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Interaction of Biebrich Scarlet with Keratoses from Performic Acid-Oxidized Wool

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Synopsis

The interaction of the acid dye Biebrich Scarlet (C.I. Acid Red 66) with α-, β-, and γ-keratoses obtained from wool fiber by performic acid oxidation was studied. The extrapolated saturation values of dye bound to protein and the association constants for the interactions were obtained. Maximum dye uptake was found for α-keratose, followed by γ- and β-keratoses, in that order. Incorporation of varying amounts of *m*-cresol in the dye solutions showed no significant effect. There was no indication of an interaction between *m*-cresol and the keratoses from direct studies.

INTRODUCTION

It has been observed that the rate of uptake and equilibrium exhaustion of acid dyes by wool fibers are markedly reduced by the presence of certain lower phenols, especially *m*-cresol, at low concentrations in the dyebaths.[1] A study of the effect of *m*-cresol on the interaction between the enzyme α-chymotrypsin and Biebrich Scarlet (C.I. Acid Red 66) has been made.[2] It was found that *m*-cresol displaces the dye from the 1:1 complex with the enzyme through allosteric competition. It was, therefore, thought that a similar study on soluble proteins from wool can provide an understanding of the effect of *m*-cresol on wool–dye interactions. The present work has been undertaken to study the interactions between keratoses (obtained from wool by oxidation) and the acid dye Biebrich Scarlet and to see if *m*-cresol has an effect on these interactions.

Both reduction and oxidation methods have been developed to obtain soluble protein fractions from wool fiber. A recent review[3] deals with these developments. The protein fractions obtained by reduction are called kerateines, and those obtained by oxidation are termed keratoses. Oxidation procedures employ peracids to attack the disulfide bonds and form cysteic acid residues on complete and irreversible oxidation.[4–6] Performic acid has been proved to completely oxidize wool this way.[5,6] Complete oxidation yields three keratoses, α, β, and γ. These have different physical and chemical characteristics. α-Keratose, which is precipitated from an

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alkaline solution of oxidized wool on acidification, is the low-sulfur fraction. γ-Keratose, which remains in solution at this stage, is the high-sulfur fraction, and β-keratose is the fraction of oxidized wool that is not soluble in alkali. The α-keratose fraction, representing about 60% of the fiber, contains 1.9% sulfur.[7] The γ-keratose fraction represents nearly 30% of the fiber and contains 5.8% sulfur. The rest, 10% of the fiber, corresponds to β-keratose, which contains 2.1% sulfur.[8,9]

In order to minimize possible coulombic effects, the present investigations on the interactions of the keratoses with the dye were carried out at the respective isoionic points of these proteins. The isoionic points were determined using a method developed by Lemin and Vickerstaff[10] and later used by Da Silva.[11] Normal methods of equilibration and filtration, followed by spectrophotometric estimation of the dye concentration, were used in the case of α- and β-keratoses. However, this could not be done in the case of γ-keratose, for it was soluble at its isoionic point. The spectrophotometric method, used in the study of interaction between enzymes and dye,[2,12] could not be used with γ-keratose as no change in the spectral character of the dye was observed. Therefore, equilibrium dialysis was used in this case.

EXPERIMENTAL

Materials

Prescoured Merino wool yarn was purified by extraction with petroleum ether and ethanol. Then it was thoroughly rinsed in deionized water and finally air dried.

Performic acid reagent for oxidation of wool was prepared according to the method of Tonnies and Hoemiller.[13] One volume of 30% hydrogen peroxide was added to 9 volumes of 98% formic acid at room temperature and allowed to stand for 1 hr.

Chromatographically pure Biebrich Scarlet was obtained from Messrs. B.D.H. Ltd. It was made free from electrolytes and other impurities by dissolving the dye in distilled water and salting out with sodium acetate five times. The final precipitate was washed repeatedly with hot aqueous ethanol to remove sodium acetate. Microanalysis of the pure sample showed that the dye was 98% pure, most of the impurity being sodium acetate. At its wavelength of maximum absorption, 505 mμ, the molar extinction coefficient of the dye was 3.360×10^4.

