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REACTION OF SILK FIBROIN WITH DIAZOMETHANE

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ABSTRACT

The treatment of silk with an ethereal solution of diazomethane resulted in a rapid decrease in tyrosine content, with no appreciable decrease in tensile strength of the fibers. The results indicate that the hydroxyl groups of tyrosine in the untreated fibers are free. The total methoxyl content after treatment of the fiber for 25 hours or longer was greater than could be accounted for by assuming that only phenolic hydroxyl groups had reacted. The "extra" methoxyl was not on either the primary amino groups or on the hydroxyl groups of the amino acid serine, but appears to have resulted from the reaction of diazomethane with groups, tentatively assumed to be carboyxl, to form the methyl esters.

CONTENTS

I. INTRODUCTION

Many of the chemical and physical properties of a protein are intimately related to the number and arrangement of its functional or reactive groups, most of which are contributed by the dibasic and diacidic amino acids. Silk fibroin is composed largely of the amino acids, glycine, alanine, and tyrosine, and of these only the last one would be expected to contribute a reactive group, namely, a phenolic hydroxyl group. This group thus becomes of special interest.

As the result of observations in this laboratory, it appeared that the hydroxyl groups of tyrosine might not be free in the untreated fiber. For example, it was found that when fibroin was treated at pH 8 with Folin's phosphomolybdate phenol reagent [1],[2] only a pale blue color, indicative of phenolic hydroxyl groups, was produced, but as the pH was increased, the solution became increasingly colored. Pretreatment of the silk with alkali also increased the depth of color obtained with this reagent. Similar results were obtained with other proteins (pepsin and wool), and it appeared that not all of the phenolic hydroxyl groups of these proteins were free. Further evidence favoring this view was obtained by Fruton and Lavin [2], who observed that the characteristic absorption bands of tyrosine were not found in

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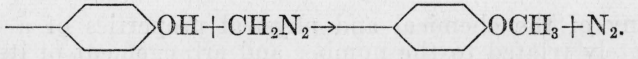
[2] Figures in brackets indicate the literature references at the end of this paper.

untreated papain, although, on acid hydrolysis, appreciable amounts of the amino acid were liberated.

The state of this hydroxyl group in the silk fiber becomes of considerable importance, since it would be expected to influence such properties of the fiber as tensile strength, extensibility, and absorption of moisture, acids, alkalies, or dyes. Unfortunately, the experiments indicating that the hydroxyl groups are not free must be considered inconclusive and open to some criticism, since they involve the use of treatments which are known to have a degradative effect on the protein. It appeared that a better approach to the problem might be made by attempting to cause the hydroxyl group to react with suitable mild chemical reagents under conditions which would minimize the possibility of degradation of the fibers.

Of the available reagents, the acid chlorides or anhydrides, ketene, and diazomethane, appear to be most suitable for inactivating the hydroxyl groups of tyrosine. The acid chlorides and anhydrides were used for this purpose by Abderhalden and Brockmann [3, 4], but they were found to produce appreciable deterioration of the fibers. Ketene was used by Kise and Carr [5, 6] with far more satisfactory results, although on prolonged treatment with this reagent the fiber was colored tan and some degradation occurred. The product of the reaction of ketene with the hydroxyl group of tyrosine is an ester, which is readily hydrolyzed in either alkaline or acid solutions. For this reason, it is not possible, by the usual tyrosine analyses, to estimate quantitatively the extent to which the ketene has reacted with the hydroxyl groups. Further, the acetyl value of the treated fiber is also inadequate for this purpose, since it is known that the ketene reacts with groups other than the phenolic hydroxyl group.

Diazomethane offers several advantages over the above-mentioned reagents. It can be used in neutral aqueous or organic solutions, and it reacts with a phenolic hydroxyl group to form an ether as follows:

(1) 

The product of the reaction is stable during the alkaline hydrolysis used in tyrosine determinations, which makes possible the quantitative estimation of the extent to which the tyrosine has reacted. The reagent was used by Herzig and Landsteiner [7], and by Abderhalden and Brockmann [3], on several proteins, including silk, but the extent of the reaction with tyrosine was not determined. The methoxyl contents of the treated fibers, however, were found to be greater than would be expected if only the phenolic hydroxyl groups were reacting.

