HEREFORD HYPOTRICHOSIS:

A MOLECULAR APPROACH

by

REBECCA ROSE

B.S., Kansas State University, 1977
D.V.M., Kansas State University, 1981

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

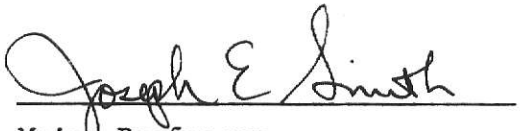
Department of Pathology

College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas 66506

1982

Approved by:



Major Professor

TABLE OF CONTENTS

Acknowledgements

I would like to thank my husband, Michael Lee Schwab, for his continuous support of my efforts in veterinary medicine. His help and insight have done much to sustain me.

I would like to thank my parents, Arthur and Wanda E. Rose, for their faith in my educational goals and for instilling in me the belief that a job once started should be finished.

For both my rigorous practical and challenging theoretical training in molecular pathology, I am deeply indebted to Dr. Joseph E. Smith, my major professor.

I wish to thank Dr. Stanley M. Dennis for sponsoring and administering the DVM/MS dual degree program. I learned much by having an orientation toward pathology while completing the clinical curriculum.

Dr. Horst Leipold generously granted me the use of his research cattle and offered much encouragement which I greatly appreciate.

While I was focused on the characteristics of hair proteins, Dr. James Cook gently reminded me by work and example that histopathology was another aspect of veterinary pathology which merited rigorous attention; my thanks go to him for that support.

For their cheerful assistance and invaluable instruction in laboratory technique I wish also to thank Kateri Moore and Donna Boyington.

LITERATURE REVIEW

Introduction

Pelage and the general process of keratinization are the focus of study for a widely divergent group of scientists. Laboratory differentiation of hair types is often required for use as legal evidence. Scientists studying Menkes' kinky hair syndrome were initially drawn to study the hair of affected human patients because the unusually twisted hair was a very characteristic sign of the syndrome. Textile industry investigators are interested in wool growth and tensile strength and have contributed much to the current knowledge about hair on the molecular level. Genetic defects in bovine hair lead to reduced viability in otherwise hardy breeds of cattle. The cosmetics industry has a substantial financial interest in the study of hair. Hair samples can conveniently be assayed for levels of trace minerals and heavy metals in nutritional and toxicological studies. The processes of chemical carcinogenesis are often studied in tissue cultures of epidermal cells and the process of keratinization in these cells is viewed as a convenient molecular process to monitor for signs of metabolic disruption.

The various studies mentioned above may be conducted on different physiological levels and by various methodological approaches. Hair follicles can be examined histologically; the rate of hair growth can be measured and the timing of hair growth cycles can be noted. Hair follicle cells can be grown in tissue culture. Hairs can be stretched under pressure and their stress-strain graphs can be compared with those of other hairs. Hairs can be subjected to solubilization procedures

followed by detailed protein analysis. The most interesting and valuable investigations often incorporate the study of hair by more than one technique.

In 1976, Paul D. Mier and D.W.K. Cotton made the following remarks while discussing hair keratins:

> As in all fields of biochemistry, the first simplistic descriptive phase is followed by a phase of intensive, multi-disciplinary research which, at first, leads to a state of maximum confusion. This phase of maximum confusion in collagen chemistry was represented by the era of gelatin investigations but has now largely been resolved. Keratin research is just beginning to emerge from such a period and within the next five or ten years we may expect a definitive description of the keratin molecule at all levels.[1]

By the end of 1981 this prediction was showing signs of being fulfilled. Information from many research laboratories was finally beginning to indicate the quaternary structure of the keratin and inner root sheath or medullary proteins. The relationships among the various keratins was also beginning to be clarified.

The study of congenital hypotrichosis, a genetic hair defect in Hereford cattle, is worthwhile in economic terms because affected cattle show reduced viability.[2] It is further believed that ultimately an investigation into this genetic problem could add useful information to the overall study of hair proteins.

Histology and Environment of the Hair Follicle

Hair follicles characteristically follow a cyclic growth pattern which is exhibited by all mammals with the possible exception of merino sheep and poodles. The stages of anagen (growing), catagen (regressing), and telogen (resting) can be identified histologically. In some species

the phases of hair growth are synchronized and, thus, all of the hair coat sheds simultaneously. In others the individual hair follicles progress through their cycles independently of one another.

Hair is a holocrine secretion. New cells are formed from cell divisions in an area of matrix cells known as the hair bulb.[3] The bulb is an onion-shaped part of the hair follicle which grows around the dermal papilla. The cells produced in the hair bulb migrate distally up the hair follicle; during this migration they continue to differentiate and are eventually filled with hardened proteins.[4]

A cross-section of a hair follicle reveals four migratory layers of cells arranged in concentric rings. At the center are the cells of the medulla. These cells are arranged in a discontinuous manner and there are often air-filled gaps between the cells of the medulla. The medulla may even be absent in very fine hairs. The cortex envelops the medulla and composes the bulk of the hair shaft. The cuticular layer is exterior to the cortex. It anchors the hair shaft in the follicle and contains two inter-locking cuticles, one from the cortex and the other from the inner root sheath. The inner root sheath is the final migratory stratum and extends only between the bulb and the duct of the sebaceous gland.[5]

The non-migratory outer root sheath is continuous with the epidermis and does not undergo complete keratinization below the duct of the sebaceous gland.[6] The outer root sheath is covered by a hyalin membrane, and external to that is a sheath of connective tissue consisting of collagen and fibrocytes.[7]

