PREPARATION OF BILIRUBIN SPECIFICALLY TRITIATED AT THE exo - VINYL GROUP

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Clarification of several important aspects of bilirubin metabolism, e.g. plasma transport, tissue distribution, intestinal reabsorption etc., requires preparation of the radioisotopically labelled pigment of high specific activity. A number of methods for introducing label into bilirubin have been reported ; they include :i) biosynthesis from [2 -^{14}C]-glycine[1] and from δ -aminolevulinic acid labelled with tritium (at C-3 and C-5) or with ^{14}C (at C-4)[2] ; chemical synthesis from 2-formyl-3-methyl-4-(β -carboxyethyl)pyrrole [formyl -^{14}C][3] ; tritiation by the Wilzbach technique[4] . However, all of these methods are subjected to a lot of limitations, e.g. low intensity of labelling, cost of the labelled precursors, laboriousness etc.

We report here a simple procedure for preparing [8b -^{3}H$_2$] bilirubin IXα (1), which requires only commercial bilirubin and tritiated water as starting materials (Scheme 1).

Such a method is based on the pyrolysis of the bilirubin derivative (2) prepared by acid-catalyzed addition of thioacetic acid to bilirubin in chloroform[5]. In fact, we found that the adduct (2), when heated under vacuum undergoes thioacetic acid elimination affording bilirubin IXα in good yield. Therefore, if all the reagents of the reaction from (1) to (2) are previously equilibrated with tritiated water for exchangeable protons, (1 , H* = ^{3}H) can be easy obtained through the above addition-elimination sequence.

To exclude significant randomisation of the label during the preparation of tritiated bilirubin, a monitoring experiment was carried out using D_2O (99.5 % D) to equilibrate bilirubin and thioacetic acid. Specific deuteration at the 8b-position of (2 , H* = ^{2}H) and (1 , H* = ^{2}H) was demontrated by their MS and NMR spectra. Particularly, the 100 MHz PMR spectrum of deuterated bilirubin IXα (1 , H* = ^{2}H) exhibited in the vinyl group region a marked change only of the ABC pattern due to the exo-vinyl protons

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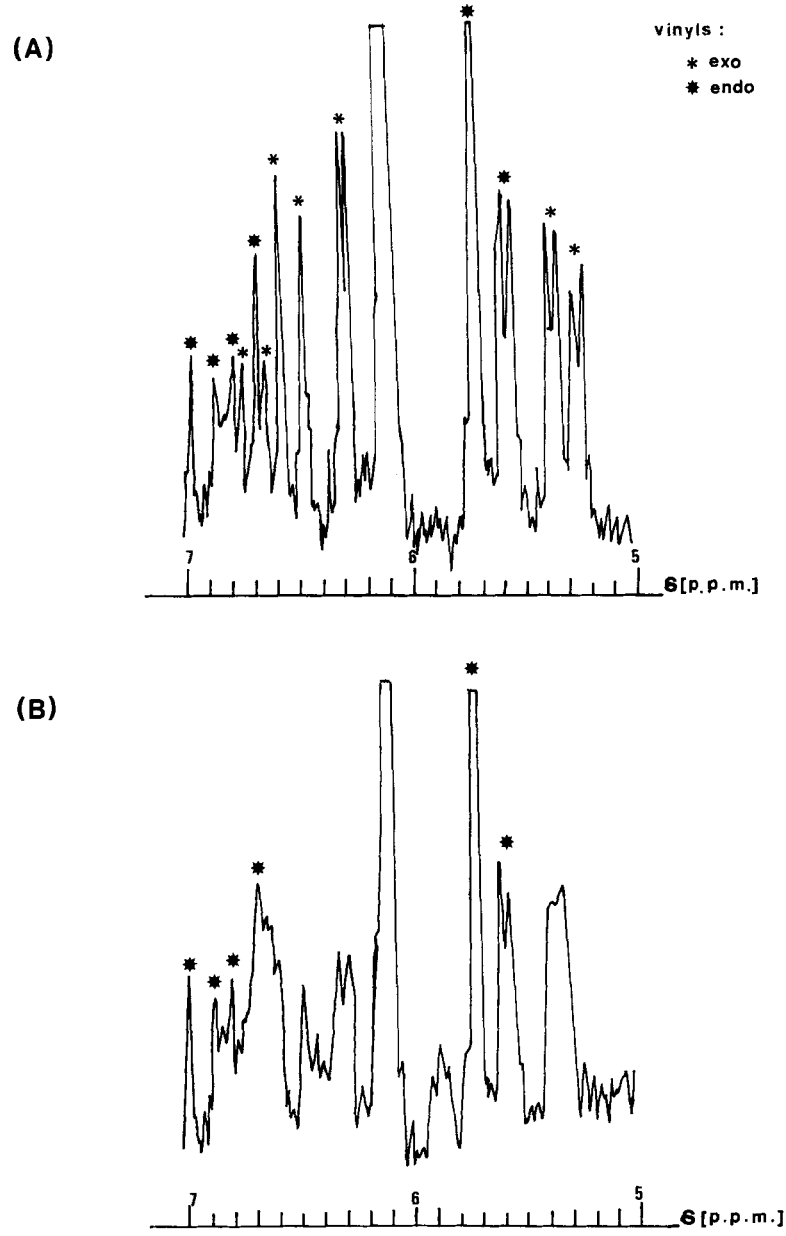
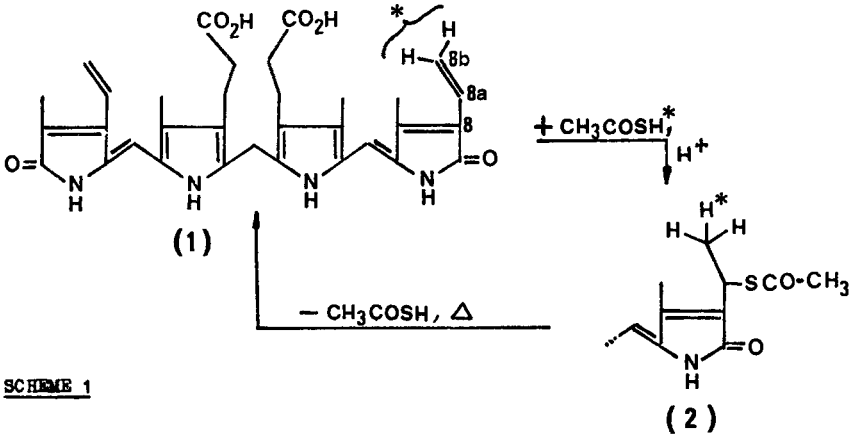


