CLXII. THE TECHNIQUE TO BE SELECTED FOR DETERMINATION OF BILIRUBIN IN BLOOD BY THE DIAZO-METHOD.

By EMANUEL GERARD GODFRIED.

From the Laboratory of Chemical Pathology, St Bartholomew's Hospital, London.

(Received April 27th, 1935.)

IN the present communication an attempt is made to decide which is the most practical method for estimating the total bilirubin in serum or plasma. To-day there are several modifications of Van den Bergh's test in use in different laboratories, and these different techniques give results which may vary quite widely, so that the findings reported by one laboratory are not comparable with those from another. Van den Bergh's direct reaction and its particular problems will not be considered now; the estimation of all the bilirubin involving treatment of the serum with alcohol, or, in other words, the "indirect quantitative test" alone will be studied.

In Van den Bergh's original description 1 volume of plasma or serum and 2 volumes of absolute alcohol are mixed and centrifuged. To 1 ml. of supernatant fluid 0.25 ml. of diazo-reagent and 0.5 ml. of absolute alcohol are added. The dilution factor is 5. The colour is compared with a standard solution of ferric thiocyanate in ether. Later on this ethereal standard was replaced by 2 % anhydrous cobaltous sulphate in water.

It was then shown that in some sera bilirubin is lost by adsorption on the protein precipitated by alcohol; this is largely prevented if the bilirubin is converted into azobilirubin before the precipitation of the proteins by alcohol, because azobilirubin is less strongly adsorbed than bilirubin itself; even more azobilirubin may be kept in solution in the alcohol if saturated ammonium sulphate is also added [Thannhauser and Andersen, 1921].

At this stage McNee and Keefer [1925] summarised the technique which might be universally adopted as follows. To 1 ml. of serum (or plasma) 0.5 ml. of diazo-reagent is added. After the maximum colour has developed 2.5 ml. of absolute alcohol and 1 ml. of saturated ammonium sulphate are added. The contents are mixed and centrifuged. The dilution factor is 4. The colour is compared with a standard solution of cobaltous sulphate (2.161 %).

Hunter [1930] then suggested that ammonium sulphate was unnecessary, and that the cobalt standard should be altered to 1.9 %, thus increasing the bilirubin estimates by some 14 %. His method simply consists in adding 0.5 ml. of diazo-reagent to 1 ml. of serum or plasma. After a short interval 2.8 ml. of absolute alcohol are added and the mixture is centrifuged, the dilution factor being about 4.

White [1932] dissolves 1.3 g. of anhydrous cobaltous sulphate in 50 ml. of distilled water, adds 40 ml. of conc. HCl (sp. gr. 1.19), mixes and cools. The volume is made up to 100 ml. and the mixture is ready for use in 24 hours.

It is more or less generally accepted that the aqueous cobalt standard is more satisfactory than the ethereal ferric thiocyanate standard; also that more accurate results are obtained if the bilirubin is azotised before the addition of alcohol. This is again clearly demonstrated by the results reported in the table below; if alcohol is added before the diazo-reagent (original method, column 2 of table) the results are nearly always lower, and may be very seriously low as compared with those where azotisation precedes the addition of alcohol (Thannhauser and Andersen's method, column 3 of table); Hunter's method gives results (column 4 of table) which are either the same as those yielded by Thannhauser and Andersen's method, or a little lower (significantly higher on one occasion only in this series), provided that the same standard is used for the two methods. Naturally the results (column 5 of table) are higher with the more dilute standard (1.9 %) recommended by Hunter. Those findings in column 5 which are a little lower than those in column 3 may be accounted for by the omission of ammonium sulphate in Hunter's modification.

