

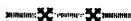
NEW AMINO ACIDS FORMED IN HAIR DURING UNHAIRING*

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ABSTRACT

Amino acid analyses of hair samples resulting from both good and bad unhairing practices have revealed that considerable change is taking place in the protein. Hair samples recovered from treatments including the use of sodium sulfide contain large amounts of lanthionine. In the presence of lime alone, lesser amounts are formed and, in addition, a new amino acid, lysinoalanine, is also formed. Both of these are probably responsible for immunization of the hair to further dissolution. The amino acid serine is partly destroyed in this process and is also converted to lanthionine and lysinoalanine. When dimethylamine is used with lime, with or without sulfide, still another new amino acid is formed, β -dimethylaminoalanine. All these data are best explained by a " β -elimination" mechanism rather than by a substitution mechanism for the action of lime and unhairing agents on the hair that does not dissolve in a hair-saving process. The results still do not clarify what is happening when the hair dissolves.



INTRODUCTION

In 1956 Henry B. Merrill (1) stated that the mechanisms of hair destruction and hair loosening were probably quite different. This is almost certainly demonstrated in the results reported here. The phenomenon of immunization was also discussed thoroughly in this chapter. This phenomenon is evidenced in two separate results of the action of alkali on hair. First, the hair that has been exposed to the action of alkali is much more difficult to remove from the skin than native hair. Second, it is also much more difficult to dissolve. Merrill came to no definite conclusions about the cause of immunization but did indicate that the formation of lanthionine (1) might be involved. Some doubt was cast on this involvement of lanthionine, however, by the results of Shaw and Lollar (2), who found lanthionine in hair that had been treated with sulfide in addition to lime. Im-

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I

munization was also discussed by Windus and Showell (3) in their paper on the mechanism of unhairing, in which they proposed a single mechanism for the action of all unhairing agents. Here, too, lanthionine was blamed for immunization but its formation was not discussed in connection with the universal mechanism.

It is the purpose of this paper to report the information we have obtained about the chemical reactions taking place in undissolved hair during good and poor unhairing practices, the changes that take place in its amino acid composition, and the implications that these make concerning the mechanism of unhairing.

EXPERIMENTAL

Unhairing Reactions

The hair used was black hair clipped from a piece of a single cattle hide after thorough washing with water. The amounts of reagents used were based on the weight of the piece of hide from which the hair was clipped. The reactions were carried out in open vessels exposed to air. After the appropriate time, the hair was removed by straining, and washed with water and then ammonium sulfate solution to neutralize the alkali. The hair was then rewashed with water and allowed to air-dry. It was next degreased with chloroform and, finally, again air-dried.

Amino Acid Analyses

The hair samples were prepared for amino acid analysis by hydrolyzing in 6N hydrochloric acid solution for 24 hrs. under an atmosphere of nitrogen. The excess hydrogen chloride was removed by repeated evaporations under reduced pressure with the intermittent addition of distilled water. The final residues were then diluted to a known volume with 0.1N hydrochloric acid solution. The analyses were run on a Piez-Morris† type ion-exchange column with a continuous, gradient-elution buffer (4). The results were calculated and tabulated on an IBM-1130 computer, using programs developed at this laboratory.

RESULTS

The results of a number of amino acid analyses of these hair samples have shown that the three amino acids that were affected by these unhairing treatments were serine, lysine, and, as expected, cystine. It was suggested by Speakman (5)

†Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

that lysine might be involved in these reactions, but this was discounted by Merrill (1) as being unlikely. No reference has been found to the involvement of serine. The results of this study also indicated that lanthionine was formed in varying amounts during the treatments and was thus involved somehow in the reaction. In addition, several other products were detected, which have been identified by comparison with model compounds and/or by analogy with the results of others.

Figure 1 shows a typical amino acid chromatogram of a sample of the hair that was used in these experiments. The positions of the amino acids formed by the unhairing treatments are included with peaks (of arbitrary size) in dashed lines.