II. EXPERIMENTAL PROCEDURE

1. MATERIALS AND METHODS

Silk fibroin in the form of a plain-woven cloth was used in this work. Before treatment with diazomethane, it was extracted for 8 hours each with alcohol and with ether, and finally washed thoroughly with cold distilled water.

Total nitrogen was determined by the micro-Kjeldahl method; tyrosine by Lugg's method [8] after an alkaline hydrolysis; amino

nitrogen by the method of Rutherford, Harris, and Smith [9]; methoxyl content by the procedure described by Vieböck and Brecher [10]; and methyl alcohol by Beyer's method [11].

2. PREPARATION OF DIAZOMETHANE

The ethereal solution of diazomethane was prepared according to the method described by Adamson and Kenner [12]. The apparatus is shown in figure 1. The temperature of the reaction flask, *B*, was maintained at 75° to 80° C in a water bath, and that of the receiver, *A*, at −10° to −15° in a brine tank. Before the distillation of the diazomethane was started, about 100 ml of ether was added to flask

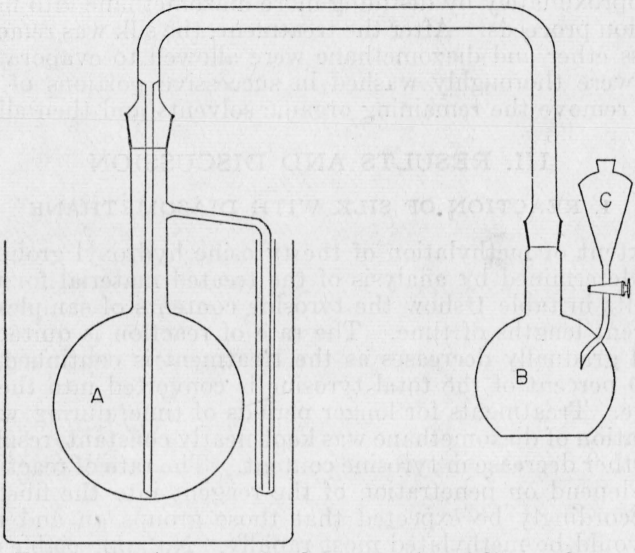


FIGURE 1.—*Apparatus for the preparation of diazomethane.*

A, and 10 ml to the trap at the side arm of *A*. 0.05-mole of the nitroso derivative of the methylamine addition product of mesityl oxide in 40 ml of ether and 6 ml of isopropanol was added dropwise from funnel *C* to 30 ml of 1-percent sodium isopropoxide in flask *B*, the rate of addition being slightly greater than that of distillation. When the addition was complete, 15 ml more of 1-percent sodium isopropoxide was added to *B*, followed by another 0.05-mole portion of the nitroso ketone, as before. Finally, ether was added to flask *B* until all the diazomethane was distilled into *A*. By this procedure about 1 g of diazomethane in 250 to 300 ml of ether was obtained. The actual amount was readily determined by the addition of an aliquot of the ether solution to a weighed quantity of benzoic acid, and subsequent titration of the excess acid with 0.1 *N* potassium hydroxide in alcohol [15].

3. METHYLATION OF FIBROIN

Preliminary experiments showed that the rate of methylation of the tyrosine hydroxyl groups of the silk was increased by the presence of a little moisture, by increased concentrations of diazomethane, or

by treating the fibers at low temperatures (−10° C or below). On the basis of these experiments, the following procedure was used. The sample was first wet thoroughly with 75-percent alcohol and then squeezed as dry as possible. This wetting served to introduce an appreciable quantity of water into the fiber, and when the sample was treated by this procedure, the subsequent separation of a water layer in the ether solution was avoided. The silk was then placed in flask *A* containing the diazomethane, and the reaction allowed to proceed at −10° to −15° C for the desired length of time. A rapid rate of reaction was obtained when the ratio of diazomethane to silk was about 1:5. This is four to five times the amount required to completely methylate the fibroin. This concentration may be maintained approximately by distilling more diazomethane into flask *A* as the reaction proceeds. After the treatment, the silk was removed and the excess ether and diazomethane were allowed to evaporate. The samples were thoroughly washed in successive portions of distilled water to remove the remaining organic solvents and then allowed to dry.

