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NATURE OF THE RESISTANCE OF WOOL TO DIGESTION BY ENZYMES

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ABSTRACT

Wool that has neither been injured mechanically nor modified chemically is completely resistant to attack by the proteolytic enzymes—pepsin, trypsin, chymotrypsin, and papain. When the cuticle or scale layer of the fibers is damaged by mechanical means, the wool becomes susceptible to attack by pepsin and chymotrypsin. Under these conditions only a small portion of the wool is digested, yet the fibers are considerably weakened and their fibrous structure is partly destroyed.

Wool in which the disulfide cross-linkages have been broken, as by reduction, or by reduction followed by methylation, is almost completely digested by pepsin and chymotrypsin, but is attacked only slightly by trypsin. When the reduced wool is reoxidized and its sulfhydryl groups are converted to disulfide groups, the wool regains its original stability. When the sulfhydryl groups of the reduced wool are converted to *bis*-thioether groups by the action of an aliphatic dihalide, the stability of the wool toward enzymes is greatly enhanced.

CONTENTS

I. INTRODUCTION

Proteins of the class called keratins, of which wool is a member, are so characteristically resistant to digestion by enzymes, that this phenomenon is usually included in the definition of the keratin group [1].[2] The exact reason for this resistance has not been entirely clear,

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[2] Figures in brackets indicate the literature references at the end of this paper.

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but on the basis of earlier work on wool it seems due to a stability resulting from the presence of disulfide cross-linkages between the polypeptide chains of the protein [2, 3], or to a resistance to diffusion of the enzyme into the fibers, brought about by an impermeable outer layer of scales or by the walls of the cells composing the fiber. For example, wool previously treated with alkali has been reported by Meunier, Chambard, and Comte [4], and by Fromageot and Porcherel [5] to be disintegrated into spindle-shaped cells by the action of preparations of pancreatic proteinases. Products resulting from the oxidation of human hair by solutions of bromine in glacial acetic acid, or by solutions of hydrogen peroxide in 0.4 *N* sulfuric acid, have been described by Stary and by Waldschmidt-Leitz and Schuckmann [6] as digestible by trypsin. An enzyme discovered by Linderstrøm-Lang and Duspiva [7] in the intestinal tract of the clothes moth was found capable of digesting wool in an alkaline and strongly reducing environment. Goddard and Michaelis [8] found that the amorphous reduction products obtained from wool by the action of alkaline thioglycolate solutions, or of other alkaline reducing agents, were digested by pepsin or trypsin. More recently, Routh and Lewis [9, 10] observed that wool powdered by prolonged grinding in a ball mill was changed chemically as well as physically, with the result that it became partly digestible.

Although all of the above-mentioned treatments affect the disulfide groups, none of them can be considered to involve these groups alone, so it cannot be finally concluded, on the basis of the earlier work, that the disulfide cross-linkages are responsible for the resistance of wool protein to digestion by enzymes; nor can the influence of histological structure be evaluated, since those treatments which destroy the fiber structure—such as grinding in a ball mill or reduction in strongly alkaline thioglycolate solution—produce other far-reaching changes as well.

There are still other barriers to drawing exact conclusions from the earlier studies. The preparations of enzymes used have usually not been pure crystalline substances, but have consisted of crude concentrates [4, 5, 8, 11]. Moreover, the methods available for detecting attack by enzymes have not been very sensitive, since they have depended on measurement of loss in weight [4], of determination of material not precipitable by sulfosalicylic acid [8], or merely of microscopic observation of the extent of disintegration [11]. Such methods are satisfactory when a considerable portion of the protein is digested, but are inadequate when the extent of digestion is small.

In the present investigation it was possible to overcome most of these objections. Chemical reactions that rupture disulfide bonds and minimize the possibility of secondary chemical reactions have recently been developed in this laboratory [3]. The development of methods for preparing crystalline enzymes by Northrop [11] and by Balls and Lineweaver [12] has made possible the use of preparations of enzymes of known identity and purity. Finally, it was possible to follow the attack of enzymes on the wool protein by noting changes in the mechanical properties of the fiber.