The four cell layers that migrate distally up the hair follicle from the bulb become filled with hardened protein as they travel. The first

cells to harden are those of the inner root sheath and it is thought that perhaps this stiffened cell layer provides a firm mold for the still-soft cortex and medulla.[8]

As the hardened protein accumulates in the cytoplasm of these four cell layers their other cellular components atrophy. The hair shaft is virtually all protein although melanin granules and nuclear remnants are sometimes identified in scanning electron micrographs.[9]

The hair follicle resides in close apposition to dermal connective tissue and is sensitive to the external environment. All human hair follicles are well-supplied with nerves.[10] A capillary network also supplies the hair follicle and the growth of the hair is extensively affected by hormonal and nutritional changes. Chronic irritation of the skin or excessive exposure to sunlight may cause hypertrichosis.

A very striking example of the degree to which the hair follicle is affected by systemic factors is telogen effluvium. Telogen effluvium is a condition in which all the hair follicles are synchronized to enter telogen at the same time; telogen effluvium can occur following childbirth, fever, or severe psychiatric shock.[11] Whether the trigger for this phenomenon is neurological, hormonal, or metabolic is unknown.

Cortex and the Keratin Proteins

The hardened cortical cells of the mature hair shaft are composed of keratin. The term, "keratin," refers not to one simple protein, rather, it refers to a whole family of structural protein molecules. Classically, the keratins have been divided into two large groups - the hard keratins and the soft keratins. The hard keratins are found in hairs, horns, wool, and nails, while the soft keratins are found in the stratum corneum. Such

a division seems subjective but is supported by differences in both chemical composition and physical properties of the various proteins. Although stratum corneum, hair and nail contain a fibrous protein with the same general helical structure and dimensions, immunological information indicates that there are subclasses of fibrous keratin proteins. Digital organs such as fingernails, by their electrophoretic pattern and immunological identity, are similar to hair, while the keratin in the epithelium of the corneum of the eye and of the mouth fall into a different category typified by the stratum corneum.[12] The feather keratins and the keratins of reptile scales differ from the keratins of mammals; their secondary structure differs from that of mammalian keratin.[13]

The current concept of keratin structure as present in the hair shaft is that of a fibrillar protein embedded in a protein matrix. The existence of this type of structure is supported both by electron microscopy[14] and by X-ray diffraction patterns. When keratin is solubilized, as studies often require, the microfibrillar subunits are found to be in a low-sulfur, high-molecular weight fraction while the matrix is in a high-sulfur, low-molecular weight fraction. The microfibrils have extensive regions of alpha-helical conformation and intermolecular disulfide bonds. The amorphous matrix is a globular protein and its disulfide bonds are intramolecular.

The quaternary structure of fibrillar keratin is one of a triple-chain coiled coil alpha-helix. The X-ray diffraction pattern is consistent with this type of structure.[15] Bovine epidermal keratin filaments have this pattern.[16] The two proteins which are subunits of mouse epidermal keratin filaments can polymerize in vitro in ratios of

1:2 or 2:1; these in vitro filaments resemble native-type keratin filaments.[17] A prekeratin has been reported to contain three major polypeptides which form a coiled-coil helical molecule.[18]

Experimentally, hairs are often stretched under quantifiable pressure; "stress-strain" curves can be drawn which plot applied stress versus the per cent extension of the hair shaft.[19] The shape of a stress-strain curve reflects interactions between the fibrillar and matrix components of keratin in the hair cortex. When hair or wool is stretched, roughly one-third of the alpha-helices in the microfibrils will elongate and assume a beta-pleated sheet conformation. The associated matrix protein concurrently goes from a solid or gel state at rest, into a liquid or sol state during extension, and returns to a gel state after extension.[20,21]

The keratins, as a group of proteins, are quite susceptible to modifying forces originating in the environment. These changes in epidermal keratin have been monitored by the sampling of serial slices through the stratum corneum. The alpha-keratin or fibrillar keratin found in the living cell layers and inner stratum corneum has been compared to that found in the more exterior tissues. As the keratin moved farther from the living cell layers, thiol groups were first oxidized to form intrachain disulfide bonds, then rearrangement occurred so that the intrachain disulfide bonds became inter-chain disulfide bonds. This oxidation and rearrangement of disulfide bonds occurred at a level in the tissue which could be compatible with the presence of an enzyme. However in the most exterior layers $\varepsilon(\gamma$-glutamyl)lysine cross-links form between the microfibrils. These unusual cross-links occur only in the outermost layers of keratin and may be formed under environmental influences.[22]

Another example of the effects of environment upon keratin involves the hair of human patients suffering from Menkes' kinky hair syndrome. In these patients, samples obtained from the proximal hair shaft differ from those taken from the distal hair shaft, and the difference is assumed to be due to environment. Patients with this syndrome appear to suffer from copper deficiency but current research indicates an overproduction of a copper-binding protein which sequesters copper in some tissues of the body.[23] Hair is deprived of adequate copper. Copper is a likely cofactor for an enzyme forming disulfide cross-links in the microfibrils of keratin of the hair. Hairs of patients with Menkes' syndrome have a decreased number of disulfide bonds. A rough measurement of the relative lack of disulfide bonds in hair samples is obtained by measuring the relative solubility of the samples in 1 N ammonia. A change in the solubility of the hair could be used to monitor therapeutic attempts. Hair from the distal hair shaft should not be used for this test because disulfide bonds will gradually form in the distal hair shafts of these patients due to exposure to the external environment.[24]

A genetic defect which could be localized to the cortex would necessarily be a keratin problem. However because of the sensitivity of the keratin fibrillar-matrix complex to the immediate milieu it would be difficult to determine whether a keratin defect was the primary lesion or whether it occurred as a secondary response to a biochemical problem elsewhere in the follicle.

