

FACTORS AFFECTING THE RATE OF COUPLING OF BILIRUBIN AND CONJUGATED BILIRUBIN IN THE VAN DEN BERGH REACTION

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Many workers have modified van den Bergh's method for determining bile pigment in serum (With, 1954). Two of the more satisfactory methods are those of Malloy and Evelyn (1937) and King and Coxon (1950). The present study was undertaken in order to explain differences in the results given by these two methods, and in an attempt to combine the desirable features of each, on a scale which was suitable for the day-to-day control of plasma bilirubin concentrations in newborn infants.

Methods

Serum.—Serum containing unconjugated, indirect-reacting bilirubin was obtained from infants at exchange transfusion. Serum containing conjugated bilirubin giving the direct reaction was obtained from patients with jaundice due to stones, cirrhosis, chlorpromazine, hepatitis, and also from infants with the "inspissated bile syndrome."

Diazo Reagent.—In most of the published procedures for determining bilirubin, the diazo reagent was prepared from 10 ml. of 0.1% (w/v) sulphanilic acid in 0.15 (or 0.25) *N*-HCl, to which 0.3 ml. of 0.5% (w/v) NaNO₂ was added. This mixture, containing 38% of the theoretical nitrite requirement of the sulphanilic acid, is referred to as the conventional diazo reagent. In preparing diazo reagents with other concentrations of diazotized sulphanilic acid, sulphanilic acid and at various reactions, the diazotization has always been conducted at a pH below 2.5.

Determination of Rate of Coupling of Bile Pigments.—The serum (0.1 ml.) was pipetted into 2.7 ml. water in the 1 cm. cell of the colorimeter. At zero time 0.7 ml. of freshly prepared diazo reagent (containing the required amount of 1.0% sulphanilic acid, 0.5% sodium nitrite, HCl, and water) was added and the contents mixed. The resultant colour was read in the colorimeter at 30 sec., 1 min., and at suitable short intervals up to 30 min. At 30 min., 3.5 ml. methanol was added, the solution mixed, and the colour read, as before, at 30 sec.,

1 min., etc., up to 30 min. A blank was determined on 0.1 ml. serum diluted with 2.7 ml. water by adding 0.7 ml. of the diazo reagent without nitrite and measuring the colour before and after the addition of methanol. In the results given in the figures account has been taken of the effect of solvent and pH on extinction. The final standard was bilirubin.

Diazo Pigments.—To compare the absorption characteristics in different solvents at various reactions a standard of azo-bilirubin was prepared. Bilirubin (B.D.H.), 2 mg., was dissolved in 1 ml. of boiling CHCl₃, 8 ml. of ethanol was added, and 1.4 ml. of concentrated diazo reagent (1 ml. 1% sulphanilic acid, 0.1 ml. *N*-HCl, and 0.3 ml. 0.5% NaNO₂). After 45 min. the reaction was stopped with 100 mg. of ascorbic acid in 0.5 ml. of water. After centrifuging, the clear supernatant was stored at -12° C. Samples for study were taken to dryness *in vacuo* and dissolved in various solvents.

The diazo pigment of bilirubin diglucuronide (azobilirubin glucuronide) was prepared from 5 ml. of gall bladder bile by adding 88 ml. of water and 7.2 ml. of diazo reagent (6.0 ml. of 1% sulphanilic acid, 1 ml. of *N*-HCl, and 0.18 ml. 5% NaNO₂) followed after 10 min. by 50 g. of (NH₄)₂SO₄ and 2 ml. of *n*-butanol. The mixture was cooled to 4° C., spun at 1,000 g, and the pigment cake removed and dried *in vacuo* for 30 min. It was extracted with 10 ml., 2.5 ml., and 2.5 ml. portions of methanol which were combined, and evaporated *in vacuo*. Brown contaminants were separated by counter current distribution in a system of CHCl₃-methanol-0.2M acetate buffer at pH 4 (4:8:5, by vol.). To recover the pigment the system was saturated with (NH₄)₂SO₄, the aqueous layers were taken to dryness *in vacuo*, extracted with methanol, and the extract was dried. Samples were taken into the solvents to be examined.

