

## FURTHER EVIDENCE IN SUPPORT OF THE ELIMINATION REACTION AS THE MECHANISM OF ALKALINE UNHAIRING

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### INTRODUCTION

In view of the continued interest in unhairing, especially as it relates to pollution from the tanning industry, it seemed imperative that we publish additional information that we have accumulated in support of the  $\beta$ -elimination (1) reaction as the mechanism of the initial reactions taking place in hair protein during alkaline unhairing. We feel that workers in the field should have the latest information available about the chemistry of the systems with which they are working.

In 1973 we published (2) the results of our research that led us to propose the  $\beta$ -elimination reaction mechanism. This proposal was based on the types and amounts of amino acids either reacting or forming during exposure of hair to various unhairing conditions. We have since accumulated additional evidence that further substantiates that the  $\beta$ -elimination reaction takes place when hair is exposed to alkali with or without additional reagents being present. This additional evidence is divided into three parts: the finding of additional reaction products, primarily amino acid derivatives; chemical studies on the reaction of cystine itself in alkali; and studies using radioactive sulfide in an unhairing reaction.

### EXPERIMENTAL

#### Reagents and Standard Compounds

All reagents and chemicals except lysinoalanine and a series of  $\beta$ -aminoalanines were purchased from commercial sources.

For the preparation of the  $\beta$ -aminoalanines, ethyl  $\alpha$ -acetamidoacrylate (3) was dissolved in an unneutralized aqueous solution of a five-fold molar excess of the appropriate secondary amine and allowed to stand at room temperature for about six hours. The resulting solutions were then evaporated to brown syrups under reduced pressure until free of excess amine. Excess 6 N hydrochloric acid solution was then added to these residues and the resulting solutions were heated at reflux for 24 hours. The resulting mixtures were evaporated under vacuum to dryness and until free of hydrogen chloride. The products

\*Retired.

were obtained from the residues as the crystalline dihydrochlorides and recrystallized from water-alcohol mixtures.

The lysinoalanine was prepared by the above procedure from  $\alpha$ -carbobenzoxy-*L*-lysine in alkaline (0.3 N sodium hydroxide) solution in place of the secondary amine. The rest of the procedure was essentially the same.

### Identifications of New Amino Acids

The manner in which the amino acid analyses was carried out has been described previously (2) with the exception that a second elution pattern of the amino acids was developed, so that the amino acid derivatives formed during the unhairing reactions could be compared with known samples of the derivatives under two sets of conditions. This required minor adjustments to the analyzer concerning buffer mixtures and running time.

### Unhairing Reactions

A sample of washed and degreased black cattle hair was suspended in the calculated amount of water (based on the weight of hide from which the hair had been clipped) to give a 200 percent float. An excess of lime was used to assure a saturated solution throughout the course of the reaction, and approximately a ten-fold molar excess of the reagent, mercaptan or secondary amine, was added, based on the amount of cystine present in the hair protein. At the end of the reaction (24 hours) the hair was washed, neutralized, hydrolyzed, and analyzed as described previously (2).

### Reaction of Cystine with Saturated Lime Solution

One gram of cystine and 119.3 mg. of valine (as an internal quantitative standard which is completely stable under these conditions) were suspended with 1.6 g. of  $\text{Ca}(\text{OH})_2$  in 50 ml. of distilled water and mixed thoroughly. A sample was removed immediately after mixing, acidified with hydrochloric acid solution, and appropriately diluted for direct amino acid analysis. The amounts of other amino acids were determined relative to valine. The suspension was sampled and analyzed again by the same procedures after 1, 2, 7, 14, and 28 days.

### Reaction of Lanthionine with Saturated Lime Solution

One hundred mg. of lanthionine and 11.4 mg. of valine were suspended with 160 mg. of  $\text{Ca}(\text{OH})_2$  in 5 ml. of distilled water. The sampling and analyzing were carried out as with cystine.

### Reactions of Cystine with Equimolar Amounts of Divalent Metal Hydroxide Solutions

The following procedure for calcium hydroxide is typical of the reaction mixtures also prepared for magnesium, strontium, and barium hydroxides: 0.503 g.

of cystine (2.1 mmol.) and 0.233 g. of  $\text{CaCl}_2$  (2.1 mmol.) were suspended in 12.5 ml. of a 0.1 N NaOH solution. (For strontium and calcium, the pH of these solutions dropped and had to be raised to 12.5 by addition of concentrated NaOH solution. The pH continued to fall over the period of time monitored, up to 16 days.) Sampling and analyses were performed as above and valine was used as an internal standard.

