

THE STABLE LINKAGES BETWEEN EPOXY
RESINS AND COLLAGEN*

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ABSTRACT

Commercial epoxy resins are known to impart a very stable tannage to collagen. A complete acid hydrolysis of the treated leather has shown that the lysine, hydroxylysine, tyrosine, and possibly methionine and arginine residues have formed extremely stable linkages with the epoxy resin. The suggestion is made that these stable linkages are responsible for the high stability of epoxy resin tannages.

INTRODUCTION

Epoxides will react theoretically with almost all nucleophilic substances, such as carboxyl, amino, hydroxyl, and sulfhydryl groups. The reactions of epoxides with proteins were extensively studied by Fraenkel-Conrat (1) in 1944. Capp and Speakman (2) used diepoxides to introduce cross bonds into animal fibers and concluded that carboxyl groups play an important role in cross-linking reactions. Immendorfer (3) and Sykes (4) made use of this cross-linking ability of difunctional epoxides to tan hides and skins. In 1956 Filachione *et al.* (5, 6) reported results of practical tanning tests using commercially available epoxide resins. The leathers produced by these resins appeared quite stable. Sykes (7) reported that there is a direct relationship between the rise in shrinkage temperature produced by tanning with epoxides and the logarithm of the number of cross linkages introduced. Sykes (8) has also shown that only multifunctional epoxides possess any cross-bonding action on collagen, and from experiments involving the chemical blocking of certain groups he has concluded that the hydroxyl residues of hydroxyproline, serine, and threonine are involved in the formation of such cross bonds.

The stability of the epoxy resin-tanned leathers indicated that the mechanism of this type of tannage might be studied by the isolation of resin-containing fragments from partial or complete hydrolyzates of epoxy resin-tanned

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collagen. As an exploratory study, the amino acids recoverable from a complete acid hydrolyzate were determined on an automatic amino acid analyzer.

EXPERIMENTAL

Collagen preparation.—Cured cowhides were washed in water and fleshed. They were then limed for five days, unhaired, and scudded. The hides were washed and neutralized with dilute acetic acid solutions to a final pH of about 5.0. They were then washed free of acid, dehydrated with several changes of acetone, air-dried, and split to obtain three layers. The center layers only were used. These were cut into small pieces and ground in a Wiley Mill in the presence of dry ice to prevent overheating of the collagen. This material contained 18.1% total nitrogen, 0.38% amino nitrogen, and 0.47% amide nitrogen on a moisture-free basis. The nitrogen was determined by the semimicro Kjeldahl method; amino nitrogen, by the method of Doherty and Ogg (9); and amide nitrogen, by the method of Mellon *et al.* (10). Shrinkage temperature was 63°C., as determined by the microscopic method of Borasky and Nutting (11).

Treating with epoxy resin.—Four hundred grams of the above purified collagen was dispersed in 10 l. of distilled water. After a thorough wetting, 84 g. of sodium carbonate and 400 g. of sodium sulfate were added, and the collagen was equilibrated for 2 hr. Then 200 g. of epoxy resin was added. This resin was obtained by solvent fractionation of commercial Epon 562 resin* and consists almost exclusively of a single component with a molecular weight of 353 which has three epoxy groups and one chlorine atom per molecule. A four-inch-square piece of limed calfskin, which had been neutralized to pH 5.0 and washed free of salts as described for the ground collagen, was also put into the reaction mixture as a reference for the degree of tannage. The mixture was stirred continuously for five days.

The supernatant solution was decanted, and the solids were washed free of dissolved salts by repeated decantations with distilled water. The solids were dehydrated with four changes of acetone and finally air-dried. The combination of water and acetone washes should remove any unbound resin or resin hydrolysis products.

This epoxy resin-tanned collagen contained 15.8% of total nitrogen, 0.027% amino nitrogen, and 1.35% chlorine. This indicates a resin content of about 13%. These fibers had a shrinkage temperature of 74°C. by the microscopic method. The piece of calfskin tanned in the same solution had a shrinkage temperature of 79°C. in a Theis Meter.

Preparation for amino acid analysis.—One-gram samples of the collagen and the epoxy resin-treated collagen were hydrolyzed by refluxing in

*This product is a condensation of glycerol and epichlorohydrin and is produced by Shell Chemical Corporation, 500 Fifth Avenue, New York 36, N. Y. The mention of specific brand or companies is not to be construed as an endorsement by the United States Department of Agriculture of these brands or companies over those not mentioned.

