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THE PHOTOCHEMICAL BINDING OF BITHIONOL TO SOLUBLE PROTEINS AND PEPTIDES

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Abstract—The photochemical reactions of the bacteriocide bithionol (a known photoallergen in man) with soluble proteins and peptides, were investigated. Solutions of human serum albumin (HSA), human γ-globulin, bovine insulin and poly-L-lysine were irradiated with ultraviolet light of wavelength 313 nm in the presence of [^{35}S]-bithionol and the extent of photochemical (covalent) binding determined. HSA bound at least four molecules of bithionol per molecule of HSA. Bithionol was also found to bind to γ-globulin to a similar extent; lower levels of binding were achieved with bovine insulin and poly-L-lysine. A bithionol–HSA photoadduct was treated with cyanogen bromide to determine the selectivity of binding. Fractionation after cyanogen bromide treatment showed that bithionol was bound to both major fragments of HSA, with a preference for the N-terminal region of the protein.

The condition of photodermatitis was first described by Wilkinson [1] as an abnormal sensitivity of the skin caused by the germicide tetrachlorosalicylanilide. This condition results from the interaction of a photoallergen with the human skin under the influence of ultraviolet light. This form of allergy has been found to be caused by many other compounds of widely varying chemical structures, for example the bacteriocides fentichlor [2–4] and bithionol [5]. Bithionol has been used as a bacteriocide in soaps, shampoos and antiseptic creams; a number of cases have been reported where users of these products have experienced photodermatitis. Photopatch testing subsequently identified bithionol as the cause of the photoallergic reaction [5].

In previous studies the ability of photoallergens to form protein conjugates when irradiated with the appropriate wavelength of light, has been demonstrated [6]. In this paper the photochemical binding of bithionol to soluble proteins and the selectivity of its binding to particular regions of the human serum albumin molecule are examined. These results are compared with those obtained for fentichlor [7] which shows no selectivity in binding to albumin, and tetrachlorosalicylanilide [8] which does.

MATERIALS AND METHODS

Materials. Human serum albumin (HSA,‡ fraction V fatty acid free), bovine insulin, human γ-globulin, poly-L-lysine and cyanogen bromide were obtained from the Sigma Chemical Co. (Poole, U.K.); 2,4-dichlorophenol and anhydrous aluminium chloride were obtained from the Aldrich Chemical Co. (Dorset, U.K.); elemental sulphur-35 (solution in toluene) was supplied by Amersham International plc (Amersham, U.K.).

Synthesis of [^{35}S]-*bithionol*. Sulphur (1 g) and iodine (0.05 g) were dissolved in carbon tetrachloride (10 ml), to which was added sulphur-35 in toluene. The mixture was cooled in an ice salt bath at −5°. Chlorine gas (2 g) (prepared by adding concentrated hydrochloric acid (5 ml) to excess potassium permanganate) was then passed into the mixture which was stirred for 30 min.

Anhydrous aluminium chloride (2.4 g) was added to carbon tetrachloride (20 ml) and the mixture heated to 50° in a water bath for 30 min. The [^{35}S]-sulphur dichloride was added together with 2,4-dichlorophenol (3.01 g) dissolved in carbon tetrachloride (20 ml) and the mixture stirred in the dark for 2 hr, then left overnight. The solution was decanted from the aluminium chloride and poured on to crushed ice. The precipitate was filtered off, dissolved in toluene, filtered hot and allowed to recrystallize. After recrystallization from toluene the yield of [^{35}S]-bithionol was 1.37 g (41.6% of theory). This had a melting point of 188–192° (Lit. 188° [9]) and a specific activity of 0.21 μCi/mg. The product showed only one component by thin layer chromatography on Polygram Silica G ($CHCl_3$:CH_3OH; 19:1, $R_f = 0.46$) and was found to be 96% pure by isotope dilution analysis.