Colorimetry.—Most of the studies of rates of colour development were made with a "unicam" S.P. 300 colorimeter using Ilford filter No. 404 (λ_{max} 525 mμ) and 1 cm. cells. The spectral curves of azo pigments were obtained with a "unicam" S.P. 500 spectrophotometer using 1 cm. cells. The pH of solvents is that recorded with a glass electrode. In calculating

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the results of experiments allowance has been made for colour differences due to pH and solvent.

Recommended Method for Infants.—The infant's heel is swabbed with 0.01% ethanolic acridine, smeared with vaseline, and punctured with a surgical needle. About 0.6 ml. blood is collected (5–10 min.) in a 6 × 50 mm. tube containing dry heparin (1 drop of 5,000 i.u. heparin delivered from a No. 12 needle). The tube, which is protected from strong light, is spun and the plasma separated.

The plasma (0.2 ml., or 0.1 ml. of plasma and 0.1 ml. of water, if a value over 15 mg. per 100 ml. of total pigment is expected) is pipetted into 5.4 ml. of water and mixed. Half of the material (2.8 ml.) is transferred to a second tube for a blank. Into the first tube 0.7 ml. of freshly prepared diazo reagent (10 ml. of 1.0% sulphanic acid in 0.2 N-HCl, to which 0.3 ml. of 0.5% NaNO₂ has been added) is delivered, and into the second 0.7 ml. of the sulphanic acid in HCl (without nitrite). The contents are mixed and read after 5 min. in a colorimeter (green filter, preferably Ilford No. 404 or 625), together with a standard. The solutions are returned to their tubes. The conjugated bilirubin is calculated from the formula $(E_{\text{test}} - E_{\text{blank}})/E_{\text{standard}} \times 10 \times 1.05$. To each tube is added 3.5 ml. of methanol. After mixing they are read in 5 min. The total pigment is calculated from $(E_{\text{test}} - E_{\text{blank}})/E_{\text{standard}} \times 20$.

A standard is prepared by weighing 10 mg. bilirubin into a hard glass volumetric flask, refluxing with chloroform until dissolved, cooling, and making to 100 ml. with chloroform. It is kept in the dark at 4° or -12° C. The standard solution (0.2 ml.) is pipetted into 3.5 ml. methanol, mixed with 0.7 ml. diazo reagent, and 2.6 ml. of water is added. The colour is read after 5 min. Water is used as a blank.

For a day-to-day check on the photometer variation a "standard" of methyl red (Haslewood and King, 1937) or Thompson's neutral grey solution (King and Wootton, 1956) may be used with the green filter. The factor which is suitable for the methyl red solution under King's conditions is not suitable under the more acid conditions described above. If this standard were used a new factor would have to be determined for each combination of instrument and filter.

Results

Factors Affecting the Light Absorption of Diazo Pigments.—Aqueous solutions of azopigment from bilirubin behaved according to Beer's law up to an extinction of 0.71, when an Ilford filter No. 404 was used. The wavelength of maximal absorption ($\lambda_{\text{max.}}$), and the relative extinction ($E\lambda_{\text{max.}}$) at this wavelength, of azobilirubin (Table I) and azobilirubin glucuronide, varied with pH and the solvent composition of the medium.

TABLE I
WAVELENGTH OF MAXIMUM ABSORPTION ($\lambda_{\text{max.}}$) AND RELATIVE EXTINCTION OF AZOBILIRUBIN IN VARIOUS SOLVENTS

Solvent	$\lambda_{\text{max.}}$ (m μ)		Relative Extinction at $\lambda_{\text{max.}}$		Relative Extinction Using Ilford 404 Filter	
	pH 1.7	pH 3.5	pH 1.7	pH 3.5	pH 1.7	pH 3.5
Water	560	520	100	65	100	73
50% methanol	559	522	106	100	105	91
68% ethanol	543	523	99	96	100	87
76% "	543	523	—	102	99	88
90% "	532*	—	—	90*	—	90

* Determined at pH 2.