Fig.1. PMR spectra (at 100 MHz) of natural bilirubin IX α (A) and of [8b - $^{2}H_{2}$] bilirubin IX α (B) in DMSO-d_6. Only vinyl group region is reported ; chemical shifts are in parts per million from internal tetramethylsilane.

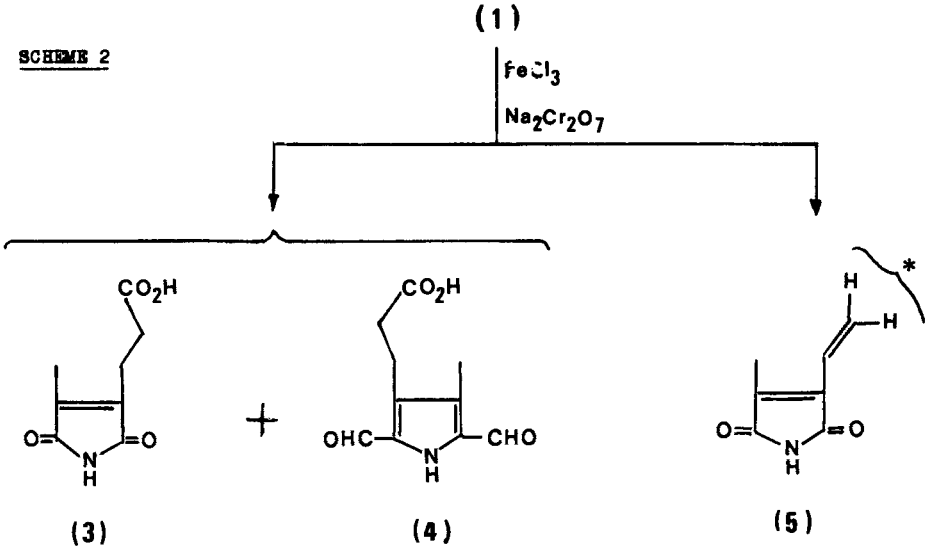
(Fig. 1). The attribution of the peaks to each vinyl group is based on a comparison of the PMR spectrum of bilirubin IX α with those of bilirubin III α (both vinyl groups in exo-position) and bilirubin XIII α (both vinyl groups in endo-position)[5].



SCHEME 1

Additional evidence for the absence of labelling in sites of the molecule other than the exo-vinyl group was obtained by chromate degradation[6] of tritiated bilirubin prepared as described above. Thus, only methylvinylmaleimide (5) arising from the outer pyrrole rings of bilirubin IX α (1) was found to be radioactive (Scheme 2).

SCHEME 2



We thank Dr. G.Severini Ricca for running PMR spectra.

