Short Communications

The Reaction between Bilirubin and Aromatic Diazo Compounds

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Bilirubin (I) reacts with diazotized aromatic amines and is cleaved at the central methylene bridge to form two isomeric azopigments (II and III). The reaction between bilirubin (I) and diazotized sulphanilic acid is used to determine the concentrations of bilirubin and its conjugates in sera [the van den Bergh reaction (Hijmans van den Bergh & Snapper, 1913)]. The structures of the azopigments derived from diazotized ethyl anthranilate and aniline have been established (Jansen & Stoll, 1971), and the reaction with diazotized ethyl anthranilate has been used in the deter-

mination of the structures of bilirubin conjugates (Compernelle *et al.*, 1970). The van den Bergh reaction is thought to occur in two steps, and it has been suggested that the central methylene bridge carbon atom of bilirubin (I) is released as formaldehyde during the second step of the reaction (Overbeek *et al.*, 1955). However, formaldehyde could not be detected in the distillate from the van den Bergh reaction by using 3-methyl-2-benzothiazolone hydrazone, which reacts with formaldehyde to form a blue colour (Fog & Jellum, 1962).

M = CH₃; V = CH=CH₂; P = CH₂-CH₂-CO₂H

Scheme 1. Suggested mechanism for the formation of formaldehyde from bilirubin (I)

Scheme 2. Reaction between dimedone and formaldehyde

A mechanism for the formation of formaldehyde from bilirubin (I) is suggested in Scheme 1; it should be noted that initial attack by the diazonium ion could occur at either of the pyrrole rings flanking the central methylene bridge.

We now report that formaldehyde is formed during the van den Bergh reaction and can be detected as formaldehyde dimethone (V), the product of the reaction between formaldehyde and 5,5-dimethylcyclohexane-1,3-dione (dimedone) (IV) (Scheme 2).

Experimental

With free bilirubin, the van den Bergh reaction can be carried out in a chloroform-methanol medium. Since both these reagents contain C_1 units that could give rise to formaldehyde, this solvent system was abandoned in favour of a 1,2-dichloroethane-2-methylpropan-2-ol medium, although the reaction was much slower in this medium.

Bilirubin (195mg, 0.33mmol) was suspended in a mixture of 1,2-dichloroethane (50ml) and 2-methylpropan-2-ol (50ml). A solution of benzenediazonium chloride (2mmol) was added and the mixture stirred in a closed flask, in the dark, at room temperature for 30min. A further quantity of benzenediazonium chloride (2mmol) was added and the solution stirred for 30min more. The reaction mixture was then evaporated to dryness, the solvents being distilled into a solution of dimedone (200mg) in 2-methylpropan-2-ol (50ml), with the receiver connected to a second vessel containing a similar solution of dimedone in 2-methylpropan-2-ol. The dimedone solu-

tions were combined and heated under gentle reflux for 30min. After cooling and removal of the solvents *in vacuo*, t.l.c. of the residue (on silica gel, with chloroform as solvent) showed the presence of formaldehyde dimethone (V), R_F 0.44. No formaldehyde dimethone (V) was obtained from two control reactions, one in which the benzenediazonium chloride was omitted and the other in which the bilirubin was omitted. The formaldehyde dimethone (V) was isolated by elution from a silicic acid column with light petroleum (b.p. 60–80°C)–diethyl ether (3:1, v/v). The isolated material was identical in all respects with an authentic sample of formaldehyde dimethone (V). The recovery of the compound (V) was up to 20% (20mg) of the theoretical amount. When 1,2-dichloroethane (50ml) and 2-methylpropan-2-ol (50ml) containing formaldehyde [10mg, equivalent to 195mg of bilirubin (I)] were distilled as above, the formaldehyde dimethone (V) recovered never exceeded 20% of the theoretical amount (100mg).

Materials

Bilirubin was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and was purified by the method of Fog (1964) before use (Found: C, 67.9; H, 6.0; N, 9.2; $C_{33}H_{36}N_4O_6$ requires C, 67.8; H, 6.2; N, 9.6%). It had a light-absorption maximum in chloroform at 454nm (ϵ 59400). 1,2-Dichloroethane and 2-methylpropan-2-ol were distilled before use, and the dimedone was used without further purification.

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