### Reactions of Cystine with Lithium, Potassium, and Sodium Hydroxide Solutions

The following procedure for lithium hydroxide is typical for all three: 0.256 g. of cystine (1.0 mmol.) was suspended in 12 ml. of distilled water and a 4 N LiOH solution was added dropwise until the pH of the solution was 12.5. Sampling and analyses were performed as above and valine was used as an internal standard.

### $^{35}\text{S}$ -Labeling

Five mg. of washed and degreased cattle hair, 8 mg. of  $\text{Ca}(\text{OH})_2$ , and 0.9 ml. of water were placed in a hydrolysis tube, and to this was added 0.1 ml. of a solution containing 0.5 mg. of  $\text{Na}_2^{35}\text{S}$  ( $2.4 \times 10^6$  c.p.m.).<sup>‡</sup> The resulting solution was allowed to stand for three days. The solution was then removed from the residual hair, and the hair was washed ten times with distilled water, using a pipette and without removing the hair from the tube. The hair was washed once with ten percent hydrochloric acid solution, and finally 2.5 ml. of 6 N hydrochloric acid solution was added. The tube was sealed under vacuum and heated at 105°C. for 18 hours. The tube was opened and the contents evaporated to dryness under a stream of nitrogen. The contents of the tube were taken up in exactly 1 ml. of 0.1 N hydrochloric acid solution for analysis. The solution was analyzed for amino acid composition and radioactivity distribution at the same time, using the method previously described.

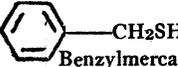
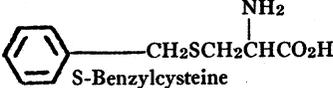
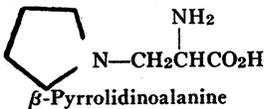
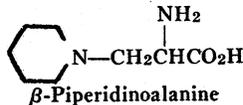
## RESULTS AND DISCUSSION

### New Amino Acid Derivatives Recovered from Unhairing Bath Hydrolysates

Using the techniques described in our earlier paper (2), we chromatographically identified a variety of additional amino acid derivatives in the hydrolysates of hair recovered from unhairing baths containing various mercaptans or secondary amines. In Table I we have listed both the names and structures of the reagents added to the unhairing baths and of the new amino acid derivatives found in the hydrolysates of the exposed hair, and in Figure 1 we have outlined

<sup>‡</sup>Caution must be exercised in the use of sulfides because of the toxic nature of the gas ( $\text{H}_2\text{S}$ ) generated on acidification. In this case the danger is compounded by the use of the radioactive isotope of sulfur.

TABLE I  
DERIVATIVES FROM REACTIONS OF THIOLS AND AMINES  
WITH DEHYDROALANYL RESIDUES

Reagents	New Amino Acid Derivatives
$\text{HO}_2\text{CCH}_2\text{SH}$ Thioglycolic Acid	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HO}_2\text{CCH}_2\text{SCH}_2\text{CHCO}_2\text{H} \\ \text{S-Carboxymethylcysteine} \end{array}$
 Benzylmercaptan	 S-Benzylcysteine
$(\text{CH}_3)_2\text{NH}$ Dimethylamine	$\begin{array}{c} \text{NH}_2 \\   \\ (\text{CH}_3)_2\text{NCH}_2\text{CHCO}_2\text{H} \\ \beta\text{-Dimethylaminoalanine} \end{array}$
 Pyrrolidine	 $\beta$ -Pyrrolidinoalanine
 Piperidine	 $\beta$ -Piperidinoalanine

the reactions involved. Addition of mercaptans to the unhairing baths resulted in the formation of thioethers (reaction c, Figure 1), the expected Michael-type reaction product. In this case they were *S*-substituted cysteine derivatives (V). Secondary amines behaved in a similar fashion (reaction d, Figure 1) forming tertiary amines (in this case  $\beta$ -aminoalanine derivatives (VI)) which were also the expected Michael-type reaction products. This latter case parallels the results obtained by Asquith and Carthew (4) with primary amines and these are the products proposed in our earlier paper (2).

These new amino acid derivatives, V and VI, are formed at the expense of the internally formed "new" amino acids, lanthionine and lysinoalanine (reactions a and b, respectively, Figure 1), described earlier (2). Formation of these latter two products results in crosslinking and stabilization of the hair protein. Addition of the reagents listed in Table I to the unhairing baths provides the competing reactions (c) and (d) which convert the reactive intermediate (II) to noncrosslinked derivatives. For this reason, dimethylamine is used as an unhairing assist.

