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Fluorescent Whitening Agents. II.* Covalent Binding to Proteins during Irradiation by Sunlight

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Abstract

Simulated solar irradiation of wool, silk, bovine serum albumin and apomyoglobin in the presence of radioactively labelled fluorescent whitening agents of the bistriazinylaminostilbene and pyrazoline types results in the irreversible binding of radioactivity to the protein. This photochemical reaction occurs both in the solid state and in aqueous solution, and is believed to result in the formation of a covalent bond between the whitener (or a photodecomposition product) and the protein.

Introduction

Fluorescent whitening agents are essentially colourless fluorescent dyes used to improve the whiteness of textiles. In the course of a study of the photodecomposition of a radiolabelled fluorescent whitening agent of the bistriazinylaminostilbene type (1), we observed that radioactive material became irreversibly bound to wool during irradiation with simulated sunlight (Milligan and Holt 1974). This binding, apparently covalent, between the whitener (or a photodecomposition product) and the wool protein is of interest, not only because fluorescent whitening agents sensitize the photoyellowing of wool, but also because of the biological implications of such a photochemical reaction between whiteners and proteins. Indeed, it has been claimed that, under certain conditions, fluorescent whitening agents are photocarcinogenic (Bingham and Falk 1970).

We have now examined another type of fluorescent whitening agent, viz. the pyrazoline (2), and other proteins, to determine whether the photochemical reaction of whiteners with proteins is a general phenomenon. The bistriazinylaminostilbene (1) was applied to both wool and silk and the pyrazoline (2) to wool for photochemical study in the solid state, while solution studies with these whiteners were carried out using bovine serum albumin and apomyoglobin. The photochemical reaction of (1) with polyamide and cellulose fibres was also examined.

Experimental

Materials

The wool fabric was woven from solvent-scoured 64s Merino wool; it weighed 130 g/m^2. The silk fabric (47 g/m^2) was an unweighted commercial sample as supplied by the Sericultural Research Station, Tokyo. The nylon-4,4 fabric (104 g/m^2), from Fibremakers Ltd., Victoria, was scoured in Alkanate D and terpolyphosphate solution before use. Bovine serum albumin (Cohen fraction V, batch 001) was obtained from Commonwealth Serum Laboratories, Melbourne. Sperm whale

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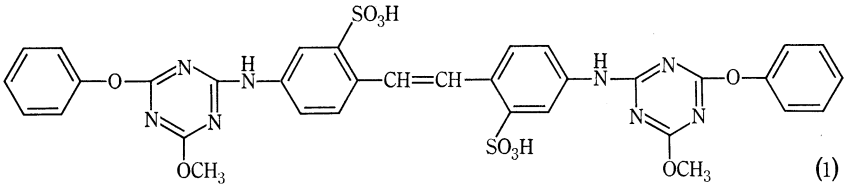
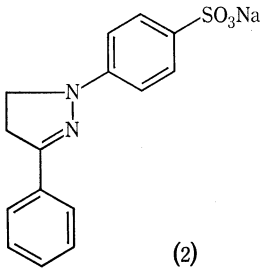
myoglobin (lot 4270-1) was purchased from Pierce Chemical Co., U.S.A., and was converted to apomyoglobin by the method of Teale (1959).



(1)



(2)

The synthesis of the collidine salt of bis-4,4′-(4″-methoxy[^{14}C]-6″-phenoxy-*s*-triazin-2″-ylamino)stilbene-2,2′-disulphonic acid is described elsewhere (Milligan and Holt 1974). Thin-layer chromatography of a 1% solution in 0·2N ammonia on silica gel (Eastman Chromagram sheet 6060), using n-hexanol–pyridine–ethyl acetate–conc. ammonia–methanol (5 : 5 : 5 : 5 : 3 v/v) as eluant, revealed one main fluorescent spot, the *trans*-isomer of (1), which contained 95·5% of the radioactivity loaded, and four minor spots. One of the latter was identified as the *cis*-isomer of (1), containing a further 1·5% of the total radioactivity. The specific activity of (1) was 51 μCi/mmol.

