Aust. J. Chem., 1977, **30**, 2277–87

Fluorescent Whitening Agents. IX* The Effect of Proteins on the Photodecomposition of Pyrazolines

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Abstract

Ultraviolet irradiation of the diaryl-2-pyrazoline (1) in water gives the corresponding pyrazole (3), the 4-hydroxypyrazole (4) and several unidentified products. Irradiation of the tetraaryl-2-pyrazoline (2) gives the pyrazole (6) as the major product. The rates of photodegradation are increased if lysozyme is present and the corresponding pyrazoles are formed preferentially. Pyrazoles are also the major products when wool containing (1) or (2) is irradiated. Both pyrazolines photosensitize the degradation of lysozyme, the tryptophan, histidine and methionine residues being the main sites of attack.

Introduction

The usefulness of fluorescent whitening agents for whitening wool is limited by the poor light stability of the resultant wool.[1,2] This behaviour is due not only to photodegradation of the whitener but also to yellowing of the wool fibre itself.[3,4] Yellowing is accompanied by photodecomposition of tryptophan and histidine residues[3,5,6] and by the uptake of oxygen.[7] Fluorescent whiteners promote both photoyellowing and the decomposition of tryptophan and histidine residues.[3,5]

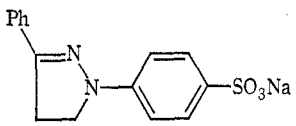
There has been much speculation about the way in which fluorescent whiteners sensitize wool photodegradation and about the relative importance of whitener destruction and fibre degradation.[4,5,8] In the present study we have used the soluble protein, lysozyme, as a model for wool in order to study the effect of the protein on whitener photodecomposition; the effect of the whitener on protein photodecomposition has also been examined.

The pyrazolines, (1) and (2), which are the least and most photostable of a series of fluorescent whitening agents used in a previous study,[4] were chosen for this work. Irradiations were carried out both in solution and in lysozyme films. The behaviour

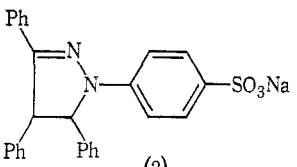
* Part VIII, *Text. Res. J.*, 1976, **46**, 539.

[1] Graham, D. R., and Statham, K. W., *J. Soc. Dyers Colour.*, 1956, **72**, 434.
[2] Fallaw, D. C., and Tucker, D. J., *Text. J. Aust.*, 1965, **40**, 46.
[3] Leaver, I. H., and Ramsay, G. C., *Photochem. Photobiol.*, 1969, **9**, 531.
[4] Evans, N. A., Rivett, D. E., and Waters, P. J., *Text. Res. J.*, 1976, **46**, 214.
[5] Holt, L. A., Milligan, B., and Wolfram, L. J., *Text. Res. J.*, 1974, **44**, 846.
[6] Holt, L. A., Milligan, B., and Savige, W. E., *J. Text. Inst.*, 1977, **68**, 124.
[7] Nicholls, C. H., and Pailthorpe, M. T., *J. Text. Inst.*, 1976, **67**, 397.
[8] Lennox, F. G., King, M. G., Leaver, I. H., Ramsay, G. C., and Savige, W. E., *Appl. Polym. Symp.*, 1971, No. 18, 353.

of these systems was then compared with that of wool that had been whitened with the same two pyrazolines.



(1)



(2)

The photodecomposition of (1) has been the subject of several papers from this laboratory.[4,9–11] In these studies the course and extent of photodecomposition was determined spectroscopically, whereas in the present work the whiteners were radiolabelled to facilitate separation of the photoproducts and quantitative determination of their yields by radioassay.

Experimental

General

Melting points were uncorrected. Microanalyses were carried out by the Australian Microanalytical Service, Melbourne. Infrared spectra were obtained on a Perkin–Elmer 257 spectrophotometer, ultraviolet spectra were obtained on a Perkin–Elmer 124 spectrophotometer and corrected fluorescence spectra were obtained on a Perkin–Elmer MPF-3L fluorescence spectrophotometer fitted with an R-446 photomultiplier. Fluorescence quantum yields were calculated as described previously.[4]

Materials

The lysozyme used was purchased from Sigma Chemical Co. (lot 75C-8483).

Sodium 4-(3′-Phenyl-2′-pyrazolin-1′-yl)benzenesulphonate (1)

The 5′-^{14}C-labelled compound, of radiochemical purity >99%, was prepared as previously described.[12] The ^{35}S-labelled compound was prepared by sulphonation[4] of 1,3-diphenyl-2-pyrazoline with chlorosulphonic[^{35}S] acid in chlorobenzene and purified by crystallization from aqueous ethanol. Its spectral and chromatographic properties were identical to those of the 5′-^{14}C-labelled compound. Its radiochemical purity was shown to exceed 99·5% by radioassay in conjunction with chromatography on Sephadex LH-20, 50% aqueous methanol being used as eluent.

