THE CLINICAL VALUE OF THE VAN DEN BERGH REACTION FOR BILIRUBIN IN BLOOD:

WITH NOTES ON IMPROVEMENTS IN ITS TECHNIQUE.

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The reaction of Hijmans van den Bergh for the recognition and estimation of bilirubin in blood serum, plasma, and other albuminous fluids of the body is now well known to clinicians. More than two years have elapsed since the method was first described in the English language, and since then ample opportunity has been afforded of testing its applications in different directions.

It is not proposed to discuss here the influence of the method on research work in connexion with hepatic disease and bile-pigment metabolism, although this influence has already been great. It seems worth while, however, to reconsider, after two years, the present position and value of the test in its purely clinical aspects, and whether it has been of much service in the routine study of hepatic disease. Certain improvements in technique, which must add greatly to any merits possessed by the test, are also referred to.

During the past two years one of us (J. W. M.) has received a great deal of correspondence from workers, both in America and Great Britain, who have made use of the test. Various points of difficulty have been raised, some easily solved by the results of experience or by experiment, and some not easily explainable at a distance without knowledge of the reagents used. From this correspondence, however, several important points have emerged, and require discussion.

Publications on the purely clinical application of the test are already numerous, especially in Germany. No full bibliography need be attempted, but ample records of clinical results are contained in the following:

Feigl and Querner (1919), Lepehne (1920, 1921), Rosenthal and Holzer (1921), Brulé, Garban and Weissmann (1922), Andrewes (1924), and Hughes (1924). Application of the test to special clinical problems has been made by: Strauss and Buerkmann (1922), Gerrard (1924), Schamberg and Brown (1924)—latent and overt salvarsan (arsphenamine) jaundice; Schiff and Eliasberg (1922)—catarrhal jaundice; Fishberg (1924)—jaundice in myocardial insufficiency; Andrewes (1924)—peculiar diazo-reaction in uraemia; Broun, Ames, Warren, and Peabody (1925)—study of the blood pigments in pernicious anaemia.

OTHER CLINICAL TESTS FOR BILIRUBIN IN BLOOD SERUM.

Since the introduction of the van den Bergh reaction a number of other colorimetric methods for the estimation of bilirubin in blood serum have been developed, most of them designed for purely clinical use. One well known and simple method is that of Meulengracht (1921), in which the yellow serum is compared with a standard solution of potassium bichromate. By this method quantitative changes in the bilirubin content of an icteric serum are easily determined, but the presence of lutein or carotin in a serum are obvious sources of error. Moreover, the Meulengracht test is unsuitable for the detection of latent jaundice, and gives no qualitative differences such as are brought out by the van den Bergh technique. Van den Bergh has stated (1924) that after an examination by Müller (one of his co-workers) of all the simple colorimetric methods, the conclusion was reached that the original diazo method is still the best, and the present writers are in agreement with this. One other test for bilirubin in blood serum of recent introduction may be referred to here.

This method, introduced by Fouchet (1918), has attracted considerable attention, particularly in France, and although not so delicate as the van den Bergh reaction, it is said to give a positive result in a dilution of 1 in 60,000 of bilirubin. It consists essentially in mixing equal volumes of the serum with a reagent made up as follows:

Trichloroacetic acid	...	...	5 grams
Ferric chloride solution	...	...	2 c.cm.
Water	...	...	20 c.cm.

The mixture of serum and reagent is stirred with a glass rod, and in the presence of bilirubin a greenish-blue colour appears. The maximum reaction is said to be obtained after twenty minutes, and an almost instantaneous coloration in concentrations of bilirubin of less than 1 in 20,000. A scale of standard tints of malachite green, diluted with pure white lead, has been devised to give a rough quantitative method for clinical use. This test does not bear comparison with the diazo-reaction of van den Bergh for clinical purposes, but is important as affording a good and delicate confirmatory test of the presence of bile pigment in blood serum.

DIFFICULTIES IN THE TECHNIQUE OF THE VAN DEN BERGH REACTION.

In an article written by one of us (J. W. M.) in 1923 time limits for the development of different varieties of the "direct" van den Bergh reaction were given as follows:

Prompt Direct Reaction.—Bluish-violet colour, beginning immediately and maximal in ten to thirty seconds.

Delayed Direct Reaction.—Reddish coloration, gradually deepening to become more violet; begins only after one to fifteen minutes, or even much longer.

Biphasic Direct Reaction.—Reddish colour appears at once, and either slowly or quite rapidly deepens to violet.