Inner Root Sheath and Medullary Proteins

The cells of the medulla and inner root sheath, like those of the cortex, begin growth as relatively undifferentiated cells in the bulb of

the hair follicle. Like the cortical cells, the cells of the inner root sheath and medulla fill with a hardened protein as they migrate up the hair follicle. However, the protein of the medullary cells and inner root sheath is not a keratin. This protein, unlike keratin, contains citrulline in peptide linkage and is also extensively cross-linked by ε(γ-glutamyl)-lysine cross-links.[25]

Both the cells of the inner root sheath and medulla contain large non-menbrane-bound electron dense granules referred to as trichohyalin granules. These granules become smaller and disappear as the inner root sheath and medullary cells migrate up the hair follicle and fill with hardened protein. The hardened protein in the inner root sheath has definite filamentous patterns under the electron microscope while the protein in the medullary cells has been described both as amorphous and as containing randomly-oriented filaments.[26] However, the proteins of the two cell types are biochemically similar.

The protein in the trichohyalin granules is rich in arginine and has an amino acid composition similar to that of the hardened protein with one exception - the hardened protein has less arginine but has a concomitant increase in citrulline. Carbon molecules found in the arginine residues in trichohyalin are later found in citrulline residues in the hardened protein. Also, an extract of hair follicle tissue is capable of acting on a substrate of trichohyalin and converting some of the arginine residues to citrulline residues.[27] Thus trichohyalin is a precursor for the hardened protein found in the inner root sheath and medulla, and as trichohyalin is transformed into hardened protein some of the arginine residues are enzymatically converted to citrulline residues.

Besides the presence of citrulline in peptide linkage, the hardened protein of the inner root sheath and medullary cells has another interesting feature - the presence of ε(γ-glutamyl)lysine cross-links. This cross-link is not unique to this protein; it is also found in insoluble fibrin and is formed in that case by the action of a plasma transamidase.[28] Some cross-links of this type are also found in the outermost layers of stratum corneum, as mentioned previously, and are presumed to occur as a result of environmental influences.[29] These ε(γ-glutamyl)lysine cross-links are present at the rate of 3 per every 100 amino acid residues in the protein of guinea pig hair medulla; this is an order of magnitude higher than the incidence of the same cross-link in insoluble fibrin.[30] The plasma transamidase and the follicle transamidase are not identical.

As is the case with several types of keratin and an isolated pre-keratin, the likely quaternary arrangement of the inner root sheath/medullary protein is that of a coiled-coil of alpha-helices.[31,32,33] The bulk of the citrulline residues and the cross-links are found on the non-helical regions of the subunits.[34]

Congenital Hypotrichosis in Hereford Cattle

Congenital hypotrichosis in Hereford cattle has been described at a histological and ultrastructural level. Hoof and horn are abnormal in isolated animals and the epidermis is noted to be unusually thin with poorly differentiated dermal papillae. However the most marked abnormalities are noted in hair shafts and follicles. Hair shafts of hypotrichotic Herefords are thin, soft, and easily broken. Trichohyalin granules of the inner root sheath are unusually large and smooth. An absence of arginine-converting-enzyme which converts arginine to citrulline has been suggested as the

cause of the abnormal trichohyalin granules and the resulting hypotrichotic condition.[35]

The purpose of this investigation is both to test for the presence of arginine-converting-enzyme in hair follicles of affected Herefords and also to probe for a cortical defect in hair shafts of affected Herefords.

References

1. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

2. Jayasekara MU, Leipold HW, Cook JE: Pathological Changes in Congenital Hypotrichosis in Hereford Cattle. Zentralblatt für Veterinärmedizin (Reihe A) 26:744-753, 1979.

3. Sato Y: The Hair Cycle and Its Control Mechanism. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

4. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

5. Montagna W: General Review of the Anatomy, Growth, and Development of Hair in Man. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

6. Hashimoto K, Shabazaki S: Ultrastructural Study on Differentiation and Function of Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

7. Langman J: Medical Embryology. Baltimore, Williams and Wilkins Company, 1975.

8. Sato Y: The Hair Cycle and Its Control Mechanism. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

9. Swift JA: A Technique for the Rapid Examination of the Gross Internal Structure of Mammalian Keratin Fibres. Journal of the Textile Institute 3:170-174, 1980.

10. Montagna W: General Review of the Anatomy, Growth, and Development of Hair in Man. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

11. Sato Y: The Hair Cycle and Its Control Mechanism. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

12. Baden HP, McGilvray LDL, Baden L, et al: Comparison of Stratum Corneum and Hair Fibrous Proteins. Journal of Investigative Dermatology 75:311-315, 1980.

13. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

14. Hashimoto K, Shibazaki S: Ultrastructural Study on Differentiation and Function of Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

15. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

16. Steinert P: Structure of the Three-Chain Unit of the Bovine Epidermal Keratin Filament. Journal of Molecular Biology 123:49-70, 1978.

17. Steinert P, Idler WW, Poirier MC, et al: Subunit Structure of the Mouse Epidermal Keratin Filament. Biochimica et Biophysica Acta, 577:11-21, 1979.