Sodium 4-(3′,4′,5′-Triphenyl-2′-pyrazolin-1′-yl)benzenesulphonate (2)

The ^{35}S-labelled compound was prepared by sulphonation of 1,3,4,5-tetraphenyl-2-pyrazoline with chlorosulphonic[^{35}S] acid as described for the unlabelled compound,[4] and converted into the sodium salt which was crystallized from benzene–light petroleum. Aqueous solvents could not be used because the compound separated as a gel. Chromatography as above revealed the presence of two radioactive impurities, which amounted to approximately 5% of the total radioactive material. They could not be removed by repeated crystallization.

Sodium 4-(3′-Phenylpyrazol-1′-yl)benzenesulphonate (3)

A stirred mixture of sodium 4-(3′-phenyl-2′-pyrazolin-1′-yl)benzenesulphonate (171 mg, 0·5 mmol) and *o*-chloranil (246 mg, 1 mmol) in dioxan (40 ml) and water (10 ml) was refluxed for 1 h, cooled and the solution evaporated to dryness under reduced pressure. The residue was triturated

[9] Evans, N. A., Rivett, D. E., and Wilshire, J. F. K., *Aust. J. Chem.*, 1974, **27**, 2267.
[10] Langley, K. F., *Aust. J. Chem.*, 1975, **28**, 1301.
[11] Leaver, I. H., Ramsay, G. C., and Stephens, L. J., *Aust. J. Chem.*, 1975, **28**, 2083.
[12] Holt, L. A., and Milligan, B., *Aust. J. Biol. Sci.*, 1974, **27**, 23.

with dioxan and then crystallized from water to yield sodium 4-(3′-phenylpyrazol-1′-yl)benzenesulphonate (3) (110 mg, 66%) which was dried under vacuum at 60° (Found: C, 54·4; H, 3·6; N, 8·3; S, 9·6. Calc. for $C_{15}H_{11}N_2NaO_3S,\frac{1}{2}H_2O$: C, 54·4; H, 3·7; N, 8·5; S, 9·7%). λ_{max} (water): 292 nm (ε 28900). Fluorescence emission maximum (water): 337 nm (quantum yield 0·95). When excited at 254 nm, this compound had a weak blue fluorescence, whereas the sample obtained by Leaver *et al.*[11] had a yellow fluorescence. It seems likely that the sample of Leaver *et al.*, isolated from an irradiated solution of the pyrazoline (1), was contaminated with a strongly yellow-fluorescent photoproduct of (1) which we believe to be the 4-hydroxypyrazole (4).

Sodium 4-(5′-Hydroxy-3′-phenylpyrazol-1′-yl)benzenesulphonate (5)

4-Hydrazinobenzenesulphonic acid (1·0 g, 5·3 mmol) was added to a solution of ethyl benzoylacetate (1·0 g, 5·2 mmol) in aqueous ethanol (50% v/v, 20 ml) containing a drop of 6 M sulphuric acid, and the mixture was then heated under reflux for 30 min. The solution was cooled, the crystalline product collected and recrystallized from water to yield 4-(5′-hydroxy-3′-phenylpyrazol-1′-yl)benzenesulphonic acid (0·8 g, 46%), m.p. 340° (dec.) (Found: C, 54·0; H, 4·3; N, 8·5; S, 9·8. Calc. for $C_{15}H_{12}N_2O_4S,H_2O$: C, 53·9; H, 4·2; N, 8·4; S, 9·6%). Menzel and Püschel,[13] using a slightly different procedure, obtained a product of m.p. 318°. The sodium salt (5) was prepared by neutralization of the free acid with sodium hydroxide solution followed by lyophilization.

Sodium 4-(3′,4′,5′-Triphenylpyrazol-1′-yl)benzenesulphonate (6)

A stirred mixture of sodium 4-(3′,4′,5′-triphenyl-2′-pyrazolin-1′-yl)benzenesulphonate (494 mg, 1 mmol) and *o*-chloranil (492 mg, 2 mmol) in dioxan (40 ml) and water (10 ml) was heated under reflux overnight, cooled and evaporated to dryness under reduced pressure. The residue was triturated with ethanol and then crystallized from aqueous ethanol to yield sodium 4-(3′,4′,5′-triphenylpyrazol-1′-yl)benzenesulphonate (6) (318 mg, 64%) which was dried under vacuum at 60° (Found: C, 65·8; H, 4·6; N, 5·4; S, 6·5. Calc. for $C_{27}H_{19}N_2NaO_3S,H_2O$: C, 65·8; H, 4·3; N, 5·7; S, 6·5%). λ_{max} (water): 274 nm (ε 18500). Both this compound and the parent unsulphonated pyrazole were found to be non-fluorescent.

