SOME ASPECTS OF THE VAN DEN BERGH TEST FOR BILIRUBIN.

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In a previous paper (M'Gowan, 1928) the jaundice occurring in iron-deficient pigs was discussed. It was emphasised that a correct buffering* and the nature, as regards strength, of the acid used in making up the Van den Bergh reagent influenced the appearance of the colour-change considerably. The present paper is a continuation and amplification of these observations.

Experiments were carried out with jaundiced pig and human sera and urines, and with solutions of bilirubin. The bilirubin used for the preparation of the solutions was that of Grübler. The B.D.H. universal buffer solution and phosphate buffer (1 per cent.) at $\rho H = 7$ were employed. Measurements were made with a teat pipette in which 1 volume equalled a certain number of drops delivered by the same pipette. The hydrogen-ion concentration, estimated in the majority of the tests, was obtained by means of the B.D.H. capillator.

It may be stated at the outset that the crucial facts, on which the Van den Bergh reaction appears to turn, seem to be the solubility of the bilirubin in alkaline, and its precipitation in acid solution, together with these and other factors which influence the velocity of the diazo reaction.

Bilirubin is acidic in character. It is very slightly soluble in distilled water. On adding powdered bilirubin to distilled water, some dispersion takes place with the production of an orange-coloured opalescent turbidity. In a zone of H-ion concentration to the alkaline side of neutrality, the bilirubin would appear to be in the colloidal state. If now a weak solution of an alkali (NaOH) be added drop by drop further dispersion and solution occur gradually, but it is not until the $\rho H$ has reached the neighbourhood of 10 or over that complete solution occurs. Its appearance is then orange or yellow, according to the concentration of the bilirubin. On diluting the orange fluid with distilled water, the colour changes to

* Davies and Dodds (Brit. Journ. Exp. Path., 1927, viii. 316) were the first to draw attention to certain aspects of the importance of buffers in the performance of the test.
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yellow, the H-ion concentration, however, remaining practically the same. If an attempt is made to dissolve the bilirubin in buffer solution (pH 7), the bilirubin goes into solution at the same H-ion concentration (about pH 10), but much more alkali has to be added before this pH is reached. The excess is absorbed by the buffer.

The Van den Bergh test may now be performed on these dispersions and solutions of bilirubin by the routine methods. In addition, a modification may be employed by the substitution of 1.5 per cent. acetic acid in solution No. 1 for the 1.5 per cent. HCl (density 1.16) usually used. This is N/4 acetic acid as compared with N/7 HCl, and a solution of a weak as compared with that of a strong acid.

Under such conditions, if the test is performed on a neutral dispersion in distilled water with hydrochloric or acetic Van den Bergh solution, the result is a very delayed direct one: if on alkaline distilled water solution, again a delayed direct is obtained, which appears earlier with the acetic Van den Bergh; while, if on the alkaline buffer solution, an immediate direct is forthcoming.

The H-ion concentrations obtained in a series of this type are of the following order:

|                      | H-ion concentration (pH) |
|----------------------|--------------------------|
|                      | Before adding Van den Bergh Solution | After adding Van den Bergh. |
|                      | HCl V. d. B. | Acet. V. d. B. |
| 1. Distilled water suspension | 7.2 | 2.0 | 3.2 |
| 2. Alkaline water solution | 9.2 | 2.0 | 3.4 |
| 3. Alkaline buffer solution | 9.2 | 4.9 | 4.7 |

On allowing this series to stand for twenty-four hours, an orange deposit of bilirubin will be found in the distilled water suspension tubes, and to a slighter extent also in those of the alkaline water solution, the acid of the Van den Bergh solution having precipitated the bilirubin. In the alkaline buffer solution, the buffer has prevented this and there is no deposit. The moiety of bilirubin in these cases which was not precipitated has gone to the formation of the coloured azo-compound.
No substance, however, is absolutely insoluble. In such tests the precipitated bilirubin is in equilibrium with a small amount of bilirubin dissolved in the supernatant fluid. As this dissolved material is used up to form the colour product, more of the precipitated material goes into solution to take part again in the reaction, and so on. This would appear to afford an explanation of the reaction appearing in suspensions of bilirubin and of the delayed direct reaction so-called.

Bilirubin may be left for long periods in suspension in neutral or acid, water or alcohol media without undergoing change. Alkaline solutions in either media, however, rapidly deteriorate.* The greater the alkalinity the greater the destruction. It is evidenced by a disappearance first of the orange and then of the yellow colour and by the failure to give the Van den Bergh reaction as time goes on. This in all probability has a bearing on the phenomenon of a serum giving an initial direct immediate result, changing on standing to one giving an indirect. In such circumstances the H-ion concentration of the serum, 7.2 to start with, rises by loss of CO₂ to the neighbourhood of 8.5, the bilirubin thus being left in a fairly alkaline solution and diminishing in amount.

