Surface Layer of Wool. II. Dityrosine in Wool

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ABSTRACT: Values for dityrosine concentration were determined in wool and its morphological components, the cortex and cuticle. Dityrosine was found to be located in the tyrosine-rich proteins of wool, cortex, and cuticle. Within the cuticle it was shown to be present in the intercellular cement and the A-layer. Within the cortex it was shown to occur in the intermicrofibrillar matrix where the tyrosine-rich proteins are located. Dityrosine was not an artifact of acid hydrolysis but a natural part of the wool. An investigation of the *in vitro* formation of dityrosine in wool fibers showed that the concentration of dityrosine increased in the wool fibers upon oxidation with hydrogen peroxide or by UV irradiation. It is suggested dityrosine may be involved in the yellowing of bleached wool fibers. Fluorescence studies showed that wool fibers had a natural blue fluorescence when excited at 365 nm. Dityrosine was unlikely to be responsible for this fluorescence. Upon oxidation of wool fibers the intensity of fluorescence was enhanced, due to the oxidation of disulfide bonds to cysteic acid. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **66:** 2365–2376, 1997

INTRODUCTION

The present authors have shown that there is a very low concentration of basic and acidic residues within the cuticular layer of wool,[1] indicating that the cuticular layer was of a low polarity, thereby accounting for the hydrophobic nature of the wool surface layer. Such hydrophobicity is an important protective property of the cuticular layer against the external environment. The fiber can still absorb water to a certain extent, affecting the physical properties of wool by reducing the mutual attraction between acidic and basic groups and breaking many hydrogen bonds, thereby softening the keratin. However, the cuticular layer also has a high sulfur and cystine content in the form of disulfide bonds that maintain the integrity of the wool fiber by curtailing any tendency for it to dissolve and by limiting diametral swelling. The importance of the cuticular layer was shown by Bradbury and Peters[2] who reported a large increase in the amount of water sorbed at saturation when the cuticular layer was removed. Bywater et al.[3] showed that the cuticular surface of the wool fiber was extremely hard as evidenced by grooves worn in the stainless steel pins of textile combing machines by the constant passage of wool fibers over the pins. Hence the cuticle layer is of importance in relation to the whole physical character of the fiber.

The chemical reactivity of the wool fiber also is affected by the cuticular layer that acts as a surface barrier to impart a certain inertness to the fiber. These properties must be taken into account when dying wool fibers, as the diffusion of the large dye molecules is inhibited by the cuticle sur-

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face layer barrier. It has been suggested that the epicuticle may act as the actual barrier,[4] but disruption of the epicuticle did not facilitate dyeing.[5] By contrast, removal of the cuticular layer enhanced dye uptake,[6] indicating that the whole cuticle acted as a barrier to the diffusion of dyes.

All of the above properties of the cuticular layer, and therefore of the wool fiber as a whole, depend strongly on the location and composition of any structural components, their hydrophobicity, and involvement in intermolecular crosslinking. Since Goddard and Michaelis[7,8] demonstrated that wool fibers consisted of two major protein fractions, there have been extensive investigations into the nature of these proteins. However, doubt still remains as to how the components are distributed within the fiber, how they contribute to the physical and chemical behavior of the fiber, and even how the cystine crosslinks are connected and located. Such uncertainty partly stems from the fact that about 100 distinct proteins can be isolated from a wool fiber, and that during the isolation procedure most crosslinks must be broken.

This study concentrates on the surface layer of wool, with particular emphasis on the identification of the crosslinking dimer, dityrosine. Dityrosine has been identified in many structural proteins, in the cuticular layer of wool, in wool keratin, and in horse hair cuticle.[9] The tyrosine dimer is thought to aid in the mechanical stability of all wool proteins. However, the dimer has not been shown conclusively to exist as an actual crosslinking agent *in vivo*.

Wool fiber is composed of three main protein fractions, the high- and low-sulfur proteins and the glycine-tyrosine-rich proteins. While the high- and low-sulfur protein types have been extensively studied and amino acid sequences determined for a number of their components,[10–13] it is only recently that the tyrosine-rich proteins have been studied in greater detail and amino acid sequencing for these proteins has been carried out.[14,15] One possible reason for the initial lack of interest in the tyrosine-rich proteins was the belief that they were only minor constituents of keratins, located in the cell membrane complex of the cuticle, and therefore not structurally important components of the keratin cortex.[16,17] However, they constitute a major fraction of some keratins, e.g., up to 12% in some wools,[18,19] and so must play a significant role in the wool structure.

The present work is concerned with determination of the content and location of dityrosine within the wool fiber and its possible link with the tyrosine-rich proteins. Results will lead to a better understanding of its role as a crosslink, and relate its presence with the physical and chemical properties of the wool fiber.

EXPERIMENTAL

Identification of Dityrosine in Wool and Its Morphological Subcomponents

Acid hydrolysates of Merino 64's wool were prepared. Dityrosine was identified within wool cortex and cuticle by analyzing each acid hydrolysate by reversed-phase high performance liquid chromatography (RP-HPLC). The amino acids were detected by absorption at 200 nm. A typical elution profile is shown in Figure 1. The hydrophobic amino acids dityrosine, tyrosine, and phenylalanine proved to be well separated in the elution profile and were easily identified by comparing the characteristic retention times to that of the corresponding amino acid standard. Dityrosine concentrations as low as 2 nmol mL^{-1} could be determined. The system was not suitable for the detection of the hydrophilic amino acids that were eluted together from the column, and therefore could not be identified.

The method was validated by spiking the keratin hydrolysate and also virgin wool prior to hydrolysis separately with dityrosine, tyrosine, and tryptophan. These latter two compounds showed that dityrosine was not an artifact of hydrolysis. In addition paper and column chromatographic separation[20] performed on the hydrolysates and a dityrosine solution confirmed the presence of dityrosine in wool and its fractions.

