Modification of Michaëlsson's method for the measurement of plasma total bilirubin

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SYNOPSIS Michaëlsson's (1961) method for the measurement of plasma bilirubin concentration was found to be complicated by conjugated bilirubin. Delaying the addition of the accelerator, diphylline, corrected this fault. The method was calibrated using serum or plasma, since reconstituted protein solutions introduced large errors.

Michaëlsson (1961), and Michaëlsson, Nosslin, and Sjölin (1965) developed a more sensitive method for the measurement of unconjugated bilirubin in infants, depending on the spectrophotometric determination of azobilirubin at an alkaline pH, using diphylline as an accelerator. This method was found to give higher results for conjugated bilirubin than for total bilirubin, and some modifications first suggested by Michaëlsson (1967) have been investigated.

METHODS

Venous blood was used throughout and was protected from light in tubes containing heparin, separated within two hours and stored in the dark at 4°C. Estimations were done the same day in a darkened laboratory.

For calibration, bilirubin dried in a desiccator for several days, of E.mol 60,600, was quickly dissolved in 0.1 ml of N/10 sodium hydroxide. Human serum which, after exposure to light, was of azo reaction equivalent to less than 0.02 mg bilirubin per 100 ml, was then added.

MALLOY AND EVELYN METHOD (1937) MODIFIED Spectrophotometry at 550 mμ was performed 15 minutes after adding methanol.

MICHAËLSSON METHOD This was performed as described by Michaëlsson (1961) and Michaëlsson *et al* (1965).

MODIFIED MICHAËLSSON METHOD The reagents are those used by Michaëlsson (1961).

Diphylline solutions Diphylline, 20 g (Diprophyl-line; 7 (2: 3-dihydroxy propyl) theophylline, of May and Baker), and 30 g anhydrous sodium acetate are dissolved in 400 ml of warm water and filtered.

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Diazo I Sulphanilic acid, 5 g, is added to 25 ml 12 N hydrochloric acid, making up to 1 litre with water.

Diazo II Sodium nitrite, 0.5 g, in 100 ml water is made up every three weeks.

Diazo reagent Diazo II, 0.25 ml, is added to 10 ml of diazo I, and is freshly made up at least twice a day.

Ascorbic acid Four g in 100 ml water.

Fehling II Sodium hydroxide, 100 g, and 350 g sodium potassium tartrate are dissolved in 1 litre of water.

PROCEDURE

<i>Modified Total Bilirubin Estimation</i>	<i>Direct</i>	<i>Blank</i>
1.0 ml plasma	1.0 ml plasma	1.0 plasma
0.5 ml diazo reagent	0.5 ml diazo	0.1 ml ascorbic acid
<i>Wait four minutes</i>	<i>Wait 10 minutes</i>	0.5 ml diazo
2.0 ml diphylline		2 ml diphylline
<i>Wait six minutes</i>		
0.1 ml ascorbic acid	0.1 ml ascorbic acid	
	2.0 ml diphylline	
1.5 ml Fehling II	1.5 ml Fehling II	1.5 ml Fehling II

Optical densities were measured within 30 minutes at 600 mμ in 1 cm cells. This method differs from Michaëlsson's method only in the addition of diphylline before the diazo reagent in the estimation of total bilirubin.

All optical densities were measured with a Unicam SP800 spectrophotometer.

RESULTS

Michaëlsson's (1961) method was used to estimate 88 plasma samples from seven patients with conjugated hyperbilirubinaemia due to intra- or extra-hepatic obstruction. Fifty-two total bilirubin values were less than the direct reacting bilirubin by as

much as 40%, and were consistently lower than those obtained simultaneously with the method of Malloy and Evelyn (1937).

Dilution of plasma with water or doubling the limiting concentration of sodium nitrite in the diazo reagent gave similar results, and the difference was sometimes observed with bilirubin levels as low as 0.6 mg per 100 ml.