Wool Whitening

A plain weave, light weight Merino wool fabric was bleached with hydrogen peroxide as described previously[4] and then fluorescently whitened by treatment with the ^{35}S-labelled whitener (1) or (2) (0·25% on the weight of wool) in 0·005 M acetic acid at 80° for 1 h. Radioassay showed that approximately 80% of each pyrazoline had been adsorbed by the wool.

Irradiations

Solutions (1 cm path length) and films were irradiated with an air-cooled Philips HOKI 2000-W mercury arc lamp fitted with glass filters which transmitted less than 2% of the radiation of wavelength below 320 nm. The pyrazolines [(1) and (2), 5×10^{-5} M] were irradiated in water which in some cases contained phosphate buffer (0·02 M) or lysozyme (1 mg/ml). Films were prepared by spreading aliquots (1·0 ml) of a solution of lysozyme (160 mg) and the ^{35}S-labelled whitener (1) or (2) (4 mg) in 50% aqueous ethanol (10 ml) on microscope slides (76 by 25 mm) and allowing the solvent to evaporate.

Fluorescently whitened wool fabrics were irradiated for 16 h on each surface. Other samples were immersed in water during irradiation (2 h each surface).

Separation and Identification of Photoproducts

(i) *Chromatography*

Irradiated solutions containing pyrazoline and lysozyme were evaporated to dryness and the residue dissolved in 25% (v/v) aqueous pyridine (1 ml), to which acetic acid (0·2 ml) was then added. The protein was then separated from the unchanged pyrazoline and its low-molecular-weight photo-

[13] Menzel, K. H., and Püschel, W., *Mitt. Forschungslab. AGFA–Gevaert AG Leverkusen–München*, 1964, **4**, 376 (*Chem. Abstr.*, 1966, **64**, 3734).

products by chromatography on a column (400 mm by 8 mm diameter) packed with Sephadex G25 (superfine) in pyridine/acetic acid/water (10 : 7 : 23, by volume). Forty fractions, each 1·0 ml, were collected. The pyrazolines and their photoproducts were detected by radioassay, and the protein was detected either by precipitation with trichloroacetic acid or by the purple colour developed with Fischl reagent.[14] Irradiated films were dissolved in 25% (v/v) aqueous pyridine (1·0 ml) to which acetic acid was then added. These solutions were chromatographed as described above.

Fractions containing pyrazolines and their photoproducts were combined, evaporated to dryness and then dissolved in 50% (v/v) aqueous methanol for chromatography on Sephadex LH-20. The column was eluted with the same solvent, 80 fractions (each 4·0 ml) being collected. Unchanged pyrazolines and their photoproducts were detected by radioassay and identified (where possible) by comparison of their ultraviolet spectra and electrophoretic behaviour with those of authentic samples. Paper electrophoresis was carried out at pH 2·3 by using a potential gradient of 30 V/cm.

Samples of wool which had been whitened with (1) or (2) and then irradiated were extracted twice with 25% (v/v) aqueous pyridine at 100° for 5 min. The combined extracts were evaporated and the residues chromatographed on Sephadex LH-20 as described above.

The extent of irreversible binding of radioactive material to lysozyme was determined by radioassay of the appropriate fractions after chromatography of the irradiated mixtures on Sephadex G-25. The extent of binding to wool was determined by radioassay of the fabric after six successive extractions with 25% (v/v) aqueous pyridine at 100°.

(ii) *Reverse Isotope Dilution Analysis*

This technique was used to analyse irradiated solutions of ^{14}C-labelled and ^{35}S-labelled (1) for the 5-hydroxypyrazole (5), and irradiated solutions of ^{35}S-labelled (1) and (2) for sodium sulphate.

The 5-hydroxypyrazole (5) (100 mg) was crystallized from water (10 ml) containing an aliquot (2·0 ml) of an irradiated solution of ^{14}C-labelled (1) (5×10^{-5} M). Two more recrystallizations from water were required to ensure constant specific activity. The amount of (5) in the irradiated solution was calculated from the activities of the pyrazoline (1) and the recovered 5-hydroxypyrazole (5).

Anhydrous sodium sulphate (100 mg) was added to aliquots (2·0 ml) of irradiated solutions of the ^{35}S-labelled whiteners (1) and (2), and then recovered by precipitation with ethanol (3 ml). Three cycles of dissolution in water followed by precipitation with ethanol yielded sodium sulphate of constant specific activity. The yields were calculated as described above.

