# THE CHEMISTRY OF PROTEINS CONTAINING CITRULLINE

A thesis submitted by

HARRY WILLIAM JOHN HARDING, B.Sc.(HONS.),

to the University of Adelaide, South Australia,

for the degree of

Doctor of Philosophy.

Department of Biochemistry,

University of Adelaide,

South Australia.

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# CONTENTS

# SUMMARY

The thesis describes studies on the chemistry of the proteins that are intracellular products in the cells of the medulla tissue of hairs and in the cells of the inner root sheaths of hair follicles. These proteins are characterized by their extraordinary insolubility in protein solvents and their unique content of citrulline in peptide linkage. The medulla and inner root sheath proteins are distinct from α-keratin which is distinguished by its high content of disulphide cross-linkages that are absent in the citrulline-containing proteins. The investigations were aimed at explaining this insolubility and its possible relationship to the presence of citrulline.

The presence of ε-(γ-glutamyl)lysine as a cross-link in guinea pig hair medulla protein has been demonstrated by a study of tryptic peptides, and this isodipeptide has been isolated from enzyme digests by ion-exchange chromatography and characterized. Measurement of 'blocked' lysine residues by a chemical method using acrylonitrile indicated that all lysine residues that are cross-linked are involved in the ε-(γ-glutamyl)lysine linkage. The cross-link was localized in citrulline-containing protein by isolation using ion-exchange and paper chromatography of peptides containing both the cross-link and citrulline residues. The cross-link occurs in hair medulla cells from a wide range of mammalian species. It is postulated that this cross-link explains the insolubility of these proteins.

Transamidases capable of forming the ε-(γ-glutamyl)lysine cross-link have been demonstrated in hair follicle homogenates. The follicle enzymes have very similar properties to those of plasma transamidase, the enzyme which insolubilizes fibrin by the formation of the same cross-link. However, the follicle enzymes can be distinguished from plasma transamidase by their enzymic and immunological properties.

## 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 when due reference is made in the text.

Signed:

Harry W.J. Harding.

# ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

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# ABBREVIATIONS

Abbreviations used in this thesis are as follows:

| | |
|---|---|
| CBZ- | carbobenzoxy- |
| DNS-(dansyl) | 1-dimethylaminonaphthalene-5-sulphonyl- |
| GEE | glycine ethyl ester |
| MW | molecular weight |
| n.d. | not determined |
| SCM- | S-carboxymethyl- |
| TCA | trichloroacetic acid |
| TPCK | L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone |

# ENZYMES

For convenience, enzymes are referred to by their trivial names throughout this thesis. They are listed by the following numbers in the *Report of the Commission on Enzymes* of the International Union of Biochemistry (1964).

| | | |
|---|---|---|
| Leucine aminopeptidase | : | 3.4.1.1 |
| Aminopeptidase M | : | 3.4.1.group |
| Carboxypeptidase A | : | 3.4.2.1 |
| Carboxypeptidase B | : | 3.4.2.2 |
| Pepsin | : | 3.4.4.1 |
| Trypsin | : | 3.4.4.4 |
| Chymotrypsin | : | 3.4.4.5 |
| Papain | : | 3.4.4.10 |
| Thrombin | : | 3.4.4.13 |
| Thermolysin | : | 3.4.4.group |
| Pronase | : | - |

CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 OCCURRENCE OF PROTEINS CONTAINING CITRULLINE

Citrulline occurs as the free amino acid in many plant and animal tissues (Greenstein and Winitz, 1961). In mammals, it is an important intermediate in urea biosynthesis (Krebs, 1964). It is not, however, commonly found as a constituent of proteins although it has been detected in simple peptides from the algae *Chondrus crispus* (Young and Smith, 1958). There have been several reports of its occurrence in proteins (Wada, 1930; Fearon, 1939; Orekhovitch, 1955) but these have not been confirmed. Recently, the presence of citrulline in a protein fraction isolated from normal human myelin has been demonstrated (Finch *et al.*, 1971).

The finding of citrulline in the protein of inner root sheath cells of hair follicles (Rogers and Simmonds, 1958; Rogers, 1958a, 1959) and in the medulla cells of hair and porcupine quill (Rogers, 1962) therefore established these proteins as unique. Bradbury and O'Shea (1969) have also demonstrated the presence of citrulline in the medulla protein of hairs from a number of species. Citrulline has been reported to be also present in the cuticle of wool (Bradbury *et al.*, 1966) and in the tissue of certain carcinomas which derive from hair follicle tissue (Holmes, 1968; Holmes *et al.*, 1968).

Citrulline in hair proteins was originally identified by paper chromatography (Rogers, 1958a) and later by ion-exchange chromatography (Rogers, 1962, 1964a). Its identity was unequivocally established by isolation of the amino acid from acid hydrolysates of porcupine quill medulla protein (Rogers, 1964a) and from guinea pig inner root sheath protein (Steinert *et al.*, 1969). The initial indications that the citrulline was covalently bound in peptide linkage in the proteins (Rogers and Simmonds, 1958; Rogers, 1958a, 1962) were confirmed by limited sequence analysis of

peptides derived from porcupine quill medulla (H.W.J. Harding, 1967; Steinert *et al.*, 1969).

In the present study the major citrulline-containing protein investigated was the protein from medulla cells of guinea pig hair, although proteins from inner root sheath cells of guinea pig hair follicles and from medulla cells of hairs and quills from a number of mammalian species were considered when appropriate. Rogers (1964a,b) has proposed that hair proteins containing citrulline be called 'citrulline-trichohyalin', and that the arginine-rich material of trichohyalin droplets be called 'arginine-trichohyalin'. However, this terminology has not come into general use.

Hair proteins containing citrulline can be distinguished from keratin by several criteria. They have a distinctive amino acid composition in that not only do they contain citrulline but also large amounts of glutamic acid (Rogers, 1962, 1964a). Early studies showed that the cystine-cysteine content was very low or nil (Barritt and King, 1931; Bekker and King, 1931; Jordan-Lloyd and Marriott, 1933; Blackburn, 1948) and these have been confirmed using more refined techniques (Rogers, 1962; Steinert *et al.*, 1969). However, reports that hair medulla protein contained a large amount of aromatic amino acids, especially tyrosine (Stoves, 1943; Matoltsy, 1953) have proved incorrect (Rogers, 1962, 1964a; Steinert *et al.*, 1969). The citrulline-containing proteins are readily digested by trypsin but they are insoluble in the usual protein solvents (Stoves, 1943, 1945; Rogers, 1962, 1964a) including those that dissolve keratin (Jordan-Lloyd and Marriott, 1933; Matoltsy, 1953). In addition, hair medulla protein does not react with antibodies prepared to hair keratin derivatives (Kemp and Rogers, 1970).

The extreme insolubility of the hair proteins that contain citrulline has hindered study of them (Rogers, 1964a) and has necessitated the use of tryptic peptides as soluble material for most investigations (Rogers, 1962, 1964a; Steinert *et al.*, 1969). The peptide mixture obtained when the proteins are solubilized by tryptic digestion is very complex and this has precluded determination of the number and size of citrulline-containing proteins present in hair tissue.

## 1.2 ORIGIN AND SIGNIFICANCE OF CITRULLINE IN PROTEINS

The presence of citrulline in proteins immediately raises two questions: (1) how does the citrulline become incorporated into protein, and (2) what is the function of citrulline in protein?

The first question is particularly interesting because no DNA triplet code is known to exist for citrulline (Crick, 1966; Jukes and Gatlin, 1971) and it therefore appears unlikely that it is incorporated directly by the usual protein synthesizing systems as described by Schweet and Heintz (1966). Furthermore, there is evidence (Rogers, 1964a) to suggest that citrulline cannot be activated for protein synthesis. It has therefore been suggested that protein-bound citrulline arises by conversion of another amino acid already incorporated into protein . Desimination of arginine has been proposed as the conversion mechanism because of the similarity in the chemical structure of arginine and citrulline (Rogers and Simmonds, 1958; Rogers, 1958a, 1959, 1963, 1964a). Rogers (1963, 1964a) has proposed that the $NH_3$ released by such desimination could be involved in the amidation of glutamic acid or aspartic acid residues or both. These residues could be located in either the trichohyalin protein or adjacent keratin. In addition, histochemical studies of hair follicle sections have indicated a precursor-product

relationship between the arginine-rich trichohyalin proteins and the citrulline-containing hardened protein into which it is transformed (Rogers, 1963).

Initial *in vivo* studies of the origin of protein-bound citrulline used [U-$^{14}$C]arginine. The interpretation of these experiments was complicated by the presence in follicles of an active urea cycle (Rogers, 1964a) which can convert free arginine to ornithine and then to citrulline (Krebs and Henseleit, 1932). However, further studies with [guanido-$^{14}$C]arginine (Allen *et al.*, 1964; Rogers, 1964a; Clarke, 1967) have overcome this problem since the urea cycle is not reversible *in vivo* (Krebs, 1964) and have indicated that protein-bound citrulline indeed arises from protein-bound arginine.

Clarke (1967) further showed that although [$^{14}$C]arginine can be detected in inner root sheath protein in less than 1 hr after intracardial injection of guinea pigs with [$^{14}$C]arginine, [$^{14}$C]citrulline was not detectable until after about 2 hr. In addition, puromycin injected 95 min after injection of the radioactive arginine largely stopped arginine incorporation and general protein synthesis, but had no effect on the formation of citrulline which still appeared at 2 hr after the original injection and increased to the expected level. The puromycin effect, together with the lag period before the appearance of citrulline, strongly suggests that arginine is first incorporated into polypeptides before it is converted to citrulline.

In this respect, the situation is analogous to the formation of hydroxylysine and hydroxyproline in collagen (Peterkofsky and Udenfriend, 1963; Juva and Prockop, 1964; Udenfriend, 1966). In the biosynthesis of this protein, lysine and proline are first incorporated into polypeptides of 10,000 - 100,000 molecular weight before they are enzymically hydroxylated

(Bhatnagar *et al.*, 1967; Rosenbloom *et al.*, 1967; Bachra and van der Eb, 1970; Miller and Udenfriend, 1970). The enzyme responsible for the conversion of proline, collagen proline hydroxylase, has been isolated from several sources and characterized (Kivirikko and Prockop, 1967; Popenoe *et al.*, 1969; Rhoads and Udenfriend, 1970; Olsen *et al.*, 1970). However, it has not been possible to demonstrate desiminase activity in hair follicle tissue *in vitro* (Rogers and Springell, 1959; Allen *et al.*, 1964; Campbell, 1965). Furthermore, it is not known if protein-bound arginine residues can participate in such reactions, since the desiminases that have been studied have utilized only free L-arginine (Petrak *et al.*, 1957).

The function of citrulline in proteins is not known. The histochemical results of Rogers (1963) indicate that the formation of citrulline is intimately involved with the fibrous transformation of trichohyalin proteins (Rogers, 1963, 1964a,b). If desimination and amidation occurred within one protein, there would be no net change in charge of the protein involved (Rogers, 1963). However, changes in conformation of the protein could be expected because possibilities for hydrogen-bonding within the protein would be increased (Rogers and Simmonds, 1958; Rogers, 1963).

## 1.3 DEVELOPMENT AND STRUCTURE OF HAIR MEDULLA AND INNER ROOT SHEATH

The tissues of particular interest in the present study of proteins containing citrulline are hair medulla and the inner root sheath of hair follicles. The relationship of these tissues to the hair fibre is shown diagrammatically in Figure 1-1. The development and structure of medulla and inner root sheath tissue is briefly described below.

*a. Medulla.* The development of hair medulla cells is illustrated by the series of electron micrographs in Figure 1-2. Medulla cells arise from the undifferentiated matrix cells just above the papilla of the hair bulb

(Auber, 1950; Roth and Helwig, 1964; Parakkal and Matoltsy, 1964) and become the central core of cells of the hair shaft (Figure 1-1). The matrix cells contain the usual cytoplasmic organelles such as mitochondria and Golgi apparatus (Roth and Helwig, 1964; Parakkal and Matoltsy, 1964). At a level about 3 - 4 cells above the papilla (level 2, Figure 1-1) the ascending matrix cells begin obvious differentiation into medulla cells with the appearance of characteristic electron-dense, amorphous granules as shown in Figures 1-2b and 1-3a. These granules are of varying size and are not bound by membrane (Roth and Helwig, 1964; Parakkal and Matoltsy, 1964). They stain strongly for arginine (Rogers, 1963) and are considered to be trichohyalin droplets (Rogers, 1964a) although they are not identical with those of inner root sheath cells (Parakkal and Matoltsy, 1964; Parakkal, 1969). Filaments arise from desmosomes but are not necessarily associated with the granules (Roth and Helwig, 1964; Parakkal, 1969).

At later stages of development (Figure 1-1, level 3) the number and size of the granules increases (Figures 1-2c and 1-3) and large amounts of glycogen are sometimes seen in the cells (Roth and Helwig, 1964). The mitochondria swell and vacuolate and vesicles appear in the cytoplasm (Figure 1-3). The nuclei degenerate and the granules tend to become irregular and coalesce (Figures 1-2d,e and 1-3c) (Roth and Helwig, 1964; Parakkal and Matoltsy, 1964; Parakkal, 1969).

In a sudden transformation, from one cell to the one above, the granules fuse giving rise to electron-dense material showing a generally granular substructure (Figure 1-2f and 1-3d) (Auber, 1950; Roth and Helwig, 1964; Parakkal and Matoltsy, 1964) which stains for citrulline (Rogers, 1963). The hardened protein in medulla cells is not overtly filamentous in contrast

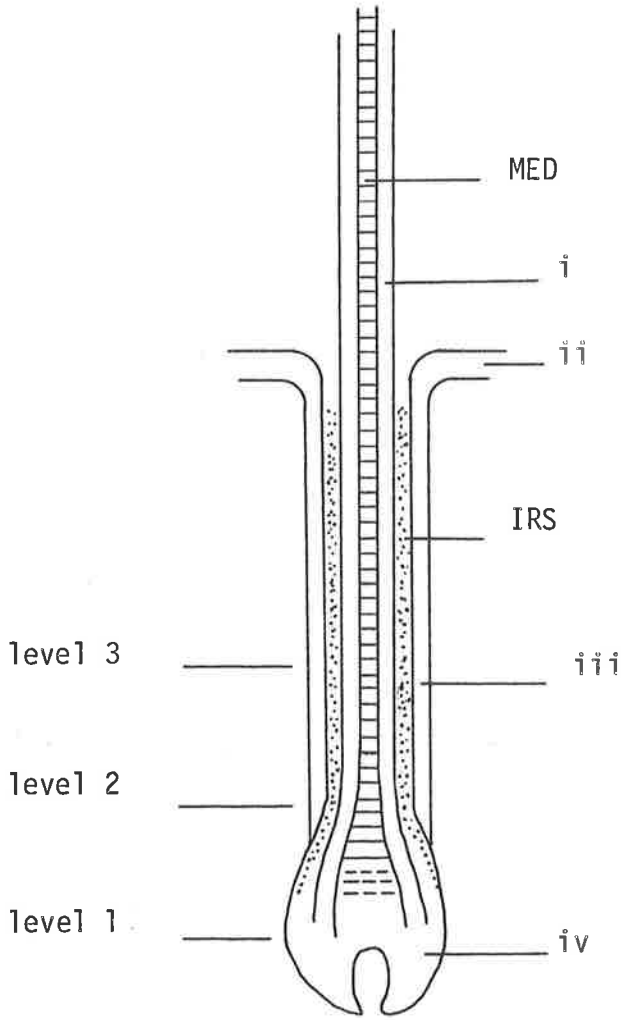



FIGURE 1-1: Diagrammatic representation of a hair follicle and emergent hair indicating the location of the cellular parts of the structure that were used in the present study: (MED) medulla layer of the hair (originates in the follicle itself), (IRS) inner root sheath (present only in the follicle). i, ii, iii, and iv respectively indicate the cortex of the hair, the epidermis of the skin, the follicle outer root sheath and the follicle bulb. Levels 1 - 3 are positions chosen in this laboratory to aid description of follicle development.

FIGURE 1-2: Series of electron micrographs of longitudinal sections of guinea pig hair follicle tissue illustrating the development of the medulla. Hair growth is in an upwards direction (a - f). Note the appearance of granules (G) and large nuclei (N) in the matrix cells (level 1;a). As development proceeds (levels 2 and 3;b;c;d), the granules increase in size and number and intracellular vesicles (IV) are seen. The granules coalesce (e) with the formation of intercellular spaces (IC) and are finally transformed into amorphous material (AM) (f). Keratin fibrils (K) can be seen in adjacent cortical cells. Stained with uranyl acetate and lead citrate.

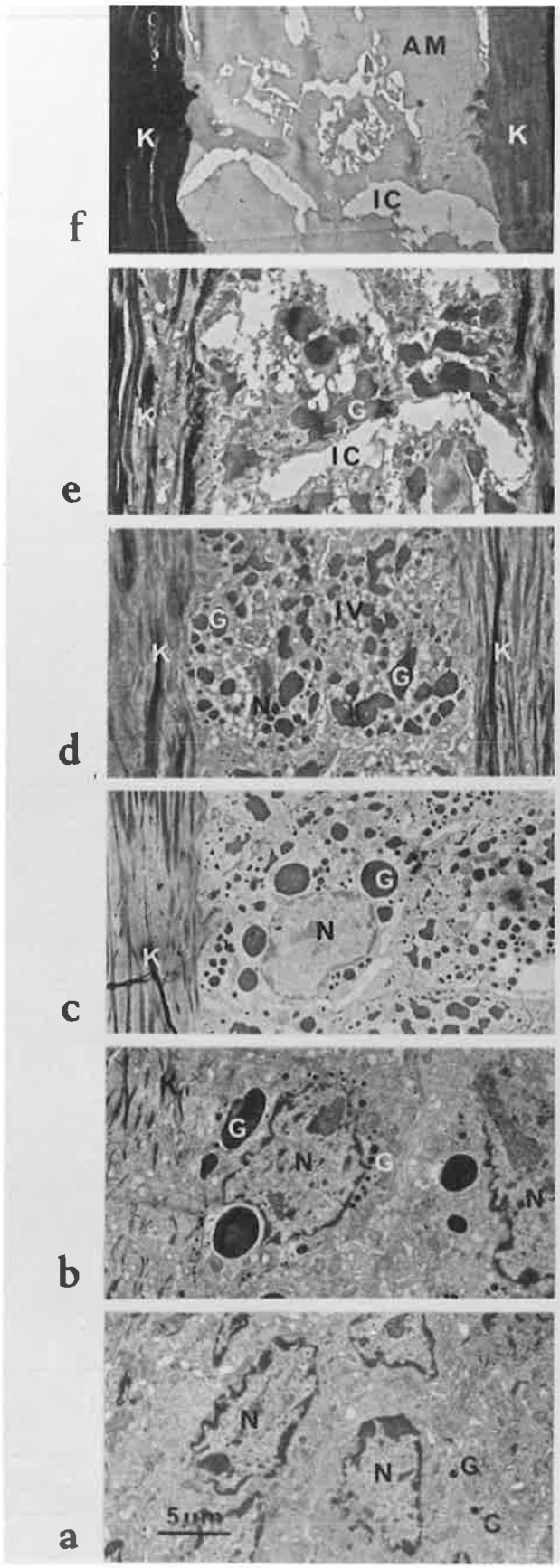
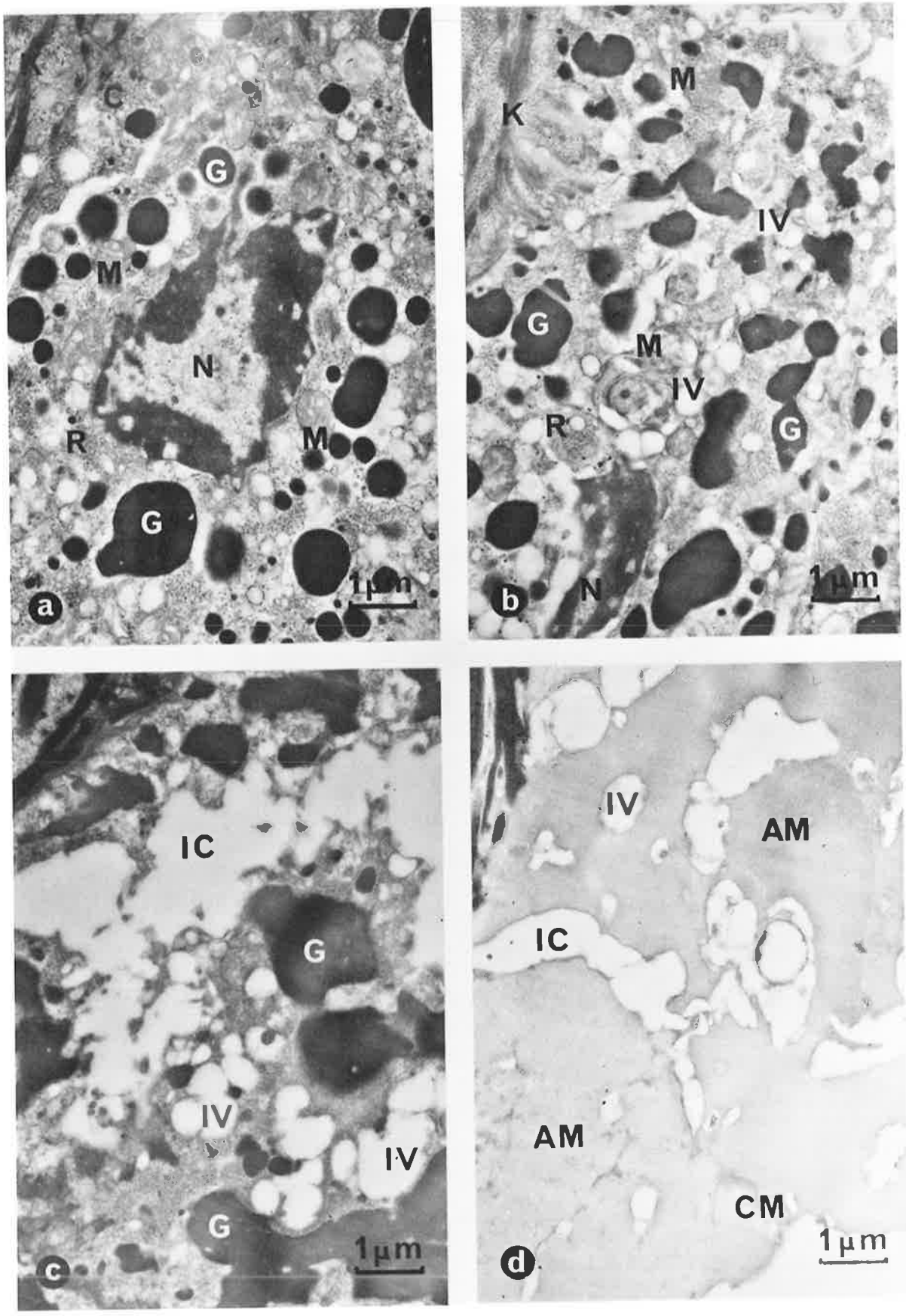

f
AM
K
K
IC
e
K
G
IC
d
G
IV
K
K
G
N
level 3
c
G
N
K
b
G
N
G
N
level 2
a
N
N
G
G
5μm
level 1

FIGURE 1-3: (a) Electron micrograph shows guinea pig hair medulla cells at an early stage of differentiation. Note the presence of granules (G) of various sizes, the large nucleus (N), mitochondria (M) and ribosomes (R). Keratin fibrils (K) are present in adjacent cortical cells (C). Stained with uranyl acetate and lead citrate.

(b) Electron micrograph of medulla cells at a later stage of development than in (a). The granules (G) have coalesced and intracellular vesicles (IV) have formed in the cytoplasm. The mitochondria (M) and nuclei (N) have begun to degenerate. Keratin fibrils (K) are present in adjacent cortical cells. Stained with uranyl acetate and lead citrate.

(c) Electron micrograph shows a guinea pig hair medulla cell in an advanced stage of differentiation. The granules (G) are very large and intercellular spaces (IC) have appeared as well as the intracellular vesicles (IV). Longitundal section, stained with uranyl acetate and lead citrate.

(d) Electron micrograph of mature medulla cells showing hardened amorphous material (AM). Note the cell membranes (CM). Hardened keratin (K) can be seen in an adjoining cortical cell. Longtidunal section, stained with uranyl acetate and lead citrate.


C
G
M
N
R
M
G
1µm
a
K
M
IV
G
M
IV
R
G
N
1µm
b
IC
G
IV
IV
G
1µm
c
IV
AM
IC
AM
CM
1µm
d

to the mature inner root sheath protein (Roth and Helwig, 1964; Parakkal and Matoltsy, 1964). On the other hand, it is not entirely amorphous since some filaments are occasionally seen (Rogers, 1964a). The protein generally shows a β-type x-ray diffraction pattern (Stoves, 1945; Rudall, 1955).

When the granules fuse large intercellular spaces form (Figure 1-3) and in some instances the cell contents tend to concentrate at the cell periphery (Auber, 1950; Roth and Helwig, 1964; Parakkal and Matoltsy, 1964). Thus the medulla has an open, yet rigid structure, producing a firm fibre whilst keeping its mass at a minimum (Auber, 1950; Mercer, 1961). Not all hairs are medullated; even when this tissue is present, the ratio of medulla to cortex is extremely variable (Auber, 1950).

Wildman (1955) has classified medullae into two main types, *viz.*, continuous, and broken. In the broken type the medulla may be broken transversely at irregular intervals by cortical material *(interrupted medullae)* or may only be present irregularly in very small amounts *(fragmented medullae)*. In addition, medullae present in up to three separate columns of cells *(multiple medullae)* have been reported for wool fibres (Jones, 1962; Doney and Smith, 1962) and human hair (Chowdhuri and Bhattacharyya, 1965).

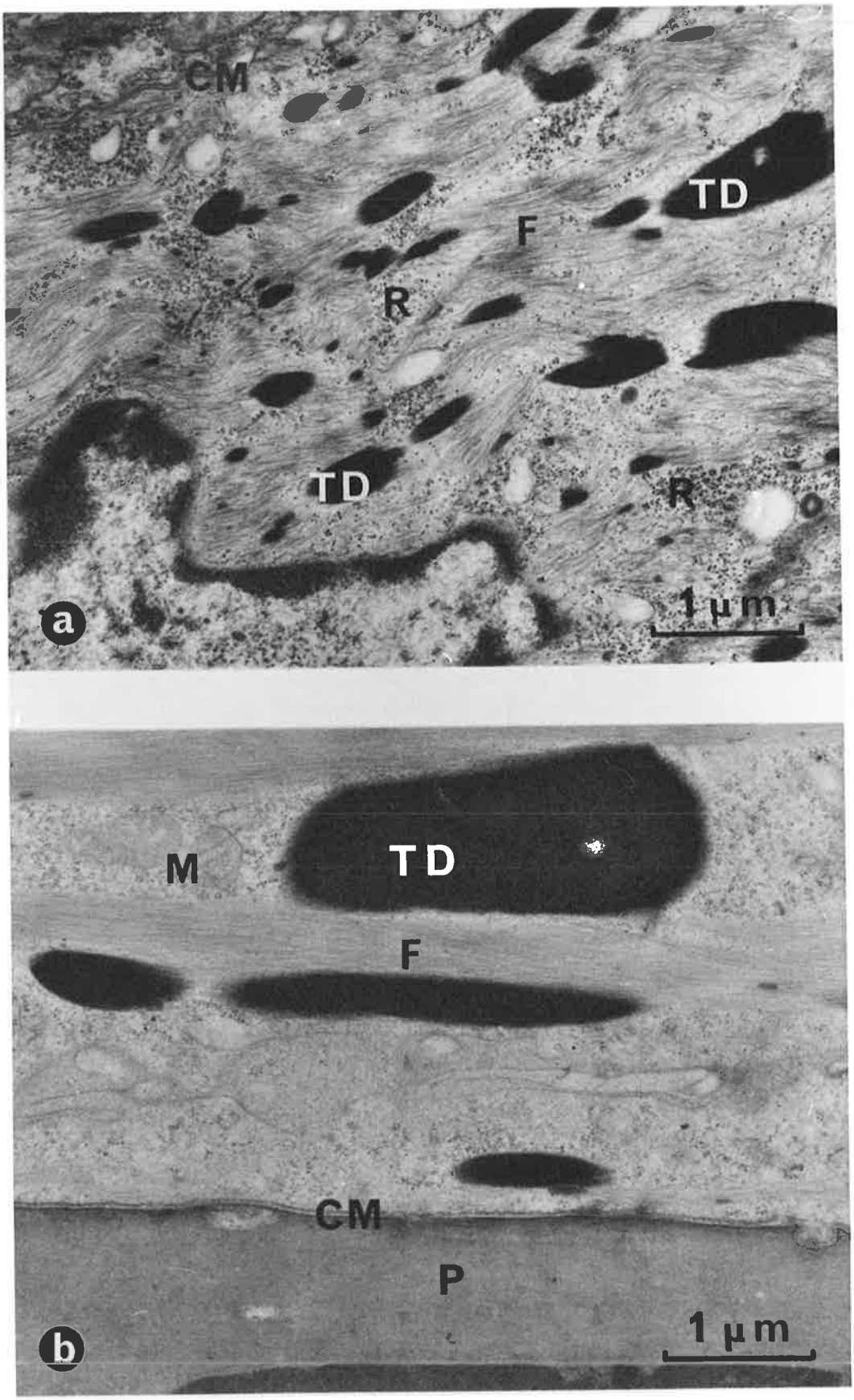
*b. Inner Root Sheath.* The inner root sheath of hair follicles (Figure 1-1) consists of three concentric cell layers. These layers are termed, in periphero-axial order, *Henle's layer, Huxley's layer* and *cuticle of the inner root sheath (*Auber, 1950).

As matrix cells differentiate, trichohyalin droplets appear in the cytoplasm (Figure 1-4a). The droplets are structureless and do not have a retaining membrane (Birbeck and Mercer, 1957). They stain strongly for arginine (Rogers, 1963). Filaments are also found in the cytoplasm at this

FIGURE 1-4: Cells from the inner root sheath of guinea pig hair follicles.

(a) Electron micrograph shows an inner root sheath cell at an early stage of differentiation. Filaments (F) and trichohyalin droplets (TD) are seen in close association. Ribosomes (R) are also present. Longitudinal section, stained with uranyl acetate and lead citrate.

(b) Electron micrograph of a longitudinal axial section of inner root sheath. A cell from the Henle layer (bottom) is already filled with mature, hardened fibrous protein (P). Cells from the Huxley layer and inner root sheath cuticle are in a late stage of differentiation. The trichohyalin droplets (TD) are enlarged and the filaments (F) are organized in the direction of the hair axis (left to right). Note the mitochondria (M) and cell membranes (CM). Stained with uranyl acetate and lead citrate.


CM
TD
F
R
TD
R
1 µm
a
M
TD
F
CM
P
1 µm
b

stage and appear to be associated with the trichohyalin droplets (Rogers, 1958b, 1964a,b; Parakkal and Matoltsy, 1964).