The values determined for dityrosine concentration within wool, cortex, and cuticle are shown in Table I.

The dityrosine concentration (2.60 μmol g^{-1}) determined for whole wool agreed with that obtained by Raven et al.[21] The concentration of dityrosine in the wool fiber was similar to that determined for the isopeptide crosslink, ϵ-(γ-glutamic) lysine, which itself is of relevance to the overall structure and property of the wool fiber.[22,23] Hence, a relatively low concentration of dityrosine in whole wool does not mean its presence can be disregarded because it would contribute to wool structure and properties in a similar way to ϵ-(γ-glutamic) lysine.

Dityrosine has previously been detected in the

cuticular layer of structural proteins. In this work it was also found to be present in the cuticular layer of wool, although at a lower concentration than the parent fiber (1.34 μmol g^{-1}). Dityrosine was also identified in the cortex where it was found to be present at a higher concentration than the parent fiber (3.20 μmol g^{-1}). It is possible that dityrosine, being mainly located within the cortex, could be of importance to the overall structure of the fiber.

Having identified dityrosine within the wool fiber and its subcomponents, the next step was to determine the location and role of the compound within the subcomponents.

Dityrosine, a tyrosine dimer, may be located within the tyrosine-rich proteins of the wool fiber. Therefore such proteins were isolated from wool fiber, cortex, and cuticle by performic acid oxidation[24] (designated δ-keratoses[25]). Table II shows the amino acid analysis results for the δ-keratose proteins isolated from wool, cortex, and cuticle. The proteins were rich in tyrosine and glycine, phenylalanine, and serine. Together these amino acids accounted for 60% of the total amino acid of the tyrosine-rich δ-keratose proteins from wool, and 40% of the δ-keratose from cortex and cuticle. The δ-keratose proteins possessed a low content of cysteic acid, lysine, histidine, isoleucine, glutamic acid, alanine, and valine. Methionine was not detected in any of the three δ-keratose proteins. The absence or low concentrations of such residues suggests that proteins isolated from the heterogeneous mixture may be lacking in some of these amino acid residues. These findings for the δ-keratose proteins isolated from the wool fiber are in agreement with previous data[19,26] and are a characteristic of tyrosine-rich proteins isolated from wool keratin fibers. Previous studies on isolated morphological components of the wool fiber have not shown the presence of tyrosine-rich proteins having the characteristic amino acid composition in structures other than the cortex.[27] Thus, in this work a tyrosine-rich protein from cuticle cells was isolated having the typical composition of high-tyrosine proteins. The yield, however, was quite low, being 2%. This may still be a quite significant

Table I Dityrosine Determination in Virgin Wool, Cortex, and Cuticle by RP-HPLC

Amino Acid	Wool (μmol g^{-1})	Cortex (μmol g^{-1})	Cuticle (μmol g^{-1})
Tyrosine	309	280	192
Dityrosine	2.60	3.20	1.34
Phenylalanine	250	220	131

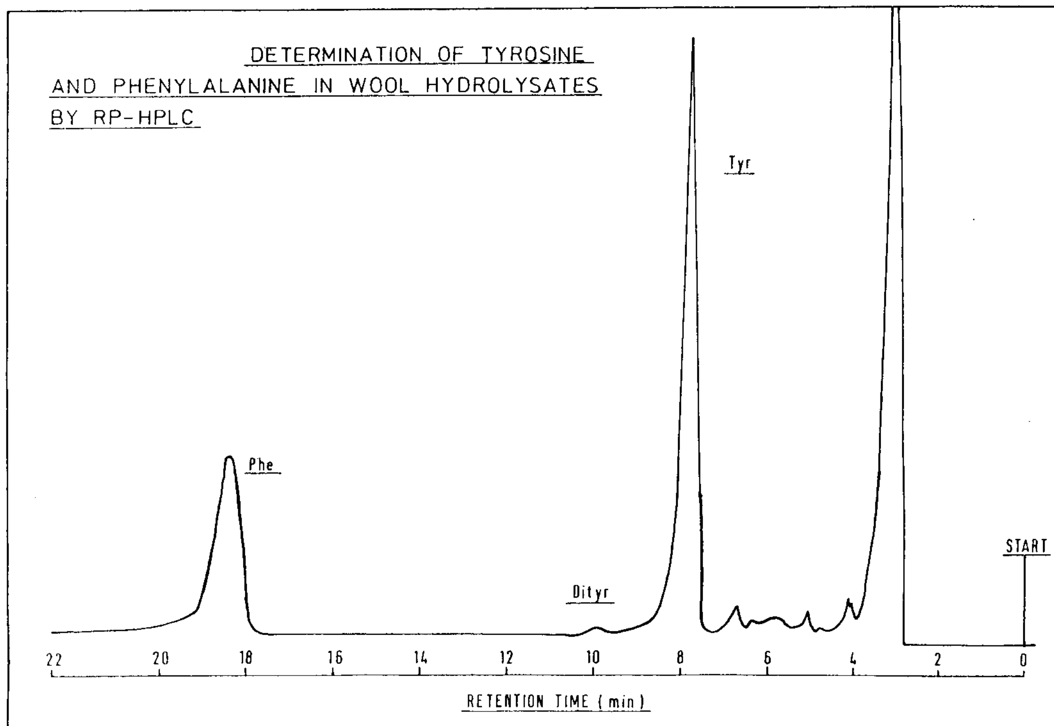

Figure 1 Determination of dityrosine in wool hydrolysate by RP-HPLC.

