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Formation of *N'*-Formylkynurenine in Proteins from Lens and Other Sources by Exposure to Sunlight

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The photo-oxidative effect of sunlight on the tryptophan residues of proteins and on free tryptophan is described. Evidence is presented that the indole ring is split to yield *N'*-formylkynurenine. The possible relation of this photo-oxidative change to changes in the lens proteins of brown cataracts is discussed.

The human lens generally becomes yellow with age, and in some types of cataract the nuclear core is golden brown or almost black. Such golden-brown lenses form a greater proportion of cataracts extracted in Shikarpur, W. Pakistan, than they do in Oxford (Pirie, 1971). It has often been suggested that these brown cataracts are due to the effect of sunlight on the lens but little work has been done to find out whether this could be so. The major pigmented material of the nucleus of the human cataractous lens is protein (Pirie, 1968). As part of a study of this material I have investigated changes that take place in proteins of the lens, in other proteins and in free tryptophan when their neutral solutions in 6M-guanidinium chloride are exposed to sunlight. Sunlight was chosen as a source, in spite of obvious difficulties, since I was concerned with a possible sun-caused change. Between 10 and 40% of the protein of the normal adult human lens is insoluble in water (Pirie, 1968; Clark, Zigman & Lerman, 1969); the percentage rises progressively with age. Rather than use only the water-soluble part of the lens protein I chose to dissolve the whole in guanidinium chloride. Aqueous solutions of ribonuclease, lysozyme, bovine serum albumin and γ-crystallin, which were used as reference proteins, partially precipitated when exposed to the sun but remained in solution in guanidinium chloride.

Most work on photochemical change in proteins is concerned with photosensitized reactions with a dye and a source of u.v. or visible light, often in strongly acid conditions (Spikes & Livingstone, 1969). In those experiments in which proteins have been exposed to the sun no products seem to have been identified. The experiments reported here suggest that sunlight in the presence of air causes oxidative cleavage of the indole ring of tryptophan to give *N'*-formylkynurenine. This is probably only one of many changes, and they will vary from protein to protein.

MATERIALS AND METHODS

Lens proteins. Human lenses obtained *post mortem* were freeze-dried *in vacuo*. They were then ground with about 10 times their weight of water and dialysed with stirring for three successive periods of 18h against 200ml of water at 4°C. Non-diffusible material was then frozen and dried *in vacuo*.

Bovine γ-crystallin. I thank Miss C. Slingsby for this preparation.

Commercial chemicals. DL-Tryptophan, chromatographically homogeneous, crystalline egg-white lysozyme and guanidinium chloride, special purified grade or laboratory grade purified by treatment with activated charcoal, were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Ribonuclease was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; DL-kynurenine was from Sigma Chemical Co., St Louis, Mo., U.S.A.

N'-Formylkynurenine. This was prepared from tryptophan by treatment in 100% formic acid with ozone (Previero & Coletti-Previero, 1967), the use of resorcinol being omitted. The solution of ozonized tryptophan was evaporated on a rotary evaporator at 20°C and the product stored at −20°C. It was used as a reference compound without further purification as it appeared spectrally homogeneous and contained 43% of *N'*-formylkynurenine, calculated from the absorption at 320nm. I thank Dr D. J. Giachardi for the use of an ozonizer.

Exposure of proteins to sunlight. All substances were exposed outside the window as near horizontally as possible on a ledge that received sunlight from both south and west. The substances were in glass tubes, of internal diameter 12mm, corked and sealed with wax paper. The glass transmitted 17% of light of 295nm and 41% of light of 310nm. Control tubes were kept in a dark cupboard or in a container on the window ledge.

Temperature did not exceed 30°C. Tryptophan (10mM) was used in water at neutrality. Proteins were used at 10mg/ml in 6M-guanidinium chloride (neutral) unless otherwise stated.

Reduction of substances with sodium borohydride. A trace of solid $NaBH_4$ was added to the solution in the spectrophotometer cell, thoroughly mixed and left at room temperature for 20min. Bubbles were removed by gentle tapping. The spectra were not affected by the slight increase in pH.

