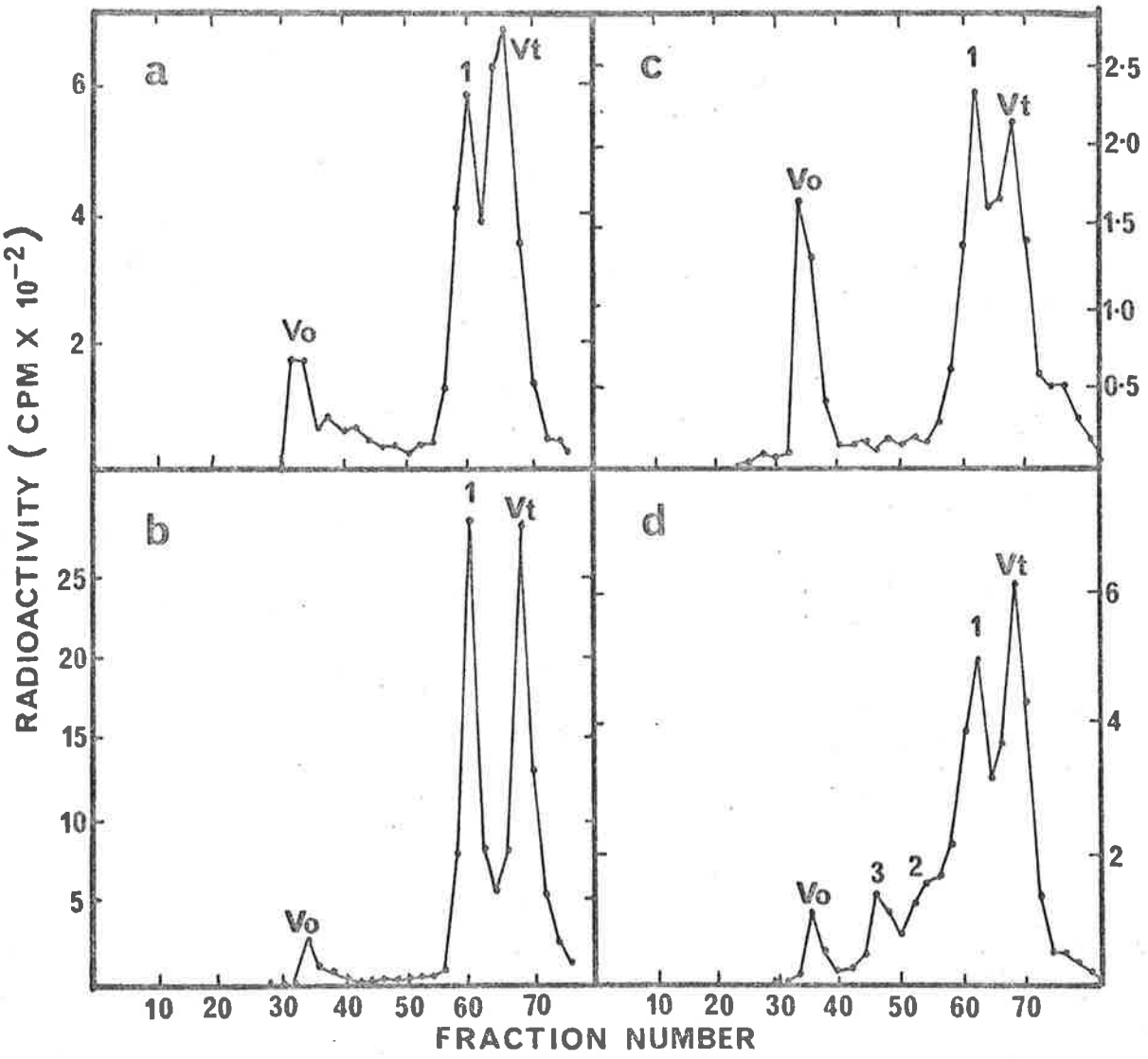

a
b
c
d
Vo
Vt
1
2
3
RADIOACTIVITY ( CPM X $10^{-2}$ )
FRACTION NUMBER
10 20 30 40 50 60 70
6
4
2
25
20
15
10
5
2·5
2·0
1·5
1·0
0·5

TABLE 6.4. PROPERTIES OF THE MATERIAL SYNTHESIZED IN FEATHER LYSATE IN THE PRESENCE OF SPARSOMYCIN AND $^{3}$H-METHIONINE.

