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Determination of Plasma Bilirubin; a Modified van den Bergh Procedure.

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The diazotization of bilirubin in the van den Bergh estimation is strictly proportioned to the intensity of the color produced.[1] However, the use of the artificial color standards and the stratification of the plasma-ammonium sulphate and alcohol in the determination afford a valid criticism of the procedures ordinarily employed. Some three years ago we adapted the Thannhauser-Anderson[2] method to avoid these difficulties. A strongly acidified solution of azobilirubin, which remains unchanged for 3 to 6 months if kept in a cold refrigerator, is employed as a standard. A proportional concentration of acid is added to the unknown. There is no stratification of the mixture, and the red reaction changes to an intense blue.[3] Publication of our procedure has been delayed until a ready

* P represents a preliminary, C a complete manuscript.

1 Hunter, G., *Brit. J. Exp. Path.*, 1930, **11**, 407.

2 Thannhauser, J. A., and Anderson, E., *Deutsch. Arch. f. klin. Med.*, **1921, 137**, 179.

3 Newman, C. E., *Brit. J. Exp. Path.*, 1928, **9**, 112.

source of bilirubin for the standard could be located. Failing in this, we have prepared our own bilirubin from bile rather simply. Foweather[4] recently has given a short procedure for obtaining bilirubin from gall stones. Our method of preparing bilirubin and the technique for the quantitative test follow:

Preparation of bilirubin. 500 cc. of bile from surgical drainage patients or 50 cc. of post mortem gall bladder bile (diluted 2-3 times) is allowed to stand a few hours in the refrigerator and the supernatant fluid is decanted. This is diluted several times with water. Barium chloride solution is added with stirring. If the precipitate of barium bilirubinate does not flocculate immediately, addition of a few drops of alkali usually suffices. When the precipitate settles the supernatant fluid is siphoned off. The precipitate is poured on a filter and is washed with water on the paper; it is air dried and pulverized in a mortar. The powder is extracted with warm alcohol and with ether or chloroform and again air dried. The precipitate is moistened with 10% sulphuric acid solution. It is then washed 3 times by suspension in a little absolute alcohol; we do this in 50 cc. centrifuge tubes, centrifuging and pouring off the alcohol. The residue is twice treated in a flask with boiling chloroform, filtered, and the chloroform evaporated with avoidance of over-heating towards the end. The bilirubin so obtained is rubbed into glacial acetic acid, centrifuged, the acid drained off, and the procedure repeated; the residue is air dried. It is again re-dissolved in boiling chloroform, filtered and evaporated to dryness. The final product (40 to 100 mg.) is brick red in color, is free from ash, and is quite stable. Standard solutions of bilirubin so prepared have checked with one another and with a supposedly pure German preparation (Schuchardt).

Preparation of the bilirubin standard. Ten mg. of bilirubin are dissolved in a little water with the aid of a few drops of 10% NaOH solution and the volume made up to 100 cc. To this is added 50 cc. of freshly prepared Ehrlich's diazo reagent, 100 cc. of saturated ammonium sulphate solution, and 400 cc. of alcohol. When the red color has developed, 100 cc. of concentrated HCl are poured in. The standard should be allowed to stand a few hours before using. It should be kept in the refrigerator and replaced after 3 months.

Determination of plasma bilirubin. To 2 cc. of plasma or serum, add 1 cc. of freshly mixed Ehrlich's reagent and mix. Note whether a "direct reaction" is obtained. Then add 2 cc. of saturated ammonium sulphate solution, 8 cc. of alcohol, and mix. When the red

[4] Foweather, F. S., *Biochem. J.*, 1932, **26**, 165.

color has developed, add 2 cc. of concentrated HCl, mix, and let stand for some minutes. Then filter through a pleated paper. The filtrate is placed in the colorimeter and the cup set at 10 mm. The standard is racked to match the unknown, the reading in mm. is the mg. of bilirubin per 100 cc. of the plasma.

Some 24 comparative determinations by our procedure and by the Thannhauser-Anderson method on the same specimen, using a cobalt standard and figuring the result as mg. of bilirubin, have given results ranging from 0.8 mg. to 30 mg. for the new method against 0.4 mg. to 12.3 mg. only for the artificial standards. Even as an "estimation" of the bilirubin content the older method is evidently progressively lower for the higher bilirubin values.

Normal values obtained with our procedure were determined on 7 subjects. The average of this series was 1.5 mg., the minimum 0.8 mg., and the maximum 2.0 mg. We have, however, obtained normals of 0.1 mg. to 0.5 mg. As in the other van den Bergh methods, values less than 2 mg. give a weak reaction and are difficult to compare in the colorimeter.

Values for plasma bilirubin in various pathological conditions have been selected for Table I.

TABLE I.
Plasma Bilirubin in Pathologic Bilirubinemia.

Diagnosis	Type of Reaction	Bilirubin mg. p.c.
1. Cardiac failure, mitral stenosis	Indirect	7.2
2. Pernicious anemia, 6 patients	"	1.5-6.3
3. Congenital hemolytic icterus, 3 pat.	"	4.0-5.2
after splenectomy, 2 patients	"	0.1-1.5
4. Acquired hemolytic icterus	"	7.2
5. Acute arsenical intoxication, lues	Direct	5.6-17.5
6. Catarrhal jaundice	"	49.2
7. Common duct stone, 2 patients	"	23.5-30.0
after operation, 1 patient	"	8.0
8. Obstructive jaundice, carcinoma	"	14.0
9. Chronic cholecystitis	"	10.6
10. Syphilis, hepatic	Indirect	2.0
11. Cirrhosis of the liver	"	2.4
12. Splenomegaly, etiology ?	"	0.5
13. Toxemia of pregnancy, 2 patients	"	1.6-3.0