These directions were founded on a moderate experience, and formed an average of the results obtained up to that time. It was not expected, however, that different observers, using different reagents, would obtain results within these exact limits, and it was believed that each observer must to some extent interpret his results from his own experience. Andrewes (1924), after considering the above directions and the time limit of thirty seconds given for the prompt direct reaction, states:

"I have hardly ever found such a reaction, and even with fresh bile the colour goes on changing after thirty seconds. If a red colour begins to appear within thirty seconds I consider that the direct reaction is positive."

One point of great importance for the development of the van den Bergh reaction has not been emphasized hitherto, but must always be considered in the interpretation of results. The interval of time between the withdrawal of blood and the carrying out of the test must be known, and should for routine clinical purposes be as short as possible—certainly not longer than two hours or thereabout. Experience has proved that serums showing a prompt direct reaction when tested at once give a slow and often long-drawn-out result when retested on the following morning. This has prevented one of us (J. W. M.) from dealing with serums forwarded by post for control examination.

Differences in reaction, either to the acid or alkaline side, are of great importance for the development of the correct colour of the azo-bilirubin compound. This can easily be proved after completion of the indirect reaction and addition of diazo-reagent to the supernatant fluid, by testing the effects of very dilute acid-alcohol or alcoholic-ammonium hydrate on the colour developed in the test. This point emphasizes the need for care and cleanliness in the reagents and glassware employed.

The occurrence of haemolysis in a serum interferes with the colour reaction in the test, and in recent work we have used oxalated plasma only. In practice, 0.2 c.cm. of a 10 per cent. solution of potassium oxalate is placed in a small wide loosely corked bottle, and evaporated to dryness during sterilization of the bottle. The oxalate is in this way finely distributed over a large surface, and will prevent coagulation of 10 to 15 c.cm. of blood, if the bottle be shaken.

IMPROVEMENTS IN TECHNIQUE.

A. Lepehne's Technique for the Qualitative Reaction.

This method has simplified the reading of results in the direct reaction, since it gives standards for comparison both in respect of the rate of development and time of completion.

Three small test tubes are taken. The first or control tube contains plasma diluted with water. The third tube contains plasma in which the diazo-reaction is allowed to develop and reach its maximum by the method described below. The second tube contains plasma to which diazo-reagent is added last of all, the character of any reaction being watched in comparison with the control tube (I) and the completed reaction (III).

Lepehne has applied an interesting observation of Adler and Strauss (1922) as an aid to completion of the reaction in tube III. These writers found, when attempting to

solve the problem of the two varieties of bilirubin apparently differentiated by the diazo-reaction, that the addition of a small amount of caffeine-sodium-salicylate induced in many icteric serums an increased rapidity in development of the maximal colour. They quote, for example, one serum giving a delayed reaction (1.6 units) only, the direct reaction being negative. Addition of a trace of caffeine-sodium-salicylate brought about a prompt direct reaction, maximal in ten seconds, while no change in colour ensued for at least two minutes in the absence of the drug. The original paper should be consulted for an account of the work leading indirectly to this observation. We have made use of caffeine-sodium-salicylate in a number of experiments, but find that the action stated above is by no means constant, at least with the samples of the drug used by us. In some serums the hastening of the colour reaction is definite, at other times no such effect was observed.

The technique of Lepehne, however, is so easy, and gives such good standards for comparison, that we recommend it strongly as the best method of carrying out the direct qualitative test.

Method.

0.25 c.cm. of the oxalated plasma is placed in each of three small test tubes. To tube I (the control) add 0.2 c.cm. of water. Drop a small flake of caffeine-sodium-salicylate into tube III, shake to dissolve completely, and then add 0.2 c.cm. of fresh diazo-reagent. In this tube a prompt or rapidly developing reaction may be obtained, quickly arriving at its maximum. Sometimes, in our experience, the effect of the drug is negative, but in any case ample time should be allowed for the colour reaction in tube III to be completed.

To tube II then add 0.2 c.cm. of fresh diazo-reagent, watching and timing the development of any prompt or biphasic reaction in comparison with the control tube I, and the completed reaction in tube III.

B. Improvement in the Quantitative Estimation of Bilirubin.

It has always been insisted on by van den Bergh that the estimation of bilirubin made possible by the indirect reaction is by no means accurately quantitative. He states (1924) that he has always called it simply "an estimation," and not a quantitative determination. This is because some bilirubin is always carried down and lost in the albuminous precipitate during the performance of the indirect reaction. This is particularly noticed in serums giving a prompt direct reaction, where the loss may be considerable, and the precipitate coloured bright yellow. In serums giving a delayed reaction the loss is much less. Where bilirubin is present abundantly the loss can be reduced by previous dilution of the serum with water, but this method is inapplicable where only small amounts of bilirubin are present.