Sodium 4-(3′-phenyl-[5-^{14}C]pyrazolin-1′-yl)benzenesulphonate (2) was prepared from [^{14}C]formaldehyde via ω-dimethylamino-[ω-^{14}C]propiophenone hydrochloride (Maxwell 1955). Condensation of equimolar amounts of the latter, sodium hydroxide and sodium 4-hydrazinobenzenesulphonate in boiling 50% aqueous ethanol for 2 h yielded the radioactively labelled fluorescent whitening agent, which crystallized as yellow plates (70% yield) from 50% aqueous ethanol (Found: C, 50·3; H, 4·7; N, 7·7. $C_{15}H_{13}N_2NaO_3S,2H_2O$ requires C, 50·0; H, 4·7; N, 7·8%). Thin-layer chromatography under the conditions described above revealed a single fluorescent component, which contained more than 99% of the radioactivity loaded onto the chromatogram. Its specific activity was 31 μCi/mmol.

Irradiations

Solutions of fluorescent whitening agents and/or proteins, in water, 0·05M ammonia or 0·05M ammonium acetate (adjusted to pH 4), were irradiated in a 20-ml cell surrounding a jacketed 100-W Hanovia medium-pressure mercury lamp. Water at 20°C was circulated through the Pyrex jacket surrounding the lamp; only wavelengths of >295 nm were transmitted. Generally, nitrogen was passed through the solutions for 15 min prior to irradiation and during irradiation. Wool, silk, nylon and cellulose samples, either in the dry or wet state, were placed 46 cm below an air-cooled Philips HOKI 2000-W mercury arc lamp fitted with a Corning glass filter 7740, so that only wavelengths of >295 nm were transmitted.

Application and Removal of Fluorescent Whitening Agents

Wool, silk and nylon-4,4 fabrics were treated with the ^{14}C-labelled stilbene (1) (2·5% on the weight of fabric) in aqueous solution (pH 3·4, 80°C) with intermittent stirring. The adsorption of (1) by the fabrics was followed by radioassay of aliquots withdrawn from the solutions. After 1·5 h, wool, silk and nylon had adsorbed 86, 85 and 70% respectively of (1). The ^{14}C-labelled pyrazoline (2) was applied to wool in the same way. Using 0·25 and 2·5% on the weight of wool, the percentages adsorbed after 1·5 h at 80°C were 90 and 86% respectively.

Cellulose (Whatman filter paper 541) was treated with (1) by soaking in a 2% solution in 2M ammonia and allowing to dry. The pyrazoline (2) was applied as a 2% solution in aqueous ethanol (1 : 1).

Fluorescent whitening agents were separated from wool, silk, nylon and paper (0·1-g samples) by five successive extractions, each of 5 min, with boiling 25% pyridine (5 ml). The residual radioactivity was determined by combusting a weighed amount of sample in a Schöniger oxygen flask. Hyamine solution was added to absorb the products of combustion; then a toluene-based scintillation mixture was added and the resultant solution radioassayed using a Packard Tri-Carb liquid scintillation spectrometer. The scintillation mixture was prepared by dissolving 1,4-bis(5-phenyloxazol-2-yl)benzene (0·05 g) and 2,5-diphenyloxazole (4·0 g) in toluene (1 litre).

Analytical Techniques

Fluorescent whitening agents were separated from bovine serum albumin or apomyoglobin by gel filtration on a column (50 cm by 1·0 cm diam.) packed with Sephadex G50 (superfine) resin equilibrated with aqueous pyridine (25%). Pyridine (0·5 ml) was added to the aqueous protein–whitener solution (1·5 ml) before loading and the column was subsequently eluted with 25% aqueous pyridine. Fractions were collected over 12-min periods, the flow rate of the column being 12 ml/h. Aliquots of effluent (0·2–0·5 ml) were added directly to a dioxan-based scintillation mixture for radioassay. The scintillation mixture was prepared by dissolving 1,4-bis(5-phenyloxazol-2-yl)benzene (0·05 g), 2,5-diphenyloxazole (4·0 g) and naphthalene (120 g) in dioxan (1 litre). Other aliquots (0·1–0·2 ml) were assayed for protein content by a modification of the Folin–Ciocalteau method (Bailey 1967); it was necessary to omit copper sulphate from the reagent to avoid the formation of precipitates.