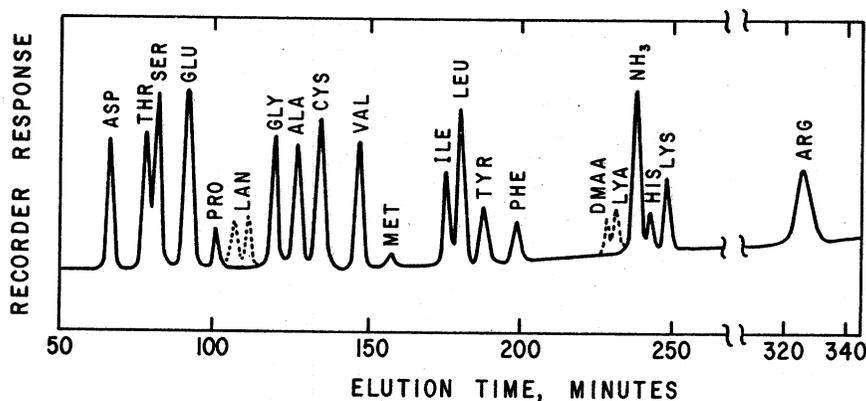


FIGURE 1.—Amino acid chromatogram of a cattle hair hydrolysate showing the positions of the new amino acids in dashed lines.

The lanthionine always appears as two peaks because of the two diastereomeric forms resulting from its mode of formation, and the ratio of the areas under the two peaks is always close to one. The formation of the two diastereomeric forms and their identification in hydrolysates of alkali-treated proteins were demonstrated in two papers by Horn, Jones, and Ringel (6, 7). The separation and identification of these two diastereomers with an amino acid analyzer were demonstrated by Ingles and Nicholls (8) after considerable trouble. The separation of the diastereomers was readily achieved on the system in use here, requiring no changes in our usual techniques.

The other two peaks are in the region of the basic amino acids and a clue to their identification came from reports of work on wool (9). The first to be identified in these hydrolysates was lysinoalanine (II) (LYA). Its original identification in the hydrolysates of proteins that have been exposed to



II

alkali is well documented in a series of papers (10-16). The amino acid was synthesized by the method of Okuda and Zahn (17) and a direct comparison was made of the eluted peak with that of the synthetic material. It is interesting that this amino acid has recently been found in alkali-treated hides (18).

The other peak, labeled DMAA, is probably β -dimethylaminoalanine (III). This peak appeared only when dimethylamine was used in the unhairing mixtures. When other amines (*e.g.*, pyrrolidine and piperidine) were used in place



III

of the dimethylamine, other peaks appeared in the same region of the chromatograms and probably represent the corresponding β -aminoalanine derivatives. Asquith and coworkers (9) hinted at the reaction of secondary amines (*e.g.*, dimethylamine) with proteins, and it remains for these compounds to be synthesized and compared with the materials responsible for these peaks.

Quantitative changes can be measured for serine, cystine, lysine, and lanthionine. The two new amino acids (LYA and DMAA) have not been measured quantitatively. The results for serine, cystine, lysine, and lanthionine are given in Table I for a number of different treatments on hair. The treatments are given in the first three columns and the results are reported as the moles of a given amino acid divided by the moles of the amino acid valine. Reporting the results in this fashion has certain advantages. First, errors made in purifying, drying, and weighing the protein sample taken for analysis are eliminated. Secondly, errors made in dilutions of hydrolysates and sampling for application to the analyzer are also eliminated. Finally, the computer is programmed to calculate these ratios directly. The values given are reliable to ± 0.06 units. This latter value was calculated for a number of amino acids not undergoing any change.

First, a few general observations about these data will be made. The serine content of the hair was decreased by all the treatments. The extent of this decrease appears to depend more on the time than on the reagents present. The one reagent that was common to all the treatments was the alkali and, therefore, this reaction would appear to be a slow, hydroxide ion-catalyzed destruction of the amino acid.