Laboratory reagent-grade *m*-cresol was used. All experimental solutions were prepared in deionized water.

Methods

Preparation of Keratoses

The method of preparation used was substantially the same as that used by earlier workers.[14] Oxidation was allowed to proceed at 0°C for 16 hr.

The oxidized wool sample was stirred with 1.5*N* ammonia and left for 30 hr. The precipitated β-keratose was removed by filtration and the filtrate was acidified to pH 4. The solution was left to stand for 24 hr and filtered. The residue was redissolved in ammonia and reprecipitated, and the α-Keratose obtained was dried under vacuum. The filtrate was dialyzed against several changes of deionized water. Then it was concentrated by evaporation under vacuum at 30°C. A large excess of ethanol was then added to this solution, and the precipitated γ-keratose was separated by centrifugation at 15000 rpm for 1 hr in a refrigerated centrifuge and dried under vacuum.

Determination of Isoionic Points

The method[10,11] is based on a measurement of pH values of a series of acid and alkaline solutions in equilibrium with the protein, in the absence and presence of a small amount of a neutral salt. The variation of pH on addition of salt is plotted as a function of the equilibrium pH value before addition of salt. The isoionic point is that equilibrium pH value which corresponds to zero change in pH on addition of salt.

A solution of known pH value, 5 ml, was taken in each case, and 25 mg of protein was added. The pH value of the solution was noted at intervals till it reached a steady value, which was taken as the equilibrium pH. Generally, the time taken for this equilibrium was 2 to 5 hr. Then an amount of sodium chloride was added to make the solution 0.1*M* in salt. The pH value of the solution was again noted till a steady value was attained. This also took 2 to 5 hr usually. The change in pH due to salt was noted. Initial solutions were so chosen as to represent pH values on either side of the expected isoionic point.

Equilibrium Dialysis Method

Visking tubing (36/32) 45 mm in diameter was treated with boiling deionized water several times before use. The tubing was dried at 50°C and cut into lengths weighing 0.4 g. γ-Keratose solution (0.2%) was prepared in a buffer of disodium hydrogen orthophosphate and citric acid. Dye solutions were also prepared in the same buffer. Five milliliters of the protein solution was pipetted into the tube, one end of which was tied tightly with a cellulose string. After the solution was transferred into the bag, the other end of the bag was also tied with the string, leaving a little empty space inside the bag. It was established after trials that these bags were completely leakproof and that protein did not leak out into the external solution. These bags were then kept in 15 ml of dye solutions of known concentration and equilibrated with constant agitation for 48 hr at 25°C. This period was found to be enough for the equilibrium to be attained. After equilibration, the external dye concentration was estimated spectrophotometrically. The solution inside the bag was also diluted as required and the dye concentration estimated using corresponding protein

solution as the blank. The increase in the amount of dye present in the bag and the decrease of dye concentration in the external solution were obtained by comparison with a blank experiment in which pure buffer solution was taken in the Visking bag and equilibrated against the same dye solution. This allowed a correction for the dilution effect as well as for the amount of dye taken up by the cellulose material of the tubing. From the changes in dye concentration due to the presence of the protein, the dye–protein interaction isotherm was calculated. Duplicate experiments were carried out in all cases.

When the effect of *m*-cresol was investigated, *m*-cresol was added to the dye solution and the above procedure was followed. The final protein–dye interaction parameters were compared with those obtained when pure dye solutions of the same concentration were used without *m*-cresol. It was established initially that the presence of *m*-cresol did not cause any change in the spectral character of the dye solutions at the range of concentrations of *m*-cresol used in these experiments.

In an experiment to examine the interaction between *m*-cresol and γ-keratose, *m*-cresol solutions in buffer alone were used as the external solution in place of dye solutions and equilibrated with γ-keratose solution in the bag. In this case, *m*-cresol was estimated spectrophotometrically at its wavelength of maximum absorption, 271 mμ.