Table II Amino Acid Analysis of Tyrosine-Rich (δ-Keratosse) Proteins from Wool, Cortex, and Cuticle

	δ-Keratose (μmol g^{-1})		
Amino Acid	Wool	Cortex	Cuticle
ASP	418	125	346
THR	302	344	333
SER	1006	715	1017
GLU	340	538	516
PRO	304	213	447
GLY	2592	1002	1180
ALA	277	330	361
VAL	217	353	269
CYA	322	179	422
1/2 CYS	0	0	0
MET	0	0	0
ILE	118	227	212
LEU	592	676	809
TYR	874	456	443
PHE	837	438	439
LYS	76	48	73
HIS	49	51	42
ARG	435	476	414

value, considering the very high-sulfur nature of the structure. Of the tyrosine-rich proteins isolated from whole wool and cortex, a total of 5 and 20%, respectively, of the original weight of the protein was isolated. The value for the proteins isolated from whole wool is in keeping with previous reports that suggested that these proteins can account for values up to 12% of fine Merino wool.[18,19]

The value of a 20% yield for tyrosine-rich proteins from wool cortex suggests that these δ-keratose proteins constitute a major part of the cortical cells and are therefore not a minor fraction as previously thought.[16,17] The question remains that the high yield could be caused by proteins other than tyrosine-rich proteins being isolated during the extraction procedure. Such could not have occurred because during the procedure the reaction time was kept to a minimum and the soluble fraction was immediately separated from the bulk fiber and dialyzed at once to precipitate out the glycine-tyrosine proteins. These experimental precautions were successful in suppressing solubilization of other proteins as evidenced by the amino acid analysis (cf. Table II) of the isolated proteins that show them to have the typical composition of tyrosine-rich proteins. Therefore, it can be concluded that tyrosine-rich δ-keratose proteins do exist as a significant component of the cortical cells of wool.

Having isolated proteins from wool and its morphological components, the next step was to analyze each protein for dityrosine by RP-HPLC. Dityrosine was identified in each of the tyrosine-rich proteins isolated from wool, cortex, and cuticle (cf. Table III). A value of 4.82 μmol g^{-1} was determined for dityrosine in the δ-keratose proteins isolated from wool, in agreement with the lower range of values determined by Otterburn and Gargan.[28]

The dityrosine concentration in the cortex and cuticle tyrosine-rich proteins were 4.95 and 2.11 μmol g^{-1}, respectively. In every case dityrosine was found to be present at a higher concentration in the δ-keratose proteins than in the respective component from which they were isolated, suggesting that dityrosine must occur mainly within these δ-keratose proteins in the wool fiber, and hence within the cortex and cuticle. Dityrosine was found to be present at a higher concentration in the δ-keratose proteins isolated from the cortex than in those isolated from the wool or cuticle. This agrees with earlier results. In the tyrosine-rich δ-keratose proteins isolated from cuticle cells, dityrosine was found to have increased by a factor similar to that for dityrosine in the δ-keratose proteins from cortical cells. Again this suggests that dityrosine must be mainly located within these proteins, albeit at a low concentration.

Dityrosine has been identified within the δ-keratose proteins of wool and its subcomponents, but where exactly are these proteins located within the fiber and what role do they play specifically within the cortex and cuticle?

Reports indicate that δ-keratose proteins are located within the cell membrane complex of the cuticle cells.[16,17,29,30] Therefore, it was decided to

Table III Dityrosine Determination in Tyrosine-Rich Proteins of Wool, Cortex, and Cuticle by RP-HPLC

	δ keratose (μmol g^{-1})		
Amino Acid	Wool	Cortex	Cuticle
Tyrosine	800	410	400
Dityrosine	4.82	4.95	2.11
Phenylalanine	780	390	390
Glycine[a]	2592	1002	1180

[a]Glycine concentration determined using LKB4101 analyzer.

Table IV Amino Acid Composition of Tyrosine-Rich Proteins Isolated by Formic Acid Treatment of Wool

Amino Acid	Cuticle (μmol g^{-1})	Tyrosine-Rich Protein (μmol g^{-1})	δ-Keratose Cuticle (μmol g^{-1})
ASP	221	485	346
THR	459	506	333
SER	1571	952	1017
GLU	988	795	516
PRO	620	ND	447
GLY	861	971	1180
ALA	570	343	361
VAL	595	273	269
CYA	80	173	422
1/2 CYS	1468	76	0
MET	42	0	0
ILE	200	136	312
LEU	448	319	809
TYR	195	500	443
PHE	150	445	439
LYS	225	290	73
HIS	79	200	42
ARG	438	518	414

Cuticle and cuticle δ-keratose compositions are shown for comparison.

analyze these proteins for dityrosine. The formic acid treatment method was employed to isolate the δ-keratose proteins from wool. A 1-h agitation resulted in the extraction of 1% (w/w). A protein content of approximately 0.3% (w/w) was present in the extract that was known to consist mainly of lipids.[30] The amino acid composition of this formic acid extract was determined (cf. Table IV), and was found to be rich in tyrosine, phenylalanine, glycine, and serine ($\sim$ 40%). The result was similar to that determined for δ-keratose proteins from cortex and cuticle. However, it was also observed that the protein consisted of considerably more lysine, histidine, and glutamic acid when compared to the amino acid composition of the δ-keratose proteins isolated from cuticle, cortex, or whole wool. This suggests that the δ-keratose proteins is of a different type from that isolated from the cuticle cells, and therefore may be of a different origin.

When the protein was analyzed by RP-HPLC, dityrosine was found to be present in a greater concentration than previously determined in any other component (7.4 μmol g^{-1}, Table V). Therefore, dityrosine must occur within the cell membrane complex where it is of some importance in maintaining the network mechanical stability.

