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Crosslinking in Keratins. III. Acid Hydrolysis of Keratins

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Synopsis

The fraction soluble during hydrolysis in an HCOOH–HCl–H_2O mixture was measured as a function of time for Lincoln wool, human hair, and kid mohair. Evaluation by crosslinking theory of the equivalent relative increase in sulfur content of the three insoluble residues shows the likely presence of two components in each with equal sulfur content although the less abundant component differs by having mainly intramolecular crosslinking. Overall variation in sulfur content entirely accounts for the variation in rate of hydrolysis among these α-keratins. The data of Leach, Rogers, and Filshie for the amino acid content of the insoluble residue following hydrolysis of wool in dilute HCl also depend in a simple way on the amount dissolved; in this case the disproportionation of amino acids between the residue and extract depends on the degree of their affiliation with the crosslink-containing cystine.

INTRODUCTION

Nonoxidizing acids act on keratins in one or more of three ways: (*a*) deamidation of the side-chain amides of aspartic or glutamic acid residues,[1,2] (*b*) rupture of peptide bonds in the main chain at various rates which depend on the environment of the bond,[3] or (*c*) equilibrium formation of intra- from interchain disulfide links via reaction with free sulfhydryl groups.[4–6] After scission of peptide linkages an increasing portion of the keratin becomes soluble, leaving a residue which can be analyzed. The sulfur content of the residue can be interpreted from the results of II[4] and the rate of solution by the results of I.[5] If the degradation involves only chain scission, interpretation is especially straightforward. Acid hydrolysis of keratins may be assumed to cause essentially pure chain scission, provided the possible disulfide–sulfhydryl interchange is kept in mind. Deamidation can be ignored because the amount of soluble material that would be involved is quite small.

For simplicity we will invoke the assumption that keratins are a collection of independent components. The further assumption that each of these components initially has a monodisperse molecular weight distribution is consistent with current notions about proteins. However, an analysis of the results in terms of an initially random distribution will be presented later to show that this distribution gives, qualitatively at least, similar results.

EXPERIMENTAL

Three keratins were chosen for the hydrolysis study: human hair, a slightly crimped Lincoln wool, and kid mohair. The samples were scoured,

TABLE I
Acid Hydrolysis of Lincoln Wool

Time of hydrolysis, min.	Soluble fraction, S	Sulfur content, %		$\frac{\phi_s}{\phi}$	$\frac{\phi_g}{\phi}$
		Solution $100\phi_s$	Residue $100\phi_g$		
0	—	—	3.62	—	1.000
50	0.018	1.98	3.65	0.547	1.008
100	0.061	2.54	3.69	0.701	1.018
150	0.149	2.02	3.90	0.557	1.077
200	0.244	1.89	4.18	0.522	1.155
250	0.315	1.81	4.45	0.500	1.230
300	0.421	2.00	4.80	0.552	1.326
350	0.482	2.00	5.12	0.552	1.414
400	0.543	2.06	5.47	0.569	1.511

TABLE II
Acid Hydrolysis of Kid Mohair

Time of hydrolysis, min.	Soluble fraction, S	Sulfur content, %		$\frac{\phi_s}{\phi}$	$\frac{\phi_g}{\phi}$
		Solution $100\phi_s$	Residue $100\phi_g$		
0	0.014	3.17	3.17	1.000	1.000
50	0.038	2.41	3.20	0.760	1.009
100	0.110	2.77	3.22	0.873	1.016
150	0.211	1.68	3.57	0.529	1.126
200	0.291	1.59	3.82	0.501	1.205
250	0.389	1.55	4.20	0.488	1.325
300	0.494	1.78	4.53	9.561	1.429
350	0.592	2.30	4.58	0.725	1.445
400	0.646	2.00	5.30	0.631	1.672