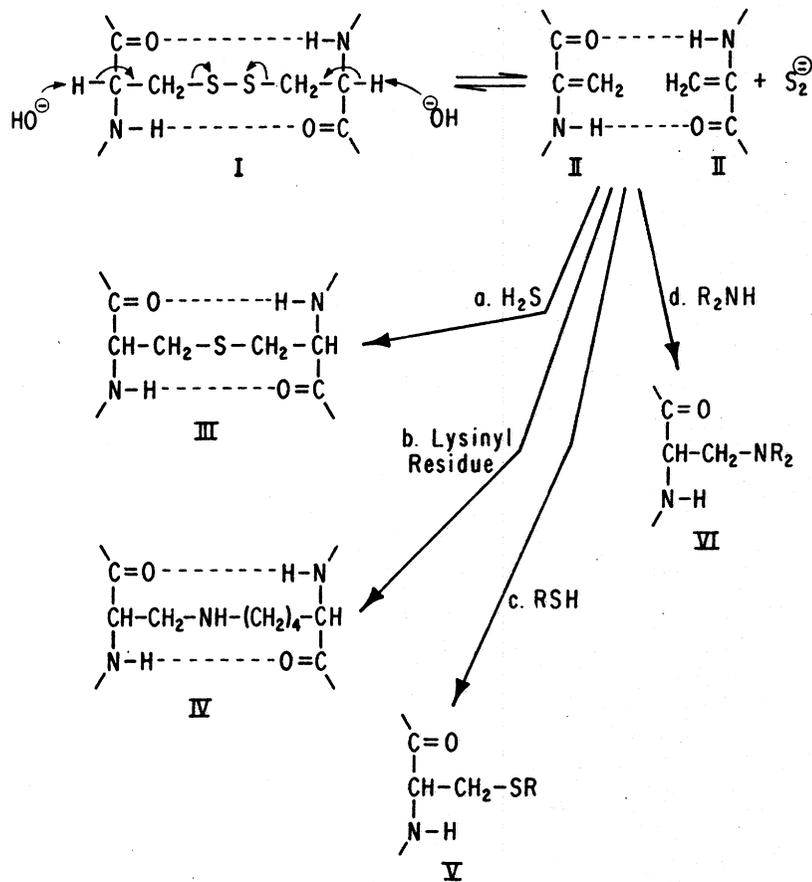
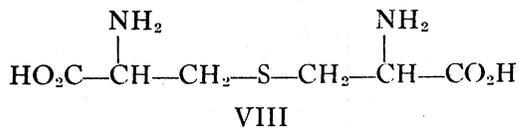
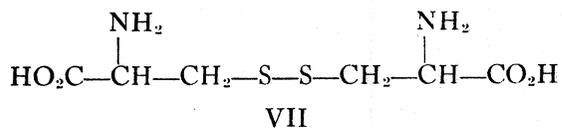


FIGURE 1—Fates of protein-bound cystinyl residues in alkaline unhairing baths.

### Cystine Chemistry

It has been stated (5) that cystine (VII), the free amino acid, does not undergo the disulfide to thioether reaction to give lanthionine (VIII) as do the protein-



(or at least peptide-) bound cystinyl residues (I) to give lanthionyl residues (III). Indeed, exposure of free cystine to sodium hydroxide solutions of the same pH (12.5) as are used for unhairing did not yield any chromatographically detectable lanthionine. Lithium hydroxide and potassium hydroxide behaved similarly. However, when saturated calcium hydroxide (lime) solutions (pH 12.5) were used, appreciable conversion to lanthionine was observable within 24 hours, accounting for 33 percent of the cystine after 48 hours (Table II). This was only an apparent maximum conversion to lanthionine, since a separate experiment

TABLE II  
REACTION PRODUCTS OF CYSTINE IN LIME SOLUTIONS  
DETECTABLE BY AMINO ACID ANALYSIS

Compound	Time (days)					
	0	1	2	7	14	28
Cystine	100%*	77	7	3	0	0
Lanthionine	0	6	33	19	10	0
Alanine	0	0	0	2	4	6
Cysteic Acid	0	0	2	4	5	5
Ammonia	0	1	4	6	10	5
Total†	100	84	46	34	29	Too high

\*Molar percentages of the moles of cystine introduced into the reaction mixture.