Protein affected the spectral characteristics. Bovine albumin (1%) shifted $\lambda_{\text{max.}}$ of azobilirubin at pH 1.5 from 560 m μ to 536 m μ , and reduced the $E\lambda_{\text{max.}}$ by 25%. With 0.2% albumin the change was almost as great, and 0.025% albumin reduced $E\lambda_{\text{max.}}$ by 13%. At a less acid reaction (pH 3.5) protein moved $\lambda_{\text{max.}}$ from 517 m μ to 521 m μ and $E\lambda_{\text{max.}}$ was increased by 10%. Human albumin and globulin had a similar effect to bovine albumin at pH 1.7 and pH 3.5. Bovine globulin, at pH 1.7, produced a slightly greater effect than albumin, while at pH 3.5 it had less influence. The effect of protein (1.0% albumin at pH 1.5) was abolished by 50% methanol. The spectral characteristics of azobilirubin glucuronide were less affected by protein than was azobilirubin.

Rate of Coupling of Bilirubin.—Sera of newborn infants, which have been shown by chromatography to contain mainly unconjugated bilirubin, were examined in the van den Berg reaction before and after the addition of an equal volume of methanol. The rate of coupling was determined at pH 1.5, with varying amounts of the conventional diazo reagent (Fig. 1) and of undiazotized sulphanic acid (Fig. 2). The rate of coupling at pH 3.5 is given in Fig. 3.

We examined the effect of using different quantities of sera in estimating the amount of pigment coupling in 30 min. in aqueous solution, and also on the total amount of pigment coupling in 50% aqueous methanol. With sera of newborn infants the calculated total pigment remained relatively constant as the serum volume was reduced, but the relative amount coupling in aqueous solution, i.e., the direct reacting component, appeared to increase, particularly at serum dilutions greater than 1 in 20. For example, one serum (total pigment 14 mg./100 ml.) appeared to have 0.7 mg. of direct reacting pigment (30 min.) when 1 ml. was used for a test in a final volume of 3.5 ml. A fivefold dilution doubled the amount

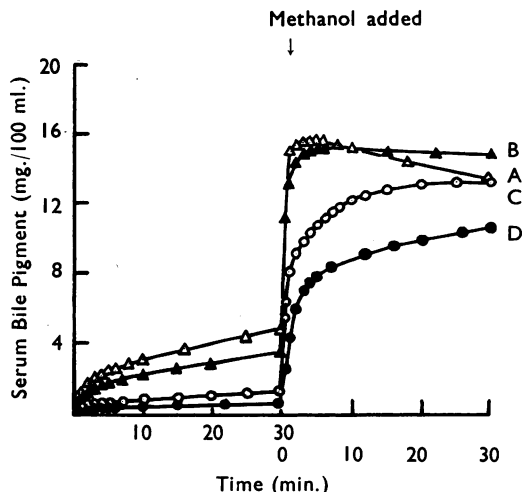


FIG. 1.—The effect of different concentrations of diazosulphanilic acid on the rate of coupling of bilirubin in serum at pH 1.5. The concentration of diazosulphanilic acid was: curve A, 0.052%; B, 0.023%; C, 0.0075%; D, 0.0037%. The concentration of undiazotized sulphanilic acid, before the addition of methanol, was twice that of diazosulphanilic acid.