The material was from peak 1 in Figure 6.15b.

| Procedure | Result |
|---|---|
| 1. Dowex-50 Chromatography | Not bound. |
| 2. HVPE at pH 1.9 | Neutral. |
| 3. N-terminal analysis by Dansyl procedure | No DNS-methionine detected. |
| 4. Amino acid analysis | No labelled methionine was present in the acid hydrolysate. Two unknown peaks were present. |

pH 1.9 and pH 3.5, as was expected.

The properties of the material synthesized in feather lysate containing $^{3}$H-methionine, in the presence of the antibiotics amicetin or sparsomycin, would not appear to clarify the nature of the mechanism of initiation of feather keratin proteins.

## D. DISCUSSION

### *1. Nature of the Proteins Synthesized in the 14-Day Lysate System*

The results from PAGE at the two different pH values, from DEAE-cellulose and Sephadex G-100 chromatography, and from the N-terminal dipeptide studies demonstrate that the major protein products of the lysate were identical by these criteria to embryonic feather keratin proteins. All radioactive protein species had optical density counterparts, as determined by each analytical technique. Thus it is concluded that all detectable protein products of the lysate were normal constituents of the fully-differentiated feather.

The specific activities of the different keratin protein bands resolved by PAGE at pH 7.5 and pH 2.7 were relatively constant in the case of proteins labelled with $^{14}$C-leucine or $^{14}$C-algal hydrolysate. This result suggests that the lysate synthesized the various different protein chains that constitute feather keratin at rates relative to each other resembling the relative rates *in vivo*. This evidence cannot be regarded as conclusive, however, as each band on the acrylamide gels is known to contain more than one

feather keratin protein (I.D. Walker; personal communication). In contrast, the specific activity of band β5 was higher than that of the other bands when the proteins were labelled with $^{3}$H-serine. As the dialysed lysate system was used in the case of the incorporation of $^{3}$H-serine but not of $^{14}$C-leucine or $^{14}$C-algal hydrolysate, the significance of the difference in results is not clear.

2. *Nature of the Proteins Synthesized in Lysates from 12- and 15-Day Feathers*

The spectrum of proteins synthesized in the 12-day feather lysate system was very different to those of the 14- or 15-day systems. In particular, the proteins in bands β2 - β5 accounted for only a minor part of the total synthesis. The results, although of a preliminary nature, indicate that in the 12-day feather lysate a similar spectrum of proteins was synthesized to that in the 12-day cultured feather system (Chapter 5). In contrast, the products of the 14- and 15-day lysate systems differered from those of the 14- and 15-day cultured feather systems, in that the rate of synthesis of keratin proteins in the lysate was far higher than that of the non-keratin proteins. The results from quantitative gel electrophoretic studies on the amount of keratin present at each embryonic age (Chapter 5), indicated that the synthesis of keratin proteins accounted for almost all net protein synthesis in feathers after day 12, and it was suggested that the observed patterns of labelling with $^{14}$C-leucine might arise in part from some effect such as

differences in pool sizes or permeability of cells in different states of maturity. The observation that keratins were the major protein products of the 14- and 15-day lysate systems would therefore appear to be consistent with the conclusion from quantitative PAGE studies on keratin synthesis (Chapter 5), namely that the synthesis of keratins accounts for the greater part of total protein synthesis in the embryonic feather after 12 days of development.