At least 50 percent of the cystine was destroyed by all these treatments and this was dependent on both the amount of time allowed and the reagents present. All these hair samples were tested for free cysteinyl residues and none was found. This is important, since Dowling and Maclaren have shown that lanthionine can be formed from cysteinyl residues during acid hydrolysis of the protein (19). Al-

TABLE I
ANALYTICAL DATA FOR THE AMINO ACIDS INVOLVED IN UNHAIRING
(Moles of amino acid/mole of valine)

| # | Treatment* | Days | Final pH | SER | CYS | LYS | LAN | Notes |
|----|---|------|----------|------|------|-----|------|----------------------|
| 1 | Control | — | — | 1.71 | 1.18 | .49 | 0 | |
| 2 | Lime (sat'd.) | 1 | 12.4 | 1.71 | .44 | .39 | .68 | LYA formed. |
| 3 | Lime (sat'd.) | 5 | 12.4 | 1.49 | .31 | .34 | .91 | LYA formed. |
| 4 | NaOH (0.1N) | 1 | 12.6 | 1.64 | .60 | .44 | .55 | LYA formed. |
| 5 | Lime (sat'd.) followed by Na ₂ S (1/2%) | 1 | 12.4 | | | | | Total time 5 days. |
| | | 4 | 12.4 | 1.47 | .29 | .35 | 1.08 | LYA formed (trace). |
| 6 | Na ₂ S (1/4%) followed by lime (sat'd.) | 1 | 10.0 | | | | | Total time 2 days. |
| | | 1 | 12.4 | 1.60 | .37 | .40 | 1.20 | LYA formed (trace). |
| 7 | Lime (sat'd.) + Na ₂ S (1/4%) | 3 | 12.4 | 1.62 | .30 | .47 | 1.38 | |
| 8 | Lime (sat'd.) + Na ₂ S (1/2%) | 3 | 12.4 | 1.52 | .27 | .45 | 1.26 | |
| 9 | NaOH (0.05N) + Na ₂ S (1/4%) | 1 | 12.0 | 1.64 | .56 | .48 | .87 | |
| 10 | Lime (sat'd.) + DMA† (2%) | 3 | 12.4 | 1.43 | .22 | .44 | .73 | DMAA and LYA formed. |
| 11 | Lime (sat'd.) + DMA (1/2%) + Na ₂ S (1/4%) | 3 | 12.4 | 1.50 | .32 | .47 | 1.29 | DMAA formed. |
| 12 | Lime (sat'd.) + Benzyl SH‡ (1/4%) | 3 | 12.4 | 1.41 | .21 | .39 | 1.32 | LYA formed. |
| 13 | Lime (sat'd.) + NaBH ₄ (1%) | 1 | 12.4 | 1.60 | .32 | .44 | 1.11 | LYA formed. |
| 14 | Lime (sat'd.) + Na ₂ SO ₃ (1%) | 1 | 12.5 | 1.69 | .46 | .34 | .80 | LYA formed. |

*These treatments are described in the Experimental Section in more detail.

†Dimethylamine.

‡Benzyl mercaptan.

most as much destruction of the cystine occurred with lime alone in five days (#3) as occurred with any other treatment. The significant exceptions were #10 and #12.

The destruction of lysine occurred chiefly in those samples that were treated with alkali alone, although there are exceptions (*e.g.*, Treatments 5, 6, 12, and 14). In all these cases, however, there was an amount of lysinoalanine formed that was roughly equivalent to the amount of lysine lost.

The amount of lanthionine formed was the first surprise. It was expected to be higher in those treatments with lime alone (immunized); but, as can be seen, there was more formed when sulfide was present. It was also high when benzyl mercaptan (#12) and sodium borohydride (#13) were used.

The treatments with alkali alone (Treatments 2, 3, and 4) were straightforward. Within the experimental error, all the losses could be accounted for by the formation of the lanthionine and lysinoalanine (assuming all the missing lysine is converted to lysinoalanine). The treatment with lime and sodium sulfite (#14) closely resembled that for lime alone for one day (#2). Also, no hair damage occurred as a result of these treatments.