Reaction of protein solutions with bithionol. A stock solution of radiolabelled bithionol in methanol was prepared and added to a solution of the protein being studied (1.50×10^{-7} M in 0.1 M Tris–HCl buffer, pH 8.1) using a microsyringe. Samples were either (a) irradiated in a quartz vial with stirring at a distance of 4 cm from the light source, or (b) stirred in the dark. The light source was a Hanovia medium pressure Hg-arc lamp fitted with Schott UG-5 (2 mm; UV transmitting) and WG-310 (1 mm) filters giving 58% transmission at 313 nm. All irradiations were carried out at room temperature. After irradiation,

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‡ Abbreviations used: HSA, human serum albumin; T_4CS, tetrachlorosalicylanilide.

Table 1. Binding of bithionol to human serum albumin

Starting ratio Bithionol : protein (m : m)	Binding ratio Bithionol : HSA (m : m) Non-irradiated	Irradiated
1:1	0.10	0.60
2:1	0.25	1.34
5:1	0.60	3.60
10:1	0.65	4.80

samples were passed through a column of Sephadex G-10 equilibrated with 0.05 M ammonium bicarbonate to separate the photoadduct from unbound bithionol.

Methods. Monomer HSA was prepared from the commercial sample by the method of Pedersen [10]. The monomer HSA–bithionol photoadduct was treated with cyanogen bromide according to the method of Meloun *et al.* [11]. The reaction mixture was desalted on a Sephadex G-25 column (2 cm^2 × 30 cm, eluted with 0.1 M ammonium formate, pH 2.9) and then freeze dried. The photoadduct was then applied to a column of Sephadex G-100 (5 cm^2 × 100 cm) eluted with 0.1 M ammonium formate, pH 2.9. The two major fractions obtained were assigned C and N respectively according to Meloun and Kusnir [12].

C and N fractions of the HSA–bithionol photoadduct were reduced and carboxymethylated [13] and maleylated [14]. Each reduced, carboxymethylated and maleylated fraction was then separated into its constituent fragments using a column of Sephadex G-100 (2 cm^2 × 100 cm eluted with 0.05 M ammonium bicarbonate).

Protein concentrations were determined using the Biorad assay [15]. Poly-L-lysine concentrations were determined using the ninhydrin assay [16]. Liquid scintillation counting was performed using a Beckman liquid scintillation spectrometer; 3 ml of National Diagnostics Liquiscint were added to 0.02 or 0.2 ml aliquots of the sample to be counted.

RESULTS AND DISCUSSION

Binding of [^{35}S]-bithionol to human serum albumin

The photochemical binding of bithionol to HSA was studied for a number of different initial ratios of photoallergen to protein. From the results presented in Table 1 it can be seen that HSA can bind at least four molecules of bithionol per HSA molecule. This is in direct contrast to the situation with T_4CS; this compound binds to HSA with a maximum mole ratio of 1:1 [8]. Fentichlor, which structurally is closely related to bithionol, also binds well to HSA; in this case, using an initial molar ratio of 10:1, fentichlor:HSA, at least seven molecules of fentichlor were bound per HSA molecule [7].

Selectivity of binding of bithionol to monomer HSA

Elution of the cyanogen bromide digest of a large preparation of [^{35}S]-bithionol–HSA (mole ratio 1:1.3 HSA:bithionol) from a Sephadex G-100 column (Fig. 1) yielded two main fragments, C, residues 299–585 and N, residues 1–123 and 124–298 (data of Meloun and Kusnir [12]) as well as some polymerized and incompletely digested protein. Radioactive counts showed that the bithionol bound to the C-fraction at a ratio of 0.2 : 1 bithionol: protein, whereas it bound to the N fraction with a ratio of 0.46:1. The [^{35}S]-bithionol was distributed between the C- and N-fractions in the ratio 1 : 2.3 respectively.

This result can be compared with those for fentichlor and T_4CS. T_4CS was found to be distributed between the C- and N-peaks in the ratio 1:2.8 [8]. The binding of T_4CS was shown to take place at a single major site in the N-fraction whereas fentichlor was found to show no such selectivity being distributed between the C- and N-fractions with a ratio of 1 : 0.98 respectively [7]. The bithionol binding ratio implies that this photoallergen, although being able to bind to HSA at more than one site, binds preferentially to the N-fraction, the part of the HSA molecule containing the T_4CS binding site.