As the cells develop and move up the follicle, the trichohyalin droplets increase in size and number and lose their spheroidal shape (Rogers, 1964a). The number of filaments increases and they become more organized (Figure 1-4b). The droplets aggregate and during the final stage of development they disappear and the cells become filled with protein filaments oriented parallel to the fibre axis (Figure 1-5a) (Birbeck and Mercer, 1957; Rogers, 1959, 1964a,b; Parakkal and Matoltsy, 1964). The appearance of the final filamentous product (Figure 1-5a,b) is very rapid, occurring from one cell to the next (Figure 1-5a), and has been described as 'crystallization' of protein chains from the amorphous trichohyalin (Birbeck and Mercer, 1957). The cells are seen to become birefringent (Auber, 1950; Rogers, 1958a, 1959) and stain for citrulline (Rogers, 1963). The hardened fibrous protein gives an $\alpha$-type x-ray diffraction pattern (Rogers, 1959, 1964b). Recently, Steinert *et al.* (1971) have isolated protein filaments (Figure 1-5c) from the inner root sheaths of guinea pig hair follicles by a brief tryptic digestion and shown that the protein contains citrulline.

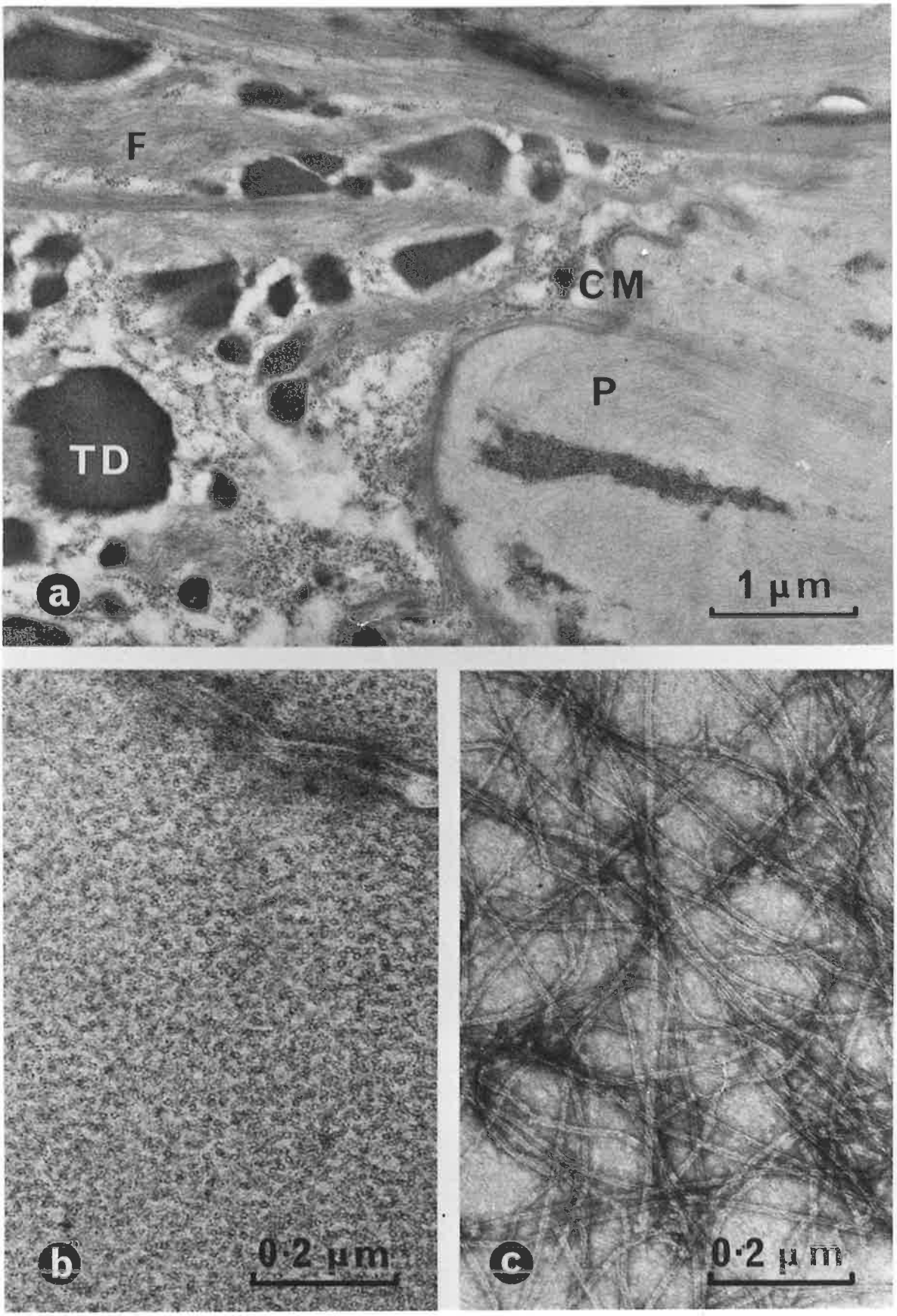
In Henle's layer, the transformation into hardened fibrous protein occurs just below the level of the constriction of the bulb (level 1, Figure 1-1), forming a hardened cylinder which acts as a 'retaining mould' for the softer tissue within (Birbeck and Mercer, 1957). The conversion to hardened material is slower and occurs higher up the follicle (level 2, Figure 1-1) in the other cell layers (Birbeck and Mercer, 1957). During further maturation, the filaments coalesce and harden further (Rogers, 1958b) and the cells fuse, presenting a homogenous appearance (Birbeck and Mercer, 1957; Rogers, 1958b).

FIGURE 1-5: (a) Electron micrograph of portion of two adjacent cells from the Henle layer of the inner root sheath of a guinea pig hair follicle showing the sudden transformation of the trichohyalin droplets (TD) and filaments (F) in the immature cell (left) into hardened fibrous protein (P) in the mature cell (right). Note the cell membrane (CM). Direction of hair growth is left to right. Stained with uranyl acetate and lead citrate.

(b) Electron micrograph of a cross-section through a cell of the Henle layer showing the hollow tubular structure of the filaments. The filaments are approximately 80 Å in diameter. The inter-filamentous regions are of very low electron density. Stained with potassium permanganate.

(c) Inner root sheath filaments prepared from whole guinea pig follicle tissue by digestion with Difco trypsin for 15 min at 20°. The majority of filaments are about 80 Å in diameter and are more than 1 micron long. They show a dense core throughout their length. Negatively stained with 2% uranyl acetate.

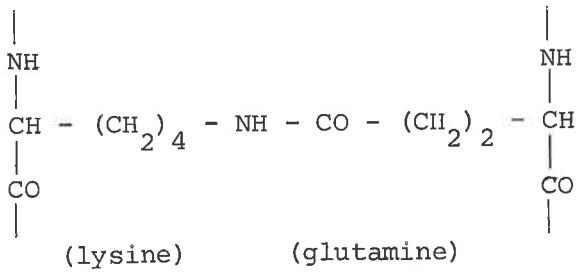
Figures 1-6(b) and (c) are by courtesy of Steinert *et al.* (1971).


F
CM
P
TD
a
1 µm
b
0·2 µm
c
0·2 µm

Thus, the process of development is similar in medulla and inner root sheath cells, but differences between the tissues are apparent, particularly in the nature of the trichohyalin droplets (Parakkal and Matoltsy, 1964; Parakkal, 1969) and in the morphologically distinct final products.

## 1.4 OCCURRENCE OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK

This thesis reports the finding of ε-(γ-glutamyl)lysine as a cross-link in citrulline-containing proteins from hair. The cross-link results from the covalent linkage of the ε-amino group of a lysine residue to the γ-carboxyamide group of a glutamine residue by an isopeptide bond.




The ε-(γ-glutamyl)lysine cross-link is widely distributed in nature. It has been detected from such diverse sources as *Escherichia coli*, *Physarum polycephalum*, chick embryo breast muscle (Loewy and Matačić, 1970) and insoluble fibrin from blood plasma (Pisano *et al.*, 1970). Loewy and Matačić (1970) also noted that 'glycerol-extracted mechanochemically active systems and membranes' were particularly rich in these bonds. It has been postulated (Loewy, 1968) that the ε-(γ-glutamyl)lysine bond is involved in muscle contraction. It has also been suggested that this cross-link occurs in collagen (for reviews, see Joseph and Bose, 1962; J.J. Harding, 1965) and some evidence for this has recently been obtained (Bensusan, 1969).

The status of the ε-(γ-glutamyl)lysine cross-link in fibrin has been studied extensively because of its important medical implications. The formation of this cross-link in fibrin is the last step in the insolubilization of blood clots (Pisano *et al.*, 1968, 1969; Matačić and Loewy, 1968; Lorand *et al.*, 1968a). A lucid account of the identification of this cross-link in fibrin has recently been given (Loewy, 1970).

The first direct demonstration of the ε-(γ-glutamyl)lysine cross-link in fibrin was reported by Pisano *et al.* (1968, 1969) who measured the cross-link directly by ion-exchange chromatography of complete enzyme digests of insoluble fibrin. They confirmed their results using a chemical procedure which detected lysines with blocked ε-amino groups. A value of 2 - 3 cross-links/mole of human fibrin was determined. Similar values were obtained by Matačić and Loewy (1968) and Lorand *et al.* (1968a).

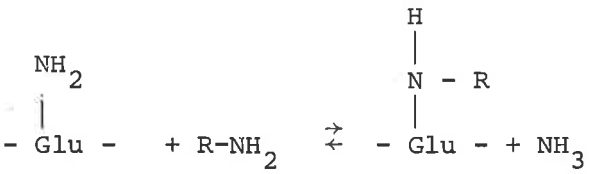
Further work demonstrated that only two kinds of fibrin chains were involved in cross-linking, *viz.*, γ-chains linked to other γ-chains, and γ-chains linked to α-chains (Chen and Doolittle, 1969; Lorand and Chenoweth, 1969; Lorand *et al.*, 1969a). The γ-γ involvement has also been demonstrated by Takagi and Iwanaga (1970, 1971). Lorand and Chenoweth (1969) further showed that the 'N-terminal disulphide knot' region of fibrin (Blomback *et al.*,1968) was not involved. The α-chain does not participate in cross-linking at all.

Recently Chen and Doolittle (1970) have isolated and characterized cross-linked peptides from bovine fibrin. It was found that in cross-linked fibrin two isopeptide bonds link overlapping C-terminal segments of neighbouring γ-chains. The amino acid sequence of the region involved in cross-linking was determined and it was shown that the two residues in each chain which participate in the cross-links are separated by only four residues.

## 1.5 TRANSAMIDASES AND THE FORMATION OF ε-(γ-GLUTAMYL)LYSINE CROSS-LINKS

Transamidases catalyze the replacement of the carboxyamide groups of protein-bound glutamine or asparagine residues by a primary amine (donor) with the concomitant release of ammonia (Neidle *et al.*, 1958; Clarke *et al.*, 1959, Mycek *et al.*, 1959; Folk and Cole, 1965).

*i.e.*,



These enzymes are found in a variety of mammalian tissues (Sarkar *et al.*, 1957; Neidle *et al.*, 1958; Tyler and Laki, 1967) and in some micro-organisms (Loewy *et al.*, 1966a).

Transamidase activity was first detected in guinea pig liver homogenates and the enzyme from this source has been extensively studied. It has been shown to react only with glutamine residues and has therefore been renamed *transglutaminase* (Mycek *et al.*, 1959). This enzyme can also catalyze deamidation (hydrolysis) of glutamine (Mycek and Waelsch, 1960; Folk *et al.*, 1968) and esterolysis of *p*-nitrophenol esters (Folk *et al.*, 1967a,b).

Calcium is essential for any reaction involving guinea pig transglutaminase (Folk *et al.*, 1967c; Folk, 1969) as well as other tissue transamidases (Neidle *et al.*, 1958). There is evidence that transglutaminase functions as a calcium-enzyme complex (Folk *et al.*, 1967c) and that the presence of calcium at both of the two calcium binding sites on the enzyme is essential for the binding of glutamine substrate (Folk *et al.*, 1967c,1968). Calcium produces a conformational change in the enzyme protein such that the essential cysteine sulphydryl group becomes available for reaction with substrate (Folk *et al.*, 1967c) or inhibitors (Folk and Cole, 1966a; Connellan and Folk, 1969). This

functional cysteine has been identified and a limited surrounding amino acid sequence characterized (Folk and Cole, 1966b).

Recently, as the result of kinetic studies Folk (1969) has shown that the mechanism of action of transglutaminase is of the ping-pong type. In such a mechanism the enzyme firstly binds to glutamine residues to form a binary complex. Ammonia dissociates with the formation of an acyl enzyme intermediate. Direct chemical evidence for such an intermediate involving the cysteine residue that is essential for all the reactions catalyzed by transglutaminase has been obtained for the esterase reaction (Folk *et al.*, 1967b). The acyl group is transferred from the enzyme to an amine to form a γ-glutamyl-amine product. The amine only binds to the enzyme in the presence of the glutamine substrate (Folk, 1969).

The enzyme in blood plasma which is involved in the final step of the blood-clotting process, *i.e.*, the stabilization of fibrin clots, is a transamidase (Loewy *et al.*, 1966b). Plasma transamidase therefore has considerable clinical importance since its inhibition or deficiency results in severe haemorrhaging (Lewis *et al.*, 1967; Lorand *et al.*, 1968b; Wong *et al.*, 1969; Godal, 1970; Lorand, 1970). Plasma transamidase exists in circulating blood in an inactive precursor form (designated Factor XIII) which is activated by thrombin (Lorand and Konishi, 1964).

The insolubilization of fibrin by transamidase is achieved by the formation of ε-(γ-glutamyl)lysine bonds (Lorand *et al.*, 1968a; Matačić and Loewy, 1968; Pisano *et al.*, 1968, 1969). Transamidases from a number of different tissues have been shown to have fibrin cross-linking activity (Loewy *et al.*, 1966a; Tyler and Laki, 1966, 1967) and therefore presumably the ability to form the ε-(γ-glutamyl)lysine isopeptide bond. Indeed, Neidle *et al.* (1958) showed that when casein was incubated with a guinea pig liver transamidase

preparation the ε-amino groups of some lysine residues became unreactive to deamination by nitrous acid and it was suggested that this indicated the formation of ε-(γ-glutamyl)lysine bonds, *i.e.*, that the ε-amino group of lysine residues could act as the donor amine in transamidase reactions.

## 1.6 AIM OF PROJECT

The occurrence of citrulline in peptide-linkage in proteins of hair medulla cells and the cells of the inner root sheaths of hair follicles is unique. The citrulline containing proteins are distinct from keratin and have very low cystine-cysteine contents. They are further characterized by their insolubility in protein solvents. The present study was aimed at explaining this insolubility and its possible relationship to the presence of citrulline.

The proteins can be released from the cells by digestion with trypsin. The tryptic products are soluble and to that extent are more suitable for study than the intact proteins. The complexity of the tryptic peptides and comparison of the proteins with other insoluble proteins such as collagen and elastin indicated that the insolubility of the native protein could be due to covalent cross-links. Accordingly, the presence of such cross-links in tryptic peptides was investigated.

CHAPTER 2

# MATERIALS AND GENERAL METHODS

## 2.1 SOURCE OF PROTEINS CONTAINING CITRULLINE

*a. Preparation of Hair.* The albino guinea pig *(Cavia porcellus)* was the main source of hair used in this work. The hair was clipped from the animals and washed sequentially in ethanol, acetone, and a 0.1% (v/v) solution of Tween 80 detergent at 45°, and rinsed twice with water. The washing procedure was repeated once and the hair dried at 40°. The dried hair was milled in a Wiley mill to through-40 mesh size.

*b. Preparation of Inner Root Sheaths.* Hair follicles of the guinea pig were exposed by the wax-sheet method (Rogers and Clarke, 1965). An animal was killed and the skin quickly removed and trimmed and the hair clipped with animal clippers (Model A-2; John Oster, Milwaukee, Wis.) fitted with size 15 cutters. The skin was cleaned by brushing and placed on an aluminium slab cooled to about 4°. A wax mixture consisting of crude beeswax-resin (2:7, w/w) at 55-60° was spread over the skin and allowed to solidify. When the skin was removed from the wax the hair roots were exposed with the shafts embedded in the wax. The sheaths were removed by brushing with a solution of 8M urea-0.2M iodoacetic acid-1M $K_2PO_4$ (pH 8.0) (Clarke and Rogers, 1970). This solution dispersed the prekeratin proteins in the follicle tissue without disrupting the inner root sheaths (Rogers, 1958a; 1959; 1964). The sheaths were collected by centrifugation, washed three times with distilled water, and either used immediately or freeze-dried.

*c. Preparation of Quills.* The medulla was dissected from quills of the African porcupine (*Hystrix cristata*) with a scalpel, milled in a Wiley mill to through-40 mesh size, and washed with water and organic solvents (Steinert *et al.*, 1969). Echidna quills were similarly treated, but the dissected medulla was not milled.

## 2.2 GENERAL METHODS

*a. Amino Acid Analyses.* Protein and peptide samples were hydrolyzed *in vacuo* with 1 ml of 6N HCl in special hydrolysis tubes (K-89685, Kontes Glass Co., N.J.) essentially according to the procedure of Moore and Stein (1963). Hydrolysis was at 110° for 20 hr and the hydrolysates were dried in a rotary evaporator.

Two main types of amino acid analysis systems were used in the course of this work. Initially, a Technicon Amino Acid AutoAnalyzer[1] was used but this was later modified to incorporate some features of the Beckman 120C Amino Acid Analyzer (see Appendix A). This modified system is referred to in the text as the modified gradient system. The various buffer gradients used are given in Appendix B. Some analyses were performed on the Beckman 120C analyzer using the standard 4 hr system[2]. For the more recent analyses an expanded scale (1mV) recorder was used.

*b. Dansylation.* Dansylation of peptides was carried out initially using the conditions of Gray (1967), but later the improved method of Gros and Labouesse (1969) was adopted. In either case the dried acid hydrolysates were extracted with water-saturated ethyl acetate, and the extracts and residues were examined separately by high-voltage paper electrophoresis at pH 4.40 (Gray, 1967) and/or by two-dimensional thin-layer chromatography on polyamide sheets (5 x 5 cm, Cheng Chin Trading Co., Taipei, Taiwan) using benzene-glacial acetic acid (9:1, v/v) and 1.5% aqueous formic acid as

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[1] Amino Acid Analyzer Instruction Manual, AAA-1, 1967, Technicon Corp., Ardsley, N.Y.

[2] Procedures Manual, A-TB-033, 1966, Beckman Instruments, Palo Alto, Calif.

solvents (Woods and Wang, 1967). In some cases, for positive identification of DNS-citrulline, the appropriate area from the electrophoretogram was eluted and chromatographed on silica-gel thin-layers (Chromagram, grade 6061, Distillation Products Industries, Rochester, U.S.A.) using benzene-pyridine-acetic acid (40:10:1, v/v) (Cole *et al.*, 1965) as solvent.

*c. Analysis with Ninhydrin.* The ninhydrin method for the analysis of column fractions was based on that of Yemm and Cocking (1955) but used the 4M sodium acetate buffer of Moore and Stein (1954). Alkaline hydrolysis (Hirs *et al.*, 1956) preceded the ninhydrin analysis of fractions that contained peptide material.

*d. Preparation of Buffers for Column Chromatography.* Pyridine, N-ethylmorpholine, and acetic acid were redistilled before use in buffers. Pyridine was refluxed with ninhydrin before redistillation (Hill and Delaney, 1967). All water used was bidistilled and ammonia-free. Unless otherwise noted, buffers were prepared according to Schroeder *et al.* (1962).

*e. Peptide Analyzer.* In several instances a Technicon Peptide AutoAnalyzer[3] was used to monitor the effluent of ion-exchange columns. The instrument was similar to that described by Catravas (1964) except that the total sample taken was hydrolyzed with alkali.

*f. High-Voltage Paper Electrophoresis.* Electrophoresis on Whatman No.3MM paper was performed on a cooled flat plate apparatus (Paton Industries, Adelaide, S.A.) similar to that described by Gross (1961).

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[3]Peptide Analyzer Instruction Manual, 1967, Technicon Corp., Ardsley, N.Y.

Amino acids and peptides were revealed by spraying the papers with a 0.1% solution of ninhydrin in ethanol.

CHAPTER 3

# INVESTIGATION OF GUINEA PIG HAIR MEDULLA: ISOLATION OF CELLS AND RELEASE OF CITRULLINE-CONTAINING PROTEINS

## 3.1 INTRODUCTION

Previous studies of hair medulla cells have involved vigorous conditions to remove the surrounding keratin (Jordan-Lloyd and Marriott, 1933; Matoltsy, 1953) or to disrupt the hair structure (Bradbury and O'Shea, 1969) to allow isolation of the cells. These methods, however, have the potential to disrupt and modify protein structure. Thus, as a preliminary to an extensive study of the structure and chemistry of hair medulla protein it was desirable to investigate milder methods for the release of medulla cells from hair fibres. Two such methods, involving solubilization of keratin by reduction, were investigated for the isolation of medulla cells from guinea pig hair, and are reported in this chapter.

The citrulline-containing proteins of medulla cells in intact hair fibres can be readily released as soluble peptides free of keratin by digestion with trypsin (Rogers, 1962; 1964a). The application of this method of isolation of protein material to guinea pig hair medulla has also been studied.

## 3.2 MATERIALS AND METHODS

*a. Reduction of Hair by Thioglycollic Acid* was performed in a solution of 8M urea and the products alkylated with iodoacetic acid as described by Gillespie (1964). The mixture was dialyzed exhaustively against 0.02M sodium borate buffer (pH 9) and the insoluble medulla and membrane material collected by centrifugation and washed with water.

*b. Reduction of Hair by Tributyl Phosphine.* Finely-milled guinea pig hair was reduced by tributyl phosphine using sodium iodide (5M) in aqueous *n*-propanol (25%, v/v) as solvent (Maclaren and Kilpatrick, 1969). Insoluble

material was collected by centrifugation and washed with aqueous propanol.

*c. Solubility Tests on Medulla Cells.* Partially purified medulla cells were treated with various solvents at room temperature for varying periods up to three weeks. The process was monitored by light microscopy using transmitted light. Where possible, samples were stained with *p*-dimethylaminocinnamaldehyde reagent (*Section e,* below). In some cases, electron microscopy was also used. Samples in suspension were negatively stained on specimen grids using uranyl acetate solution and examined in the Siemens Elmiskop I electron microscope.

*d. Digestion of Hair with Trypsin.* Finely milled guinea pig hair was evenly suspended in water to a concentration of 5% (w/v) by degassing, and then digested with trypsin (0.5%, w/w) in a pH-stat (Radiometer, Model TTT 1c) at pH 8 and 37$^{o}$ using 1N NaOH as titrant. A steady flow of nitrogen gas was maintained through the reaction mixture to avoid absorption of carbon dioxide. Generally, crystalline trypsin[1] of minimal chymotrypsin content (Mann Research Laboratories, New York) was used, but in some specified instances TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.) was employed. At the completion of the reaction the insoluble keratin and cellular debris were removed by centrifugation. The supernatant was clarified by filtration through a 0.65 µm Millipore filter and freeze-dried.

*e. Histochemical Stain for Citrulline.* Tissues were stained for citrulline with a solution of *p*-dimethylaminocinnamaldehyde (1%, w/v) in 0.5N HCl. The solution was made 4M with respect to NaCl to help reduce disruption of the tissues (Rogers, 1963). Specimens on microscope slides were flooded with the reagent, covered with a coverslip, and immediately examined for the appearance of the deep red color indicative of citrulline.

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[1] This trypsin had not been treated with TPCK and is referred to as 'untreated trypsin'.

## 3.3 RESULTS

*a. Isolation of Medulla Cells from Hair.* Reduction of guinea pig hair by either thioglycollic acid or tributyl phosphine solubilized about 50% of the dry weight of the hair as keratin derivatives. The reduction did not in itself result in the separation of medulla cells because the cortical and cuticle cell membranes did not dissolve (Fraser and Rogers, 1953), and held the fibres more or less intact, although they were very swollen (Figure 3-1). Therefore, the hair was mechanically disrupted, and attempts made to separate the products of the latter process.

A variety of homogenization and sonication procedures were tried as a means of releasing the medulla cells. Similarly, a number of methods for separating the medulla cells from the contaminating membranes were investigated. These latter methods mainly involved using sucrose density gradients, but electrofocussing of cells suspended in sucrose solution was also attempted. In general, the following procedure proved to be the most successful.

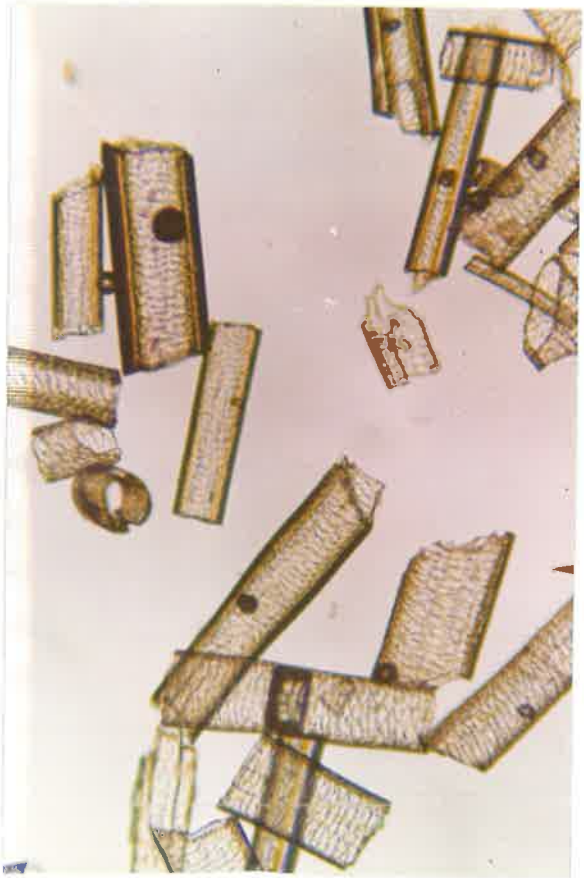
Reduced hair in aqueous suspension (about 5%, wet w/w) was sonicated lightly for 10 - 20 sec at Power 5 (Soniprobe Type 1130A, Dawe Instruments, London) and the larger cuticle membranes and medulla cells sedimented at 1 *g* for 30 min. At this stage medulla cells were mainly present as discs, which were one cell thick and composed of several cells (Figure 3-2). These discs of cells result from the ready breakage of the medulla core in a plane transverse to the length of the hair. The 1 *g* pellet containing the discs was then layered on to a 40% (w/v) sucrose solution and centrifuged at about 500 rpm in an MSE Super Minor centrifuge for 5 min. After this step the main contaminant of the medulla preparation was cuticle membrane and this proved very difficult to remove. The medulla cells tended to be very sticky and adhered to both glassware and membranes. However, some of the membrane could be

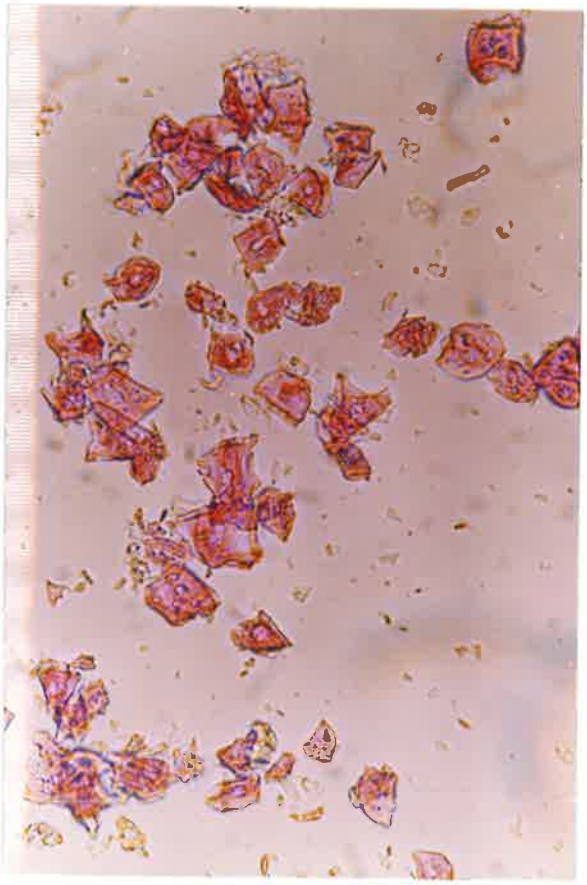
FIGURE 3-1: Light micrograph of guinea pig hair after removal of the bulk of the keratin by reduction in 8M urea - 0.2M thioglycollate at pH 10.5 and subsequent alkylation with iodoacetate at pH 8.5. Stained with *p*-dimethylaminocinnamaldehyde. Note the intense red coloration of the citrulline-containing protein of the medulla or central core. The membranes of the cortical and cuticle cells of the fibre do not stain but have a yellowish coloration. x 120

FIGURE 3-2: Light micrograph of discs of medulla cells liberated by sonication from guinea pig hair after treatment as described in Figure 3-1. The aggregates of medulla cells stain red with *p*-dimethylaminocinnamaldehyde because of their content of the citrulline-containing protein. x 480

FIGURE 3-3: Light micrograph of guinea pig hair residue after removal of medulla by digestion with trypsin. The absence of any red staining with *p*-dimethylaminocinnamaldehyde indicates complete removal of the citrulline-containing medulla protein by the trypsin treatment. The residual keratin does not stain. The pieces of hair are hollow tubes of cortical and cuticle cells. x 120

FIGURE 3-4: Light micrograph of medulla cells purified from the 'debris layer' of a TPCK-trypsin digest of guinea pig hair. The red coloration produced by staining with *p*-dimethylaminocinnamaldehyde shows that some citrulline-containing protein remains within the cells. x 480





removed by homogenizing the pellet in the tight Dounce homogenizer in the presence of 0.003% Tween 80 detergent and then centrifuging the cells through a discontinuous sucrose gradient composed of layers of 20, 40 and 60% (w/v) sucrose solutions using conditions similar to the previous centrifugation. Homogenization broke down the majority of the discs of medulla cells into single cells, which were recovered from the 40% layer.

All stages of the separation procedures were monitored by light microscopy to obtain optimal conditions, but complete removal of all membrane material was never achieved. Preparations stained with *p*-dimethylaminocinnamaldehyde gave a deep red color with cells that had citrulline-containing protein, thus making these components readily recognizable (Figure 3-1). In the best preparations, the membrane contamination was visually estimated at less than 10%. Yields of the partially purified medulla cells were generally low and variable, ranging from about 10 - 25% of the crude preparation, *i.e.*, about 5 - 10% by weight of the whole fibre. The amino acid composition of partially purified medulla cells is given in Table 3-I. The composition of the whole hair fibre and the solubilized keratin is also given.