EXPERIMENTAL

T.l.c. was carried out on plates coated with Merck silica gel GF_{254} . I.r. spectra were recorded for solutions in chloroform on a Perkin-Elmer 257 spectrometer; p.m.r. spectra for solutions in hexadeuterodimethylsulfoxide on a Varian XL-100-FT spectrometer at 100 MHz ; u.v. spectra for solutions in chloroform on a Perkin-Elmer spectrophotometer Mod. 402 . Mass spectra were determined with an LKB 9000 simple-focusing mass spectrometer by direct inlet system.

Radioactive samples were counted on a Packard Tricarb liquid scintillation spectrometer Mod. 3320 (internal standard [^{3}H] toluene) ; the composition of the solvent-scintillator was : POP (6.5 g) , dimethylPOPOP (130 mg), and naphthalene (104 g) in toluene (500 ml) and dioxan (500 ml).

To reduce the color quenching of bilirubin and its thioadduct, a chloroform solution of the sample (30 -100 μg in 0.5 ml) was placed into a glass counting vial and evaporated under a slow stream of nitrogen. After addition of 0.2 ml of a solution of ammonium persulfate in 0.1-N NaOH (1 mg/ml) the vial was closed with its cap and kept at r.t. overnight . The above liquid-scintillator was then added to the resulting colorless solution before counting.

Preparation of [8b -$^3H_2$] bilirubin IXα

Bilirubin (500 mg) and thioacetic acid (1ml) in chloroform (500 ml) were shaken with tritiated water (80 ml, specific activity 6.4 mCi/ml) for 2 h. After separating the water layer , a few crystals of p-toluensulfonic acid were added to the chloroform solution previously dried on anhydrous sodium sulfate. The reaction mixture was kept at r. t. overnight, then the excess of p-toluensulfonic acid was filtered off and pure ($\underset{\sim}{2}$, $H^* = {}^3H$)(412 mg) was recovered by evaporation of the solvent under vacuum and by washing of the residue with methanol. Such a product was characterized and checked for its purity by p.m.r. , i.r. , u.v. spectra and t.l.c.[5] ; its spec. activity was $915 \cdot 10^5$ disint. $min.^{-1}$ $mmol^{-1}$. 20 mg of the so prepared compound was pyrolyzed by heating at 280° for 45 min. under vacuum ($6 \cdot 10^{-4}$ mmHg). The crude product of pyrolysis was then extracted with chloroform and the insoluble residue separated by centrifugation. After adding methanol to the chloroform solution and evaporating to a small volume, [8b -$^3H_2$] bilirubin IXα crystallized as orange needles (11 mg ; pure in t.l.c. : benzene - chloroform-

methanol 100:50:1.2 as eluent ; m.s., i.r. and p.m.r. spectra identical with those of an authentic radioinactive sample; u.v. 452 nm , ε 60,000 $\pm$ 1,000 [7,8] ; specific activity 452 . 10^5 disint. $min.^{-1}$ $mmol^{-1}$, which was shown to be constant after repeated crystallization).

Chromate degradation of bilirubin IXα

Tritiated bilirubin (ca. 1 mg) prepared as described above was dissolved in dimethylsulfoxide (1 ml). When a few crystals of $FeCl_3 . 6H_2O$ were added, the mixture turned green immediately. After 1 h at r. t. this mixture was added to 1.0 ml of an aqueous solution of potassium hydrogen sulfate (1%) and sodium dichromate (1%), stirred for 3 h at r.t., diluted with water (20 ml), and extracted with ethyl acetate (3 x 5 ml). The organic layer was washed with saturated sodium hydrogen carbonate solution (2 x 10 ml), dried on anhydrous $MgSO_4$, and evaporated to leave a residue , which was shown to be pure methylvinylmaleimide by t.l.c. comparison with an authentic sample[6] (eluent : benzene - diethyl ether 9:1 ; detection by the color reaction of the imides with chlorine benzidine[6]). Such a residue, when counted, exhibited 218 .10^2 disint. $min.^{-1}$.

The sodium hydrogen solution used to wash the above ethyl acetate extract was made acid with conc. HCl and extracted with ethyl acetate (3 x 10 ml). The organic extract was washed with water (2 x 5 ml), dried on anhydrous $MgSO_4$, and evaporated to give a mixture of hematinimide (3) and pyrrole-2,5-dicarbaldehyde (4) as shown by t.l.c. comparison with authentic samples[6] (eluent : ethyl acetate - ethanol 8.5:1.5 ; detection by spraying with 2,4-dinitrophenylhydrazine[6]). The activity of the acid mixture resulting from bilirubin degradation was ca. 580 disint. min^{-1}.

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