TABLE III
Acid Hydrolysis of Human Hair

Time of hydrolysis, min.	Soluble fraction, S	Sulfur content, %		$\frac{\phi_s}{\phi}$	$\frac{\phi_g}{\phi}$
		Solution $100\phi_s$	Residue $100\phi_g$		
0	—	—	4.60	—	1.000
180	0.067	4.60	4.60	1.000	1.000
360	0.257	1.43	5.35	0.311	1.163
480	0.351	2.07	5.97	0.450	1.297
720	0.471	1.83	6.18	0.398	1.344
960	0.538	2.52	7.02	0.548	1.526
1200	0.578	2.01	6.78	0.437	1.476
1440	0.730	3.50	7.57	0.761	1.646

then washed successively with Skellysolve B (cyclohexane was used for the hair), ethanol, and deionized water. After drying, the samples were Wiley-milled and given another deionized water wash. The water solubility of the wool after milling was 0.043%.

Hydrolysis, with simultaneous extraction, was performed at 40°C. in a 1:1 mixture of 88% formic acid and 37% hydrochloric acid. After hydrolysis, further extraction was made with 88% formic acid, followed by washing with deionized water.

The filtrate was analyzed by a micro-Kjeldahl method for total nitrogen, which was compared to the total initial nitrogen to determine the amount of keratin dissolved. Direct weighing established the validity of this method. When the dried residue had equilibrated in ambient air conditions it was analyzed for sulfur. A few analyses were done by standard methods, but most were done with an x-ray fluorescence technique calibrated against a wool with known sulfur content. Although extreme accuracy is not a virtue of this method, rapidity is. Agreement with standard analytical methods was within ±0.2% sulfur, adequate for the present problem.

Results of the hydrolysis experiments are given in Tables I, II, and III for Lincoln wool, kid mohair, and human hair, respectively. To make sure that the formic acid treatment did not contribute further to the hydrolysis, a series of solubility–time measurements for Lincoln wool were made in 88% formic acid alone, at various temperatures. These results, given in Table IV, show this method to be quite gentle, at least at temperatures up to 40°C.

TABLE IV
Lincoln Wool Dissolved by 88% Formic Acid

Temp., °C.	Time, min.	Wool dissolved, %
24	120	0.25
	360	0.28
40	120	0.39
	360	0.40
70	30	0.68
	60	0.89
	120	2.18
	240	2.79
	360	4.16

DISCUSSION

Sulfur Content of the Insoluble Residue

The following expression [eq. (13) of II] predicts that the sulfur content of the gel fraction should always increase with increasing S:

$$\phi_0 = \phi(1 + fS). \tag{1}$$

Figure 1 shows the relative increase in sulfur content of the gel fraction, ϕ_g/ϕ, plotted against the extent of solubilization S for all three keratin samples. The behavior of human hair, wool, and kid mohair is uniform on this plot.

Within the content of a two-component model, Figure 1 can be interpreted in the following way. At low solubilities a fraction dissolves which contains an extraordinarily large amount of sulfur, so that the remaining gel shows little increase in sulfur content. Two possibilities suggest themselves. First, there might be a substantial amount of a form of keratin that dissolves in the mixture, in spite of the known negligible solubility of wool in formic acid alone. Because this hypothesis involves postulation

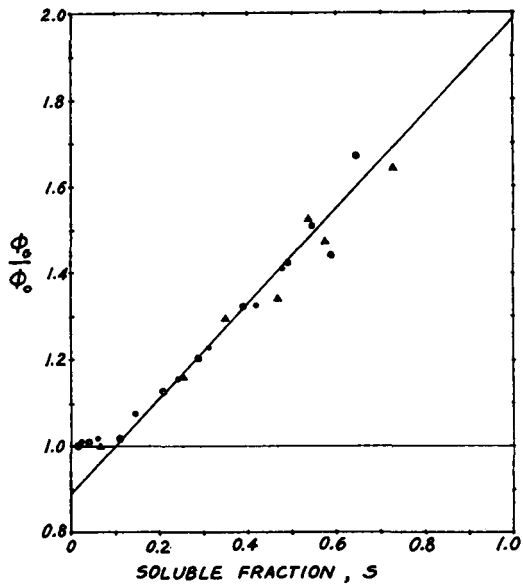

Fig. 1. Relative increase in sulfur content of the insoluble residue from HCl–HCOOH hydrolysis at 40°C. vs. the fraction soluble: (●) Lincoln wool; (○) kid mohair; (▲) human hair.

