CLIII. A STUDY OF THE BILE PIGMENTS IN RELATION TO THE VAN DEN BERGH REACTION.

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(Received August 8th, 1930.)

INTRODUCTION.

JAUNDICE is a condition consequent upon an excess of bile pigments in the blood; of these pigments the most thoroughly investigated is bilirubin. In the course of a study of the bilirubin content of blood-serum, van den Bergh [1923] made the important discovery that two forms of bilirubin are to be met with; these are distinguished by their behaviour towards Ehrlich's diazo-reagent. Bilirubin forms a coloured azo-compound with this reagent in the presence of alcohol, and icteric blood-sera also react under these conditions, but, as van den Bergh was able to show, in some cases of jaundice the sera can form the coloured compound in the absence of alcohol. Sera, therefore, can be divided into two main classes, *viz.* (i) those giving a prompt reaction when reagent only is added: the so-called van den Bergh direct reaction, and (ii) those in which the coloured compound is produced only after addition of alcohol: the so-called van den Bergh indirect reaction. A very delayed reaction (20 minutes or more) with reagent alone is not considered a direct reaction.

Exactly wherein lies the difference between the pigment giving the direct reaction, and that giving the indirect, has been a matter of much investigation. Notwithstanding, there is still no unanimity of opinion on the subject. Van den Bergh was inclined to the view that the difference depends either upon some slight variation in the bilirubin molecule, or, in the case of the indirect reaction, upon the fact that the bilirubin is in combination with protein, which combination must be broken down by the addition of alcohol before the reaction can proceed. Thannhauser and Andersen [1921], on the other hand, postulated that the pigment giving the indirect reaction was not bilirubin, whereas Collinson and Fowweather [1926] hold that, whilst free bilirubin is responsible for the indirect reaction, it is ammonium bilirubinate which gives the direct. Davies and Dodds [1927] have suggested that the indirect reaction is due to oxidation products of bilirubin, which is the pigment of direct-reaction serum. Further explanations have been advanced by Roberts [1928], Newman [1928] and others. In this paper we present what we believe to be new and important observations on this subject. It will be apparent that, whilst bilirubin is

responsible for the indirect reaction in serum, the pigment in direct-reaction serum is in many respects quite dissimilar to bilirubin or to its ordinary salts.

METHODS.

The original van den Bergh reagent was used, and in carrying out the test equal quantities of reagent and of solution or serum to be tested were taken. Merck's bilirubin was used in the main, but certain experiments were performed with a sample of bilirubin prepared from gallstones by Küster's method [1909] which gave results identical with the pure Merck's product. Colorimetric quantitative determinations were not made, as both cobalt and permanganate colour-ranges were represented in various experiments, the colour of the azo-compound apparently varying with the nature of the solvent and the final p_H of the reaction mixture. p_H determinations were made with the glass electrode [Kerridge, 1925]. The fallacies of colorimetric methods are well-known, and tests of the quinhydrone electrode against the glass electrode showed the former to give fallacious values in the presence of alcohol and the nitrite of the reagent.

EXPERIMENTAL.

Davies and Dodds [1927] and Newman [1928] state that bilirubin prepared from gallstones, or from bile (by acidification and extraction with chloroform), dissolves in serum to yield a solution giving a prompt direct diazo-reaction. Andrewes [1924], however, observed that the residue from the chloroform extract of bile, after evaporation to dryness and solution in weak alkali, gave only an indirect reaction. That bilirubin gives only an indirect reaction has also been recorded by Roberts [1928] who describes the formation of a colloidal solution when a small volume of a chloroform-alcohol solution of bilirubin is poured into water; this colloidal solution remained unchanged for weeks and gave a greatly delayed diazo-reaction. A similar reaction was given by serum containing colloidal bilirubin. Addition of alcohol produced an instantaneous reaction. The bilirubin did not dialyse, but was capable of extraction with chloroform.