Reduction, carboxymethylation and maleylation of the HSA cyanogen bromide digestion products followed by separation on a Sephadex G-100 column yields seven protein fragments, three produced from the N-fraction (CB1-3) (Fig. 2) and four from the C-fraction (CB4-7) (not shown). Thirty-two per cent of the N-fraction activity applied to the G-100 column was eluted with the CB1 fragment, 16% with the CB2 fragment and 33% with the CB3 fragment. The CB3 fragment therefore appears to be important in the binding of bithionol (as it is in the binding of T_3CS to HSA). However, whereas T_4CS binds very selectively to the CB3 fragment, bithionol binds almost equally well to both the CB1 and CB3 fragments.

Separation of the fragments produced from the C-fraction of the cyanogen bromide digest was poor, even after remaleylation; the radioactivity was spread throughout the fractions collected from the column.

Photochemical binding of bithionol to soluble proteins and peptides

A range of widely differing soluble proteins/peptides was used in this study, the results of which are presented in Table 2. It can be seen that bithionol binds strongly to all four proteins under the influence of ultraviolet light. Bithionol has a greater affinity for HSA than for any of the other proteins/peptides tested; it binds least well to poly-L-lysine. Like fentichlor [7], the affinity of bithionol for human γ-globulin is almost as great as its affinity for HSA. This is in contrast to the binding abilities of T_4CS which binds to γ-globulin 100 times less efficiently than it does to HSA*.

General discussion

The results presented above demonstrate that, like fentichlor, bithionol has the ability to bind covalently to HSA and other soluble proteins on irradiation with ultraviolet light. Bithionol shows greater selectivity in binding to particular regions of HSA than

* M. D. Barratt, unpublished observations.

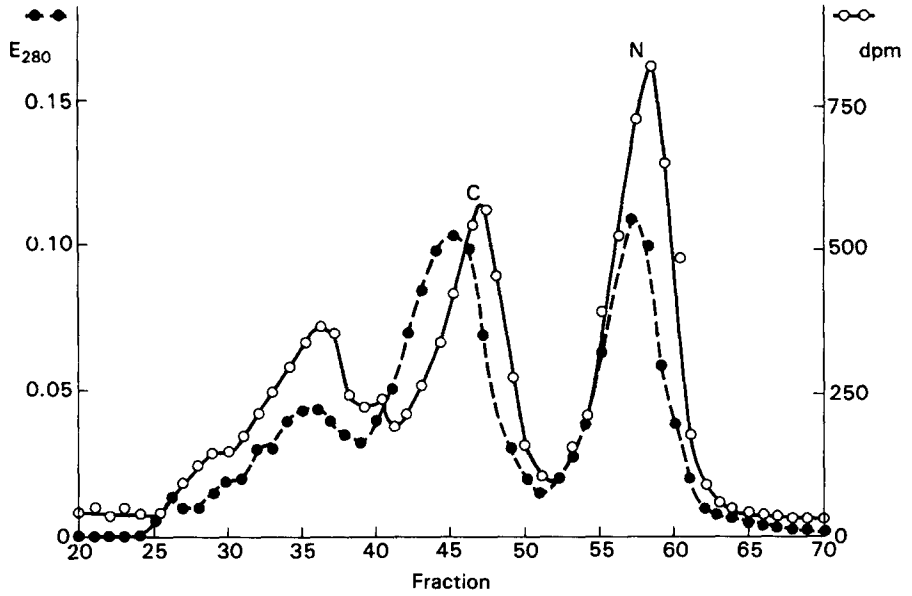


Fig. 1. Elution profile of cyanogen bromide-treated [^{35}S]-bithionol-HSA on Sephadex G-100 in 0.1 M ammonium formate pH 2.9.