18. Lee LD, Baden HP: The Structure of Epidermal Keratin. Federation Proceedings 37(6):1813.

19. Baden HP: Genetic Basis for Structural Protein Changes in Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

20. Danilatos G, Feughelman M: Dynamic Mechanical Properties of α-Keratin Fibers During Extension. Journal of Macromolecular Science - Physics, B16(4):581-602, 1979.

21. Denilatos G, Feughelman M: The Microfibril-Matrix Relationships in the Mechanical Properties of Keratin Fibers. Textile Research Journal 50:568-574, 1980.

22. Steinert PM, Idler WW: Postsynthetic Modifications of Mammalian Epidermal α-Keratin. Biochemistry 18(25):5664-5669, 1979.

23. Olson RE: On the Pathogenesis and Clinical Expression of Menkes' Kinky Hair Syndrome. Nutrition Reviews 39(11):391-393, 1981.

24. Kahler JP, Goldsmith LA: Extractable Hair Protein in Menkes' Syndrome. Journal of Pediatrics 92(4):675, 1978.

25. Rogers GE: The Localization and Significance of Arginine and Citrulline in Proteins of the Hair Follicle. Journal of Histochemistry and Cytochemistry 11:700-705, 1963.

26. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

27. Rogers GE, Harding HWJ, Llewellyn-Smith IJ: The Origin of Citrulline-Containing Proteins in the Hair Follicle and the Chemical Nature of Trichohyalin, an Intracellular Precursor. Biochimica et Biophysica Acta 495:159-175, 1977.

28. Harding HWJ, Rogers GE: Formation of the ε(γ-glutamyl)lysine Cross-link in Hair Proteins - Investigations of Transamidases in Hair Follicles. Biochemistry 11(15):2858-2862, 1972.

29. Steinert PM, Idler WW: Postsynthetic Modifications of Mammalian Epidermal α-Keratin. Biochemistry 18(25):5664-5669, 1979.

30. Rogers GE, Harding HWJ: Molecular Mechanisms in the Formation of Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

31. Steinert P: Structure of the Three-Chain Unit of the Bovine Epidermal Keratin Filament. Journal of Molecular Biology 123:49-70, 1978.

32. Steinert P, Idler WW, Poirier MC et al: Subunit Structure of the Mouse Epidermal Keratin Filament. Biochimica et Biophysica Acta 577:11-21, 1979.

33. Lee LD, Baden HP: The Structure of Epidermal Keratin. Federation Proceedings 37(6):1813.

34. Steinert PM: Structural Features of the α-Type Filaments of the Inner Root Sheath Cells of the Guinea Pig Hair Follicle. Biochemistry 17(23):5045-5052, 1978.

35. Jayasekara MU, Leipold HW, Cook JE: Pathological Changes in Congenital Hypotrichosis in Hereford Cattle. Zentralblatt für Veterinärmedizin (Reihe A) 25:744-753, 1979.

PAPER 1: HYPOTRICHOSIS IN HEREFORDS IS NOT DUE TO AN ABSENCE OF ARGININE-CONVERTING-ENZYME

INTRODUCTION

At levels near the bulb of the hair follicle, cells of the inner root sheath and medulla contain trichohyalin granules. The granules are associated with filaments which appear at the borders of the granules and are oriented parallel to the axis of the hair follicle. As the cells of these layers migrate up the follicle toward the epidermis, fewer trichohyalin granules are seen. Eventually the cells in both the inner root sheath and medulla are filled with a hardened protein and no trichohyalin granules are seen.[1] The protein of the inner root sheath and medullary cells is distinct from keratin proteins and the protein in the hardened cells of these layers comes from a precursor protein found in the trichohyalin granules.[2,3,4]

An enzyme present in hair follicle extracts converts some of the arginine residues in trichohyalin to citrulline. This enzyme is referred to as arginine-converting-enzyme.[5]

When hypotrichotic Hereford follicular tissue was examined by electron microscopy, trichohyalin granules in the inner root sheath cells were larger than normal and no filaments were noted at the periphery of these granules. It was hypothesized that the large smooth trichohyalin granules could be due to a lack of arginine-converting-enzyme. The lack of this enzyme would prevent the conversion of the non-filamentous trichohyalin protein into the hardened protein found in normal inner root sheath and medullary cells.[6]

In order to test that hypothesis, frozen sections of hair follicles from both healthy and hypotrichotic Herefords were stained for citrulline. The presence of citrulline should indicate the presence of arginine-converting-enzyme.

MATERIALS AND METHODS

Skin biopsies of healthy and hypotrichotic Herefords were taken and immediately frozen. They were mounted on metal block holders with OCT[a] and were sliced to a thickness of 5-10 micrometers using an Ames Cryostat.[b] The resulting frozen sections of tissue were placed on cover slips coated with Mayer's Albumin Glycerol.[7]

Citrulline was detected by the Archibald reaction as modified by Guthohrlein and Knappe.[8]

The adherent tissue sections on the coverslips were stained for 20 minutes at 90° C. Pieces of filter paper spotted with citrulline, arginine, or polyarginine were used as controls.

RESULTS AND DISCUSSION

The frozen sections of follicular tissue from both healthy (Fig. 1) and hypotrichotic Herefords (Fig. 2) stained positively for citrulline, as evidenced by the development of orange coloration in the layers of inner root sheath.

To determine which layers of the inner root sheath are staining positively, it should be noted that the outermost layer which is stained presents a smooth and continuous outer border; this indicates that it is Henle's layer of the internal root sheath. Henle's layer and Huxley's layer are each one cell thick.[9] The positively staining cells are noted to be two cell layers thick indicating that Huxley's layer as well as Henle's layer are staining positively for the presence of citrulline.

