in vivo since it produces thrombosis when injected alone.

The application of a water-insoluble trypsin preparation has proved very useful, because centrifugation ensures its rapid removal and the risk of instability arising from prolonged contact is greatly decreased. Trypsin coupled to agarose was selected as this form is more permeable to proteins (Porath, Axén & Ernback, 1967). It was obtained as a 25% (v/v) suspension from Miles-Serevac Ltd. (Maidenhead, Berks., U.K.), and before use was resuspended in distilled water and sedimented by centrifugation. The barium sulphate-adsorbable proteins of pig serum were prepared as described by Howell & Scott (1964) and utilized as a source of Factor X precursor. The insoluble trypsin preparation was first standardized against soluble trypsin by estimating their respective activities towards *N*^α-benzoyl-L-arginine ethyl ester. The solution of barium sulphate-adsorbable proteins was diluted 1:10 in 50mM-imidazole buffer, pH 7.4, and equilibrated at 37°C, and 2ml was added to the insoluble trypsin from 0.5ml of the original suspension (0.5ml on the basis of its activity towards *N*^α-benzoyl-L-arginine ethyl ester). The mixture was agitated continuously over a contact period of 2min and centrifuged twice, and the clotting activity of the supernatant was determined in a Factor X assay (Denson, 1961). At the concentration used activation of 2ml of the diluted solution of barium sulphate-adsorbable proteins was comparable with that produced by 5μg of soluble trypsin or by a 1:600 000 dilution of Russell's-viper venom. In another experiment clotting times were plotted on double-logarithmic paper against increasing concentrations of barium sulphate-adsorbable proteins activated with either Russell's-viper venom or insoluble trypsin, and in each case typical straight lines were obtained.

Insoluble trypsin can therefore replace Russell's-viper venom in the controlled activation of Factor X and possesses the additional advantage that it may be recovered and used again.

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Separation and Structural Analysis of Vinyl and Isovinyl Azobilirubin Derivatives

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Compernelle, Jansen & Heirwegh (1970) reported the separation of ethyl anthranilate azobilirubin into two components, which are the vinyl and isovinyl isomers. The methyl derivatives of these products each consisted of two isomers of ethyl anthranilate azobilirubin methyl ester, which were considered possibly to be *syn-anti* isomers.

This paper describes the preparation and identification of vinyl and isovinyl ethyl anthranilate azobilirubins derived from bilirubin and from biological sources, in order to establish which half of the bilirubin molecule is involved in monoglycosidic conjugation.

Bilirubin was coupled with the diazonium salt of ethyl anthranilate (Heirwegh, Van Hees, Leroy, Van Roy & Jansen, 1970). The products were separated as methyl esters by t.l.c. Similar quantities of two major products were obtained (93–95% yield), which were shown to be homogeneous by further t.l.c. Mass spectrometry showed that the two products were isomeric, but differed slightly in their fragmentation pattern. N.m.r. spectrometry demonstrated that the two compounds differed in the positions of the substituents in the pyrrolenone end ring; in one compound the methyl and vinyl groups were interposed compared with the other compound. Methylation studies, together with evidence obtained from i.r. spectra, showed that azobilirubin exists in the lactam form.

These model compounds prepared from bilirubin were used as reference standards for investigation of the specificity of glycosidic conjugation of bilirubin in rats and in an *in vitro* system, composed of liver homogenate incubated with bilirubin and UDP-glucuronic acid (Black, Billing & Heirwegh, 1970), in which bilirubin monoglucuronide is formed. Analysis of the derived azobilirubin and azobilirubin conjugates showed that conjugation at the propionic acid side chain of both sides of the bilirubin molecule can occur. Azopigments prepared with phenyldiazonium chloride from bilirubin and bile gave similar results. The physical properties of one of these derivatives were in agreement with those of the azopigment prepared from vinylneoxanthobilirubin acid (Kuenzle, 1970).

During preparation of ethyl anthranilate reference compounds a series of minor components were

isolated by t.l.c. Analysis of mass spectra of many of these components showed that three side reactions can occur: (1) methylation of the imide carbonyl group, (2) addition of methanol to the vinyl substituent and (3) transmethylation of the ethoxycarbonyl group.

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