Treatment #5 was run to demonstrate a poor unhairing process in which immunization would occur. As can be seen, it closely resembles treatment with lime alone for five days (#3). The addition of the sulfide after one day did not alter the final outcome except that there is slightly more lanthionine formed. Again, very little hair was destroyed and all the amino acids are accounted for.

Treatments 7 and 8 represent "good" unhairing processes and were essentially identical. Some hair damage did occur in #8. There was no lysinoalanine formed in these treatments. The balance between the losses and the gains is not good. There was more lanthionine formed than can be accounted for by the loss of cystine. Some of this can be accounted for by the loss of serine but not all. This is also the case with Treatments 11, 12, and 13. Treatment with sodium hydroxide and sodium sulfide (#9) is analogous to those where lime was the source of alkali.

Treatment with lime and dimethylamine (#10) resulted in extensive changes in these amino acids. There was still some lanthionine and lysinoalanine formed, but not enough to account for all the losses. The formation of the new amino acid, suspected to be β -dimethylaminoalanine, probably accounts for the additional losses. There was no visible hair damage.

The combination treatment with lime, dimethylamine, and sodium sulfide (#11) closely resembles treatment with sulfide and lime alone, with the exception of the formation of DMAA. There was some slight hair damage and, again, the amount of lanthionine formed exceeded that expected by the losses. Unhairing with lime and mercaptans has been known for a long time (20) and Treatment 12 is an example of this. Just as with lime and sodium sulfide, extensive formation of lanthionine occurred. Surprisingly, some lysine was lost.

Sodium borohydride has been demonstrated to be an unhairing agent (21) and was included here (#13). It also resembled sodium sulfide.

Lime and sodium sulfite (#14), as has already been mentioned, resembled lime alone. At lower pH's, this reagent has been shown to cause the formation of lanthionine (22).

DISCUSSION

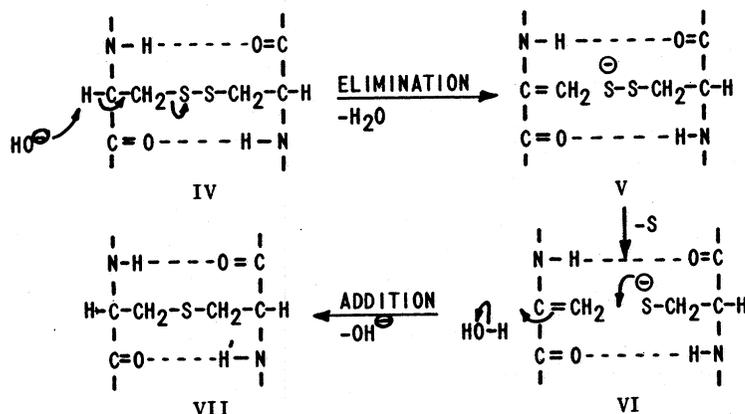
First, it should again be pointed out that the reactions and products under discussion here are those involved in the hair that does *not* dissolve. These may or may not be the same as those in the hair that dissolves or in the epidermis. These latter are under investigation now. Also, in hair-saving processes, the material attacked may not be the hair itself but rather the epidermis, as indicated by Bowes and Elliot (23). However, the same amino acids are present, although in different relative amounts (23), and the same types of reagents are responsible for loosening the hair as for dissolving ("pulping") it. Therefore, it may very well be a matter of degree, as indicated by Windus and Showell (3).

In general, there is more lanthionine formed in the presence of the more active unhairing agents than in their absence. In the reverse sense, lysinoalanine is formed in the processes that would cause "immunization" with respect to hair-loosening, and very little or none is formed when sodium sulfide is present. On the surface these facts would appear to indicate that lysinoalanine was responsible for immunization and that lanthionine was not, even though both represent crosslinks. These results could be explained by assuming that lanthionine formation results only in intramolecular crosslinks, while lysinoalanine formation results in intermolecular crosslinks. This assumption is probably not correct, however. Again, these results are for the hair that did *not* dissolve, regardless of the treatment. There is evidence that a direct relationship exists between the lanthionine and lysinoalanine contents and the further solubility of the hair in an excess of reagents. This has been demonstrated for wool (24) and can be considered the second type of immunization—immunization to dissolution. It is under investigation now and preliminary results would seem to indicate that both amino acids are involved.