that hydrochloric acid frees molecules in an unknown, nonhydrolyzing way, we reject it at this time for lack of evidence. The second hypothesis, rather more attractive, is that a small fraction of the keratin holds most of its sulfur in the form of intramolecular crosslinks along the chain. These crosslinks could either be present originally in a separate component of wool, or could be formed as a result of a disulfide–sulfhydryl exchange mechanism[6–8] on a one-component keratin which forms a pseudo-component deficient in intermolecular linkages. In any case, we will take the keratin to be effectively a two-component mixture, one part of which, designated by subscript 2, will substantially vanish from the gel at some point of the hydrolysis.

Equation (24) of II, written for two components, is as given in eq. (2)

$$\phi_g = \phi_1 X_1(1 + f_1 S_1)G_1/G + \phi_2 X_2 (1 + f_2 S_2)G_2/G \tag{2}$$

Now, allowing component 2 to disappear by setting $G_2 = 0$, we have

$$\phi_g = \phi_1 X_1(1 + f_1 S_1)G_1/G \tag{3}$$

Equation (26) of II for two components is

$$S = S_1 X_1 + S_2 X_2$$

or

$$G = G_1 X_1 + G_2 X_2 \tag{4}$$

Again, putting $G_2 = 1 - S_2 = 0$ in eq. (4) and eliminating S_1 in eq. (3), we obtain

$$\phi_g/\phi = (\phi_1/\phi)\ [1 - f_1(X_2/X_1)] + (\phi_1/\phi)(f_1 S/X_1) \tag{5}$$

where both sides have been divided by the initial total sulfur concentration ϕ. This expression, linear in S, has a slope

$$b = -\frac{d}{dS}\left(\frac{\phi_g}{\phi}\right) = \frac{\phi_1 f_1}{\phi X_1} \tag{6}$$

and an intercept when $S = 0$ of

$$a = (\phi_1/\phi)\ [1 - f_1(X_2/X_1)] \tag{7}$$

Combining, we obtain

$$\phi_1/\phi = a + bX_2 \tag{8}$$

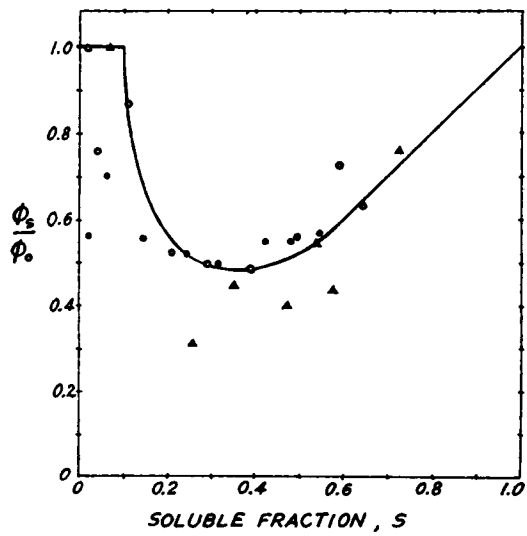

Fig. 2. Relative increase in sulfur content of the soluble portion on HCl–HCOOH hydrolysis at 40°C. vs. the fraction soluble: (●) Lincoln wool; (○) kid mohair; (▲) human hair. The line is calculated from eqs. (22) and (23) of the text.