[a,b] Ames Cryostat Company, Elkhart, Indiana 46514

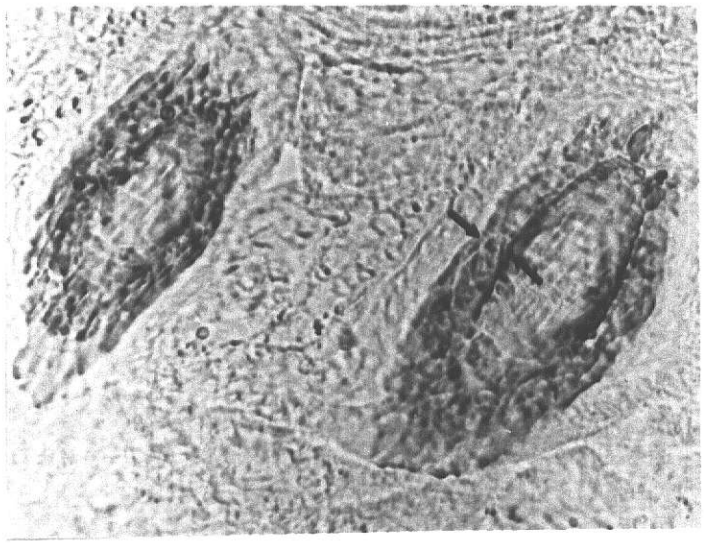


Figure 1. Follicular tissue of healthy Herfords. Although follicles were cut obliquely, arrows indicate where staining is clearly noted to be two cell layers thick.

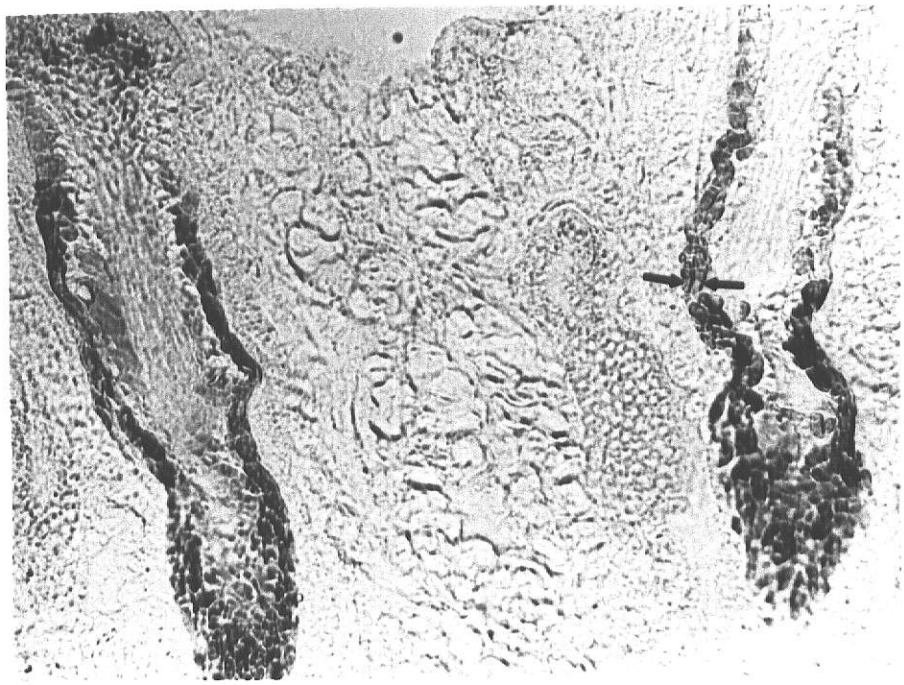


Figure 2. Follicular tissue from hypotrichotic Herfords. Arrows indicate that positive staining is two cell layers thick.

The demonstration of the specific end-product, citrulline, in both Henle's and Huxley's layers of the internal root sheath of hypotrichotic Herefords indicates that arginine-converting-enzyme is present. A defect in the amount of arginine-converting-enzyme present is not ruled out by this procedure.

REFERENCES

1. Hashimoto K, Shibazaki S: Ultrastructural Study on Differentiation and Function of Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

2. Rogers GE, Harding HWJ: Molecular Mechanisms in the Formation of Hair. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

3. Rogers, GE: Some Observations on the Inner Root Sheath Cells of Hair Follicles. Biochimica et Biophysica Acta 29:33-43, 1958.

4. Rogers GE: The Localization and Significance of Arginine and Citrulline in Proteins of the Hair Follicle. Journal of Histochemistry and Cytochemistry 11:700-705, 1963.

5. Rogers GE, Harding HWJ, Llewellyn-Smith IJ: The Origine of Citrulline-Containing Protein in the Hair Follicle and the Chemical Nature of Trichohyalin, an Intracellular Precursor. Biochimica et Biophysica Acta 495:159-175, 1977.

6. Jayasekara MU, Leipold HW, Cook JE: Pathological Changes in Congenital Hypotrichosis in Hereford Cattle. Zentralblatt für Veterinärmedizin (Reihe A) 26:744-753, 1979.

7. Thompson SW: Selected Histochemical and Histopathological Methods. Springfield, Illinois, Charles C. Thomas Company, 1966.

8. Guthorhlein G, Knappe J: Modified Determination of Citrulline. Analytical Biochemistry 26:188-191, 1968.

9. Birbeck MSC, Mercer EH: The Electron Microscopy of the Human Hair Follicle. Journal of Biophysical and Biochemical Cytology 3(2): 1957.