Filtration Method

Experiments with α- and β-keratoses were simpler, since they were insoluble at their respective isoionic points. In each case, 25 mg of the protein sample was equilibrated for 48 hr at 25°C with 15 ml of dye solutions of various concentrations in buffers of disodium hydrogen orthophosphate and citric acid. After the equilibration period, the solutions were filtered

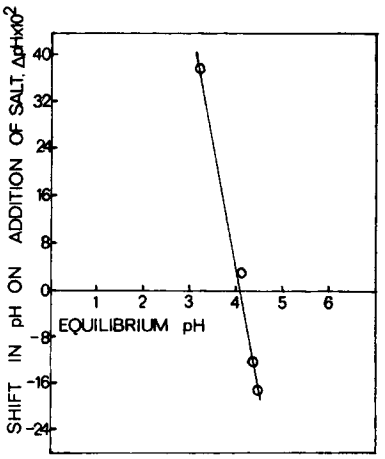

Fig. 1. Relationship between the shift in pH on addition of salt and the pH value of solutions in equilibrium with α-keratose.

through G3 funnels. The filtrate was diluted as required and the dye concentration measured spectrophotometrically. The dye–protein interaction isotherms were calculated from the exhaustion data.

As in the experiments with γ-keratose, here, too, when the effect of *m*-cresol was investigated, the dye solutions containing different amounts of *m*-cresol were used, and the results were compared with those obtained when pure dye solutions of the same dye concentrations were used. Experiments were also carried out with pure *m*-cresol solutions to study directly the interaction between these keratoses and *m*-cresol. The same buffers giving the isoionic pH of the proteins were used. *m*-Cresol concentrations were determined by spectrophotometry. All spectrophotometric estimations of dye concentrations were made on a Unicam SP 600 Series 2 spectrophotometer, and the estimations of *m*-cresol concentrations were carried out on a Hilger-Uvispec UV spectrophotometer.

RESULTS AND DISCUSSION

Isoionic Points

Figure 1 shows typically the variation of shift in pH, caused by addition of salt, plotted against the equilibrium pH of solutions in contact with α-keratose. The straight line intersects the equilibrium pH axis at a pH value of 4.05. This, therefore, is the isoionic point of α-keratose. Similar plots were made for γ- and β-keratoses, and the isoionic points obtained in this way are 2.25 and 6.6 for γ- and β-keratoses, respectively. β-Keratose was found to have the highest isoionic point, the same as that reported for the wool fiber.[11] This is consistent with the fact that the amino acid composition of β-keratose is similar to that of wool.[15] γ-Keratose is very acidic and its isoionic point is close to the pH of minimum solubility, 2.9, reported for the S-carboxymethyl derivative of the high-sulfur fraction of wool.[16] In the only other study of this kind on γ-keratose, it has been reported that γ-keratose was negatively charged at a pH of 4.0, indicating an isoionic point below pH 4.0.[17] This is in conformity with the present results. The higher isoionic point found for α-keratose agrees well with the pH of minimum solubility of 4.4, reported for the S-carboxymethyl derivative of low-sulfur fraction of wool.[3]

Keratose–Dye Interaction

The isotherm for the interaction of α-keratose with Biebrich Scarlet is shown in Figure 2. Here the amount of dye bound per kg of α-keratose has been plotted against the equilibrium concentration of the dye in solution. Similar plots were obtained for β- and γ-keratoses and are shown in Figures 3 and 4.