II. EXPERIMENTAL PROCEDURE

1. MATERIALS

The wool fibers used in the present study were a portion of the lot used in previous investigations in this laboratory [3, 13]. This wool had been subjected to no chemical or mechanical treatment other than successive extraction with alcohol and with ether, followed by washing with water at 40° C.

Crystalline pepsin, trypsin, chymotrypsin, and papain [3] were used in this work. The papain preparation consisted of a suspension of the crystalline enzyme in a 0.02 *M* solution of sodium cyanide.

The solutions of pepsin with which the fibers were treated contained 1 mg of crystalline enzyme per milliliter of a solution of 0.1 *M* potassium chloride, and were adjusted to a pH value of 1.5 by the addition of hydrochloric acid. The solutions of crystalline trypsin and chymotrypsin each contained 1 mg of the crystalline enzyme per milliliter of a 0.1 *M* solution of potassium phosphate at a pH value of 7.0. The solutions of crystalline papain were prepared from the suspension described above, by diluting it with water to a concentration of 0.16 mg of protein nitrogen per milliliter and adjusting to pH 7.0 by the addition of dilute acetic acid. The addition of small amounts of merthiolate effectively prevented the growth of bacteria in all of these solutions.

A commercial trypsin[4] was also used in some experiments. In these experiments, 10 mg of enzyme preparation per milliliter of 0.05 *M* potassium phosphate at pH 7.0 was used. The solutions of commercial trypsin were kept sterile by the addition of small amounts of thymol.

2. METHODS

a. DETECTION OF ATTACK BY ENZYMES

The attack by the enzymes was followed by measuring the stress-strain characteristics of the fibers at different stages of treatment. This method has the advantage that it can detect amounts of digestion too small to be detectable by the usual methods. It[5] depends upon the fact that a wool fiber that has been immersed in water or a neutral buffer solution and stretched under load until it is elongated 30 percent will return almost to its original length when the load is removed. If the fiber is allowed to relax in the unstretched state in water or in the neutral buffer solution for about 24 hours and then again stretched until elongated 30 percent, it is found that the same amount of energy (within about 1 percent) is required for the second stretching as for the first. However, if some treatment that weakens the fiber is interposed between the two stretchings, it is found that a lesser amount of energy is required the second time. In such a case, the ratio of the energy required for the second stretching to that required for the first is known as the *30-percent index* of the fiber. A fiber that has been stretched the first time is hereafter referred to as a *calibrated fiber*.

[3] Acknowledgment is made to J. H. Northrop, of the Rockefeller Institute for Medical Research, for a gift of crystalline trypsin and chymotrypsin, and to A. K. Balls, of the U. S. Department of Agriculture, for a gift of crystalline chymotrypsin and papain.

[4] The term "commercial trypsin" is used in this paper to indicate a relatively crude preparation of enzyme material prepared from pancreatic juice and available from commercial sources.

[5] The mechanical details are presented elsewhere [13].

All stress-strain measurements were made on fibers in 0.2 *M* phosphate buffer solution at pH 7.0. Calibrated fibers that had been treated with solutions of enzymes were always washed thoroughly with distilled water, and then kept in the phosphate buffer solution at pH 7.0 for at least 20 hours before stretching them a second time.

b. CHEMICAL TREATMENTS

Methods for the preparation of derivatives of wool in which the disulfide cross-linkages have been ruptured without visibly affecting the fiber structure have previously been described in detail [3]. The methods involve reducing the wool with thioglycolic acid solutions followed by treating the reduced product with an alkyl halide. The reactions appear to involve only the disulfide groups of the cystine in wool, and may be represented by the following equations:

$$W-S-S-W+2HS-CH_2-COOH\longrightarrow 2W-SH+(HOOC-CH_2-S)_2 \quad (1)$$

$$W-SH+RX\longrightarrow W-SR+HX \quad (2)$$

$$W-SH+CH_2X_2\longrightarrow W-S-CH_2-S-W+2HX, \quad (3)$$

where W represents the portions of the wool molecule connected by the disulfide groups, R an alkyl group, and X a halogen atom.