### 3. *Evidence for Initiation in the Lysate*

From the evidence that the N-terminal serine residue of keratin proteins was labelled in the lysate system, it is concluded that a large percentage of the protein chains completed and released in the system were also initiated in the system. At least two other interpretations, although considered unlikely, are nevertheless possible. One is that the keratins are synthesized in a "zymogen" form - i.e., they could have an N-terminal sequence which is cleaved from the finished products. Thus the serine residue isolated as the N-terminus could in fact be an internal residue of the nascent chains. Such a process does in fact occur in the synthesis of another fibrous protein, collagen (Lenaers *et al.*, 1971; Lapiere *et al.*, 1971). The polysomes on which the keratins are synthesized contain about 4 - 6 ribosomes however, (G.A. Partington, personal communication), which is about the size expected for proteins of MW 10,500 (Heywood *et al.*, 1968). It would therefore appear that if such a precursor does exist, its molecular weight could not be much

greater than that of the completed proteins.

The other possibility is that the acetyl-serine or a large peptide is added to newly synthesized precursor molecules by an enzymic, non-ribosomal, mechanism. A mechanism of this type has been suggested to account for the heterogeneity of the high-sulphur proteins from α-keratins (Gillespie, 1965), but recent evidence is not in support of this hypothesis (Steinert, 1972).

No evidence exists which supports either of these alternative hypotheses. Furthermore, the incorporation of $^{14}$C-leucine into protein in the lysate was inhibited by sodium fluoride and aurintricarboxylic acid (G.A. Partington, personal communication), which are known to be specific inhibitors of initiation (Lodish *et al.*, 1971; Hoerz and McCarty, 1971). This result supports the conclusion that initiation of keratin synthesis occurred in the 14-day feather lysate system.

The observation that Ac-($^{3}$H-Ser)-SCMC could be isolated from proteins synthesized in the lysate system also indicated that the presumed enzymic system responsible for N-acetylation of keratin proteins was functional in the lysate system. The incorporation of $^{14}$C-acetate, derived from acetyl-CoA, into the N-terminus confirmed this result, implicating acetyl-CoA as the *in vivo* acetyl donor, as could be expected (Nohara *et al.*, 1968; Allfrey *et al.*, 1966).

## 4. *Nature of the Initiator of Feather Keratin Synthesis*

Attempts to demonstrate the nature of the initiator of feather keratin synthesis did not give conclusive results, in part due to the failure to find suitable conditions for the accumulation of short nascent chains. It is possible that the products obtained in the presence of antibiotics which selectively inhibit translation were in fact related to the initiation of keratin synthesis, but were isolated in some chemically modified form. Such modification of the N-termini of nascent chains has been invoked (Hunter and Jackson, 1971) to explain the apparent involvement of N-formyl methionine in the initiation of globin synthesis observed by Yoshida *et al.* (1970). The present results could therefore be taken to suggest that methionine is in fact the initiator of keratin synthesis .

The possibility that Ac-aminoacyl-tRNAs are ever involved in the initiation of protein synthesis has been made remote by recent work in which methionyl $tRNA_F$ has been shown to be involved in the initiation of N-acetyl proteins (Straus *et al.*, 1971; Berns *et al.*, 1972). Thus it would appear that the acetyl moiety of such proteins is in fact a post-translational modification of the proteins, presumably catalyzed by enzymes able to specifically recognize certain N-terminal sequences. Demonstration of such an enzymic activity in the chick reticulocyte has been claimed (Marchis-Mouren and Lipmann, 1965) but refuted (Moss and Thompson, 1969). The stimulation by "de-acetylated keratin" of incorporation of

$^{14}$C-acetate from acetyl CoA into the N-termini of peptides would appear to constitute some evidence for the presence of such an enzyme in 14-day feather lysate. This result was not definitive, as an increase in Ac-Ser-SCMC was not demonstrated. Unsuitability of the substrate and broad specificity of the polypeptide acetylase could be invoked to explain the results, however.

# CHAPTER 7

# CONCLUDING DISCUSSION

## A. SALIENT FEATURES OF THE RESULTS

The aims of the work reported in this thesis were directed towards the eventual understanding of the control of keratin synthesis at the molecular level during development of the embryonic chick feather. Although the achievements to this end have been limited, it is considered that the results obtained will unquestionably facilitate the continuation of studies in this direction on the feather system. The most significant findings and some questions arising from these findings will be highlighted in the ensuing discussion.