(iii) *Photochemical Synthesis of the 1,3-Diarylpyrazole (3) and the 1,3-Diaryl-4-hydroxypyrazole (4)*

The lithium salt of 4-(3′-phenyl-2′-pyrazolin-1′-yl)benzenesulphonic acid was prepared by passing a 50% aqueous ethanolic solution of the sodium salt through Zeocarb 225 (acid form), neutralization of the effluent with lithium hydroxide solution and lyophilization. The lithium salt was used in large-scale irradiations because of its greater solubility in water. A solution of this salt (300 mg) and ^{35}S-labelled (1) (1 mg) in water (80 ml) was irradiated with a medium-pressure mercury arc lamp; a sodium vanadate solution was used to filter out radiation of wavelengths below 320 nm. The solution was irradiated until its absorbance at 360 nm fell to 25% of the initial value. It was then evaporated to dryness under reduced pressure and the residue subjected to gel permeation chromatography on Sephadex LH-20 in aqueous methanol (50% v/v); the elution of products was followed by radioassay. A similar separation to that from small-scale irradiations (see Fig. 1, p. 2282) was obtained. The fractions corresponding to photoproduct D were combined and evaporated to dryness to yield a colourless solid (49 mg), which was converted into the sodium salt by chromatography on Zeocarb 225 (acid form) followed by neutralization with sodium hydroxide solution and evaporation under reduced pressure. The resulting solid was identified as sodium 4-(3′-phenylpyrazol-1′-yl)benzenesulphonate (3) by comparison (infrared and ultraviolet spectra) with the authentic sample (see above). The yellow fluorescent product (peak E) (40 mg) could not be recrystallized and was characterized as follows: it was dissolved in 10% sodium carbonate solution (5 ml), Pauly solution (5 ml, prepared by mixing equal volumes of 1% sulphanilamide and 5% sodium nitrite solutions and allowing to stand 5 min at room temperature) was added and the mixture stirred for 1 h at room temperature. The yellow solid (55 mg) was collected, crystallized from water and dried at 70° under vacuum (Found:

[14] Opienska-Blauth, J., Charezinski, M., and Berbec, H., *Anal. Biochem.*, 1963, **6**, 69.

C, 46·3; H, 3·4; N, 12·9. Calc. for $C_{21}H_{16}N_5NaO_6S_2,H_2O$: C, 46·7; H, 3·4; N, 13·0%). Its infrared spectrum was quite different from that of the azo dye prepared by treating the 5-hydroxypyrazole (5) with Pauly solution.

(iv) *Amino Acid Analyses*

Irradiated protein samples were hydrolysed with 4 M methanesulphonic acid containing tryptamine,[15] and analysed on a Beckman–Spinco amino acid analyser.

(v) *Analysis of Tryptic Peptides of Lysozyme*

Lysozyme (100 mg) was reduced and carboxymethylated,[16] and then incubated at 38° with a solution of TPCK-trypsin (Worthington, 2 mg) in a solution containing ammonia (0·1 M) and 2-amino-2-hydroxymethylpropane-1,3-diol (0·05 M) for 2 h. The digest was chromatographed at 50° on a column of Technicon Chromobeads (type C) by using a buffer gradient formed by mixing 25% (v/v) pyridine and 25% (v/v) acetic acid with an LKB Ultrograd gradient mixer (the volume fraction of 25% pyridine in the eluent is shown in Fig. 3, p. 2285). The tryptophan-containing peptides in the collected fractions (7·5 ml) were located colorimetrically by treating evaporated aliquots (2·0 ml) with Fischl reagent (total volume 1·5 ml).[14]

The appropriate fractions were examined by paper electrophoresis at pH 1·9, a potential gradient of 50 V/cm being used. The tryptophan-containing peptides were located by dipping the paper into a solution of 4-dimethylaminobenzaldehyde (2%) and 10 M hydrochloric acid (20%) in acetone. Some of the resultant pink and purple colours, which developed within 5 min, could be intensified by exposure of the paper to nitrous fumes. The identity of the peptides was established by the following procedure: (*1*) extraction from appropriate areas of a paper electrophoretogram; (*2*) hydrolysis (6 M hydrochloric acid, 110°, 24 h); (*3*) amino acid analysis; (*4*) comparison of amino acid contents with structures of the tryptic peptides of lysozyme[17] (see Fig. 3).

This procedure was also carried out with lysozyme that had been irradiated for 16 h in either water or 5×10^{-5} M ^{35}S-labelled pyrazoline (2).

Reversible Binding of Pyrazolines to Lysozyme

The extent of binding of the pyrazolines to lysozyme was determined by dialysing the protein (10 mg) in water (10 ml) against a 5×10^{-5} M solution (20 ml) of ^{14}C-labelled (1) or ^{35}S-labelled (2) in water at 20° until no further change in pyrazoline concentration occurred. Samples were then taken from inside and outside the dialysis bag for radioassay.