The reduced and the reduced and alkylated wool fibers used in the present investigation contained between 4 and 6 percent of unchanged cystine. The cystine content of the untreated wool was 12.4 percent. The reduced and reoxidized wool fibers were prepared by reducing wool to about this same cystine content, and reoxidizing with oxygen as previously described [3].

c. MECHANICAL TREATMENTS

The mechanically "injured" fibers were prepared by placing calibrated fibers on a glass plate, and tapping them at about six places along their length with a rounded glass rod. Such fibers, on examination under the microscope, appeared to be injured, since crevices were formed and cuticle scales lost in the tapped regions. At this point in the procedure, the mechanically injured fibers were again calibrated, and those that had 30-percent indices less than 0.96 were discarded.

d. ENZYME TREATMENTS

Each fiber, after the calibration and the subjection to either mechanical or to chemical treatments, was placed in a small test tube. One milliliter of the solution of enzyme was added and permitted to act for the specified period at 20° to 25° C. In order to be sure that the effects observed were the results of the action of enzymes, control experiments were always carried out, in which another group of calibrated fibers was exposed to the action of identical solutions, except that no enzyme was present.

III. RESULTS AND DISCUSSION

The results of the action of the different enzymes upon wool fibers are summarized in the following sections.

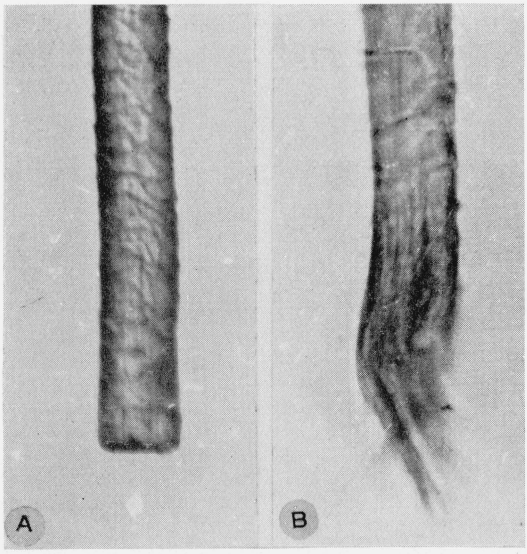

FIGURE 1.—*Broken ends of untreated wool fibers (A) and mechanically damaged woo fibers (B) after treatment with pepsin.* ×250.