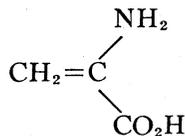
It is felt that the major reaction that is taking place upon the action of alkali alone, and perhaps with dimethylamine, is the β -elimination reaction, which was apparently first proposed by Nicolet and Shinn (25), as illustrated in the paper by Mizell and Harris (26). It is shown in Scheme I, along with the further steps resulting in formation of a lanthionyl residue in place of the original cystinyl residue. As can be seen in Scheme I, this sequence of steps results in the cleavage of a carbon-sulfur bond as opposed to cleavage of the sulfur-sulfur bond, which would take place in a substitution type reaction (3). The H-bonds shown are not meant to imply that this cystinyl residue must be bonded in this fashion—it most probably is not. The amide groups are H-bonded, but to other residues than

SCHEME I

β -ELIMINATION: LANTHIONINE FORMATION



those shown. They are drawn this way only for convenience. The activation of the hydrogen atom attached to the α -carbon atom (amino acid terminology) by the adjacent amide groups is very well documented and discussed adequately by Neuberger (27). Abstraction of this proton by base is certainly not out of the realm of possibility. The first step is probably a reversible step in the sequence and the remaining steps do not necessarily follow every proton abstraction. This, of course, could result in racemization of the amino acid and it is interesting that a part of the lanthionine isolated by Horn, Jones, and Ringel (7) was racemic. The reason for the inertness of the free amino acid to these conditions or their decomposition by other means is that in place of the two activating amide groups there is a deactivating carboxylate ion and free amino group attached to the α -carbon atom. The residue which is formed in Structure V is called a dehydroalanyl residue. The hypothetical free amino acid would have Structure VIII.

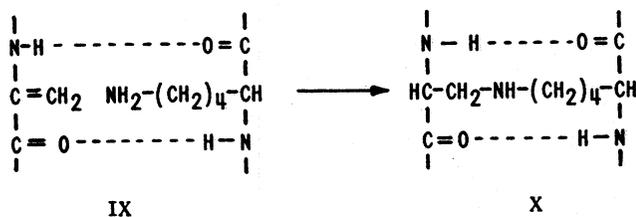


VIII

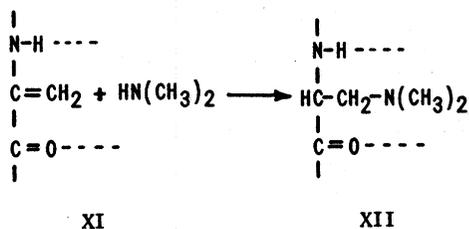
If there is a lysyl residue in close proximity to an intermediate, such as V or VI, it can compete with the cysteinyl residue for the dehydroalanyl residue and result in formation of a lysinoalanyl residue (Scheme II).

Also, in the presence of an added amine, *e.g.*, dimethylamine, other alanine derivatives could be formed (Scheme III). While it has not been established beyond all doubt, it is believed that the peak in the chromatogram (Fig. 1) labeled DMAA is the β -dimethylaminoalanine (Treatments #10 and 11).

SCHEME II
LYSINOALANINE FORMATION

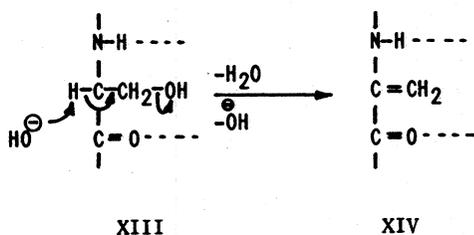


SCHEME III
 β -DIMETHYLAMINOALANINE FORMATION



The destruction of serine is presumed to take place by the same mechanism (Scheme IV); and, of course, once the dehydroalanyl residue is formed it can react by any of the above to form lanthionine, lysinoalanine, or β -dimethylaminoalanine. This destruction of serine, which was dependent only on time and not on the addition of other reagents, was the clue to the mechanism. This destruction almost certainly has to be an elimination reaction and there is no reason to suppose any different reaction is taking place with cystine. Evidence for this type of reaction on substituted seryl residues in proteins is discussed in the review chapter

