Photoactivated Covalent Binding of [^3H]Bilirubin to Human Serum Albumin

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A one-step procedure has been developed for the preparation of [^3H]bilirubin IX- α in good yield from unlabelled bilirubin. Irradiation of an aqueous solution of [^3H]bilirubin IX- α in the presence of human serum albumin results in the covalent attachment of the bilirubin to the protein. Preliminary degradation studies have been carried out to locate the site of attachment of the bilirubin to the albumin.

Bilirubin is reversibly bound to serum albumin in plasma and is transported to the liver where it is conjugated and then excreted. Many experiments have been aimed at locating which domain of the albumin molecule binds bilirubin, but no conclusions have yet been made as to the exact site of binding. For example, affinity labelling of human albumin with bilirubin that had been activated with Woodward's reagent K (2-ethyl-5-*m*-sulphophenylisoxazolium hydroxide) (Gitzelmann-Cumarasamy *et al.*, 1976; Kuenzle *et al.*, 1976) indicates that two regions (residues 124–297 and residues 446–547) are attached to the bilirubin. However, objections can be raised to the activation of bilirubin by this reagent as the latter interacts initially with the carboxy groups of the bilirubin and for covalent attachment a suitably nucleophilic amino acid side chain of the albumin must be present in a favourable position in the binding site. Thus purely hydrophobic interactions between the albumin and the bilirubin are unlikely to be revealed by this method.

In the present paper we report the photoactivated covalent binding of [^3H]bilirubin to human serum albumin. This activation, which is brought about by light from a tungsten lamp, presumably involves free radicals and may not require the presence of nucleophilic groups in the binding site before a covalent attachment of the bilirubin to the albumin can occur. Thus this technique may reveal amino side chains that are involved in hydrophobic bonding to the bilirubin.

Materials and Methods

n-Butyl-lithium, di-isopropylamine and hexamethylphosphoric triamide were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and CNBr was obtained from Eastman Kodak Co., Kirkby, Liverpool, Lancs., U.K. Bilirubin IX- α and human serum albumin (fraction V) were obtained from Sigma (London) Chemical Co., Kingston upon

Thames, Surrey, U.K. The albumin was defatted before use by the method of Chen (1967).

Synthesis of [^3H]bilirubin

To a solution of di-isopropylamine (0.2 g, 2.0 mmol) in anhydrous tetrahydrofuran (80 ml) under an N_2 atmosphere was added *n*-butyl-lithium in hexane (1.0 ml, 1.6 M) and the mixture was stirred at -78°C for 30 min. Bilirubin (58.4 mg, 0.1 mmol) was added and the mixture was stirred at -78°C for 30 min after which hexamethylphosphoric triamide (1.5 ml, 8.55 mmol) was added. The mixture was stirred for a further 30 min and then trifluoro[^3H]acetic acid (0.546 ml, 4.8 mmol) was prepared by treating trifluoroacetic anhydride with an equimolar amount of $^3\text{H}_2\text{O}$ (5 Ci/ml; sp. radioactivity approx. 100 mCi/mmol). The reaction was stirred for 4 h at 21°C and then acidified with 0.1 M-HCl at 0°C . The aqueous solution was extracted with chloroform (3×100 ml), the chloroform extracts were combined, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The residual bilirubin was dissolved in chloroform (5 ml) and purified by preparative t.l.c. on silica gel with chloroform/methanol/water (40:9:1, by vol.) as solvent. The yellow band (R_f 0.75) was excised and eluted from the silica with chloroform. The chloroform was removed *in vacuo* and the residual bilirubin was crystallized from chloroform/methanol (1:1, v/v) to yield 19.5 mg. This bilirubin was then suspended in water and brought into solution by adding sufficient conc. aq. NH_3 (sp.gr. 0.88). After 5 min the solution was acidified with 1 mM-HCl and extracted with chloroform (100 ml). The chloroform was dried (Na_2SO_4) and evaporated *in vacuo* to give [^3H]bilirubin (19.3 mg, 33% yield from unlabelled material). The bilirubin ran as a single spot on t.l.c. (McDonagh & Assissi, 1971), had a light-absorption maximum in chloroform at 454 nm (ϵ 59 650 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$) and had a sp. radioactivity of 1.3 mCi/mmol. Diazotization (Hutchinson *et al.*, 1972) of the [^3H]bilirubin

gave azopigments with at least 99% of the specific radioactivity of the bilirubin.

Covalent binding of [^3H]bilirubin to human serum albumin

A solution of [^3H]bilirubin ($65\mu\text{M}$) was prepared by dissolving 3.8mg (sp. radioactivity 1.3×10^9 c.p.m./mmol) in 0.2M-NaOH (0.2ml) and diluting to 100ml with 0.1M-sodium phosphate buffer, pH 7.4. A mixture of this solution (5ml) and a solution of human serum albumin (5ml, $65\mu\text{M}$) in 0.1M-sodium phosphate buffer, pH 7.4, was irradiated at 40cm from a tungsten lamp (150W) for 1 h.