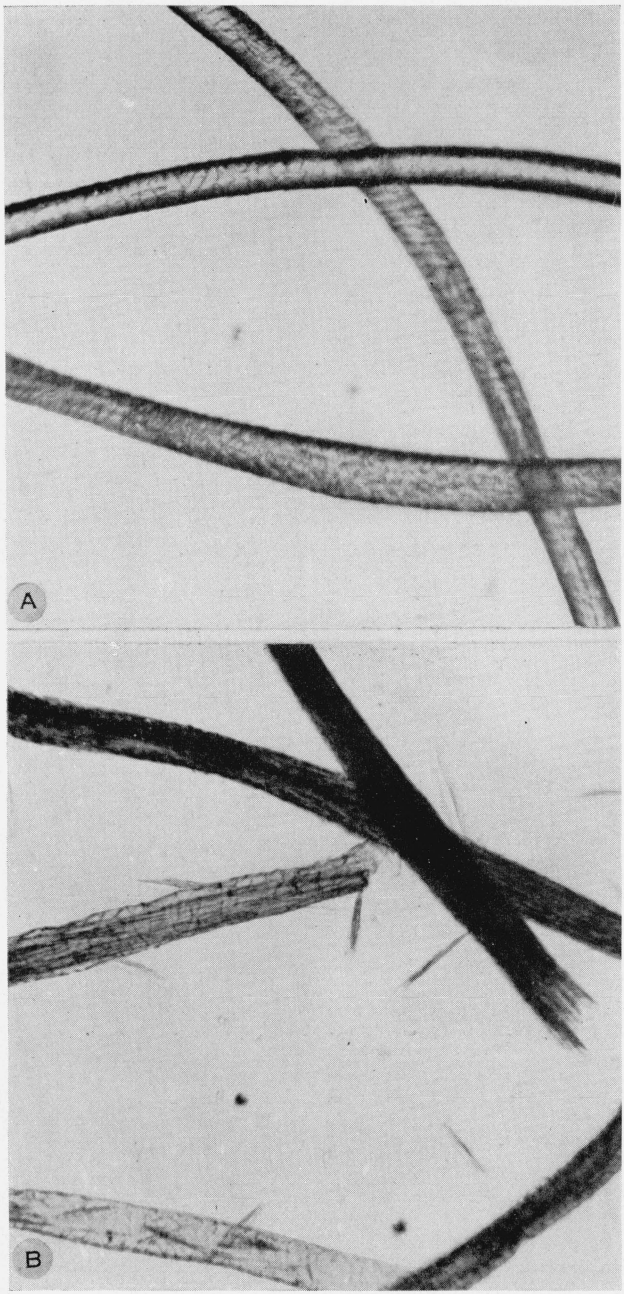

FIGURE 2.—*Untreated wool fibers (A) and reduced and methylated wool fibers (B) after treatment with pepsin.* ×150.

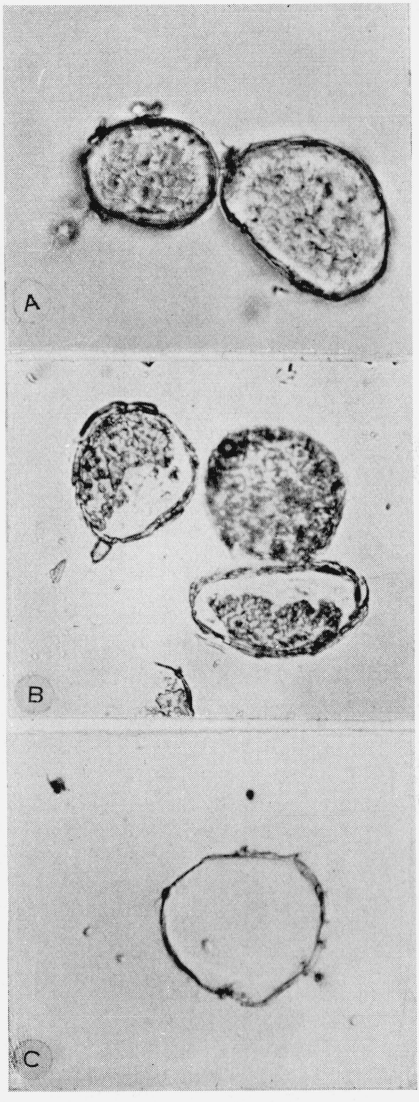

FIGURE 3.—*Cross sections of wool fibers treated with pepsin.* ×450.

A, Sections of untreated fibers treated with pepsin for 30 days; *B*, sections of reduced and methylated fibers treated with pepsin for 10 days; *C*, sections of reduced and methylated fibers treated with pepsin for 30 days.

1. CRYSTALLINE PEPSIN

During periods up to 10 days, solutions of crystalline pepsin had no appreciable effect upon the untreated fibers, as indicated by the fact that the 30-percent indices remained high, averaging 0.96 (table 1). When the treatment was prolonged to 30 days, the fibers disintegrated at the slightest touch. However, fibers in a similar but enzyme-free solution adjusted to a pH value of 1.5 were found to be considerably weakened, the 30-percent index falling to 0.77; and such fibers, when transferred to pepsin solution, were almost immediately attacked by the enzyme. It is thus seen that undamaged fibers are not attacked by pepsin, but that solutions acid enough for the optimum activity of pepsin gradually attack the fibers and make them susceptible to the action of the enzyme.