III. RESULTS AND DISCUSSION

1. REACTION OF SILK WITH DIAZOMETHANE

The extent of methylation of the tyrosine hydroxyl groups of the silk was determined by analysis of the treated material for tyrosine. The results in table 1 show the tyrosine contents of samples treated for different lengths of time. The rate of reaction is quite rapid at first, and gradually decreases as the treatment is continued. In 10 hours, 80 percent of the total tyrosine is converted into the methyl derivative. Treatments for longer periods of time, during which the concentration of diazomethane was kept nearly constant, resulted in a slight further decrease in tyrosine content. The rate of reaction may, in part, depend on penetration of the reagent into the fiber, and it might accordingly be expected that those groups on and near the surface would be methylated most rapidly. No appreciable decrease in tensile strength of the fibers occurred even on prolonged treatment. Since practically all of the tyrosine in silk is so readily methylated under these conditions, it appears that the hydroxyl groups of this amino acid are free in the untreated fiber.[3] A possible explanation of the discrepancy between this conclusion and that of the experiments with Folin's phenol reagent, previously described, might be found in a consideration of the nature of the reaction in the Folin method. This reaction depends on the oxidation of a phenolic hydroxyl group by the reagent, the latter in turn being reduced to the highly colored state. The depth of color developed by a known amount of tyrosine is used as a standard. If, however, the oxidation potential of the hydroxyl group of the tyrosine, as it exists in the protein, is different from that of the tyrosine itself, then the apparent tyrosine values obtained for the protein will not be true values. On this assumption, it would appear that the oxidation potential of the hydroxyl group is greater when the tyrosine is part of the protein molecule.

[3] It is of interest to note that the tyrosine contents of silk sericin, crystalline pepsin, and wool were reduced from 6.3, 7.8, and 5.8 percent to 0.5, 1.8, and 1.4 percent, respectively, after treatment for 20 hours with diazomethane.

TABLE 1.—*Tyrosine and methoxyl contents of silk fibroin after treatment for different lengths of time with an ethereal solution of diazomethane*

Duration of treatment	Tyrosine content	Methoxyl content of—		
		Fibroin	Hydrolysate	Hydrolysate (calculated from decrease in tyrosine content)
hr	%	%	%	%
0	12.1			
1	6.6	0.92	0.94	0.94
1.5	4.1	1.94	1.49	1.37
3	3.6	1.97	1.54	1.45
5	3.2	1.99	1.52	1.52
10	2.4	2.32	1.60	1.66
20	2.3	2.35	1.58	1.68
35	2.0	2.55	1.76	1.73
50	1.6	2.62	1.86	1.80

An examination of the data in columns 3 and 5 of table 1 shows that the methoxyl content of the treated silk is greater than can be accounted for, if it is assumed that only the hydroxyl groups of the tyrosine had reacted. Presumably, the "extra" methoxyl results from the reaction of diazomethane with other groups in the fiber. Conceivably, diazomethane might react with silk in several ways, as shown in the following equations:

$$P-CH_2-C_6H_4OH+CH_2N_2\rightarrow P-CH_2C_6H_4OCH_3+N_2 \quad (1)$$

$$P-CH_2NH_2+CH_2N_2\rightarrow P-CH_2NHCH_3+N_2 \quad (2)$$

$$P-CH_2COOH+CH_2N_2\rightarrow P-CH_2COOCH_3+N_2 \quad (3)$$

$$P-CH_2OH+CH_2N_2\rightarrow P-CH_2OCH_3+N_2, \quad (4)$$

where, P represents the protein molecule to which the above groups are attached. The equations then represent the reaction of diazomethane with (1) the hydroxyl groups of tyrosine, (2) the free amino groups, (3) the free carboxyl groups, and (4) the hydroxyl groups of serine.