From Figure 1, $b = 1.10$ and $a = 0.89$. We also see from Figure 1 that most of component 2 is in solution when $S = 0.1$; hence we would expect that fraction X_2 is in the range of 0.1 also. Using this value in eq. (8) gives $\phi_1/\phi = 1.00$ and $f_1 = 0.99$, which indicate that the larger fraction 1 has the same sulfur content as the whole keratin and that the sulfur is all intermolecularly crosslinked. This simplified approach further implies that none of fraction 1 dissolves until 2 is gone, and that ϕ_2/ϕ is also unity

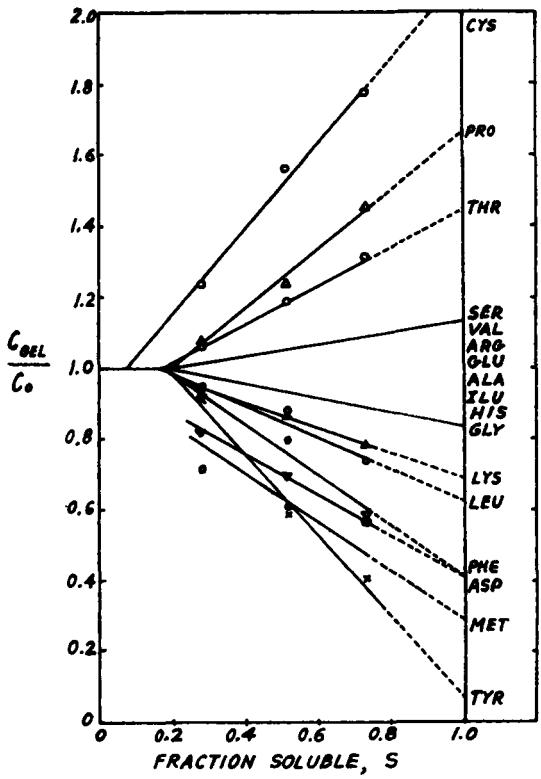

Fig. 3. Relative increase in concentration of amino acids in the insoluble residue on hydrolysis in 0.01M HCl at 100°C. vs. the fraction soluble. Data on a Merino wool by Leach, Rogers, and Filshie.[9]

although $f_2 \cong 0$, indicating intramolecular crosslinking of the smaller fraction.

Allowing X_2 to go as high as 0.20 gives $\phi_1/\phi = 1.11$ and $f_1 = 0.79$, indicating that the crosslinks in fraction 1 are still predominately intermolecular and that this fraction contains only 11% more sulfur than the original material.

Although we gain no new information, it is interesting to see how the sulfur content of the soluble fraction varies with increasing solubilization. Figure 2 shows ϕ_s/ϕ calculated by weight balance from ϕ_g/ϕ, as shown in

Tables I, II, and III. The line is calculated by considering $S_2 = 1$ when $S \leqslant 0.10$. The expressions used in the calculation are

$$\phi_s/\phi = 1 \qquad (S \leqslant 0.1) \qquad (9)$$

and

$$\phi_s/\phi = [1-(a + bS)(1 - S)]/S \quad (S > 0.1) \qquad (10)$$

More detailed calculations based on eqs. (11) or (28) of I, which relate S_1 and S_2 to the extent of hydrolysis, would be likely to eliminate the sharp break at $S = 0.1$ and provide a better fit to the experimental data; they remain to be made, however.

An interesting recent paper by Leach, Rogers, and Filshie[9] deserves mention here. By hydrolyzing wool in 0.01*M* (pH 2) HCl at 100°C. and successively analyzing the residues and a nondialyzable part of the solution, they obtain progressive changes in amino acid composition which they use to show the residues and solutions to be respectively approaching the so-called γ- and α-keratoses. However, from the results of II we would expect the sulfur (or cystine) content of the residue to increase linearly with the solubility. In addition, if we say that some of the amino acids are more or less closely affiliated with cystine in the chain, then these too will change linearly with solubility, but with a different slope. Thus, we can write for the ratio of the concentration of the various amino acids in the residue to that in the original material.