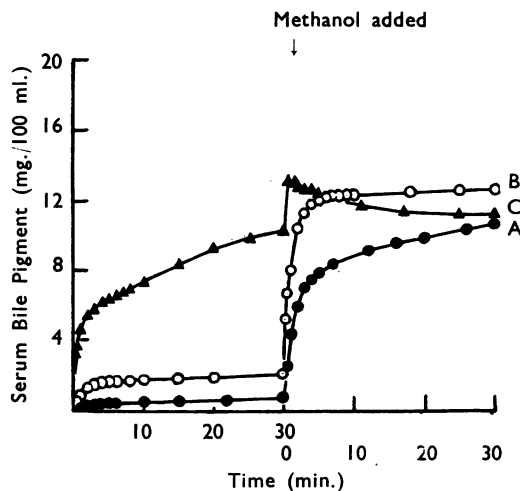


FIG. 3.—The rate of coupling of bilirubin in serum at pH 1.5 and 3.5, with low and high concentrations of diazotized sulphanilic acid. The excess sulphanilic acid was twice the concentration of the diazotized sulphanilic acid. The following were the pH values and the concentrations of diazotized sulphanilic acid before the addition of methanol: curve A, pH 1.5, 0.0037%; B, pH 3.5, 0.0037%; C, pH 3.5, 0.052%.

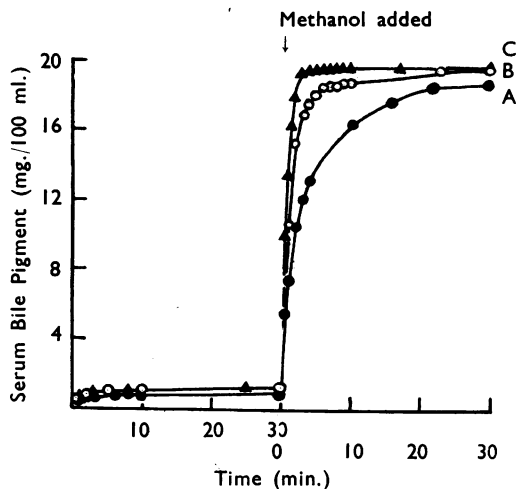


FIG. 2.—The effect of varying concentration of excess sulphanilic acid on the rate of coupling of bilirubin in serum at pH 1.5. The concentration of diazosulphanilic acid was 0.007% throughout. The concentration of the undiazotized sulphanilic acid, before the addition of methanol, was: curve A, 0.013%; B, 0.053%; C, 0.193%.

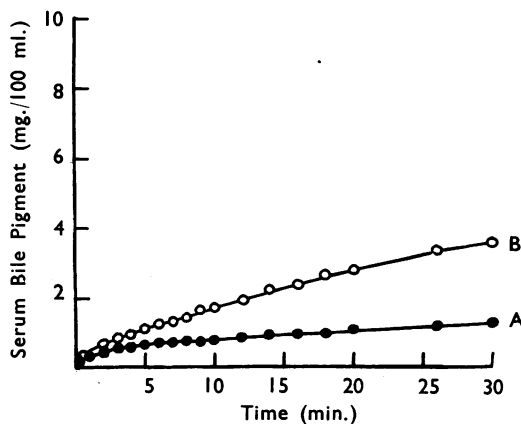


FIG. 4.—The rate of coupling of serum bilirubin in the presence and absence of added human serum protein. The initial reaction mixture (curve A) contained 0.1 ml. icteric serum, 0.007% diazotized sulphanilic acid, 0.013% excess sulphanilic acid, at pH 1.5. Curve B contained, in addition, 0.4 ml. of normal human serum. In calculating the amount of colour formed in curve B that due to the normal serum alone has been subtracted.

coupling in water and a twentyfold dilution increased it by 4.5 times. This effect was several times greater when the dilutions were made in normal serum, the protein being kept constant. This enhancement of the relative amount of coupling in aqueous solution could also be demonstrated by the addition of normal serum to a

reaction mixture in which the amount of pigmented serum was constant (Fig. 4), provided that the protein content was initially low (not much higher than 0.2 ml. in 3.5 ml.). The effect could not be reproduced with human and bovine albumin, but it was given by human and bovine sera and by globulin.

