ESTIMATION OF BILIRUBIN IN SERUM

BY

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Most methods of estimating the concentration of bilirubin in serum are based on the van den Bergh reaction in which bilirubin couples with diazotized sulphanilic acid to give red "azobilirubin," the reaction being facilitated by the addition of an activator or catalyst such as ethanol. The many modifications of this reaction can be classified into two groups: (a) those in which ethanol is used as the catalyst, with consequent precipitation of the serum proteins, and (b) those in which some substance, other than ethanol, acts as the catalyst and protein precipitation is avoided. Precipitation methods, such as those described by McNee and Keefer (1925), Haslewood and King (1937), and King and Coxon (1950), suffer from the disadvantage that some "azobilirubin" is lost on the protein precipitate; and further, the volume of coloured supernatant fluid or filtrate is unknown and varies from test to test. Consequently such methods cannot give really accurate results.

Non-precipitation methods include those published by Jendrassik and Cleghorn (1936) modified by Jendrassik and Grof (1938), Malloy and Evelyn (1937), Rappaport and Eichhorn (1943), Powell (1944), and Patterson, Swale, and Maggs (1952). The technique of Jendrassik and Cleghorn has been criticized by a number of workers on account of clouding of the final test solution. In Malloy and Evelyn's method the colour of the test solution, except in cases of moderate or severe jaundice, is too pale for accurate matching with the standard. The technique of Rappaport and Eichhorn is unsatisfactory for two reasons: the colour of the test solution is unstable and secondly, with some sera, full colour development of the "azobilirubin" compound is delayed for several hours. In Powell's method, serum, treated with diazo and sodium benzoate urea solutions, is matched against an alcohol-activated azobilirubin standard; the use of a standard differing so greatly in composition from that of the test is very undesirable. We have no experience of the method of Patterson, Swale, and Maggs which was published when the present

work was almost complete, but we feel that it may be criticized on the ground that the use in the routine laboratory of such a corrosive reagent as 90% phenol is undesirable.

In the method which we have developed the serum is treated with diazo reagent and a mixture of caffeine sodium benzoate and a phosphate buffer; the azobilirubin is formed rapidly and it is measured in a photo-electric colorimeter and compared with that of an azobilirubin standard previously prepared from bilirubin dissolved in N/10 sodium carbonate solution. A blank is used to compensate for any slight cloudiness or any colour other than bilirubin, which may be present in the test serum. This method, which is based on Goodson and Sheard's (1940) technique for estimating urinary bilirubin, is rapid, simple to perform, and gives good colours, the final test and standard solutions being buffered to a pH of between 5.7 and 6.3. Estimations of serum bilirubin levels by this technique compare well with estimations by the protein-precipitation method of King and Coxon. We have also made some observations on the factors required to bring about satisfactory coupling of bilirubin and diazo solution.

Experimental

Reagents.—The following reagents are required:

Diazo Reagent

Diazo A.—Sulphanilic acid	...	...	1 g.
Concentrated hydrochloric acid	...	...	15 ml.
Distilled water to	...	...	1,000 ml.
Diazo B.—Sodium nitrite	...	...	0.5 g.
Distilled water to	...	...	100 ml.

For use 10 ml. of diazo A is mixed with 0.3 ml. of diazo B.

Diazo Blank

Concentrated hydrochloric acid	...	...	15 ml.
Distilled water to	...	...	1,000 ml.

Caffeine Buffer Mixture.—Caffeine, 25 g., and 25 g. sodium benzoate are dissolved in about 400 ml. warm distilled water, cooled, and 4.1 g. potassium dihydro-

gen phosphate and 3.4 ml. N sodium hydroxide are added. The mixture is diluted to 500 ml. with water. The mixture, which should be filtered if not clear, will keep for at least a month.

Bilirubin Solution.—About 20 mg. of pure bilirubin (Hoffman la Roche) is weighed accurately and 0.2 ml. wetting agent added (10% "lissapol" N or 10% "tec-pol"). When wetting is complete 7 ml. of N/5 sodium hydroxide is added and the mixture is stirred until the bilirubin is completely dissolved. It is diluted with N/10 sodium carbonate to a volume of 500 ml. It is important that this preparation should be carried out rapidly (within five minutes). Immediately after this solution has been made it should be used for the preparation of the standard azobilirubin and standard blank solution as below.

Azobilirubin Standard.—This standard solution should be prepared in duplicate. To 50 ml. of caffeine buffer mixture 5 ml. of diazo solution is added, followed by 10 ml. of bilirubin solution, mixed, and allowed to stand for 10 minutes. Then its optical density (S) is measured using a green filter. If stored in the dark this solution can be used for checking the photometer in the subsequent serum determinations made during the following two weeks.

Standard Blank Solution.—This solution also should be prepared in duplicate. To 10 ml. of caffeine buffer mixture 1 ml. water is added, followed by 2 ml. of bilirubin solution. The solution is mixed and allowed to stand for 10 minutes, then its optical density (SB) is measured using the same green filter as employed in measuring the density of the standard.