Bilirubin comports itself with regard to alcohol in much the same way as to water. There are some significant differences, however, which may be noted. Bilirubin is much more soluble in alkaline alcohol than in alkaline water solution. On adding a drop of a weak solution of alkali to an alcohol suspension of bilirubin, the yellow or orange colour of solution appears like a flash, differing from the long-drawn-out result with water. Precipitation of the bilirubin in alcoholic solution is less easily effected by means of acid; and the velocity of the azo-reaction is greater in alcohol than in water. For these reasons the colour product can appear much more easily. This is possibly the explanation of the uniformity with which the indirect reaction is obtained whenever bilirubin is present. As a corollary to this, however, it would seem that the ordinary method of estimating bilirubin must be inaccurate because there is a race here between the acid to precipitate the bilirubin

* A watery solution of bilirubin in alkali of just sufficient strength to dissolve it (pH = 10) and of a strength of 150 units, on standing at room temperature for forty-eight hours loses nearly all its colour and ceases to give a positive Van den Bergh reaction even when buffered.
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and the other factors to change it into a soluble colour product before it can be precipitated.

Buffering substances in the alcoholic media, as in the water, favour the appearance of the colour-change.

It was suggested in the previous paper (McGowan, 1928) that the action of the alcohol might be to prevent the dissociation of the acid and so keep the H-ion concentration at a level favourable for the appearance of the colour-change. This does not appear to be the correct explanation, as the presence of alcohol does not effect this but seems to have rather an opposite tendency.

Where alcohol is used in the test, as in the indirect method, the pink colour obtained at first often goes through a series of colour-changes. At times the pink may change to purple and then more slowly to blue, green, and finally yellow; at others there may be rapid decolorisation followed by a blue-green coloration, green, and finally yellow. These changes of colour are dependent on the presence of alcohol, and the more alcohol present the more likely they are to occur. A low H-ion concentration favours their appearance, but this of itself in the absence of alcohol will not cause them. The presence of a large amount of diazo reagent relatively to the amount of bilirubin is also of importance. Where a small amount of diazo reagent is present in relation to a small amount of alcohol and a large amount of bilirubin the pink coloration may persist for days or at most change to purple.

When the test is performed in aqueous solution, there is a tendency for the original pink to change slowly by intermediate orange tints into a golden yellow. This occurs especially if the H-ion concentration of the test mixture is high comparatively speaking, or round neutrality. Whether the final yellow arrived at in aqueous and alcoholic solution is the same substance has not been determined.

In both aqueous and alcoholic tests, the presence of small amounts of bilirubin relative to large amounts of diazo reagent added may give rise to difficulties in interpretation of results. In both media there occurs a fleeting pink blush, often not seen, followed by immediate decolorisation and the appearance of a pellucid limpidity. In the case of water, this slowly becomes yellow, while with alcohol it changes to blue, green, etc. Bilirubin, actually present, may therefore be missed, and it may be that these late appearing yellow and green products
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may yet come to acquire a significance in the determination of the presence of bilirubin.

The factors influencing the appearance of the pink of the Van den Bergh reaction may be briefly summarised. There are those which speed up the appearance of the colour-change, or the reaction velocity, by the provision of a large amount of reacting bilirubin. There may be thus a large amount of bilirubin present initially, and this may be protected from inactivation (by precipitation) by the presence of buffers, solution in alcohol, and the use of weak acids in the preparation of the Van den Bergh test solution. Weak acids, apart from less liability to precipitation, allow of a much larger amount of the reagent being added for a given amount of precipitation and so increase the velocity of the reaction by a mass effect. Conversely a just sufficient amount of the reagent may be added in such circumstances with much less precipitation of the bilirubin present. The actual H-ion concentration of the solution would appear to affect the appearance of the reaction only very slightly. It can be obtained in a region extending from a pH on the alkaline side of neutrality down to one in the neighbourhood of two, if the favouring conditions enumerated above are present in an optimum degree. The crucial desideratum would appear to be to have the conditions so that the bilirubin is changed into the soluble colour product as rapidly as possible before it can be precipitated.

