Short Communication

Tautomerism and Hydrogen Bonding in Bilirubin

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The tautomerism of bilirubin has been the subject of recent investigations (Kuenzle, 1970; Nichol & Morell, 1969) and we now present additional evidence that the lactam form is the predominant tautomer in solution and in the solid state.

The ^1H n.m.r. spectrum of bilirubin (40 mg, $70\ \mu\text{mol}$) in carefully dried $[\text{}^2\text{H}_6]$ dimethyl sulphoxide (0.5 ml) exhibits broad singlets at $\delta = 11.90$ (2H), 10.50 (2H), 10.10 (1H) and 9.95 (1H) p.p.m. (Kuenzle, 1970), but no signal at 5.25 p.p.m., which has been attributed (Nichol & Morell, 1969) to the enolic protons of the lactim tautomer. We have found that, although all low-field signals collapse on addition of deuterium oxide ($20\ \mu\text{l}$, 1 mmol), when water ($10\ \mu\text{l}$, 0.5 mmol) is added to a similar solution of bilirubin in $[\text{}^2\text{H}_6]$ dimethyl sulphoxide only the signal at 11.90 p.p.m. disappears. The n.m.r. spectra of solutions of *N*-benzoylglycine or 2,4-dimethylpyrrole-3-propionic acid (Morsingh & MacDonald, 1960) in dry $[\text{}^2\text{H}_6]$ dimethyl sulphoxide are also affected by the addition of water. In these cases the signals due to hydroxylic protons disappear, whereas signals due to protons attached to nitrogen atoms persist. These results suggest that in bilirubin two protons are attached to oxygen atoms and that the lactam tautomer is the predominant form in solution in dry $[\text{}^2\text{H}_6]$ dimethyl sulphoxide.

Tritiated bilirubin was prepared by direct exchange (Brodersen, Flodgaard & Krogh-Hansen, 1967) in an atmosphere of dry nitrogen. The specific radioactivity of two preparations, carefully dried to constant radioactivity, indicated that 5.95 (average of seven determinations) and 6.03 (average of six determinations) atoms of tritium had been incorporated into each molecule of bilirubin. An earlier report that only four atoms of tritium are incorporated per bilirubin molecule (Brodersen *et al.* 1967) may be explained by the extreme rapidity of exchange of the hydroxylic protons with adventitious water.

Additional evidence that all six protons in bilirubin can undergo exchange is provided by i.r.

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spectroscopy. The i.r. spectrum (Nujol mull or potassium chloride disc) of fully deuterated bilirubin, prepared as described above by direct exchange in an atmosphere of dry nitrogen, has no absorption maxima in the region $3000\text{--}3600\text{ cm}^{-1}$ due to O-H or N-H vibrations. If a potassium bromide disc of deuterated bilirubin is prepared, deuterium-proton exchange with the slightly hygroscopic matrix produces absorption maxima in the region of 3400 and 3225 cm^{-1} , as found for undeuterated bilirubin (Newbold & LeBlanc, 1964). The

Fig. 1. Tautomeric forms of bilirubin.

carbonyl stretching vibration of the propionic acid side chains of undeuterated bilirubin appears in the i.r. spectrum at 1690cm^{-1} (Brodersen *et al.* 1967; Fog & Jellum, 1963; Newbold & LeBlanc, 1964). We have found that the position of this band does not alter whether the sample is prepared as a Nujol mull, potassium bromide disc or in solution (chloroform or dioxan). In contrast, the carbonyl stretching vibration of 2,4-dimethylpyrrole-3-propionic acid is strongly dependent on the medium, ranging from 1690cm^{-1} (Nujol mull) to 1735cm^{-1} (dioxan solution).

These results suggest that the carbonyl groups of the propionic acid side chains in bilirubin participate in strong intramolecular hydrogen bonds and that the hydrogen-bonding does not alter with medium. Two structures (I and II, Fig. 1) are possible on steric grounds (Brodersen *et al.* 1967) and we believe that the conformation of the molecule is the same in solution and in the solid state. Hydrogen-bonding between the hydroxyl groups of the propionic side chains and the π -electron system of the pyrrole rings (Kuenzle, 1970) should not lower the carbonyl stretching frequency to 1690cm^{-1} and we consider that this form of bonding is unlikely in bilirubin.

Materials. Bilirubin was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and recrystal-

lized from chloroform-methanol (Found: C, 67.9; H, 6.0; N, 9.2; $\text{C}_{33}\text{H}_{36}\text{N}_4\text{O}_6$ requires C, 67.8; H, 6.2; N, 9.6%). It had a light-absorption maximum in chloroform at 454nm (ϵ 59400). Deuterated bilirubin had an absorption maximum in chloroform at 450nm (ϵ 58300).

Infrared spectra were recorded with a Perkin-Elmer 621 spectrometer and ^1H n.m.r. spectra were recorded with a Perkin-Elmer R12 spectrometer. The tritium content of samples was measured with a Packard 4000 scintillation counter by using a dioxan-based scintillation medium containing naphthalene (60g/l), 2,5-diphenyloxazole (4g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l).

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- Brodersen, R., Flodgaard, H. & Krogh-Hansen, J. (1967). *Acta chem. scand.* **21**, 2284.
Fog, J. & Jellum, E. (1963). *Nature, Lond.*, **198**, 88.
Kuenzle, C. C. (1970). *Biochem. J.* **119**, 395.
Morsingh, F. & MacDonald, S. F. (1960). *J. Am. chem. Soc.* **82**, 4377.
Newbold, B. T. & LeBlanc, G. (1964). *Can. J. Biochem.* **42**, 1697.
Nichol, A. W. & Morell, D. B. (1969). *Biochim. biophys. Acta*, **177**, 599.