This difficulty has been solved by Thannhauser and Andersen (1921), who have pointed out that all the azo-bilirubin can be retained for estimation in the supernatant fluid if diazo-reagent be first mixed with the test plasma to allow "coupling" to take place, and then alcohol and saturated ammonium sulphate solution be added. In this way, after shaking and centrifugalizing, a layer of clear ammonium sulphate solution is left at the bottom of the centrifuge tube, above this a white albuminous precipitate, and on the surface the clear reddish-violet alcoholic solution of azo-bilirubin. By this technique all the bilirubin from plasma giving a direct prompt reaction is available for estimation. This method is unnecessary for serums giving the delayed reaction only, where the loss of bilirubin in the albuminous precipitate is slight.

Method.

The method may be summarized as follows: To 1 c.cm. of serum add 0.5 c.cm. of diazo-reagent. After a minute or two add 2.5 c.cm. of 95 per cent. alcohol and 1 c.cm. of a saturated solution of ammonium sulphate. Mix and centrifugalize. If the colour of the supernatant fluid is too intense for quantitative estimation, dilute with alcohol 2 parts, water 1 part.

The rationale of this method need not be discussed in detail. It may be mentioned, however, that pure bilirubin is scarcely soluble in alcohol, and in the ordinary indirect reaction it is astonishing how much bilirubin remains in the alcoholic supernatant fluid after precipitation of the protein. Azo-bilirubin, on the other hand, is freely soluble

in alcohol. Moreover, Adler and Strauss have pointed out that in serums from cases of obstructive jaundice associated with a prompt direct reaction the globulin content of the serum is always diminished. They found that salting out the globulin fraction with magnesium sulphate hastened the development of the direct diazo-reaction under experimental conditions. It seems likely that the removal of globulin, or of water, or both, by ammonium sulphate in the method suggested by Thannhauser and Andersen has something to do with the results obtained, but this question requires further investigation. In this method some water certainly goes over into the saturated ammonium sulphate solution, so that the dilution of the serum in the supernatant alcoholic solution is really slightly less than 1 in 4. For practical purposes, however, the dilution of the serum in making the estimation may safely be taken as 1 in 4.

Table contrasting the Results of the Original Method of van den Bergh (I) and the Thannhauser and Andersen Modification (II).

Disease.	Type of Reaction.	Bilirubin Units.	
		I.	II.
Post-salvarsan jaundice...	Direct—either prompt or b. phasic	1.5	6.0
Post-salvarsan jaundice...		1.0	5.5
Carcinoma—head of pancreas		3.0	17.0
Carcinoma of liver—metastatic		7.0	15.0
Catarrhal jaundice ...	Indirect only	4.5	5.5
Ditto—end stage ...		0.5	0.5

C. Improved Standard Solution.

The standard ethereal solution of iron rhodanate, originally used by van den Bergh for estimation of bilirubin in "units," has also been a difficulty and source of trouble. In a review on jaundice (1923) some of the criticisms of this standard solution were briefly discussed, and it must be admitted that even when the test is carried out as carefully as possible with clean glassware and carefully prepared reagents, the colours of the test solution and of the standard may not accurately match. Slight changes in reaction of the various reagents may, as already noted, account for this, and one practical point noticed by us is that for diluting the test fluid, in order to bring it within the range of the standard solution, alcohol must be used and not water, or a change in colour tone at once ensues.

The practical difficulties with the ethereal standard solution do not end here. It must be freshly prepared for each set of estimations, and evaporation must, of course, be prevented. During the past summer Professor Hijmans van den Bergh has informed us of a new artificial standard solution which he and Müller have found can replace the iron rhodanate ethereal solution. So far as we are aware this standard has not been published, so we wish to acknowledge its source. The new standard is made up by dissolving 2.161 grams of anhydrous cobaltous sulphate in 100 c.cm. of distilled water. This solution gives a colour equivalent to 1 unit of bilirubin (1 in 200,000), and is almost identical in colour with the original iron rhodanate standard. It is permanent if kept in the dark, and the writers have found it entirely satisfactory in practice, and in direct comparison experiments with the original standard. The cobalt salt must be anhydrous, and at first the supply of the new cobalt standard should be compared with the old ethereal standard and any necessary adjustments in colour made. We have found slight adjustments necessary with the cobalt salt used by us. The ideal thing would be to compare the standard with the diazo-reaction given by a solution of 1 in 200,000 of pure bilirubin, but pure bilirubin is practically unobtainable.