SCHEME IV
DESTRUCTION OF SERINE



added sulfide ion can compete with the lysinyl residues for the dehydroalanyl residues. This would result in a sequence of steps shown in Scheme VI and the end result of the reaction would be the same. The early work of Nicolet and Shinn (25) would support this proposal and it is further supported by the fact that, in the combination reaction (#11), DMAA was still formed. Also, results of work with wool, even at a lower pH, indicated that the β -elimination reaction was the reaction taking place (15).

Therefore, it would appear that the reactions taking place in the hair that remains in a hair-saving process can be explained by a single mechanism, β -elimination followed by addition. Some of these addition steps result in crosslinking, which is the cause of immunization to further dissolution. Whether this is also the cause of immunization to hair-loosening remains to be proven. Others of these addition steps result in the introduction of new groups into the hair. In any case, the "hair" resulting from a hair-saving process is certainly chemically different from the native hair.

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DISCUSSION

MR. SHIVAS (Barrie Tanning Ltd.): This has been a most interesting paper. It is really quite a breakthrough to learn what happens to the hair during unhairing procedures. Most practical tanners have experienced some good and some bad effects from immunization during unhairing. Sometimes even a very brief exposure to alkali can have a great effect on the ability to unhair. But in enzyme unhairing the stock can be pretreated with lime and, therefore, the hair is immunized; yet the hair comes off. So there must be a different chemical breakdown with unhairing. Have you done any work with the effect on hair in enzyme unhairing?

DR. FEAIRHELLER: No, we haven't. We believe that this also involves the other proteins that are in the hair follicle and not just the hair keratin itself. These are the proteins we want to look at next to find out what is happening to these in the normal hair loosening process and possibly also with enzyme unhairing.

MR. SHIVAS: To get the hair off in a hair saving procedure, there must be a chemical breakdown of the epidermis. Have you done any work to determine what happens to the epidermis or what by-products are produced during unhairing?

DR. FEAIRHELLER: Not yet. This is again what we want to look at. As I mentioned, the proteins are similar, and the amino acid residues that are involved are probably the same; certainly the reagents that are used are the same and it may be just a matter of degree. We hope to look into this further, though.

MR. SHIVAS: Are there any questions from the floor?

DR. R. G. DONOVAN (Canada Packers Ltd.): Did you notice any change in the amount of threonine after the treatment of your hair?

DR. FEAIRHELLER: No, we didn't. We looked at this when we discovered that serine was involved. We thought that threonine should be also, but it isn't. I think it can be explained theoretically but we don't have time to go into that now.

DR. DONOVAN: Can I also ask if there is any evidence of carbohydrate-linked *O*-seryl or *O*-threonyl residues in hair?

DR. FEAIRHELLER: We haven't found any. Of course, the dehydration of seryl residues in proteins is not uncommon. However, in all those cases where it has been found, the hydroxyl group has been linked to some other residue, such as a carbohydrate or a phosphate group. In these cases the dehydration occurs quite readily. We have no evidence as yet that there is carbohydrate or anything else involved. I suppose it is something we should find out.

DR. HOLLOWAY (Rohm and Haas Co.): In the early days of dimethylamine and investigation of its effect on unhairing, people were uncertain whether it was monomethylamine or dimethylamine, and so I suppose that if you used different types of amines you might get a whole series of substituted aminoalanines. Would you expect to?

DR. FEAIRHELLER: Very much so. Ammonia or any primary or secondary amine would produce the appropriate aminoalanine derivative. We have some evidence along these lines already.

MR. SHIVAS: Any more questions? If not, we'll thank Dr. Feairheller and turn the meeting over to Malcolm.