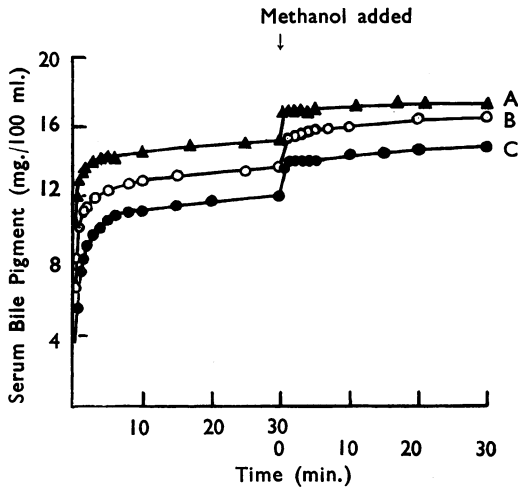


FIG. 5.—The rate of coupling of conjugated bilirubin in serum at pH 1.5, using different concentrations of diazosulphanilic acid. The excess sulphanilic acid was twice the concentration of diazosulphanilic acid. The following were the concentrations of diazotized sulphanilic acid before the addition of methanol: curve A, 0.023%; curve B, 0.0075%; curve C, 0.0037%.

Rate of Coupling of Conjugated Bilirubin.—

Sera in which the main pigment was conjugated bilirubin were examined at pH 1.5 with varying amounts of the conventional diazo reagent (Fig. 5). An increase in the undiazotized sulphanilic acid from 0.008 to 0.046% did not alter the rate of coupling of conjugated bilirubin in water and 50% methanol significantly.

In a series of tests the apparent total pigment and the direct-reacting (30 min.) pigment were determined, using 1 to 0.05 ml. of pigmented serum which contained much conjugated bilirubin. The final volume of the aqueous mixture was 3.5 ml. The apparent total pigment remained relatively constant as the serum volume was reduced, and the apparent rise in direct-reacting pigment was attributable to the effect of dilution on the coupling of unconjugated bilirubin in aqueous solution. If the protein concentration was kept constant at 1 ml. by adding normal serum the 30 min. direct pigment increased slightly as with bilirubin, but there was a marked drop in the apparent total pigment (12.2, 10.1, and 8.8 mg. with 0.2, 0.1, and 0.05 ml. of pigmented serum). Protein had no effect on the rate of coupling of conjugated bilirubin in aqueous solution.

Results Compared by Recommended and King and Coxon's Methods.—Ten sera have been analysed by both methods, and the results are given in Table II.

TABLE II
TOTAL PIGMENT LEVELS COMPARED IN NEONATAL SERA DETERMINED BY KING AND COXON'S AND THE RECOMMENDED PROCEDURES

Serum No.	1 Bile Pigments Determined by King and Coxon Method (mg./100 ml.)	2 Bile Pigments Determined by Recommended Method (mg./100 ml.)	Comparative Values (1 as % of 2)
1	15.6	16.3	96
2	12.7	13.3	95
3	9.9	9.7	102
4	9.6	10.4	92
5	9.0	10.0	90
6	8.9	10.0	89
7	5.9	6.7	88
8	5.6	6.4	88
9	5.3	6.1	87
10	3.4	4.5	76

Discussion

Conditions for Distinguishing between Direct and Indirect Reactions.—Van den Bergh (1918) used the term "direct" in referring to a rapid colour development which occurred when diazotized sulphanilic acid was added to some sera in the absence of alcohol. "The direct reaction occurs in all cases in a few seconds, at the most 30 seconds." He distinguished this from a slow reaction in the absence of alcohol—"... reaction also occurred without addition of alcohol, but this commenced considerably later—there was an interval of 2, 3, 4 minutes or longer before the reaction began to become definite and still longer until it reached its greatest intensity." He identified this with the "indirect" reaction.

Nevertheless, others were less critically observant, and it was frequently held that bilirubin did not couple at all in simple aqueous solution, in this respect differing qualitatively from the direct reacting pigments, which are now known to be conjugated bilirubin. A review of the conditions of the original van den Bergh reaction, and some of its modifications (Table III), indicates that there have been extensive changes in the conditions under which the reaction is carried out in aqueous solution, and some authors have not taken account of the way this affects the rate of coupling of bilirubin and of conjugated pigments.