No.	Original method.	Thannhauser-	Hunter's method.	Hunter's method.
	Cobalt standard 2.161 %	Andersen method. Cobalt standard 2.161 %	Cobalt standard 1.9 %	Cobalt standard 2.161 %
1	1.0	6.0	11.4	10.0
2	0.5	1.2	1.8	1.6
3	4.0	14.0	16.0	14.0
4	2.5	6.4	7.3	6.4
5	0.7	2.8	2.3	2.0
6	12.0	18.0	17.0	15.0
7	—	8.0	7.4	6.5
8	20.0	25.0	27.4	24.0
9	2.0	6.0	6.8	6.0
10	2.0	6.4	7.3	6.4
11	0.7	1.8	1.7	1.5
12	—	6.0	6.3	5.5
13	—	12.0	11.5	10.0
14	—	5.8	5.7	5.0
15	—	4.5	5.1	4.5
16	—	8.5	8.2	7.2
17	0.6	2.0	2.1	1.8
18	—	22.0	20.5	18.0
19	—	8.4	9.6	8.4
20	1.2	1.2	1.4	1.2
21	2.0	2.1	2.4	2.1
22	1.0	1.2	1.4	1.2
23	1.5	2.4	2.7	2.4
24	0.5	3.5	4.1	3.6
25	0.8	2.1	2.4	2.1
26	0.7	1.0	1.1	1.0
27	0.6	0.8	0.8	0.7
28	0.7	1.8	2.1	1.8
29	0.8	1.3	1.6	1.4
30	1.3	2.2	—	—
31	0.6	2.0	2.1	1.8
32	4.2	4.2	4.1	3.6

All values are in Van den Bergh units. The first 19 cases showed a "direct positive reaction", *i.e.* a red colour developed within a few minutes after the addition of diazo-solution to the plasma.

White's standard again gives different results. A few comparisons were made, but the standard shows no advantage.

The following table indicates that when the same final solution of azobilirubin is compared with the 2.161 % cobalt standard and with White's standard the latter will give a result some 25 % higher than the former.

Cobalt standard 2·161 %	White's standard	Cobalt standard 2·161 %	White's standard	Cobalt standard 2·161 %	White's standard
2·0	2·5	8·5	11·0	14·0	19·0
2·1	2·7	14·0	17·0	2·5	3·0
1·0	1·3	1·5	2·0	6·4	8·0
1·2	1·5	2·4	3·0	1·8	2·3
6·0	8·0	0·5	0·6	2·4	3·0
1·2	1·5	3·5	4·5	0·7	0·9
1·6	2·0	4·0	4·8	2·8	3·5

The writer concludes therefore that Hunter's and White's modifications are no improvement and suggests the universal adoption of Thannhauser and Andersen's technique as outlined by McNee and Keefer, unless and until further work results in the introduction of an undeniably more accurate method.

As pointed out by Harrison [1930] however the Thannhauser and Andersen technique is unsatisfactory if there is gross haemolysis, because the haemoglobin is not completely precipitated by the addition of alcohol and ammonium sulphate. In that case it is best to repeat the test with fresh non-haemolysed serum; alternatively the original Van den Bergh's method may be used, in which the 2 volumes of added alcohol do precipitate the haemoglobin, but, as has been shown, such results may be seriously low.

SUMMARY.

Out of the several procedures suggested for Van den Bergh's estimation of bilirubin in blood the method described by Thannhauser and Andersen and the concentration of cobalt sulphate (2·161 %) used by McNee and Keefer are selected as the most suitable for clinical purposes.

I am indebted to Dr G. A. Harrison for his suggestions, his help in compiling this paper and for the facilities provided in his laboratory. The work was done with the aid of the Prof. Stokvis' funds, University of Amsterdam, and with a grant from the Cassel Foundation.

REFERENCES.

- Harrison (1930). Chemical methods in clinical medicine, p. 247. (London.)
 Hunter (1930). *Brit. J. Exp. Path.* **11**, 407.
 McNee and Keefer (1925). *Brit. Med. J.* ii, 52.
 Thannhauser and Andersen (1921). *Deutsch. Arch. klin. Med.* **137**, 179.
 White (1932). *Brit. J. Exp. Path.* **13**, 76.