The reaction mixture was then applied to a column (0.90cm $\times$ 15.0cm) of Sepharose-albumin (Kuenzle *et al.*, 1976). The column eluate was dialysed against 2 litres of 12.5mM-sodium salicylate, pH 7.4, for 48 h and then against 2 litres of 0.1M-HCl for 24 h, after which time a non-diffusible bilirubin-albumin complex (5×10^7 c.p.m./mmol of albumin) was obtained. In a typical experiment, after irradiation for 1 h a solution of albumin (5.0mg/ml) had an absorption of 0.50 at 450nm after chromatography on Sepharose-albumin. When the experiment was repeated with degassed solutions and with N_2 flushing during the irradiation, the residual specific radioactivity was virtually the same. If the irradiation step was omitted the specific radioactivity of the bilirubin-albumin complex was 2×10^6 c.p.m./mmol of albumin.

Degradation of bilirubin-albumin complex by CNBr

The bilirubin-albumin complex was dissolved in formic acid and degraded with CNBr (McMenamy

et al., 1971). After chromatography, three peptides were obtained [A (residues 298–585), B (residues 1–124) and C (residues 125–297)], the identities of the peptides being confirmed by the determination of their *N*-terminal amino acids. For the complex obtained after irradiation for 60min in the air, the maximum radioactivity (62%) was found in peptide B with lesser amounts in peptides C (32%) and A (3%). When the albumin obtained from the non-irradiated sample was degraded with CNBr no radioactivity could be detected in any of the peptides.

Discussion

Treatment of the lithium derivative of acetophenone with trifluoro[^3H]acetic acid has been described recently as a method for the synthesis of [^3H]acetophenone in high yield, the latter being used for the preparation of phenyl[2- ^3H]glyoxal (Augustus & Hutchinson, 1979). We have now adapted this method for the one-step preparation of [^3H]bilirubin from unlabelled material. This method is preferable to the two-step procedure involving the preparation of bilirubin dimethyl ester (Hancock *et al.*, 1976) as the overall yield is higher and the specific radioactivity of the [^3H]bilirubin obtained is greater. By analogy with other reactions of metallated carboxylic acids (Creger, 1977), we assume that only one ^3H atom is introduced into each α -methylene group of the bilirubin. No ^3H is introduced into the bridge methylene group as diazotization of the [^3H]bilirubin yields azopigments that contain at least 99% of the initial radioactivity.

Irradiation of a mixture of [^3H]bilirubin together with one molar equivalent of human serum albumin with light from a tungsten lamp resulted in the bilirubin becoming firmly bound to the protein. Flushing the reaction mixture with N_2 before and during the irradiation did not have an appreciable effect on the amount of radioactivity that is firmly bound to the albumin. This suggests that either the binding is not an oxidative process, but may involve the addition of an amino acid side chain of the albumin to one or both of the *exo*-vinyl groups of the bilirubin (Manitto & Monti, 1972), or that trace amounts of O_2 remaining in the system are sufficient to allow the photo-oxidation of bilirubin to take place (Bonnett, 1976). The photo-activated bilirubin could then react with an amino acid side chain in the albumin molecule. The relative concentrations of albumin and bilirubin in these preliminary experiments were chosen so that high-affinity binding site in the albumin is just fully occupied. Thus it is hoped that complications due to the binding of bilirubin at high- and low-affinity sites do not arise to a significant extent.

We have found that it is difficult to remove final traces of radioactive bilirubin from the albumin even when no irradiation has taken place. A combination of chromatography on an affinity column of human serum albumin-Sepharose (Kuenzle *et al.*, 1976) and exhaustive dialysis against sodium salicylate and 0.1 M-HCl failed to remove the final traces of radioactivity. However, the covalent attachment of bilirubin to the albumin after irradiation could be clearly demonstrated by degradation of the latter with CNBr (McMenamy *et al.*, 1971). No radioactivity was associated with the degradation products unless irradiation of the bilirubin/albumin mixture had taken place.

The majority of the radioactivity (62%) is associated with the peptide corresponding to residues 1–124 and 32% was associated with residues 125–297. This contrasts with the observations of Gitzelmann-Cumarasamy *et al.* (1976), who found that two regions in the albumin (residues 124–297 and 446–547) become bound to the bilirubin. This difference may be due to the difference in manner in which the bilirubin is activated. After activation with Wood-

ward's reagent K, the carboxy groups of the bilirubin may be unable to react with any amino acid side chains in the binding site, but may react with nucleophilic groups that are remote from this site. Alternatively, in our experiments the *Z,Z*-isomer of bilirubin IX- α may undergo a configurational change during irradiation to one of the three other geometric isomers of bilirubin IX- α (McDonagh *et al.*, 1979) before reaction with the albumin takes place. We have been unable to detect chromatographically the formation of other isomers of bilirubin, e.g. the III- α or XIII- α isomers, during our irradiation experiments. It is relevant that Geisow & Beaven (1977) found that bilirubin bound non-covalently to two albumin-derived peptides corresponding to residues 1–386 and 49–307 respectively.

Further experiments are required to investigate the nature of the binding reaction and to locate exactly the site of attachment of the bilirubin to the albumin.

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