Figs. 1-3 show that a sharp distinction between the rate of reaction of bilirubin and of conjugated bilirubin in aqueous solution can be drawn only at a low pH and a low concentration of diazotized sulphanilic acid. Bilirubin may react almost completely in 30 min. in aqueous solution at pH 3.5 and 0.052% diazosulphanilic acid (Fig. 3, curve C). These conditions must be avoided or a substantial error in estimating the proportion of unconjugated and conjugated bilirubin may result.

TABLE III
CONDITIONS UNDER WHICH BILIRUBIN AND CON-
JUGATED BILIRUBIN HAVE BEEN DETERMINED

	Reac- tion	Serum (%)	Diazotized Sulphanilic Acid (%)	Excess Sulphanilic Acid (%)	pH
van den Bergh and Snapper (1913)	Total	29	0.007	0.013	2.0
van den Bergh and Muller (1916)	Direct	31 or 44	0.004 or 0.003	0.007 or 0.005	5.3
Malloy and Evelyn (1937)	Direct Total	4 4	0.0037 0.0037	0.0063 0.0063	1.5 2.1
Ducci and Watson (1945)	Direct Total	4 4	0.0037 0.0037	0.0063 0.0063	1.5 2.1
Lawrence and Abbott (1956)	Total	1.66	0.003	0.0055	1.8
King and Coxon (1950)	Direct Total	10 10	0.0018 0.0018	0.0032 0.0032	2.6 4.2
White and Duncan (1952)	Total	4	0.011	0.019	1.8
Hsia <i>et al.</i> (1956)	Direct Total	1.66 0.83	0.006 0.003	0.044 0.022	1.5 1.8
Recommended method	Direct Total	2.86 1.43	0.007 0.0035	0.193 0.097	1.5 1.8

Figures in the body of the table are the final concentrations attained.

Aqueous Reaction of Bilirubin.—It is observed in Figs. 1, 2, and 3 that neonatal sera shown by chromatography to contain negligible conjugated bilirubin will couple with aqueous diazosulphanilic acid at pH 1.5 in two phases, a fast (0–3 min.) and a slow (3–30 min.). Both phases of the aqueous reaction of bilirubin can be increased by high diazosulphanilic acid concentration as in Fig. 1, curves A and B (under similar conditions to those of White and Duncan, 1952), and by a high pH (Fig. 3, curve C) until they amount at 30 min. to more than 70% of the total. In view of this fact, it seems probable that some or all of the small amount of colour which is developed at pH 1.5 and 0.0037% diazosulphanilic acid (conditions used by Malloy and Evelyn, 1937, as in Fig. 1, curve D) is also due to bilirubin and not to conjugated bilirubin.

Protein has its main effect on the slow phase of colour development (apparent after 3 min. in Fig. 4). A comparison of the aqueous reaction curve C of Fig. 1, which was obtained with a modification of the Malloy and Evelyn method previously used in this laboratory and circulated privately, and curve C of Fig. 2, obtained with the modification suggested in this paper, shows that in water high concentration of undiazotized sulphanilic acid affects neither the fast nor the slow phases of bilirubin coupling.

Although pH 1.5 is preferable to 3.5 for reducing the aqueous coupling of bilirubin (compare