Results

Pyrazoline Photodegradation

Irradiation of the diarylpyrazoline (1) in water causes spectral changes characterized by isosbestic points at 226, 241 and 304 nm. These values do not agree well with those published previously by Leaver *et al.*,[11] viz. 228, 255 and 312 nm. Much better agreement was obtained when we irradiated (1) in phosphate buffer (pH 7·0), and Leaver (personal communication) has since confirmed that his spectral studies[11] were carried out in aqueous solution buffered at pH 7 and not in water.

Chromatography showed that a different distribution of photoproducts resulted when (1) was irradiated in buffered solution (pH 7) rather than in water. Irradiation of ^{14}C-labelled (1) in water at $\lambda > 320$ nm resulted in at least four products (peaks A, B, D and E) in addition to unchanged (1) (peak C); all four products were present in similar amounts (see Fig. 1*a*). In contrast, peak D was the major product when

[15] Simpson, R. J., Neuberger, M. R., and Liu, T. Y., *J. Biol. Chem.*, 1976, **251**, 1936.
[16] Weil, L., and Seibles, T. S., *Arch. Biochem. Biophys.*, 1961, **95**, 470.
[17] Canfield, R. E., *J. Biol. Chem.*, 1963, **238**, 2691.

^{14}C-labelled (1) was irradiated for the same time in aqueous solution buffered at pH 7, peaks A, B and E being quite small.

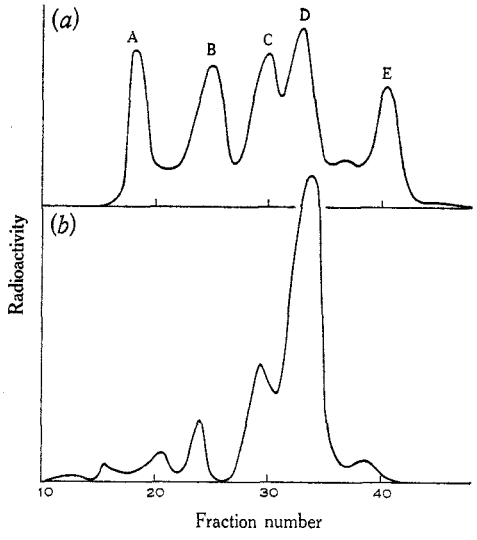
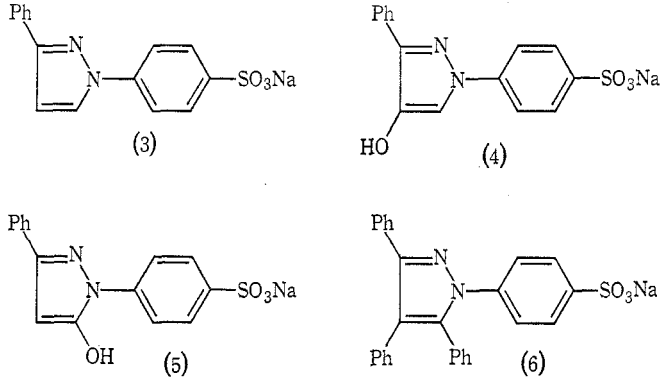


Fig. 1. Chromatograms of the ^{14}C-labelled whitener (1) after irradiation (3 h) at a concentration of 5×10^{-5} M in water in the (*a*) absence of lysozyme; (*b*) presence of lysozyme (1 mg/ml). The irradiated solutions were concentrated and chromatographed on Sephadex LH-20 with aqueous methanol (50% v/v) as eluent.

Peak D was shown to contain the pyrazole (3) by comparison of its ultraviolet spectrum and its chromatographic properties with those of an authentic sample. Also, the infrared spectrum of a sample obtained from a large-scale irradiation was shown to be identical with that of the authentic sample. The material in peak E gave a yellow fluorescence upon excitation at 254 nm. Its colour reaction with diazotized sulphanilamide and its ultraviolet and proton magnetic resonance spectra are consistent with its formulation as (4) or (5). Although E could not be isolated in pure form due to its high solubility in water, the azo dye prepared by reaction with diazotized



sulphanilamide gave elementary analyses consistent with the expected molecular formula. An authentic sample of the 5-hydroxypyrazole (5) was prepared and found to differ in its spectral properties and chromatographic behaviour from the material in peak E. Thus, by inference, we assign the structure (4), a 4-hydroxypyrazole, to peak E. Reverse isotope dilution analysis of the irradiated solution of ^{14}C-labelled (1) showed that some 5-hydroxypyrazole (5) was present, but only as a minor photoproduct (1–2%). We were unable to identify the products in peaks A and B. The

