Fluorescence of *N'*-Formylkynurenine and of Proteins Exposed to Sunlight

By ANTOINETTE PIRIE

Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.

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When solutions of proteins are exposed to sunlight the tryptophan in their molecules is photo-oxidized to *N'*-formylkynurenine (Pirie, 1971). Jori & Galliazo (1971) have found the same change in proteins exposed to u.v. light in the presence of a photosensitizing dye. *N'*-Formylkynurenine can be detected by a change in the absorption spectrum of the protein and by chromatography and electrophoresis after acid hydrolysis. This releases kynurenine from photo-oxidized proteins (Pirie, 1971).

A further method of detection and identification of *N'*-formylkynurenine in the intact photo-oxidized proteins is through the determination of their fluorescence spectrum. Proteins containing tryptophan normally fluoresce maximally near 360 nm, but after photo-oxidation another emission peak develops near 435 nm in neutral solution and the tryptophan fluorescence is diminished. I have compared the fluorescence spectrum of *N'*-formylkynurenine with that of proteins photo-oxidized in sunlight, considering that this might prove a sensitive method of detection and identification. The fluorescence spectrum of *N'*-formylkynurenine does not seem to have been reported previously.

Experimental

N'-Formylkynurenine was prepared by the method of Dagleish (1952). The product was purified by re-precipitation from hot water; different preparations were 85–90% pure judged by their absorption at 321 and 260 nm (Mehler & Knox, 1950). Chromatography and electrophoresis, as described by Pirie (1971), showed traces of kynurenine as the only detectable fluorescent impurity; the amount present was too small to be detected spectrofluorimetrically. Proteins (10 mg/ml) in 6 M-guanidinium chloride were photo-oxidized in sunlight in stoppered tubes placed outside the window at ambient temperature (Pirie, 1971). Fluorescence spectra were measured with an Aminco Bowman spectrofluorimeter, and I thank Dr. P. Brunet, Department of Zoology, University of Oxford, for the use of this machine. The fluorescence maxima are uncorrected for instrumental error and were recorded manually. α -Crystallin and γ -crystallins from bovine lenses were a gift from Miss C. Slingsby. Protein from normal human lenses (post-mortem specimens) was prepared by thorough dialysis of a water dispersion of proteins from the

ground lenses. Egg-white lysozyme (three-times crystallized) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results and discussion

Fluorescence of *N'*-formylkynurenine. In neutral or acid solution *N'*-formylkynurenine fluoresces at 440 nm. The main exciting wavelength is 330 nm, with a small peak of excitation at 265 nm. Above pH 10 the emission peak changes to 400 nm, excited at 315 nm, with a subsidiary excitation peak at 240 nm, and the intensity of fluorescence increases at least 20-fold. Fig. 1 shows the spectra of solutions of *N'*-formylkynurenine (*a*, 20 μ g/ml at pH 7.4, and *b*, 1.0 μ g/ml at pH 11). The change in the emission peak is immediate, but maximum intensity of fluorescence in alkali takes about 15 min to develop, possibly owing to enolization of the carbonyl group gradually reaching an equilibrium. When the solution is neutralized after about 20 min in alkali the fluorescence reverts to the original spectrum, with emission maximum at 440 nm.

More-concentrated alkali (0.1 M-NaOH) gradually destroys *N'*-formylkynurenine. The absorption spectrum is irreversibly changed, with loss of both maxima at 321 and 260 nm and development of a shoulder at 350 nm. The fluorescence spectrum in 0.1 M-NaOH is at first unchanged, but later becomes complex and does not revert to the original when the solution is neutralized.

Reduction of *N'*-formylkynurenine to γ -(2-formylaminophenyl)homoserine by NaBH₄ at pH 10 followed by neutralization changes the fluorescence. The usual spectrum is replaced by a peak of emission at 345 nm excited at 285 nm. This excitation peak corresponds to the absorption peak at 280 nm of reduced *N'*-formylkynurenine.

Treatment with 1 M-HCl to remove the formyl group, followed by neutralization, changes the fluorescence to that of kynurenine (excitation maximum at 370 nm, emission maximum at 480 nm).

Fluorescence of sunlight-treated proteins. After exposure to sunlight α -crystallin and γ -crystallins from bovine lens, the mixed proteins from normal human lenses and egg-white lysozyme fluoresce in neutral solution at 430–440 nm excited at 320–325 nm, with subsidiary excitation at 260 nm. In alkaline

Fig. 1. Fluorescence spectra of *N'*-formylkynurenine in neutral and alkaline solutions

(a) Emission at 440nm and excitation at 265 and 330nm. The concentration of *N'*-formylkynurenine was 20 $\mu\text{g/ml}$, in 50mM-potassium phosphate buffer, pH7.4. The meter multiplier setting was 0.003. (b) Emission at 400nm and excitation at 315 and 240nm. The concentration of *N'*-formylkynurenine was 1.0 $\mu\text{g/ml}$. The solution was adjusted to pH11.0 with NaOH. The meter multiplier setting was 0.003.

solution the fluorescence emission shifts to 390–400nm excited at 315 and 240nm, and the intensity increases. Before being illuminated these proteins show only the fluorescence of tryptophan (emission near 360nm, excited at 285nm). This diminishes during illumination, but does not disappear. Ribonuclease and salmine, which contain no tryptophan,

Fig. 2. Fluorescence spectrum of bovine γ -crystallin after photo-oxidation in sunlight

The concentration of γ -crystallin was 5 $\mu\text{g/ml}$. The solution was adjusted to pH10.5 with NaOH. The meter multiplier setting was 0.001.

do not change their fluorescence when exposed to sunlight.

Fig. 2 shows the fluorescence of γ -crystallin after exposure to sunlight. The record was made at high instrument sensitivity to find the lowest concentration of protein to give a good spectrum. The sample of photo-oxidized γ -crystallin whose spectrum is recorded was found by R. H. Buckingham (personal communication) to have polymerized and to contain covalent cross-links other than disulphide bonds. It is possible that *N'*-formylkynurenine or some derivative of it participates in cross-linking and, if this is so, correlation of loss of tryptophan with formation of *N'*-formylkynurenine is unlikely to be possible. In a single experiment there was progressive loss of tryptophan fluorescence with time of photo-oxidation of γ -crystallin, whereas the fluorescence at 440nm rose to a plateau and then did not increase further as the tryptophan fluorescence decreased. *N'*-Formylkynurenine itself, exposed to sunlight in neutral solution, loses both its characteristic absorption spectrum and its fluorescence emission at 435nm, but the products formed have not been identified.

The resemblance of the fluorescence spectra of proteins photo-oxidized in sunlight to that of *N'*-formylkynurenine confirms the evidence given previously (Pirie, 1971) that photo-oxidation of tryptophan residues yields this compound. Such fluorescence is absent from proteins before photo-

oxidation. It is possible to record the fluorescence of *N'*-formylkynurenine in a photo-oxidized protein by using 2 μ g of protein/ml at pH 11.0. If, therefore, photo-oxidation of the tryptophan residues in a protein is suspected it is worth while taking the fluorescence spectrum.

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