curves A and B of Fig. 3) even at the low pH about 4% of the bilirubin couples in 30 min. when the diazosulphanilic acid is 0.0037% (Fig. 1, curve D) as in the technique of Malloy and Evelyn (1937). These authors were largely responsible for the introduction of 30 min. as a period for the direct reaction, though there is nothing in the rate of colour development of conjugated bilirubin (Fig. 5, curve C) to make this period distinctive. A period of 30 min. was selected in order to include most of the direct pigment, though the authors mentioned that there was a slow development of colour over some hours. Ducci and Watson (1945) worked under similar conditions to Malloy and Evelyn and suggested the use of a "1 min. direct" to exclude the slow phase of bilirubin conjugation in water. It appears from curve C of Fig. 5, which was studied under the conditions of Ducci and Watson, that 1 min. is too short a time to measure the extent of the fast component due to conjugated bilirubin. It has a second disadvantage that the extinction is changing so rapidly that reproducible results would be difficult to obtain. It seems desirable that the time selected for the direct reading should include coupling of as little of the bilirubin and as much of the conjugated bilirubin as possible. We suggest that the colour development at 5 min. be taken as an estimate of the amount of conjugated bilirubin. In sera containing predominantly conjugated pigment the difference between the values at 5 and 10 min. is less than 10% of that at 10 min. (Fig. 5, curve B).

It might be thought that in the determination of direct-reacting pigment according to King and Coxon (1950) substantial amounts of bilirubin would react, as their usual procedure for total pigment is carried out at pH 4.2. This has been avoided, however, since the omission of $(\text{NH}_4)_2\text{SO}_4$ and ethanol reduces the pH to 2.6, where bilirubin is much less reactive.

Effect of Changes in Spectral Characteristics.—The differences in spectral curves and extinctions, produced by variations in solvent and pH (Table I), as well as protein concentration, indicate that caution is needed in using a standard under conditions which differ from the test for which it was designed. Thus the methyl red standard of Haslewood and King (1937) matches the colour of azobilirubin in their test (pH 4.2) but not that of the azobilirubin in 50% methanol under the more acid conditions (pH 2.1) of the Malloy and Evelyn test. Nor is the use of a filter satisfactory, since this will vary from instrument to instrument. If one departs from the conditions of King's test

it is essential to calibrate the method with bilirubin.

Effect of Protein.—Protein may alter the spectral characteristics of azobilirubin and the rate of reaction of bilirubin. In aqueous solution at pH 1.5, serum protein (1–3%) reduced the colour by 10%, or by 15% if 0.8 ml. of serum was used in the Malloy and Evelyn test. However, at pH 2.6 (direct reaction of King and Coxon, 1950) the extinction is scarcely altered by protein. The total amount of pigment shown by the Malloy and Evelyn test is approximately correct, because the effect of protein is abolished by the presence of 50% methanol.

Under special circumstances (Fig. 4) serum protein will facilitate the colour reaction of bilirubin in aqueous solution. It shares this property with many polar substances, e.g., ethanol, but the effect is minor, and cannot account for the “direct” reaction, as some authors have suggested. It is noted in Fig. 4 that the protein effect is negligible during the rapid phase of aqueous coupling of bilirubin (0–3 min.) and for this reason does not invalidate the suggested method which is based on a reading at 5 min.

The only circumstance in which dilution is likely to alter substantially the amount of apparent direct-reacting pigment would be the use of very small amounts (0.05 ml.) of serum in a modified Malloy and Evelyn technique. This would double the apparent conjugated pigment, and might erroneously suggest the development of obstruction in the infant.

Conjugated Bilirubin by King's Method

It may be anticipated that laboratories will select a method on the basis of its general usefulness, rather than its adaptability to the needs of the newborn infant alone. Since most determinations are made on adult sera, many of which have low concentrations of pigment, the King and Coxon method (Table III) has the great advantage of a low serum dilution. It has not been found possible to devise a method for determining conjugated bilirubin under King's conditions, because turbidity can only be overcome at a low pH, which makes the colour match with the methyl red standard unsatisfactory. If water is used in place of ammonium sulphate and ethanol, as suggested by King and Coxon (1950) for the determination of direct-reacting pigment, turbidity is frequently so great that a “blank” determination is insufficient to compensate for it. To avoid these difficulties the following technique may be used.