Table 1. Photodegradation of the radiolabelled pyrazoline (1) during irradiation in the absence and presence of proteins

Yields and radioactivity bound to protein as percentages

Conditions	Label in (1)	Time (h)	Pyrazoline recovered	Pyrazole formed	4-Hydroxypyrazole formed	Radioactivity bound to protein
			No proteins			
Water	^{14}C	1	65	7	9	—
Water	^{14}C	3	19	24	15	—
Water[A]	^{14}C	3	13	71	7	—
Water	^{35}S	3	19	25	17	—
Water	^{14}C	15	0	27	19	—
			Lysozyme			
Water	^{14}C	1	49	42	0	2
Water	^{14}C	3	14	72	0	5
Water	^{35}S	3	7	64	0	5
Water	^{14}C	16	0	89	0	7
Film	^{14}C	3	80	16	0	2
Film	^{14}C	16	37	39	0	9
			Wool			
Dry fabric	^{35}S	16[B]	16	62	0	5
Wet fabric	^{35}S	2[B]	4	81	0	8

[A] Aqueous buffer (pH 7·0). [B] Each surface.

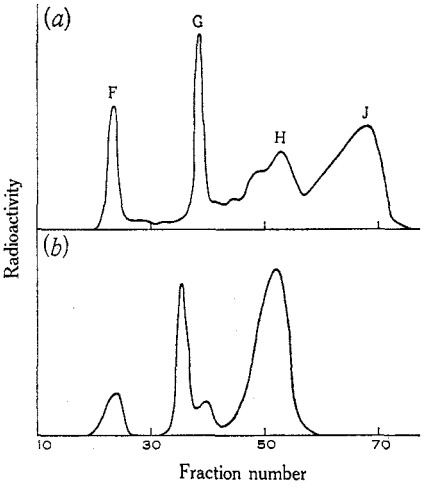


Fig. 2. Chromatograms of the ^{35}S-labelled whitener (2) after irradiation (8 h) in water in the (*a*) absence of lysozyme; (*b*) presence of lysozyme. (See Fig. 1 for details.)

Table 2. Photodegradation of ^{35}S-labelled pyrazoline (2) during irradiation in the absence and presence of proteins

Protein	Conditions	Time (h)	Pyrazoline recovered (%)	Pyrazole formed (%)	Bisulphate formed (%)	Radioactivity (%) bound to protein
—	solution	8	43	20	10	—
—	solution	16	17	42	7	—
Lysozyme	solution	8	0	60	8	6
Lysozyme	solution	16	0	55	8	8
Wool	dry fabric	16[A]	50	22	5	6
Wool	wet fabric	2[A]	65	26	1	4

[A] Each surface.

amounts of starting material recovered and of the pyrazole (3) and 4-hydroxypyrazole (4) produced after irradiating (1) under various conditions are shown in Table 1.

Chromatography of an irradiated solution of ^{35}S-labelled (1) revealed the presence of an additional photoproduct which eluted between peaks A and B. Since it is not detected in irradiated solutions of the ^{14}C-labelled compound, its formation must involve C–N or C–S fission. Reverse isotope dilution analysis with sodium sulphate indicated that some sulphate (or, more likely, bisulphate) was also formed (3–4% yield). Sodium 4-hydroxybenzenesulphonate may also be present but it could not be positively identified as a photoproduct of the ^{35}S-labelled whitener by reverse isotope dilution analysis due to the difficulty in separating it from (1) and other photoproducts.

Our results confirm that the tetraarylpyrazoline (2) is more stable to light than the diarylpyrazoline (1), as previously reported.[4] However, if ^{35}S-labelled (2) was irradiated in water for 8 h, at least three photoproducts (peaks F, G and H, Fig. 2*a*) were formed. Reverse isotope dilution analysis with sodium sulphate showed that peak F consisted almost entirely of sodium sulphate or (more likely) sodium bisulphate. Peak H was identified as the tetraarylpyrazole (6) by comparison of its spectral and chromatographic properties with those of an authentic sample. Peak G was not identified and peak J comprised unchanged starting material. Table 2 shows the amounts of pyrazoline recovered and of pyrazole and bisulphate produced after irradiation of (2) under various conditions.

The Influence of Proteins on Pyrazoline Photodegradation

Addition of lysozyme to an aqueous solution of the pyrazoline (1) increases the extent of photodegradation of the whitener, and selectively increases the yield of the pyrazole (3) (see Fig. 1*b* and Table 1). These effects are even more marked for the tetraarylpyrazoline (2) than for the diarylpyrazoline (1), possibly because more (2) than (1) is bound to the protein; equilibrium dialysis experiments show that approximately 30% of (1) and 90% of (2) are bound (reversibly) to the protein under the conditions used for irradiation. The selective increase in pyrazole yield is not due to an increase of the pH of the solution, even though the latter has the same effect. Irradiation of lysozyme films containing (1) also yields the corresponding pyrazole (3) as a major product (see Table 1).

