The ^3H -Labelling of Bilirubin

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A simple method has been developed for the preparation of ^3H -labelled bilirubin IX- α from bilirubin dimethyl ester. The label is located in the propionic acid side chains, and there is no isomerization of the bilirubin during the preparation.

Methods currently available (Manitto *et al.*, 1975; Plieninger *et al.*, 1972; Howe *et al.*, 1970; Lester & Klein, 1966; Grodsky *et al.*, 1962) for the preparation of radioactively labelled bilirubin are often difficult, and do not give good yields of material with a high specific radioactivity. We now report a simple method for the preparation of ^3H -labelled bilirubin which relies on the base-catalysed exchange of hydrogen α to the carbonyl groups in the propionic acid side chains of bilirubin dimethyl ester.

Materials and Methods

Bilirubin was obtained from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and was converted without further purification into the dimethyl ester by using 1-methyl-3-*p*-tolyltriazene (Hutchinson *et al.*, 1973). *NN*-Dimethylformamide was purified by distillation *in vacuo* from barium oxide, and was de-gassed before use.

To a suspension of bilirubin dimethyl ester (280 mg, 0.48 mmol) in *NN*-dimethylformamide (5 ml) was added sodium methoxide (100 mg, 1.9 mmol) and $^3\text{H}_2\text{O}$ (100 μl , 500 mCi), and the mixture shaken under N_2 in the dark at 4°C for 24 h. Chloroform (100 ml) was then added and the solution washed with 1 M-HCl (50 ml) followed by water (3×100 ml). The chloroform was dried (Na_2SO_4) and the solvent removed *in vacuo*; the residual bilirubin was crystallized from chloroform/methanol (1:1, v/v), the yield being 110 mg. The bilirubin was then suspended in water and brought into solution by adding sufficient conc. NH_3 (sp.gr. 0.88). After 5 min the solution was acidified with 1 M-HCl and extracted with chloroform (100 ml). The chloroform was dried (Na_2SO_4) and evaporated *in vacuo* to give bilirubin (110 mg; 40% overall yield). The bilirubin ran as a single spot on t.l.c. (McDonagh & Assissi, 1971), had a light-absorption maximum in chloroform at 454 nm ($\epsilon = 60000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and had a specific radioactivity of 300 $\mu\text{Ci}/\text{mmol}$.

Results and Discussion

Under suitable conditions, bilirubin dimethyl ester can be converted in one reaction vessel into

^3H -labelled bilirubin. The yield of bilirubin obtained in this manner is comparatively high, provided that air and light are excluded from the reaction. An alternative method in which the ^3H -labelled bilirubin dimethyl ester was isolated before hydrolysis was rejected, because of lower yields and increased experimental manipulation. The ^3H exchange and ester hydrolysis are both second-order reactions, and the conditions described above (bilirubin/methoxide/water, mol.prop. 1:4:5.5) were selected so that appreciable exchange took place in the dimethyl ester before hydrolysis. Crude bilirubin obtained from the exchange reaction contains non-covalently bound ^3H , possibly owing to the intramolecular hydrogen-bonding in bilirubin (Kuenzle *et al.*, 1973; Knell *et al.*, 1975; Manitto & Monti, 1976). This is removed by adding to an aqueous suspension of bilirubin sufficient NH_3 to obtain solution. Immediate acidification of the aqueous solution so obtained, followed by extraction with chloroform, gives a quantitative recovery of bilirubin with a constant specific radioactivity. The bilirubin can be recrystallized from chloroform/methanol (10:1, v/v) with unchanged specific radioactivity. The ^3H is presumed to be located α to the carboxyl groups of the propionic acid side chains and not in the methylene bridge of bilirubin, as diazotization (Hutchinson *et al.*, 1972) of ^3H -labelled bilirubin gave azopigments with more than 99% of the specific radioactivity of the bilirubin.

In view of the known instability of bilirubin in alkaline (De Ewenson *et al.*, 1966) and in acidic solution (McDonagh & Assissi, 1972), it is possible that the bilirubin might isomerize under the conditions of the exchange or isolation. However, the only species that could be detected by nuclear-magnetic-resonance spectroscopy in C^2HCl_3 or by t.l.c. was bilirubin IX- α .

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