Table V RP-HPLC Analysis of Tyrosine-Rich Protein Isolated from Wool by Formic Acid Treatment

Amino Acid	Tyrosine-Rich Protein (μmol g^{-1})
Tyrosine	524
Dityrosine	7.40
Phenylalanine	440

In an attempt to determine more specifically where dityrosine was located within the cuticle cells, an enzymatic study was carried out. It involved the papain dithiothreitol digestion of the cuticle cells. Samples of digested cuticle were removed at 1.5 and 2.5 h during the course of the digestion, and the total amino acid composition as well as the dityrosine content were determined (cf. Table VI). The results show that as digestion proceeded the concentration of cystine increased, implying that initial digestion removed the endocuticular layer, exposing the more resistant layers to attack by the enzyme.[31] Further digestion resulted in attack of the exocuticle layer until after a digestion time of 2.5 h, only the A-layer and the

Table VI Amino Acid Analysis of Enzymatic Digests of Wool Cuticle

Amino Acid	Cuticle (μmol g^{-1})	Enzyme Digest (μmol g^{-1})	
		1.5 h	2.5 h
ASP	221	156	100
THR	459	473	609
SER	1571	1183	2940
GLU	988	781	923
PRO	620	479	666
GLY	861	622	723
ALA	570	498	607
VAL	595	482	565
CYA	80	56	36
1/2 CYS	1468	1436	1752
MET	442	17	16
ILE	200	147	182
LEU	448	371	437
TYR	195	230	252
PHE	150	170	205
LYS	225	168	220
HIS	79	63	67
ARG	438	367	399

cell membrane complex remained. The amino acid composition of the digest after 2.5 h was rich in cystine, and also in the amino acids such as serine, threonine, and proline known to be present in cystine-rich proteins. When the helix to nonhelix index was determined for this protein and for the protein fraction isolated after a digestion time of 1.5 h, values of 0.38 and 0.54 were determined, respectively (compared with the value of 0.63 for cuticle cells). Thus, as the time of digestion increased, first the endocuticle and then the exocuticle were digested leaving a protein fraction of a high sulfur content, as indicated by amino acid analysis, and a low helix to nonhelix index. The results indicate that this fraction consisted of the A-layer. The cell membrane complex must also be present because it is greatly resistant to attack by the enzyme.[31] When the protein fractions were then analyzed for dityrosine, values of 2.10 and 1.84 μmol g^{-1} were determined for the 1.5 and 2.5 h fractions, respectively. Therefore dityrosine was present in both fractions at approximately the same concentration, suggesting that dityrosine was present in the A-layer of the cuticle cells. However, the A-layer could not be isolated free from the cell membrane complex, and it was difficult to determine just how much was actually present in the A-layer protein and how much in the cell membrane complex. The extremely high-sulfur content and low tyrosine content would suggest that the A-layer was the main protein constituent of the fraction, and therefore dityrosine must be present to some extent in this component of the cuticular layer.

Values for dityrosine content in wool and its morphological subcomponents were determined. The location of this dimer within the glycine-tyrosine rich proteins was identified, indicating that within the cuticular layer, dityrosine occurs within the A-layer and cell membrane complex.

DISCUSSION

This work showed that dityrosine was present at a greater concentration within the cortex of the wool fiber, and that it occurred in the tyrosine-rich δ-keratose protein of the cortex. Originally the tyrosine-rich proteins were thought to be only within the cuticle component of the fiber.[17] However, later studies showed that the tyrosine-rich proteins could account for up to 12% of the wool fiber[18,19] (the present work has indicated a value of 5%) and could not all be accommodated within the cell membrane complex of the cuticle cells because this component only accounted for approximately 3% of the total fiber.[16,30] It was shown that the tyrosine-rich proteins occurred to a great extent in the intermicrofibrillar matrix, along with the high sulfur proteins, as a structural component of the keratin.[32] The location of dityrosine within the cortical proteins implies that it acted as a crosslink within this nonhelical intermicrofibrillar matrix, and therefore aided in maintaining the overall conformation of the matrix. In so doing, the dityrosine crosslink would play a role, along with that of the disulfide bonds, in providing some degree of orientation to the matrix of the intact fiber. Evidence for the role of the disulfide bonds in this function has already been reported.[33,34]

The cuticular membrane proteins were also shown to contain dityrosine that provided mechanical stability by crosslinkage. These cuticular membrane proteins, although tyrosine-rich proteins, were different in their amino acid composition from the tyrosine-rich proteins isolated from whole cuticle by oxidation. They were found to be richer in glutamic acid, histidine, and lysine, implying that the tyrosine-rich proteins isolated by oxidation were of a different type and origin than proteins of the membrane complex. Such differences in composition have been reported previously.[26] Dityrosine was identified in both tyrosine-rich proteins of the cuticle, showing that dityrosine present in the cell membrane complex must also occur elsewhere within the cuticle cell. The exact location of the tyrosine-rich proteins isolated by oxidation of the cuticle cells was sought by using enzymatic digestion. The results suggested that dityrosine occurred within the A-layer where it could exist as a crosslink. However the A-layer is already highly crosslinked with disulfide bonds, and therefore the role of dityrosine as a crosslink would be minimal by comparison. Work on the hair cuticle by Swift and Bews[31] showed that the concentration of the aromatic amino acids within the A-layer was greater than expected, suggesting that the A-layer was probably a complex mixture of different types of proteins. In this work the concentration of tyrosine and phenylalanine was shown to increase moderately with time of digestion in agreement with the results of Swift and Bews.[31] It is possible that a type of tyrosine-rich protein exists in the A-layer, which is associated with the proteins isolated by performic acid oxidation of the cuticle and serves as a source of dityrosine. The values determined

Table VII Effects of Oxidation on Dityrosine Levels in Virgin Wool Fibers

Amino Acid	Virgin Wool (μmol g^{-1})	Virgin Wool (μmol g^{-1})		
		Oxidized at 80°C	Oxidized at 25°C	Control
Tyrosine	303	196	203	229
Dityrosine	2.66	4.03	3.30	2.56
Phenylalanine	276	224	244	234

for dityrosine in the tyrosine-rich proteins isolated by oxidation and in the 2.5-h enzymatic digestion fraction would support this suggestion, but the fraction isolated enzymatically is known to be contaminated with cell membrane complex proteins. So the point can only be proven conclusively by a study on a homogeneous fraction of the A-layer.