Reaction (1) is shown to occur by the decrease in tyrosine content of the treated silk. Reaction (2) would result in the formation of a methylamino compound which would not give methoxyl under the analytical conditions used, and would therefore not account for any "extra" methoxyl. Moreover, amino-nitrogen determinations as shown in table 2 indicate that this reaction does not occur. Reaction (3) undoubtedly occurs since diazomethane is known to readily methylate carboxylic acids with the formation of methyl esters. Upon hydrolysis of the fibers under the conditions generally used in the tyrosine determination (6 *N* NaOH for 20 hours at 105° C), such esters would be expected to decompose liberating methyl alcohol with the regeneration of the carboxylic acid. The former would be evolved, and thus the methoxyl content of the hydrolysate should represent those methoxyl groups which resist the hydrolytic conditions used in these experiments. Reactions (1) and (4) would give this type of methoxyl.

TABLE 2.—*Total and amino-nitrogen contents of silk after treatment for different lengths of time with diazomethane*

Duration of treatment	Total N	Amino N
hr	%	%
0 (untreated)	18.78	0.10
20	[a] 18.82	.10
50	[a] 18.81	.10

[a] Corrected for increase in weight due to the addition of methyl groups.

The methoxyl contents of the hydrolysates are shown in column 4 of table 1. As was expected, the values are lower than those obtained for the treated silk (column 3). The agreement of the hydrolysate values with the methoxyl values calculated from the decrease in tyrosine contents (column 5), however, leaves little likelihood that reaction (4) occurs to any appreciable extent, especially since complete methylation of the serine of silk (1.8 percent [13]) would yield 0.53 percent of methoxyl groups, or about 30 percent of the total found.

The liberation of methyl alcohol from methylated silk during hydrolysis was readily demonstrated by refluxing several different samples for 1 hour with 0.1 *N* NaOH, and then distilling about half the solution into a receiver maintained at 0° C (to avoid loss of CH_3OH). The methyl alcohol content of the distillate was determined colorimetrically by Beyer's method. The results, calculated as methoxyl, are shown in table 3.

TABLE 3.—*Effect of a 1-hour treatment with a boiling 0.1 N solution of sodium hydroxide on the methoxyl content of methylated silk*

Duration of diazomethane treatment	Total methoxyl content	Methoxyl liberated during hydrolysis as methyl alcohol	Residual methoxyl content of silk
hr	%	%	%
3	1.97	0.30	1.55
10	2.32	.50	1.85
50	2.62	.73	2.08

If it is tentatively assumed that the "extra" methoxyl in the treated silk is in the form of esterified carboxyl groups, the present results indicate that silk fibroin contains an appreciable quantity of dicarboxylic amino acids, though this seems not to have been reported in the literature. From the data in table 1, it is estimated, on the basis of the above assumption, that silk may contain as much as 0.26 millimole per gram of free acidic groups. Such an appreciable amount of acidic groups would be expected to considerably influence the acidic and basic properties of the silk, and it should be readily detectable in the titration curve of the fiber.[4]

During these studies it was observed that an appreciable quantity of alkali was liberated when the fibroin was washed with water subsequent to the diazomethane treatment. A possible explanation for this is that cations, held by the acidic groups of the fiber, are freed as

[4] The titration curve of silk is now being determined as part of a separate investigation in progress in this laboratory.

these groups react with diazomethane. Since the reaction was carried out in ethereal solution, the alkali was not removed from the fibers until they were washed with water. If such an explanation is correct, no alkali should be liberated from ash-free silk under similar conditions. Furthermore, the rate of reaction of ash-free silk with diazomethane might be very different than that of the untreated silk, since at least a portion of the acidic groups of the latter would already be combined with inorganic substances.

In order to test this explanation and its consequences, a sample of ash-free fibroin was prepared by electrodialysis and then treated with diazomethane for different lengths of time. It was found that no measurable quantity of alkali was liberated when the treated sample was washed with water. Furthermore, the rate of increase in methoxyl content of the electrodialyzed silk during the first hour of treatment was greater than that of the untreated fibers, as shown by the results plotted in figure 2. After one hour of treatment, no "extra"