*b. Dissolution of Isolated Medulla Cells.* In all, about 40 different solvents and chemical treatments were used with little success in an attempt to solubilize the medulla cells. The conditions tested included the following: organic solvents, such as *n*-butanol, *t*-butanol, *n*-propanol and pyridine in aqueous solution; detergents such as Triton X-100, Lubrol W, sodium lauryl sulphate and deoxycholate; oxidizing agents (NaOCl, $H_2O_2$); reducing agents ($Na_2S$, in addition to those used in the isolation of the cells); chelating agent (EDTA); acids, including formic, acetic, trifluoroacetic and hydrochloric acids at various strengths; alkali (KOH solutions); high salt concentrations (saturated $LiClO_4$ and KSCN solutions); and strong dissociating

TABLE 3-I: AMINO ACID CONTENT OF GUINEA PIG HAIR FRACTIONS[a].

| Amino Acid | Residues/1000 Residues | | | |
|---|---|---|---|---|
| | Medulla Cells | 'Solubilized' Medulla Material[b] | Whole Fibre | Solubilized Keratin |
| SCM-Cysteine | 47.5 | 88.0 | | 178.6 |
| Aspartic acid | 55.3 | 62.7 | 49.2 | 50.0 |
| Threonine | 29.2 | 50.3 | 49.9 | 61.5 |
| Serine | 49.8 | 80.6 | 87.2 | 106.7 |
| Glutamic acid | 269.3 | 149.9 | 150.1 | 116.6 |
| Citrulline[c] | 98.7 | 31.6 | 23.5 | |
| Proline | 39.2 | 64.2 | 77.6 | 83.7 |
| Glycine | 56.6 | 72.8 | 72.2 | 71.8 |
| Alanine | 39.2 | 56.4 | 50.5 | 44.0 |
| Valine | 36.4 | 58.0 | 47.1 | 46.4 |
| Half-cystine | trace | trace | 137.0 | |
| Methionine | 8.3 | 12.4 | 5.9 | 4.1 |
| Isoleucine | 21.4 | 30.8 | 25.9 | 26.9 |
| Leucine | 94.2 | 84.0 | 71.0 | 60.9 |
| Tyrosine | 15.3 | 24.9 | 26.0 | 31.3 |
| Phenylalanine | 26.3 | 28.9 | 22.4 | 20.8 |
| Lysine | 53.5 | 45.5 | 33.4 | 21.2 |
| Histidine | 14.0 | 14.2 | 12.0 | 10.8 |
| Arginine | 45.9 | 45.1 | 59.2 | 63.9 |

[a]Uncorrected for hydrolytic losses, except as noted. [b]Remains in supernatant at 38,000 *g* after disruption of medulla cells in French Pressure Cell.
[c]Includes citrulline and ornithine.

agents such as 8M urea, 6M guanidine hydrochloride, dimethyl formamide, cupriethylene diamine and phenol-acetic acid-water (1:1:1, v/v).

Chemical treatments that were tested included methylation, succinylation, and reaction with cyanogen bromide, hydroxylamine and sodium borohydride. In some instances the cells were disrupted by extrusion through a French Pressure Cell, in which case material remaining in the supernatant after centrifugation at 38,000 *g* for 1 hr was regarded as being soluble. However, filtration on Millipore membranes and differential centrifugation revealed that this so-called soluble material was in fact a polydisperse suspension of very small particles of hardened protein material. These particles were seen by electron microscopy to have no distinctive structure and were themselves insoluble in many solvents. The amino acid composition of these particles is given in Table 3-I. Vigorous sonication also had little effect on solubilizing the protein. In fact, the only conditions which caused any significant degree of solubilization were those involving $Na_2S$, NaOCl and $H_2O_2$, and these also solubilized the contaminating membranes.

*c. Tryptic Digestion of Hair.* Tryptic digestion of the hair by Mann trypsin was complete in 4 hr with 18% of its weight being solubilized. Amino acid analysis of the residual keratin showed that all the citrulline-containing material had been removed as soluble tryptic peptides (Table 3-II). This was confirmed by examination of the preparation with the light microscope (Figure 3-3).

In contrast, digestion of the hair by TPCK-trypsin was slower and required 5 hr. Centrifugation of the digest produced three fractions; the supernatant and a pellet composed of two layers. The soluble tryptic peptides in the supernatant amounted to 15% of the weight of the hair. The lower layer of the pellet contained undigested keratin, as before. However,

TABLE 3-II: AMINO ACID CONTENT OF GUINEA PIG HAIR PROTEINS[a].

| Amino Acid | Residues/1000 Residues | | | |
|---|---|---|---|---|
| | Whole Fibre[b] | Residue (Keratin) | Soluble Tryptic Peptides of Medulla | Medulla Cells[b] |
| Cysteic acid | | 4.2 | | |
| SMC-Cysteine | | | | 47.5 |
| Aspartic acid | 49.2 | 47.4 | 68.1 | 55.3 |
| Threonine | 49.9 | 59.1 | 28.5 | 29.2 |
| Serine | 87.2 | 106.0 | 45.0 | 49.8 |
| Glutamic acid | 150.1 | 110.1 | 302.1 | 269.3 |
| Citrulline[c] | 23.5 | trace | 127.8 | 98.7 |
| Proline | 77.6 | 82.8 | 12.7 | 39.2 |
| Glycine | 72.2 | 84.4 | 48.1 | 56.6 |
| Alanine | 50.5 | 45.1 | 42.3 | 39.2 |
| Valine | 47.1 | 50.6 | 33.3 | 36.4 |
| Half-cystine | 137.0 | 175.8 | 7.6 | trace |
| Methionine | 5.9 | 4.5 | 8.6 | 8.3 |
| Isoleucine | 25.9 | 28.1 | 20.3 | 21.4 |
| Leucine | 71.0 | 60.5 | 100.0 | 94.2 |
| Tyrosine | 26.0 | 28.9 | 17.5 | 15.3 |
| Phenylalanine | 22.4 | 20.5 | 28.5 | 26.3 |
| Lysine | 33.4 | 22.7 | 52.2 | 53.5 |
| Histidine | 12.0 | 10.5 | 13.4 | 14.0 |
| Arginine | 59.2 | 58.9 | 43.6 | 45.9 |
| Tryptophan[d] | n.d. | n.d. | 7.8 | n.d. |

[a]Uncorrected for hydrolytic losses, except as noted. [b]Taken from Table 3-I. [c]Includes citrulline and ornithine. [d]Colorimetric assay (Opiénska-Blauth *et al.*, 1963).

the upper layer of the pellet (termed 'debris layer') was found by light microscopy to consist mainly of swollen medulla cells, produced in a yield of about 5% (w/w). The cells gave a positive reaction for citrulline when tested with *p*-dimethylaminocinnamaldehyde (Figure 3-4). The protein was readily released from the cells by digestion with chymotrypsin. Amino acid analyses of the debris layer (cells) and of the soluble TPCK-tryptic peptides before and after dialysis against water are given in Table 3-III. A quantitative recovery of material was obtained from the dialysis of the peptides with 70% by weight of the peptides being retained by the dialysis membrane.

## 3.4 DISCUSSION

Published methods for the isolation of hair medulla cells have involved solubilization of the keratin or breakdown of the hair structure using vigorous conditions which could damage or modify the proteins. The alkali method (Jordan-Lloyd and Marriott, 1933; Matoltsy, 1953) has, in fact, been shown to alter the x-ray diffraction pattern of porcupine quill medulla protein (Stoves, 1945) and also to alter the amino acid composition of kangaroo hair medulla protein (Bradbury and O'Shea, 1969). It is not inconceivable, either, that the alternative formic acid procedure proposed by Bradbury and O'Shea (1969) could cause random peptide bond breakage even though no effect on the amino acid content is apparent. Two mild methods for solubilization of the keratin were investigated in this report. These methods, both incorporating steps of reduction and alkylation, are well characterized, and are commonly used to solubilize keratin (Rogers, 1969).

The heterogeneity of the products of the reduction is not entirely unexpected because of the heterogeneity of the original hair fibres. The difficulty in obtaining good yields of purified medulla cells was further

TABLE 3-III: AMINO ACID CONTENT OF GUINEA PIG HAIR PROTEINS[a].

| Amino Acid | Residues/1000 Residues | | | |
|---|---|---|---|---|
| | TPCK-Tryptic Peptides of Medulla (Before Dialysis) | TPCK-Tryptic Peptides of Medulla[b] (Dialyzed) | Tryptic Peptides of Medulla (Mann Trypsin)[c] | Debris Layer (Cells) |
| Aspartic acid | 67.0 | 61.9 | 68.1 | 48.5 |
| Threonine | 29.8 | 28.3 | 28.5 | 28.5 |
| Serine | 41.0 | 41.5 | 45.0 | 49.3 |
| Glutamic acid | 295.4 | 293.3 | 302.1 | 333.1 |
| Citrulline[d] | 107.0 | 126.4 | 127.8 | 155.8 |
| Proline | 23.9 | 30.5 | 12.7 | 38.7 |
| Glycine | 47.8 | 45.7 | 48.1 | 50.7 |
| Alanine | 44.3 | 41.3 | 42.3 | 35.4 |
| Valine | 36.4 | 34.8 | 33.3 | 34.7 |
| Half-cystine | 6.4 | 7.9 | 7.6 | 58.0 |
| Methionine | 12.3 | 9.6 | 8.6 | 5.5 |
| Isoleucine | 27.8 | 25.6 | 20.3 | 19.7 |
| Leucine | 92.1 | 104.1 | 100.0 | 88.3 |
| Tyrosine | 17.1 | 15.3 | 17.5 | 15.0 |
| Phenylalanine | 28.6 | 27.5 | 28.5 | 27.4 |
| Lysine | 56.1 | 51.7 | 52.2 | 48.9 |
| Histidine | 12.2 | 12.0 | 13.4 | 12.4 |
| Arginine | 54.9 | 42.7 | 43.6 | 23.3 |
| Tryptophan[e] | n.d. | n.d. | 7.8 | n.d. |

[a]Uncorrected for hydrolytic losses, except as noted. [b]Material retained by dialysis membrane: 70% (w/w) of total. [c]Taken from Table 3-II. [d]Includes citrulline and ornithine. [e]Colorimetric assay (Opiénska-Blauth *et al.*, 1963).

compounded by the presence of membranes of similar size and sedimentation properties to those of the cells. A similar problem was experienced by Bradbury and O'Shea (1969) who overcame it by preferentially depositing gold in the medulla cells, thereby increasing the density differential. This procedure was not used in the present work because of the interest in obtaining the medulla protein in an unmodified form for solubility and structural studies.

The methods used in this work did, however, allow the partial purification of some medulla cells and made it possible to test the effect of numerous solvents on cells not protected by keratin-containing cortical cells. Solvents tested were chosen for their known ability to solubilize tissues and proteins such as cell membranes, silk, and collagen which go into solution only with difficulty. Medulla cells were soluble only in vigorous conditions such as $Na_2S$, NaOCl and dilute acid hydrolysis; in these conditions peptide bond breakage is known, or is likely to occur (O'Donnell and Woods, 1955). This fact, together with the insolubility of the protein fragments produced when the cell membrane is disrupted, indicates that the extreme insolubility is an inherent property of the protein itself and is not due to surrounding keratin or membranes.

The development of a simple and rapid histochemical staining procedure for citrulline was of considerable use in studying the isolation and solubility of medulla cells. The stain was adapted from Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) (Dent, 1948) as used by Rogers (1958a, 1963) in histochemical studies of the origin of citrulline-containing proteins. The new stain had the advantage that it gave a far more intense and distinctive color than Ehrlich's reagent (Rogers, 1963), and at the same time was more rapid and easier to apply than the method of Holmes (1968, 1970)

which used very strong acid conditions. However, at the present stage of development of the staining procedure, the color produced is not stable for long periods. The mechanism of the reaction between *p*-dimethylaminocinnamaldehyde and substituted ureas has recently been described in a different context by McCormick and Roth (1970).

Medulla cells produced either by reduction of keratin or by TPCK-trypsin treatment have a higher half-cystine content than is expected from the studies of Rogers (1962), and also higher than that found for the soluble tryptic peptides. This result can be ascribed to the presence of keratin material from the contaminating cuticle and cortical cell membranes and perhaps the medulla cell membranes as well. However, it could also indicate that the cells contain some true keratin protein that is not released by trypsin. The material 'solubilized' by the Pressure Cell process is enriched in this keratin material rather than the citrulline-containing protein. Bradbury and O'Shea (1969) have also reported high cysteic acid-half cysteine values in analyses of whole medulla cells.

In common with other citrulline-containing proteins from hairs and quills (Rogers, 1962; Bradbury and O'Shea, 1969) the medulla protein released from guinea pig hair by either TPCK-trypsin or untreated trypsin is characterized by a high content of citrulline, glutamic acid and leucine, and a low proline and cystine-cysteine content (Tables 3-II, 3-III). Dialysis of the peptides produced by TPCK-trypsin yields an enrichment in citrulline, leucine and proline and a reduction in arginine, giving an analysis essentially identical to that of the peptides produced by untreated trypsin. This is presumably due to the loss through the dialysis membrane of small peptides relatively rich in arginine but which contain only low levels of citrulline, leucine and proline. Except for the very high values for glutamic acid (almost one residue in three is glutamic acid) and the presence

of citrulline, the analyses of hair medulla protein are similar to those of other structural proteins (Mazia and Ruby, 1968).

Digestion with TPCK-trypsin can release medulla cells from hair. These cells are apparently intact, since their amino acid analysis (Table 3-III) shows that they still contain the citrulline-containing protein, thus confirming the histochemical staining results. These cells are very susceptible to chymotryptic attack in which the protein is rapidly released without further detectable alkali uptake in the pH-stat. These results would suggest that pure (TPCK) trypsin solubilizes the protein within the cells but cannot readily degrade the medulla cell membrane to release the peptides. The soluble peptides that are released by TPCK-trypsin digestion may derive from broken and damaged medulla cells in which the protein is readily accessible to the enzyme. Other proteolytic activities such as that of chymotrypsin can rapidly disrupt the cell membrane (perhaps already weakened by trypsin) and release the protein. Thus, when untreated (Mann) trypsin is used, the solubilization and release of protein (as peptides) would be concurrent processes.

CHAPTER 4

# CHARACTERIZATION OF TRYPTIC PEPTIDES OF CITRULLINE-CONTAINING PROTEINS

## 4.1 INTRODUCTION

It has not been possible to obtain the citrulline-containing proteins of hair or quill medulla cells in an intact and unmodified condition suitable for physicochemical study because of their extreme insolubility in protein solvents (Rogers, 1964a; see also Chapter 3). However, the proteins are readily solubilized by tryptic digestion and most of the experimental work on these proteins has therefore used the tryptic peptides as a source of soluble material (Rogers, 1962, 1964a; Steinert *et al.*, 1969).

Rogers (1962) has noted that such peptides from porcupine quill medulla have molecular weights greater than about 10,000 since they are not readily dialyzable through 'Cellophane' membranes. Resolution of the complex mixtures of peptides by ion-exchange chromatography on DEAE-cellulose columns was not successful, but a crude fractionation, yielding a citrulline-enriched fraction (formerly referred to as *tryptic core*, but now termed *acid-insoluble peptides)* was achieved by acid fractionation (Rogers, 1962). In the present study the molecular weight and size distributions of the tryptic peptides from guinea pig hair medulla were examined by gel filtration chromatography. Disc gel electrophoresis and isoelectric focussing were investigated as possible means of monitoring separation of the peptides.

## 4.2 MATERIALS AND METHODS

*a. Acid Fractionation of Tryptic Peptides.* Tryptic peptides of guinea pig hair medulla (Chapter 3.2) were dissolved to a concentration of 1% (w/v) in aqueous pyridine (pH 7) and the acid-insoluble peptides precipitated by lowering the pH to 3.5 with glacial acetic acid (Rogers, 1962). The precipitate was collected by centrifugation, washed with pyridine-acetate buffer (pH 3.5) and finally redissolved in aqueous pyridine and freeze-dried. The combined

supernatants containing the acid-soluble tryptic peptides were also freeze-dried.

*b. Gel Filtration Chromatography*. Sephadex G-200 (Pharmacia, Uppsala, Sweden) was prepared by swelling in 0.3M pyridine (pH 8.1) with careful removal of fines. The column was fitted with an adjustable flow adapter designed in this laboratory and was operated by upward flow. A Mariotte flask maintained a constant pressure head of 18 cm. Chymotrysinogen (MW 25,000), pepsin (MW 35,000), glutamate dehydrogenase (MW 250,000) and urease (MW 480,000) were used for the molecular weight calibration of the column by the method of Whitaker (1963).

Chromatography on columns of Sephadex G-150, G-75 and G-50 was carried out by downward flow according to the usual procedures. Pepsin (MW 35,000), soybean trypsin inhibitor (MW 21,500), bovine ribonuclease A (MW 13,700) and bovine insulin (MW 5,700) were used as markers on the columns of G-75 and G-50.

*c. Disc Electrophoresis* in acrylamide gels was performed basically according to Davis (1964). The protein bands were stained with either 0.5% Amido black in 7% aqueous acetic acid or with Coomassie blue in 10% TCA (Chrambach *et al.*, 1967).

*d. Isoelectric Focussing* was performed in tubes of acrylamide gel as described by Wrigley (1968). The protein bands were detected as white precipitation bands by immersing the gels in 5% TCA. 'Ampholine' carrier ampholytes were obtained from LKB-Producter AB, Sweden.

*e. N-Terminal Analyses* were performed by the dansyl technique (Chapter 2.2*b*). The intensity of the fluorescent spots was estimated semi-quantitatively.

*f. Tryptophan Analyses.* The tryptophan content of protein fractions was determined by the colorimetric assay of Opiénska-Blauth *et al.* (1963).

## 4.3 RESULTS

*a. Acid Fractionation of Tryptic Peptides.* The amino acid composition of peptide fractions obtained from a tryptic digest of guinea pig hair medulla by acid precipitation is given in Table 4-I. As is the case for porcupine quill tryptic peptides, the acid-insoluble peptides (12.5% (w/w) of the total) are enriched in citrulline when compared to the total tryptic digest. The phenylalanine content is also increased, while the values for proline, and arginine in particular, are reduced.

*b. Gel Filtration Chromatography.* The elution patterns obtained by chromatography of the tryptic peptide fractions of guinea pig hair medulla on the Sephadex G-200 column are shown in Figure 4-1. No fractionation into discrete components was achieved, but it can be seen that the peptides belong to two main molecular weight classes. The high molecular weight material was excluded and was shown to have a molecular weight greater than 500,000. This material is concentrated in the acid-insoluble peptide fraction but is virtually absent from the acid-soluble peptides. In all three fractions, the bulk of the peptides eluted in the low molecular weight peak and for these peptides the molecular weight was calculated to be in the range of 10,000 - 30,000. These molecular weight values were confirmed by chromatography on the G-150, G-75 and G-50 Sephadex columns. No significant fractionation of the main peak occurred even when these more highly cross-linked Sephadex gels were used.

Rechromatography of pooled fractions from high and low molecular weight peaks showed that the higher molecular weight material did not arise by

TABLE 4-I: AMINO ACID CONTENT OF GUINEA PIG HAIR PEPTIDE FRACTIONS[a].

| Amino Acid | Residues/1,000 Residues | | |
|---|---|---|---|
| | Total Tryptic Digest of Medulla | Acid-Insoluble Tryptic Peptides | Acid-Soluble Tryptic Peptides |
| Aspartic acid | 68.1 | 70.9 | 65.7 |
| Threonine | 28.5 | 25.8 | 29.7 |
| Serine | 45.0 | 34.1 | 45.9 |
| Glutamic acid | 302.1 | 302.1 | 287.6 |
| Citrulline[b] | 127.8 | 180.0 | 120.5 |
| Proline | 12.7 | 2.4 | 15.0 |
| Glycine | 48.1 | 40.0 | 51.3 |
| Alanine | 42.3 | 35.6 | 42.9 |
| Valine | 33.3 | 32.6 | 36.3 |
| Half-cystine | 7.6 | 12.7 | 9.0 |
| Methionine | 8.6 | 7.7 | 9.6 |
| Isoleucine | 20.3 | 24.9 | 21.0 |
| Leucine | 100.0 | 106.1 | 105.2 |
| Tyrosine | 17.5 | 16.6 | 17.1 |
| Phenylalanine | 28.5 | 39.1 | 28.5 |
| Lysine | 52.2 | 43.6 | 54.0 |
| Histidine | 13.4 | 12.2 | 14.4 |
| Arginine | 43.6 | 13.6 | 46.5 |
| Tryptophan[c] | 7.8 | 9.1 | 6.3 |

[a]Uncorrected for hydrolytic losses, except as noted. [b]Includes citrulline and ornithine. [c]Colorimetric assay (Opiénska-Blauth *et al.*, 1963).

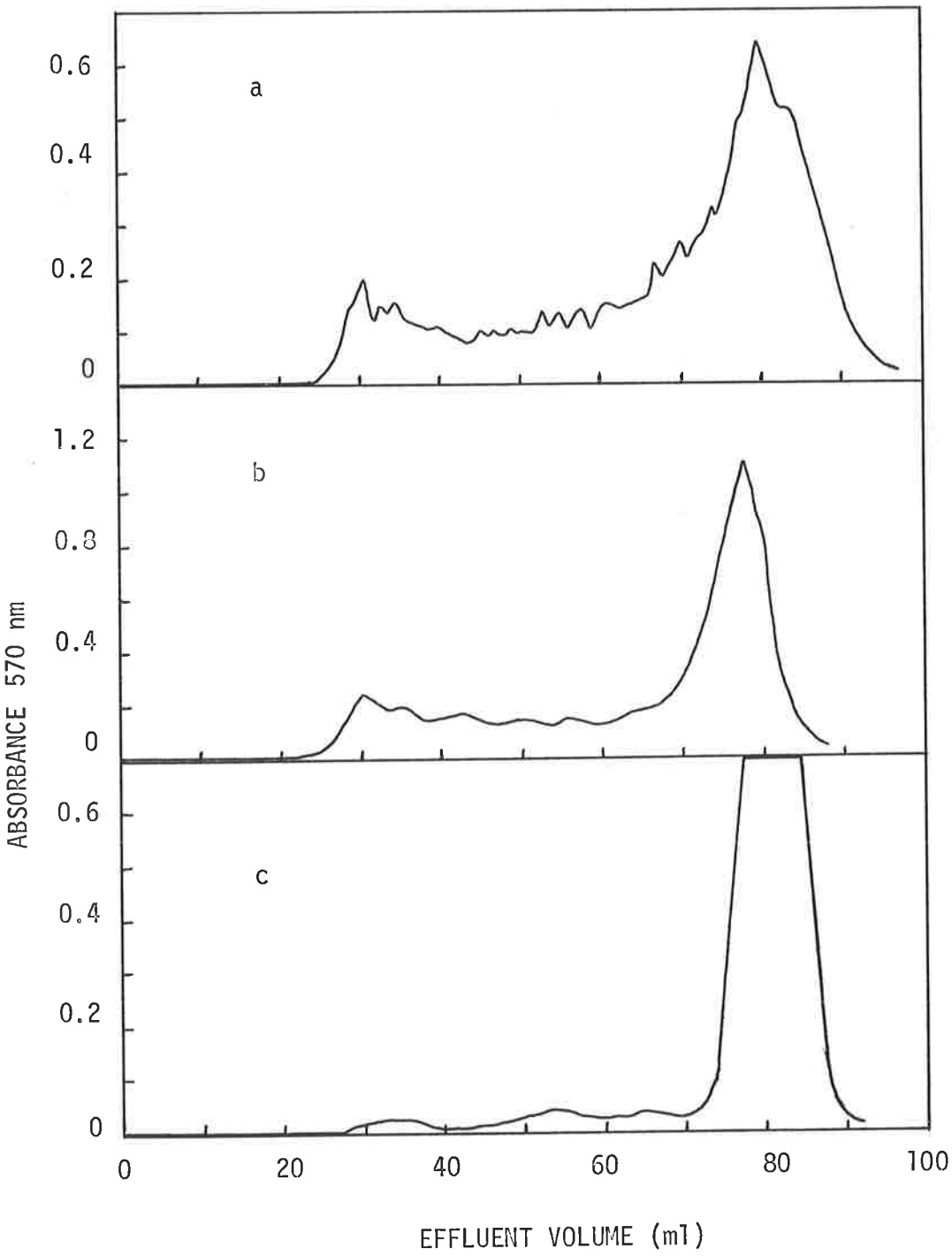



FIGURE 4-1: Gel filtration chromatography of tryptic peptide fractions from guinea pig hair medulla on a column, 1.1 x 85 cm, of Sephadex G-200, equilibrated with 0.3M pyridine (pH 8.1). The flow rate was 4 ml/hr and 1 ml fractions were collected. Aliquots from each fraction were analyzed by the ninhydrin method after alkaline hydrolysis. (a) Total tryptic digest (8 mg). (b) Acid-insoluble tryptic peptides (15 mg). (c) Acid-soluble tryptic peptides (10 mg).

aggregation of the smaller peptides. However, aggregation of the acid-insoluble peptides to give material with apparent molecular weights greater than about 100,000 was observed when chromatography on the G-200 column was performed using buffers containing added salt (NaCl) or 8M urea or buffer near neutral pH. No such effect was seen for the acid-soluble peptides.

The elution pattern for the total tryptic digest of inner root sheath (Figure 4-2) was similar to that for hair medulla peptides (Figure 4-1). However, in this case acid precipitation caused a definite fractionation into the two molecular weight classes under the conditions used. The acid-insoluble peptides were all of high molecular weight (greater than 500,000) (Figure 4-2b) whereas the acid-soluble fraction was composed almost entirely of peptides with lower molecular weights comparable to those of the medulla peptides (Figure 4-2c).

Peptides in the total tryptic digest of porcupine quill medulla (Figure 4-3) also gave a similar elution pattern to those from hair medulla and inner root sheath, except that under the conditions used the amount of high molecular weight material was relatively small. The bulk of the peptides had molecular weights of the same order as those from the other tissues.

*c. Disc Electrophoresis and Isoelectric Focussing.* Disc gel electrophoresis was attempted with and without 6.25M urea in the gels, with gel concentrations varying from 3.75% up to 15% at several pH values in the range 1.9 to 9.5. Staining of the gels showed that a large percentage of the material did not even enter the stacking gel (2.5% gel concentration). Some of the peptides that did enter the gels moved with the tracker dye (bromophenol blue) when electrophoresed at pH 8.9 in 7.5% gels. At high loadings it was also possible to see two poorly resolved, slow-moving bands in samples of both the acid-insoluble and acid-soluble peptides. A serious problem with the technique

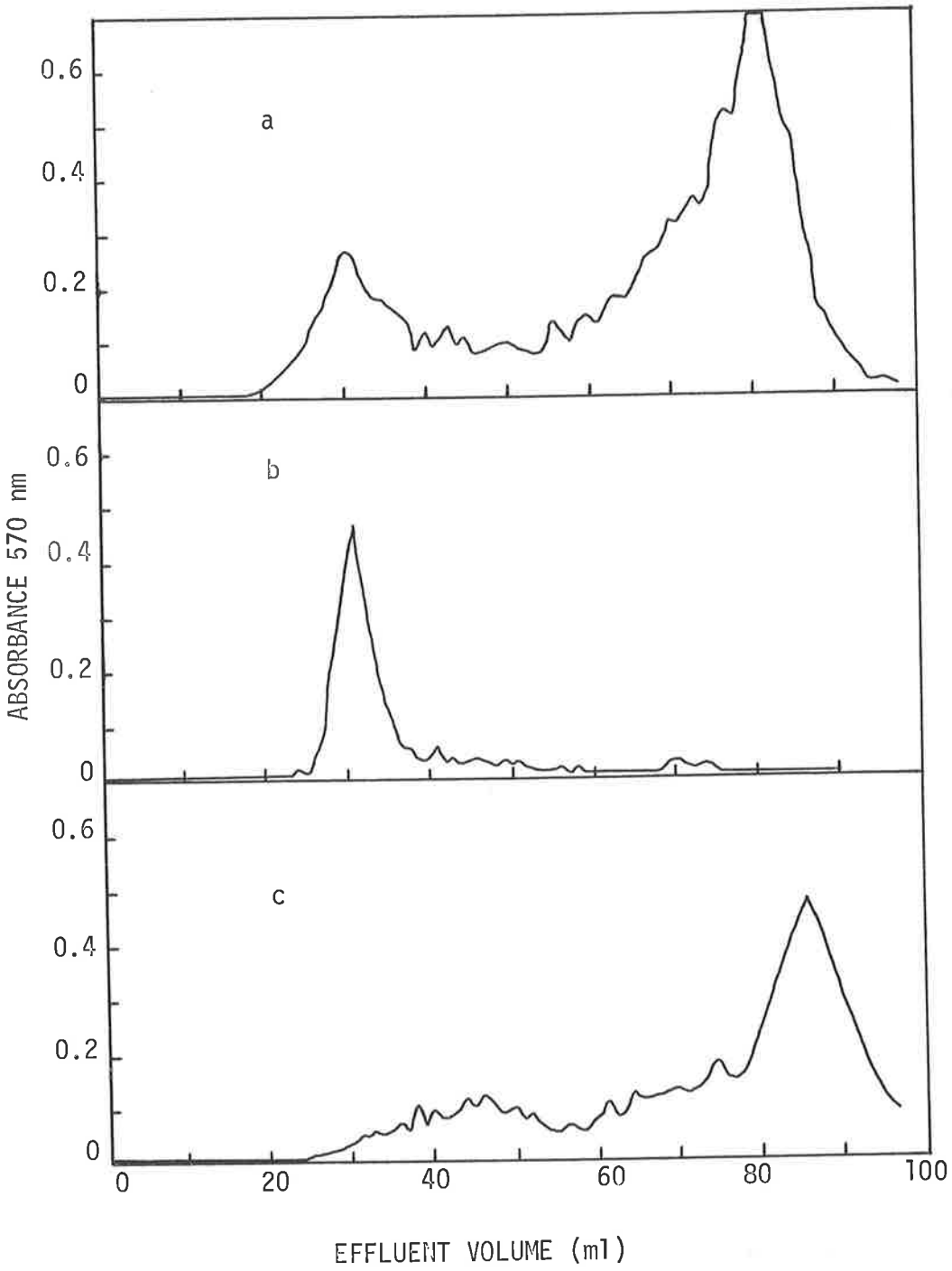



FIGURE 4-2: Gel filtration chromatography of tryptic peptide fractions from inner root sheaths of guinea pig hair follicles on Sephadex G-200. The column and conditions are as for Figure 4-1. (a) Total tryptic digest (20 mg). (b) Acid-insoluble tryptic peptides (5 mg). (c) Acid-soluble tryptic peptides (10 mg).