Similar results were obtained on irradiation of wool fabrics containing (1) or (2). The presence of water greatly increases whitener photodegradation, more (1) being degraded in 4 h when the fabric was irradiated wet, than in 32 h when it was irradiated dry (see Tables 1 and 2).

Another consequence of irradiating radiolabelled pyrazolines in the presence of proteins is the irreversible binding of radioactive material to the protein (see Tables 1 and 2). In the case of lysozyme, the bound radioactivity could not be removed from the protein by dialysis or by gel filtration in a variety of solvent systems chosen for their disaggregating properties. In the case of wool, the bound radioactive material resisted extraction with a range of solvents which extracted pyrazolines from unirradiated fabrics. Irreversible binding of fluorescent whitening agents or their photoproducts to proteins has been reported for several whitener–protein systems.[12]

The Influence of Pyrazolines on Protein Photodegradation

Both pyrazolines photosensitize the degradation of tryptophan, histidine, methionine and, to a smaller extent, tyrosine residues when lysozyme solutions are irradiated

(see Table 3). Approximately half of the tryptophan destroyed is converted into kynurenine (Kyn) and β-(3-oxindolyl)alanine (Oia) and most of the methionine is converted into the corresponding sulphoxide. An attempt was made to compensate for the different photostabilities of the pyrazolines by adjusting the concentration of (1) periodically. Even so, (2) was more effective in photosensitizing the degradation of the protein, possibly because lysozyme reversibly binds more (2) than (1) (see above).

Table 3. Changes in amino acid content (residues mol^{-1}) of lysozyme after irradiation in the absence and presence of pyrazolines (1) and (2)

Lysozyme (1 mg/ml) was irradiated in water for 16 h. Only those amino acids showing a significant change in content after irradiation are shown

Amino acid	Unirradiated Expected	Found	Irradiated alone	Irradiated with (1)[A,B]	(2)[A]
His	1	1·00	0·93	0·60	0·10
Trp	6	5·68	4·94	4·43	2·65
Kyn	0	0	0·07	0·25	0·85
Oia	0	0	0·32	0·36	0·63
Met	2	1·92	1·86	1·65	1·35
Met(o)	0	0	0·09	0·20	0·39
Tyr	3	3·04	2·94	2·99	2·58

[A] The initial pyrazoline concentration was 5×10^{-5} M.
[B] Additional pyrazoline was added after 4, 8 and 12 h to restore the concentration to 5×10^{-5} M.

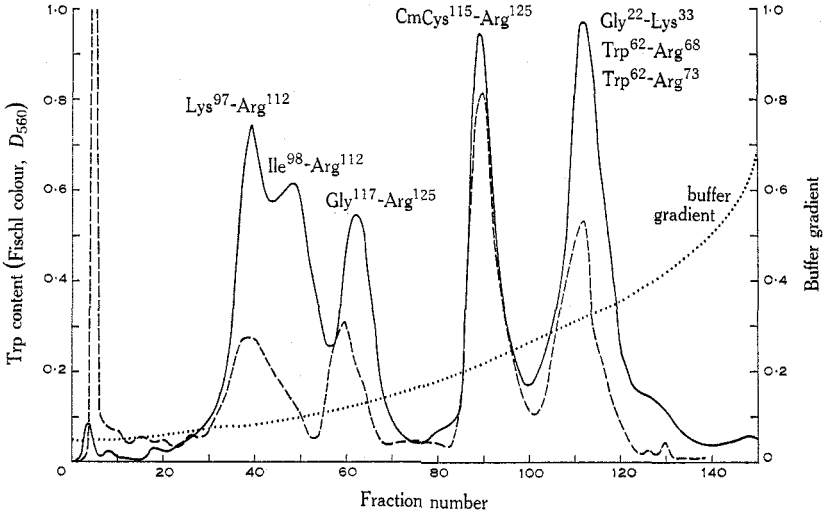


Fig. 3. Ion-exchange chromatograms of the tryptophan-containing peptides obtained by tryptic digestion of reduced and carboxymethylated lysozyme (—) and lysozyme which had been irradiated for 16 h in water in the presence of the ^{35}S-labelled tetraaryl pyrazoline (2) and then reduced and carboxymethylated (---).