Crystalline pepsin readily attacked mechanically damaged fibers, and weakened them so that they broke at 5- to 10-percent elongation. The broken ends of the mechanically damaged, pepsin-treated fibers showed ragged breaks, at which clearly outlined cortical cells were visible, and not the sharp, straight break characteristic of untreated fibers, as may be seen by comparison of figure 1 (*A*) with figure 1 (*B*).

TABLE 1.—*Effect of solutions of crystalline pepsin upon wool fibers subjected to various treatments*

Previous treatment	Duration of enzyme treatment	30-percent indices	
		Enzyme-treated	Controls
	Days		
None	10	0.96	0.95
Do	30	(1)	.77
Mechanical injury	10	(1)	.96
Reduction	4	(1)	.62
Reduction and reoxidation	10	.94	.94
Reduction and CH_3I	2	(1)	.48
Reduction and CH_2I_2	14	.93	.93

[1] Fibers broke before or during second calibration. See text.

The effect of reduction, or of reduction followed by methylation, was to render the fibers extremely susceptible to digestion by crystalline pepsin. Fibers that had been subjected to either of these chemical treatments and then to the action of pepsin seemed, while still immersed in the enzyme solution, not to have changed greatly in appearance, but were so fragile that attempts to remove them caused them to break into small pieces. A comparison of figure 2 (*A*) with figure 2 (*B*) shows the marked changes produced by the enzyme, including digestion of cortical material, liberation of some cortical cells, and formation of empty tubes of scales. The photographs of cross sections, figure 3 (*A*, *B*, and *C*), demonstrate progressive digestion by pepsin of the cortex of reduced and methylated wool fibers.

Fibers that had been reduced and reoxidized were as stable as untreated fibers toward solutions of crystalline pepsin. Fibers into which new cross-linkages had been introduced by reduction followed by treatment with methylene iodide (according to eq 3) were likewise not attacked.

2. CRYSTALLINE CHYMOTRYPSIN

Pepsin is most active in acid solution, but acid solutions slowly attack the wool and increase its susceptibility to the action of the enzyme. To avoid this complication, it appeared desirable to study the effect upon wool of an enzyme active at a neutral pH value. Crystalline chymotrypsin, which occurs in pancreatic juice along with crystalline trypsin and other enzymes, is active at pH 7.0. Although this enzyme was found to attack reduced and reduced and methylated wool somewhat less rapidly than does pepsin, its action resembled that of pepsin in that it digested the cortex but not the cuticle. Furthermore, untreated wool fibers and those that had been reduced and alkylated with methylene iodide were not attacked (table 2).

TABLE 2.— *Effect of solutions of crystalline chymotrypsin upon wool fibers subjected to various treatments*

Previous treatment	Duration of enzyme treatment	30-percent indices	
		Enzyme-treated	Controls
	Days		
None	7	0.99	0.99
Mechanical injury	7	(1)	.95
Reduction	4	(1)	.55
Reduction and reoxidation	7	.97	.99
Reduction and CH_3I	4	(1)	.41
Reduction and CH_2I_2	7	.89	.91

1 Fibers broke before or during second calibration.

3. CRYSTALLINE PAPAIN

Neither untreated wool, nor any derivative of wool studied, was attacked by solutions of crystalline papain. In all cases, the 30-percent indices of the enzyme-treated fibers were very close to those of the "control" fibers. The derivatives included wool that had been reduced, reduced and reoxidized, reduced and methylated, and reduced and treated with methylene iodide (table 3).