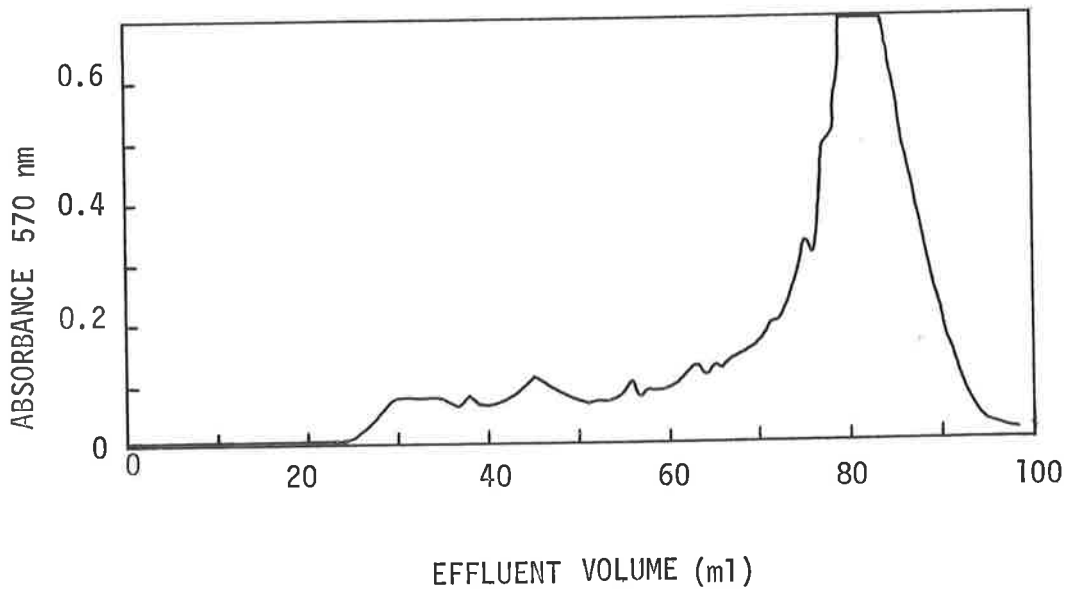



FIGURE 4-3: Gel filtration chromatography of tryptic peptides (10 mg) from porcupine quill medulla on Sephadex G-200. The column and conditions are as for Figure 4-1.

in this application was high background after staining. This made the bands that appeared very indistinct and interpretation of the gels required a fair degree of imagination.

Similarly, isoelectric focussing was largely unsuccessful because of precipitation of the peptides in the gels even when the gels contained 8M urea. A number of diffuse bands (up to 10) could be seen in the gels, especially those containing the acid-soluble peptides, in which the precipitation was less severe. Isoelectric points found were in the range pH 3.5 - 5.5, but were mainly at the lower values, where the pH gradient was least reproducible. This made the band patterns unreliable even when ampholytes in the range pH 3 - 6 were used.

*d. N-Terminal Analyses.* Within the limits of detection of the technique, the range and extent of N-terminal amino acids were identical for the high and low molecular weight fractions of the acid-insoluble peptides and also the acid-soluble tryptic peptides of guinea pig hair medulla. All the amino acids occurring in these peptides, except histidine, proline and tryptophan, were detected as N-termini; those present in largest amounts being leucine, phenylalanine, valine, alanine, glutamic acid and aspartic acid, in that order. Other amino acids were present in much smaller amounts. Only traces of DNS-citrulline and DNS-cysteine were detected. Histidine, proline and tryptophan would not have been detected even if they were present because of the lability of their DNS-derivatives under the conditions employed for acid hydrolysis. A similar N-terminal analysis was obtained for the disrupted purified medulla cells described in Chapter 3.3*b*.

The N-terminal analyses for the acid-insoluble and acid-soluble tryptic peptides of inner root sheath were identical to each other and very similar to those of the guinea pig hair medulla fractions, except that DNS-glycine,

DNS-isoleucine, α-ε-bis-DNS-lysine and α,O-bis-DNS-tyrosine were also present in large amounts. A similar study of the tryptic peptides of porcupine quill medulla (H.W.J. Harding, 1967) produced a similar analysis with DNS-glutamic acid predominating. In all cases, the typical reactions of DNS-chloride with the side-chains of lysine and tyrosine were seen. Attempts to quantitate the N-termini of guinea pig hair medulla tryptic peptides as the phenylthiohydantoins by the method of Thompson (1957) were frustrated by high optical density values for the controls and were abandoned.

## 4.4 DISCUSSION

The gel filtration experiments presented in this chapter show that the molecular weights of the bulk of the tryptic peptides of guinea pig hair medulla are of the order of 10,000 - 30,000; values which are in accord with preliminary ultracentrifuge data. The tryptic peptides of guinea pig inner root sheath and porcupine quill medulla were found to be of similar size, thus confirming the earlier work of Rogers (1962).

It is interesting that the acid-insoluble tryptic peptides of guinea pig hair medulla aggregate at high ionic strength. Such an effect has been noted for several proteins, *viz.*, wool keratin derivatives (Harrap, 1955), bovine pancreatic carboxypeptidase A (Bethune, 1963), and ox liver sulphatase A (Nichol and Roy, 1965). In all these cases this phenomenon has been interpreted to mean that at low ionic strength the proteins are kept in the disaggregated or monomer state by electrostatic repulsive forces which are consequently reduced as the conductivity of the solvent is increased. In the acid-insoluble medulla peptides, such repulsive forces could be caused by the large number of glutamic acid residues, the γ-carboxyl groups of which would be ionized under the conditions used. At pH 6.5 these forces would be reduced, and this would

allow the molecules to approach each other sufficiently for short range attractive forces to operate and cause the observed aggregation. This effect has been demonstrated with the keratin derivative, kerateine 2 (Harrap, 1955).

Separation of the medulla tryptic peptides on the basis of size and charge by disc gel electrophoresis proved unsatisfactory because a large proportion of the peptides failed to enter the gel. Very high background staining was also a problem. Separation of the peptides on the basis of their isoelectric points (isoelectric focussing, Haglund, 1967) was also unsatisfactory because of precipitation of peptides in the gels. Such precipitation immediately precluded the use of preparative isoelectric focussing in sucrose solutions. The results of the electrophoresis and chromatography experiments indicate that the majority of peptides produced by trypsin from guinea pig hair medulla are very similar in size and charge and will thus require techniques of extremely high resolving power, together with a greater understanding of the structure of the peptides, to achieve separation of individual components in high yield. Rogers (1962) was forced to a similar conclusion concerning porcupine quill medulla tryptic peptides.

If it is assumed that trypsin solubilizes hair medulla by the specific cleavage of peptide bonds involving the carboxyl group of lysine and arginine residues as is found for other proteins (Desnuelle, 1960), then the results of the N-terminal analyses of the tryptic peptides would suggest that in the intact protein these basic residues are commonly followed by hydrophobic residues. By similar reasoning, a basic residue is only rarely followed by a citrulline residue. The finding that the N-termini are the same for the high and low molecular weight acid-insoluble peptides and for the acid-soluble peptides serves to emphasize the complexity of these fractions.

The large number of N-terminal amino acids in the hardened cornified

protein obtained from the disrupted cells is reminiscent of the situation in wool keratin where N-terminal residues were detected using both fluorodinitrobenzene and phenyl isothiocyanate. The results showed an array of amino acids similar to that presently found for medulla protein, but quantitation showed them to be stoichiometrically insignificant (Thompson, 1957). The actual N-termini were subsequently found to be blocked by acetyl groups (O'Donnell and Thompson, 1964). Preliminary results (obtained in conjunction with D.J. Kemp and I.D. Walker) from experiments, similar to those of O'Donnell and Thompson (1964) on wool keratin, indicate that the N-termini in hair medulla protein may, in fact, be N-acetylserine.

CHAPTER 5

# ISOLATION AND CHARACTERIZATION OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK FROM GUINEA PIG HAIR MEDULLA PEPTIDES

## 5.1 INTRODUCTION

In view of the large size of the tryptic polypeptides of guinea pig hair medulla, their wide range of N termini (Chapter 4) and the insolubility of the original protein (Chapter 3) it was postulated that the proteins were covalently cross-linked. Since the insolubility of the proteins in strong reducing and dissociating agents and the low value for half-cystine (about 10 residues/1000) excludes disulphide bonds as being significant in cross-linking, a search for other cross-links was undertaken. In initial studies analyses of acid hyrolysates of samples that had been reduced with sodium borohydride by analogy with the method applied to collagen by Blumenfeld and Gallop (1966) and to elastin by Lent and Franzblau (1967), failed to reveal the presence of an aldehyde-type cross-link. However, the presence of a group blocking the ε-amino groups of some lysine residues was indicated since some of these residues failed to react when the tryptic polypeptides were treated with acrylonitrile. This technique has been recently used with success by Pisano *et al.* (1969) for the detection of ε-amino cross-links in insoluble fibrin.

This chapter reports the detection and isolation of ε-amino cross-links in the citrulline-containing protein fractions of medulla cells from hair. For identification of the cross-link, tryptic polypeptides of guinea pig hair medulla protein were extensively degraded with proteolytic enzymes and the products were separated by ion-exchange chromatography. A new compound, which initially was present as a shoulder on the trailing edge of the leucine peak, was purified and subsequently characterized as ε-(γ-glutamyl)lysine.

## 5.2 MATERIALS AND METHODS

*a. Preparation of Tryptic Peptides.* Tryptic peptides of guinea pig hair were prepared as described in Chapter 3.2*d*. The solution of peptides was fractionated at pH 3.5 according to the procedure in Chapter 4.2*a* to give the acid-soluble and the acid-insoluble tryptic peptides.

*b. Digestion with Pepsin.* The digestions with pepsin (three-times crystallized, Sigma Chemical Co., St. Louis, Mo.) were carried out in 5% (v/v) aqueous formic acid at a substrate concentration of 0.5% (w/v) and an enzyme to substrate weight ratio of 1.5:100. Incubations were for 18 - 24 hr at 37°. The reactions were terminated by freeze-drying.

*c. Digestion with Chymotrypsin.* For the preparation of chymotryptic peptides for cyanoethylation experiments, substrates and enzyme (α-chymotrypsin, Worthington Biochemical Corp., Freehold, N.J.) were incubated at 37° at a weight ratio of 1:20 in 0.2 M N-ethylmorpholine acetate (pH 8.3). The digests were freeze-dried after 26 hr. When chymotryptic digestion was used as a step in the complete enzymic digestion of the protein samples, the digestions were performed in 0.1 M Tris-Cl (pH 8.0) at an enzyme to substrate weight ratio of 1:125.

*d. Amino Acid Analyses* were performed on the Beckman 120C analyzer using either the standard 4 hr system, or the modified gradient system (Appendix A). Analyses for ε-(γ-glutamyl)lysine were performed in the gradient system using the modified gradient M4 (Appendix B). Norleucine was not used as an internal standard with the gradient system in any analysis that involved carboxyethyllysine or ε-(γ-glutamyl)lysine since all these compounds eluted in the same region. In these cases α-amino-β-guanidinopropionic acid was used as an internal standard. Synthetic γ-L-glutamic-ε-L-lysine $H_2O$ (Grade 1) and α-L-glutamic acid acetate (Grade 1) were both obtained from Cyclo Chemical Company, Los Angeles, Calif.

*e. Detection and Determination of Cross-Linking.*

i. REACTION WITH ACRYLONITRILE. This was carried out essentially according to the method of Pisano *et al.* (1969). The chymotryptic digests of the tryptic peptides were employed except in one case of hair medulla protein for which a peptic digest of the tryptic peptides was used. Samples (2 - 5 mg) were dissolved in 0.5 - 1 ml of aqueous triethylamine (3%, v/v) (Ajax Chemicals, Sydney, Australia). Acrylonitrile (0.2 ml) (BDH, Poole, England) was added and the mixtures were incubated in sealed glass tubes on a shaker at 37° for 100 - 144 hr. The contents were then evaporated twice at 50° under a stream of nitrogen and hydrolyzed for amino acid analysis. Controls used were native ribonuclease A and the chymotryptic peptides of heat-denatured chymotrypsin.

ii. COMPLETE ENZYMIC DIGESTION. For the direct determination of ε-(γ-glutamyl)lysine on an analytical level, approximately 3 mg samples of tryptic peptides were digested with pepsin and then chymotrypsin as described above. The chymotryptic digest (in 0.1 M Tris-Cl, pH 8.0) was then treated with pronase (2 mg) and $Mg^{2+}$-activated leucine aminopeptidase (0.2 mg) (hog kidney, Type III, Sigma Chemical Co., St. Louis, Mo.) as described by Pisano *et al.* (1969). Following digestion with these enzymes, the mixture was made 0.2 M with respect to NaCl and 0.1 - 0.2 mg of carboxypeptidase A (bovine pancreas, Sigma Chemical Co., St. Louis, Mo.) (solubilized according to Ambler, 1967) and 0.1 mg of carboxypeptidase B (porcine pancreas, Sigma Chemical Co., St. Louis, Mo.) were added, and the digestion was continued at 37° for a further 24 hr. Each sample was then deproteinized with 1% (w/v) picric acid (Stein and Moore, 1954). After centrifugation to remove precipitated protein, the picric acid was removed from the supernatant on a small column of Dowex 2 - X8 in 0.02 N HCl. The eluate was rotary evaporated to dryness.

*f. Isolation of ε-(γ-Glutamyl)lysine.* The acid soluble tryptic peptides of guinea pig hair medulla were used for the isolation of ε-(γ-glutamyl)lysine. Enzymic digestion of 500 mg followed that described for the analytical determinations except that only one-half the relative amounts of pronase, leucine aminopeptidase, and carboxypeptidases A and B were used. The digest was deproteinized as above, rotary evaporated and redissolved in water. A control was run which contained all the enzymes but no substrate. Another control used native ribonuclease A as a substrate.

After samples had been removed for analysis, the solution was made 0.1 N with respect to HCl and applied to a 2.4 x 117 cm column of Bio-Rad AG 50W - X8 (100 - 200 mesh) (Bio-Rad Laboratories, Richmond, Calif.) equilibrated at room temperature in 0.2 M sodium citrate buffer (pH 2.96). Elution was carried out with 0.2 M sodium citrate (pH 3.85) at a flow rate of 150 ml/hr and 10-ml fractions were collected. An aliquot from each fraction was analyzed with ninhydrin. Although most of the material eluted as a large, tailed peak, amino acid analyses of aliquots from a number of fractions showed that some purification of the isodipeptide had been achieved. Fractions thus shown to contain this compound were desalted on a small Dowex 50 - X8 column using 5 N $NH_4OH$. These eluates were rotary evaporated at 45°, dissolved in 0.1 N HCl and chromatographed on the Technicon amino acid analyzer column (Chromobeads, type A, 0.6 x 130 cm, Technicon Corp., Ardsley, N.Y.) at 60°. The column had previously been calibrated with a standard mixture of amino acids. The sodium citrate gradient M4a (Appendix B) was used. For this preparative work the citrate buffers were made up without either Brij 35 or thiodiglycol. The total effluent was collected in 3 ml fractions and aliquots were removed for analysis with ninhydrin. Fractions containing the new ninhydrin-positive peak in the position of ε-(γ-glutamyl)lysine (after leucine) were pooled, desalted, and dried.

Trace impurities arising from the citrate buffer were removed by chromatography on a 0.6 x 68 cm column of Dowex 50-X8 (-400 mesh) operated at 50 ml/hr at 50°. Elution was carried out using a linear gradient of pyridine-acetic acid from pH 2.98 (0.19 M pyridine) to pH 5.0 (2.0 M pyridine). The buffers were adapted from those developed by Schroeder *et al.* (1962). After detection with ninhydrin, fractions containing the purified material were pooled, rotary evaporated at 45° and freeze-dried.

*g. Dansylation.* Dansylation of synthetic and purified isolated ε-(γ-glutamyl)lysine was carried out using the conditions suggested by Gros and Labouesse (1969) (see Chapter 2.2*b*). The ethyl acetate extracts and residues were examined separately by both high-voltage paper electrophoresis at pH 4.40 and two-dimensional polyamide thin-layer chromatography. Samples of the dansylated compounds were also chromatographed and electrophoresed before hydrolysis.

*h. Further Characterization of Isolated ε-(γ-Glutamyl)Lysine.* In addition to the two ion-exchange systems already described for amino acid analysis, the isolated ε-(γ-glutamyl)lysine was characterized by high-voltage paper electrophoresis at pH 4.0 (Kornguth *et al.*, 1963) and descending paper chromatography in a system of 1-butanol-acetic acid-water (4:1:5, v/v).

## 5.3 RESULTS

*a. Chemical Determination of Cross-Links.* The major reaction product of protein-bound lysine and acrylonitrile is hydrolyzed to ε-N,N-bis(β-carboxyethyl) lysine (Cavins and Friedman, 1967). The elution position of this compound in the gradient system was determined using a marker prepared by reacting poly-L-lysine under the same conditions, including hydrolysis, as those used for the protein

samples. Using the routine gradient, the product eluted between, but well separated from leucine and tyrosine, *i.e.*, in the position normally occupied by norleucine (internal standard). The colour value was not calculated. The number of lysines involved in cross-links in the protein samples was therefore determined from the number of lysines remaining following alkylation with acrylonitrile and hydrolysis and the number of lysines (based on the alanine content) expected from the control hydrolysate. Table 5-I shows the results obtained for the tryptic peptides of guinea pig hair medulla. The values shown have been corrected using the average of the values found for the controls, chymotrypsin and ribonuclease, the absolute values of which are given.

*b. Enzymic Determination of Cross-Links.* Amino acid analysis of aliquots of the enzymic digestions in general showed the appearance of only one new peak in significant amounts. This peak occurred as a shoulder on the trailing edge the leucine peak using the routine gradient. By lowering the pH of the buffers in chambers 5 and 6 of the gradient (modified gradient M4) the new peak was made to appear slightly later, well resolved from all other amino acids. Chromatography of an authentic sample indicated that the new peak was indeed ε-(γ-glutamyl)lysine. The amount of this material in digests was therefore quantitated using the colour value calculated from a known amount of the authentic compound. The results are shown in Table 5-I. In the 4 hr system the new peak eluted between methionine and isoleucine.

The enzymic digest of the acid soluble hair medulla tryptic peptides showed a second significant peak which was also present in trace amounts in the other digests. This peak, which appeared as a fairly broad peak immediately before valine in analyses in the gradient system was shown by chromatography of an authentic sample to be α-L-glutamyl-L-glutamic acid. In the Beckman 4 hr system this compound eluted between valine and methionine.

TABLE 5-I: COMPARISON OF CROSS-LINKS MEASURED BY CHEMICAL AND ENZYMIC METHODS.

| | | Moles of Cross-Link/1000 Moles of Amino Acid Residues | | | | | |
|---|---|---|---|---|---|---|---|
| | | Guinea Pig Hair Medulla | | | Controls | | |
| | | Total Tryptic Digest | Acid-Insoluble Tryptic Peptides[a] | Acid-Soluble Tryptic Peptides[b] | Chymotrypsin | Ribonuclease | Enzyme |
| Chemical[c] | (1) | 16.2[d] | 20.7 | 18.3 | 2.3 | 1.2 | |
| | (2) | 17.2 | 19.9 | 17.0 | 0.9 | | |
| Enzymic | (1) | 23.0[e] | 19.9[e] | 16.7[e] | | | 0 |
| | (2) | 13.2 | 14.5 | 12.6 | | | 0 |
| | (3) | | | 15.4 | | 0 | 0 |

[a]Tryptic peptides insoluble at pH 3.5 : 12.5% (w/w) of total. [b]Tryptic peptides soluble at pH 3.5 : 87.5% (w/w) of total. [c]Values corrected by the average of control values, except for control values, which are absolute. [d]Peptic digest of tryptic peptides. [e]Not completely resolved from leucine in these analyses.

The enzymic control digest showed no trace of peaks in the positions of either of the above peaks. Neither of the peaks was affected by dialysis of the various proteins prior to enzymic digestion. The components present in only trace amounts were not investigated.

*c. Isolation and Characterization of the Cross-Link.* The ε-(γ-glutamyl)-lysine released by the complete enzymic digestion of 500 mg of acid soluble tryptic peptides of hair medulla was initially isolated by chromatography on a large Dowex 50 - X8 column using citrate buffer of constant pH and concentration. This procedure separated the isodipeptide from all the basic amino acids, tyrosine, phenylalanine, and the bulk of the acidic amino acids and citrulline. The ε-(γ-glutamyl)lysine was then purified from the major contaminant, leucine, by further chromatography on the Technicon column using a pH-concentration elution gradient of citrate buffers.

At this stage the product was essentially pure, but to remove small amounts of peptide-like material it was re-chromatographed on a Dowex 50 - X8 column using pyridine-acetic acid buffers. The product of the final purification step was pure as judged by chromatography in both the amino acid analysis systems.

The pure material cochromatographed with authentic ε-(γ-glutamyl)lysine in both analytical systems. It was identical to the synthetic material by high-voltage paper electrophoresis at pH 4.0 and by paper chromatography in 1-butanol-acetic acid-water (4:1:5, v/v). Acid hydrolysis of the isolated material in 2N HCl at 110° for 2.5 hr yielded equal amounts of glutamic acid and lysine.

The dansylated product was identical with dansylated ε-(γ-glutamyl)lysine according to two-dimensional polyamide thin-layer chromatography and high-voltage paper electrophoresis at pH 4.40. Both compounds produced a major and a minor

fluorescent spot. The nature of the minor spot has not been determined. Hydrolysates of the dansylated material showed DNS-glutamic acid and α-DNS-lysine by high-voltage paper electrophoresis.

## 5.4 DISCUSSION

The presence of ε-amino cross-links in citrulline-containing protein fractions from guinea pig hair has been demonstrated by both chemical and enzymic means. Acrylonitrile reacts with the free amino groups of amino acids to form the cyanoethyl derivatives which are converted to the acid-stable carboxyethyl derivatives by acid hydrolysis (Riehm and Scheraga, 1966; Cavins and Friedman, 1967). Thus, following reaction with acrylonitrile and hydrolysis, lysines not involved in an ε-amino cross-link would yield α-N,N-bis(β-carboxyethyl)lysine (Cavins and Friedman, 1967) whereas lysines that are involved in such a cross-link would not react and would yield free lysine (Pisano *et al.*, 1969). Therefore, the amount of lsyine remaining in such a hydrolysate is a measure of the amount of ε-amino cross-links in the original protein, provided that precautions are taken to see that the reaction of the lysines with acrylonitrile is not sterically hindered. Cross-linked lysines that are N terminal are a special case, producing α-N-(β-carboxyethyl)lysine, not free lysine, thus possibly introducing a small discrepancy. In the present work, either the peptic peptides or the chymotryptic peptides of the original tryptic peptides were used to ensure that all noncross-linked lysines were free to react. Even so, all the protein fractions tested showed the presence of nonreacting (*i.e.*, blocked) lysines (Table 5-I). Since the citrulline-containing protein fractions are rich in glutamic acid residues it seemed likely at the outset that a cross-link between the γ-carboxyls of glutamic acid residues and the ε-amino groups of the lysine residues was responsible

for the observed blockage of some of the lysine sidechains.

Definitive evidence for the nature of the cross-link has been obtained by isolation of a new compound appearing in a complete enzymic digest of hair medulla protein and its characterization as ε-(γ-glutamyl)lysine. Good agreement is seen between the values obtained by both chemical and enzymic methods (Table 5-I), indicating that all the lysines that are cross-linked are involved in the ε-(γ-glutamyl)lysine linkage.

The ε-(γ-glutamyl)lysine is present in hair medulla at very high levels compared to insoluble fibrin which has been shown by the same method to contain about 1 mole of ε-(γ-glutamyl)lysine/1000 residues (Lorand *et al.*, 1968); Matačić and Loewy, 1968; Pisano *et al.*, 1968, 1969). The value of 13 moles of ε-(γ-glutamyl)lysine/1000 moles of amino acid residues for hair medulla corresponds to 25% of the lysine residues being cross-linked. It may be noted here that the determination of cross-linked lysines in this case demonstrates the stability of the ε-(γ-glutamyl)lysine to hydrolysis by pepsin. Its resistance to several other proteolytic enzymes has already been reported (Kornguth *et al.*, 1963; Pisano *et al.*, 1969).

The presence of significant amounts of the dipeptide α-glutamylglutamic acid in the enzymic digest of the acid soluble tryptic peptides of hair medulla is interesting yet perhaps not surprising in view of the high glutamic acid content of the proteins. The occurrence of this sequence in these proteins was predicted by Rogers (1962). It is probable that only small amounts of this dipeptide are seen in the digests of the other protein fractions studied because of the higher enzyme concentrations used.

The present results show directly that citrulline-containing fractions of guinea pig hair medulla protein are cross-linked by the ε-(γ-glutamyl)lysine cross-link. The fact that no other unknown peaks were found at significant levels in analyses of enzymic digests of the protein fractions, suggests that this linkage is the major cross-link involved and is therefore, at least in part, responsible for the insolubility of these proteins.

CHAPTER 6

# OCCURRENCE OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN KERATINIZING TISSUES

## 6.1 INTRODUCTION

It has been shown in Chapter 5 that protein from the medulla cells of guinea pig hair is cross-linked by the isopeptide link, ε-(γ-glutamyl)lysine. The presence and amount of this cross-link in the medulla protein from the hairs and quills of a number of mammalian species was investigated. The presence of the cross-link in protein from inner root sheaths of guinea pig hair follicles and in keratin of guinea pig hair was also studied. The present chapter describes these studies.

## 6.2 MATERIALS AND METHODS

*a. Source of Tissues.* Hairs from seal (species unknown), possum (species unknown), red kangaroo (*Megaleia rufa*), corgi (Pembrokeshire Welsh Corgi) and camel (*Camelus dromedarius*) were a gift from Mr. J.M. O'Shea, Australian National University, Canberra. Echidna quills and all other hairs were taken from laboratory animals (Department of Zoology, University of Adelaide). The preparation of inner root sheaths from guinea pig hair follicles and African porcupine quill medulla has been described earlier (Chapter 2.1). The finely-milled hair, quill medulla or inner root sheaths were digested with trypsin in the pH-stat as described in Chapter 3.2*d*. After filtration the digests were dialyzed against distilled water for 18 hr at 4° then freeze-dried. SCM-keratin was isolated from guinea pig hair as described in Chapter 3.2*a*. The method of extraction obviated the need for tryptic digestion. The soluble SCM-feather proteins from chicken were a gift from Mr. D.J. Kemp.

*b. Determination of Cross-Link.* Cross-link in inner root sheath and porcupine quill preparations was determined by both the chemical and enzymic

methods (Chapter 5.2e). Cross-link in the other samples was determined using only a modification of the enzymic method in which carboxypeptidases were omitted and aminopeptidase M (Rohm & Haas, Darmstadt, Germany) replaced leucine aminopeptidase in some cases. All enzymic digestions were carried out in 0.2M N-ethylmorpholine acetate buffer (pH 8.3) at 37° and contained a crystal of thymol to inhibit bacterial growth. Cross-link in a particular tissue, liberated by protease digestion and determined on the amino acid analyzer, was quantitated by relating the amount to the total amino acid analysis carried out on a total acid hydrolysate. The alanine content was used as the basis for normalizing the quantitative data.

## 6.3 RESULTS

*a. Yields of Hair Medullae.* The yields of medulla solubilized by trypsin from the various hair preparations are given in Table 6-I. The high yield of material from guinea pig hair makes this tissue an ideal source of protein for the major study. No material was solubilized from samples of human head hair and merino wool, confirming the absence of medulla in these two types of hair.

*b. Amino Acid Composition of Hair Medulla.* The amino acid composition of the solubilized medullae of the hairs and quills studied is given in Table 6-II. The compositions of the other tissues studied are given in Table 6-III.

*c. Determination of Cross-Link.* The results for the determination of cross-link in the medulla of hairs or quills from ten mammalian species are presented in Table 6-IV. The values for citrulline, taken from Table 6-II, are also shown. It will be noted that in the preparation of guinea pig hair

TABLE 6-I: YIELD OF SOLUBILIZED MEDULLA FROM HAIRS.

| Species | Yield (%, v/v) |
|---|---|
| Camel | 7.3 |
| Corgi | 2.3 |
| Guinea pig | 16.0 |
| Kangaroo | 5.8 |
| Possum | 4.6 |
| Rabbit | 7.6 |
| Rat | 5.9 |
| Seal | 1.0 |

TABLE 6-II: AMINO ACID COMPOSITION OF HAIR AND QUILL MEDULLA PROTEINS[a].

| Amino Acid | Residues/1000 Residues | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Camel | Corgi | Echidna[b] | Guinea Pig | Kangaroo | Porcupine[b] | Possum | Rabbit | Rat | Seal |
| Aspartic acid | 80.2 | 92.4 | 106.3 | 60.0 | 111.3 | 59.0 | 101.6 | 71.3 | 73.1 | 104.6 |
| Threonine | 30.2 | 45.5 | 50.7 | 25.1 | 46.5 | 17.7 | 59.7 | 33.9 | 28.0 | 49.9 |
| Serine | 56.5 | 75.4 | 74.2 | 38.3 | 79.2 | 33.2 | 76.3 | 56.3 | 42.9 | 86.6 |
| Glutamic acid | 314.1 | 223.0 | 172.5 | 334.3 | 200.3 | 354.6 | 184.0 | 299.2 | 337.3 | 177.3 |
| Citrulline[c] | 66.1 | 59.1 | 4.9 | 155.3 | 59.7 | 191.4 | 21.8 | 98.3 | 122.5 | 19.2 |
| Proline | 30.9 | 25.0 | 29.9 | 26.0 | 26.8 | 13.5 | 28.8 | 23.2 | 22.4 | 41.7 |
| Glycine | 60.8 | 88.7 | 104.7 | 44.2 | 89.9 | 38.5 | 93.7 | 60.9 | 48.3 | 98.6 |
| Alanine | 47.9 | 65.1 | 81.7 | 38.0 | 68.9 | 42.0 | 69.7 | 55.9 | 37.9 | 68.2 |
| Valine | 37.3 | 51.0 | 59.7 | 30.4 | 52.8 | 36.2 | 55.2 | 36.3 | 31.3 | 54.7 |
| Half-cystine | 1.3 | 14.8 | 14.1 | 1.5 | 9.7 | 6.9 | 15.6 | 4.7 | 3.3 | 15.1 |
| Methionine | 8.5 | 17.2 | 16.3 | 8.7 | 14.3 | 4.4 | 21.2 | 16.0 | 12.5 | 14.3 |
| Isoleucine | 26.2 | 37.4 | 30.6 | 21.7 | 42.3 | 15.2 | 42.2 | 25.8 | 21.5 | 42.2 |
| Leucine | 87.1 | 91.6 | 97.9 | 88.0 | 86.8 | 79.6 | 86.7 | 90.8 | 77.4 | 82.7 |
| Tyrosine | 11.2 | 21.6 | 21.7 | 11.0 | 21.7 | 11.1 | 29.2 | 14.4 | 8.4 | 23.0 |
| Phenylalanine | 38.8 | 22.8 | 29.3 | 22.5 | 26.8 | 27.4 | 34.4 | 21.3 | 35.4 | 33.2 |
| Lysine | 46.6 | 67.7 | 60.4 | 53.4 | 41.1 | 29.8 | 40.4 | 42.9 | 40.7 | 40.4 |
| Histidine | 6.4 | 10.7 | 8.7 | 13.2 | 9.3 | 12.1 | 12.2 | 11.4 | 9.2 | 11.4 |
| Arginine | 50.3 | 22.7 | 36.7 | 34.4 | 24.6 | 27.4 | 27.9 | 37.8 | 48.0 | 28.7 |

[a]Uncorrected for hydrolytic losses, except as noted. Values are the average of at least two determinations. [b]Quill medulla. [c]Total citrulline; includes both citrulline and ornithine.