20 ml. of 6*N* hydrochloric acid for 16 hours. The products were evaporated to dryness on a steam bath under a current of air, and then further evaporated three successive times from distilled water solutions to expel the hydrochloric acid. The products were diluted to exactly 50 ml. with distilled water and filtered to remove slight traces of insoluble material. Five ml. of these solutions was diluted to 25 ml. with chromatographic buffer (pH 3.25), and one-ml. aliquots of this solution were put on a chromatographic column.

Amino acid analysis.—The analyses for the amino acid contents of hide collagen and epoxy resin-treated collagen were performed according to the techniques of Moore, Spackman, and Stein (12, 13), using Amberlite IR 120 resin and the Phoenix Amino Acid Analyzer, Model K5000. Chromatographic columns, 150 cm. in length, were used for the acidic and neutral amino acids, and 15 cm. in length for the basic amino acids. Hydroxyproline was determined by the Martin and Axelrod (14) modification of the Neuman and Logan method.

RESULTS AND DISCUSSION

The analysis of cowhide collagen and epoxy resin-treated collagen are given in Table I. In the first two columns, which compare the recovered amino acid nitrogen as a percentage of the total nitrogen, four outstanding differences due to the resin treatment are shown: free amino nitrogen is reduced from 2.1 to 0.2%; the lysine value changes from 4.1 to 0.4%; the hydroxylysine value decreases from 1.0 to 0.05%; and the tyrosine value is reduced from 0.3% to a negligible amount.

All the other amino acids were recovered to the same degree from both the tanned and untanned samples except for methionine and arginine, which were recovered in slightly reduced amounts from the tanned sample. These latter reductions may be questionable since the methionine content of collagen is initially very low, and the decrease in arginine content is close to the experimental error for the method of analysis. Even if these changes in methionine and arginine content are significant, it is not clear whether only a fraction of these amino acids have formed highly stable bonds with the resin or whether a large fraction reacted initially to form bonds which later hydrolyzed significantly. Although the amount of tyrosine in native collagen is small, its total disappearance in these experiments is believed to indicate that it forms an extremely stable bond with the resin.

To arrive at a more meaningful expression of the magnitude of the reaction of the epoxy resin with the five amino acids in question, recoveries of these amino acids were calculated on a basis of moles per 10,000 moles of total nitrogen. The values are given in Table II. The table shows that a total of 328 moles of amino acids have reacted with the resin.

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TABLE I
AMINO ACID COMPOSITION OF COWHIDE COLLAGEN AND
EPOXY RESIN-TREATED COLLAGEN

| | Amino Acid N as Percent of Total N | | Grams Amino Acid Per 100 Grams | |
|----------------|---------------------------------------|-------------------------|-----------------------------------|-------------------------|
| | Cowhide Collagen | Epoxy Resin Collagen | Cowhide Collagen | Epoxy Resin Collagen |
| Total nitrogen | | | 18.1 | 15.8 |
| Amino nitrogen | 2.1 | 0.2 | | |
| Amide nitrogen | 2.6 | 2.4 | | |
| Glycine | 25.9 | 24.8 | 25.2 | 21.0 |
| Alanine | 8.7 | 8.2 | 10.0 | 8.3 |
| Leucine | 2.0 | 1.9 | 3.4 | 2.8 |
| Isoleucine | 1.0 | 1.0 | 1.7 | 1.5 |
| Valine | 1.7 | 1.7 | 2.6 | 2.2 |
| Serine | 2.6 | 2.7 | 3.5 | 3.2 |
| Threonine | 1.3 | 1.4 | 2.0 | 1.9 |
| Methionine | 0.5 | 0.2 | 1.0 | 0.3 |
| Proline | 10.8 | 10.3 | 16.1 | 13.4 |
| Hydroxyproline | 7.9 | 8.3 | 13.4 | 12.3 |
| Phenylalanine | 1.0 | 1.0 | 2.1 | 1.9 |
| Tyrosine | 0.3 | Trace | 0.7 | Trace |
| Arginine | 14.1 | 12.7 | 7.9 | 6.2 |
| Histidine | 1.3 | 1.2 | 0.9 | 0.7 |
| Hydroxylysine | 1.0 | 0.05 | 1.0 | 0.05 |
| Lysine | 4.1 | 0.4 | 4.0 | 0.3 |
| Aspartic acid | 3.2 | 3.1 | 5.5 | 4.7 |
| Glutamic acid | 5.4 | 5.1 | 10.3 | 8.5 |
| Total* | 95.4 | 86.5 | | |

*This total does not include the value for amino nitrogen.