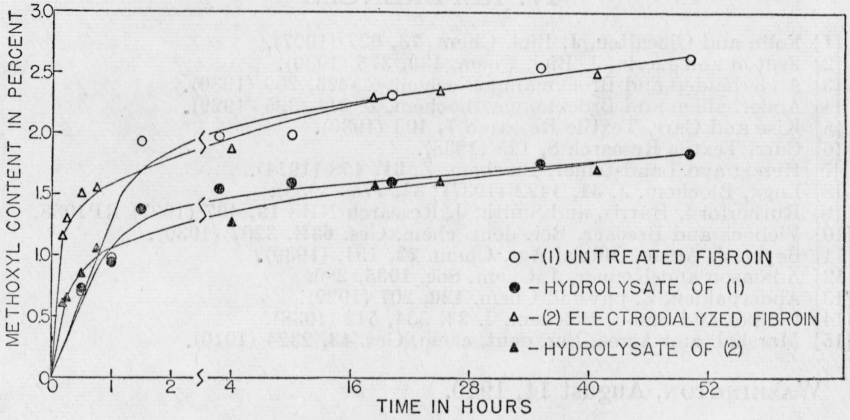


FIGURE 2.—*Rate of reaction of untreated and electrodialyzed fibroin with diazomethane.*

methoxyl was found in the latter, whereas treatment of the electrodialyzed fibers for the same length of time resulted in the formation of about 65 percent of the maximum "extra" methoxyl. After about 20 hours, there was no further increase in "extra" methoxyl content. As in the case of the untreated silk, the difference between the total and hydrolysate methoxyl contents of the electrodialyzed sample becomes approximately constant and equal to about 0.26 millimole per gram.

Although no further work on this phase of the investigation has been done, it appears from the rate studies that the specificity of diazomethane for the hydroxyl groups of tyrosine might be increased by appropriate pretreatment of the protein with some inorganic substance for the purpose of blocking the carboxyl groups.

2. REACTION OF METHYLATED SILK WITH NITROUS ACID

The methylated and untreated samples of silk were found to behave quite differently toward nitrous acid. The untreated silk assumed the usual dark-brown color, while the depth of color formed in the diazo-

methane-treated sample appeared to be dependent on the extent of methylation. A sample in which the tyrosine content had been reduced to a very low value was colored only slightly by nitrous acid.

Philpot and Small [14] have suggested that the color resulting from the reaction of pepsin with nitrous acid may be due to a nitrosation of the tyrosine, and that the first product of the reaction is an ortho-nitroso derivative. Assuming a similar reaction for silk, it appears that the methylation of the tyrosine prevents the formation of this derivative, either by decreasing the reactivity of the ortho position, or by preventing reaction of the nitrous acid with the free hydroxyl group to form a compound which subsequently rearranges to the ortho position. Regardless of which of the above mechanisms prevails, the difference in the behavior of methylated and untreated silks toward nitrous acid may be considered additional evidence that hydroxyl groups of the tyrosine in untreated silk are free.

IV. REFERENCES

[1] Folin and Ciocalteu, J. Biol. Chem. **73,** 627 (1927).
[2] Fruton and Lavin, J. Biol. Chem. **130,** 375 (1939).
[3] Abderhalden and Brockmann, Biochem. Z. **226,** 209 (1930).
[4] Abderhalden and Brockmann, Biochem. Z. **211,** 395 (1929).
[5] Kise and Carr, Textile Research **7,** 103 (1936).
[6] Carr, Textile Research **8,** 125 (1938).
[7] Herzig and Landsteiner, Biochem. Z. **61,** 458 (1914).
[8] Lugg, Biochem. J. **31,** 1422 (1937); **32,** 775 (1938).
[9] Rutherford, Harris, and Smith, J. Research NBS **19,** 467 (1937) RP1038.
[10] Vieböck and Brecker, Ber. deut. chem. Ges. **63B,** 3207 (1930).
[11] Beyer, J. Assoc. Official Agr. Chem. **22,** 151, (1939).
[12] Adamson and Kenner, J. Chem. Soc. **1935,** 286.
[13] Abderhalden, Z. physiol. Chem. **120,** 207 (1922).
[14] Philpot and Small, Biochem. J. **32,** 534, 542 (1938).
[15] Marshall and Acree, Ber. deut. chem. Ges. **43,** 2324 (1910).

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