It seems certain that this new and permanent standard must replace the older ethereal solution. If a Hellige wedge colorimeter be used, one wedge can be kept full and sealed, and is then available at any time.

SUMMARY AND CONCLUSIONS.

1. The van den Bergh reaction is the best method so far available for the clinical investigation of the bilirubin

content of blood serum or plasma. It has numerous advantages over other tests which have subsequently been proposed: (a) its extreme delicacy; (b) is not given by other yellow substances which may colour the plasma; (c) gives important qualitative distinctions between certain forms of icterus; (d) can now be used for a satisfactory quantitative estimation of bilirubin.

2. The qualitative reaction may enable a positive diagnosis of obstructive or of haemolytic icterus to be made. Pernicious anaemia, except in the stage of rapid temporary improvement, can be distinguished from secondary anaemias. In some diseases (for example, catarrhal jaundice, subacute liver atrophy, cardiac failure) the reaction may pass through all the stages from completely delayed to prompt direct, as the disease progresses. As a rule, however, in the commonest type of jaundice (the toxic and infective hepatic group) no information of any diagnostic or prognostic value is given to the clinician.

3. The test is of great practical value for the detection of "latent" jaundice.

4. Various alterations in the original technique have improved and simplified its use in clinical medicine.

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RADIOLOGY OF THE GALL BLADDER BY GRAHAM'S METHOD.*

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It is generally admitted that the routine examination by x rays of the gall-bladder area for the presence of stones is subject to serious limitations as regards diagnostic value, for even by means of good radiographs and skilful interpretation their existence can only be clearly demonstrated in, at the outside, 35 to 40 per cent. of the cases where they are present. Though an x-ray finding if positive is of definite diagnostic value, a negative finding does not exclude the presence of gall stones and must be ignored.

In the interpretation of shadows found in the gall-bladder area there are two types sufficiently characteristic to be practically diagnostic of gall stones. These are the annular or ring-like shadow (Fig. 1) and the cluster of shadows resembling a bunch of grapes (Fig. 2). The finding of these shadows in a radiograph of the right upper abdomen is practically certain evidence of the presence of gall stones; especially if, by radiographs taken in different positions of the body, they are shown to be situated nearer the front of the abdomen than the back. Other atypical shadows may be found in the region of the gall bladder which call for more serious thought and skill in interpretation before they can be taken as evidence of gall stones.

A large number of gall stones, however, are not sufficiently opaque to x rays to throw any shadow at all when they are in the body. Some, indeed, are so translucent that even when the gall bladder containing them is removed and radiographed they are no more opaque than the bile or the walls of the gall bladder by which they are surrounded; in

fact, they may be even less opaque, and may show in the radiograph of the free gall bladder as translucent areas in the shadow of the more opaque gall bladder and bile.

In 1913 Holland and Williams investigated the factors underlying this varying degree of opacity of gall stones. They found that the opacity depended on the proportion of the pigment and other salts of calcium. Those containing a large proportion of cholesterol were the most translucent, while those containing a large proportion of pigment or other salts of calcium were the most opaque. The calcium salts are often deposited at the periphery of the stone and so give rise to the characteristic annular shadow.

Apart from the direct x-ray evidence of gall stones, the existence of a pathological condition of the gall bladder may often be suspected from abnormalities of contour, position, or function, of the stomach or duodenum, found during the examination of the gastro-intestinal tract by the opaque meal; or by a process of elimination, by means of x rays, of gastric, duodenal, and appendicular lesions, and of urinary stones.

GRAHAM'S METHOD OF EXAMINATION.

The method of radiologically investigating the gall bladder, observations on which we wish to record in this paper, is attributable to Graham of St. Louis, U.S.A. It is based on the use of a salt opaque to x rays which, when injected into the blood stream, is excreted by the liver, enters the gall bladder, and is there concentrated, thus rendering the gall bladder under normal conditions opaque to x rays.

Rowntree has shown that certain dyes—for example, phenol-tetrachlorophthalein—when injected into the blood stream, are excreted almost entirely by the liver. The rate of disappearance of this dye from the blood

stream has been used as a test of liver function. Graham and Cole showed that tetrabrom-phenolphthalein salts—of both calcium and sodium—were excreted by the liver almost entirely and were opaque to x rays. The iodine compound, although more opaque, was, in their opinion, too toxic for general use. More recently Whitaker and Milliken, after a careful clinical and

FIG. 1.—Two gall stones, showing opaque periphery and nucleus, the intermediate portion being more translucent. Annular type of shadow.

* Read at a meeting of the Liverpool Medical Institution.