†Additional products detected by other means, such as sulfide ions, free sulfur, and oxalate ions, were not quantitated and are not included in these totals.

showed that the lanthionine itself was being destroyed by the alkali under these same conditions. Strontium hydroxide gave results similar to those with calcium hydroxide, whereas magnesium and barium hydroxides gave results similar to those obtained with the monovalent hydroxides.

In addition to the products listed in Table II, which were detected and quantitated by amino acid analysis, we also detected sulfide ions, free sulfur, and oxalate ions in the reaction mixtures. The formation of the lanthionine as well as the formation of these other products, except alanine, all lend themselves to support of the elimination reaction (Figure 2). The reaction undoubtedly involves complex formation between cystine (VII) and divalent metallic ions of calcium and strontium. The monovalent metals did not catalyze the reaction because of their inability to form the stable complexes. Magnesium and barium did not catalyze the formation of these products, probably because of spatial requirements. Magnesium was presumably too small and barium too large.

The formation of the complex, IX, neutralizes the high electron density around the  $\alpha$ -carbon atoms (amino acid terminology) caused by the adjacent carboxyl anions and the free amino groups in the free amino acid in alkaline

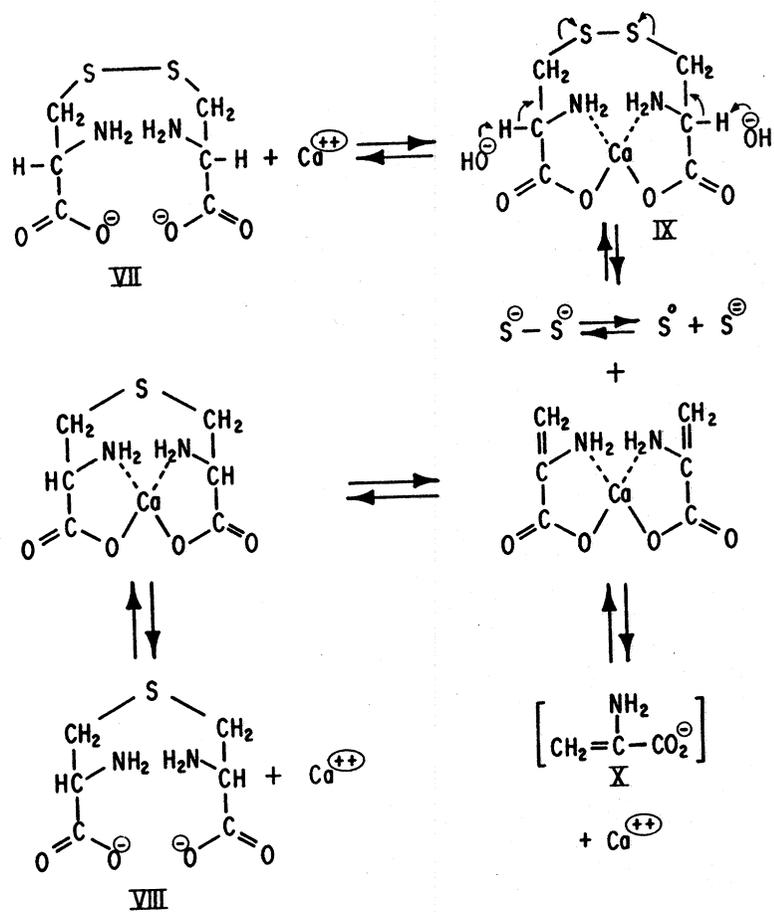


FIGURE 2.—Calcium ion catalyzed alkaline reactions of cystine — major pathway.

solution. This reduction in electron density about the  $\alpha$ -carbon atom allows abstraction of the  $\alpha$ -hydrogen and the subsequent steps of the  $\beta$ -elimination to proceed. The same effect is achieved in the protein by peptide bond formation, and, as we have shown (2), any source of alkali including monovalent metal hydroxide will bring about the conversion of protein-bound cystinyl residues to lanthionyl residues.