Existing limited evidence suggests that dityrosine functions as a crosslink by bridging peptide chains into a stable 3-dimensional network with unique chemical properties, including adhesion.[35,36] However rigorous proof is missing, and in only one system (the fertilized sea urchin egg) has a peroxidase been identified whose major function is the formation of dityrosine.[37] The fact that dityrosine occurs in proteins rich in glycine and tyrosine (i.e., the tyrosine-rich proteins), in which glycine is present at a greater percentage, favors the proposal that dityrosine exists as a crosslink. The formation of dityrosine crosslink would require reaction between protein-bound tyrosine radicals, which would have to be in close contact for the reaction to occur. Such contact would be difficult if the neighboring amino acid residues carried bulky side chains, and would be more likely to occur if the residues surrounding the tyrosine residues did not have bulky side chains. The presence of a high concentration of glycine in these proteins favors dityrosine crosslink formation, because the higher the proportion of amino acids with small side chains (such as glycine), the greater will be the number of peptide chains allowed to come into close proximity. Therefore, in such a protein, which also has a high tyrosine content, the probability of dityrosine crosslink formation will be increased. However, only by isolating a dityrosine-containing peptide and carrying out amino acid sequence studies will more conclusive evidence be obtained for the function of dityrosine as a crosslink.

Anderson[38] stated that for dityrosine to exist as a crosslink in the protein resilin, the oxidation of tyrosine residues was necessary. He believed it to be an enzymatic process, and since peroxidase together with hydrogen peroxide was able to perform such an oxidation *in vitro,* it was possible that a peroxidase oxidized this reaction *in vivo.* Anderson[38] proposed that the enzyme that oxidized the tyrosine residues was located near to or in the cell membrane in such a way that the chains were oxidized while being secreted. A similar enzymatic process with wool keratin is possible during keratinogenesis with the formation of dityrosine crosslinks. These dityrosine crosslinks and possibly isopeptide crosslinks[22,23] would aid in stabilizing the protein prior to formation of disulfide bonds. To prove that this did occur it would be necessary to identify a peroxidase in the wool follicle capable of such a reaction. As mentioned above, a peroxidase enzyme capable of forming dityrosine *in vivo* has been identified in the fertilized sea urchin egg. Dityrosine has also been identified in human periodontal ligament collagen[39] where a lactoperoxidase enzyme known to be in the oral environment is involved in the reaction. However, while studies have shown the *in vitro* oxidation of human periodontal ligament collagen in the presence of the enzyme does result in dityrosine formation, it is still not known if the same route can occur *in vivo.*[39] However, final proof will only be obtained by isolating a dityrosine-containing peptide that could then be sequenced, and by determining the presence of a peroxidase that would be capable of oxidizing tyrosine residues to dityrosine.

Oxidation and UV Irradiation of Merino Virgin Wool and Salt's Serge Wool Fabric

It has been shown that the oxidation of some structural and nonstructural proteins with peroxidase and hydrogen peroxide resulted in the *in vitro* formation of dityrosine.[38–40] Dityrosine formation has also been observed when solutions of tyrosine were exposed to UV irradiation.[41] Therefore, it was decided to oxidize virgin wool fibers and Salt's Serge fabric with a typical hydrogen

Table VIII Effect of Oxidation and Irradiation on Dityrosine Levels in Wool Fabric

Amino Acid	Wool Fabric (μmol g^{-1})	Wool Fabric (μmol g^{-1})		
		Oxidized	UV Irradiated	Oxidized and Irradiated
Tyrosine	293	165	226	190
Dityrosine	2.32	3.78	3.10	3.98
Phenylalanine	271	158	250	200

peroxide bleaching solution used in the industrial treatment of wool fabrics, to determine if the process resulted in the formation of dityrosine. Samples of virgin wool were oxidized at 80° and 25°C for 3 h. A control sample in which hydrogen peroxide was absent was also analyzed for dityrosine. The results of these oxidative treatments (cf. Table VII) show that when virgin wool fibers were oxidized the level of dityrosine within the fibers increased. More dityrosine was formed when the fibers were treated at 80° than at 25°C. When the fibers were treated at 80°C in the absence of hydrogen peroxide, dityrosine levels did not increase. When samples of wool fabric were oxidized using the same methodology, the level of dityrosine was also found to increase (cf. Table VIII).

Bleaching wool fabric with hydrogen peroxide accelerates subsequent yellowing of the fabric.[42] Dityrosine levels also increased in bleached wool fabric, so dityrosine may be involved in the yellowing process. It has been suggested that the yellowing of wool when exposed to radiation was connected with photodegradation of the aromatic amino acid residues (particularly tryptophan[43]) in a reaction in which tyrosine was involved in the internal transfer of excitation energy.[44] Some of the tyrosine-rich proteins contain tryptophan[19] and therefore could play a part in wool yellowing. To determine if dityrosine levels were affected by UV irradiation, samples of wool fabric and bleached wool fabric were irradiated and the level of dityrosine determined. Dityrosine levels were found to increase in irradiated fabric, the increase being greater in fabric samples bleached prior to irradiation (cf. Table VIII). The bleached samples were also noticeably more yellow than the unbleached irradiated samples. Röper and Finnimore[45] also found that dityrosine levels increased in wool as a result of irradiation. Therefore dityrosine levels were increased in wool fabrics by irradiation as well as by oxidation, the largest increase being when the wool fabric was oxidized and then UV irradiated. The resultant increased levels of dityrosine in UV irradiated wool fabric suggest that dityrosine could play a part as a photoproduct, contributing to the yellowing of wool and/or as an initiator of secondary photochemical reactions. However, the actual mechanism is unknown.

