THE SIGNIFICANCE OF THE VAN DEN BERGH
REACTION

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Introduction

A REASSESSMENT of the clinical significance of the van den Bergh reaction
is rendered highly desirable by the development during the last 15 years of
new methods for the determination of bilirubin in body fluids as well as for
the estimation of the proportions of the alleged direct and indirect forms
of bilirubin. Moreover, modern photo-electric devices permit an accurate
measurement of the rate of formation of colour in the van den Bergh diazo
reaction, and thereby offer a more objective means of distinguishing between
so-called prompt, biphasic, and delayed direct van den Bergh reactions given
by jaundiced sera. Van den Bergh and Muller (1916) showed that the
presence of alcohol was not necessary in the production of the red pigment
azobilirubin by the action of a solution of diazotized sulphanilic acid (the
Ehrlich diazo reagent) on bile or sera from patients with obstructive
(regurgitation) jaundice. Such sera were said to give a direct reaction in
contrast to sera from normal persons or from patients with haemolytic
(retention) jaundice, which formed azobilirubin with the diazo reagent only
in the presence of alcohol or other hydroxylic substances, and which were
said therefore to give an indirect reaction. Van den Bergh and Muller (1916)
observed that sera giving the indirect reaction either completely failed to
react with the diazo reagent in the absence of alcohol, or gave only a delayed
direct reaction, but they did not regard these differences as of clinical signi-
ficance. Later workers (Feigl and Querner, 1919; Lepehne, 1920; McNee,
1922) subdivided the direct reaction into prompt and biphasic types according
to whether the formation of azobilirubin rapidly approached its maximum
or whether the initial period of rapid formation was followed by a more
gradual deepening of the colour. Such biphasic reactions, which are common
in hepatogenous jaundice, have hitherto been regarded as due to the presence
in the serum of two forms of bilirubin, one (direct bilirubin) giving a prompt
reaction, and the other (indirect bilirubin) the delayed reaction character-
istic of retention jaundice. Attempts to determine the rate of formation of
azobilirubin in the direct reaction have been made by Malloy and Evelyn

1 Received February 26, 1947.
2 In the present article the nomenclature used will be that of Rich (1930) who classi-
fied jaundice into retention and regurgitation types. Retention jaundice corresponds
to haemolytic jaundice, but regurgitation jaundice is subdivided into mechanical and
parenchymal types corresponding to obstructive and hepatogenous jaundice respectively.

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Numerous methods of determining the proportion of directly and indirectly reacting bilirubin have therefore been described, some depending on an alleged difference in solubility in chloroform of the two forms of bilirubin (Newman, 1928; Sepulveda and Osterberg, 1942). Ducci and Watson (1945) have shown that the chloroform-soluble bilirubin is regularly less in amount than the indirect bilirubin fraction as determined by another procedure, and it seems reasonably certain that a variable loss of bilirubin must result when attempts are made to extract the pigment in the presence of protein. Others have measured the relative proportions of azobilirubin formed with and without the addition of alcoholic substances. Malloy and Evelyn (1937) compared the amount of azobilirubin formed in aqueous solution in 30 min. with the total amount formed in the presence of methyl alcohol. Rappaport and Eichhorn (1943) measured so-called direct bilirubin by azobilirubin formation in the presence of a phosphate buffer containing urea, and total bilirubin in the presence of citric acid and caffeine. Ducci and Watson (1945) differentiated prompt-reacting bilirubin and delayed direct-reacting bilirubin by measuring the azobilirubin formed in 1 min. and 15 min. under the conditions of the Malloy and Evelyn method. It therefore appears necessary to consider critically the relative merits of each of these methods of investigation, and to decide which, if any, are likely to be of greatest value in clinical diagnosis.

**Prompt, Biphasic, and Delayed Reactions and their Clinical Significance**

A photo-electric method for investigating the direct van den Bergh reaction by following the rate of formation of the red pigment was first described by Malloy and Evelyn (1937). Lepehne (1941), however, found this method to be of little value in diagnosis. Gray and Whidborne (1946) used a modification of the method of Rappaport and Eichhorn (1943) and followed the course of the direct reaction by measuring the azobilirubin formed after varying times. It was thus shown that sera from patients with acute hepatitis or obstructive jaundice gave a prompt, biphasic, or delayed reaction according to the amount of bilirubin present and not according to the type of jaundice. These different appearances were found to be due to the failure of strong azobilirubin solutions to absorb light proportionately to their concentration (that is, they fail to obey Beer's Law). The classification of direct van den Bergh reactions into prompt, biphasic, and delayed types is therefore of no value whatever in the differentiation of acute hepatitis from obstructive jaundice.

