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# THE REACTION OF SILK FIBROIN WITH OXIDIZING AGENTS

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The general effect of oxidizing agents on proteins is to oxidize side-chains and bring about main-chain degradation. During an investigation to determine the latter by studying the viscosity of solutions of oxidized silk fibroin, it was found that many agents rendered much of the protein insoluble in the usual solvents.

Although it is known that fibroin may be made insoluble by cross-linking agents such as formaldehyde[14]; 1:3-difluoro-4:6-dinitrobenzene[19] and bis-(4-fluoro-3-nitrophenyl)sulphone[21], only in a review by HOWITT[8] is it stated that fibroin may be rendered insoluble in cupriethylenediamine by light irradiation, or reaction with chemical reagents. No experimental details are given.

In this paper results are presented which show that fibroin may be insolubilized by reaction with simple oxidizing agents such as chlorine, potassium permanganate, chlorine dioxide and iodine. It is shown also that the solubility of fibroin is highly dependent on the state of the tyrosine residues, and oxidation or substitution in the benzene nucleus of this side-chain may render fibroin insoluble.

## EXPERIMENTAL

Raw silk (*Bombyx mori*) was freed from sericin and dirt by washing in warm soap solution, several changes of warm distilled water, and then standing in distilled water overnight. After removal of foreign matter by teasing, the dried silk was extracted with ethyl alcohol and then ether in a Soxhlet apparatus.

*Oxidation reactions*

The general procedure was to react 1 g of the purified fibroin with the reagent dissolved in 200 ml

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of water in a closed flask, the quantity of oxidant being calculated as a percentage by weight of the dry fibroin. Reactions were performed in the dark at 20° C, with occasional agitation. When reaction was complete, or a known quantity of oxidant had reacted, the fibroin was washed and dried at 95° C. Analytical determinations were made using standard volumetric methods. Reaction times varied widely but the majority of reactions were completed within 24 hours.

Details of the different reactants are as follows:

*Chlorine.* Reactions were performed either in 0.10 *N* $H_2SO_4$ of pH 1.4 or 0.05 *M* borax of pH 9.2. Chlorine was added as NaOCl solution, all quantities of chlorine being calculated as positive or available chlorine (1 litre *N* $Na_2S_2O_3$ = 35.5 g available chlorine). After reaction in acid solution, the fibroin liberated iodine from KI due to sorbed chlorine. The latter was determined by iodometric titration of the oxidized fibroin, which was then discarded and a repeat experiment performed, the active chlorine being removed from the fibroin by standing in dilute $NaHSO_3$ solution. This material was used for viscosity determinations. The fibroin did not sorb chlorine from alkaline solution.

*Potassium permanganate.* $KMnO_4$ was dissolved in either 0.1 *N* $H_2SO_4$ or 0.05 *M* borax. Sorbed $MnO_2$ was removed from the fibroin by standing in dilute acid $H_2O_2$ for a few minutes, followed by washing.

*Chlorine dioxide.* $ClO_2$ was freed from chlorine by passing through $AgNO_3$ solution prior to dissolving in water.

*Peracetic and permonosulphuric acids.* 10% w/v peracetic acid or $H_2SO_5$, prepared by allowing a mixture of 10 g $K_2S_2O_8$ and 18 g $H_2SO_4$ to stand in air for 4 hours, were diluted to the required concentrations with water.

*Iodine.* Fibroin (0.4 g) was iodinated with the following solutions:

(a) 0.06 g iodine in 10 ml conc. KI solution and 70 ml 0.05 *M* borax added.

(b) 0.10 g iodine in 10 ml KI solution and 70 ml 0.5 *N* $NH_4OH$ added, *i.e.* the conditions of MICHEL AND RIVERS[10].

(c) 0.10 g iodine in 8 ml KI solution, followed by the addition of 20 ml ethyl alcohol and 52 ml 0.05 *M* borax.

When the solutions were decolourized, sorbed iodine was removed from the fibroin with $Na_2S_2O_3$ solution.