Plasma samples were then first treated with diazo reagent alone, after which the diphylline was added at various times. Optical density was measured 10 minutes after starting the reaction with the diazo reagent. The colour initially developed rapidly, suggesting ready reaction of the conjugated pigment. When the diphylline was thus added between 30 seconds and six minutes after the diazo reagent the total bilirubin values were considerably increased, and now always exceeded the values for the direct pigment.

Analyses of total and direct reacting bilirubin were then made by Michaëlsson's (1961) method of a further 122 plasma samples from 13 patients with conjugated hyperbilirubinaemia. In addition, a duplicate analysis of total bilirubin of each sample was made by the modified method in which the diphylline was added four minutes after the diazo reagent. These 'modified' analyses for total bilirubin gave values of an average about 20% higher than the standard analyses.

The standard deviation of multiple readings made on the same plasma sample with the modified method was less than 0.05 mg per 100 ml with coefficient of variation of 3%, and of 18 different samples estimated in duplicate less than 0.06 mg per 100 ml for values over 1.0 mg per 100 ml, and less than 0.03 mg per 100 ml for lower values. Analyses made by the 'modified' method were also compared with those made by the method of Malloy and Evelyn (1937) on the same 10 samples. The mean difference between the readings was 7%.

Plasma from a patient with unconjugated hyperbilirubinaemia gave slightly higher results with the modified procedure than those obtained with Michaëlsson's standard method (mean 2.7%).

CALIBRATION

The slopes of the calibration curves of the two methods were compared using free bilirubin dissolved in 5% aqueous bovine and human albumin, and in bovine albumin dialysed for 24 hours against tap water. The slope obtained using Michaëlsson's (1961) method was unexpectedly higher than that with the 'modified' method, and this difference increased as the interval between the addition of azo reagent and diphylline was increased. When human

serum or plasma was used, however, the slopes were identical. The addition of bovine albumin to bilirubin already dissolved in serum or to the plasma from the patient with unconjugated hyperbilirubinaemia did not produce this discrepancy. The modified method was therefore calibrated with bilirubin dissolved in human serum and dilutions were made using the same serum. Beer's law was followed to an optical density of 0.8. A calibration factor was obtained from two separate preparations, and for undiluted plasma samples optical density $\times 4.5$ = bilirubin concentration in mg per 100 ml.

DISCUSSION

During this work Baer and Wood (1967) reported similar results in a small series in which they added diphylline two minutes after the diazo reagent. They attributed the difficulties with the Michaëlsson method to the reaction of free bilirubin with diphylline to form a compound not reacting with the diazo reagent. But this explanation is inconsistent with the similar results obtained using the standard and modified methods on the plasma samples from the patient with unconjugated hyperbilirubinaemia; moreover, the absorption spectrum of the azo pigment and of the diazo-diphylline complex during the reaction was not different, and was the same with samples containing the free or conjugated pigment. For the same reason it is unlikely that conjugated bilirubin forms a diazo-inactive, diphylline-conjugated bilirubin complex.

Alternatively diphylline inhibits the reaction of the conjugated pigment with the diazo reagent. Delaying the addition of diphylline after the diazo reagent gave a more acceptable estimation, and four minutes was established as a suitable delay. Presumably conjugated bilirubin first reacts rapidly with the diazo in four minutes (Lathe and Ruthven, 1958), and then the free pigment does so in the presence of diphylline. The total bilirubin estimated in this way showed better agreement with the method of Malloy and Evelyn and permits measurement of normal plasma total bilirubin concentrations as low as 0.04 mg per 100 ml.

The effect of bovine or human albumin in reducing the reaction of unconjugated bilirubin with the diazo reagent is not explained. Inhibition of the diazo reaction by the protein is unlikely since the addition of albumin to unconjugated bilirubin samples in serum had no effect. It is essential, therefore, to prepare calibration curves using serum or plasma and not reconstituted solutions (Billing, 1959; American Academy of Pediatrics, 1963).

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