TABLE II
AMINO ACID RECOVERIES INDICATING REACTION WITH EPOXY RESIN

| Amino Acid | Recovery From | | Indicated Reaction | |
|---------------|----------------------|--------------------------|--------------------|-----|
| | Cowhide Collagen* | Epoxy Resin Collagen* | Amount* | % |
| Lysine | 205 | 20 | 185 | 90 |
| Hydroxylysine | 50 | 2 | 48 | 95 |
| Tyrosine | 30 | 0 | 30 | 100 |
| Methionine | 50 | 20 | 30 | 60 |
| Arginine | 352 | 317 | 35 | 10 |
| Total | | | 328 | |

*Moles per 10,000 moles of total nitrogen.

The amount of resin uptake by the collagen can be estimated from the nitrogen and chlorine analyses of the epoxy resin-treated collagen and the original collagen. The nitrogen analyses indicate that the resin content of the product is 12.7%, which is equivalent to 319 moles of resin per 10,000 moles of total nitrogen. The chlorine analyses indicate that the resin content of the product is 13.8%, which corresponds to 349 moles of resin per 10,000 moles of total nitrogen. The average of these two calculated figures is 334 moles, which is in remarkable agreement with the value of 328 moles given in Table II. However, since each molecule of resin contains three reactive epoxy groups, this should only be interpreted that one-third of the epoxy groups have reacted to form linkages extremely resistant to acid hydrolysis.

The work of Zahn and Wegerle (15) on the cross linkages produced by *p,p'*-difluoro-*m,m'*-dinitrodiphenyl sulfone indicates that at least 55% of both the lysine and hydroxylsine residues of collagen are cross-bonded to other lysine or hydroxylysine residues. The fact that both lysine and hydroxylysine are cross-bonded to the same degree would indicate that cross-bonding may be limited by the hydrolysis of the second fluorine atom before it can react to form the cross bond. The more slowly hydrolyzing epoxy group may, therefore, be able to produce a higher number of cross bonds between lysines and hydroxylysines than the difluorinated compound.

The high molar ratio (2:3) between the resin reacted and the two lysines present and the possibility that more than 50% of the lysines can be expected to be cross-bonded to other lysines would argue that about half of the resin must be bound to groups other than the two lysines. It is true that epoxides react with many groups of collagen, but certain of these groups will form linkages which are more stable than others. Sykes (8), as a result of experiments with deaminated and acetylated collagen, suggested that the hydroxyl groups of serine, threonine, and hydroxyproline are responsible for the increase in thermal stability produced by epoxy-type tanning materials. This is debatable, for the deamination reaction employed by Sykes converted the free amino groups of the lysine and hydroxylysine residues to hydroxyl groups which can also react with epoxy groups. Since the spacing between these hydroxyl groups is almost the same as the spacing between the amino groups which they replace, it is quite possible that the number of cross linkages formed between the modified lysines and hydroxylysines in the deaminated collagen is still close to the number formed with native collagen. Therefore, the reduction of the shrinkage temperature following deamination of collagen cannot be used to prove that these cross bonds between lysines are not important in maintaining the elevated shrinkage temperature. Consequently, the great reduction in shrinkage temperature noted by Sykes for the acetylated collagen cannot be attributed solely to the blocking of the hydroxyl groups, for the amino groups are also successfully blocked by this reaction.

The resistance to acid hydrolysis exhibited by the linkages between the epoxy resin and the lysine, hydroxylysine, tyrosine (and possibly methionine and arginine) present in epoxy resin-tanned collagen indicates that these linkages are extremely stable. It is, therefore, suggested that the stability of the epoxy resin-tanned leathers results from the formation of these extraordinarily stable linkages.