### *1. Nature of the Embryonic Feather Keratin Proteins*

The results presented in Chapter 3 support and extent the earlier findings of Harrap and Woods (1964a,b; 1967) which indicated that feather keratin consists of a heterogeneous family of proteins.

Four major and different groups of keratinized tissues from the chicken, namely embryonic feathers and adult feather barbs, adult feather rachis and calamus, adult and embryonic scales, and embryonic feather sheaths can be conclusively distinguished because they each contain a distinct set of keratin proteins, unique to each group. The alternative possibility that different amounts of the *same* proteins occur in the different tissues is not acceptable on the evidence from gel electrophoresis. Among the feather

tissues examined, two distinct sets of proteins were apparent - the rachis-calamus pattern and the embryonic feather pattern. There is some qualification to these conclusions in that adult barbs are a mixture of barb- and rachis-type cells and thus contain the proteins unique to both the cell types involved. Further, scales and sheaths each contain a different set of proteins.

The results from N-terminal sequence studies (Chapter 4) confirmed and extended the results of O'Donnell (1971). The data demonstrated the presence of more than two different N-terminal tryptic peptide sequences, and therefore demonstrated that at least some of the observed heterogeneity of the feather keratin proteins arises from the presence of multiple genes for feather keratin proteins. However, the possibility that additional heterogenetiy results from post-translational modification of the proteins has not been excluded in the present studies.

Rogers (1959) postulated post-translational modification by an enzymic mechanism to account for the heterogeneity of the high-sulphur proteins of α-keratins, and this concept was extended by Gillespie (1965). It is now clear however that these earlier notions are untenable, and that the heterogeneity of α-keratins results from the presence of multiple genes, apparently derived by duplications and mutational divergence of (an) ancestral gene(s) (Haylett and Swart, 1969; Haylett *et al.*, 1971; Elleman, 1972) and the same situation would appear to occur in the case of feather keratin.

Recent work (I.D. Walker; personal communication) has shown that most embryonic feather keratin bands resolved by PAGE in the present work are themselves heterogeneous, and

a total of over 30 distinct components have been detected, each presumably reflecting the presence of a different gene. It is clear from the present work that some of the different feather type tissues examined so far contain different keratin proteins; if each of these sets of proteins are as grossly heterogeneous as the proteins from embryonic feathers, over 100 genes for feather keratin could conceivably be present in the genome. It is noteworthy that a different situation appears to prevail in the case of the proteins from emu rachis. Sequence analysis of a major component from this tissue indicated only very limited heterogeneity (I.J. O'Donnell; personal communication).

The biological significance of the observed heterogeneity of feather keratin proteins is somewhat difficult to assess. An attractive possibility is that the expression of a large number of genes in each cell is required to allow a total rate of keratin synthesis sufficient to fill the cell with keratin. The heterogeneity of feather keratin proteins could therefore be considered to be a genetic control of the rate of keratin synthesis, analogous to the presence of multiple genes for ribosomal RNA in higher organisms (reviewed by Maden, 1968). The precise number of different feather keratin proteins synthesized in any individual cell is not at present known, however. The present work would suggest at least 4-6 keratin proteins per cell type, while subsequent studies (I.D. Walker; personal communication) would suggest a minimum of 10.

In support of the above hypothesis, certain other

structural proteins exhibit marked heterogeneity, for example, the α-crystallins (Slingsby and Croft, 1972). On the other hand, muscle proteins do not appear to exhibit such marked heterogeneity (see for example Rees and Young, 1967; Woods, 1967). The major protein of muscle, myosin, has a polypeptide subunit of molecular weight around 200,000, however (Gazith *et al.*, 1969). Polysomes synthesizing myosin are about 10 times the size of those synthesizing feather keratin (Heywood *et al.*, 1967). If it is assumed that the rates of peptide bond formation for feather keratin and myosin are similar, one myosin mRNA molecule could therefore instruct protein synthesis at 10 times the rate of one feather keratin mRNA molecule. Less myosin mRNA than feather keratin mRNA would therefore be needed for the cells to synthesize the same mass of these proteins, and the presence of multiple genes for feather keratin would allow a greater total rate of feather keratin mRNA synthesis.