PAPER 2: INCREASED SOLUBILITY OF HAIR FROM HYPOTRICHOTIC HEREFORDS

INTRODUCTION

Human and animal hair can both be solubilized with appropriate solvents. The keratin proteins are solubilized by reducing agents which break the disulfide bonds between fibrillar keratin proteins.[1] Oxidation of the sulfide groups with agents such as peracetic acid will also solubilize keratins. Hair treated with β-mercaptoethanol and sodium dodecylsulfate (SDS) solubilizes both the fibrillar and matrix proteins of the keratin in hair.[2]

Medullary proteins are less soluble than keratins and require peptide bond cleavage before they will dissolve.[3]

Keratin can be solubilized from hair shafts using 1N ammonia but this process does not yield large amounts of protein.[4] Tetramethylammonium hydroxide (TMAH) will solubilize some keratins and the solubilized keratin shows less degradation than it does with other keratin solvents.[5]

The hair follicles of Hereford cattle affected by congenital hypotrichosis have been described both histologically and on an ultrastructural level. The hair shafts of hypotrichotic Herefords were also described as being different from those of healthy Herefords. Affected hair shafts have been described as being thin, soft, curly and easily broken.[6]

To test the hypothesis that hairs of healthy and hypotrichotic Herefords have different protein structure, the extractability of these different hair shafts in TMAH was determined. The hairs were also solubilized in SDS and β-mercaptoethanol and the resulting protein solutions were compared by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Hairs were collected from healthy Herefords, from hypotrichotic Herefords, and from healthy Holsteins. The hairs were cleaned by first soaking in petroleum ether for 12 hours and then subjecting them to alternate washings and rinsings with a common laboratory soap and with distilled water until the odor of petroleum ether was no longer detectable. The hairs were then allowed to air-dry and were then ground in a Wiley mill[a] and filtered through #20 mesh.

Cleaned and ground hair samples from six hypotrichotic Herefords, six healthy Herefords, and six healthy Holsteins were solubilized in 10% TMAH as described by Ogawa et al[7] except that 150 mg of hair was homogenized with 6 ml of 10% TMAH. After centrifugation the residue was reextracted with an additional 2 ml of 10% TMAH. The two extracts were pooled for further analysis. The absorbances of the extracts were measured spectrophotometrically at 280 nm and 340 nm. Because cattle hairs contain variable amounts of melanin even when the hair appears white, the absorbances at 340 nm were measured as an indication of background absorbance due to the presence of melanin. The amount of protein in each solution was calculated by $(A_{280} - A_{340})$. Analysis of variance was used to evaluate the data.[8] The significant treatments were located by the Student-Newman-Keul's multiple-range test.[9]

Cleaned and ground hair samples from one hypotrichotic Hereford, one healthy Hereford, and one healthy Holstein were solubilized in SDS and β-mercaptoethanol as described by Tezuka.[10] The proteins were separated by electrophoresis on 7.5% SDS-polyacrylamide gels at a

[a]Scientific Products, McGaw Park, Illinois 60085

current of 2 milliamps. The gels were stained with Coomassie blue dye overnight and were then destained with 10% acetic acid. The gels were examined spectrophotometrically at 540 nm with a gel scanner.[b] The area under each peak was determined by the paper-weighing method of integration and was expressed as the per cent of total area for that gel.

RESULTS

Hypotrichotic hair shafts were more soluble in 10% TMAH. Values for hypotrichotic solubilized protein were different from values for healthy Herefords and healthy Holsteins ($p<.01$). The amount of protein solubilized from healthy Hereford and healthy Holstein hair did not differ significantly (Table 1).

The soluble protein from hair treated with SDS-β-mercaptoethanol had 6 peaks (Figures 1-3) when separated on SDS-polyacrylamide gels and examined spectrophotometrically at 540 nm. No obvious differences were observed between healthy and hypotrichotic samples. The three peaks designated B were collectively elevated in hypotrichotic hair (48.7%) as compared to healthy Hereford hair (38.6%) or healthy Holstein hair (37.8%) (Table 2). Peak B_3 appears to be more prominent in the gel from the hypotrichotic Hereford hair.

[b]Gilford Instruments, Oberlin, Ohio 44074

Table 1 Solubilization of Hair Shafts

by 10% Tetramethylammonium Hydroxide

Sample	Number	$A_{280} - A_{340}$
		Mean ± standard error
Holstein	6	$1.72 \pm .0574^{a}$
Hereford	6	$1.85 \pm .0888^{a}$
Hypotrichotic	6	$2.56 \pm .261^{b}$

^{a,b}Means with different superscripts vary ($p<.01$)