The major course of the reaction is that which leads to formation of free sulfur and lanthionine (VIII). Free dehydroalanine (X) is incapable of existence and decomposes presumably to pyruvic acid. This latter product is known to react with free cysteine to give 2-methylthiazolidine-2,4-dicarboxylic acid which has been shown to be one final product of the alkaline destruction of both cystine and lanthionine (6). We have chromatographically demonstrated its presence in



TABLE III  
REACTION PRODUCTS OF LANTHIONINE IN LIME SOLUTIONS  
DETECTABLE BY AMINO ACID ANALYSIS

Compound	Time (days)					
	0	1	2	7	14	28
Lanthionine	100%*	99	86	20	0	0
Cystine	0	2	5	0	0	0
Alanine	0	0	0	4	5	5
Cysteic Acid	0	0	0	4	6	6
Ammonia	0	0	7	20	33	Too high
Total†	100	101	98	48	44	

\*Molar percentages of the moles of lanthionine introduced into the reaction mixture.

†Additional products detected by other means, such as sulfide ions, free sulfur, and oxalate ions, were not quantitated and are not included in these totals.

### Radioisotope Studies

In order to gain more insight into the reactions taking place in these complex systems, we repeated one of our earliest reactions — the simple exposure of hair to lime/sulfide mixtures followed by amino acid analysis of the recovered hair. This time, however, we incorporated a small amount of radioactive sulfur,  $^{35}\text{S}$ , into the reaction mixture in the form of sodium sulfide  $^{-35}\text{S}$ . The effluent from the amino acid analyzer column was split into two streams. One portion was analyzed by conventional means and the remaining portion was analyzed for radioactivity using a flow-through cell in a liquid scintillation counter. Three amino acids accounted for 87 percent of the total radioactivity in the recovered hair: lanthionine, 40 percent; cystine, 20 percent; and cysteic acid, 27 percent. As we anticipated, the lanthionine was labeled with the  $^{35}\text{S}$ . This agrees with the course of the reaction proposed in the  $\beta$ -elimination reaction (Figure 1).

However, even stronger evidence in support of this mechanism was given by the extensive labeling of the cystine in the recovered hair. Its activity was half as high as that of the lanthionine. This indicates that the reaction is reversible and that the protein-bound cystine reversibly undergoes the elimination reaction during exposure to alkali. What happens to it next depends on the additional materials present. It can reform and in so doing pick up disulfide anion (and label since disulfide ions are in equilibrium with the labeled sulfide ions under these conditions) from solution. Added sulfide ion promotes the formation of lanthionyl residues (III). Reagents like mercaptans and amines can add to form thioethers or amine derivatives as shown earlier (Table I). In the absence of other reagents, lysinyl residues can react to form lysinoalanyl residues (IV).

## CONCLUSIONS

All of the evidence supports the  $\beta$ -elimination mechanism as the initial reaction which takes place at the disulfide crosslinks in hair exposed to alkaline conditions, including, specifically, those conditions encountered in tannery unhairing treatments. The amino acid derivatives observed, the reactions of cystine and lanthionine in alkaline solutions of calcium and strontium ions, and the radioactive labeling experiment all support this conclusion.

It must be stressed to those scientists looking for uses for the hair protein recovered from tannery unhairing effluents, whether the protein originates from a hair-saving process or a hair-burning process, that the recovered protein does contain chemically modified peptide units which on hydrolysis yield amino acid derivatives whose chemical and physiological properties have not been well characterized. This has been demonstrated in this paper and our earlier paper (2) for recovered intact hair, and has also been shown for recovered pulped hair protein (7). Although to our knowledge it has not been investigated, it is entirely possible that hair protein recovered from processes using recycled unhairing liquors could possess an even more complex amino acid derivative mixture due to the formation and accumulation in the liquors of compounds capable of taking part in the reactions discussed in this paper.

## REFERENCES

1. Gould, E. S. "Mechanism and Structure in Organic Chemistry," Chapter 12, Henry Holt and Company, New York, 1959.
2. Fairheller, S. H., Taylor, M. M., Filachione, E. M., and Windus, W. *JALCA*, **67**, 98-110 (1972).
3. Hellmann, H., Teichmann, K., and Lingens, F. *Chem. Ber.*, **91**, 2427-2431 (1958).
4. Asquith, R. S., and Carthew, P. *Biochim. Biophys. Acta*, **278**, 8-14 (1972).
5. Danehy, J. P. *Int. J. Sulfur Chem., B*, **6**, 103-114 (1971).
6. Dann, J. R., Oliver, G. L., and Gates, J. W. *J. Amer. Chem. Soc.*, **79**, 1644-1649 (1957).
7. Happich, W. F., Happich, M. L., Cooper, J. E., Fairheller, S. H., Taylor, M. M., Bailey, D. G., Jones, H. W., Mellon, E. F., and Naghski, J. *JALCA*, **69**, 50-63 (1974).