CXXIII. THE ESTIMATION OF BILIRUBIN IN BLOOD PLASMA

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IN spite of a great deal of published work, the present methods of estimation of blood bilirubin by the diazo-reaction still remain unsatisfactory. Difficulties arise from three main causes: the use of unsatisfactory artificial standards for matching the colour; the presence of extraneous coloured substances; and inaccurate assumptions in the calculation of volume relationships.

By comparing coloured solutions against a neutral grey screen, interposing a series of monochromatic light filters [King & Haslewood, 1937], it is possible to obtain curves corresponding to the amount of light of different wave-lengths transmitted by the solutions [cf. Heilmeyer & Krebs, 1930]. In Fig. 1, such curves
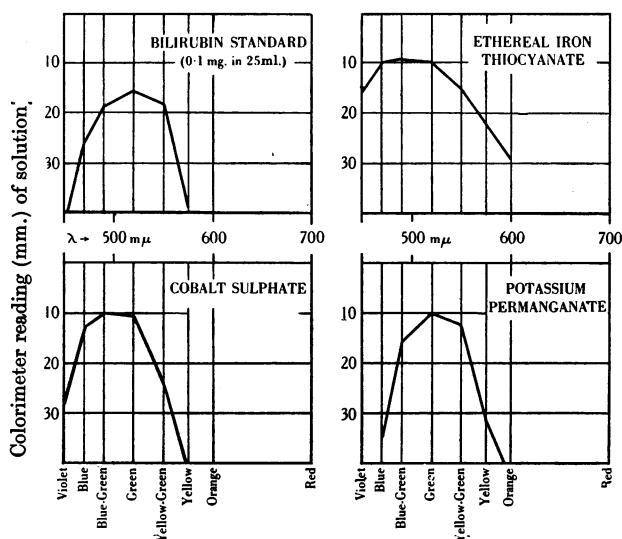

Fig. 1. Light absorption curves of standards for bilirubin estimation by the diazo-method—spectral filters; neutral grey screen density 0·50.

are given for the common artificial bilirubin standards—ethereal ferric thiocyanate, potassium permanganate and cobalt sulphate. Comparison of the graphs for these solutions with that for the red solution given by coupling a solution of pure bilirubin [Hunter, 1930] with diazotized sulphanilic acid[1] shows that each of the artificial standards differs markedly from the bilirubin-azo-dye

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[1] This coupling must be carried out at pH 3·0–4·0. Above pH 4·0, the colour of the coupled mixture fades rapidly to a greenish tint. At a pH much below 3·0, a permanent blue is obtained (the colour used by many workers, particularly in Germany, for estimation of bilirubin [cf. Krupski & Almasy, 1935]). At pH 3·0–4·0, the maximum amount of red colour is produced [Hunter, 1930].

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solution in its light absorption. It is therefore not surprising that the colorimetric estimations with these artificial standards should be difficult, because the colours are seen to differ quantitatively and qualitatively.

On theoretical grounds it seemed possible that an azo-dye, having the same type of chemical structure as that given by bilirubin itself in the reaction, might show closely similar light absorption. In Fig. 2 are shown the graphs for methyl red (*o*-carboxybenzeneazodimethylaniline) at *p*H 4·8, 4·7 and 4·6. The curves for methyl red at *p*H 4·7 and 4·6 almost exactly correspond with that given by the bilirubin dye. Methyl red is readily available in the pure state, and moreover its solution is easily buffered to *p*H 4·6–4·7 by acetic acid-sodium acetate. Such a solution is therefore very suitable for a permanent artificial standard and theoretically it should be possible to compare a methyl red standard directly with the bilirubin azo-dye from blood. In spite of these considerations, such a comparison is not universally reliable, for some observers have difficulty in matching the colour obtained from blood plasma with the methyl red standard. The light absorption curves of bilirubin azo-dye from blood plasma are not identical with that of the bilirubin standard; and further, the colour varies greatly with different blood

Fig. 2. Light absorption curves of bilirubin-azobenzenesulphonic acid and of methyl red in acetate buffer.

samples. It is not certain that all the pigment in blood is bilirubin; and the position is undoubtedly complicated by the presence in blood of other substances (for example, histidine and ergothioneine) which give colours with the diazo-reagent. For quantitative estimation, however, it is most convenient to determine the total red colour and to express this in terms of "bilirubin" in the plasma.

The graphs show that with a green light filter the maximum light absorption takes place, that is, the minimum amount of bilirubin-dye is needed to match the neutral grey screen [*v*. also Van den Bergh & Grotepass, 1934]. This means that if a green light filter is used, the maximum effect obtained is due to the red bilirubin-azo-dye or to dyes of a similar colour. Further, the effect of extraneous colours is minimized. These considerations, of course, also apply to the methyl red standard, because this solution shows almost the same light absorption curve as the bilirubin-dye. Hence, if the colorimetric determination is carried out with the use of a green light filter, the value obtained will correspond closely to the amount of bilirubin in the tested solution. From the graphs it is clear that any filter passing light which is virtually monochromatic and of a wave-length 490–550 m*μ* may be employed, since the curves for methyl red and bilirubin-dye coincide on this range (a consideration which does not apply to the other artificial standards).