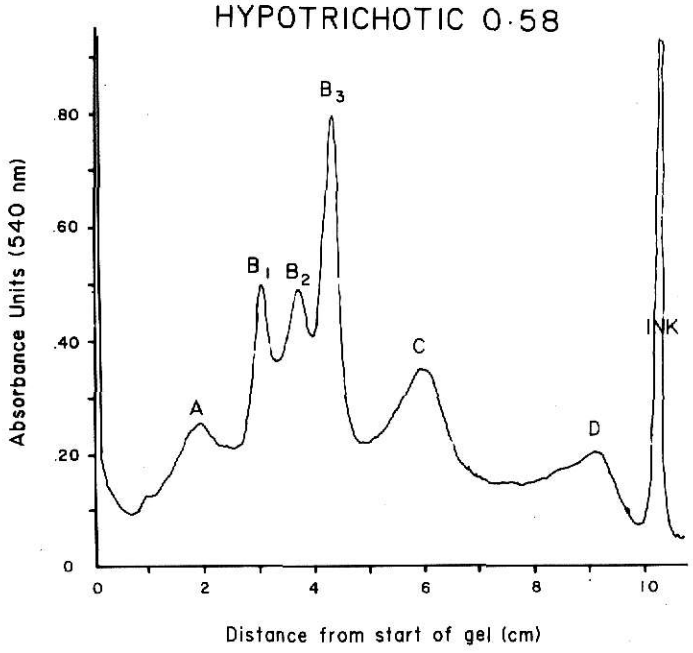


Figure 1. Spectrophotometric scan of electrophorogram of soluble hair proteins from a hypotrichotic Hereford. Nomenclature follows that proposed by Tezuka[11].

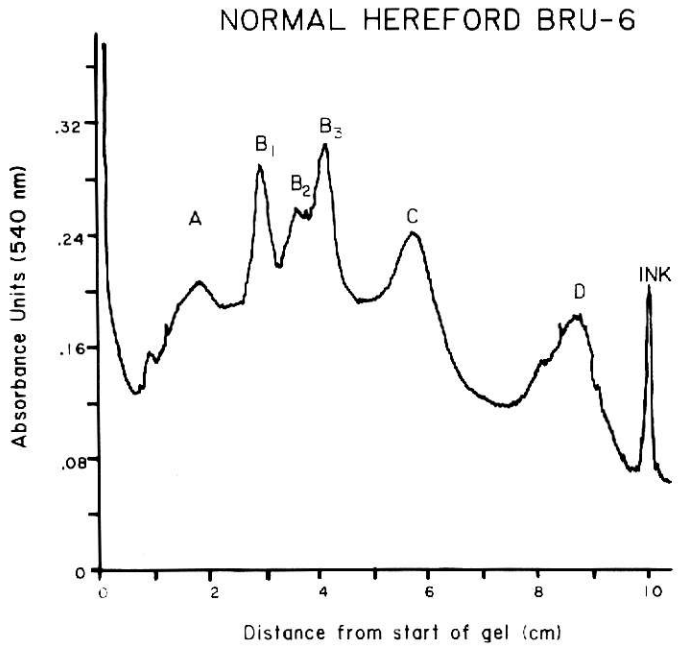


Figure 2. Spectrophotometric scan of electrophorogram of soluble hair proteins from a healthy Hereford. Nomenclature follows that proposed by Tezuka[11].

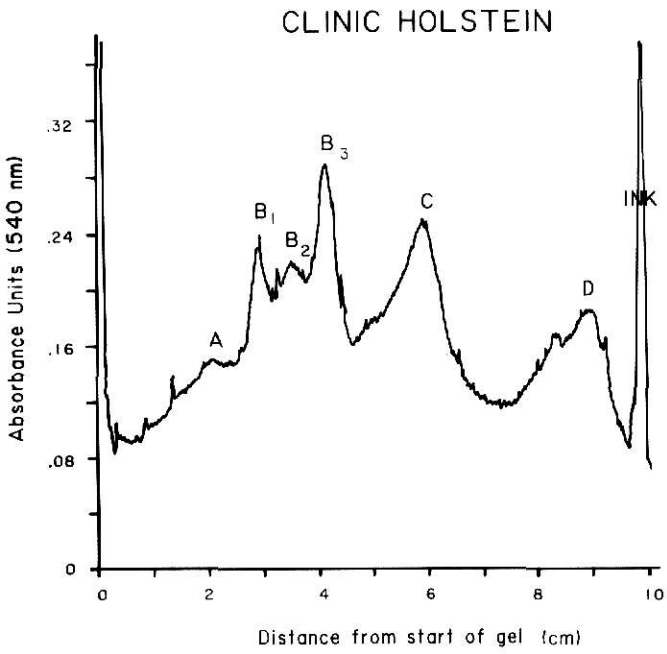


Figure 3. Spectrophotometric scan of electrophorogram of soluble hair proteins from a healthy Holstein. Nomenclature follows that proposed by Tezuka[11].

Table 2. Peaks from Figures 1-3 Expressed as Per Cent of Total Gel Area

Peak	Hypotrichotic Hereford	Healthy Hereford	Healthy Holstein
A	13.2%	18.5	11.1
B_1	13.1	12.6	10.1
B_2	13.2	9.2	11.1
B_3	22.4	16.8	16.7
(total B)	(48.7)	(38.6)	(37.8)
C	26.3	26.1	16.7
D	11.8	16.8	18.9

DISCUSSION

The protein solubilized by β-mercaptoethanol and SDS is probably keratin protein containing both fibrillar and matrix components. Hardened medullary protein is not solubilized by β-mercaptoethanol and SDS.[12] Any trichohyalin protein persisting in the medullary cells will be solubilized and appear in the polyacrylamide gels as a matrix-type protein.[13] It is unlikely that mature hair shafts contain significant trichohyalin protein.

The three prominent peaks designated B_1, B_2, and B_3 represent proteins derived from the fibrillar keratins of the cortex. The two rapidly-migrating bands C and D represent matrix proteins.[14] The origin of peak A has not been identified.

The protein bands of the polyacrylamide gels present no striking differences when the three gels are compared to each other. That is, there is not one band that is missing from one gel, and there is not an extra band on one gel. Hypotrichotic hair shaft qualities do not result from an absence of a keratin filament subunit or a gross deficit of matrix protein.