Bilirubin is very soluble in chloroform*: the alkaline salt, however, is much less soluble. These facts may be applied to an understanding of a test which has been used in an attempt to differentiate hæmolytic from obstructive jaundice. Hæmolytic bilirubin is said to be soluble in chloroform, while obstructive bilirubin is regarded as insoluble. The test may be employed by diluting the test fluid (serum, bile, urine, etc.) with alcohol, filtering, then adding chloroform, mixing and subsequently adding water. The chloroform separates out in a layer at the bottom of the tube, coloured or uncoloured with

* Chloroform may be used, like a buffer, to assist the appearance of the Van den Bergh colour-change. The addition to the test fluid of a drop of chloroform and shaking produces a fine chloroform suspension. The chloroform particles dissolve up the bilirubin as it is precipitated by the acid of the reagent and so keep it in a reactive form and its minute subdivision allows of the reagent attacking it. This reaction may be employed as a means of estimating bilirubin in a fluid, cobaltous nitrate solution being used as a standard.

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bilirubin according as the latter is soluble or insoluble in chloroform. If this test is performed on a fluid, such as bile, without the reaction of the bile being taken into account, very irregular results will be obtained. Bile may vary in reaction so much as to be in one sample acid to litmus, in another alkaline, with a pH range of 4.0 to 9.0, and the result obtained will depend on the reaction present. If, however, the bile is made distinctly acid, thus precipitating the bilirubin, then a constant result, solution of the bilirubin by the chloroform, is obtained. The same would appear to hold in regard to "haemolytic" and "obstructive" jaundice sera in the sense that "obstructive" bilirubin occurs in a medium which is more buffered and so less liable to acid precipitation than "haemolytic" bilirubin. The problem seems to resolve itself into the question of the alkaline reserve present in obstructive jaundice serum cases as compared with haemolytic jaundice ones. Bile salts, the combination of a strong base and a weak acid, would seem to increase the alkaline reserve in obstructive cases. Experimentally they act as buffers when tested by their effect on H-ion concentration and on the Van den Bergh reaction. Other substances increasing the alkaline reserve may be present in such cases.

It would seem, however, that there exists in addition a decreased alkaline reserve in haemolytic jaundice conditions. Haldane (1927), Haldane, Kellas, and Kennaway (1919), and Yandell Henderson (1925) have shown that the titration alkalinity or alkali reserve of the blood is greatly diminished in conditions of increased breathing caused by diminution of oxygen. This diminished alkalinity is brought about by the kidneys excreting more alkali than usual and so meeting the threat of the dangerous condition of alkalosis, or hypocapnia arising. Dill, Bock, Van Caulert, Fölling, Huxthhal, and L. J. Henderson (1928) have found in cases of pernicious anaemia a definite slight alkalosis indicative of a potentiality which needs to be compensated. Gettler and Lindeman (1920) found the alkali reserve subnormal in pernicious anaemia, and Ashby (1925) obtained the same result using the method of Van Slyke and Fitz. The latter also observed that there was a tendency for it to increase with increase of total number of red blood cells present for each unit of body weight.

In regard to what has just been discussed it is significant that acidification of an obstructive jaundice serum renders
the bilirubin present soluble in chloroform and that pernicious anæmia sera, when buffered and especially when the test is reinforced by the addition of multiple doses of Van den Bergh reagent prepared with a weak acid, give a reaction which falls within the limits regarded as characteristic of the direct reaction. Liver treatment of pernicious anæmia cases by the patient's private medical attendant has made it increasingly difficult to obtain hospital patients for observation purposes. Iron-deficient pigs with their anæmia and bilirubin at times in the serum would appear to be well adapted, however, for testing out the significance of the alkali reserve under the conditions just discussed. Observations are at present being carried out of this nature on such cases, the alkali reserve being estimated by Van Slyke's titration method (Journ. Biol. Chem., 1922, 50, xvi).

Summary and Conclusions.—The crucial factors in regard to the performance of the Van den Bergh test are the liability of the bilirubin to be precipitated by the acid of the reagent and the use of measures to hasten the formation of the soluble colour product before precipitation can occur.

The bilirubin present in obstructive and hæmolytic jaundice would appear to be identical chemically. In optimal favourable conditions, however, it would seem to be possible to differentiate the obstructive form from the hæmolytic type of jaundice by means of the Van den Bergh test owing to the occurrence of differences in the buffer reserve of the serum in the two cases. Nevertheless, because of the many varying factors affecting the reaction, as just discussed, numerous indeterminate results are to be expected and do occur. Cases of true obstructive jaundice are especially liable to be mistaken for hæmolytic ones, and cases of either variety with a small amount of bilirubin present are liable to be missed.

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