CONCLUSIONS

1. A triepoxide has reacted almost to completion with the lysine, hydroxylysine, and tyrosine residues of collagen to form linkages extremely resistant to acid hydrolysis.
2. A fraction of the methionine and arginine in collagen may also have formed linkages with the epoxy resin which are resistant to acid hydrolysis.
3. One-third of the total epoxy groups of the epoxy resin are utilized in forming these stable bonds.
4. The stability of the epoxy resin-tanned leathers results from the presence of these highly stable linkages.

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DISCUSSION

DR. J. H. HIGHBERGER: Our knowledge of the structure of collagen has been growing by leaps and bounds during the last few years. Unfortunately, I think it is fair to say that knowledge of the chemical modifications produced in collagen by tanning agents has not kept pace with the increase in our understanding of collagen, itself. We know, in a general way, as Dr. Bitcover has indicated, that most tanning agents do introduce cross links, but in most of these cases the exact location of these and their detailed nature are still unknown.

Aside from the work of Zahn and his collaborators in Germany, to which Dr. Bitcover also referred, on the demonstration of certain specific cross links in collagen, I am not aware of any major effort in this direction in the case of collagen.

The present work is certainly, therefore, to be welcomed as an original attempt in this direction.

It is now possible to isolate, by chromatography or other process, the various cross links and single-chain components of native, untanned collagen, after mild denaturization. It occurs to me that a similar approach might be fruitful in the case of tanned materials. I would like to ask Dr. Bitcover whether consideration has been given to this approach in this case.

DR. BITCOVER: Yes, we are continuing this study of partial and complete hydrolysates of epoxy resin-tanned collagen by chromatography and electrophoresis. We are making progress, but it is a little premature to report the results.

DR. HIGHBERGER: One further question: I am a little surprised that the shrinkage temperature of this material is not higher than it is. Do you have any explanation for that, if all of these cross links are introduced? I believe you said it was 79°.

DR. BITCOVER: I think Dr. Filachione has had better results and can probably tell what shrinkage temperatures have been realized.

DR. FILACHIONE: You used a ground-up collagen, didn't you?

DR. BITCOVER: Yes.

DR. FILACHIONE: We treat the intact skin and do get shrink temperatures in the neighborhood of 85°, but maybe the ground-up collagen might suffer some partial effects due to the increased surface characteristics of the intact skin.

Your shrink temperature was also determined by the microscopic technique, while ours was on a specimen such as the Theis Meter uses.

DR. BITCOVER: The Theis Meter gives a reading 2°-5° higher than the microscopic measurement. Maybe that is the difference.

DR. JAMES M. CASSEL (National Bureau of Standards, Washington, D.C.): I thought I heard you say that Dr. Sykes from South Africa had inferred the cross-linking was with the hydroxyl group of serine and threonine. Wouldn't the concentration of these amino acids remain unchanged in your work, if they cross-link and then hydrolyze?

DR. BITCOVER: Yes, they would decrease only if they formed linkages stable to the acid hydrolysis. We are not saying these are the only linkages formed. What we are saying is that these five linkages are very stable linkages.

DR. CASSEL: Maybe more stable than some others?

DR. BITCOVER: Yes.

DR. FILACHIONE: Dr. Sykes didn't prove those linkages. He just speculated more or less, if I remember correctly. He even postulated linkages including the carboxyl group. But I don't think he had any recovery of amino acids from the hydrolysate.

DR. EDWARD F. MELLON (Eastern Utilization Research and Development Division, Philadelphia, Pa.): In the previous studies, Sykes used acetylation to cover up the amino and hydroxyl groups and inferred that the drop in the shrinkage temperature was due to the hydroxyl groups, because he did not get the drop when he removed only the amino groups by deamination procedure.

If you deaminate, you are only converting the amino groups to hydroxyl groups. If the two amino groups are close enough to bridge, and you convert the amino groups to hydroxyl groups, which can also react with the epoxy resin, these may also form cross links, because the spacing between them is still correct. We think that is what happens. Sykes still has the cross bond which he thinks he has removed by his acetylation procedure, and therefore we think he is just misinterpreting the results that he has.

If the hydroxyl group reacted with the epoxy group, the combination should be an ether and therefore we should have a decrease in the amount of serine, threonine, and hydroxyproline recovered. The fact that we did not get them is an indication that the hydroxyl groups are not forming the ether linkages. They may be forming other linkages on which we don't want to comment further at the present time.