A subtle difference in the gel protein bands is present, however. Peak B_3 is much higher on the hypotrichotic gel than on the other two. This indicates that the fibrillar protein constitutes a greater proportion of the total soluble proteins in the hypotrichotic gel than in the other two gels.

Hair from hypotrichotic Herefords is significantly more soluble in 10% TMAH than is the hair of healthy Herefords or healthy Holsteins ($p<.01$). Increased solubility implies altered structure. The hair shaft contains

both cortical keratins and mature medullary protein. While it would seem feasible that a strong base such as TMAH should be capable of hydrolysis of medullary protein, in fact it is the keratin which is dissolved by TMAH.[14]

Initial biochemical examination of hair shafts of hypotrichotic Herefords reveals that there is no striking absence or alteration of proteins resulting in abnormal patterns after polyacrylamide gel electrophoresis. The cortical proteins of hypotrichotic hair shafts are significantly more soluble in 10% TMAH than are the cortical proteins of healthy Herefords. The increased solubility implies a structural difference. Whether this structural difference is a primary or secondary defect and whether it is present in the fibrillar or matrix cortical proteins has not been determined.

REFERENCES

1. Mier PD, Cotton DWK: The Molecular Biology of Skin. London, Blackwell Scientific Publications, 1976.

2. Ogawa T, Tsuda M, Kawanishi Y: Chemical Treatment and Fractionation of Hair Protein. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

3. Harding HWJ, Rogers GE: ε(γ-glutamyl)lysine Cross-linkage in Citrulline-Containing Protein Fractions from Hair. Biochemistry 10(4):624-630, 1971.

4. Kahler JP, Goldsmith LA: Extractable Hair Protein in Menkes' Syndrome. The Journal of Pediatrics 92(4):675, 1978.

5. Ogawa T, Tsuda M, Kawanishi Y: Chemical Treatment and Fractionation of Hair Protein. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

6. Jayasekara MU, Leipold HW, Cook JE: Pathological Changes in Congenital Hypotrichosis in Hereford Cattle. Zentralblatt für Veterinärmedizin (Reihe A) 26;744-753, 1979.

7. Ogawa T, Tsuda M, Kawanishi Y: Chemical Treatment and Fractionation of Hair Protein. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

8. Snedecor GW, Cochran WG: Statistical Methods. Ames, Iowa, Iowa State University Press, 1980.

9. Rohlf FJ, Sokal RR: Biometry. San Fransisco, W.H. Freeman and Company, 1969.

10. Tezuka T: Extraction of the Fibrous and Matrix Components of Rat Hair and Their Disc Gel Electrophoretic Profiles, Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

11. Tezuka T: Extraction of the Fibrous and Matrix Components of Rat Hair and Their Disc Gel Electrophoretic Profiles. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

12. Harding HWJ, Rogers GE: ε(γ-glutamyl)lysine Cross-linkage in Citrulline-containing Protein Fractions from Hair. Biochemistry 10(4):624-630, 1971.

13. Tezuka T: Extraction of the Fibrous and Matrix Components of Rat Hair and Their Disc Gel Electrophoretic Profiles, Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

14. Ogawa, Tsuda M, Kawanishi Y: Chemical Treatment and Fractionation of Hair Protein. Biology and Disease of the Hair. Baltimore, University Park Press, 1976.

HEREFORD HYPOTRICHOSIS:

A MOLECULAR APPROACH

by

REBECCA ROSE

B.S., Kansas State University, 1977
D.V.M., Kansas State University, 1981

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology

College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas 66506

1982

Hereford hypotrichosis has been characterized histologically and ultrastructurally. Unusually large and smooth trichohyalin granules have been noted in the inner root sheaths of affected animals. An absence of arginine-converting-enzyme, which converts arginine to citrulline, has been hypothesized.

Frozen sections of follicular tissue of both healthy and hypotrichotic Herefords were stained for the presence of citrulline using the Archibald reaction in which the presence of citrulline is detected by an orange color.

Hair shafts of six hypotrichotic Herefords, six healthy Herefords, and six healthy Holsteins were solubilized in 10% TMAH and the resulting solubilized protein was measured spectrophotometrically. Hair shafts from a hypotrichotic Hereford, a healthy Hereford, and a healthy Holstein were solubilized in a SDS-β-mercaptoethanol mixture and were then separated by electrophoresis in SDS-polyacrylamide gels. These gels were placed in a gel scanner and the resulting graphs were compared.

Frozen sections stained for the presence of citrulline indicated that citrulline was present in the inner root sheaths of both hypotrichotic and healthy Hereford follicular tissue. Hereford hypotrichosis is not due to a lack of arginine-converting-enzyme.

Solubilization of hair shafts in 10% TMAH indicated that hair of hypotrichotic Herefords was significantly more soluble than was the hair of healthy Herefords and Holsteins ($p<.01$). TMAH is a solvent for keratins and increased solubility of hypotrichotic hair in this solvent implies a

structural difference in the cortical keratin protein. Whether this difference is a primary or secondary defect and whether it is due to a structural change in the fibrillar or matrix component of cortical keratin has not been determined.

Comparison of electrophoretic scanning patterns after solubilization of hair shafts in SDS-β-mercaptoethanol and electrophoresis indicated that there were no major differences between hypotrichotic hair and hair from healthy Hereford and Holstein. Hypotrichosis is not due to a lack of a keratin subunit or the gross deficit of matrix protein.