Results and Discussion

Most fluorescent whitening agents for wool can be regarded as acid dyes, which bind to the protein by a combination of electrovalent, hydrophobic, hydrogen bond and van der Waals interactions. These are disrupted by boiling aqueous pyridine, and therefore it is not surprising that this solvent removes virtually all of the radioactivity from wool that has been fluorescently whitened with radiolabelled (1) or (2) (see Table 1). However, if these fluorescently whitened wool fabrics are first exposed to simulated

Table 1. Photochemical reaction in the solid state between radiolabelled fluorescent whitening agents and various fibres

About 2% whitening agent on the weight of substrate was used in each case. Values are percentage of applied radioactivity remaining after pyridine extraction (see Methods)

Whitening agent	Conditions	Wool	Silk	Nylon-4,4	Cellulose
Bistriazinylamino-stilbene (1)	Not irradiated	2	1	2	0
	Irradiated wet[A]	31	62	7	—
	Irradiated dry[B]	19	29	22	10
Pyrazoline (2)	Not irradiated	0	—	—	0
	Irradiated wet[A]	15	—	—	—
	Irradiated dry[B]	13	—	—	6

[A] Each side was exposed to simulated summer sunlight for 8 h.
[B] Each side was exposed to simulated summer sunlight for 24 h.

sunlight, either in the dry or the wet state, then a substantial proportion of the radioactive material present resists extraction with boiling aqueous pyridine. This behaviour parallels that of wool that has been dyed with reactive dyes. These dyes are linked to

wool not only by the interactions listed above, but also by covalent bonds, and resist extraction by aqueous pyridine and similar solvents (Cockett *et al.* 1969; Beech 1970). This suggests that the fluorescent whitening agents (1) and (2), or their photodegradation products, are bound covalently to wool during irradiation. Other solvents used for the detection of covalently bound dye on wool, viz. an aqueous solution containing formic acid and pyridine (Cockett *et al.* 1969) or aqueous urea containing a nonionic detergent (Christoe and Datyner 1970), failed to remove any more radioactivity from the irradiated whitened wool than did aqueous pyridine.

The fluorescent whitening agent (1) also appears to bind covalently to silk, nylon and cellulose during irradiation with simulated sunlight. Whereas virtually all of the radiolabelled whiteners can be extracted before irradiation, significant amounts of radioactive material resist extraction after irradiation (see Table 1).

The photochemical reaction of fluorescent whitening agents with proteins occurs not only in the solid state, but also in aqueous solution, as shown by experiments using bovine serum albumin (BSA) and the bistriazinylaminostilbene (1). Fig. 1*c* shows that the two components of the mixture, before irradiation, were completely separable by gel filtration on Sephadex G50 resin, provided 25% pyridine was used as solvent. Both (1) and (2) give much sharper bands when subjected to gel filtration in 25% pyridine than in water, presumably due to disaggregation by the former solvent. Folin–Ciocalteau reagent was used to determine the position of the protein peak and the fluorescent whitening agent was detected by radioassay. Complete separation of the protein and the whitener was obtained, regardless of whether gel filtration was carried out immediately or after the mixture had been stored for several days.

After irradiation of the solution of BSA and (1) in an atmosphere of nitrogen, gel filtration revealed two peaks of radioactivity (Fig. 1*b*). The new peak of radioactivity coincided with the protein peak, which is good evidence for the covalent binding of (1) to BSA. However, it could be argued that irradiation of (1) produces polymeric photodegradation products which are of similar size to the protein, or which bind strongly to the protein by electrostatic, hydrophobic, hydrogen bond and van der Waals interactions, because this would also lead to a peak of radioactivity coincident with the protein peak. This explanation seems to be unlikely in view of the result shown in Fig. 1*a*. In this experiment a solution of (1) was irradiated alone and BSA was then added. Although (1) gave rise to a little material of higher molecular weight in this case, virtually all of the radioactive material was separable from the BSA by gel filtration. In another control experiment, radiolabelled (1) was added to an irradiated solution of BSA; again, gel filtration completely separated radioactive material from protein. Thus, only when the labelled fluorescent whitening agent and the protein are irradiated together is radioactive material bound to the protein. The binding was independent of pH within the range studied (pH 4–9) and was unaffected by the presence of air.