Hydrolysis of substances with 1M-hydrochloric acid to remove formyl groups. A solution, about 10 times more concentrated than needed for recording a spectrum, was made 1M with respect to HCl and left at room temperature for 20min. A sample was neutralized with 1M-K_2HPO_4 and diluted to a suitable concentration for the spectrum to be recorded.

Before analysis, proteins were separated from guanidinium chloride by precipitation with 10 vol. of chilled ethanol, washed with 50ml of 95% ethanol three times and then freeze-dried *in vacuo*.

Tryptophan in protein. This was determined by the method of Spies & Chambers (1949) without hydrolysis.

Acid hydrolysis of proteins. This was carried out with 6M-HCl (Aristar: BDH Chemicals Ltd.) for 18h at 108°C in tubes sealed under vacuum.

Chromatography. The following solvents were used: butan-1-ol–acetic acid–water (40:9:20, by vol.); 20% (w/v) KCl; 3% (w/v) NH_4Cl. Whatman papers nos. 1 and 3MM were employed.

Electrophoresis. This was carried out at pH2 by using a ridge-pole tank with 90% (v/v) formic acid–acetic acid–water (1:3:16, by vol.) as solvent, on Whatman no. 3MM paper, at 10V/cm.

Identification tests. The Ehrlich reaction was used for indoles and related compounds and the Ekman reaction for diazotizable amines (Smith, 1969).

Fluorescence. This was examined on paper at 260nm and 360nm.

Spectroscopic measurements. These were made with an Optica CF4 recording spectrophotometer in a 1.0cm-light-path silica cell.

RESULTS

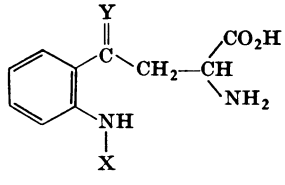
Exposure of proteins and of tryptophan to sunlight causes the solutions to turn first yellow and then golden brown. After some days of intermittent sun, tryptophan in aqueous solution may turn a deep brown and deposit a black precipitate. Table 1 shows that there is a loss of tryptophan from normal human lens proteins, bovine γ-crystallin and lysozyme and from bovine serum albumin both in guanidinium chloride and in water. The loss of tryptophan varied, probably owing to variability of sunlight. Table 1 gives representative examples. The absorption at 280nm falls and that over 300nm and near 260nm rises. A shoulder near 320nm develops, suggesting the formation of *N'*-formylkynurenine (Knox & Mehler, 1950), but there is no increase at 360nm, which rules out kynurenine. Ribonuclease, which does not contain tryptophan, did not show these spectral changes, although the solution became yellow. These changes did not occur in the dark or *in vacuo*, or if ascorbic acid or cysteine was added to the protein solutions before illumination.

N'-Formylkynurenine was recognized by its absorption spectrum [$\lambda_{max.}$ 321nm, ϵ 3750, and 260nm, ϵ 10980, at pH7 (ϵ being the molar extinction coefficient; Mehler & Knox, 1950)] and by changes in this spectrum on treatment with 1M-hydrochloric acid or sodium borohydride (Previero & Bordignon, 1964; Previero, Coletti-Previero & Jolles, 1966). Treatment with 1M-hydrochloric acid for 20min at room temperature changes the

Table 1. *Loss of tryptophan after sunlight*

Proteins were exposed to sun, as described in the Materials and Methods section (10mg of protein/ml of 6M-guanidinium chloride at neutrality unless otherwise stated). Exposure was continued until the proteins showed a decrease in absorption at 280nm and a rise at 320nm.

Expt. no.	Preparation	Tryptophan (g/kg) Dark	Sunlight
1	Total normal human lens protein	47	7
2	Total normal human lens protein	42	26
3	Egg-white lysozyme	36	18.5
4	Bovine γ-crystallin	37	nil
5	Serum albumin	6	3
6	Serum albumin	5	1
7	Serum albumin in aqueous solution	5.8	2.6



Formula (I)

Substance	X	Y
N'-Formylkynurenine	CHO	O
Kynurenine	H	O
γ-(2-Aminophenyl)homoserine	H	HOH
γ-(2-Formylaminophenyl)homoserine	CHO	HOH