Mechanisms have been proposed for the activation of the same gene in different cell types (Britten and Davidson, 1968). In the case of feather keratin, this apparently does not happen. Why different feather keratin proteins with, presumably, similar quaternary structures and functional demands should be synthesized in the different tissues is not clear. Similar situations exist with other cell types however. For example, different haemoglobin chains are synthesized during the life of a human individual (Inman, 1965) and the light chains of myosin differ in different tissues (Sarkar *et al.*, 1971; Weeds and Pope, 1971).

2. *Keratin Synthesis During Embryonic Feather Development*

The studies described in Chapter 5 demonstrate that quantitative PAGE can be utilized as a suitable assay for feather keratin proteins during embryonic development, thereby achieving a major aim of the present work. It must be emphasized however, that the procedures do not measure the amounts of individual polypeptide chains. Considering the similarity of the various keratin proteins and the large number of these, it would appear that no currently available technique would be capable of quantitatively resolving all of them. Two dimensional PAGE (Kaltschmidt and Wittman, 1970; Martin and Gould, 1971) may provide an approach to the problem, but at the expense of simplicity of operation and ease of quantitation.

The results from all PAGE systems demonstrated that the relative amounts of all the major keratin bands remained approximately constant during development. As the techniques do not measure individual polypeptide chains, it is possible that changes in the relative amounts of different polypeptide chains within particular bands were not detected. As the basis of fractionation is different at the high and low pH values, it is considered unlikely that such undetected changes occur.

Malt and Bell (1965) had concluded that there was a two-phase synthesis of feather keratin during embryonic development. However, they did not identity the keratin proteins and the conclusions were reached in part by analogy with the synthesis of hair keratins. For hair keratins, there is

a clear increase in the relative rate of synthesis of high-sulphur matrix proteins to that of the low-sulphur fibrous proteins as the cells mature (Downes *et al.*, 1963, 1966; Fraser, 1969a,b).

It would appear from the present results that keratin synthesis follows different courses in hair and feather and that this difference is related to the differing structural organizations of α- and feather keratins. α-Keratin contains two distinct types of structural proteins and there is a difference in the time course of synthesis of these two components. Feather keratins on the other hand do not have this two-component structure, and there are no distinct phases in the synthesis of the closely related proteins present.

### 3. *Protein Synthesis in Cultured Feathers*

The results of Chapter 5 demonstrate that embryonic feathers maintained in culture for short periods continue to synthesize keratin. In particular, the spectrum of proteins synthesized reflected the stage of development of the tissue. Although many such systems have been available for the study of the synthesis of other proteins (for example, Kabat and Attardi, 1967; Reeder and Bell, 1967; Patton *et al.*, 1969; Berger and Kafatos, 1971) a comparable system has not previously been available for the study of keratin synthesis for the reasons discussed previously (Chapter 5.A). Bell (1964), Malt and Bell (1965) and Bell and Merrill (1967) had previously studied protein synthesis in cultured

feathers, but lacked an assay for keratins.

It should be realized that this system has been investigated in only a very preliminary manner in the present work. The influence of different culture media was not investigated and optimal culture conditions were not defined. It is well known that such factors as hormones, serum and embryo extract can have dramatic effects on the behaviour of cultured tissues and cells (for example, Coon, 1966; Cahn and Cahn, 1966; de la Haba and Amundsen, 1972). Recent studies (P. Gibbs; personal communication) have not so far revealed any great differences in the behaviour of 14-day feathers cultured in different media.

Whether cultured 12-day feathers can be enabled to develop to the stage of maximal keratin synthesis is perhaps the outstanding question bearing on the potential utility of the system. If this was found to be the case, the system would be eminently suited to the study of the control of the onset of keratin synthesis under experimentally manageable conditions. Systems in which maturation of tissues or cells *in vitro* occurs have been described, for example, muscle cells (Yaffe, 1968), erythroid cells (Wilt, 1965) and the embryonic mouse pancreas (Rutter *et al.*, 1967).