Finally, in estimating blood bilirubin, it is essential to obtain the red colours in a constant known volume of solution. This point requires emphasis, because, for example, in one of the standard text-book methods for this reaction [cf. Godfried, 1935], the sum of the volumes of reagents added is 5·0 ml. The dilution factor
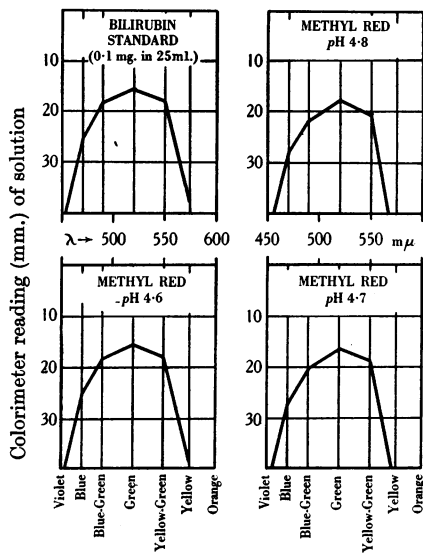
for the plasma is taken as 1 in 5 or, sometimes, as 1 in 4, whereas actually about 3·2 ml. of coloured solution are obtained from 1 ml. of plasma. By use of the method described below, a constant volume of almost exactly 4·0 ml. of coloured solution is obtained, while the precipitate, from bloods very rich in bilirubin, contains not more than 10 % of the total red colour. The acidity of the diazo-reagent has been given a definite value by the use of standard acid, instead of the commonly used "concentrated hydrochloric acid," whose strength varies in different countries. With this diazo-reagent, the final pH of the coloured solution is about 4. The colour of these solutions from blood plasma is much less sensitive to changes in acidity than is the case with the bilirubin-dye standards and the pH may be varied from about 3·0 to 4·5 without affecting the quantity permanence or quality of the colour obtained.

<center>EXPERIMENTAL</center>

Diazo-reagent.

Solution A. 1·0 g. sulphanilic acid dissolved in 250 ml. N hydrochloric acid and the volume made to 1 l. with water.

Solution B. 0·5 g. sodium nitrite in 100 ml. aqueous solution.

The diazo-reagent is made immediately before use by mixing 10 ml. of A with 0·3 of B.

Standard bilirubin-dye solution (0·1 mg. bilirubin in a volume of 25 ml.) [cf. Hunter, 1930].

10 mg. pure bilirubin were dissolved in chloroform and the volume made to 100 ml. 1·0 ml. of the solution, in a 25 ml. volumetric flask, was treated with 19 ml. 95 % ethyl alcohol and 5 ml. of a fresh mixture of diazo-reagent and sodium phosphate buffer (6 g. Na_2HPO_4, $12H_2O$ per 100 ml.). The solution was mixed, allowed to stand for a few moments and made to the mark with 95 % alcohol.

The pH of the solution was varied by altering the proportions of diazo-reagent and buffer employed. The solutions were evaluated photometrically in a Duboscq colorimeter against a neutral grey screen, the light filters being placed on the eyepiece of the colorimeter, according to the technique described by King *et al.* [1937].

Methyl red standard (2·9 mg./l. at pH 4·63).

0·29 g. pure methyl red was dissolved in glacial acetic acid and the volume made to 100 ml. 1·0 ml. of this solution in a 1-l. flask was treated with 5·0 ml. glacial acetic acid. Water was added and 14·4 g. crystalline sodium acetate ($CH_3COONa, 3H_2O$) washed into the mixture and dissolved. The volume was made to 1 l. with water.

The colour of the solution was equal in quantity (measured as above) to the average amount of colour given by four different samples of bilirubin (from Prof. H. Fischer, Prof. G. Hunter, The Eastman Kodak Co. and Messrs Fraenkel and Landau) when treated as described. The amounts of colour given by these different bilirubin specimens were almost identical.

<center>*Estimation of plasma bilirubin*</center>

1·0 ml. plasma is carefully layered with 0·5 ml. diazo-reagent. A coloured ring at the liquid junction shows a positive "direct" reaction. The solutions are mixed and treated with 0·5 ml. saturated ammonium sulphate solution, and,

finally, with 3·0 ml. absolute ethyl alcohol. The mixture is stoppered, shaken, allowed to stand 1 min. and filtered. The clear filtrate is compared against the methyl red standard, a green light filter being employed.

Calculation:

$$\text{Bilirubin (mg./100 ml. of plasma)} = \frac{\text{reading of standard}}{\text{reading of test}} \times 0\cdot1 \times \frac{4}{25} \times \frac{100}{1\cdot0}$$

$$= \frac{\text{reading of standard}}{\text{reading of test}} \times 1\cdot6.$$

Monochromatic "spectral" light filters (gelatin, mounted in "A" quality glass, $\frac{3}{4}$ in. diameter), and neutral grey screens (densities 0·25, 0·50 and 0·75) were obtained from Messrs Ilford Ltd., London. The authors gratefully acknowledge gifts of pure bilirubin from Prof. G. Hunter and Prof. Hans Fischer.

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