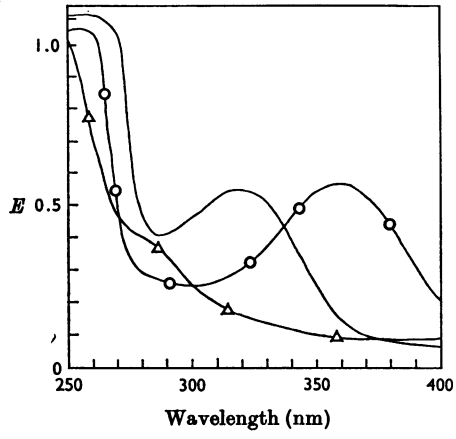


Fig. 1. Absorption spectrum of *N'*-formylkynurenine and the effect of hydrolysis with 1 M-HCl and of reduction with sodium borohydride. ——, *N'*-Formylkynurenine (0.03 mg/ml) in water (pH 7) calc. from ϵ 3750 at 321 nm; ○, *N'*-formylkynurenine treated with 1 M-HCl for 20 min and then neutralized (final concn. 0.03 mg/ml in potassium phosphate buffer, pH 7); △, *N'*-formylkynurenine (0.03 mg/ml) in water, treated with sodium borohydride for 20 min.

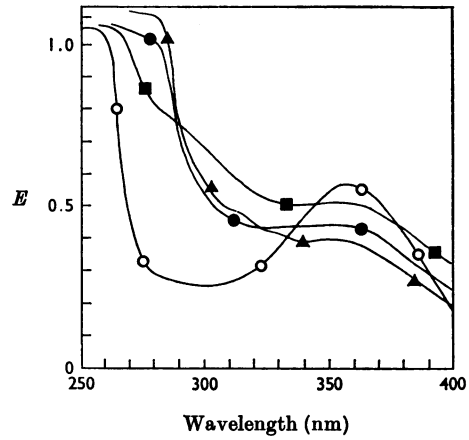


Fig. 3. Effect of hydrolysis for 20 min with 1 M-HCl at room temperature on: ○, *N'*-formylkynurenine (0.03 mg/ml) in phosphate buffer, pH 7; ▲, photo-oxidized tryptophan (0.1 mg/ml) in phosphate buffer, pH 7; ■, photo-oxidized lysozyme (0.4 mg/ml) in 6 M-guanidinium chloride containing phosphate buffer, pH 7; ●, photo-oxidized normal human lens proteins (1.0 mg/ml) in 6 M-guanidinium chloride containing phosphate buffer, pH 7.

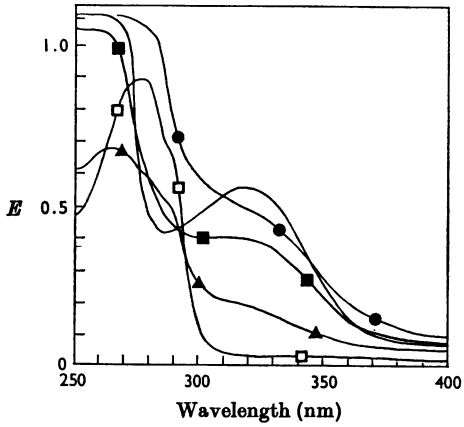


Fig. 2. Absorption spectrum of photo-oxidized substrates compared with that of *N'*-formylkynurenine and of lysozyme. ——, *N'*-Formylkynurenine (0.03 mg/ml) in water, neutral; ▲, tryptophan photo-oxidized in sunlight (0.03 mg/ml) in water, neutral; ■, lysozyme photo-oxidized in sunlight (0.3 mg/ml) in 6 M-guanidinium chloride; ●, normal human lens proteins photo-oxidized in sunlight (0.4 mg/ml) in 6 M-guanidinium chloride; □, lysozyme, kept in dark (0.3 mg/ml) in 6 M-guanidinium chloride.