*Determination of insoluble fraction and degree of degradation of soluble fraction*

Oxidized fibroin was extracted for 40 mins. with 90% aqueous formic acid (w/w) containing 10 g anhydrous $CaCl_2$ per 100 ml[5], the ratio of solvent to fibroin being 200:1 (v/w). The solution was filtered through a sintered glass crucible and the washed and dried residue determined. The relative viscosity of the fibroin solution was determined in an Ostwald No. 2 B.S.S. viscometer at 25° C, using the simple expression $\eta \text{ rel} = \frac{\text{Time of flow of solution}}{\text{Time of flow of solvent.}}$

Viscosity determinations were commenced within one hour of entry of the fibroin into the solvent.

The reduced viscosity of the solution was taken as a measure of the degree of degradation of the soluble fibroin.

$$\eta \text{ reduced} = \frac{\eta \text{ rel} - 1}{c} \quad \text{where } c = \text{concn. of fibroin in g/100 ml solution.}$$

Ideally, intrinsic viscosities $[\eta]$ should be used, *i.e.* $c \to 0$ to determine degradation, since $\eta$ red. is dependent somewhat on concentration. However, over the range of concentrations studied, which varied mainly from 0.25–0.5%, this effect was not serious and a value of 1.4 for $\eta$ red. may be taken for untreated fibroin.

*Infra-red spectroscopy*

Specimens were examined on a Grubb Parsons S3A double-beam spectrometer over the wavelength range 2–15 $\mu$, using the KBr pressed disc technique[3] for their preparation.

*X-Ray diffraction photographs*

The instrument used was a Newton Victor Raymax X-ray apparatus, using high intensity copper K$\alpha$ radiation from a rotating copper anode.

## RESULTS

### *The effect of oxidizing agents on the solubility of fibroin*

It is seen from Table I that considerable reaction occurred between fibroin and solutions of potassium permanganate, chlorine, chlorine dioxide and iodine. Apart from chlorine in acid solution, these reagents rendered from 50 to over 90% of the protein

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TABLE I

THE REACTION OF FIBROIN WITH CHLORINE, PERMANGANATE, CHLORINE DIOXIDE AND IODINE

| *Reagent* | *% reagent on weight of fibroin* | *% wt. loss of fibroin after reaction* | *Insoluble fraction %* | *$\eta$ red. of soluble fraction* |
|---|---|---|---|---|
| | 1.0 | 3.2 | 16.8 | 1.06 |
| | 2.0 | 3.2 | 21.8 | 1.10 |
| $KMnO_4$ | 4.0 | 3.7 | 31.4 | 0.94 |
| pH 1.4 | 10.0 | 4.1 | 42.5 | 0.90 |
| | 15.0 | 4.4 | 48.7 | 1.03 |
| | 25.0 | 6.9 | 46.5 | 0.89 |
| | 35.0 | 9.5 | 35.8 | 0.65 |
| | 1.0 | 2.9 | 32.0 | 1.45 |
| | 3.0 | 3.6 | 44.1 | 1.24 |
| $KMnO_4$ | 5.0 | 4.7 | 52.7 | 1.24 |
| pH 9.2 | 10.0 | 4.7 | 57.3 | 0.78 |
| | 15.0 | 6.1 | 63.4 | 0.50 |
| | 20.0 | 5.6 | 59.6 | 0.36 |
| | 30.0 | 11.5 | 49.7 | 0.28 |
| | 0.94 | 2.2 | 4.5 | 0.96 |
| | 2.4 | 2.7 | 5.7 | 1.00 |
| Chlorine | 4.5 | 2.6 | 5.7 | 0.87 |
| pH 1.4* | 6.1 | 2.9 | 7.5 | 0.61 |
| | 9.1 | 1.7 | 4.5 | 0.45 |
| | 11.5 | 2.5 | 5.8 | 0.32 |
| | 15.0 | 9.5 | 4.8 | 0.25 |
| | 0.96 | 1.5 | 19.0 | 1.54 |
| | 2.9 | 2.7 | 31.2 | 1.60 |
| Hypochlorite | 4.9 | 3.5 | 50.1 | 1.58 |
| pH 9.2 | 9.7 | 5.0 | 76.6 | 1.09 |
| | 14.5 | 7.7 | 88.5 | 0.71 |
| | 19.5 | 7.8 | 89.1 | 0.63 |
| | 29.3 | 11.8 | 93.3 | 0.21 |
| | 1.0 | 1.3 | 52.0 | 1.26 |
| | 3.0 | 0.5 | 92.0 | 0.78 |
| $ClO_2$ | 5.0 | 0.9 | 96.5 | 0.23 |
| | 10.0 | 0.3 | 91.5 | 0.08 |
| | 18.0 | 0.2 | 74.8 | 0.04 |
| Iodine | | | | |
| Soln. (a) | 15.0 | — | 90.2 | 0.24 |
| Soln. (b) | 25.0 | — | 53.8 | 0.52 |
| Soln. (c) | 25.0 | — | 90.0 | 1.25 |