The radioactively labelled pyrazoline (2) behaved similarly to (1), being separable from BSA by gel filtration before irradiation. However, after irradiation of the mixture the protein peak became radioactively labelled (Fig. 2*b*). The apparent occurrence of a second protein peak in these experiments is due to the fact that the pyrazoline gives a weak colour reaction with Folin–Ciocalteau reagent. As may be seen in Fig. 2*a*, gel filtration of an irradiated solution of (2), to which BSA had been added subsequently, gave quite separate protein and radioactivity peaks, indicating

Fig. 1

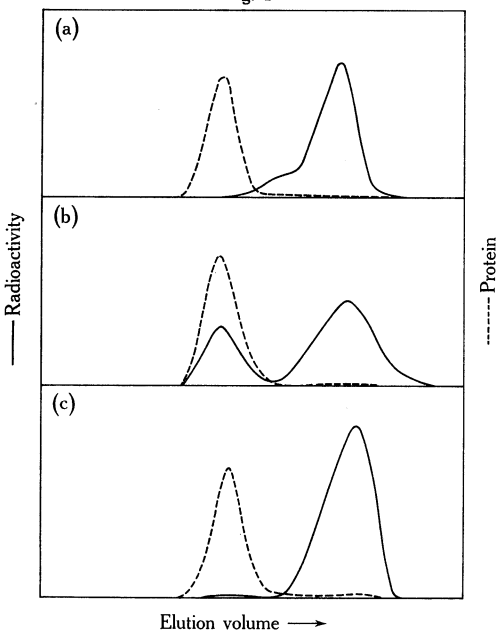


Fig. 2

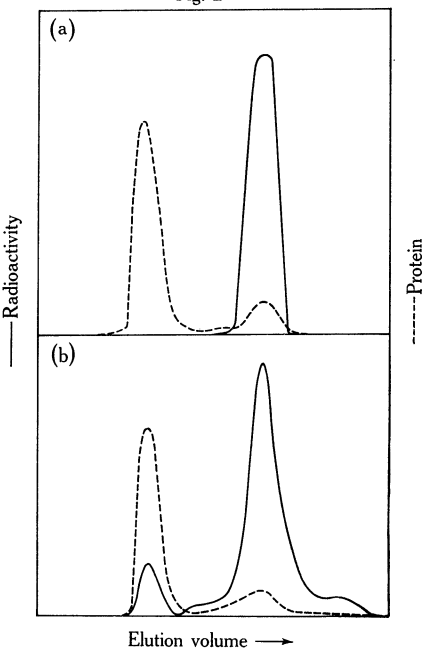


Fig. 1. Elution patterns obtained by gel filtration of mixtures of ^{14}C-labelled bistriazinylaminostilbene (1) (0·48 mM) and BSA (0·13 mM), using 25% pyridine as eluant. (*a*) BSA + irradiated (1) (90 min); (*b*) irradiated mixture (90 min); (*c*) unirradiated mixture.

Fig. 2. Elution patterns obtained by gel filtration of mixtures of ^{14}C-labelled pyrazoline (2) (0·86 mM) and BSA (0·13 mM), using 25% pyridine as eluant. (*a*) BSA + irradiated (2) (90 min); (*b*) irradiated mixture (90 min).

that no interaction had occurred between the irradiated fluorescent whitening agent and the protein.

Further evidence to support the claim that fluorescent whitening agents (or their photodecomposition products) become covalently bound to BSA during irradiation was obtained from exchange and electrodialysis experiments. In the former type of experiment the radioactively labelled protein derivatives, isolated from irradiation mixtures by gel filtration, were treated with a large excess of unlabelled fluorescent whitening agent in dissociating solvents such as anhydrous formic acid and 1N ammonia–pyridine (3 : 7). They were then subjected again to gel filtration. The sole peak of radioactivity still coincided with the protein, demonstrating that no exchange of the radioactive label from the protein derivative to the fluorescent whitening agent had occurred. Electrodialysis of the radioactively labelled BSA derivative also failed to remove any radioactive material from the protein. These experiments undoubtedly demonstrate that the bonds introduced between BSA and (1) or (2) (or their photodecomposition products) by irradiation are very strong, providing good, although circumstantial, evidence for their covalent nature.