The above data have been subjected to a Langmuir type relation,

$$\frac{1}{D_k} = \frac{e^{\Delta\mu^0/RT}}{D_{ks}D_s} + \frac{1}{D_{ks}} \tag{1}$$

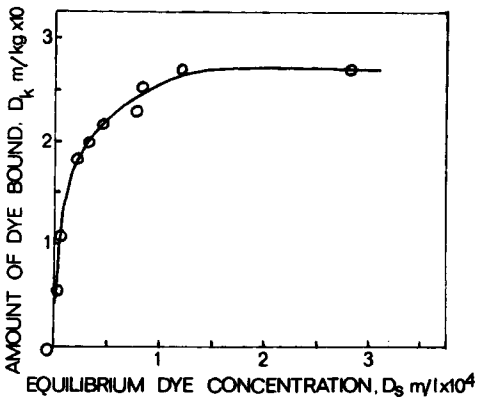

Fig. 2. Isotherm for the interaction of Biebrich Scarlet with α-keratose.

where D_k is the amount of dye bound to the protein in moles per kg of the protein, D_{ks} is the saturation value of dye bound to protein in moles per kg of protein, D_s is the concentration of equilibrium dye solution in moles per liter, $\Delta\mu^0$ represents the standard free-energy change for the interaction, and e, R and T have the usual significance.

The term $e^{\Delta\mu^0/RT}$ is in fact the equilibrium constant and therefore the reciprocal of the association constant K_a given in the Klotz equation.[18] Substituting $1/K_a$ in place of $e^{\Delta\mu^0/RT}$ in eq. (1) gives

$$\frac{1}{D_k} = \frac{1}{D_{ks}D_sK_a} + \frac{1}{D_{ks}}. \tag{2}$$

Hence, values of $1/D_k$ were plotted against $1/D_s$ values in all cases. Linear regression gave correlation coefficients of not less than 0.996 in all cases. Values of the intercept and slope were taken from the linear regression analysis. From these values, the saturation value D_{ks} and the association constant K_a were calculated for the three keratoses. These results are given in Table I.

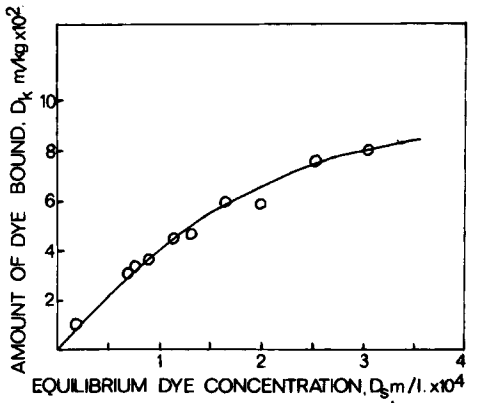

Fig. 3. Isotherm for the interaction of Biebrich Scarlet with β-keratose.

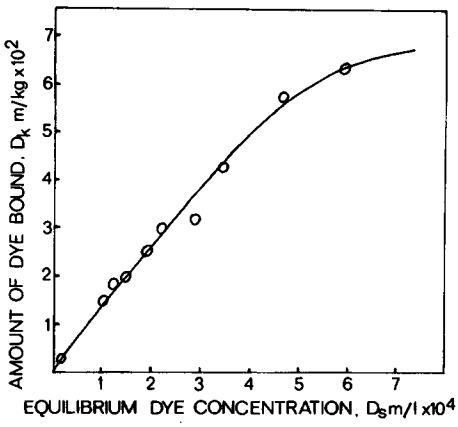

Fig. 4. Isotherm for the interaction of Biebrich Scarlet with γ-keratose.

Clearly, α-keratose is the fraction that shows maximum dye binding. The saturation value for α-keratose is double that for β-keratose and substantially more than that for γ-keratose. The association constant for the dye with α-keratose is also higher than for the interactions of the dye with β- and γ-keratoses. β-Keratose shows a higher association constant than γ-keratose. Since all these experiments were carried out at the respective isoionic points of the keratoses, the effect of charge is eliminated to a large extent. The trends observed are, therefore, a reflection of the affinities of the keratoses toward the dye. α-Keratose, thus, has a larger affinity to the dye, compared to β- and γ-keratoses. It is interesting to examine these observations in the light of the origin of these keratoses in the wool fiber. Considerable amount of indirect evidence[19-21] and some direct evidence[22] has been given to support the view that α-keratose comes mainly from the microfibrils and γ-keratose from the matrix of the wool fiber. One is inclined to the idea that the behavior of α keratose might reflect that of the microfibrils, while γ-keratose might behave like the matrix. Microfibrils have been shown to have highly organized structures approaching crystallinity,[23] while the matrix has been described as consisting of "pseudoglobular" units,[24] but with very little evidence of the structural organization obtained.[3] The matrix is generally described as