* The quantity of chlorine reacted is given as that lost from solution less that sorbed by the fibroin.

insoluble in calcium chloride-formic acid solutions. Peracetic and permonosulphuric acids, however, reacted but little. After 24 hours only 1.5% peracetic acid reacted out of 100% originally present. Fibroin, however, is nearly devoid of amino acid residues which readily undergo oxidation by aliphatic peracids[18]. Although none of the fibroin was rendered insoluble, its reduced viscosity increased to 2.0. Under the same conditions 4.2% permonosulphuric acid reacted. The fibroin after reaction, however, gelled in the solvent and filtration was impossible, whereas after oxidation by the other reagents no difficulties in filtration were experienced.

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*Reactions with modified fibroin*

If the phenolic hydroxyl groups of the tyrosine residues in fibroin take part in cross-linking reactions, as suggested by HOWITT[8], blocking these groups should inhibit such reactions. Three methods were employed to block the hydroxyl groups of the tyrosine residues in fibroin, methylation with dimethylsulphate[7], or diazomethane[16] and formation of the dinitrobenzene ether using 2:4 dinitrofluorobenzene[20]. Methylation was complete since the fibroin failed to form a red nickel nitroso complex[13]. However, methylation with dimethylsulphate produced material of little mechanical strength, methylation with diazomethane caused no change in the mechanical properties of the fibroin but its reduced viscosity was only 60% that of untreated, whilst the dinitrobenzene ether of fibroin itself was 66% insoluble in the calcium chloride-formic acid solvent. This ether is insoluble also in 50% lithium bromide solution at 100° C[20].

Attempts to produce methylated fibroin without a fall in viscosity by modifying the reaction conditions with diazomethane were unsuccessful.

Although no inferences could be drawn regarding the solubility of methylated fibroin after oxidation, it was significant that methylated fibroin reacted more slowly with oxidizing agents. With permanganate in solution of pH 1.5, reaction rates were approximately three times slower than with untreated fibroin.

*The indicator effect of oxidized fibroin*

It has been observed previously that fibroin oxidized with solutions of permanganate behaves as an indicator[1], being yellow in alkaline solutions and becoming colourless when acid. The same effect was observed to varying degrees in the reactions studied here, with the exception of peracetic acid which produced no colour change. The effect was a minimum with permonosulphuric acid, the fibroin becoming only very pale yellow in alkaline solution, whereas after reaction with chlorine dioxide it became a deep rust colour in alkaline solution and then yellow-brown in acid solution.

*Effect of other solvents on the residues*

The residues from fibroin after oxidation with solutions of potassium permanganate, alkaline hypochlorite or chlorine dioxide, followed by extraction with calcium chloride in formic acid were insoluble after standing for 20 hours in 90% aqueous phosphoric acid or cupriethylenediamine reagent. Untreated fibroin dissolved in these solvents in less than 20 minutes.

*Infra-red and X-ray spectra*

The infra-red absorption tracings of fibroin oxidized by the different reagents were essentially the same as those of untreated fibroin. The X-ray diffraction pattern of the fibrous residue obtained after chlorine dioxide treated fibroin had been extracted showed a typical silk pattern, indicating that the crystalline regions of the fibroin had not been affected to a marked degree.

## DISCUSSION

It is shown in this work that solutions of hypochlorite, acid and alkaline permanganate, chlorine dioxide and iodine react readily with fibroin rendering it insoluble in the usual

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solvents. Chlorine in acid solution reacts but does not produce insolubility, whereas peracetic and permonosulphuric acids react but little. Although fibroin is not insolubilized by the two latter reagents, its viscosity is appreciably increased. Over 90% of fibroin may be rendered insoluble by oxidation with suitable reagents. Viscosity measurements show that, in general, the soluble fraction is degraded, and after maximum insolubility has been attained further oxidation produces a decrease in the insoluble fraction and progressive degradation of the soluble fibroin.