TABLE 6-III: AMINO ACID COMPOSITION OF PROTEINS FROM KERATINIZING TISSUES[a].

| | Residues/1000 Residues | | | |
|---|---|---|---|---|
| | Inner Root Sheath | SCM-Hair Keratin | SCM-Feather Medulla | SCM-Embryonic Feather[b] |
| SCM-cysteine | 0.0 | 159.8 | 73.0 | 81 |
| Aspartic acid | 93.2 | 51.7 | 60.4 | 60 |
| Threonine | 47.5 | 59.2 | 40.0 | 40 |
| Serine | 69.1 | 96.5 | 131.2 | 120 |
| Glutamic acid | 207.6 | 127.3 | 105.7 | 76 |
| Citrulline[c] | 43.7 | 0.0 | 0.0 | 0 |
| Proline | 30.5 | 52.0 | 152.0 | 113 |
| Glycine | 78.8 | 83.7 | 114.5 | 125 |
| Alanine | 62.1 | 49.7 | 59.9 | 45 |
| Valine | 44.4 | 53.8 | 64.7 | 84 |
| Half-cystine | 10.4 | 0.0 | 2.9 | 0 |
| Methionine | 44.4 | 0.0 | 0.0 | 0 |
| Isoleucine | 37.1 | 30.5 | 28.9 | 46 |
| Leucine | 88.6 | 64.0 | 63.3 | 75 |
| Tyrosine | 25.0 | 30.2 | 5.8 | 25 |
| Phenylalanine | 28.3 | 34.1 | 47.5 | 42 |
| Lysine | 43.1 | 24.1 | 4.4 | 3 |
| Histidine | 15.9 | 12.0 | 1.7 | 10 |
| Arginine | 30.5 | 71.4 | 42.2 | 55 |

[a]Uncorrected for hydrolytic losses, except as noted. [b]Analysis provided by Mr. D.J. Kemp. [c]Total citrulline; includes citrulline and ornithine.

TABLE 6-IV: DETERMINATION OF ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN HAIR AND QUILL MEDULLA[a].

| Subclass | Species | Moles/1000 Residues | |
|---|---|---|---|
| | | Cross-Link | Citrulline[b] |
| Eutheria (Placental) | Camel | 24.3 | 66.1 |
| | Corgi | 9.2 | 59.1 |
| | Guinea pig | 28.1 | 155.3 |
| | Porcupine[c] | 2.3 | 191.4 |
| | Rabbit | 25.9 | 98.3 |
| | Rat | 29.5 | 122.5 |
| | Seal | 6.6 | 19.2 |
| Metatheria (Marsupial) | Kangaroo | 5.5 | 59.7 |
| | Possum | 5.6 | 21.8 |
| Prototheria (Monotreme) | Echidna[c] | 10.1 | 4.9 |
| Controls | | | |
| | No substrate | 0 | 0 |
| | SCM-Chymotrypsin | 0 | 0 |
| | SCM-Ribonuclease A | 0 | 0 |

[a]Values are the average of at least two determinations. Duplicate determinations generally agreed within 10%. [b]Total citrulline; taken from Table 6-II.
[c]Quill medulla.

medulla used in this series of experiments the value for the cross-link is higher than that reported in Chapter 5.3. The reason for the increase is not clear; control experiments indicated that dialysis did not significantly affect the value. However, chemical determination of the cross-link using acrylonitrile showed that the number of blocked lysines in the preparations is 24.6 residues/1000 residues, a value which agrees well with the values of the cross-link determined here by the enzymic method.

The results of the cross-link determinations on inner root sheath protein, SCM-keratin and the feather proteins are given in Table 6-V. When determined by the chemical method a value of 1.3 cross-links/1000 residues was obtained for the sheath protein.

## 6.4 DISCUSSION

The amino acid composition of the trypsin-solubilized medullae of the various hairs and quills studied show an overall similarity (Table 6-II). They all contain citrulline to some extent and have a relatively high glutamic acid content and a low cystine-cysteine content, although this latter value is generally somewhat higher than that found for guinea pig hair medulla. The values obtained for rabbit hair and porcupine quill medullae compare well with those reported by Rogers (1962) and by Bradbury and O'Shea (1969). Bradbury and O'Shea (1969) have also reported a similar analysis for kangaroo hair medulla, but these workers used a different method for separating the medulla protein from keratin and have obtained higher values for cystine-cysteine than those reported here.

Cross-link was determined by a modification of the enzymic method described in Chapter 5.2e. In this work it was found that digestion by carboxypeptidases A and B could be omitted without affecting the value for

TABLE 6-V: DETERMINATION OF ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN KERATINIZING TISSUES.[a]

| Tissues | Species | Moles/1000 Residues | |
|---|---|---|---|
| | | Cross-Link | Citrulline[b] |
| Inner root sheath | Guinea pig | 2.4 | 43.7 |
| SCM-hair keratin | Guinea pig | 0.8 | 0 |
| SCM-feather medulla | Chicken | 0 | 0 |
| SCM-embryonic feather | Chicken | 0 | 0 |

[a] Values determined as for Table 6-IV. The same controls apply.

[b] Total citrulline; taken from Table 6-III.

the cross-link. This result is not unexpected, since it has been shown that pronase contains enzymes with activities similar to that of these carboxypeptidases (Narahashi and Fukunaga, 1969; Trop and Birk, 1970).

All the medulla tissues investigated contained the ε-(γ-glutamyl)lysine cross-link and all contained citrulline (Table 6-IV). As indicated in the table, the species studied included examples from the three subclasses of mammals. Four orders of the subclass Eutheria are represented. The finding of the cross-link in all these proteins suggests that the occurrence of the ε-(γ-glutamyl)lysine cross-link in hair and quill medulla of mammalian species is a general phenomenon, and strengthens the hypothesis (Chapter 5.4) that the cross-link plays a major role in the insolubility of these tissues.

It is interesting to note the relationship between the two quill medullae studied. The amino acid compositions of the proteins from porcupine and echidna quills are different, although generally they follow the same pattern (Table 6-II). The differences are highlighted by their citrulline and cross-link contents (Table 6-IV). The citrulline content of porcupine quill medulla is high and the cross-link content is low, while the reverse situation holds for echidna quill medulla.

The ε-(γ-glutamyl)lysine cross-link was not found in SCM-feather medulla or SCM-embryonic feather from chicken (Table 6-V). However, it was found in small amounts in the citrulline-containing protein from the inner root sheath cells of guinea pig hair follicles. It would be of interest to determine the relationship of the cross-link to the citrulline-containing protein filaments recently isolated from inner root sheath cells by Steinert *et al.* (1971).

Very small amounts of the ε-(γ-glutamyl)lysine cross-link were found in SCM-keratin prepared from guinea pig hair by reduction and alkylation.

The value obtained of 0.8 moles/1000 residues (Table 6-V) was about one-half the value recently obtained by Asquith *et al.* (1970) for this cross-link in oxidized merino wool keratin, and thus the present work confirms the presence of the cross-link in keratin.

The two major types of protein in hair therefore utilize different cross-links for the stabilization of the mature, hardened protein. In citrulline-containing proteins of hair medulla and inner root sheath cells of hair follicles the predominant cross-link is the presently established ε-(γ-glutamyl)lysine bond, whilst keratin proteins of the adjacent cortical cells are mainly cross-linked by disulphide bonds (Rogers, 1969) with a small amount of the isopeptide bond.

CHAPTER 7

# STRUCTURE OF HAIR MEDULLA PROTEIN: LOCALIZATION OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN PROTEINS CONTAINING CITRULLINE

## 7.1 INTRODUCTION

The presence of the ε-(γ-glutamyl)lysine cross-link has been demonstrated in citrulline-containing protein fractions derived by tryptic digestion from medulla cells of hairs and quills and from cells of inner root sheaths of guinea pig hair follicles (Chapters 5, 6). In this chapter it is demonstrated that the ε-(γ-glutamyl)lysine cross-link is localized in citrulline-containing proteins. This was achieved by the isolation of cross-linked petides that contained citrulline. Chosen fractions of tryptic peptides from guinea pig hair medulla were then digested with thermolysin and the mixture fractionated on ion-exchange resins. A number of fractions from the columns were purified and then analyzed for both citrulline and cross-link.

## 7.2 MATERIALS AND METHODS

*a. Preparation of Tryptic Peptides.* Finely-milled hair (15 g) from albino guinea pigs was digested with TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.) as described in Chapter 3.2*d*. After freeze-drying, the soluble tryptic peptides were redissolved in 100 ml bidistilled water and dialyzed for 24 hr against two changes of water. The dialyzed solution was centrifuged at 38,000 *g* for 30 min to remove a small amount of insoluble material and the supernatant freeze-dried.

*b. Digestion with Thermolysin.* For digestion with thermolysin, the tryptic peptide fraction (585 mg) was dissolved in 100 ml of 0.2M N-ethyl-morpholine acetate (pH 8.3) containing 2 x $10^{-3}$M $CaCl_2$ and 6 mg of thermolysin (Daiwa Kasei K.K., Osaka, Japan) was added. Digestion was allowed to proceed at 40$^{o}$ for 4 hr when an additional 2 mg of thermolysin was added. After a

further 3 hr the reaction was terminated by freeze-drying.

*c. Chromatography on DEAE-Sephadex.* The mixture of tryptic peptides was chromatographed on a column (4.6 x 50 cm) of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden, lot no. 1517) at 22°. After equilibration of the gel with 10mM Tris-Cl buffer (pH 7.4), the peptides were applied and eluted with starting buffer. Elution was then continued using a linear salt gradient by increasing the KCl concentration to 1M over a total volume of 5 l. of the Tris-Cl buffer. The optical density of the effluent was monitored at 230 nm. Appropriate effluent fractions were pooled, desalted by dialysis against distilled water, and freeze-dried.

*d. Chromatography on Dowex 1.* Chromatography of the thermolytic peptides was performed on a column (2 x 150 cm) of Dowex 1-X2, 100-200 mesh (Bio-Rad Laboratories, Richmond, Calif.). The column was maintained at 40° with a constant temperature circulating bath. The resin was prepared for chromatography as described by Schroeder *et al.* (1962) with pyridine-N-ethylmorpholine-acetic acid buffer (pH 9.0). The water for all the buffers used during preparation of the resin and elution of the peptides was freed of carbon dioxide by degassing under reduced pressure and bubbling with nitrogen (Schroeder *et al.*, 1962). After equilibration with pH 9.0 buffer, 580 mg of thermolytic peptides in 27 ml of buffer at pH 9.5 were flushed with nitrogen and allowed to flow into the column under gravity.

The gradient making device consisted of two measuring cylinders of 2 l. capacity. After passing 900 ml of pH 9.0 buffer through the column, gradient elution was begun. The following gradients were used sequentially, each gradient involving 1.5 l. of each solution: Gradient A, pyridine-N-ethylmorpholine-acetic acid (pH 8.0) to 0.05N acetic acid; Gradient B, 0.05N to 0.5N acetic acid and Gradient C, 0.5N to 2.5N acetic acid. Elution was

completed with 5N acetic acid. From alternate fractions, 0.2 ml was removed and analyzed by the ninhydrin method after alklaine hydrolysis (Hirs *et al.*, 1956). Appropriate fractions were pooled, concentrated to dryness in a rotary evaporator at 40° and finally freeze-dried. The peptides were solubilized with 35% (v/v) aqueous acetic acid.

*e. Purification of Dowex 1 Column Fractions.* The composition of a number of the fractions from the Dowex 1 column was assessed by peptide mapping with two-dimensional descending paper chromatography. The following solvent systems were used with Whatman 3MM paper: Solvent I, 1-butanol-glacial acetic acid-water (40:6:15, v/v) and Solvent II, 1-butanol-pyridine-glacial acetic acid-water (15:10:3:12, v/v). The peptides were located by spraying the maps with 0.15% (w/v) ninhydrin in ethanol.

Depending on the results of the maps of the fractions, further purification was accomplished by one or a combination of the following methods. (a) Chromatography on a column of cation exchange resin (Chromobeads, type P, Technicon Corp., Ardsley, N.Y.) Ten per cent of the column effluent was monitored by the peptide analyzer. (b) Descending paper chromatography using Solvent I. (c) Descending paper chromatography using Solvent II. The Whatman 3MM chromatography paper was washed extensively with 35% (v/v) aqueous acetic acid and dried before use. The peptides, located by ninhydrin on guide strips, were eluted by centrifugation with 35% (v/v) aqueous acetic acid or pyridine-N-ethyl-morpholine-acetic acid buffer (pH 9.2) (Schroeder *et al.*, 1962), using a device similar to that described by Edstrom (1968). The eluates were dried by rotary evaporation. (d) Gel filtration on a column of Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) in pyridine-N-ethylmorpholine-acetic acid buffer (pH 9.2). Elution of peptides was followed by ninhydrin estimations on aliquots of each fraction after alkaline

hydrolysis (Hirs *et al.*, 1956) and the appropriate fractions pooled, and dried in the rotary evaporator.

*f. Cross-Link Determinations.* Cross-link in purified peptides was determined by two methods (Chapter 5). The chemical method with acrylonitrile was performed as described previously and was used for quantitation of the cross-link whereas the enzymic method was used only to demonstrate that the cross-link measured was actually ε-(γ-glutamyl)lysine. The latter was a modification of the earlier work in that the peptides were dissolved in 0.2M N-ethylmorpholine acetate buffer (pH 8.3) and digested with pronase (B grade, Calbiochem, Los Angeles, Calif.) for a total of 36 hr and with aminopeptidase M (Rohm and Haas GmbH, Darmstadt, Germany) for a further 12 hr. Determination of the cross-link was performed using the amino acid analyzer as described in Chapter 5.2*d*.

*g. Digestion with Papain.* Digestions of purified peptides with papain were carried out at 37° in 100 µl of 0.2M pyridine acetate (pH 6.0) containing 5 µl of 2,3-dimercapto-1-propanol (Bornstein, 1967). Approximately 0.7 µmole of the peptides were digested using 6.5 µg mercuripapain (Sigma Chemical Co., St. Louis, Mo.).

*h. Partial Acid Hydrolysis.* Peptides (0.7 µmoles) were hydrolyzed *in vacuo* with 0.5 ml 2N HCl at 110°. After 1 hr the samples were frozen and quickly dried in the rotary evaporator.

*i. Amino Acid Analyses* of peptide hydrolysates were performed using the modified gradient system (Chapter 2.2*a*).

*j. Amino-Terminal Analysis* of peptides was carried out by using the dansyl procedure (Chapter 2.2*b*).

*k. Nomenclature.* Since a complex mixture of peptides from medulla cells

was used in these studies, the relationship of peptides to one another and to the amino acid sequence of the original proteins is not known. Therefore, the nomenclature for the peptides is based on the procedures by which they were separated and purified. The Roman numerals refer to the position of the pooled fraction from the Dowex 1 column. Fractions chromatographed on the peptide analyzer are indicated, in parentheses, by the letters PA and the position of the chromatographic peak investigated, *e.g.*, III(PA6). Peptides purified by paper chromatography were labelled sequentially from the origin (O); Arabic numbers are used for Solvent I and Greek letters for Solvent II, *e.g.*, VI/4β, V/3α. Peptides remaining at the origin in both solvents are designated 0*. Peptides derived from cross-linked peptides by papain digestion and partial acid hydrolysis are denoted by the suffixes '-Pap' and '-A' respectively. This designation is also followed by a number which describes the properties of the peptide on paper using Solvent II.

## 7.3 RESULTS

*a. Preparation of Tryptic Peptides.* An amino acid analysis of dialyzed TPCK-tryptic peptides is given in Table 3-III. An analysis of tryptic peptides produced by trypsin that had not been treated with TPCK and used in earlier work (Chapters 4, 5, 6) is also given in the table for comparison.

*b. Column Chromatography of Tryptic Peptides.* The elution pattern of the TPCK-tryptic peptides from the DEAE-Sephadex column is presented in Figure 7-1. All the material applied to the column (1200 mg) was eluted with the gradient used. No discrete separation of the bulk of material was obtained. The amino acid analyses of pooled fractions 1, 6 and 7 are given in Table 7-I. The analysis of fraction 1 was low in citrulline and thus was not considered further. Analyses of the two major fractions across the main peak

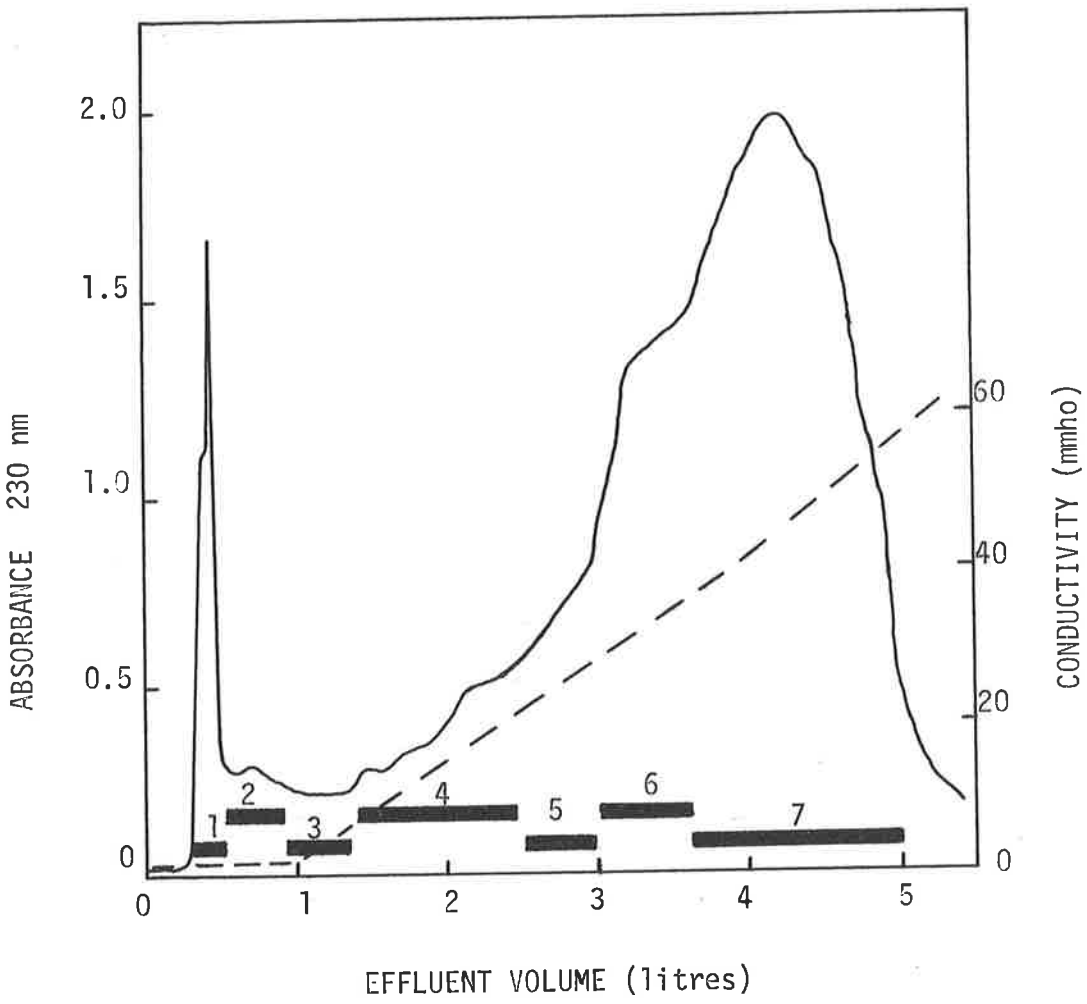



FIGURE 7-1: Elution of peptides from a tryptic digest of guinea pig hair medulla chromatographed at 22° on a column, 4.6 x 50 cm, of DEAE-Sephadex A-25. Starting buffer was 10mM Tris-Cl (pH 7.4). After 0.35 l. a linear salt gradient was commenced and the limiting salt concentration was 1M KCl. Fractions of 20 ml were collected at a flow rate of 200 ml/hr. The solid bars indicate the fractions pooled and the conductivity is shown by the dashed line.

TABLE 7-I: AMINO ACID COMPOSITION OF TRYPTIC PEPTIDE FRACTIONS.[a]

| Amino Acid | Residues/1000 Residues | | |
|---|---|---|---|
| | Fraction 1 | Fraction 6 | Fraction 7 |
| Aspartic acid | 71.3 | 95.3 | 56.8 |
| Threonine | 73.5 | 43.4 | 22.6 |
| Serine | 134.7 | 55.3 | 31.8 |
| Glutamic acid | 108.0 | 254.8 | 376.6 |
| Citrulline[b] | 22.6 | 61.9 | 191.5 |
| Proline | 58.1 | 31.8 | 0 |
| Glycine | 165.4 | 61.2 | 34.4 |
| Alanine | 66.7 | 58.8 | 27.7 |
| Valine | 64.5 | 40.8 | 19.6 |
| Half-cystine | 0 | 1.6 | 0 |
| Methionine | 9.6 | 20.9 | 0 |
| Isoleucine | 43.2 | 30.2 | 13.4 |
| Leucine | 70.7 | 86.3 | 83.8 |
| Tyrosine | 12.5 | 18.8 | 10.7 |
| Phenylalanine | 33.1 | 26.3 | 25.9 |
| Lysine | 42.4 | 52.2 | 48.8 |
| Histidine | 9.9 | 11.4 | 10.1 |
| Arginine | 13.8 | 39.7 | 35.1 |

[a] Pooled fractions from the DEAE-Sephadex column.

[b] Includes citrulline and ornithine.

shows that some fractionation had occurred. Fraction 7 is particularly rich in glutamic acid and citrulline when compared to both fraction 6 and to the unfractionated material (Table 3-III). The total absence of proline, half-cystine, and methionine in fraction 7 is to be noted. Fraction 7 amounted to 585 mg and was chosen for the thermolytic digestion.

*c. Chromatography of Thermolytic Peptides on Dowex 1.* The thermolytic digest of tryptic fraction 7 was chromatographed on the Dowex 1 column and the elution pattern is shown in Figure 7-2. The pooled fractions I - XXII are shown by the solid bars. The fractions that were selected for investigation were I, III, IV, V, XIV - XVI, XVIII, XIX and XXII. These fractions were chosen because they represent the regions of best separation on the profile and their high yield as measured by the ninhydrin assay. Each fraction was analyzed for citrulline, glutamic acid and lysine, the latter two amino acids being particularly important because they are components of the cross-link.

In the following discussion only the investigation of the fractions from which cross-linked peptides were finally obtained will be given in detail. These fractions are III, IV, V, XVIII and XIX. In all, a total of 24 peptides were purified and analyzed during the search for the cross-linked peptides. Some fractions proved to be so complex that they could not be purified.

*d. Purification of Cross-Linked Peptides.*

FRACTION III. Fraction III yielded 32 mg of peptides when freeze-dried. This fraction was not mapped but was applied directly to the peptide analyzer in two portions (16 mg) and the elution pattern is shown in Figure 7-3. Peak 6 (III/(PA6)) was subjected to preparative paper chromatography using Solvent I. In this system, the bulk of the ninhydrin positive material

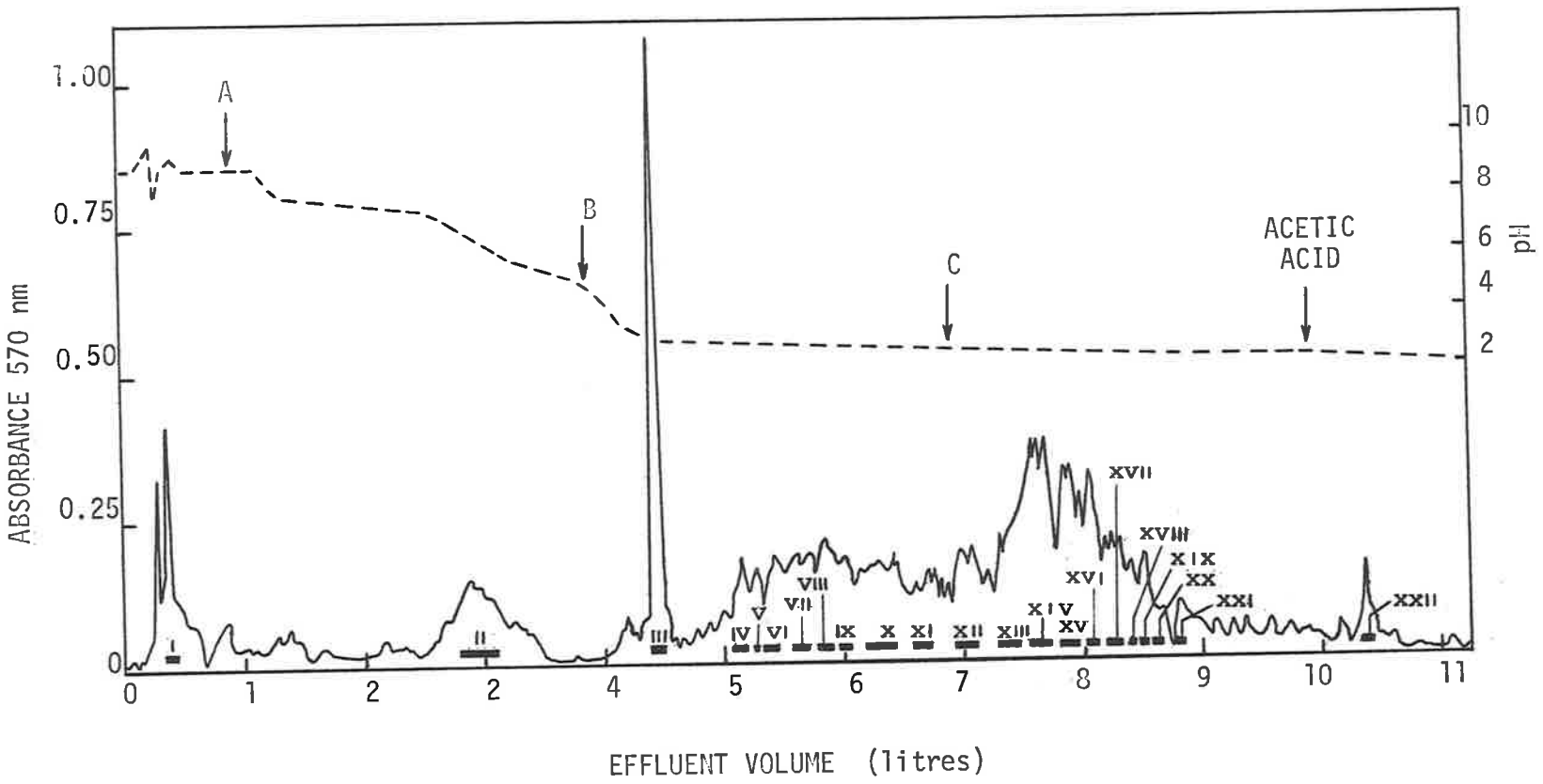



FIGURE 7-2: Fractionation of the thermolytic digest of the DEAE-Sephadex fraction 7 at 40° on a column, 2 x 150 cm, of Dowex 1-X2. Pyridine-N-ethylmorpholine-acetic acid buffer and acetic acid gradients as described in the text were applied at the points indicated. Fractions of 12.5 ml were collected at a flow rate of 150 ml/hr. From alternate fractions aliquots of 0.2 ml were analyzed by the ninhydrin method after alkaline hydrolysis. The fractions were pooled according to the solid bars and the pH gradient is shown by the dashed line.

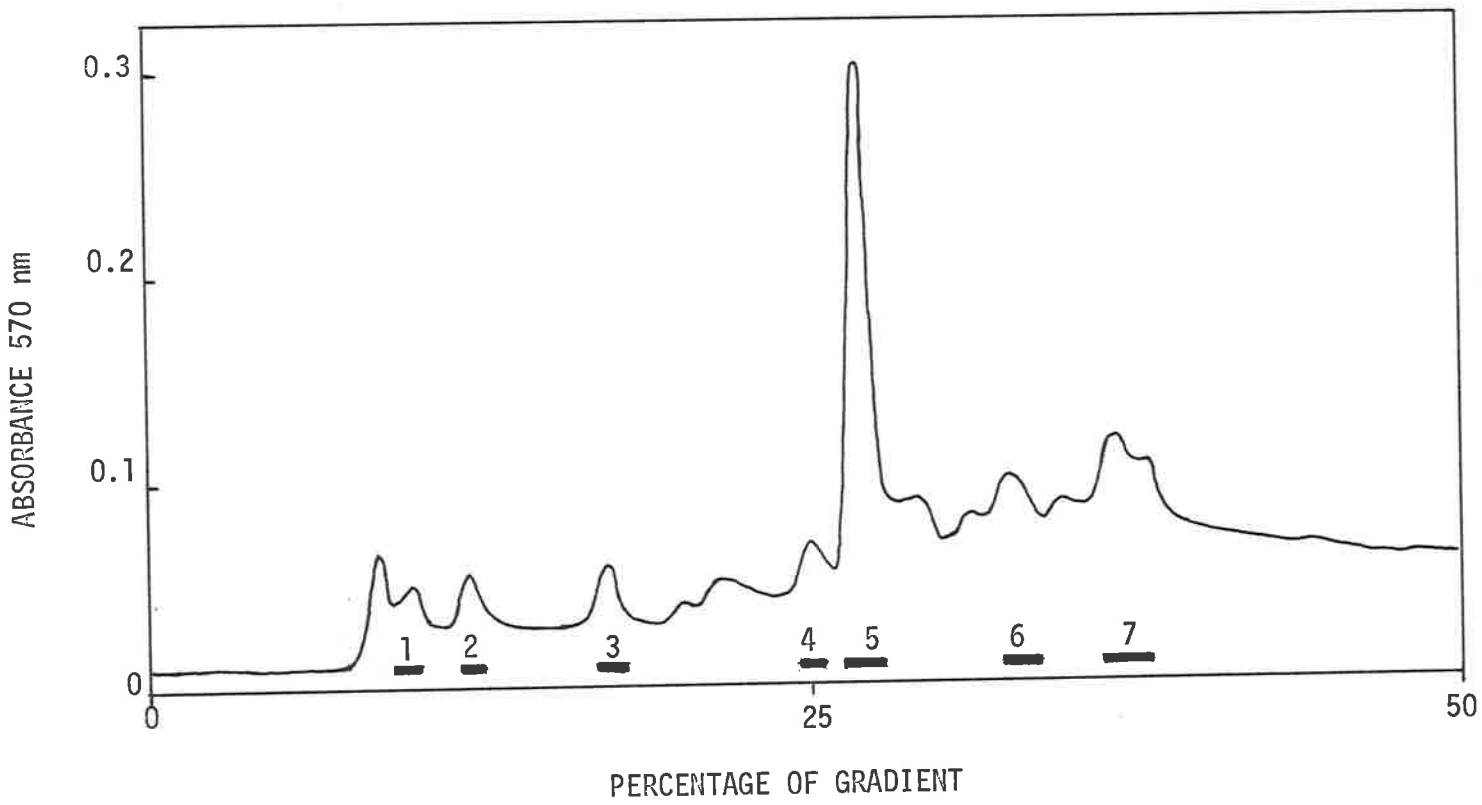



FIGURE 7-3: Cation-exchange chromatography of Dowex 1 fraction III. Conditions were: column, 0.6 x 23 cm, 60°, 30 ml/hr, pyridine acetate gradient from 0.2M pyridine (pH 3.1) to 2M pyridine (pH 6.6). The effluent was monitored by the peptide analyzer. The horizontal bars under the peaks indicate the fractions pooled.

remained at the origin and was designated Peptide III/(PA6)0. It was only slightly retarded on the Sephadex G-25 column. The amino acid analysis of the peptide is given in Table 7-II. Peaks 2-5 of the peptide analyzer separation were also investigated and two noncross-linked peptides that contained lysine, were purified. The analyses of these peptides, III/(PA4)1 and III/(PA5)α are given in Table 7-III.