This study has shown that dityrosine occurs naturally in wool fibers, and that it was increased artificially either by oxidation or irradiation. Such a phenomenon is not unusual as the isopeptide crosslinks that were also shown to occur naturally in wool were found to increase in concentration upon heat treatment.[46]

Fluorescence of Wool Fibers

When a molecule is irradiated with visible or UV light, it may undergo an electronic transition during which the molecule absorbs a quantum of energy, and an electron is excited from the orbital it occupies in the ground state to another orbital of higher energy. For the molecule to return to the unexcited state, photon emission occurs in the form of fluorescence or phosphorescence.[47]

When wool is irradiated with UV light, in the range of 290–320 nm, it emits fluorescence in the invisible UV region.[48] The aromatic amino acids phenylalanine, tyrosine, and tryptophan are responsible for wool's absorption of UV light below 320 nm. The energy absorbed by phenylalanine and tyrosine is transferred to tryptophan residues, exciting them to the singlet and triplet excited states. Fluorescence emission then occurs as the singlet and triplet excited states of tryptophan decay to the ground state. Hence the reactions of these three amino acids explain the fluorescence of wool when excited at 290–320 nm.

However, wool is also known to emit visible fluorescence when excited by near UV light ($\geq$ 350 nm), but the actual chromophore(s) and the reactions responsible have not been established. Smith and Melhuish[49] proposed that part of the fluorescence from wool excited by near UV light arose from *N*-formylkynurenine residues present as a result of tryptophan oxidation. The

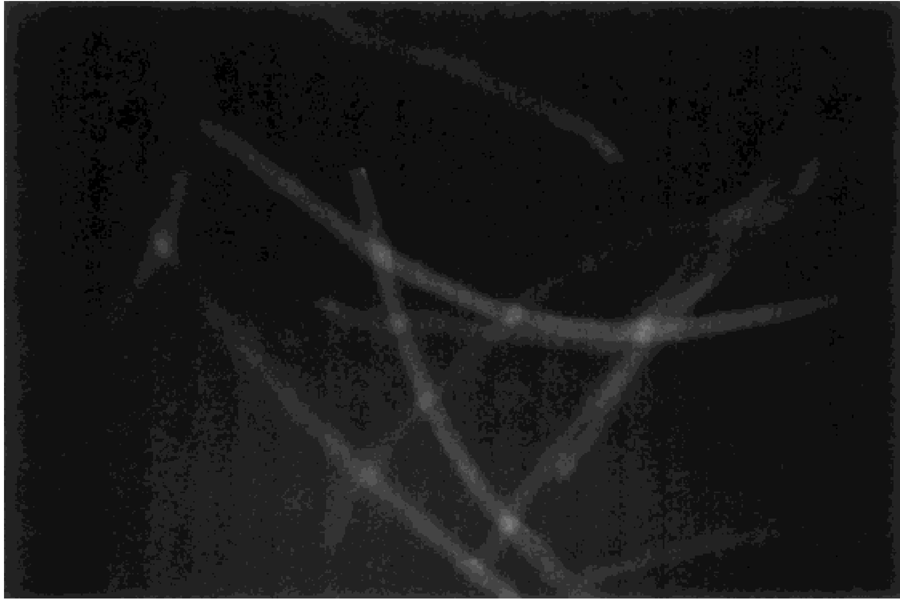

Figure 2 Fluorescent micrographs of Merino 64's virgin wool. Wool fibers excited at 345 nm.

natural visible fluorescence of other proteins has also been reported. For example, Fujimori[50] studied the effect of UV light on collagen, and showed that the blue fluorescence at 450 nm increased following irradiation of collagen at 253.7 nm. This was accompanied by a decrease in the UV fluorescence from tyrosine and phenylalanine. The irradiated collagen showed a fluorescence maxima at 350 nm that was attributed to the photoproducts formed from the irradiation of tyrosine and phenylalanine. Tryptophan could not be involved because collagen is devoid of this amino acid. Briza et al.[51] reported that when yeast ascospore walls were irradiated at 340–380 nm, they displayed a strong bluish fluorescence attributed to dityrosine present in the yeast ascospore wall.

Figure 3 Fluorescent micrographs of Merino 64's virgin wool. Wool fibers excited at 365–400 nm.

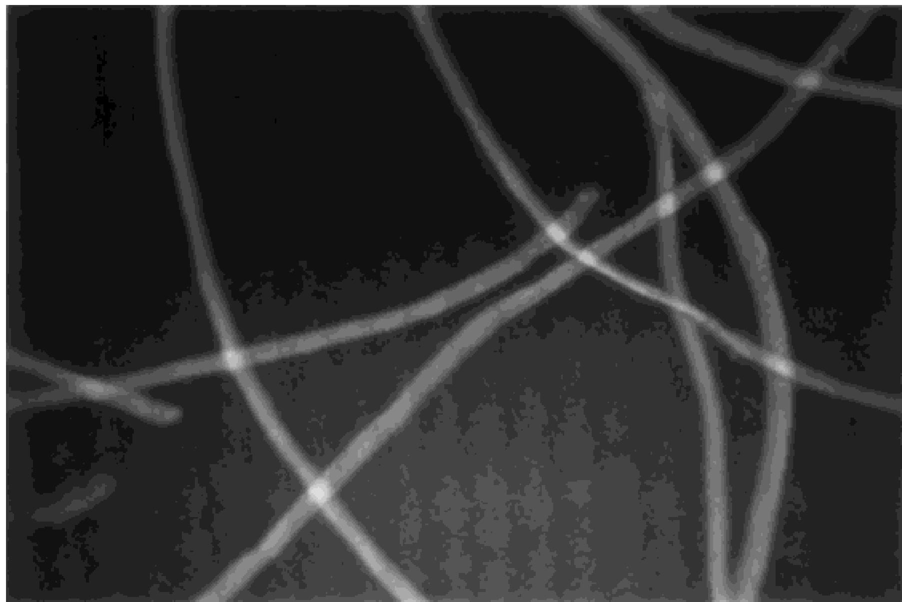

Figure 4 Fluorescent micrograph of Merino 64's virgin wool. Wool fibers oxidized at 80°C, excited at 365 nm.