**The Direct-Indirect Quotient (D.I.Q.)**

The biphasic reaction given by some sera was formerly attributed to the presence of both direct and indirect reacting bilirubin, but this explanation
is no longer tenable. Moreover, the existence of two distinct forms of bilirubin has never been demonstrated unequivocally, so that the significance of alleged measurements of direct and indirect bilirubin requires reinterpretation. Methods of investigation depending on the alleged solubility in chloroform of indirect bilirubin are useless owing to incomplete extraction in the presence of protein, and need no further consideration. Measurements of the relative proportions of azobilirubin formed with and without the addition of alcoholic substances might be more satisfactory, and Gray and Whidbome (1947) have suggested that the use of the terms direct and indirect bilirubin should be abandoned in favour of the term direct-indirect quotient. This direct-indirect quotient, conveniently abbreviated to D.I.Q., may be defined as:

\[
\frac{\text{final amount of azobilirubin formed in the direct reaction}}{\text{final amount of azobilirubin in the indirect reaction}} \times 100.
\]

It has long been known that sera from patients with retention jaundice may give delayed direct van den Bergh responses, and since similar reactions may be obtained in regurgitation jaundice as well as chronic liver damage when the serum-bilirubin is only slightly raised, it appeared important to investigate more fully the possibility of distinguishing these conditions by determination of the rate of the direct reaction, or by suitable measurements of D.I.Q. The value of D.I.Q. will vary with the pH at which the reaction is investigated and with the time at which the extent of the direct reaction is measured, as well as with the composition of the reaction mixture of the particular method used. The problem therefore presents itself as to which of the various methods of measuring D.I.Q. is of the greatest value in clinical work.

Methods

Of the methods available, only those are worthy of consideration in which the risk of adsorption of pigment on to precipitated protein is avoided. The methods of Rappaport and Eichhorn (1943) and Malloy and Evelyn (1937) fulfil this condition, and it has been shown that for the determination of total bilirubin these two methods agree well, provided precautions are taken in the preparation of the calibration curves. The direct reaction was investigated by a photo-electric modification of the method of Rappaport and Eichhorn, the course of the direct reaction being followed by measuring the azobilirubin formed after varying times in the presence of a phosphate buffer containing urea. Since with certain sera the presence of urea in the reaction mixture has a profound effect upon the course of the direct reaction, similar investigations have been made using a phosphate buffer without urea. Similarly, the course of the indirect reaction was followed by measuring the azobilirubin formed after varying times in the presence of a buffer containing

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3 The final concentration of urea in the reaction mixture is 18.6 gm. per 100 c.c. and the effect described above is not seen when urea is present in the much smaller concentrations found in the body fluids in clinical uraemia.
cafeine and sodium citrate. Measurements by the Malloy and Evelyn method were made at 1, 15, and 30 min. after mixing the reagents. These investigations have been described in detail elsewhere (Gray and Whidborne, 1946, 1947). All measurements of azobilirubin were made in the Spekker photo-electric absorptiometer.

Results

Rappaport and Eichhorn method. A detailed analysis of most of the results has been given elsewhere, but for clinical purposes the direct reaction may be described as rapid when it is 90 per cent. complete within 30 min., and slow when it is not. These terms have been used in preference to the more usual prompt and delayed direct reaction, because there is no correlation between the diazo reaction in the presence of urea and the ordinary van den Bergh reaction.

Urea present in the reaction mixture. In regurgitation jaundice the direct reaction was always rapid, and the D.I.Q. high, that is, above 55. With 59 sera from 30 cases of retention jaundice the D.I.Q. was equally high and the direct reaction rapid, whereas it was slow in only six sera from five cases of retention jaundice. In the last six sera the D.I.Q. was significantly lower than that encountered with sera from cases of regurgitation jaundice.