### 4. *Protein Synthesis in Feather Lysates*

Studies on the nature of the protein products synthesized in the 14-day lysate system (Chapter 6) demonstrated that the major products were keratins. Furthermore, the results demonstrated that a large proportion of these keratin

molecules were initiated and also acetylated in the system. The polysomes present in the lysate therefore must contain functional mRNAs for keratin proteins and it should therefore be possible to isolate the mRNAs. In a number of comparable instances the isolation of mRNAs and their translation in heterologous cell-free systems has been achieved (for example, Lockard and Lingrel, 1969; Heywood, 1970; Nienhuis *et al.*, 1971; Pemberton *et al* ., 1972; Means *et al.*, 1972; Matthews *et al.*, 1972).

The availability of purified mRNA preparations provides a potentially powerful tool for the study of control mechanisms, utilizing the technique of nucleic acid hybridization (see, for example, Davidson and Hough, 1971; Birnstiel *et al.*, 1972; Melli *et al.*, 1971). The potential of this approach has been greatly extended by the recent use of RNA dependent DNA polymerase to obtain complementary copies of the mRNAs (Verma *et al.*, 1972; Kacian *et al.*, 1972). Isolation of the mRNAs for feather keratin proteins would therefore provide a valuable approach to the study of the control of keratin synthesis during embryonic development.

As the lysate system was shown to be active in the initiation of keratin synthesis, the system is now directly amenable to the investigation of possible controls of keratin synthesis acting at the level of translation, as mentioned earlier. The possible involvement of acetyl-seryl-tRNA in a specific initiation mechanism for keratin proteins was investigated in the present work. The nature of the initiator of keratin synthesis was not successfully demonstrated.

However, the addition of a partial acid hydrolysate of keratin proteins to the lysate resulted in a stimulation of incorporation of $^{14}$C-acetyl from $^{14}$C-acetyl-CoA to form blocked peptides. This observation constitutes evidence for the presence of an enzyme of broad specificity in the lysate capable of N-acetylating proteins at the post-translational level. Although the evidence is far from definitive, it is in agreement with the recent observations of Straus *et al.*, (1971), who demonstrated that the N-acetylation of α-crystallins must occur at the post-translational level.

It was observed that the 12-day lysate system was not as active as the 14-day system, and that only a small portion of the total protein synthesized appeared to be keratin. It would therefore be of interest to examine the reasons for the relative inactivity of the 12-day system. Possible explanations for the relative inactivity could include a dearth of mRNA or of specific initiation factors or tRNAs. In a comparable system, Ilan and Ilan (1968) have presented evidence that lack of (a) specific tRNA(s) is the factor limiting synthesis of a specific protein rather than lack of the mRNA for this protein. Developmental stage specific initiation factors have also been found (Ilan and Ilan, 1971). Such a result in the present system would be of obvious interest with regard to the question of whether or not keratin mRNA is stored in an inactive form for some time before it is utilized.

If it is found that heterologous systems can translate feather keratin mRNA, it would be possible to

approach this question directly (Rosenfeld *et al.*, 1972).

## B. A CRITICAL EVALUATION OF THE DEVELOPING EMBRYONIC FEATHER SYSTEM

Within the context of the aims of the project, it is relevant to consider the potential of the system at the present stage for the study of the control of protein synthesis as compared with the potential of other systems.

Obvious disadvantages of the feather system include the facts that embryonic feathers are composed of several cell types, not precisely phased in development and synthesisizing a large number of different keratin proteins. The situation contrasts with, for example, the avian erythroid system, where populations of one cell type, well-phased in development, and synthesizing a very limited number of gene products are available (Williams, 1971). On the other hand, the cell types and gene products of the feather are closely related and it would seem reasonable to assume that the control of synthesis of the different gene products in the different cells are equally closely related. Although the cells are not precisely phased in development, the bulk population of cells in the 11- and 12-day feather are vastly different in developmental stage to those in the 14-day feather.