The extents of binding of (1) and (2) to BSA under various conditions are shown in Table 2. As might be expected, the extent of reaction of the fluorescent whitening agent with the protein is greatest when a large molar excess of whitener to protein is

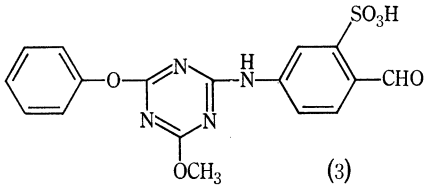
used. In general, most reaction occurs in the first half-hour of irradiation, 1–2 mol of whitener (or photodegradation product) per mole of protein being bound.* By comparison, (1) reacts much less readily with apomyoglobin, only 0·05 mol/mol of protein being bound under conditions where BSA binds 1·2 mol/mol.

Table 2. Photochemical reaction in aqueous solution between radiolabelled fluorescent whitening agents and bovine serum albumin

Irradiation time (h)	Protein conc. (mM)	Extent of reaction[A] between whitener and protein (mol/mol): (1)[B]	(2)[B]
0·5	0·013	1·7	1·1
0·5	0·130	0·8	0·1
1·5	0·013	1·1	1·5
1·5	0·130	1·2	0·7
3·0	0·130	1·3	—[C]

[A] Based on the proportion of radioactivity bound to the protein.
[B] Initial concentrations of whiteners: bistriazinylaminostilbene (1), 0·48 mM; pyrazoline (2), 0·86 mM.
[C] Precipitation occurred after irradiation for more than 1·5 h.

The protein derivatives isolated by gel filtration of irradiated solutions of BSA and either (1) or (2) were examined spectroscopically to determine the way in which binding occurs. Much of the fluorescent whitener seems to react with the protein in such a way as to disrupt its original chromophoric system. Although the u.v. spectra of the protein derivatives have similar absorption peaks to those of the original whiteners, optical density measurements show that no more than 5% of the radioactive material bound to the protein can be present in a form retaining the original chromophoric system. This is consistent with the observation that the protein derivatives were only weakly fluorescent. In the case of the bistriazinylaminostilbene (1), this marked decrease in absorptivity could occur as the result of photochemical addition of a variety of groups in the protein to the olefinic bond of the whitener. Alternatively, photochemical binding to the protein may involve reaction with a photodecomposition product of (1), rather than with the whitener itself. Indeed, the aldehyde (3), which has been identified as a photoproduct of (1) (Milligan and Holt 1974), binds irreversibly



(3)

* The amount of whitener bound to the protein at the lower concentration (0·013 mM) was <5% of the total whitener present in all cases. Consequently, the experimental error of these determinations is much higher than that of those using the higher protein concentration. This may account for the observation that more (1) was bound to BSA after 0·5 h than after 1·5 h at the lower protein concentration.

to wool during irradiation with simulated sunlight, but this does not necessarily mean that (1) binds to proteins via (3). Binding of the pyrazoline (2) could occur via photochemical addition of protein side-chains to the corresponding pyrazole, as pyrazoles are known to arise from photo-oxidation of pyrazolines (Schrader 1971).

The photochemical reaction of fluorescent whitening agents with proteins probably occurs at many different sites. There is evidence that, as the result of an energy migration, the free radicals produced by u.v. irradiation of proteins are distributed among virtually all of the residues present (Miller *et al.* 1971). Each of these radicals may then undergo addition to (1), which would lead to many different products. No interaction occurs when solutions of (1) are irradiated in the presence of various amino acids or their derivatives, e.g. glycine, *N*-acetyltryptophan, polylysine. This suggests that the reaction between whiteners and proteins or other substrates (nylon and cellulose) only occurs during irradiation if the two molecules are first held in intimate contact, e.g. by cooperative electrovalent, hydrophobic and hydrogen bond interactions.

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