The fluorescence of any substance can be quenched with certain other substances called quenchers. In brief,[47] a quencher is a substance that robs the energy-rich singlet state molecules of their excess energy that is transferred from the fluorophor to the second absorbing species. The quencher may give off the energy as heat or it may emit its own fluorescence. Some examples of quenching agents include oxygen, iodide, and metal ions.[47] With respect to the wool fiber, it is known that the cystine disulfide bonds quench the short wavelength emission from tryptophan.[52,53]

To determine if dityrosine was involved in the visible fluorescence of wool, samples of wool fibers were excited with both UV light (365 nm) and UV-violet light (350–400 nm). The possible effects

Figure 5 Fluorescent micrograph of Merino 64's virgin wool. Wool fibers oxidized at 80°C, excited at 350–400 nm.

on fluorescence from the oxidation treatment, any photooxidation products, and metal ion quenches present in the wool were investigated.

The wool fibers have a natural blue fluorescence at excitation wavelengths of 365 nm (cf. Fig. 2); and when excited at 350–400 nm, a green fluorescence was observed (cf. Fig. 3). The former blue fluorescence at 365 nm was in contradiction to the known properties of dityrosine. Dityrosine was determined as having a maximum absorbance at 315 nm under alkali conditions and at 284 nm under acid conditions; at a wavelength of 365 nm, dityrosine had negligible absorption and thus could not fluoresce at this wavelength. Therefore, dityrosine was not responsible for the observed blue fluorescence of wool at 365 nm. However during photooxidation tyrosine residues in wool, like those of collagen fibers,[50] form tyrosine radicals. Dityrosine could therefore be produced and function as an intermediate step in the reactions leading to the blue fluorescent property of wool.

When the fibers were oxidized at room temperature there was no significant change in the fluorescence intensity of the fibers. However, when oxidized at 80°C there was quite a significant change in the intensity of fluorescence at both wavelengths of excitation (cf. Figs. 4 and 5). Because dityrosine is not the main cause of fluorescence, the previously observed increase of dityrosine in oxidized wool fibers would not account for the increase in fluorescent intensities. Instead this increase in intensity appears to be related to the oxidation of the surface disulfide bonds to cysteic acid.[54] The disulfide bonds are known to quench fluorescence; cysteic acid is a nonquencher. When the cystine and cysteic acid concentration of the wool fibers oxidized at 80°C and at room temperature were determined, it was found that at the higher temperature there was a greater increase in cysteic acid content. Therefore the reduced concentration of disulfide bonds would account for the higher fluorescence intensity of the wool fibers oxidized at 80°C. The increase in fluorescence may also be a result of the formation of new chromophores from the oxidation procedure. Because oxidation did increase the level of dityrosine in wool fibers, this increase could be relevant if dityrosine was part of the process involved in the production of the blue fluorescence.

Wool fibers were boiled in solutions of EDTA and oxalic acid to determine if the presence of the metal ions, e.g., copper and iron, act as quenching agents. EDTA treatment resulted in a decrease in the intensity of fluorescence of the fibers excited at both wavelengths. Oxalic acid treatment did not appear to have any effect on the intensity of fluorescence when the fibers were excited at 365 or 350–400 nm. Therefore, the presence of the metal ions in wool fibers did not have a significant effect on wool fluorescence, because treatment with the strong chelating agent, oxalic acid, which removes any metal ions, did not affect fluorescence.

CONCLUSION

Dityrosine has been identified in wool fiber and its morphological components. It was located within the tyrosine-rich proteins of these structures, and within the cuticle it was located within the cell membrane complex and the A-layer. In all these structures dityrosine apparently occurs as a crosslink, providing mechanical stability.

Dityrosine levels were increased artificially in the wool fiber as a result of oxidation or UV irradiation and was probably involved in the yellowing of the wool fiber.

It has also been shown that dityrosine is not responsible for the natural blue fluorescence of wool fiber.

REFERENCES

1. K. Stewart, P. L. Spedding, M. S. Otterburn, and D. M. Lewis, *J. Soc. Dyers Col.,* **113,** 32 (1997).
2. J. H. Bradbury and D. E. Peters, *Textile Res. J.,* **42,** 248–250 (1972).
3. N. E. Bywater, T. Buckley, A. Hepworth, and J. Sikorski, *Proc. Evaporation Congr. Electron Microsc.,* **5,** 384–396 (1972).
4. J. D. Leeder and M. Lipson, *J. Appl. Polym. Sci.,* **7,** 2053–2066 (1963).
5. K. R. Makinson, *Textile Res. J.,* **38,** 831–842 (1968).
6. Y. J. Lim, S. H. Kim, W. K. H. Rye, and I. D. Park, *J. Korean Soc. Textile Eng. Chem.,* **10,** 53–55 (1984).
7. D. R. Goddard and L. Michaelis, *J. Biol. Chem.,* **106,** 605–614 (1934).
8. D. R. Goddard and L. Michaelis, *J. Biol. Chem.,* **112,** 361–371 (1935).
9. P. E. Gargan, Ph.D. thesis, Queen's University of Belfast, 1984.
10. J. C. Elleman, *Biochem. J.,* **128,** 1229–1239 (1972).
11. J. C. Elleman and T. A. A. Dopheide, *J. Biol. Chem.,* **247,** 3900–3909 (1972).