It is also relevant to ask whether the really important events in control of protein synthesis in the developing feather occur at stages before the tissue is amenable to study by the procedures established in the present work. As was indicated earlier, it can be presumed that the

inductive events mediated by dermis earlier in development are obligatory steps in the succession of events by which the genes for keratin synthesis are eventually activated. Holtzer (1970) however, has developed the concept of a "quantal mitosis" being an important fundamental control point in the pathway of differentiation. It would appear likely from the present results that many of the feather cells proceed through such a step at around 12 days, and therefore, that the molecular events occurring in this step are amenable to study. Demonstration of the development of feathers in culture through this stage until the stage of maximal keratin synthesis is the outstanding technical necessity in this area.

For the effective study of control phenomena, a stimulus/response situation is preferable. An outstanding recent achievement in this field, at the molecular level, is that of Rosenfeld *et al.*, (1972). A quantitative response in ovalbumin mRNA synthesis to hormonal stimulation of the immature chick oviduct was observed. The work of Yatvin (1966a,b) has demonstrated a reversible response in feather development to hypophysectomy. Feather growth appeared relatively normal, but keratin synthesis (as evidenced by polysome profiles) was inhibited in the hypophysectomized embryos. Administration of pituatory hormones apparently restored keratin synthesis towards normal. It is possible that this approach may fulfill the need for a stimulus/response experimental system for the study of the control of keratin synthesis, at both the transcriptional and translational

levels.

The potential advantages of studying control phenomena in higher systems amenable to fine-structural genetic analysis have been pointed out (for example, Lederberg, 1966; Watson, 1970; Scheiderman and Bryant, 1971). The feather system does not have this advantage. Furthermore, the tendency to diversify even further the number of higher cell control systems under study rather than to direct the weight of effort to a particular system has been criticized (Lederberg, 1966; Watson, 1970).

It is concluded that the feature of the system which particularly justifies further study is the immediate potentiality of isolating the mRNAs for feather keratin proteins and using such preparations as tools for the analysis of control mechanisms.

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# APPENDICES

## APPENDIX A. PREPARATION AND INCUBATION OF FEATHER LYSATES.

Feathers were plucked as described in Chapter 4 at room temperature and placed in a solution containing 200 mM KCl, 10 mM Tris/HCl and 5.3 mM $MgCl_2$ pH 7.4, at 0°. The feathers were washed 4 times in the same solution by quickly pelleting and resuspending, collected by centrifugation at 2,200 g for 5 min and the supernatant discarded. The feathers were then allowed to stand for 10 min at 0° in 2 volumes of the same solution, containing in addition DTT at concentration of 4 mM. The feathers were homogenized in a 2 ml hand-held Potter-Elvejhem homogenizer, generally using 6-7 strokes. The homogenate was centrifuged at 10,200 g for 10 min at 0° and the supernatant collected. All operations after plucking the feathers were carried out in a cold-room at 2-4°, with the feathers standing on ice where possible.

The incubation mixture for cell-free protein synthesis ("lysate") contained 0.75 volumes of the supernatant fraction prepared as described above. In addition, the mixture contained amino acids (0.01 mM) ATP (1.0 mM), GTP (0.25 mM) $MgCl_2$ (4 mM), phospho-creatine (15 mM) and creatine kinase (100 µg/ml). The final concentrations of Tris and KCl were 10 mM and 150 mM respectively. The amount and nature of radioactive precursor used for each experiment is described in the captions to figures. The mixture was incubated at 37° for the stated times.

In experiments in which $^3$H-serine was used as the radioactive precursor, the supernatant fraction of the feather homogenate was dialyzed against the buffer containing

DTT at 0° for 2.5 hr, with a change of buffer after 1.5 hr, in order to lower the large pool of serine present. The incubation mixture prepared from this dialyzed supernatant is referred to as "dialyzed lysate" in the text.

# APPENDIX B. PUBLICATIONS.

## 1. PAPERS PUBLISHED.

(a) Describing Studies Not Included in this Thesis.

Immunological and Immunofluorescent Studies on Keratin of the Hair Follicle. (with G.E. Rogers)
J. Cell. Sci. 7, 273 (1970).

(b) Describing Studies Included in this Thesis.

Differentiation of Avian Keratinocytes.
Characterization and Relationships of the Keratin Proteins of Adult and Embryonic Feathers and Scales.
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