$$C_G/C = 1 - S + Y(1 + f)S \qquad (11)$$

where Y is the fraction of the total amino acid affiliated with cystine. Figure 3 is a plot of the data of Leach et al. in terms of C_G/C for each amino acid of the insoluble residue. The line for cystine resembles closely that already illustrated in Figure 1, constructed from the data of the present paper. The other amino acids also follow fairly linear plots, from which the Y of eq. (11) can be calculated. This set of Y values provides a sort of map showing the nonrandom environment of the cystine. We plan to discuss further in a later paper the meaning of these and other related amino acid analyses, with special reference to the distribution of molecular weights remaining in the gel fraction, a subject entirely omitted from the present series of papers. Variation in the rate constants for hydrolysis of different amino acids is, of course, a further complication in this analysis.

Ratios of the amino acids after 96 hr. of hydrolysis were omitted from Figure 3 because the value for S is uncertain. The data of Leach et al. show that after 72 hr. $S = 0.73$ and after 96 hr. $S = 0.75$; however, the change in amino acid compositions for both residue and solution is much too large to be compatible with such a small solubility change. It should be mentioned also that their procedure of using only the nondialyzable part of the extract as characteristic of the soluble fraction as a whole is cause for error. It is in effect fractionation analogous to the sol–gel split, where the insolubility of a gel is replaced by impermeability of a membrane.

The same argument applies to other procedures[10-13] in which keratin is dissolved and then fractionally precipitated by changing the pH or other variables of the solution; these again can be special cases of the sol–gel split. Both the last examples will be treated in detail in paper VI.

Kinetics of Hydrolysis

Figure 4 shows the fraction of insoluble residue G as a function of hydrolysis time. The data on wool, mohair, and human hair are taken from Tables I, II, and III. There is an apparent induction period for the reaction. Study of the numerical results of eqs. (13) or (28) of I revealed that if the number of peptide links were to decrease exponentially, then no feasible set of adjustable parameters that would fit all the experimental data could be found for a one-component system. Since this problem is still under study, we will mention two additional hypotheses that could account for the initial period of hydrolysis: (*a*) there is a considerable induction period; (*b*) the system contains two components, the minor one possibly acting as an acid-resistant sheath to the major one. An explanation also might be postulated around a disulfide–sulfhydryl exchange mechanism, but the requirements are too ill-defined to discuss at present. We tentatively reject (*a*) because the apparent induction period is much longer than the time required for full swelling in the hydrolyzing solution. This leaves hypothesis (*b*), which, pending more detailed study, we feel is a good working model.

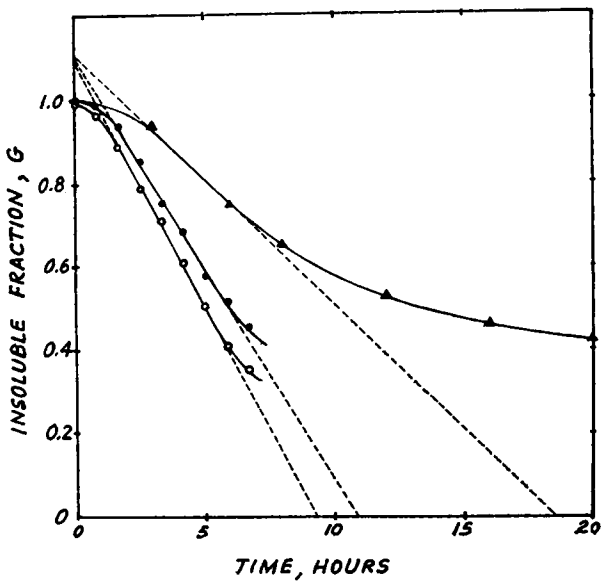

Fig. 4. Fraction of insoluble residue on HCl–HCOOH hydrolysis at 40°C. vs. time: (●) Lincoln wool; (○) kid mohair; (▲) human hair.