Method

Test and Blank.—Fresh serum, 1 ml., is pipetted into each of two test-tubes. To one tube (the test) 0.5 ml. diazo reagent is added and to the other tube (the blank) 0.5 ml. diazo blank. The tubes are shaken and stood for approximately one minute and it is noted if a red develops in the test solution indicating a positive direct van den Bergh reaction. To both tubes 5 ml. of caffeine-buffer mixture is added. The contents are mixed well and stood for 15 minutes to allow for full colour development. The colour is stable for at least one hour.

The optical densities of the test (T) and the blank (B) are measured in a photoelectric colorimeter using a green light filter (e.g., Ilford bright spectrum green 624, or spectrum green 604, or, less good, Ilford tri-colour green 404) or in a spectrophotometer at 525 m μ , making the zero setting with water.

Calculation.—Concentration of bilirubin in serum = $\frac{T-B}{S-SB} \times \frac{W}{5}$ in mg. per 100 ml. serum where W = weight of bilirubin in mg. taken in preparing the bilirubin solution, and T, B, S, and SB, the optical densities of the solutions indicated above. If exactly 20 mg. of bilirubin was weighed out the standard

azobilirubin solution is equivalent to a serum bilirubin concentration of 4 mg./100 ml.

With strongly jaundiced sera (above 6 mg. per 100 ml.) the test solution is diluted without delay after coupling with a mixture containing 10 vol. caffeine-buffer solution and 1 vol. diazo reagent. This diluted solution is allowed to stand for 15 minutes before measuring its optical density.

If only 1 ml. (or less) of serum is available sufficient caffeine-buffer mixture is added to make a total volume of 6 ml., the optical density of this mixture is measured, and then 0.5 ml. diazo reagent is added and the density measured again. This method suffers from the disadvantage that it gives no measure of the direct van den Bergh reaction.

Three or four preparations of standard azobilirubin should be made from the solid bilirubin, and the average value of the factor $\frac{1}{S-SB} \times \frac{W}{5}$ determined; this value is then used for calculating all serum determinations.

In order to check the photometer some stable standard red solution should be measured each time a serum bilirubin level is determined. The azobilirubin standard can be used for this purpose, but its density very gradually increases. An alternative and more permanent standard which may be used is the methyl red solution prepared according to the method of King and Coxon (1950), although the colour density of this solution is much less than that of the azobilirubin standard and its tint less blue than the latter.

Results

After this method had been chosen it was necessary to determine (1) the purity of samples of bilirubin commercially available, and (2) to investigate the stability of the standard solutions of bilirubin and azobilirubin. As regards the purity of bilirubin, two preparations of bilirubin were examined by chromatographic adsorption of the chloroform solution on alumina and eluting with chloroform containing 1% acetic acid. It was found that the preparation made by Hoffman la Roche was very nearly pure and this was chosen as the standard.

As regards the stability of the bilirubin and azobilirubin solutions, it was found that standard bilirubin solutions prepared in the manner described above showed no alteration in concentration when examined five, 10, and 15 minutes after preparation. While we did not care to use bilirubin solutions more than 10 minutes after preparation, we observed that on some occasions they remained stable for at least one hour, but at other times the standard was found to have lost 5% of its bilirubin half an hour after preparation.

In contrast the standard solutions of azobilirubin were found to be much more stable. After adding the bilirubin to the mixed caffeine buffer and diazo

FIG. 1.—Relationship between the concentration of bilirubin (mg. per 100 ml.) and the optical density of azobilirubin formed.

solutions, the colour developed rapidly and reached its full intensity within a minute. Occasionally a very small decrease in optical density occurred during the next few minutes, but from 10 minutes onwards the extinction remained constant for two weeks, and thereafter very slowly increased.

Relationship between Bilirubin Concentration and Optical Density of Azobilirubin.—A graph (Fig. 1) was made by plotting the optical density of azobilirubin solutions, prepared from a series of dilutions of a 20 mg. per 100 ml. bilirubin standard against the concentration of bilirubin expressed in mgm. per 100 ml. The graph was practically a straight line between 2 mg. and 20 mg. bilirubin per 100 ml. Further experiments showed that even up to concentrations of 40 mg. per 100 ml. the departure from the straight line was only slight.

Recovery Experiments.—The addition of alkaline solutions of bilirubin in concentrations ranging from 2 to 8 mg. per 100 ml. to both normal and jaundiced sera gave “recoveries” of 95% to 103%.

Comparison with King and Coxon (1950) Method.—Parallel serum bilirubin estimations were made by the proposed caffeine method and the modification of the Haslewood-King technique described by King and Coxon (1950). The results from 32 consecutive sera obtained from non-jaundiced patients are shown in Table I. The results from 42 cases of hyperbilirubinaemia are shown in Table II.