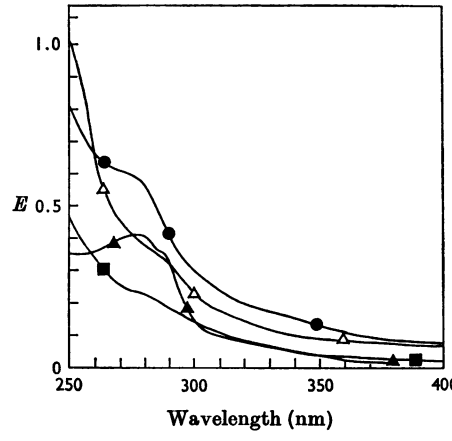


Fig. 4. Effect of reduction with sodium borohydride on: △, *N'*-formylkynurenine (0.03 mg/ml) in water; ▲, photo-oxidized tryptophan (0.03 mg/ml) in 0.03 M-potassium phosphate, pH 7; ■, photo-oxidized lysozyme (0.4 mg/ml) in 6 M-guanidinium chloride; ●, photo-oxidized normal human lens proteins (0.4 mg/ml) in 6 M-guanidinium chloride.

spectrum to that of kynurenine ($\lambda_{max.}$ 360 nm) by hydrolysis of the formyl group, whereas reduction with borohydride reduces the oxo group and removes the peaks at 321 nm and at 260 nm. Fig. 1 illustrates these spectral changes. Formula (I) shows the various derivatives.

Figs. 2, 3 and 4 compare spectra of *N'*-formylkynurenine with tryptophan, lens proteins and lysozyme after exposure to sunlight. Fig. 2 shows that all have a shoulder at 320 nm, with a higher

one near 260nm. Lysozyme before illumination is included for comparison. After brief hydrolysis with 1M-hydrochloric acid a shoulder at 360nm replaces that at 320nm (Fig. 3) and after reduction with sodium borohydride the peaks at 320 and 260nm disappear (Fig. 4). It is clear that not all tryptophan is photo-oxidized, as considerable absorption at 280nm remains and can be seen particularly after removal of the *N'*-formylkynurenine peaks with sodium borohydride (Fig. 4). Before photo-oxidation none of the proteins showed any peak other than that at 280nm caused by aromatic amino acids.

This spectroscopic evidence has been strengthened by results obtained from electrophoresis and chromatography. Acid hydrolysis, of proteins that had been exposed to the sun, with 6M-hydrochloric acid, in the usual way, will remove the formyl group and release kynurenine. This was recognized by comparison with the authentic compound, by using fluorescence, colour reactions and known R_F values for identification. Or, if the acid hydrolysates were reduced with sodium borohydride before chromatography, the blue fluorescent spot of kynurenine, which gives an orange Ehrlich reaction, disappears. A new compound, which slowly is coloured yellow with the Ehrlich reagent, which is non-fluorescent and which corresponds in R_F to γ-(2-aminophenyl)homoserine, can be detected. Neither free tryptophan nor lens proteins nor lysozyme before exposure to sun releases kynurenine on acid hydrolysis. Table 2 gives R_F values of *N'*-formylkynurenine and its derivatives, and of the corresponding spots on chromatograms of sun-exposed tryptophan and acid hydrolysates of sun-exposed proteins before and after reduction, together with tests of identification. Table 3 shows that *N'*-formylkynurenine and its derivatives can be separated by electrophoresis at pH 2.0. A control test showed that the acetone–hydrochloric acid mixture used in the Ekman reagent (Smith, 1969) would hydrolyse *N'*-formylkynurenine to give kynurenine. Hence a slow false positive may develop (Tables 2 and 3).

Taken together this evidence gives a reasonable certainty that *N'*-formylkynurenine is formed when free tryptophan, or proteins containing tryptophan, are exposed to the sun.

DISCUSSION

N'-Formylkynurenine is the first product in the metabolism of tryptophan (Knox & Mehler, 1950). It is also formed, either free or combined in peptide links, when tryptophan, tryptophan peptides or proteins are oxidized with ozone in 100% formic acid (Previero & Bordignon, 1964) and by the action of X-rays in neutral solution (Jayson, Scholes & Weiss, 1954). It has not previously been described as a product of the photo-oxidation of free or combined tryptophan by sunlight but has been identified as a product of u.v. irradiation of tryptophan (Deschreider & Renard, 1955). Yoshida & Kato (1954) found that free tryptophan yielded kynurenine and 3-hydroxykynurenine, and Takase (1966) reported that tryptophan in saké, a Japanese fermented liquor made from rice, formed β-carbolines in the sun. In

Table 2. *Chromatographic behaviour of* N'*-formylkynurenine and other preparations*