TABLE 3.— *Effect of solutions of crystalline papain upon wool fibers subjected to various treatments*

Previous treatment	Duration of enzyme treatment	30-percent indices	
		Enzyme-treated	Controls
	Days		
None	7	0.99	0.99
Reduction	7	.55	.55
Reduction and reoxidation	7	.94	.94
Reduction and CH_3I	7	.43	.43
Reduction and CH_2I_2	7	.83	.89

4. CRYSTALLINE TRYPSIN

Untreated wool fibers were not attacked by trypsin, even after prolonged exposure of the fibers to sterile solutions of this enzyme. Similarly all of the treated fibers, except those that had been reduced

alone, were unaffected. Reduced fibers, however, appeared to be slightly attacked, since many of them broke at 20- to 25-percent elongation (table 4).

Although the optimum pH value for the action of trypsin is nearer 8 than 7, the trypsin solutions were adjusted to the latter pH value in most of the experiments in order to avoid extraneous effects due to alkali. Further experiments have now shown that solutions of crystalline trypsin do not digest untreated or reduced and methylated wool even at pH 8.0.

TABLE 4.—*Effect of solutions of crystalline trypsin upon wool fibers subjected to various treatments*

Previous treatment	Duration of enzyme treatment	30-percent indices	
		Enzyme-treated	Controls
	Days		
None	14	0.98	0.98
Mechanical injury	10	.95	.96
Reduction	7	(1)	.60
Reduction and methylation	7	.48	.55

[1] Fibers broke after 20- to 25-percent extension.

5. COMMERCIAL TRYPSIN

Commercial trypsin is a relatively crude preparation made from pancreatic juice and consists of a mixture containing proteolytic enzymes active at a pH value of 7.0. Since it has been used extensively by earlier investigators, it appeared advisable to conduct a few experiments with it in the present investigation. The effects of commercial trypsin upon the fibers are much like those of crystalline chymotrypsin. Fibers that were untreated, reduced and reoxidized, or reduced and treated with methylene iodide were not affected by the enzyme. Reduced and methylated fibers were readily attacked (table 5). Commercial trypsin acted more slowly, however, and never produced as complete digestion of the cortex of the fibers as did crystalline pepsin. Since commercial trypsin attacks wool and crystalline trypsin does not do so, it is evident that some constituent other than crystalline trypsin, possibly crystalline chymotrypsin, is responsible for the action of the commercial product upon the fiber.

TABLE 5.—*Effect of solutions of commercial trypsin upon wool fibers subjected to various treatments*

Previous treatment	Duration of enzyme treatment	30-percent indices	
		Enzyme-treated	Controls
	Days		
None	7	0.99	0.99
Mechanical injury	7	(1)	.96
Reduction and reoxidation	7	.89	.89
Reduction and CH_3I	2	(1)	.47
Reduction and CH_2I_2	7	.91	.91

[1] Fibers broke before or during second calibration.

IV. DISCUSSION

The experiments described in the preceding sections show that wool fibers, when chemically unchanged and mechanically intact, are not attacked by sterile solutions of any of the enzymes studied. The present methods permit more exact interpretations and reveal some of the reasons why earlier experiments have sometimes indicated that wool is digestible by enzymes. Since detailed information has generally not been given in earlier published accounts, no attempt is made to consider each report individually. Results based upon wool that has previously been treated with alkali [4, 5, 15, 16] or with other chemical agents which do not react solely with disulfide groups [4, 6, 9, 10], or that has received mechanical treatment [11], do not provide clear evidence regarding its stability to enzymes. Particularly noticeable in the literature is the absence of statements regarding the sterility of the enzyme solutions. That this is important is shown by the fact that wool kept in a solution of commercial trypsin to which a small crystal of thymol has been added is not noticeably attacked after several weeks, and that wool in a similar, but thymol-free solution shows rapid digestion and evolution of hydrogen sulfide. The effect is apparently a result of the action of microorganisms which attack disulfide groups as well as hydrolyze peptide linkages.