TABLE I
BILIRUBIN CONCENTRATION OF 32 CONSECUTIVE SERA FROM NON-JAUNDICED PATIENTS

Diagnosis	Method of King and Coxon (mg./100 ml.)	Proposed Caffeine Method (mg./100 ml.)
Abdominal pain	0.5	0.6
Bronchiectasis	0.4	0.5
Headache	0.2	0.3
Eosinophilia	0.3	0.5
Ankylosing spondylitis	0.7	0.8
Tarsal arthritis	0.9	0.7
Alopecia areata	0.5	0.4
Menopause	0.4	0.4
Rheumatoid arthritis	0.6	0.7
Pregnancy	0.2	0.4
Carcinoma of stomach	0.2	0.3
Pregnancy	0.4	0.5
Erysipelas	0.4	0.5
Spontaneous pneumothorax	0.2	0.3
Urinary infection	0.3	0.4
Lymphatic leukaemia	0.1	0.2
Chronic nephritis (blood urea, 190 mg.%)	0.2	0.1
Chronic nephritis (blood urea, 162 mg.%)	0.1	0.2
Subacute combined degeneration	0.6	0.7
Anaemia ?cause	0.5	0.5
Sarcoidosis	0.5	0.4
Carcinoma of rectum	0.8	0.8
? Pernicious anaemia	0.5	0.5
Post-infective hepatitis, cirrhosis	0.8	0.6
Hepatic cirrhosis	0.6	1.0
Anaemia	1.0	0.9
Hepatomegaly	0.4	0.3
Leucoerythroblastic anaemia	0.8	0.9
Biliary peritonitis	0.4	0.6
Cholecystectomy	0.7	0.4
? Subacute hepatitis	0.7	0.8
Normal	0.6	0.5

Discussion

During our search for a good bilirubin standard we carried out many preliminary experiments with a view to determining the conditions governing satisfactory coupling of bilirubin with diazotized sulphanilic acid.

It was found desirable to have a buffer present in order to regulate the rate of coupling. The reaction proceeds only slowly in strongly acid solution, but is very rapid in neutral or alkaline solution. Further, the colour of the azobilirubin formed varies with reaction, being violet in the presence of mineral acid, the usual red or pink at pH 4–7, and greenish blue in alkaline solution. In the case of the ordinary van den Bergh test the serum itself is a sufficient buffer to reduce the acidity of the diazo solution to the useful pH range.

The stability of the azobilirubin formed is dependent on the reaction of the medium (it decomposes rapidly in alkaline solution) and on the presence of other substances, e.g., caffeine, in the solution. In fact caffeine appears both to facilitate coupling and to stabilize the product.

of King and Coxon is more stable and was used as an artificial standard in spite of the fact that it is weaker and less blue in tint than the 4 mg. azobilirubin standard. Another solution that may be used as an artificial standard is a 4% anhydrous cobalt sulphate solution to which an equal volume of 8 N hydrochloric acid has been added (White, 1932).

For accurate determination it is necessary to compensate for the optical density of the bilirubin which is present in the serum blank (B) but absent from the coupled test solution (T). Consequently $T - B$ is not a true measure of the azobilirubin in the test solution but is, in fact, too low, and if the

expression $\frac{T - B}{S}$ were used in calculating, the result

would also be too low (by about 8%). The extent of the correction to be applied is determined by preparing a solution containing the same concentration of bilirubin as used in making the standard azobilirubin solution, but water is substituted for diazo reagent. The optical density of this yellow standard blank solution (SB) is measured with the green light filter. It is found that SB is about 8% of S. The correction for the bilirubin present in the serum blank (B) but not in the test (T) can be made by subtracting SB from S and thus using the

expression $\frac{T - B}{S - SB}$ in calculating the bilirubin content.

The method which we have described is suitable for routine laboratory use as it is rapid and simple to perform, and no unusual or corrosive reagents are required. The method can be carried out satisfactorily on slightly haemolysed specimens of serum. Another advantage of the technique is that with strongly jaundiced sera the extra dilution is made after the addition of the diazo solution. In some methods involving protein precipitation it is necessary to dilute the serum with buffer before adding the diazo solution, and considerable experience is required in assessing the degree of pre-

liminary dilution necessary to obtain a satisfactory result when using these methods.

We are not aware of any serious disadvantage in the method we present. The most difficult part of the estimation seems to be the preparation of azobilirubin standards of consistent optical density; we found that variations of up to $\pm 3\%$ from the mean may occur.

The main criticism of caffeine catalysed methods in general is the fact that the final solution sometimes clouds, perhaps due to separation of benzoic acid derived from the catalyst solution. In our method benzoic acid never separated, and the final solutions were nearly always satisfactorily clear. On rare occasions the final solution has a very slight milkiness, possibly due to a globulin or lipid component in the serum. In the three cases so far encountered the milkiness appeared to be present to an equal extent in the test and blank solutions, and there was no discrepancy between the results obtained by this method and the method of King and Coxon.

Summary

A simple and rapid method for the estimation of bilirubin in serum is described. The method is suitable for routine use in laboratories using photoelectric colorimeters. A number of observations are made on the conditions necessary for satisfactory reaction between bilirubin solutions and diazotized sulphanilic acid.

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