After establishing the possibility of a minor protective component, we now will ignore it and proceed to the main portion of the degradation curve. This slope will be considered to involve only the major component, subscript 1. After the minor component vanishes we have for the gel fraction

$$G = G_1X_1 \tag{12}$$

In relating G_1 to the extent of hydrolysis we may use the series expansion, eq. (15) of I, in which we take $G_{10} \cong 1$,

$$G \cong X_1[1-(2P_1U_1/\delta_1^2) + \ldots] \tag{13}$$

If the degradation is first-order, and we assume that the various rate constants can be lumped into a single one, k_1, we have

$$P_1 = 1 - \exp\{-k_1t\}$$

which may be expanded at small k_1t to consider only the early part of the hydrolysis. Equation 13 then becomes

$$G = X_1 - (2X_1U_1k_1t/\delta_1^2) + \ldots \tag{14}$$

From eq. (19) of II we know that $\delta_1 = \phi_1f_1M_1/32$, and that $U_1 = M_1/m$, where m for keratins is about 109. Hence, we may take the derivative of eq. (14) and eliminate δ_1 to find approximately for the early rate of hydrolysis

$$-dG/dt = 19X_1k_1/M_1\phi_1^2 \tag{15}$$

Table V gives the apparent rates, from Figure 4, of the three keratins, along with their sulfur concentrations, ϕ. Calculated quantities are ϕ^2 and $-(dG/dt)\phi^2$. The latter shows a constancy within the experimental error of the whole experiment. This constancy implies that sulfur variation alone explains the apparently different rates of acid hydrolysis among the three keratins. It supports the previous assumption that the sulfur content of the major fraction is the same as that of the whole keratin; that is, $\phi_1 = \phi$. A further tentative conclusion would be that X_1, k_1, and M_1 are constant among the α-keratins.

TABLE V
Relation of Hydrolysis Rates to Sulfur Content

Sample	Rate $-dG/dt$, hr.$^{-1}$	Sulfur content ϕ, %	ϕ^2	$-\phi^2dG/dt$
Kid mohair	0.120	3.17	10.1	1.21
Lincoln wool	0.101	3.62	13.1	1.32
Human hair	0.060	4.60	21.2	1.27

Dusenbury[14] carried out hydrolysis of keratins in boiling dilute sulfuric acid and followed it by alkali extraction. His placements of the rates of hydrolysis of kid mohair, wool, and human hair agree well with the findings

of this paper. However, his rate curves show no sign of the slow start seen in Figure 4. The difference presumably lies either in his use of an alkali treatment or in the lower probability of disulfide–sulfhydryl exchange in sulfuric acid[8] although, as mentioned before, the latter is a doubtful explanation.

Effect of a Random Distribution of Molecular Weights

For uniformity, we again follow the approach of Charlesby[15] as we did in I for the uniform distribution. If the assembly is random initially it will still be so after a fraction P of random chain breakage. If crosslinking now takes place until a fraction Q of monomers is crosslinked, then the solubility will be given as

$$S + \sqrt{S} = 1/QU'_1 \tag{16}$$

where U'_1 is the post-fracture number-average degree of polymerization; that is, the total number of residues (amino acids) divided by the total number of molecules. Writing it as

$$U'_1 = 1/[P + (m/\bar{M}_n)] \tag{17}$$

where $\bar{M}_n$ is the initial number-average molecular weight, we have for eq. (16)

$$S + \sqrt{S} = [P + (m/\bar{M}_n)]/Q \tag{18}$$

Using $P = 1 - \exp\{-kt\}$, we can write eq. (18) as

$$-kt = \ln\{[1 + (m/\bar{M}_n)] - Q(S + \sqrt{S})\} \tag{19}$$

By using this expression with $m/\bar{M}_n \cong 0$ and average Q values calculated from the overall sulfur concentration by the relation $Q = \phi m/32$, we can obtain a reasonable fit for a good portion of the hydrolysis data. There are two reasons why this should be so. First, the large $\bar{M}_n$ required makes the initial part of the random distribution resemble the uniform distribution. Second, after hydrolysis proceeds all distributions will approach randomness. As pointed out before, the occurrence of a random distribution in natural proteins is contrary to most current experimental findings and is incompatible with the notion that a unique sequence of amino acids exists, even for keratins. Therefore we will not treat the random distribution further, in spite of its inherently simpler mathematical form.

