human serum at 5.0×10^{-4} M inhibitor using the same incubation conditions as those employed for choline acetylase. Further details on the synthesis of related compounds, as well as the biochemistry and pharmacology, will be reported in a later publication.

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Intramolecular Hydrogen Bonding in Bilirubin

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Fog and associates,[1,2] after studying infrared spectra of bilirubin, mesobilirubin, and dimethyl-mesobilirubin, have suggested that bilirubin contains intramolecular hydrogen bonds, as shown in formula I, and have pointed out that this structure would explain the relative stability of bilirubin in chloroform solution. This possibility has been investigated further after exchange of hydrogen with tritium or deuterium in aqueous alkaline solutions of bilirubin sodium salt.

Bilirubin (Sigma, sigma grade) 50 mM dissolved in 0.25 M sodium hydroxide solution at room temperature, with HTO added to 2 mC/ml, was precipitated after one minute by addition of hydrochloric acid. The precipitate was washed twice with HTO in water, dried *in vacuo*, dissolved in toluene, and the radioactivity was measured in a liquid scintillation spectrometer (Packard, Tricarb 2002). The concentration of bilirubin was determined by spectrophotometry. In six experiments it was found that 4.5 — 3.5 — 3.6 — 4.1 — 4.2 and 4.6 atoms of hydrogen had been exchanged. Further drying did not alter the results. The four hydrogen atoms exchanged are presumably two in the carboxyl groups and two connected to nitrogen in the end rings, since all these are acidic protons.

Exchange with deuterium was done under identical conditions with final enrichment 99.3 % D. Infrared spectra (recorded on Beckman IR 7) of the deuterated bilirubin, and of similarly treated bilirubin without deuterium, in KBr discs are seen in Fig. 1. The sharp band at 3420 cm^{-1} has the frequency expected for the NH stretching mode in a cyclic γ-lactam, when this is not hydrogen-bonded.[3] In a bonded compound this band would be found at 3175 cm^{-1}. Half of this absorption shifts to 2550 cm^{-1} on exchange with deuterium. This corresponds to the expected shift for deuteration of the NH-groups. The formula [4]

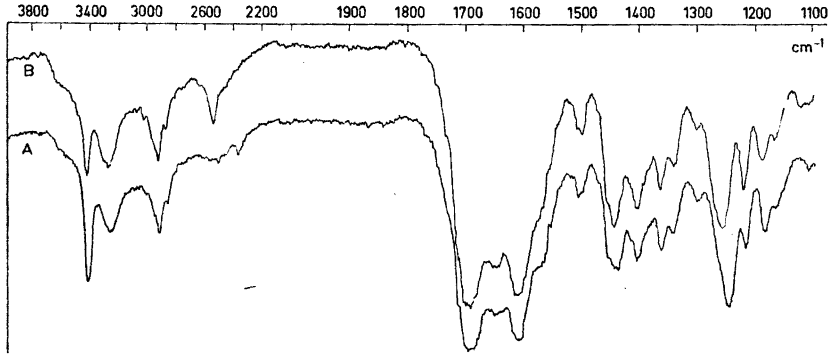


Fig. 1. IR-spectra of bilirubin (A) and deuterated bilirubin (B) in KBr discs.

$$\frac{\nu_{N\text{-}D}}{\nu_{N\text{-}H}} = \left(\frac{\frac{M_N M_H}{M_N + M_H}}{\frac{M_N M_D}{M_N + M_D}} \right)^{\frac{1}{2}}$$

gave the frequency 2500 cm^{-1}. The place of deuteration must be the two NH of the end rings.

The remaining part of the absorption at 3420 cm^{-1} is presumably due to the two NH of the central (pyrrol) rings. All four NH-groups are thus un-bonded in the solid state. The carboxyl groups appear to be hydrogen-bonded, as seen from the strong CO-band at 1695 cm^{-1} and the lack of a free OH-stretching frequency.

Formula II, with double hydrogen bonds between the carboxyl groups, is suggested for bilirubin in a solid (amorphous) state, freshly precipitated with acid from an aqueous solution of its salt. This molecule has been built from Courtauld atomic models (Baird & Tatlock) and is free from strain. Dimer or polymer forms with intermolecular hydrogen bonds between carboxyl groups would likewise explain the findings.

Chloroform solutions of deuterated and non-deuterated bilirubin were prepared by extraction of the freshly precipitated substances. Infra-red spectra were recorded with chloroform in reference cells (Fig. 2). In these spectra the NH-band at

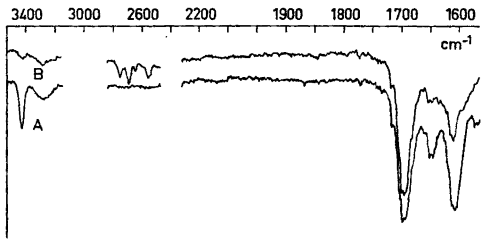
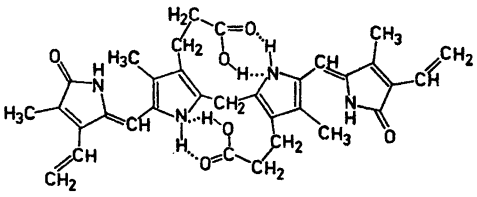


Fig. 2. IR-spectra of bilirubin (A) and deuterated bilirubin (B) in chloroform solution, 3 mM, light path 0.5 mm, with chloroform in reference cells. Ranges with strong absorption due to chloroform are omitted.

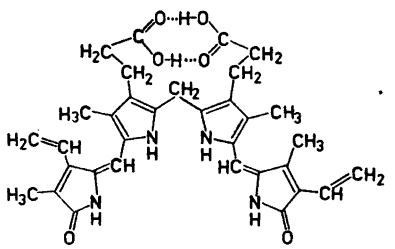
3420 cm^{-1} is shifted totally by exchange with deuterium and therefore must represent the NH-groups of the end rings only. The two central NH-groups are probably hydrogen-bonded and their bands are hidden by the chloroform absorption around 3100 cm^{-1}. Fog's formula (I) ex-



Formula I

plains these findings. Working with the Courtauld models it has not been possible to construct any other monomer bilirubin molecule with the end rings free and both central NH-groups and carboxyl groups hydrogen-bonded. The structure shown is free from strain.

Summary. Infra-red spectra of bilirubin, and of bilirubin with four acidic protons exchanged with deuterium, are in agreement with the presence of intramolecular hydrogen bonds, as shown in formula II in the solid state, and in formula I in chloroform solution.



Formula II

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