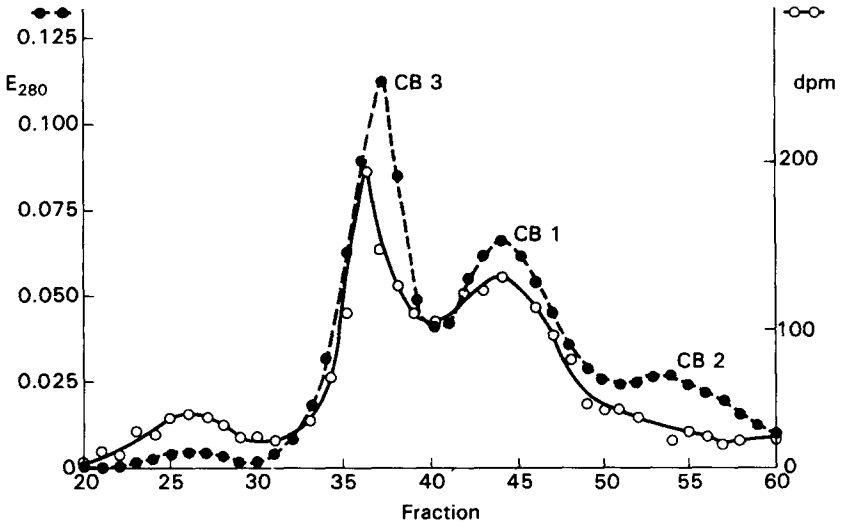
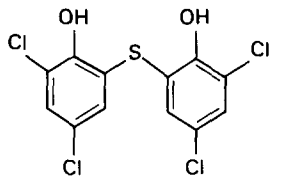


Fig. 2. Elution profile of reduced, carboxymethylated and maleylated [^{35}S]-bithionol-HSA N-fraction on Sephadex G-100 in 0.1 M ammonium bicarbonate (showing fragment positions).

Table 2. Binding of bithionol to soluble proteins

Protein	Starting ratio Bithionol : protein (m : m)	Binding ratio Bithionol : protein (m : m) Non-irradiated	Irradiated
HSA	10 : 1	0.65	4.80
Insulin	10 : 1	0.12	1.20
γ-Globulin	10 : 1	0.37	4.30
Poly-L-lysine	10 : 1	0.35	0.70



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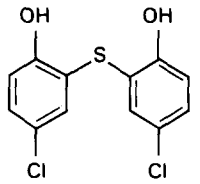

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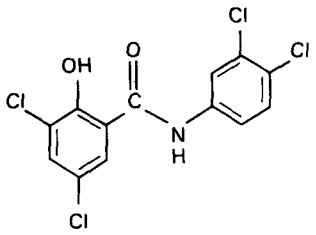

III

Fig. 3. Chemical structures of (I) bithionol, (II) fentichlor and (III) tetrachlorosalicylanilide (T_4CS).

does fentichlor; twice as much bithionol was found to bind to the N-fragment than to the C-fragment, whereas fentichlor was shown to bind with equal preference to C- and N-fragments of HSA [7]. The similarity in the binding capabilities of bithionol and fentichlor are perhaps not surprising considering their structural similarity (Fig. 3).

Both fentichlor and bithionol produce radicals upon UV irradiation [17, 18] and are believed to react photochemically with proteins via free radical reactions.

The high selectivity exhibited by T_4CS in its photochemical binding to one region of the HSA molecule is believed to be due to the reactive T_4CS species having an extremely short lifetime, hence non-covalent association with the protein prior to irradiation is essential for photoconjugate formation [19]. Bithionol is very much less selective in its binding to HSA than is T_4CS, indicating a reactive species with a longer lifetime than for T_4CS, and indeed bithionol radicals have been shown to possess lifetimes of several minutes [17].

The fact that some bithionol remains bound to HSA in the absence of irradiation suggests the possibility that bithionol is able to associate non-covalently with HSA. If the binding site(s) for this association is in the same region of the HSA molecule as that for T_4CS [8], this would be a possible explanation for the preferential binding of bithionol to the N-fragment of HSA. Occupation of the T_4CS binding site on HSA by bithionol is quite feasible because of the close similarity in chemical structure between T_4CS and bithionol, i.e. both are substituted 2,4-dichlorophenols. Since the lifetime of the bithionol radical is of the order of a few minutes [17], the binding selectivity is much less than is observed for T_4CS.

The significance of differences in the binding selectivity of photoallergens to proteins has recently been linked to differences in photoallergic potential [20]. The greater photoallergic potential of T_4CS compared with that of fentichlor is attributed to the affinity of the former to bind photochemically to a single site on serum albumin.

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