TABLE I
Extrapolated Saturation Bound Dye Values and Association Constants for Biebrich Scarlet on α-, β-, and γ-Keratoses at 25°C

Keratose	Saturation value of bound dye D_{ks}, moles/kg of protein	Dye association constant K_a
α-Keratose	0.229	2.052×10^5
γ-Keratose	0.161	1.086×10^3
β-Keratose	0.109	5.812×10^3

amorphous. There has been some x-ray evidence to show that most of the sorbed water in the wool fiber is in the matrix,[25] and this is consistent with an amorphous character. It is, therefore, reasonable to expect that, in wool–dye interactions, the dye molecules would mainly be associated with the matrix proteins. Furthermore, in native state in the wool fiber, the high-sulfur proteins in matrix are strongly basic, while the low-sulfur proteins of the microfibrils are predominantly acidic.[26] From the above considerations, it would be expected that the bulk of dye–protein interactions in wool fiber would be in the matrix.

The results of the present study are in direct contrast to this situation. Maximum uptake of the dye was found for the low-sulfur fraction, α-keratose. The high sulfur fraction, γ-keratose, interacts with the dye to a significantly smaller extent and is more like β-keratose, the cell wall protein fraction.[27,28] It is difficult, of course, to try and draw parallels between behavior of the proteins in the native state in the fiber and the oxidized derivatives. The oxidation process has clearly disrupted the physical structure of the fiber, thereby eliminating the mutual effects of the structural units. The ordered substructure of the microfibrils might also be disturbed. One very important factor would be the influence of hydrophobic environment, which is considerably reduced. All these might alter the way in which the individual proteins interact with the dye molecules.

Effect of *m*-Cresol

The present studies failed to detect any effect of the presence of *m*-cresol on the dye–keratose interactions. Dye solutions of the same dye concentration but varying concentrations of *m*-cresol all gave the same amounts of dye bound to the protein within the limits of experimental error. The concentration range of *m*-cresol used was from 5×10^{-4} to 1.4×10^{-2} moles/l., corresponding to 7.5×10^{-6} to 2.15×10^{-4} moles of *m*-cresol in equilibrium with 0.01 g of γ-keratose or 0.025 g of α- or β-keratose. These weights of keratoses would correspond to 5×10^{-7} moles of γ-keratose or 1.25×10^{-6} moles of α- or β-keratose, if the molecular weight of 20,000 is assumed in all cases.[17,29,30] The present ratios of *m*-cresol to protein covered the range, over which considerable influence of *m*-cresol was reported on the α-chymotrypsin–Briebrich Scarlet interaction.[2]

The attempts to investigate directly the interaction between *m*-cresol and keratoses failed to detect any. The concentration ratios of *m*-cresol to keratose were the same as described earlier. No detectable difference was found between initial and equilibrium concentrations of cresol.

The absence of any interaction between keratoses and *m*-cresol is not difficult to understand. *m*-Cresol is able to interact with wool and proteins such as α-chymotrypsin leading to competitive inhibition of dye–protein interaction. This is possible because the structures of wool fiber and α-chymotrypsin are conducive to extensive hydrophobic bonding, and the interaction of phenols with these is probably one of hydrophobic bonding.[2,12,31] In the case of keratoses, however, the complex physical structure

of the wool fiber is absent. Furthermore, numerous hydrophilic groups (sulfonic acid groups) have been introduced in the keratoses, reducing the probability of extensive hydrophobic interactions in the molecular chain. These keratoses do not have the specific hydrophobic sites as in the case of α-chymotrypsin.[12] In view of the above considerations, it is very likely that the extent of hydrophobic environment in the keratoses would be small. This could explain the lack of any effect of *m*-cresol on the interaction with the dye as well as the lack of interaction between *m*-cresol and the keratoses.

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