Urea not present in the reaction mixture. In regurgitation jaundice the direct reaction was just as rapid and the D.I.Q. just as high as was found in the presence of urea. In retention jaundice a slow direct reaction was

### Table I

**The Nature of the Diazot Reactions and the Direct-Indirect Quotient (D.I.Q.) of Sera in Regurgitation and Retention Jaundice**

<table>
<thead>
<tr>
<th>Type of jaundice</th>
<th>Speed of reaction</th>
<th>Number of sera</th>
<th>D.I.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct reaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of urea</td>
<td>Regurgitation</td>
<td>Rapid</td>
<td>42 (33 cases)</td>
</tr>
<tr>
<td></td>
<td>Retention</td>
<td>Slow</td>
<td>59 (30 cases)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (5 cases)</td>
<td>53 ± 0.1 (30-74)</td>
</tr>
<tr>
<td>Absence of urea</td>
<td>Regurgitation</td>
<td>Rapid</td>
<td>12 (9 cases)</td>
</tr>
<tr>
<td></td>
<td>Retention</td>
<td>Slow</td>
<td>11 (9 cases)</td>
</tr>
<tr>
<td>Indirect reaction</td>
<td>Regurgitation</td>
<td>Slow</td>
<td>42 (33 cases)</td>
</tr>
<tr>
<td></td>
<td>Retention</td>
<td>Rapid</td>
<td>41 (33 cases)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 (13 cases)</td>
<td></td>
</tr>
</tbody>
</table>

### Table II

**D.I.Q. Values Obtained at 1, 15, and 30 Minutes by the Malloy and Evelyn Method**

<table>
<thead>
<tr>
<th>1 min. reading</th>
<th>Type of jaundice</th>
<th>Number of sera</th>
<th>D.I.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Regurgitation</td>
<td>12</td>
<td>32 ± 1.0 (34-41)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>12</td>
<td>61 ± 1.3 (55-69)</td>
</tr>
<tr>
<td>6</td>
<td>Retention</td>
<td>12</td>
<td>65 ± 1.0 (60-71)</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>6</td>
<td>2.3 ± 1.2 (0-9)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>6</td>
<td>11 ± 1.8 (0-18)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>18</td>
<td>30 ± 5.4 (8-69)</td>
</tr>
</tbody>
</table>
usually observed, while the D.I.Q. was generally much lower than in regurgitation jaundice. In one case of retention jaundice, however, the direct reaction in the absence of urea was rapid on three different occasions, and the D.I.Q. was well within the limits found for regurgitation jaundice.

The course of the indirect reaction. The indirect reaction was rapid in all cases of regurgitation jaundice examined, but in retention jaundice it was slow in about two-thirds of the cases.

These results are summarized in Table I.

*Malloy and Evelyn method.* Table II shows the D.I.Q. values given by the Malloy and Evelyn method when measurements were made at 1, 15, and 30 min. after the addition of the diazo reagents. The Figure shows the distribution of 30 min. D.I.Q. values found in cases of acute hepatitis, obstructive jaundice, haemolytic jaundice, and cirrhosis of liver.

**Discussion**

The visual direct van den Bergh reaction. It is generally recognized that the ordinary direct van den Bergh reaction is of only limited value in diagnosis. A delayed direct reaction is often seen with sera from patients with retention jaundice partly owing to the frequent coexistence of liver damage, and
partly because a weak direct reaction is very readily detected since the serum is only diluted with half its volume of diazo reagent. With considerable experience of the visual van den Bergh reaction, it is possible to judge with a fair degree of accuracy whether a given delayed direct reaction is likely to be associated with regurgitation or with retention jaundice if it is considered in relation to the total amount of bilirubin present. Thus the direct reaction associated with a total serum bilirubin of 2·0 mg. per 100 c.c. in regurgitation jaundice, may be stronger than that observed with a total bilirubin of 4·0 mg. per 100 c.c. in retention jaundice. Such distinctions are difficult and may be misleading when the retention jaundice is accompanied by liver damage.