Modified wool fibers may be attacked by enzymes in at least two different ways, depending upon whether the fibers have been modified by mechanical or by chemical means. Mechanically injured wool was attacked by pepsin, but was only broken down into spindle-shaped cortical cells. Even after several months over 90 percent of the wool could be recovered in the form of these cells. However, chemical modification that involves rupture of the disulfide cross-linkages of the wool renders the cortex of the fibers completely digestible. For example, reduced and methylated wool (cystine content, 2 percent) was almost completely digested by pepsin after 2 weeks. The residue, which consisted principally of scale substance, amounted to only 5 percent of the weight of the wool. Since the effects of the two modes of attack are very different, they are discussed separately in the following sections:

1. DIGESTION OF MECHANICALLY INJURED WOOL FIBERS

Pepsin and chymotrypsin attack mechanically injured fibers, but in a way best discussed after considering the histology of the fibers. Wool fibers are not homogeneous but consist of cellular units, arranged in three layers: an outer cuticle consisting of scale cells; a middle region called the cortex, made up of spindle-shaped cortical cells; and a central core or medulla. In many types of wool, the medulla may be very small or absent. After mechanical injury the fibers are attacked and cortical and scale cells are liberated, but these do not appear to be digested by the enzymes. An attack of this type apparently does not result in the digestion of the principal fibrous protein of the wool; rather the appearance of the product suggests that an intercellular substance may have been digested. Such an explanation has already been offered for the action of commercial trypsin on wool by Stakheeva-Kaverzneva and Gavrilov [15], and by Speakman and McMahon [16].

Apparently the impermeability of the cuticle is an important factor in the resistance of chemically unchanged fibers to digestion by enzymes. Mechanical injury of the cuticle or, as will be discussed later, rupture of the disulfide cross-linkages, increases its permeability and facilitates attack. Further evidence of this impermeability is shown by the fact that the cut ends of fibers (either chemically unchanged or reduced and methylated) are more readily attacked than other parts [17].[6] The permeability of the cuticle undoubtedly varies considerably with different types of wool, since these are attacked by enzymes and also absorb dye at very different rates under identical conditions. Damage to the cuticle during chemical or mechanical processing of commercial wools probably contributes to the conflicting results reported in the literature concerning the attack of wool by enzymes.

2. DIGESTION OF CHEMICALLY MODIFIED WOOL FIBERS

The digestion of cortical material was almost complete after the disulfide cross-linkages had been broken by reduction, or by reduction followed by methylation. When the cross-linkages were rebuilt, either by reoxidation of the sulfhydryl groups to disulfide groups, or by their conversion to *bis*-thioether groups ($-S-CH_2-S-$), the resistance to digestion of the original wool was restored.[7] Thus, the resistance appears to be due, not to the chemical nature of the groups involved, but to the existence within wool of a compact three-dimensional polymeric structure, which is a result of the presence of disulfide cross-linkages between polypeptide chains.

A more detailed consideration of the chemistry of polymerization supports this hypothesis. Reduced wool probably consists of polypeptide chains containing cysteine residues. In the sense of Carothers' [19] definition, these polypeptides are linear polymers bearing functional groups capable of reacting with each other to yield a three-dimensional polymer. That is, oxidation of the reduced wool would form disulfide cross-linkages between different polypeptide chains and yield polymers of high molecular weight.

The formation of polymers of high molecular weight from molecules that bear more than one functional group is favored when the concentration of a reactant is high. At lower concentration, lower degrees of polymerization and even reactions to form monomers are to be expected [20]. This means that the formation of disulfide cross-linkages between different polypeptide chains should be favored in reduced wool *fibers*, because the concentration of the reactant (reduced protein) is extremely high. The reoxidation of wool by the method of Goddard and Michaelis, in which reduced wool is *dissolved* in alkali and then treated with an oxidizing agent, should result in a lower degree of polymerization and should favor the formation of disulfide links between different parts of single folded chains. That the amorphous products obtained by these authors may be of this nature is suggested by their high solubility in dilute solutions of acids or alkalies, in which reoxidized wool fibers of the type obtained in the present study are almost completely insoluble.