12. J. C. Elleman, *Biochem. J.,* **130,** 83–845 (1972).
13. L. S. Swart and H. J. Hayle, *Biochem. J.,* **133,** 641–654 (1973).
14. T. A. A. Dopheide, *Euro. J. Biochem.,* **34,** 120–124 (1973).
15. R. C. Marshall, J. M. Gillespie, A. S. Inglis, and M. J. Frenkel, *Proc. Int. Wool Textile Res. Conf.,* **6,** 147 (1980).
16. J. H. Bradbury, J. D. Leeder, and I. C. Watt, *Appl. Polym. Symp.,* **18,** 227–236 (1971).
17. R. A. Dedeuwaerder, W. G. Dobb, and B. J. Sweetmaon, *Nature,* **203,** 48–49 (1964).
18. J. M. Gillespie, *Comp. Biochem. Physiol.,* **41B,** 723–734 (1972).
19. J. M. Gillespie and R. L. Darkus, *Aust. J. Biol. Sci.,* **24,** 1189–1197 (1971).
20. K. Stewart, P. L. Spedding, M. S. Otterburn, and D. M. Lewis, *J. Appl. Polym. Sci.,* **66,** 2359–2363 (1997).
21. D. J. Raven, C. Earland, and M. Little, *Biochem. Biophys. Acta,* **251,** 96–99 (1971).
22. H. W. J. Harding and G. E. Rogers, *Biochemistry,* **11,** 2858–2863 (1972).
23. M. S. Otterburn, Ph.D. thesis, University of Bradford, 1971.
24. H. Brunner, A. Brunner, and J. Gerendas, *Appl. Polym. Symp.,* **18,** 55–64 (1971).
25. P. Alexander and C. Earland, *Nature,* **166,** 396–397 (1950).
26. J. M. Gillespie and M. J. Frenkel, *Proc. Int. Wool Textile Res. Conf.,* **5,** 265 (1975).
27. R. C. Marshall, J. M. Gillespie, H. S. Inglis, and M. J. Frenkel, *Proc. Int. Wool. Textile Res. Conf.,* **6,** 174 (1980).
28. M. S. Otterburn and P. F. Gargan, *J. Chromatog.,* **303,** 429–432 (1984).
29. J. D. Leeder and R. C. Marshall, *Textile Res. J.,* **52,** 245 (1982).
30. J. H. Bradbury, E. V. Chapman, and N. L. R. King, *Int. Congr. Recherches Textile Lainiere.* Paris, **3,** 359–366 (1965).
31. J. H. Swift and B. Bews, *J. Soc. Cosmet. Chem.,* **25,** 355–366 (1974).
32. R. D. B. Fraser, J. M. Gillespie, and J. P. MacRae, *Comp. Biochem. Physiol.,* **44B,** 943–947 (1973).
33. R. D. B. Fraser and J. P. MacRae, *Polymer,* **14,** 61–67 (1973).
34. C. J. Bailey, C. N. Tyson, and H. J. Woods, *Proc. Int. Wool. Textile Res. Conf.,* **3,** 105 (1965).
35. S. O. Anderson, *Biochim. Biophys. Acta,* **93,** 213–215 (1964).
36. D. P. De Vore and R. J. Gruebel, *Biochim. Biophys. Res. Comm.,* **80,** 993–999 (1978).
37. T. Decets, M. Farrance, E. S. Kay, L. Medill, E. F. Turner, P. J. Weidman, and B. M. Shapiro, *J. Biol. Chem.,* **259,** 13525–13533 (1954).
38. S. O. Anderson, *Acta Physiol. Scand.,* **66,** Suppl 263, 1–81 (1966).
39. J. Tenovuo and K. Paunio, *Arch. Oral. Biol.,* **24,** 591–594 (1979).
40. R. Aeschbach, R. Amado, and H. Neurkom, *Biochim. Biophys. Acta.,* **439,** 292–301 (1976).
41. S. S. Lehrer and G. D. Fusman, *Biochemistry,* **6,** 757–767 (1967).
42. R. Levene, *Textile Res. J.,* **55,** 477–483 (1985).
43. A. S. Inglis and F. G. Lennox, *Textile Res. J.,* **35,** 104–109 (1965).
44. F. W. J. Teale and G. Weber, *Biochem. J.,* **65,** 476–482 (1957).
45. H. Röper and E. Finnimore, *Proc. Int. Wool Textile Res. Conf.,* **7,** 21 (1985).
46. R. S. Asquith and M. S. Otterburn, *Appl. Polym. Symp.,* **18,** 277 (1971).
47. G. G. Guilbault, *Fluorescence Theory, Instrumentation and Practice,* Dekker, New York, 1967.
48. C. H. Nicholls and M. T. Paolthorpe, *J. Textile Inst.,* **67,** 397–403 (1976).
49. G. J. Smith and W. H. Melhuish, *Textile Res. J.,* **55,** 304–307 (1985).
50. E. Fujimori, *Biochemistry,* **5,** 1034–1040 (1966).
51. P. Briza, G. Winkler, H. Kalchhauser, and M. Breitenbach, *J. Biol. Chem.,* **261,** 197–199 (1986).
52. G. M. Bhatnagar and L. C. Gruen, *Aust. J. Biol. Sci.,* **21,** 1325–1326 (1968).
53. I. M. Leaver, *Photochem. Photobiol.,* **21,** 197–199 (1975).
54. J. H. MacLaren and B. Milligan, *Wool Scene—The Chemical Reactivity of the Wool Fibres,* London, Science Press, 1981.