The location of the tryptophan residues which were destroyed was determined by comparing the tryptic peptides derived from unirradiated and irradiated lysozyme after reduction/carboxymethylation. Ion-exchange chromatography of the digest from unirradiated lysozyme gave four tryptophan-containing peaks (see Fig. 3),

which were shown by paper electrophoresis to contain the same six tryptophan peptides observed first by Canfield.[17] When this procedure was applied to lysozyme that had been irradiated in the presence of the tetraarylpyrazoline (2), the same four tryptophan-containing peaks were obtained, but in smaller amounts (see Fig. 3). All six tryptophan peptides were still present. Thus there appear to be no marked differences between the six tryptophan residues in lysozyme with regard to sensitized photodegradation by (2).

Discussion

Our study of the photoproducts from radiolabelled pyrazoline (1) shows that its photochemistry is more complex than previously thought.[11] Although irradiations of solutions of (1) buffered at pH 7 give the corresponding pyrazole in about 80% yield (see Table 1 and ref.[11]), several other products are formed in unbuffered solutions. There is indirect evidence that one of these photoproducts is the 4-hydroxypyrazole (4) and that products containing the sulphur atom but not the C 5′ atom are formed. Sodium bisulphate (or, less likely, sodium sulphate) has been identified as one of these, and it is possible that sodium 4-hydroxybenzenesulphonate is another, since phenol and also chalcone have been identified as photoproducts of 1,3,5-triphenylpyrazoline.[18] The more complex distribution of products resulting from irradiation in water (pH *c.* 5·5) than in buffer solution (pH 7) may be due to the formation of a protonated form of an excited state of (1) at the lower pH. Alternatively, it is conceivable that the buffer ions preferentially quench one or more reactive excited states and thus simplify the course of photodegradation.

Both pyrazolines are degraded more rapidly when irradiated in lysozyme solutions than in water, and yield the corresponding pyrazoles as major products. This simplification of product distribution is not due to a change in the pH of the solution but may be due to the less ready protonation of an excited state of the pyrazoline when bound to the protein than when free in solution. Alternatively, it may be due to quenching of some excited state(s) of the pyrazoline by the protein. It is surprising that the pyrazole (3) is such a major photoproduct when the pyrazoline (1) is irradiated in lysozyme solution, if we consider that only 30% of the pyrazoline is bound to the protein under the conditions of irradiation. One possible explanation is that the pyrazoline bound to the protein is degraded more rapidly than the free pyrazoline and that the observed products are derived principally from the reactions in the bound state. In this regard, it is significant that lysozyme accelerates the photodecomposition of (2) (90% of which is bound to the protein) much more than the photodecomposition of (1) (cf. Tables 1 and 2).

Although it is difficult to compare, quantitatively, photodecomposition rates in solution with those in protein films, because of light-scattering effects, it appears that the pyrazoline (1) is less stable in lysozyme solutions than in lysozyme films. Possibly non-radiative deactivation of pyrazoline excited states is enhanced in the solid state, leading to higher photostability.

Irradiation of wool fabrics containing the two pyrazolines also gives a simpler distribution of whitener photoproducts than does irradiation in water (see Tables 1 and 2). The pyrazolines are degraded much faster when the fabrics are irradiated wet than dry, a result previously recorded for wool containing fluorescent whiteners of the

[18] Lin, J., Rivett, D. E., and Wilshire, J. F. K., *Aust. J. Chem.*, 1977, **30**, 629.

stilbene type.[19,20] This effect has been attributed[20] to physical swelling of the fibre by water, which reduces the steric constraints that hinder molecular orientation and promotes diffusion of oxygen through the fibre.

It has recently been postulated that singlet molecular oxygen is involved in the photoyellowing of unwhitened[7] and whitened[21] wool. Our data for the pyrazoline-sensitized photodegradation of lysozyme are also consistent with the involvement of singlet oxygen in that tryptophan, histidine and methionine residues are degraded. These are known to be the amino acid residues most prone to react with singlet oxygen;[22,23] they are also the ones degraded when wool fibres containing various fluorescent whiteners are irradiated.[3,21]

Although some dyes photosensitize the degradation of specific tryptophan residues in lysozyme,[24–26] this does not seem to be true when the pyrazoline (2) is used. In this case no one particular tryptophan residue is degraded selectively, even though only one molecule of pyrazoline is bound per lysozyme molecule under the conditions of irradiation. There are two possible explanations for the above results: either the pyrazoline binds to several different sites on the lysozyme molecule or the reactive species is able to diffuse to various sites within the lysozyme molecule. The latter hypothesis is consistent with the involvement of singlet oxygen as the reactive intermediate.

In conclusion, our results show that the photochemical behaviour of pyrazolines is qualitatively the same in lysozyme solutions or films as in wool fabrics, and that fluorescent whitener/lysozyme systems are useful models for whitened wools.

Acknowledgments

The authors thank Dr C. M. Roxburgh for the amino acid analyses and Miss Jenny A. Hawker for technical assistance.

Manuscript received 1 April 1977

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