[6] The penetration of large dye molecules into wool fibers seems to be analogous, since, as has been shown by Millson, Royer, and Wissemann [18], the rate of penetration is increased in injured fibers. Likewise, the rate of penetration of dye into wool in which disulfide cross-linkages have been ruptured is also increased.

[7] Wools of this type have now been shown to be even more resistant to enzymes, bacteria, fungi, and moths than the original wool [22].

The resistance of the cortex of wool to digestion by enzymes, then, appears to be due to its compact three-dimensional polymeric structure. A structure of this sort might resist attack by enzymes through two mechanisms: (1) by keeping the molecules of enzyme from penetrating the structure, (2) by interfering with free rotation of the units of the peptide chains into the special configurations believed to be favorable for enzyme action [21].

Goddard and Michaelis have suggested that a definite physical pattern imparts to wool protein its resistance toward enzymes. The present work indicates that all that may be required is the formation of polymers of extremely high molecular weight and compact structure.

V. REFERENCES

[1] R. J. Block in C. L. A. Schmidt, Chemistry of the Amino-Acids and Proteins, p. 278 (Charles C. Thomas, Springfield, Ill., 1938).
[2] W. T. Astbury and A. Street, Trans. Roy. Soc. (London) **230** [A] 333 (1931).
[3] W. I. Patterson, W. B. Geiger, L. R. Mizell, and M. Harris, J. Research NBS **27,** 89 (1941) RP1405.
[4] L. Meunier, P. Chambard, and H. Comte, Compt. rend. **184,** 1208 (1927).
[5] C. Fromageot and A. Porcherel, Compt. rend. **193,** 738 (1931).
[6] E. Waldschmidt-Leitz and G. V. Schuckmann, Ber. deut. chem. Ges. **62,** 1891 (1929).
[7] K. Linderstrøm-Lang and F. Duspiva, Z. physiol. Chem. **237,** 131 (1935).
[8] D. R. Goddard and L. Michaelis, J. Biol. Chem. **106,** 505 (1934); **112,** 361 (1935).
[9] J. I. Routh and H. B. Lewis, J. Biol. Chem. **124,** 725 (1938).
[10] J. I. Routh, Proc. Am. Soc. Biol. Chemists, J. Biol. Chem. **133,** 82 (1940); J. Biol. Chem. **135,** 175 (1940).
[11] R. Burgess, J. Textile Inst. **25,** T289 (1934).
[12] J. H. Northrop, The Crystalline Enzymes (Columbia University Press, New York, N. Y., 1939).
[13] A. K. Balls and H. Lineweaver, J. Biol. Chem. **130,** 669 (1939).
[14] A. M. Sookne and M. Harris, J. Research NBS **19,** 535 (1937) RP1043; Am. Dyestuff Rptr. **27,** 179 (1938).
[15] C. Stakheeva-Kaverzneva and N. W. Gavrilov, Bul. soc. chim. (Mém.) 4me sér. **4,** 647 (1937).
[16] J. B. Speakman and P. R. McMahon, Nature **141,** 118 (1938).
[17] C. W. Hock, R. C. Ramsay, and M. Harris, J. Research NBS **27,** 181 (1941) RP1412.
[18] H. E. Millson, G. L. Royer and M. E. Wissemann, Am. Dyestuff Reptr. **28,** 632 (1939).
[19] W. H. Carothers, Chem. Rev. **8,** 353 (1931).
[20] G. Salomon, Trans. Faraday Soc. **32,** 153 (1936).
[21] M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider, and H. Sleich, J. Biol. Chem. **109,** 325 (1935).
[22] W. B. Geiger and Milton Harris [work not yet published].

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