CONCLUSIONS

Although some of the conclusions of this study appear to contradict much of the literature on the composition of wool and other α-keratins,[16–20] we believe that the contributions of these and other workers will be considerably unified by a more general use of crosslinking theory.

The conclusions of this paper, which partially agree with the results of Leveau[21] and Blackburn,[22] are as follows.

(*1*) The α-keratins (human hair, wool, and kid mohair) behave on acid hydrolysis as though they consist of two components. One making up about 10% of the material, has sulfur bound mainly in the form of intramolecular crosslinks. The other has sulfur crosslinks that are mostly intermolecular.

(*2*) The sulfur content of both components is essentially the same.

(*3*) The minor component may act as an acid-resistant sheath to the major one.

(*4*) Differences in rates of acid hydrolysis are determined solely by the crosslink density (and therefore by the total sulfur concentration) and depend mainly on the major component.

(*5*) Selective removal of amino acids during hydrolysis largely depends on the degree of their affiliation with the crosslink-containing cystine.

The assistance of H. C. Lukens and Dr. K. J. Palmer in performing the x-ray fluorescence sulfur analysis is gratefully acknowledged. A number of most helpful conversations were held with Dr. W. H. Ward and with Dr. H. P. Lundgren.

Reference to a company or product name does not imply approval or recommendation of the product by the Department of Agriculture to the exclusion of others that may be suitable.

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Résumé

On a mesuré en fraction du temps, la fraction soluble qui se forme pendant l'hydrolyse dans un mélange HCOOH–HCl–H_2O de la laine Lincoln, du cheveu humain et du mohair de chevreau. L'évaluation au moyen de la théorie de la réticulation de l'augmentation équivalente relative de la teneur en soufre des trois résidus insolubles montre la présence probable de deux composants dans chaque résidu, possédant une teneur en soufre égale, tandis que le composé le moins abondant diffère dans le fait qu'il possède principalement des pontages intramoléculaires. La variation totale de la teneur en soufre explique entièrement la variation de la vitesse d'hydrolyse de ces α-kératines. Les résultats de Leach, Rogers et Filshie concernant la teneur en acide aminé du résidu insoluble aprés hydrolyse de la laine par HCl dilué dépendent également d'une façon simple de la quantité disolute; dans ce cas, la disproportion des acides aminés entre le résidu et l'extrait dépend du degré de leur affiliation avec la cystine participant à la réticulation.

Zusammenfassung

Die während der Hydrolyse in einer HCOOH–HCl–H_2O-Mischung lösliche Fraktion wurde an Lincolnwolle, menschlichem Haar und Angoraziegenwolle als Funktion der Zeit gemessen. Auswertung der äquivalenten relativen Zuhahme des Schwefelgehaltes der drei unlöslichen Rückstände nach der Vernetzungstheorie zeigt, dass wahrscheinlich in jedem zwei Komponenten mit gleichem Schwefelgehalt vorhanden sind, wobei sich die weniger häufige Komponente durch ihre hauptsächlich intramolekulare Vernetzung unterscheidet. Die Gesamtänderung des Schwefelgehalts kann die Unterschiede in der Hydrolysengeschwindigkeit dieser α-Keratine vollständig erklären. Auch die Ergebnisse von Leach, Rogers und Filshie für den Aminosäuregehalt des unlöslichen Rückstandes nach der Hydrolyse von Wolle in verdünnter HCl hängen in einfacher Weise von der gelästen Menge ab; in diesem Fall besteht eine Abhängigkeit der ungleichen Vertelung der Aminosäuren zwischen Rückstand und Extrakt von dem Ausmass ihrer Vereinigung nit dem vernetzungshältigen Cystin.

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