The Rappaport and Eichhorn method and its modifications. The rapid rate of the direct reaction in the presence of urea observed in almost all cases of jaundice clearly renders such investigations useless for the differentiation of retention from regurgitation jaundice. In the absence of urea the slow reaction and significantly lower D.I.Q. in retention jaundice suggest that such investigations might be of greater value, but the occasional occurrence of a rapid reaction and a high D.I.Q. in such cases limits the significance of these findings. The considerable percentage of rapid indirect reactions in retention jaundice similarly limits the value of following the course of the indirect reaction. Thus a slow indirect reaction is almost diagnostic of retention jaundice, but a rapid indirect reaction may occur in either retention or regurgitation jaundice. It is probable that such determinations of the rate of formation of azobilirubin give no less information than do the D.I.Q. determination at 30 min. by the Malloy and Evelyn method. The infinitely greater rapidity and simplicity of this latter method, however, renders it unquestionably the method of choice.

The Malloy and Evelyn method. A superficial consideration of the 1 min. and 15 min. Malloy and Evelyn values for D.I.Q. would suggest that these might be of considerable value in the differential diagnosis of the two forms of jaundice, but it must be remembered that few cases of retention jaundice show bilirubin contents greater than 4 mg. per 100 c.c. and the D.I.Q. readings at 1 min. and 15 min. therefore would correspond to about 0·2 and 0·5 mg. per 100 c.c. respectively. At this level measurements of optical density by many photo-electric devices are relatively inaccurate unless inconveniently large depths of solution are used, and even then measurements may be rendered inaccurate by minor variations in clarity of the solutions. For routine work, therefore, it is preferable to measure the Malloy and Evelyn D.I.Q. at 30 min., that is, at the same time as the total bilirubin. With this technique, the two forms of regurgitation jaundice, as typified by acute hepatitis and obstructive jaundice, give almost identical distributions of D.I.Q. and are quite indistinguishable by such measurements. Neither the direct van den Bergh reaction nor any of its modern modifications enable one to distinguish between the jaundice of acute hepatitis and that due to obstruction. In retention jaundice the D.I.Q. is generally, but not
always, lower than in regurgitation jaundice. The series of cases of chronic liver damage examined by the Malloy and Evelyn technique gave, as was expected, similar results to those observed in regurgitation jaundice.

Conclusions

It is clear that even with the refined modifications of the van den Bergh reaction now available, it is impossible to distinguish between the parenchymal and mechanical forms of regurgitation jaundice, and it is not always possible to distinguish with certainty between retention and regurgitation jaundice. Retention jaundice is frequently complicated by liver damage and in such circumstances can be distinguished from regurgitation jaundice only by more elaborate hepatic function tests. It is possibly worthy of mention that in one case of constitutional non-haemolytic jaundice the typical findings of retention jaundice were observed, although it is generally considered that the inability of the liver to clear bilirubin is the principal factor in the causation of this condition.

Of the methods studied, the Malloy and Evelyn measurement of D.I.Q. at 30 min. is the simplest and most helpful. The Malloy and Evelyn method is specially valuable since it gives more accurate results for the determination of total bilirubin than most of the earlier methods. A D.I.Q. below 40 is diagnostic of retention jaundice, and a D.I.Q. above 50, although usually indicative of regurgitation jaundice, can occur in retention jaundice complicated by liver damage. A D.I.Q. between 40 and 50 is highly suggestive of, but not absolutely diagnostic of, retention jaundice. In cases of difficulty a reticulocyte count, erythrocyte fragility estimation, search for spheroctosis, or determination of the faecal stercobilin excretion may be essential for the clinician to establish the diagnosis.

Summary

1. The significance of the various types of direct van den Bergh reaction is discussed, and it is emphasized that the so-called biphasic reaction is not due to the presence in the serum of two forms of bilirubin, but to the failure of strong solutions of azobilirubin to follow Beer's Law.

2. The terms direct and indirect bilirubin should be abandoned because there is no proof that these two forms really exist.

3. The use of the term direct-indirect quotient (D.I.Q.) is recommended since it merely gives an expression of the extent of the direct reaction under the conditions of the experiment.

4. Neither the direct van den Bergh reaction nor any of its modern modifications enable one to distinguish between the jaundice of acute hepatitis and that due to obstruction.

5. For clinical purposes, the method of Malloy and Evelyn is the simplest and best of those examined. A D.I.Q. below 40 is then diagnostic of retention jaundice, and a D.I.Q. above 50, although usually indicative of
regurgitation jaundice, can occur in retention jaundice complicated by liver damage.

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