One millilitre of serum (or a mixture of serum and buffer) is mixed with 0.5 ml. of the conventional diazotized sulphanilic acid (pH now 2.2) and after 5 min. (longer will produce a false high direct reaction) 0.1 ml. of 5% ascorbic acid (or a knife tip, since an excess of up to 50 mg. may be used) is added to destroy the remaining diazotized sulphanilic acid. The procedure may then be continued in the usual way with $(\text{NH}_4)_2\text{SO}_4$ and ethanol, as bilirubin will not be coupled. This is recommended for laboratories which routinely use King's method, but occasionally need to know the actual amount of bilirubin and conjugated bilirubin in infant sera or in those of adults.

Recommended Rapid Procedure

For clinical use, and particularly for directing the treatment of jaundiced newborn infants, it is desirable to have a rapid method for estimating both bilirubin and conjugated bilirubin. The method should be accurate at 10 to 30 mg. of bilirubin/100 ml. of serum and should provide a compensation for the haemolysis which is frequently found in heel prick specimens of infant blood.

Although at pH 1.5 and 0.007% diazotized sulphanilic acid the aqueous reaction, which includes almost all of the conjugated pigment, could be read at 5 min., bilirubin coupling being minimal (Fig. 1, curve C), the coupling of unconjugated pigment in 50% methanol would be too slow. This could be accelerated by adding more diazotized sulphanilic acid, but would give a false high direct reading (Fig. 1, curve B) as in the White and Duncan (1952) procedure. This disadvantage can be overcome by increasing the excess sulphanilic acid to 0.19% rather than by increasing the diazotized sulphanilic acid. This accelerates the reaction of bilirubin in methanol but not in water (Fig. 2, curve C). The amount of apparent conjugated bilirubin was increased by less than 10%. For this reason it is recommended that the diazo reagent be prepared from 1% sulphanilic acid, but only the usual amount (0.3 ml./10 ml.) of nitrite should be added, as this gives a suitable concentration of both diazotized and undiazotized sulphanilic acid in the final mixture.

In jaundiced newborn infants the apparent conjugated bilirubin given by this method seldom exceeds 1.5 mg./100 ml. This is probably an artifact, due to coupling of bilirubin, and may be ignored. A final understanding of the significance of the minor proportion of indirect-reacting pigment, shown by the van den Bergh test in adult sera, must await a more detailed investigation of pigment I (bilirubin monoglucuronide) which is

usually the chief pigment of these sera. Although it gives a direct reaction, qualitatively, there is some evidence that an increase in colour development results when methanol is added (Billing, Cole, and Lathe, 1957). This may possibly account for some of the complicated time curves found in the literature.

The results given by this method are approximately 5–15% higher than those by the method of King and Coxon (1950) (Table II). This difference, which is present after account has been taken of pH and solvent effects, is due to incomplete coupling in King and Coxon's method. This effect of reagent concentration on the total amount of pigment which couples is shown in Fig. 1, curves B and C, for bilirubin, and in Fig. 5, for bilirubin glucuronide. The method which is recommended here also gives results for total pigment which are low, probably by about 3%. However, this cannot be corrected by increasing the concentration of diazo reagent, without producing greater errors in estimation of the conjugated bilirubin and producing fading in methanol (Fig. 1, curve A).

In the recommended method colour development (total pigment) may not be complete at 5 min. if the concentration of bilirubin exceeds 15 mg. per 100 ml. This can be overcome by starting with 0.1 ml. of serum, or reading the final colour until it is constant (7–9 min.).

Summary

The effect of pH, solvent, and protein on the spectral characteristics and optical extinctions of

azobilirubin and azobilirubin glucuronide has been examined. The extinction was increased in aqueous solution by a drop in pH and was further raised by the addition of methanol.

The influence of pH, protein, concentration of diazotized sulphanilic acid, and of sulphanilic acid on the rate of coupling of bilirubin and conjugated bilirubin has been investigated. The rate of coupling of bilirubin in water and methanol was increased by a rise in diazo concentration and in pH.

A high concentration of undiazotized sulphanilic acid increased the coupling of bilirubin in 50% methanol at pH 1.8 without significantly increasing the rate of reaction in water.

A rapid method for estimating conjugated and unconjugated bilirubin in a small amount of serum has been described.

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