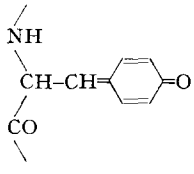
The reagents studied which reacted to an appreciable degree with fibroin are all powerful oxidizing agents with the exception of iodine. It is known, however, that iodine readily substitutes in the phenolic groups of the tyrosine residues of proteins[11]. Thyroxine has been isolated also from hydrolysates of iodinated fibroin[10]. Although the mechanism of their formation is obscure, it seems highly probable that thyroxyl side-chains are present in iodinated fibroin prior to hydrolysis, since RIVERS[15] has isolated N-acetylthyroxylglutamic acid by incubating N-acetyldiiodotyrosylglutamic acid under physiological conditions.

It has been shown that when hydrophobic groups such as 2:4-dinitrobenzene are incorporated into fibroin it becomes insoluble in the usual solvents. In a similar manner the insolubility of iodinated fibroin could be due to the introduction of iodotyrosyl and thyroxyl groups into the structure in place of the more hydrophilic tyrosine residues.

The other reagents which produced insolubility of fibroin are all known to react readily with tyrosine residues in proteins (1. 2). Chlorine dioxide is specific for oxidizing tyrosine residues in fibroin[17], and since this is the most effective agent for producing insolubility, the solubility of fibroin must be highly dependent on the state of these residues.

Further evidence has been obtained which shows the important part played by tyrosine residues in the reactions studied. The X-ray diffraction pattern of fibroin insolubilized with chlorine dioxide differs little from ordinary fibroin. It is known that the regions containing tyrosine have little or no influence on this pattern in the case of the silk fibre (6.9). Further, modification of the tyrosine side-chains by methylation reduced the rate of oxidation of fibroin to a marked degree.

Little is known concerning the reaction products when tyrosine residues in proteins are oxidized. It has been assumed from the indicator behaviour of oxidized proteins that the tyrosine is oxidized to a quinone structure (1.4), but there is no direct evidence that oxidized tyrosine residues can be represented by structures such as I.



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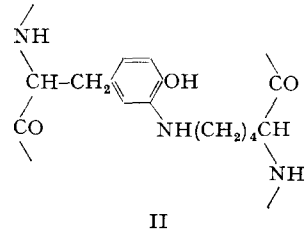

II

Although this work shows the important influence tyrosine residues may have on the solubility of fibroin, it was not possible to determine whether the oxidized residues themselves are the important factor, or if they are able to cross-link adjacent protein

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chains. Such a cross-link could be produced by reaction between a quinone and a free amino group (II). HOWITT, on the other hand, has tentatively suggested[8] that ether-type cross-links could be formed by the elimination of water from hydroxyl groups of tyrosine residues of adjacent main chains. A preliminary examination of the infra-red absorption spectrum of the insoluble fibroin failed to show the presence of possible cross-links.

The failure of chlorine in acid solution to render fibroin insoluble is due most probably to the fact that it can cause general oxidation of amino acids[2] and effect main-chain degradation of the protein.

The reduction in the viscosity of solutions of fibroin methylated with diazomethane using the procedure of RUTHERFORD *et al.*[16], is of interest since these workers found no appreciable decrease in the tensile strength of the fibres after reaction. Although the simplest explanation for the reduction in viscosity is that diazomethane produces main-chain degradation, it is possible that replacement of the hydroxyl groups of the tyrosine residues by methoxy groups inhibits intra-chain hydrogen bonding, which reduces the rigidity of the fibroin chains, thus permitting coiling. There is some evidence that such a mechanism produces the variation in viscosity of cellulose acetates of differing acetyl contents[12].

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SUMMARY

After reaction with solutions of hypochlorite, acid and alkaline permanganate, chlorine dioxide or iodine, silk fibroin becomes insoluble in the usual solvents. Chlorine in acid solution reacts but does not render fibroin insoluble.

It is shown that the solubility of fibroin is highly dependent on the state of the tyrosine residues and that fibroin may be rendered insoluble when the residues undergo oxidation or substitution reactions.

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