FRACTION IV. Peptide mapping of this fraction showed six major components. Paper chromatography with Solvent 1 produced four bands in significant amounts. When the material from the origin (IV/0) was rechromatographed in Solvent II, it again remained at the origin and was thus designated Peptide IV/0*. The peptide eluted as a sharp peak from the Sephadex G-25 column, followed by a trace of smaller peptide material. The amino acid composition of the purified peptide is given in Table 7-II. N-terminal analysis revealed only leucine residues.

FRACTION V. The peptide map of fraction V showed that this fraction from the Dowex 1 column was relatively pure. Most of the material remained at the origin and was further purified by paper chromatography using Solvent I. The peptide eluted from the paper (Peptide V/0) was pure as judged by high voltage paper electrophoresis at pH 6.5, N-terminal analysis, gel filtration and amino acid analysis (Table 7-II). No other peptides were isolated in significant yields from this fraction.

FRACTION XVIII. Fraction XVIII also showed only one major component at the origin when mapped. Purification through paper chromatography in both solvents and gel filtration yielded Peptide XVIII/0*. The composition of this peptide is given in Table 7-II.

FRACTION XIX. Fraction XIX contained at least five components as revealed by peptide mapping. Paper chromatography with Solvent I produced three major bands. The peptide material that remained at the origin, and which

TABLE 7-II: AMINO ACID COMPOSITION OF CROSS-LINKED PEPTIDES.[a]

| | III/(PA6)0 | IV/0* | V/0 | XVIII/0* | XIX/0 |
|---|---|---|---|---|---|
| Aspartic acid | 1.20 (1) | 0.77 (1) | 0.59 (1) | 0.91 (1) | 0.71 (1) |
| Threonine | 0.28 | | | | |
| Serine | | 0.22 | | | |
| Glutamic acid | 14.68 (15) | 14.92 (15) | 13.42 (13) | 15.39 (15) | 10.02 (10) |
| Citrulline[b] | 9.95 (10) | 5.18 (5) | 6.18 (6) | 10.22 (10) | 9.45 (9) |
| Alanine | 0.36 | 0.31 | | 0.21 | |
| Valine | 0.24 | 0.22 | | | |
| Leucine | 1.96 (2) | 1.84 (2) | 1.92 (2) | 2.09 (2) | 1.91 (2) |
| Phenylalanine | | | | | 0.23 |
| Lysine | 2.57 (2) | 2.03 (2) | 2.02 (2) | 1.46 (1) | 1.08 (1) |
| Histidine | 0.65 (1) | 0.40 | 0.30 | | |
| Arginine | 1.16 (1) | 2.38 (2) | 2.09 (2) | | |
| Total residues | (32) | (27) | (26) | (29) | (23) |
| Cross link[c] | 0.94 (1) | 2.00 (2) | 1.17 (1) | 0.91 (1) | 0.70 (1) |
| N Terminal | n.d. | Leucine | Leucine | n.d. | Leucine |

[a] Values reported are amino acid residues per peptide after corrections for impurities in a 'paper blank' analysis. Values of 0.2 or less are omitted. Estimated number of residues are in parentheses.

[b] Includes citrulline and ornithine.

[c] Determined using the chemical method.

TABLE 7-III: AMINO ACID COMPOSITION OF NONCROSS-LINKED PEPTIDES FROM FRACTION III[a].

| | III/(PA4)1 | III/(PA5)α |
|---|---|---|
| Aspartic acid | 0.96 (1) | - |
| Glutamic acid | 2.94 (3) | 4.80 (5) |
| Citrulline[b] | 5.09 (5) | 3.84 (4) |
| Leucine | 0.88 (1) | 1.02 (1) |
| Lysine | 0.97 (1) | 0.95 (1) |
| Total residues | (11) | (11) |

[a]Values reported are amino acid residues per peptide after correction for impurities in a 'paper blank' analysis. Values of 0.2 or less are omitted. Estimated number of residues are in parentheses.

[b]Includes citrulline and ornithine.

had been well separated from the rest of the components, was again isolated. This was chromatographed on the Sephadex G-25 column and the Peptide XIX/0 eluted as a sharp peak and showed only a trace of contaminating lower molecular weight material. The amino acid analysis of the purified peptide is presented in Table 7-II.

*e. Amino Acid Composition of Peptides Derived from Cross-Linked Peptides.* Peptides IV/0* and XIX/0 were digested with papain and the products were analyzed by paper chromatography using Solvent II. Only peptides present in sufficient yield to obtain an amino acid analysis are reported (Table 7-IV). Note that Peptide XIX/0-Pap1 contains a lysine residue.

Peptides IV/0*, V/0 and XIX/0 were subjected to partial acid hydrolysis and the peptides obtained were separated as for the papain peptides. The composition of these derived peptides is given in Table 7-V.

## 7.4 DISCUSSION

In previous work, peptide-bound citrulline was found to be present in all the tryptic digests of hair medulla tissue in which the ε-(γ-glutamyl)lysine cross-link was determined (Chapter 6). However, to establish that the isopeptide cross-link was present in polypeptide chains that also contained citrulline residues it was necessary to isolate appropriate peptides from the heterogeneous mixture of tryptic digestion products. The present isolation from guinea pig hair medulla tissue of such peptides unequivocally demonstrates that the cross-link has an abundant occurrence in proteins of this tissue that contain citrulline. On the other hand, it is still possible that the cross-link is present in a protein component or components that do not contain citrulline residues. This possibility has not been subjected to study.

TABLE 7-IV: AMINO ACID COMPOSITION OF PAPAIN PEPTIDES[a].

| | IV/0*-Pap2 | XIX/0-Pap1 | XIX/0-Pap2 | XIX/0-Pap5 | XIX/0-Pap6 |
|---|---|---|---|---|---|
| Aspartic acid | | 0.82 (1) | | | |
| Glutamic acid | 2.69 (3) | 8.60 (9) | 1.23 (1) | 0.73 (1) | 0.87 (1) |
| Citrulline[b] | 1.07 (1) | 4.48 (4) | 1.00 (1) | 1.19 (1) | |
| Glycine | 0.55[c] | 0.26 | | | |
| Leucine | | 0.26 | | 0.84 (1) | 1.00 (1) |
| Lysine | 0.27 | 0.89 (1) | | | |
| Arginine | 0.93 (1) | | | | |
| Total Residues | (5) | (15) | (2) | (3) | (2) |

[a] Values reported are amino acid residues per peptide after correction for the 'blank' analysis. Values of 0.2 or less are omitted. Estimated number of residues is given in parentheses.

[b] Includes citrulline and ornithine.

[c] Contaminant arising from chromatography paper: not present in original cross-linked peptide IV/0* (Table 7-II).

TABLE 7-V: AMINO ACID COMPOSITION OF PEPTIDES DERIVED BY PARTIAL ACID HYDROLYSIS[a].

| | IV/0*-$A_2$ | IV/0*-$A_3$ | V/0-$A_2$ | V/0-$A_3$ | V/0-$A_8$ | XIX/0-$A_2$ | XIX/0-$A_3$ | XIX/0-$A_6$ | XIX/0-$A_7$ |
|---|---|---|---|---|---|---|---|---|---|
| Aspartic acid | 0.79 (1) | | 0.91 (1) | | | 1.15 (1) | | | |
| Serine | | | | 0.26 | | | | | |
| Glutamic acid | 2.95 (3) | 7.00 (7) | 4.45 (4) | (8)[c] | 0.60 (1) | 3.61 (4) | 2.69 (3) | 0.95 (1) | 0.61 (1) |
| Citrulline[b] | 1.08 (1) | 1.69 (2) | 1.77 (2) | 2.04 (2) | | 2.18 (2) | 0.81 (1) | 1.20 (1) | |
| Glycine | | | | 0.54 | | | 0.22 | | |
| Leucine | | | | | 1.00 (1) | | | 0.76 (1) | 1.00 (1) |
| Lysine | 1.10 (1) | 0.22 | 1.28 (1) | | | 0.83 (1) | | | |
| Arginine | 0.87 (1) | 0.95 (1) | 0.94 (1) | 1.00 (1) | | | | | |
| Total residues | (7) | (10) | (9) | (11) | (2) | (8) | (4) | (3) | (2) |

[a]Values reported are amino acid residues per peptide after correction for the 'blank' analysis. Values of 0.2 or less are omitted. Estimated number of residues is given in parentheses. [b]Includes citrulline and ornithine. [c]Peak off-scale in analysis: estimate only.

The hydrolysates of a large number of purified peptides obtained from a thermolytic digest of tryptic peptides of the medulla protein were analyzed for the components of the cross-link (glutamic acid and lysine) as well as citrulline. Peptides containing these amino acids were then examined for the presence of the intact cross-link. At the present time, this stochastic approach is the only method that can be applied to the detection of such peptides because there are no means of specifically labelling the cross-link for easy identification. By way of comparison, studies on this cross-link in fibrin have shown it possible to label the acceptor (glutamine) sites in chains of the noncross-linked protein by the enzymic incorporation *in vitro* of [$^{14}$C]glycine ethyl ester (Chen and Doolittle, 1969; Lorand and Chenoweth, 1969). In this way these workers were able to identify which fibrin chains are involved in cross-linking. Chen and Doolittle (1970) were able to isolate and determine the amino acid sequence of the peptides involved. However, noncross-linked precursors of the medulla proteins have not been isolated. Furthermore, the number, size, and composition of the constituent chains in the cross-linked proteins is not known.

In retrospect, it may have been useful to have isolated by molecular sieving a high molecular weight fraction of peptides as was done by de Luque *et al.* (1970) in their study of collagen peptides containing the cross-link, hydroxylysinonorleucine. Analyses of peptides purified during the present study but not reported here, have shown that it is not invariably true that the cross-linked peptides are larger than noncross-linked ones. Moreover, it is desirable to obtain as small a peptide as possible since small peptides are more easily characterized. During the course of the work, it was found that the cross-linked peptides tended to remain at the origin in the paper chromatographic systems used. The explanation is not readily apparent but it was not entirely a size effect. The adoption of a wider range of

separation solvents was not possible. This stemmed from the serious limitations imposed by the quantities of partially purified peptide fractions containing both the cross-link and citrulline and the necessity for frequent amino acid analysis during the course of purification.

The amino acid analyses of the cross-linked peptides isolated (Table 7-III) show that they are similar in both size and composition. It is clear that they are unique in their extraordinarily high content of glutamic acid (and/or glutamine) and citrulline. The main contaminants from the chromatography paper, and for which the analyses have been corrected were serine, glutamic acid and glycine. This contamination problem has been noted by other workers (see, for example, Stevens *et al.*, 1967) and is particularly accentuated when operating the analyzer at high sensitivity with low levels of peptides, as as in the present case. The values reported for cross-link in the peptides were measured by a chemical method which actually measures ε-amino-blocked lysines (Pisano *et al.*, 1969, see also Chapter 5). The results were confirmed in some cases by direct measurement, on the amino acid analyzer, of ε-(γ-glutamyl)lysine released by extended protease digestion. The yields of cross-link from the enzyme method were generally low, probably due to incomplete hydrolysis as a result of the high glutamic acid content of the peptides. Chen and Doolittle (1970) have also reported low yields when they investigated the cross-link in this way in the peptides from fibrin.

It is not practicable to sequence cross-linked peptides unless some means of obtaining the peptides in the noncross-linked form is available (Chen and Doolittle, 1970). Consequently, other procedures were investigated in an attempt to more precisely define the location of the cross-links in the peptides. Thus papain was chosen to fragment some of the original peptides since α-N-benzoyl-L-citrulline methyl ester has been shown to be an excellent substrate for this enzyme (Cohen and Petra, 1967) and all the peptides were

rich in citrulline. Only one of the derived peptides investigated (Peptide XIX/0-Papl, Table 7-V) contained a lysine residue and was therefore assumed to be cross-linked, since the cross-link is not hyrolyzable by papain (Kornguth *et al.*,1963). Although this peptide is reduced in size by eight residues, five of which are citrulline, it is still too large to identify the residues that are immediately adjacent to the cross-link. The amino acid composition remains similar to that of the parent peptide (Table 7-III) and it is clear that the cross-link is located in an environment that is rich in glutamic acid and citrulline residues. The other papain peptides derived from Peptide XIX/0 do not give any further information.

Peptides IV/0*, V/0 and XIX/0 were also subjected to partial acid hydrolysis in an attempt to preferentially hydrolyze the cross-link with a minimum of main chain breakage. The HCl concentration (2N) and conditions used were based on the studies of Kornguth *et al.* (1963) on the hydrolysis of the ε-(γ-glutamyl)lysine bond, and on preliminary experiments in this laboratory using the pure isodipeptide. The composition of the peptides obtained (Table 7-V) show that there was, however, some α-peptide bond cleavage. The high content of glutamic acid/glutamine residues in these smaller peptides further indicated the tendency of these residues to occur in groups, *e.g.*, Peptides IV/0*-A3, V/0-A2 and V/0-A3.

Some structural features of the cross-linked medulla peptides characterized in the present study can be inferred from the known specificity of the enzymes used to produce them (Table 7-II). The N-terminal leucines of peptides IV/0*, V/0 and XIX/0 are expected as a result of thermolytic digestion (Matsubara *et al.*, 1966) and it can be assumed that the leucine residues would also be N-terminal in Peptides III/(PA6)0 and XVIII/0*. Similarly, lysine residues in a peptide that are not accounted for by the cross-link can be assumed to be C-terminal as a result of the tryptic digestion of the intact

protein. Thus, in Peptide III/(PA6)0 the C-terminal residues are lysine and arginine whilst in Peptide IV/0* they are both arginine. Peptides XVIII/0* do not have any particular residue that can be unequivocally assigned to the C-terminal position. These peptides could therefore have been C-terminal in the original proteins or they could derive from a large tryptic peptide by thermolytic hydrolysis at several sites to produce new N and C termini. Peptide V/0 contains one extra basic residue which cannot be accounted for as C-terminal or cross-link. It may be either one of the lysine residues or one of the arginine residues, and has presumably arisen from the incompleteness of the initial tryptic digestion. This may well result from the proximity to this basic residue of glutamic acid residues. For example, Keresztes-Nagy *et al.* (1969) found that a Lys-Glu bond in alfalfa ferredoxin was resistant to tryptic hydrolysis because of an adjacent Glu-Glu-Glu sequence.

Each of the cross-linked peptides studied contained two N-terminal leucine residues and therefore consisted of two chains. On the present evidence no conclusions can be drawn as to whether the cross-link in the native protein is *inter-* or *intra*chain or *both*. Only a small proportion of the total cross-linked peptides have been examined, and in any case the proteolytic preparation of the peptides could convert an intrachain link to one that is apparently between chains.

CHAPTER 8

# FORMATION OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN HAIR PROTEINS. INVESTIGATION OF TRANSAMIDASES IN HAIR FOLLICLES

## 8.1 INTRODUCTION

Fibrin is insolubilized by the formation of ε-(γ-glutamyl)lysine cross-links which are produced by the action of plasma transamidase (activated Factor XIII). As described in Chapter 1.5, a number of transamidases from different mammalian tissues also have fibrin cross-linking activity. Following the finding that the ε-(γ-glutamyl)lysine cross-link occurs extensively in hair proteins, particularly the medulla proteins (Chapter 6) it was of interest to determine whether transamidase and fibrin cross-linking activity was present in hair follicle tissue. Although a number of studies of enzymes in wool roots and hair follicle tissue have been made (Ellis *et al.*, 1950; Rogers, 1959; Rogers and Springell, 1959) transamidase activity has not previously been investigated.

In this chapter experiments are reported which demonstrate the presence in hair follicle tissue of an enzyme which has properties similar to those of plasma transamidase. These properties include the incorporation of [$^{14}$C]GEE into casein by forming γ-glutamyl derivatives and the cross-linking of fibrin clots. However, the follicle enzymes can be distinguished from plasma transamidase.

## 8.2 MATERIALS AND METHODS

*a. Preparation of Hair Follicle Enzymes.* Albino guinea pigs of 4-5 weeks of age were used as a source of follicle tissue unless otherwise noted. The skin of a guinea pig was removed taking particular care to keep the skin free of blood. Hair follicles were exposed by the wax method (Rogers and Clarke, 1965). The follicle cells were collected by brushing with 15 ml of a cold solution of 5mM Tris-Cl buffer (pH 7.1) containing 2mM EDTA. The resulting brei was homogenized with 20 tight strokes in a Dounce homogenizer

and centrifuged at 38,000 *g* for 20 min to remove inner root sheaths and hair fibres. The supernatant was further centrifuged at 100,000 *g* for 90 min, and this high-speed supernatant was dialyzed for 4 hr at 4° against two changes of Tris-EDTA buffer (pH 7.1). The dialyzed homogenate was used as the crude enzyme preparation and usually contained 20 - 30 mg of total protein.

*b. Purification of Plasma Transamidase.* Guinea pig blood was allowed to clot at room temperature for 2 hr. The serum was collected by centrifugation and was cooled to 4°. An equal volume of cold, saturated ammonium sulphate solution was added, and the mixture stood at 2° for 1 hr. The precipitate was collected by centrifugation, redissolved in distilled water, and dialyzed against two changes of 0.0175M sodium phosphate buffer (pH 7.2) for 1 hr. The dialyzed solution was then chromatographed on a column of DEAE-cellulose essentially according to Loewy *et al.* (1961a). The protein fraction eluted by 0.20M sodium phosphate buffer (pH 7.2) contained the transamidase activity and was dialyzed against the Tris-EDTA solution used for the hair follicle enzyme preparations.

*c. Enzyme Assays.* Fibrin cross-linking activity was determined semi-quantitatively using the system described by Tyler and Laki (1966). Clot solubility was assessed visually at 20 and 60 min after stopping the reaction with urea solution.

Coupling of hydroxylamine to CBZ-L-glutaminylglycine was assayed by the method of Folk and Cole (1965) as modified by Tyler and Laki (1966).

Transamidase activity was assayed by the incorporation of [$^{14}$C]GEE (New England Nuclear, Boston, Mass.) into casein as described by Tyler and Laki (1967) except that a 2% casein solution was used.

*d. Preparation of Purified Fibrinogen Substrate.* Human fibrinogen

(Cohn fraction number I-4, Batch No. 827, Commonwealth Serum Laboratories, Melbourne, Australia) was treated to remove transamidase activity by using specific precipitation of the contaminating Factor XIII with rabbit anti-Human Factor XIII-serum (Batch No. 1494 H, Behringwerke AG, Marburg, Germany). The fibrinogen was dissolved in 0.2M borate-saline (pH 7.8) to a concentration of 5 mg/ml (Tyler and Laki, 1966). Antiserum, as determined by preliminary experiments to be just sufficient to remove all activity, was added and the mixture incubated at 37° for 4 hr then at 4° for 12 hr. The antibody-antigen complex was removed by centrifuging at 100,000 *g* for 1 hr. The purified fibrinogen did not form an insoluble clot after clotting in the presence of $Ca^{2+}$ and cysteine under the conditions of the assay.

*e. Preparative Incorporation of [$^{14}$C]GEE into Casein.* The following mixture was used to incorporate [$^{14}$C]GEE into casein: 50 mg of casein; 50 µCi [$^{14}$C]GEE (300 µmoles); 100 µmoles $CaCl_2$ and 30 µmoles reduced glutathione all in 4 ml of 0.1 M Tris-Cl buffer (pH 7.5). Thymol was added to inhibit bacterial growth. Enzyme extract (2.5 mg protein in 5 ml), prepared from rat hair follicles, was added and the mixture was incubated at 37° for 12 hr. A further 3 ml of enzyme extract (0.8 mg protein) was added and the incubation continued for a further 24 hr. The labelled casein was precipitated, washed and dried according to Matačić and Loewy (1968). The dried casein was redissolved in 2 ml of 0.2M N-ethylmorpholine acetate buffer (pH 8.3) and digested sequentially with α-chymotrypsin, pronase and leucine aminopeptidase for a total of 163 hr (Chapter 5). The digestion mixture was deproteinized by the picric acid procedure (Stein and Moore, 1954), and dried in the rotary evaporator.

A preliminary purification of the deproteinized digestion mixture was

performed on a column of Bio-Rad AG50W-X8 (100 - 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) equilibrated at 45° with 0.1M pyridine acetate buffer (pH 2.95). The column was loaded and then washed with this buffer and the radioactive material was eluted with 1M pyridine acetate (pH 5.0). The column effluent was monitored for radioactivity by dissolving aliquots from each fraction in dioxane scintillation fluid (Bray, 1960) and counting in the Packard Scintillation Spectrometer. Additional column purification of the radioactive fraction was carried out on a column of Technicon Chromobeads, type P (Technicon Corp., Ardsley, N.Y.). The column was operated at 45° using 0.2M pyridine acetate buffer (pH 3.1). Fractions were collected and 1% of the effluent was monitored by the peptide analyzer. Aliquots from the fractions were counted for radioactivity as before. This purification step was repeated for the major radioactive peak. At this stage a sample was removed, hyrolyzed with 6N HCl at 110° for 12 hr, and analyzed on the Beckman 4-hr amino acid analysis system using the expanded scale recorder.

The remainder of the labelled material was further purified by high-voltage electrophoresis on acid-washed Whatman 3MM paper. The electrophoresis was performed on the flatplate apparatus at 50 v/cm for 100 min using pyridine acetate buffer (pH 6.5) (Michl, 1951). After drying, a strip of the paper was cut into pieces 1 cm square and counted in the scintillation counter using a toluene scintillation fluid containing 0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazolyl)benzene. The labelled material from the area thus located was eluted with 0.02N acetic acid, hydrolyzed as above, and analyzed for amino acids.

*f. Chromatography of Follicle Homogenate on Anti-Factor XIII-Serum-Sepharose Column.* Purification of the transamidase activity in follicle extracts was undertaken using affinity chromatography. Thus, anti-Factor XIII-

serum (50 mg protein, 1 ml) was coupled with Sepharose 4B (20 ml, lot no. 4969, Pharmacia, Uppsala, Sweden) according to Cuatrecasas *et al.* (1968). Approximately 45% of the serum protein bound to the support. Chromatography was performed at 22° using 0.0175M sodium phosphate buffer (pH 7.2) and approximately 5 mg of follicle protein in 2 ml was loaded. The unadsorbed follicle protein was washed from the column with the phosphate buffer and then the follicle protein that was bound to the column was eluted using 0.2M glycine-HCl buffer (pH 2.2) (Avrameas and Ternynck, 1967). Acidic fractions were neutralized with NaOH and separately dialyzed against 0.0175M sodium phosphate buffer (pH 7.2) before activity was assayed. Pooled fractions were concentrated in a collodion bag (Sartorius-Membranfilter GmbH, Göttingen, Germany) at 2°.

*g. Immunodiffusion Procedure.* Double diffusion in 1% agar gel was carried out by the method of Ouchterlony (1962) in 0.1M sodium phosphate (pH 7.4) containing 0.16M NaCl. The precipitin lines were stained with Coomasie Blue in 10% trichloroacetic acid (Chrambach *et al.*, 1967).

*h. Protein Determinations.* The protein content of enzyme preparations was determined by the method of Lowry *et al.* (1951) modified as reported by Bailey (1967) using bovine serum albumin as standard. Protein in column effluents was determined from the absorbancy at 280 nm.

## 8.3 RESULTS

*a. Transamidase and Fibrin Cross-Linking Activity.* Enzyme preparations from guinea pig hair follicles generally contained 20 - 30 mg of total protein per animal and readily incorporated labelled GEE into casein (Figure 8-1). For guinea pig preparations the incorporation was linear with time for at

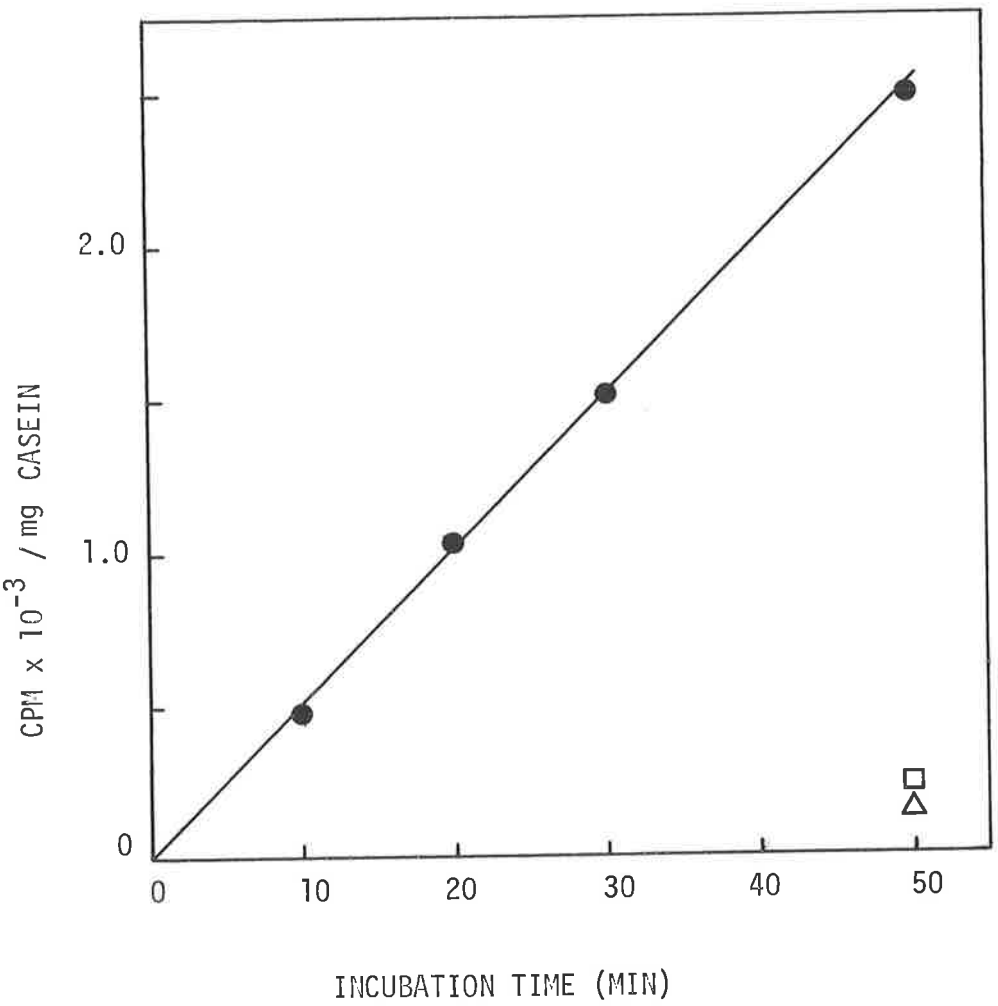



FIGURE 8-1: Incorporation of [$^{14}$C]GEE into casein by guinea pig hair follicle homogenate. The reaction mixture contained 10 µmoles of $CaCl_2$ or EDTA, 5 µmoles of reduced glutathione or 1 µmole of iodoacetate, 5 µCi (10 µmoles) of GEE, 2 mg of casein, and 1.2 mg of total follicle protein made up to 1 ml with 0.2M Tris-Cl buffer (pH 7.9). Aliquots were removed at various times and the amount of GEE incorporated was determined as described by Tyler and Laki (1967). Complete assay (●); + Iodoacetate (□); + EDTA (Δ).

least 50 min. The reaction was inhibited by iodoacetate and EDTA (Figure 8-1). It will be noted that the enzyme does not require preliminary activation by thrombin. The transamidase activity could be partially purified by the method of Clarke *et al.* (1959) but this procedure was not routinely employed. The guinea pig hair preparation did not couple hydroxylamine to CBZ-L-glutaminylglycine; nevertheless, as little as 20 µg of total protein from such preparations could stabilize a 1 mg fibrin clot. Both the transamidase and the fibrin cross-linking activity in these follicle preparations were inhibited by the anti-Factor XIII-serum. The antibody-enzyme complex did not precipitate and it was found sufficient to merely add the antiserum to the enzyme. This is in sharp contrast to the reaction of the antiserum with the human Factor XIII contamination in the fibrinogen substrate in which case the antibody-antigen complex had to be removed from the solution to remove the activity.

Rat hair follicle preparations had very similar properties to those from the ginea pig. However, the properties of enzyme preparations from wool follicles of a cross-breed merino sheep were very different. Although such preparations incorporated [$^{14}$C]GEE into casein and also cross-linked fibrin, the transamidase activity was not inhibited by the addition of EDTA, *i.e.*, the removal of calcium ions from the reaction mixture (Figure 8-2). Furthermore, iodoacetamide inhibited only in the presence of calcium, and the enzyme did not cross-react with the anti-Factor XIII-serum. Neither the rat nor the sheep enzyme required activation by thrombin.

*b. Binding Site of [$^{14}$C]GEE in Casein.* The labelled casein prepared using rat follicle homogenate had an activity of 40,000 cpm/mg before the enzymic digestion. On the peptide analyzer the bulk of the labelled material eluted as the first peak, but not completely resolved from the large aspartic

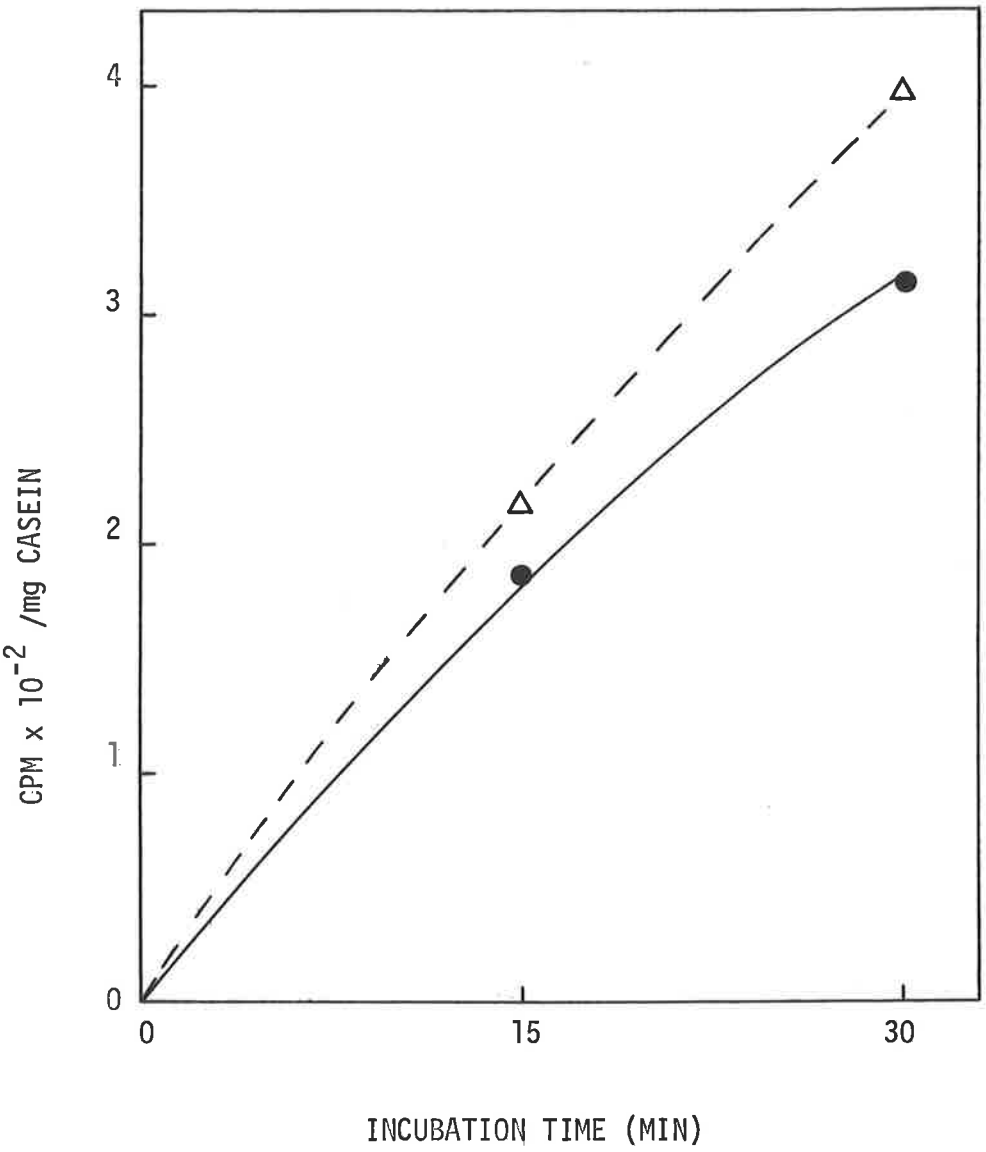



FIGURE 8-2: Incorporation of [$^{14}$C]GEE into casein by a homogenate of cross-breed merino wool follicle tissues. Total follicle protein added was 200 µg. Experimental details as for Figure 8-1. Complete assay (●); + EDTA (Δ).

acid peak. A minor radioactive peak corresponded to the glycine peak. Amino acid analysis at this stage showed that the product was contaminated by several amino acids, particularly aspartic acid and methionine (Table 8-I). On electrophoresis the radioactivity migrated as a single spot just behind aspartic and glutamic acid, well clear of the neutral amino acids. This purification step produced a large reduction in the amount of aspartic acid present while the glycine and glutamic acid were still in essentially equal amounts (Table 8-I). The chromatographic properties of the labelled compound were comparable to those of γ-glutamylglycine as reported by Matačić and Loewy (1966).

