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THE SOLUBILITY OF BILIRUBIN.

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The titration curve of a suspension of bilirubin with NaOH has been determined and analysed. A value of 5×10^{-11} mole/l for the solubility of undissociated bilirubin has been derived from the titration.

During an investigation on the diazo reaction of bilirubin [1]) we became interested in the solubility of this bile pigment. It was known [2]) that bilirubin is easily soluble in alkaline solutions, but that a decrease of the pH below 7 precipitates it almost completely. Apparently the acid form of bilirubin is very insoluble, whereas its salt is soluble.

Potentiometric titration of a suspension of this substance proved to be a good method for determining its solubility, which is strongly dependent on the pH.

The results of a potentiometric titration with the glass electrode of an aqueous suspension of 50 mg of bilirubin with 0.0510 N NaOH and back-titration with 0.0510 N HCl have been plotted in fig. 1. The total volume of the solution was 250 ml.

The bilirubin had been obtained from Hoffmann-La Roche, and had been recrystallized from chloroform. Its purity was tested by chemical analysis (N-content), chromatography (no biliverdin present), and by the absorption spectrum.

The first titration, indicated by A, started from a crude suspension of bilirubin in water. The reaction with NaOH was slow, and this curve therefore does not represent equilibrium states. In the back-titration B, the bilirubin precipitated below pH 8. The precipitate consisted of very fine flocculae and was quite reactive, as is proved by

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1) *A. A. Hijmans van den Bergh* and *I. Snapper*, Deut. Arch. klin. Med. **110**, 540 (1913); *A. A. Hijmans van den Bergh* and *P. Muller*, Biochem. Z. **77**, 90 (1916); *C. L. J. Vink*, thesis Utrecht, 1954.

2) *V. A. Najjar*, Pediatrics **10**, 1 (1952).

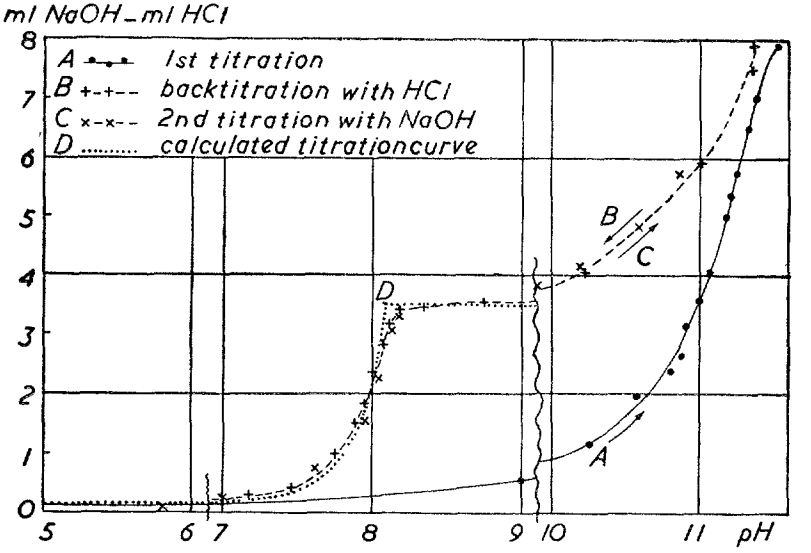

Fig. 1. Titration of 50 mg of bilirubin, suspended in 250 ml of water, with 0.0510 N NaOH and 0.0510 N HCl.

the fact that the second titration with NaOH (curve C) gave a curve practically coincident with the back-titration B. The titrations B and C are therefore considered to represent true equilibria. Oxygen and carbon dioxide were excluded during the titration. The temperature was 20° C.

The titration curve shows a number of interesting features. It is definitely not symmetrical, shows only one step, although bilirubin contains two carboxylic groups (fig. 2), and the step is situated at

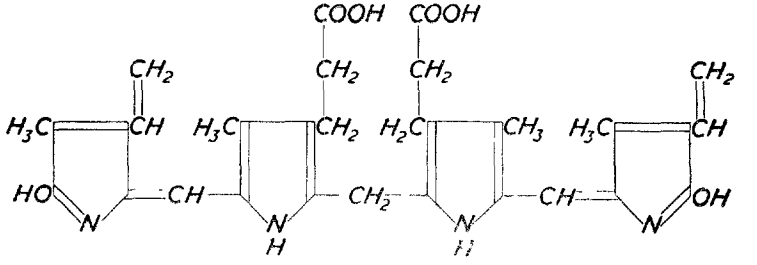

Fig. 2. Bilirubin.

pH 8, while the titration exponent for a carboxylic group on an aliphatic side chain is about 5.