	$100 \times R_F$					
	Butanol–acetic acid–water	20% KCl	3% NH_4Cl	Fluorescence	Ehrlich reaction	Ekman reaction
N'-Formylkynurenine	44	77	73	Blue–white	Orange	Negative*
Photo-oxidized tryptophan	44	—	76	Blue–white	Orange	Negative*
Kynurenine	41	71	69	Blue	Orange	Purple
Photo-oxidized lens proteins, acid hydrolysate	38	—	71	Blue	Orange	Purple
Photo-oxidized lysozyme, acid hydrolysate	38	71	—	Blue	Orange	Purple
γ-(1-Aminophenyl)homoserine	39	84	81	Nil	Yellow	Purple
Photo-oxidized lens proteins, acid hydrolysate, reduced with borohydride	—	—	81	Nil	Yellow	—
γ-(1-Formylaminophenyl)homoserine	44	87	84	Nil	Yellow	Negative*
Photo-oxidized tryptophan reduced with borohydride	44	—	86	Nil	Yellow	Negative*
Tryptophan	50	59	—	Purple (260nm)	Purple	Negative

* A slight positive may slowly develop as the formyl group is hydrolysed by the reagent on the paper.

Table 3. *Electrophoresis of N′-formylkynurenine and derivatives*

Electrophoresis was carried out for 2.5h at 10V/cm, 50mA, on Whatman 3MM paper, at pH2.

	Distance travelled (cm)	Ehrlich reaction	Ekman reaction
N′-Formylkynurenine	10	Orange	Negative*
Photo-oxidized tryptophan	10	Orange	Negative*
Kynurenine	11.8	Orange	Purple
Reduced *N′*-formylkynurenine	10.2	Yellow	Negative*
Reduced photo-oxidized tryptophan	9.8	Yellow	Negative*
Reduced kynurenine	14, 14.8	Yellow	Purple
Tryptophan	10.5	Purple	Negative

* A false positive may develop as the formyl group is hydrolysed by the reagent on the paper.

my own experiments the amount of *N′*-formylkynurenine formed from tryptophan varied with the time of illumination, rising to a maximum and then decreasing. *N′*-Formylkynurenine itself is unstable in sunlight at neutrality.

Photo-oxidation of the tryptophan residues of proteins by sunlight to yield *N′*-formylkynurenine may be of general interest apart from its possible relevance in cataract formation. Yellowing of wool and silk in the sun is well known, and Lennox & Rowlands (1969) correlated loss of tryptophan with yellowing of various keratins during their photo-oxidation by light of 300–310nm, but they described no product.

Teale & Weber (1959) conclude that light of 295–310nm is almost specific for excitation of tryptophan in proteins, and Koller (1965) estimates that the energy of sunlight below 313nm is 175μW/cm^2 on a clear midsummer noon at latitude 39°N, falling to 40μW/cm^2 in December; it constitutes less than 1% of the total light energy. Light of these wavelengths reaches the lens. Kinsey (1948) found that 50% of light of 310nm and 15% of light of 300nm reached the anterior surface of the lens and was all absorbed on passage through its substance. The proteins of the lens, particularly those of the nuclear core, are rich in tryptophan and are also long-lived. The lens grows from the periphery inwards, so that the nuclear core is the oldest part (Mann, 1964). If *N′*-formylkynurenine is formed *in vivo* through the action of sunlight, further reactions may follow during the many years that may elapse between start of nuclear sclerosis and development of a brown nuclear cataract.

The brown insoluble proteins of these cataracts have less tryptophan than proteins of normal lenses (Pirie, 1971), but only traces of kynurenine are present in acid hydrolysates. Lerman, Tan, Lewis & Hollander (1970) describe a 'fluorogen' in acid hydrolysates of γ-crystallin and other lens proteins, which they suggest may be derived from one of the tyrosine residues. They do not, however, consider the possibility that the 'fluorogen' may be a β-carboline formed from tryptophan during the acid hydrolysis of the proteins (Tschetsche, Jenssen & Rangachari, 1958). Ether extraction of the acid hydrolysate of bovine γ-crystallin removes fluorescent substances that give a typical β-carboline fluorescence at 450nm excited by light of 250, 310 and 370nm at pH2.

I am pleased to acknowledge helpful discussions with colleagues in the Laboratory.

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