*c. Chromatography on the Anti-Factor XIII-Serum-Sepharose Column.* When a guinea pig hair follicle preparation was chromatographed on the anti-Factor XIII-serum-Sepharose column the transamidase and fibrin cross-linking activity was bound to the column and was eluted with the acid buffer (Figure 8-3a). Preliminary experiments had shown that the enzyme was stable in this buffer at room temperature for at least 1 hr. When an enzyme preparation was chromatographed on a column of Sepharose 4B (no antiserum) the enzyme activity eluted with the bulk of the protein at the void volume of the column (Figure 8-3b), indicating that binding to the antiserum column was specific.

*d. Immunodiffusion.* Only guinea pig follicle preparations were analyzed by immunodiffusion. The plasma transamidase used for comparison was prepared from fresh guinea pig serum and partially purified on the DEAE-cellulose column as described in Section 8.2*b*. This method of preparation of plasma transamidase has the advantage that it is simple and rapid and the enzyme is obtained in the activated form ready for assay. Problems can occur from over-digestion by thrombin (Kiesselbach and Wagner, 1966) so only short clotting times were employed in the preparation of the serum.

TABLE 8-I: AMINO ACID COMPOSITION OF LABELLED DERIVATIVE BEFORE AND AFTER ELECTROPHORESIS.[a]

| Amino Acid | Before | After |
|---|---|---|
| Aspartic acid | 7.06 | 0.35 |
| Threonine | 0.42 | - |
| Serine | 0.49 | - |
| Glutamic acid | 1.50 | 0.99 |
| Glycine | 1.00 | 1.00 |
| Alanine | 0.23 | - |
| Methionine | 4.14 | - |

[a]Values are expressed relative to the glycince content: glycine = 1.00.

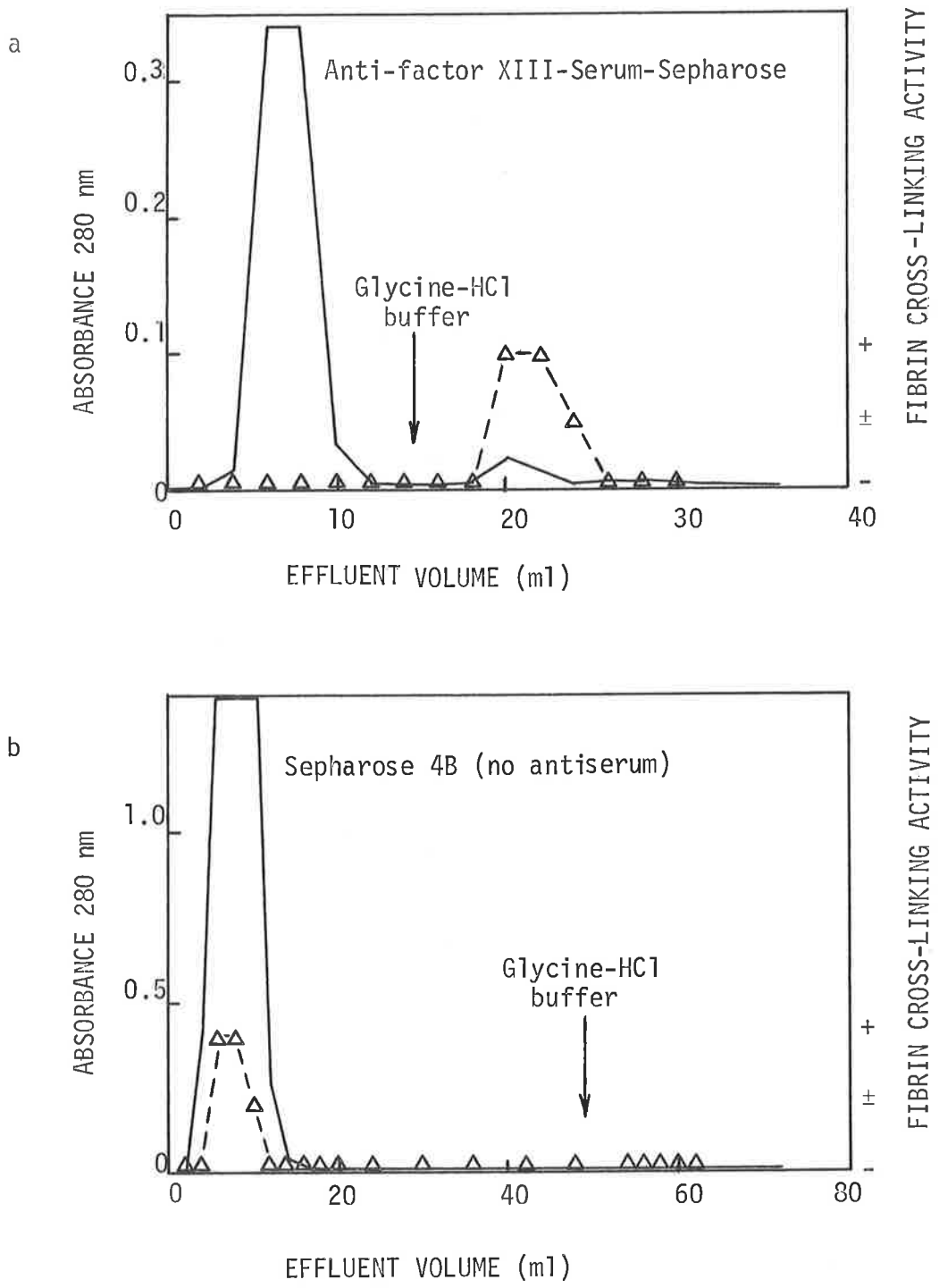



FIGURE 8-3: Chromatography of guinea pig hair follicle homogenate preparations on Sepharose columns. Columns were 1 x 9 cm and were operated in 0.0175M sodium phosphate buffer (pH 7.2). Bound follicle protein was eluted with 0.2M glycine-HCl buffer (pH 2.2). Fibrin cross-linking (Δ---Δ) was measured semi-quantitatively as described in Materials and Methods (Chapter 8.2*c*). (a) Sepharose 4B to which anti-Factor XIII-serum had been coupled. (b) Underivatized Sepharose 4B.

Immunodiffusion of hair follicle enzyme and plasma enzyme was carried out over a wide range of concentrations of both the antiserum and the enzymes. In no case did a follicle preparation give a precipitin line, even when a preparation purified on the anti-Factor XIII-serum-Sepharose column was used (Figure 8-4). The minor band sometimes seen for plasma enzyme is continuous with a band between the enzyme well and the well containing normal rabbit serum (control), and is therefore not due to a specific antibody.

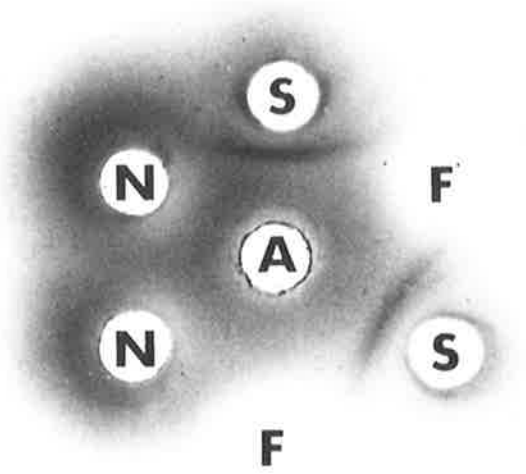
## 8.4 DISCUSSION

Transamidases catalyze the replacement of the carboxyamide groups of protein-bound glutamine or asparagine residues by another amine group, with the concomitant release of ammonia (Clarke *et al.*, 1959; Folk and Cole, 1965). A variety of soluble amines have been used as donors to demonstrate this incorporation activity (Neidle *et al.*, 1958; Lorand *et al.*, 1968b; 1969b). The incorporation of [$^{14}$C]GEE has, however, been found to be a convenient assay for demonstrating both the rate and extent of transamidase activity in plasma preparations (Lorand and Ong, 1966) and also tissue homogenates (Tyler and Laki, 1967). Tissue transamidases are sulphydryl enzymes and require calcium for activity (Neidle *et al.*, 1958; Folk *et al.*, 1967c). These characteristics are also displayed by the plasma enzyme (Loewy *et al.*, 1961b).

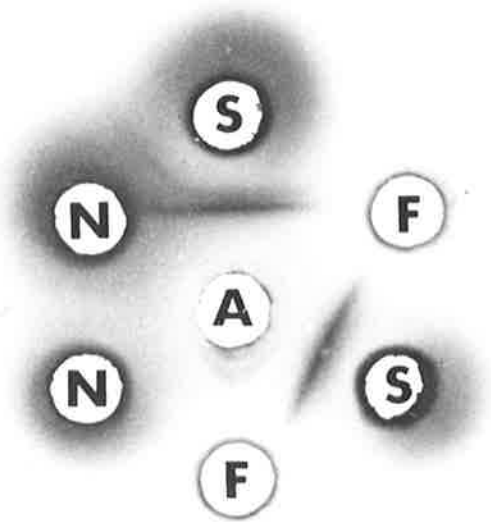
The presence of transamidase activity in hair follicle tissue has not previously been reported, although both transaminase and transpeptidase activities have been detected (Rogers and Springell, 1959). The experiments presented in this chapter show that homogenates of guinea pig hair follicle tissue have a transamidase activity similar to that of plasma transamidase. The follicle preparations readily incorporate [$^{14}$C]GEE into casein, and as

FIGURE 8-4: Double diffusion tests of transamidases in agar, as described in Materials and Methods. Wells were loaded with the following solutions: A, anti-Factor XIII-serum; N, normal rabbit serum; F, guinea pig hair follicle homogenate preparation; S, guinea pig serum preparation. Precipitin bands were stained with Coomassie Blue. (a) Diffusion of antiserum against partially purified follicle and plasma transamidases. (b) Diffusion of antiserum against crude follicle homogenate and unfractionated serum.

a




b



is also the case for plasma transamidase, this activity requires calcium and is inhibited by an SH-blocking agent (iodoacetate). In addition, the follicle homogenates do not couple hydroxylamine to CBZ-L-glutaminylglycine but they do show fibrin cross-linking activity. This again is similar to the plasma enzyme but distinguishes the follicle enzyme from the transamidase found in guinea pig liver homogenates which can use the CBZ-L-glutaminylglycine as substrate (Folk and Cole, 1965). Homogenates of rat hair follicle tissue also show similar transamidase activity. Furthermore, with the rat preparations it was shown that the GEE is incorporated mostly, if not entirely, as the γ-glutamyl derivative. The presence of aspartic acid in the hydrolysate of the isolated derivative (Table 8-I) means that the possibility of the formation by the enzyme of some β-aspartylglycine cannot be completely excluded.

The transamidase activity in wool follicle homogenates has distinctive properties. Although the homogenates have both transamidase and fibrin cross-linking activities as for the follicle preparations from rat and guinea pig, the wool enzyme is not inhibited by EDTA. In fact, the activity was slightly stimulated in the absence of calcium ions (Figure 8-2). It is unlikely that this unexpected result could be explained by the removal of inhibiting metal ions by the EDTA since the homogenate was prepared using solutions containing EDTA.

It has been directly shown that fibrin is insolubilized by plasma transamidase with the formation of ε-(γ-glutamyl)lysine cross-links (Pisano *et al.*, 1968, 1969; Matačić and Loewy, 1968a; Lorand *et al.*, 1968b) between specific glutamine and lysine residues (Chen and Doolittle, 1970). All the hair follicle transamidase preparations tested in this work were also able to cross-link fibrin and therefore the enzymes in these homogenates would be capable of forming the ε-(γ-glutamyl)lysine bonds found in hair proteins. Presumably, the transamidase in the sheep preparations only forms

the small number of cross-links in the inner root sheath proteins and in wool keratin (Chapter 6) since the wool fibres used were not medullated. Hairs from both guinea pig and rat have large medullae, the protein of which is extensively cross-linked (Chapter 6).

None of the hair follicle transamidases apparently required activation by thrombin as does plasma transamidase (Lorand and Konishi, 1964). A commercial preparation of anti-Factor XIII-serum was used to demonstrate that the hair follicle enzyme activites were not due to contaminating plasma enzyme that had already been activated. Upon addition of antiserum transamidase from both the follicle and serum of the guinea pig are inactivated as measured by both radiochemical and fibrin cross-linking assays. It was not necessary to remove the antibody-enzyme complex from the mixture as was required for the removal of activity of the human plasma transamidase during the purification of the fibrinogen substrate. On immunodiffusion, the guinea pig plasma enzyme produced strong precipitin bands in the gels but under no conditions was a precipitin line formed by the follicle preparations. Notwithstanding the lack of precipitation on immunodiffusion, chromatography of follicle preparations on a column of insolubilized antiserum showed that the transamidase and fibrin cross-linking activity was specifically bound by the antibody. Some cross-reaction with the antiserum is to be expected (Clausen and Heremans, 1960) even though the antibody was raised against human Factor XIII. The inactivation of the guinea pig hair follicle enzyme by the antiserum without precipitation indicates that antibody binds to the enzyme at only one site and in such a way as to block the active site. The sheep follicle enzyme is not inhibited by the antiserum, confirming the difference of this enzyme from that of the guinea pig.

Thus it has been demonstrated that the transamidases capable of forming the $\varepsilon$-($\gamma$-glutamyl)lysine cross-links in hair proteins occur in follicle tissue. It is

of prime interest to establish the location of the glutamine and lysine residues that are involved in the cross-link, and to determine the relationship, if any, of the cross-linking to the occurrence of citrulline in these proteins.

CHAPTER 9

# GENERAL DISCUSSION

The protein of hair medulla cells has in the past been called keratin because it is an intracellular product in a keratinizing structure. However, it is not keratin in the sense that the term is commonly applied and as defined by Mercer *et al.* (1963), *i.e.*, 'Keratins are proteins produced by epithelial cells, and usually retained within the cell. They are insoluble in the usual protein solvents, *due to the presence of numerous disulphide bonds between peptide chains*. Keratins may be predominantly filamentous, predominantly amorphous, or mixtures of filamentous and amorphous elements'.

Medulla protein is indeed similar to keratin in that it is insoluble and chemically resistant; in fact, it is even insoluble in solvents that dissolve cortical keratin (Matoltsy, 1953; Rogers, 1964a). Initial indications that it was different from keratin were demonstrations that the sulphur content of medulla protein was very low or nil (Barritt and King, 1931; Bekker and King, 1931; Jordan-Lloyd and Marriott, 1933). The medulla protein also gave an abnormal reaction for tyrosine when stained with Pauly reagent and this was taken by Bekker and King (1931) to indicate that it contained some unidentified constituent which was peculiar to medulla. This view was confirmed in part when Rogers (1962) demonstrated the presence in medulla of the amino acid citrulline, a component not recognized by earlier workers (Blackburn, 1948). Rogers and Simmonds (1958) and Rogers (1958a) had previously shown this amino acid to be present in the protein from inner root sheath cells of hair follicles.

Bekker and King (1931) noted the low sulphur content and since they described keratinization as 'cystinization', or the formation of disulphide bonds, these authors suggested that the cornification (insolubilization) of medulla was not due to keratinization. They stated that 'the nature of the keratinizing agent must await further chemico-histological study of the

follicle and skin tissues'.

Rudall (1955) claimed that the insolubility of medulla protein in solvents such as NaOH could be ascribed to the β-structure of the protein and its lipid content. Rogers (1958a) suggested that citrulline could be involved in the insolubility of the protein of inner root sheaths. The inner root sheath protein is very similar to medulla protein and the suggestion could be extended to the latter protein as well. In this thesis, however, it is shown that the insolubility of medulla protein under strong dissociating conditions can be explained by the presence of ε-(γ-glutamyl)lysine cross-links. These cross-links can therefore be taken to be the 'keratinizing agent' predicted by Bekker and King (1931). It was further shown in the present study that these isopeptide bonds are formed by distinctive follicle transamidases and they cross-link the peptide chains of the citrulline-containing protein of medulla cells.

While the finding of these covalent cross-links in the medulla protein does not remove the possibility that citrulline is involved in the insolubility of these proteins, it does reduce its importance.

The presence of the isopeptide cross-link in citrulline-containing proteins raises questions on the biosynthesis and structure of these proteins further to those already posed (Chapter 1.2). In addition to asking how citrulline becomes incorporated into protein and what its function might be in the protein, and indeed in the follicle itself, it is also pertinent to consider the relationship of the cross-link and the cross-linking process to the presence of citrulline. More specifically, it may be asked: does cross-linking occur before the modification of arginine to citrulline, or *vice versa*? Are follicle transamidases involved in the formation of citrulline as well as cross-linking? What defines the particular lysine and glutamine residues that eventually become involved in cross-links? What factor(s) defines

the particular arginine residues which are converted to citrulline? Why does the ε-(γ-glutamyl)lysine bond cross-link the citrulline-containing proteins in medulla and inner root sheath cells whereas keratin in adjacent cortical cells is stabilized by disulphide bonds? The answers to these questions must await further experimental work, but at the present time it is worthwhile considering similarities in the chemistry and synthesis that exist between citrulline-containing proteins of hair and the protein chains of collagen. Such considerations could act as guidelines in the devising of future investigations. The similarities include the presence of unique or rare amino acids which are formed from more common amino acids after their incorporation into protein, a high content of a particular amino acid and the presence of cross-links. As indicated earlier in this thesis (Chapter 1.2) the conversion of arginine to citrulline in hair proteins by some desimination mechanism is analogous to the hyroxylation of proline and lysine in protocollagen. It has been suggested that hyroxylation of these residues is necessary for extrusion of the protein from the cells (Juva *et al.*, 1966) but the evidence is equivocal (Gribble *et al.*, 1969). In contrast, the reason for the modification of arginine residues to citrulline in the medulla is not understood although a number of suggestions have been made (Rogers and Simmonds, 1958; Rogers, 1958a, 1959, 1963, 1964a) and have been discussed (Chapter 1.2). In citrulline-containing proteins, the glutamic acid content is usually high (Rogers, 1962, 1964a; Steinert *et al.*, 1969) and can reach about 30 moles per cent. A characteristic of collagen is that glycine accounts for approximately one-third of the total residues (for review, see Seifter and Gallop, 1966).

The covalent cross-links present in the citrulline-containing protein result from the action of a transamidase on the side-chains of lysine and glutamine residues, whereas in collagen, cross-links arise by the oxidative

deamination of lysine residues (Pinnell and Martin, 1968) by lysyl oxidase (Siegel *et al.*, 1970) to produce the corresponding δ-semialdehyde. These aldehyde residues condense, probably spontaneously (Kang and Gross, 1970; Shiffman and Martin, 1970) to form covalent cross-links such as hydroxylysinonorleucine (Bailey and Peach, 1968), syndesine (from hydroxylysine) (Bailey *et al.*, 1969) and lysinonorleucine (Kang *et al.*, 1970; Tanzer and Mechanic, 1970). A variety of other bonds that could potentially serve as cross-links in collagen have been reviewed by J.J. Harding (1965).

The biosynthesis of collagen occurs in discrete, sequential steps in which hydroxylation is followed by glycosylation (Blumenkrantz *et al.*, 1969) and then cross-linking (Bailey *et al.*, 1969). The temporal relationship of the formation of citrulline and cross-linking in hair proteins is unknown at present, but the isolation and characterization of a precursor protein (trichohyalin) should yield information in this respect. It is interesting to speculate on the possible relationships of these two phenomena, however. Firstly, it is possible that these two events are indepenent. On the other hand, cross-linking may occur before modification of the arginine residues thereby defining the secondary and tertiary structure of the protein so that the (presumed) converting enzyme can selectively carry out the conversion of these residues to citrulline. The inverse is an equal possibility; that is, the conversion is a prerequisite for cross-linking.

In the cross-linking of collagen, specific lysines near the N-terminus of each α-chain are converted to aldehydes which react by aldol condensation to form *intra*molecular cross-links (Bornstein and Piez, 1966; Kang *et al.*, 1969). By alignment of the chains in the molecule (Tanzer, 1968) these residues can also form *inter*molecular cross-links (Kang and Gross, 1970). Peptides isolated from the cross-linking region of rat skin collagen show that the amino acid sequence of this part of the chain is not typical of

collagen (Kang *et al.*, 1967) but a more explicit relationship between the specific lysine of the cross-link and the sequence is not obvious. Isolation of peptides containing the cross-link hydroxylysinonorleucine from calf skin collagen (de Luque *et al.*, 1970) has so far yielded no further information. However, it has been suggested that the lysines involved in desmosine formation in elastin are defined by a region of five alanine residues (Sandberg *et al.*, 1971); definitive evidence on this point is lacking at present. Thus for citrulline-containing protein it would be of interest to at least determine sequences of small cross-linked peptides in the immediate vicinity of the cross-link to see if any information can be obtained concerning the specificity of the cross-linking transamidase. The high content of glutamic acid residues found in the present study of such peptides suggests that these residues may be functionally significant. The possible importance of a confluence of glutamic acid and/or glutamine residues in the defining of the glutamic acid and lysine residues to be cross-linked or the arginine residues to be converted to citrulline, cannot be overlooked. In pursuing sequence studies in this context, technical difficulties can be anticipated. A large number of glutamic acid residues could hinder sequencing, because of a large number of repetitious residues. A resistance to proteolytic attack to produce smaller peptides could be anticipated. Further, these problems are compounded by the lack of a cleavage method specific for ε-(γ-glutamyl)lysine cross-links. Acid hydrolysis conditions studied during the present work were not sufficiently selective and an enzyme, peptidoglutaminase II, which can deaminate peptide-bound glutamine residues (Kikuchi *et al.*, 1971) was tested but did not attack the cross-link.

The complexity of the tryptic peptides of medulla protein (Chapter 4) may also derive from circumstances that pertain to collagen. Thus, Bornstein (1967) has shown in peptides isolated from rat skin and tail collagen that the hydroxylation of certain proline residues is incomplete. By analogy then, an incomplete conversion of arginine to citrulline would produce varying tryptic

cleavage positions (arginine residues) giving peptides of a range of sizes. An entirely random conversion would obviously give the same effect. Similarly, incomplete or random cross-linking involving lysine residues would produce variations in the number and position of tryptic cleavage points. In addition, complexity of the peptide mixture may arise from microheterogeneity of the original proteins due to amino acid replacements. There would be little selective pressure against such replacements in a protein like the medulla protein, the function of which at present is recognized only as being structural and thus relatively non-specific.

The mechanism of the conversion of arginine to citrulline remains an enigma. However, the present finding of transamidase activity in follicle tissue is encouraging in this regard, since Rogers (1963) has suggested a transamidation reaction between arginine and glutamic acid as being an intermediate step in the formation of citrulline. This suggestion was based on the postulation by Erlanger (1960) of an interaction of similar type between arginine and aspartic acid at the active sites of some proteolytic enzymes. It is interesting to note that Joseph and Bose (1962) have suggested an interaction of arginyl guanido groups and glutamyl γ-carboxyl groups as a possible cross-link in collagen. It may therefore be particularly significant that the follicle transamidases studied have distinctive properties, especially the wool enzyme which is the only transamidase known that does not require calcium for activity.

It is of fundamental importance to note that the cells of the hair cortex, inner root sheath and medulla all have their origin from the same region of dividing cells in the follicle (the matrix) and develop in close association with one another (Rogers, 1964a; see also review by Rudall, 1968). Each cell line develops its own distinctive intracellular protein product. Thus, the cortical cells become packed with keratin which is stabilized by numerous disulphide bonds (Rogers, 1969). The medulla cells contain a protein

that is very low in sulphur-containing amino acids but contains citrulline and is stabilized by the presently established ε-(γ-glutamyl)lysine cross-links. A similar protein and the same cross-link is also found in cells of the inner root sheath.

Thus, it can be seen that all the proteins noted above pose interesting questions as to their structure and mode of synthesis. Moreover, the great difference in the chemical nature of these specialized gene products produced by the differentiated cells of the hair follicle point up this cellular system as a particularly interesting one in the wider context of cellular differentiation in eukaryote cells. *A priori* one might have expected the proteins of the medulla and inner root sheath to be closely related to the protein family of homologous chains that are known to constitute the α-keratins. Clearly, this is not the case; not only must a different gene or set of genes be activated in the medulla and inner root sheath cells, but ancillary processes must coordinately develop for conversion of the primary product to the final protein.

# APPENDIX A

## MODIFICATION OF THE BECKMAN 120C AMINO ACID ANALYSER

The Beckman 120C amino acid analyzer is capable of fast and sensitive analyses of acid hydrolysates, but the system had a number of disadvantages as far as the present work was concerned. These disadvantages were as follows: (1) Ordinarily lysine and ornithine are not separated on the short (basics) column (0.9 x 15 cm). For proteins containing citrulline such a separation is required to allow calculation of 'total citrulline' figures, since citrulline is degraded to ornithine during acid hyrolysis (Rogers, 1958). Furthermore, this resolution is required to obtain an accurate value for lysine, which was also of particular importance in this work. (2) The 2-hr and 4-hr long column (acidics/neutrals) systems both give crowded chromatograms which make the determination and separation of new unknown peaks difficult. Alteration of parameters such as buffer pH to improve separation is tedious. (3) The long column will not resolve ε-(β-aspartyl)lysine and ε-(γ-glutamyl)lysine (B. Milligan, 1969, personal communication).

However, these problems are readily overcome using the single column gradient elution technique of Piez and Morris (1960) by appropriate simple manipulation of the gradient. Buffer gradients used are given in Appendix B. This system is the basis of the Technicon Amino Acid AutoAnalyzer. Modifications were therefore carried out on the Beckman analyzer by the addition of Technicon

components to incorporate features of both systems. The Beckman analyzer was fitted with an expanded scale (1mV) recorder. The flow diagram for the modified system is given in Figure A-1.

The nine-chambered Technicon Autograd was connected to an extra Beckman 'Accu-Flo' pump via two three-way solenoid valves (Technicon) and a bubble trap. High pressure nylon tubing was used to connect the pump outlet to the Technicon sample injection device at the top of the column. The pressure gauge (Esdaile, 0-800psi) was protected from the buffer by a Gauge-saver (Rast Industries, South Australia). A Beckman stainless steel screen resin support replaced the teflon sinter at the base of the Technicon column. The outlet fitting (Technicon) was threaded to accept a 12mm ball connector (Beckman) to give a direct connection of the total column effluent to the Beckman analytical system using the socket connector for the 'Orange' column.

Operation of the combined system is by a modified Technicon Automatic Column Shutdown Programmer in conjunction with the Beckman control systems. A schematic diagram is given in Figure A-2. The programmer was modified for automatic switching of the ninhydrin pump and recorder, as well as its normal functions of operating the solenoid valves for selection of buffers. A four-pole three-position (ON - OFF - ON) switch was incorporated in the circuit on the control side of the fuses for pumps 1 and 2 and the recorder, to select the controlling system. Automatic control of the temperature change at

the end of an analysis can be achieved by appropriate arrangement of the Beckman Temperature and Master Timers. Details of the operation program are given in Table A-I. Operating parameters are listed below:

Resin bed: 0.6 x 126 cm

Resin type: Chromobeads, type A (Technicon)

Buffer flowrate: 70 ml/hr

Ninhydrin flowrate: 35 ml/hr

Pressure: 360 - 450 psi

Temperature: $60^{o}$

Total analysis time: 630 min.