These features are quite naturally explained by assuming that the acid form of bilirubin (RH_2) is very insoluble, whereas both the monovalent (RH^-) and the bivalent ($R^=$) ions are reasonably soluble. Then the following equilibria have to be considered.

$$RH_2 \leftrightarrows RH^- + H^+ \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

$$RH^- \leftrightarrows R^= + H^+ \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Assuming the dissociating tendencies of the two carboxylic groups to be identical, the equilibrium constants are

$$K_1 = \frac{[H^+]\,[RH^-]}{[RH_2]} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

and

$$K_2 = \frac{[H^+]\,[R^=]}{[RH^-]} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

The symbols between brackets represent activities or, with sufficient accuracy, concentrations in solution.

In the region of the step (pH $\sim$ 8) both equilibria, (1) and (2), are strongly shifted to the right. The monovalent ion is therefore practically absent from the system, and the amount of bivalent ion is equivalent to the NaOH added. Eliminating the concentration of the monovalent ion from equations (3) and (4), we obtain:

$$[R^=] = \frac{[RH_2]\,K_1K_2}{[H^+]^2} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

As long as bilirubin is present as a solid, the numerator of the right-hand side of eq. (5) is constant. Eq. (5) enables us to draw a theoretical titration curve by putting the amount of bilirubinate ion equivalent to the NaOH added (or rather to the excess of NaOH over HCl). By taking 2.15×10^{-20} mole^3 l^{-3} as the value of the numerator of eq. (5), the theoretical and experimental curves are made to coincide at half-neutralization and pH = 7.95. As the molecular weight of bilirubin ($C_{33}H_{36}O_6N_4$) is 584, 50 mg should consume 3.36 ml of 0.0510 N NaOH for neutralization of its two carboxylic groups. The theoretical curve D is drawn between the limits 0.12 ml and 3.48 ml. The rise of the experimental curve beyond pH 9 corresponds to the amount of OH^- ions necessary to bring an unbuffered solution to these pH's.

The agreement between the calculated and the experimental curves is very satisfactory.

The solubility of the acid form is thus equal to

$$[RH_2] = \frac{2.15 \cdot 10^{-20}}{K_1K_2} \text{ mole/l} \quad . \quad . \quad . \quad . \quad . \quad (6)$$

If the interaction between the two carboxylic groups is neglected, the dissociation constants K_1 and K_2 are expected to be equal to twice and

one-half resp. of the dissociation constant of pyrrole-propionic acid (carboxy ethyl pyrrole), which should be about 2×10^{-5} mole/l.

The solubility of acid bilirubin can therefore be estimated at 5×10^{-11} mole/l = 0.03 γ/l, a very low solubility indeed. Table I gives the solubility of the acid and the ionized forms together as a function of the pH, as estimated from eqs (4) and (5), and putting K_2 equal to 10^{-5}.

Table I.

Solubility of bilirubin in its different forms in mole/l.

pH	$[RH_2]$	$[R^=]$	$[RH^-]$	total = $[RH_2]+[RH^-]+[R^=]$
4	5×10^{-11}	2×10^{-12}	2×10^{-11}	7×10^{-11}
5	"	2×10^{-10}	2×10^{-10}	4×10^{-10}
6	"	2×10^{-8}	2×10^{9}	2×10^{-8}
7	"	2×10^{-6}	2×10^{-8}	2×10^{-6}
8	"	2×10^{-4}	2×10^{7}	2×10^{-4}

Apart from evident chemical implications, this strong dependence of the solubility on the pH may have physiological consequences, too. The occurrence of gallstones might be explained by a pathological lowering of the pH. The pH of liver bile [3]) is 8.2 or higher, but in the gall-bladder bile [4]) it is decreased to 6.9—7.7, which depresses the solubility of bilirubin below the usual bilirubin concentration. The pH may be lowered even further by bacterial activity in case of inflammation.

The method described here for the determination of the solubility of bilirubin can be quite easily applied to the solubility of any sparingly soluble acid or base. It has in fact already proved its value for the determination of the solubility of long-chain amines [5]).

The reverse procedure, the calculation of a dissociation constant from the solubility of a (not too) sparingly soluble acid or base, has been described by *Krebs* and *Speakman* [6]), where earlier literature is also cited.

Acknowledgement.

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[3]) *C. H. Best* and *N. B. Taylor*, The physiological basis of medical practice, Baillière, Tindall and Cox, London, ed. 4., p. 457 (1945).

[4]) *J. G. Reinhold* and *L. K. Ferguson*, J. Exptl. Med. **49**, 681 (1929).

[5]) Unpublished results obtained at the Metallurgy Department of the Massachusetts Institute of Technology by *D. Brown* and one of the present authors.

[6]) *H. A. Krebs* and *J. C. Speakman*, J. Chem. Soc. **1945**, 593.