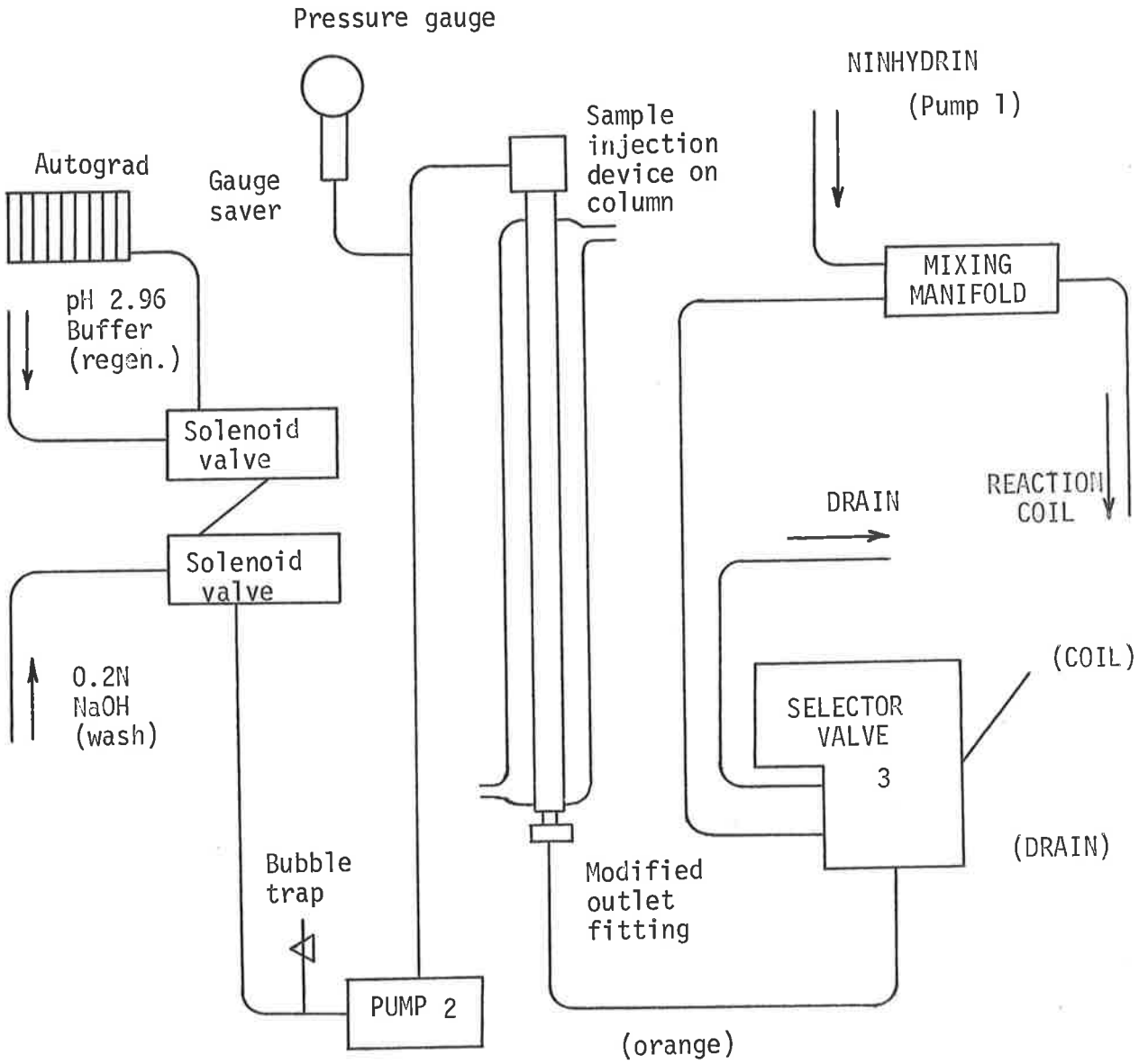



FIGURE A-1: Flow diagram for the modified Beckman amino acid analyzer, set up for 'GRADIENT' analysis.

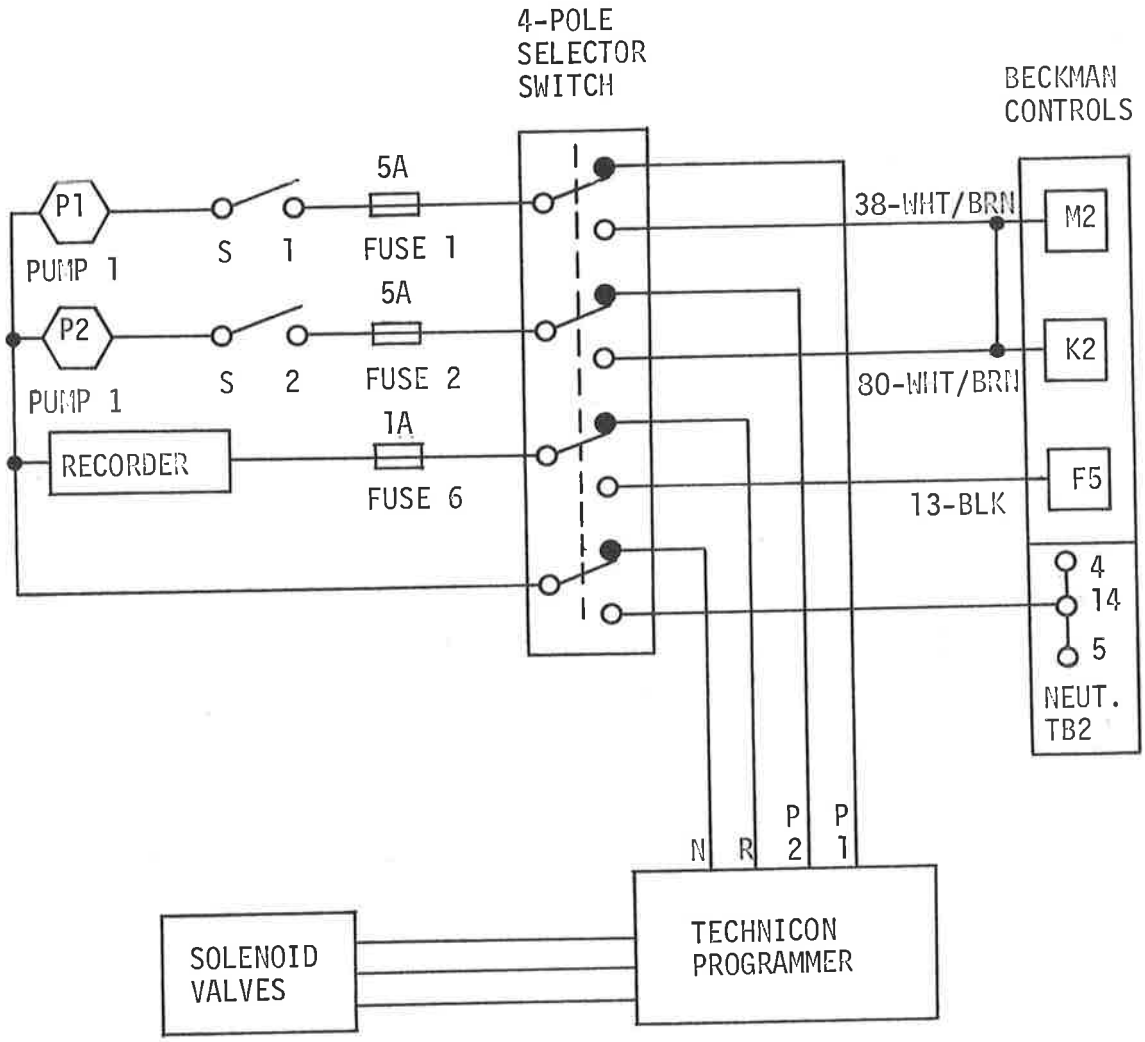



FIGURE A-2: Schematic circuit diagram for modifications to the Beckman amino acid analyzer. The system as shown here is set up for 'GRADIENT' analysis. Abbreviations are those used in the Beckman Instruction Manual for the Model 120C (Supplement A-TB-030, 1966).

TABLE A-I: OPERATION PROGRAM FOR THE MODIFIED GRADIENT SYSTEM.

| Operational Mode | Time Before Shutdown (min) | Components Activated |
|---|---|---|
| ANALYSIS | 630[a] - 100 | Pump 1[b], Pump 2[c], Recorder, Autograd valve OPEN |
| WASH + ANALYSIS | 100 - 80 | Pump 1[b], Pump 2[c], Recorder, NaOH valve OPEN |
| WASH | 80 - 70 | Pump 2[c], NaOH valve OPEN |
| REGENERATION | 70 - 0 | Pump 2[c], Regeneration buffer valve OPEN |
| OFF | - | Temperature =LO, Autograd valve OPEN |

[a]Maximum time for 75 ml buffer/Autograd chamber. [b]Ninhydrin pump.

[c]Buffer pump.

# APPENDIX B

## PRODUCTION OF BUFFER GRADIENTS FOR THE MODIFIED GRADIENT ANALYSIS SYSTEM

Sodium citrate buffers were prepared as described in the Technicon Amino Acid Analyzer Manual, AAA-1 (1967, Technicon Corp., Ardsley, N.Y.) except that the low pH buffer was adjusted to pH 2.96 and the composition of the pH 5.00 buffer was modified according to the procedure of Wells (1967). The pH 10.00 buffer was prepared from pH 5.00 buffer by titration with anhydrous sodium carbonate. In all gradients, chambers 1 and 2 of the Autograd also contained 5 ml and 3 ml of methanol, respectively. The details of the gradients are given in Table B-1.

TABLE B-I: PRODUCTION OF BUFFER GRADIENTS FOR AMINO ACID ANALYSIS.

| | Routine Gradient MO | | | Modified Gradient M4 | | | | Modified Gradient M4a | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Buffer (ml) | | | Buffer (ml) | | | | Buffer (ml) | | |
| Autograd Chamber | pH 2.96 | pH 3.80 | pH 5.00 | pH 2.96 | pH 3.80 | pH 5.00 | pH 10.00 | pH 2.96 | pH 3.80 | pH 5.00 |
| 1[a] | 70 | | | 70 | | | | 70 | | |
| 2[b] | 72 | | | 72 | | | | 72 | | |
| 3 | 75 | | | 75 | | | | 75 | | |
| 4 | 75 | | | 75 | | | | 75 | | |
| 5 | | 70 | 5 | | 75 | | | | 75 | |
| 6 | 6 | 9 | 60 | | 75 | | | | 75 | |
| 7 | | | 75 | | | 75 | | | | 75 |
| 8 | | | 75 | | | 75 | | | | 75 |
| 9 | | | 75 | | | | 75 | | | 75 |

[a]Chamber 1 also contains 5 ml of methanol. [b]Chamber 2 also contains 3 ml of methanol.

# PUBLICATIONS

# APPENDIX C

1. PAPERS PUBLISHED*

The Characterization of Protein-Bound Citrulline (with P.M. Steinert and G.E. Rogers).

*Biochim. Biophys. Acta, 175* (1969) 1.

ε-(γ-Glutamyl)lysine Cross-Linkage in Citrulline-Containing Protein Fractions from Hair (with G.E. Rogers).

*Biochemistry, 10* (1971) 624.

2. PAPERS PRESENTED AT MEETINGS*

An Initial Study of Sequences in Peptides Derived from a Protein Containing Citrulline (with G.E. Rogers).

*Proc. Aust. Biochem. Soc. 1* (1968) 5.

Characterization of the Gamma-Link in a Citrulline-Containing Protein (with G.E. Rogers).

*Proc. Aust. Biochem. Soc. 3* (1970) 56.

The ε-(γ-Glutamyl)lysine Cross-Link in Keratinizing Tissues: Its Occurrence, Formation and Localization in Citrulline-Containing Proteins (with G.E. Rogers).

*Proc. Aust. Biochem. Soc. 4* (1971) 15.

3. PAPERS IN PRESS

The Occurrence of the ε-(γ-Glutamyl)lysine Cross-Link in the Medulla of Hair and Quill (with G.E. Rogers).

*Biochim. Biophys. Acta.*

*Reprints bound at back of thesis.

4. PAPERS SUBMITTED

Structure of Hair Medulla Protein: Occurrence of the ε-(γ-Glutamyl)lysine Cross-Link in Peptides Containing Citrulline (with G.E. Rogers).

*(Biochemistry)*

Formation of the ε-(γ-Glutamyl)lysine Cross-Link in Hair Proteins. Investigation of Transamidases in Hair Follicles (with G.E. Rogers).

*(Biochemistry)*

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# THE CHARACTERISATION OF PROTEIN-BOUND CITRULLINE

P. M. STEINERT, H. W. J. HARDING AND G. E. ROGERS
*Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5001 (Australia)*

(Received August 8th, 1968)

## SUMMARY

1. The amino acid citrulline, generally not found as a constituent of proteins, has been isolated and purified from the medulla protein of the quill of the African porcupine and from the inner root sheath protein of the hair follicle of the guinea pig.

2. The properties of the amino acid isolated from the two sources were found to be identical to those of L-citrulline. The identity of the amino acid has therefore been unequivocally established.

3. The proteins of the medulla and of the inner root sheath isolated as tryptic polypeptides contain, respectively, 760 and 235 $\mu$moles of citrulline per g.

4. Citrulline has been released almost completely from the proteins by digestion with subtilisin. Furthermore, limited amino acid sequences containing citrulline have been determined in purified peptides released by combined tryptic and peptic digestion of the proteins. Earlier indications that citrulline is covalently bound in peptide linkage in the proteins have been confirmed.

5. The possible origins of the citrulline in the proteins are briefly discussed.

## INTRODUCTION

Citrulline is widely distributed as a free amino acid in plants and animals but it has not been found as a general constituent of proteins. The occurrence of citrulline in protein and polypeptide material has been reported[1–6], but these reports have either not been fully substantiated or must be considered unlikely in view of the more recent studies on the amino acid composition of many proteins.

An apparently unique occurrence of citrulline in proteins was established when ROGERS[7] isolated inner root sheath material from hair follicles and demonstrated substantial quantities of this amino acid in the cell-proteins of this tissue. Citrulline has also been shown to be present in the protein (or proteins) that occur in the cells of the central canal or medulla of hair fibres of several species[8] and of modified hairs, namely the quills of the porcupine[9].

Abbreviations: DNS, dansyl-group or 1-dimethylamino-5-naphthalenesulfonyl-group; PTH, phenylthiohydantoin.

The inner root sheath and medulla proteins, like keratins, are insoluble in protein solvents but they are dissimilar from keratins in being readily hydrolysed by trypsin and contain only trace amounts of cystine. Several experiments have already indicated[7–12] that the citrulline present in these proteins is covalently bound in high-molecular-weight polypeptides and not merely adsorbed to the proteins. More recently, citrulline has been detected in the protein of the cuticle layer of the hair fibre[13].

Before an amino acid can be accepted as a new constituent of a protein, the evidence for its occurrence should meet certain criteria[14]. In earlier work, insufficient quantities of protein were isolated to permit full characterisation of the citrulline[8–10,15]. It is the purpose of this paper to report the isolation and characterisation of citrulline and the establishment of citrulline residues in amino acid sequences of particular peptides isolated from the cell-proteins of both inner root sheath and medullary cells.

## MATERIALS AND METHODS

### *The source of proteins containing citrulline*

A protein or group of proteins containing this amino acid has not been isolated intact from either inner root sheath or medullary tissue nor is it known how many protein species containing it might be present in the cells of these tissues. Consequently, investigations of the chemistry of the proteins concerned were performed on the whole tissues (inner root sheath or medulla). Alternatively, the proteins were quantitatively removed by digestion with trypsin and separated from the cell debris and keratin contamination. This proteolytic procedure reduces the proteins to soluble polypeptides which still contain all of the protein-bound citrulline.

In the present paper where reference is made to 'inner root sheath protein' or 'medulla protein' this does not connote isolation of the 'native' protein or proteins from them.

### *Isolation of inner root sheaths and medulla tissue*

Hair follicles of the guinea pig (*Cavia porcellus*) were exposed by the wax–sheet method[9] and the sheaths removed according to the established procedure[10]. The medulla was dissected from quills of the African porcupine (*Hystrix cristata*) with a sharp scalpel, ground in a Wiley mill to through-40 mesh size and washed with water and organic solvents to remove ninhydrin-positive material and lipid.

### *Isolation of citrulline*

L-Citrulline (Calbiochem) was used as a standard throughout.

*Separation of citrulline from protein hydrolysates.* The protein contained in the inner root sheath and medulla material was removed as polypeptides by digestion with crystalline trypsin (Mann Research Lab.; minimal chymotrypsin content). Digestion was conducted at 37° in 0.01 M $NH_4HCO_3$ (pH 8.3) using an enzyme: protein ratio of 1:100. Digestion was terminated at 3 h by freeze-drying the supernatant after centrifuging at 5000 × *g*.

The water-soluble tryptic peptides derived from the medulla and inner root sheath preparations were hydrolysed by refluxing in glass-distilled constant-boiling HCl. The hydrolysis time for the isolation of citrulline was 12 h to minimise destruction

of the amino acid. A protein to acid ratio of 1:200 (w/v) was used. Acid was removed by passing the hydrolysate through a short column of Dowex 50 X4 ($H^+$ form).

Citrulline was separated from the mixture of amino acids according to HIRS, MOORE AND STEIN[16]. The amino acids were adsorbed on a column of Dowex 1 X8 (acetate form). The fractions containing the neutral and basic amino acids were pooled and then applied to a column of Dowex 50 X4 ($H^+$ form). Separation of the mixture was initiated at 20° with 1.00 M HCl and completed at 30° using a linear acid gradient between 1.00 and 2.50 M HCl. Citrulline was eluted at an acid concentration of about 2.3 M HCl and was recovered from the acid by adsorption on a small column of Dowex 50 X4 ($H^+$ form) and eluted with 0.5 M pyridine–acetate buffer at pH 5.3.

Amino acids in fractions from the ion-exchange columns were determined by the procedure of YEMM AND COCKING[17]; citrulline was estimated quantitatively according to ARCHIBALD[18] or the modification of MCLEAN, NOVELLO AND GURNEY[19]. Assays of acid hydrolysates of both free and protein-bound citrulline showed a potentiation of colour by approx. 50% but the factor responsible has not been identified. The extent of potentiation was determined from standard amounts of citrulline to which had been added optimal amounts of a 120-h hydrolysate of citrulline in which this amino acid had been completely destroyed.

*Purification of citrulline.* Contaminating basic amino acids and some salts were removed by passing the crude citrulline through a short column of Dowex 50 X4 ($NH_4^+$ form) with water. Traces of contaminating salts were removed from the citrulline on a column of Sephadex G-10 using de-ionised water as eluant and an impurity of $NH_4^+$ detected by nesslerisation was removed by heating at 40° *in vacuo* over $P_2O_5$ for 2 days. The remaining impurities were removed by the formation of the copper complex[14]. The citrulline was recovered from the copper citrullinate and crystallised by evaporating to dryness over $P_2O_5$ in a desiccator. Traces of water from the product were removed by heating at 45° *in vacuo* over $P_2O_5$.

*Characterisation of the isolated citrulline*

Descending paper chromatography was performed using Whatman No. 1 paper and ascending thin-layer chromatography was conducted on Eastman 'Chromagram' sheets (grade K301R2). In both instances a minimum of five solvent systems[20] were applied. Amino acids were located by spraying with 0.1% (w/v) ninhydrin in ethanol. Ehrlich's reagent, 1% (w/v) *p*-dimethylaminobenzaldehyde in 1 M HCl, was used for the detection of citrulline[21].

Oxidation of the isolated citrulline samples with L-amino acid oxidase was performed according to the method of MALMSTADT AND HADJIOANNUO[22] using an oxygen-electrode. The reaction mixture contained 1.0 μmole citrulline, 5 μg catalase (Calbiochem, B. grade) and 1 mg L-amino acid oxidase (snake venom; Worthington Chemical Corp.) in 3.40 ml of 0.1 M Tris–HCl buffer at pH 7.4. Reaction was complete in 10 min. The extent of oxidation was used to determine the configuration of the isolated citrulline.

*Proteolytic release of citrulline from citrulline-proteins*

The medulla and inner root sheath tissue and tryptic peptides derived from them, were hydrolysed with subtilisin (Nutritional Biochemicals Corp.) at an enzyme: protein ratio of 1:100 in 0.01 M ammonium acetate at pH 7.4 for 7 h at 37°. The ci-

trulline released was collected by adsorption on Dowex 50 X8 ($H^+$ form) and quantitatively determined.

*Sequence of citrulline peptides*

Citrulline-containing peptides were prepared from the proteins of porcupine quill medulla by the action of crystalline trypsin[8]. The tryptic peptides, precipitated by adjusting the pH to 3.5 ('tryptic core') were further digested with pepsin (Sigma, 5 times crystallised) using an enzyme:protein ratio of 1.5:100 in 5% (v/v) aqueous formic acid at pH 2 for 24 h at 37°. The peptides in this mixture were fractionated on a column of Dowex 50 X2 with a pyridine–formic acid gradient (pH 2.6 to 9.1) and the elution of peptides was followed by ninhydrin estimations on aliquots of each fraction after alkaline hydrolysis[23]. Samples were quantitatively estimated for the presence of citrulline by the method of McLEAN, NOVELLO AND GURNEY[19]. Fractions were pooled and submitted to two-dimensional high-voltage paper electrophoresis at pH 6.5 and 3.7, and those containing a minimal number of peptides and a favourable citrulline content were studied. Peptides were purified by separate steps of one-dimensional paper electrophoresis and paper chromatography; their purity was checked by N-terminal amino acid analysis using the dansyl (DNS) technique of GRAY AND HARTLEY[24].

Amino acid analyses of the peptides were carried out using an automatic analyser. The ornithine that arises from the hydrolysis of the citrulline side-chain during acid hydrolysis was estimated in the analysis and the value was added to the citrulline estimate to give a 'total citrulline' value.

Amino acid sequence analyses were performed using a combination of the DNS end-group method with the stepwise Edman degradative procedure[24] using trifluoroacetic acid for cyclisation[25]. Preliminary studies on authentic citrulline and 1,9-citrulline bradykinin showed that the degradative method could be applied satisfactorily and caused only a minimal breakdown of citrulline. The phenylthiohydantoin (PTH) derivatives of asparagine and glutamine were extracted according to the method of NEDKOV AND GENOV[26] and identified by thin-layer chromatography. The DNS-derivatives of the N-terminal amino acids were identified by one-dimensional thin-layer chromatography on silica gel G layers and polyamide layers[27]. α-DNS-citrulline undergoes partial breakdown to α-DNS-ornithine during the acid hydrolysis so that spots corresponding to both these compounds were seen when citrulline residues were N-terminal.

## RESULTS AND DISCUSSION

*Amino acid content of the proteins*

The destruction of the ureido-group of citrulline occurs under hydrolytic conditions with constant-boiling HCl, yielding ornithine, $CO_2$ and $NH_3$. Consequently, in the direct colorimetric determination of the citrulline content of the proteins it is necessary to correct for this breakdown by performing citrulline assays during the course of acid hydrolysis and extrapolate to zero time. The extrapolated values were corrected for the potentiation effect that occurs in the colorimetric analysis of hydrolysates of citrulline. The time course of digestion is shown in Fig. 1.

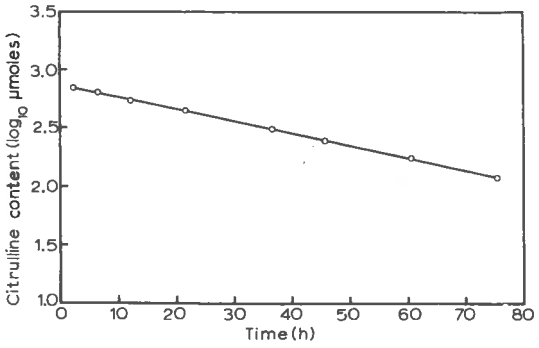


Fig. 1. The rate of hydrolytic destruction of free and bound citrulline. Authentic L-citrulline and the tryptic polypeptides of the medulla and inner root sheath preparations were separately hydrolysed (105°) at a ratio of 1:200 of protein (or equivalent quantity of L-citrulline) and constant-boiling HCl. The citrulline contents were determined at various times. Since the rates of destruction were found to be identical the results are presented as a single plot. Extrapolation to zero time yielded a recovery figure of 99.5% for the L-citrulline and enabled calculation of the citrulline contents of the two proteins.

TABLE I

AMINO ACID COMPOSITION OF THE MEDULLA AND INNER ROOT SHEATH PROTEINS

All values are expressed as μmoles/g of tryptic peptides of the tissue. Arginine, aspartic acid and the amide contents were not determined in the present work; their values presented here are taken from other studies (unpublished).

| *Amino acid* | *Protein source* | |
|---|---|---|
| | *African porcupine quill: medulla* | *Guinea pig: inner root sheath* |
| Arg | 260 | 265 |
| Ammonia | [945] | [1160] |
| Asp | 460 | 510 |
| Ala | 370 | 440 |
| Citrulline | 760* | 235* |
| Cys | Trace | Trace |
| Glu | 2625 | 1510 |
| Gly | 310 | 520 |
| His | 95 | 100 |
| Ile | 135 | 250 |
| Leu | 710 | 680 |
| Lys | 675 | 630 |
| Orn | 90 | 30 |
| Met | 45 | 160 |
| Phe | 215 | 220 |
| Pro | 165 | 245 |
| Ser | 260 | 505 |
| Thr | 140 | 225 |
| Tyr | 175 | 170 |
| Val | 330 | 345 |

* Corrected for citrulline breakdown.

The citrulline contents of tryptic peptides prepared from medulla and inner root sheath material determined by this procedure were respectively 760 and 235 $\mu$moles per g (dry wt.).

The quantitative analyses of all amino acids in the proteins with the exception of arginine and aspartic acid were determined from the column chromatograms and the values are given in Table I. The similarities that are evident between the medulla and inner root sheath analyses indicate a close relationship between the proteins from the two tissues. It can be seen that the values for citrulline are identical with those obtained by the direct assays discussed above.

*Isolation and properties of citrulline*

Ion-exchange chromatography of acid hydrolysates of the medulla and inner root sheath protein yielded crude fractions of citrulline that required further purification. Citrulline assays showed that the purified citrulline isolated from both tissues was $> 99.5\%$ pure. The elemental analysis of it (Table II) agreed with the calculated values and with analyses performed on authentic citrulline which had been recrystallised from water.

TABLE II

ELEMENTAL COMPOSITION OF ISOLATED CITRULLINE

Analyses were performed by the Australian Microanalytical Service, C.S.I.R.O., Melbourne.

| | *Element (%)* | | | |
|---|---|---|---|---|
| | *C* | *H* | *O* | *N* |
| Calculated for: $C_6H_{13}N_3O_3$ | 41.1 | 7.4 | 27.5 | 24.0 |
| Observed values for citrulline from: | | | | |
| Medulla | 41.1 | 7.4 | 27.9 | 23.7 |
| Inner root sheath | 40.8 | 7.4 | 27.6 | 24.1 |
| Authentic | 40.9 | 7.6 | 27.2 | 23.6 |

Melting points were determined on isolated and authentic preparations of citrulline (219–220°, decomp.), their crystalline copper complexes (259–260°, decomp.) and DNP-derivatives (139–140°, decomp.). There were no significant differences in the values between the isolated and authentic materials. Furthermore, examination by paper and thin-layer chromatography showed without exception that the citrulline preparations were indistinguishable from authentic citrulline.

The infrared spectra given by the isolated material and authentic citrulline are shown in Fig. 2. No significant difference between preparations was observable.

The values of dextrorotation of citrulline isolated from the medulla and inner root sheaths were identical with the authentic material and they were all increased in the positive direction on the addition of acid, in conformity with the rule for L-amino acids[14]. In addition, the preparations of citrulline were submitted to the action of L-amino acid oxidase and the results showed that the isolated citrulline from both inner root sheath and medulla was of the L-configuration in both instances.

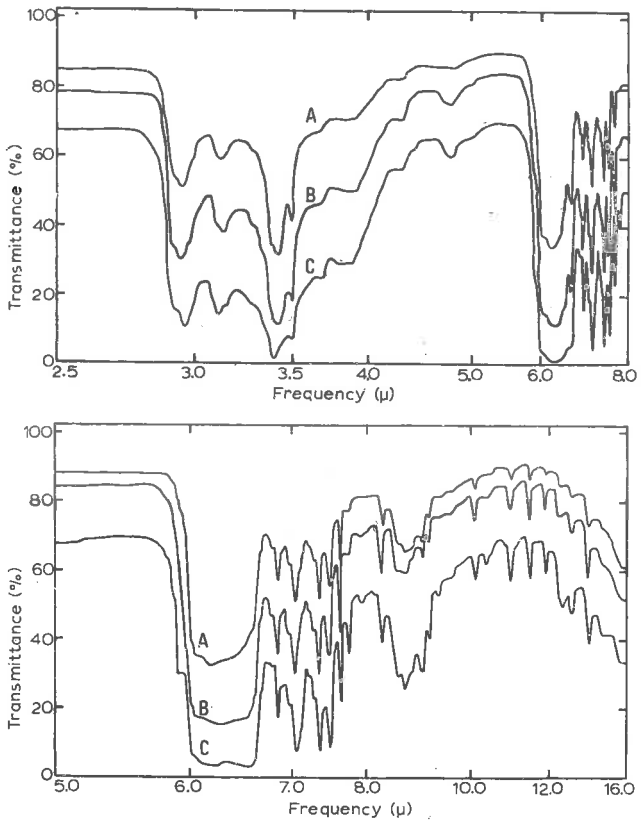


Fig. 2. Infrared spectra of citrulline. (A) authentic L-citrulline, (B) citrulline from medulla and (C) citrulline from the inner root sheath. Nujol mull; Perkin-Elmer "Infracord-237" spectrometer.

*The evidence for* L-*citrulline in peptide linkage*

The results of earlier work indicated that citrulline is covalently-bound in the proteins investigated in the present study. Thus vigorous washing techniques with a variety of solvents will not release free citrulline[9] even though the tissues themselves give intense colour reactions for this amino acid with both Ehrlich's reagent[11] and the Fearon reagent[9]. Further, the protein material in the medulla and inner root sheath tissues is remarkably resistant to extraction with most reagents, although readily accessible to proteolytic attack[7–10,12]. Moreover, it was shown that exopeptidases liberate citrulline from tryptic and peptic peptides. In the present work subtilisin was found to release free citrulline almost quantitatively from the tryptic peptides as seen in Fig. 3.

The identification of amino acid sequences containing citrulline in purified peptides (Table III) together with the foregoing evidence, firmly establish that L-citrulline occurs in peptide linkage in high molecular weight polypeptides.

The precise mechanism by which citrulline arises in the proteins is as yet unclear. It could be incorporated *de novo* but it would appear unreasonable to suppose that keratinising tissues should alone possess this capability. A more attractive hypothesis

TABLE III

N-TERMINAL AMINO ACID SEQUENCES

| *Peptide** | *Sequence* |
|---|---|
| I M-TP4/6a | Asp–Cit–Phe–Cit– |
| I M-TP4/7d | Cit–Cit–Val–Cit–Cit–(Glu, Gln)–Val** |
| I M-TP6/7b | Leu–Leu–Glu–Cit–Cit– |
| I M-TP7/1 | Phe–Cit–Glx–Glx– |
| II M-TP5/3b | Leu–Cit–Gln– |
| II M-TP10/2a | Asp–Cit–Cit–Phe– |
| 1,9-Citrulline bradykinin | Cit–Pro–Pro– |

Abbreviation: Cit, citrulline.

* The notation adopted for the peptides refers to the type of digestion used and their order of elution from the initial column chromatograms. The relationships between the peptides and to the primary structure of the protein is not known at present.

** The C-terminal valine was determined from carboxypeptidase A digestion of the peptide. The presence of the Gln residue in this peptide was deduced from the electrophoretic mobility of the DNS-peptide[30].

for which evidence has already been adduced[10] is that citrulline residues are produced by desimidation of arginine residues after their activation and incorporation[7–11,28]. A process of this kind would be unique although precedents for amino acid modification after incorporation into a polypeptide are known, such as the hydroxylation of proline during collagen biosynthesis[29]. The origin of citrulline in the proteins and the relationship of its mode of formation with the process of keratinisation is being investigated.

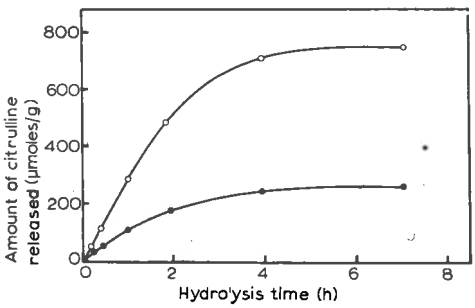


Fig. 3. Release of citrulline from the tryptic peptides of the medulla and inner root sheath proteins by subtilisin. The citrulline released from both proteins was in excess of 95% of the total after 7 h incubation. ○—○, medulla tryptic peptides; ●—●, inner root sheath tryptic peptides.

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