We have found that if Merck's bilirubin be ground up with normal human or ox-serum and centrifuged, a clear brown solution remains. This apparently contains bilirubin in colloidal state, since it cannot be removed by further centrifuging, and no dialysis occurs through a collodion membrane. The solution gives no immediate direct diazo-reaction. A faint reddish-brown coloration develops in the reaction mixture after standing for some 20 minutes. On addition of alcohol, however, an immediate violet-pink reaction is obtained. These observations confirm the findings of Roberts [1928] quoted above.

Treatment of the bilirubin with a little $N/2$ sodium hydroxide prior to solution in serum (with a view to forming sodium bilirubinate) yields a solution still giving no direct diazo-reaction, provided the sodium hydroxide is not in excess: the bilirubin is still soluble in chloroform. Indeed, bilirubin does not

become insoluble in chloroform until sufficient sodium hydroxide has been added to raise the initial p_H of the solution to 10.7, which is well beyond physiological limits for serum. We found that addition of bile salts, cholesterol and lecithin to this solution of bilirubin did not induce a reaction.

Since sodium hydroxide, added in sufficient amount, has been found to convert an indirect-reaction serum into one giving a direct reaction [Newman, 1928], we proceeded to determine the conditions of this reaction as affects a suspension in serum of pure bilirubin. By adding graduated amounts of $N/2$ or $N/5$ sodium hydroxide to a solution of Merck's bilirubin (5 mg. per 100 cc.), the concentration of the bilirubin being kept constant by the addition of compensating amounts of serum, it was found that a cherry-red direct reaction was obtained when the final p_H of the mixture (serum and reagent) was in the region of 6.0. When the p_H was 5.0, a faint colour only was produced; and at p_H 7.9 no red colour appeared, the mixture taking on a brownish tint (Table I).

Table I. *Addition of sodium hydroxide to bilirubin in serum.*

Exp.	Bilirubin in serum cc.	$N/5$ NaOH cc.	Serum cc.	Initial p_H	Final p_H	Colour changes
1	1.0	0.2	0.8	9.17	4.29	Slight reddish tint after 20 minutes
2	1.0	0.4	0.6	9.96	5.00	Reddish-brown in 10 minutes
3	1.0	0.6	0.4	10.57	6.18	Immediately reddish-brown: cherry-red in 10 minutes
4	1.0	0.8	0.2	11.14	7.87	No colour developed

It will be seen from this table that the p_H of the serum must be approximately 10.0 before addition of an equal quantity of reagent will induce a definite prompt reaction. With normal serum the final p_H of a mixture of equal parts of serum and reagent is 2.30–2.40, a value which is too low to enable coupling to take place in the case of pure bilirubin.

M'Gowan [1930] has postulated that the type of van den Bergh reaction given by a serum is related to its alkali reserve; that is to say, an indirect reaction results if the alkali reserve of the serum is diminished. This explanation did not seem to be adequate since the serum used by us had a normal alkali reserve. However, in order to test this point further, we added varying amounts of a 5 % solution of sodium bicarbonate in serum to fixed quantities of a suspension of bilirubin in serum (5 mg. per 100 cc.). A cherry-red reaction was obtained only when the p_H of the mixture of serum and reagent was 6.05, and a rose reaction at p_H 5.56. These values for p_H are those actually measured and the respective alkali reserves were approximately equal to a combining power of 310 and 285 cc. of CO_2 per 100 cc. of serum. It is obvious that such changes in alkali reserve as are encountered in the circulating blood cannot influence the reaction.

As we have seen, the p_H of serum containing bilirubin must be raised very considerably before prompt coupling occurs on addition of an equal quantity of reagent. At this p_H bilirubin exists largely in the form of a salt, soluble in

water, but practically insoluble in chloroform; in fact, it is fairly certain that free bilirubin, being insoluble in water, is incapable of rapid coupling in this medium. At the p_H of blood the greater part of the bilirubin must be in the free condition as a result of hydrolysis, and this is probably the principal reason for its inability to react. This view is strengthened by the fact that addition of reagent sufficient to lower the p_H to about 6.0 does not induce a reaction in serum containing bilirubin.

As a result of many experiments on these lines, we were forced to conclude that pure bilirubin is identical neither with the pigment of direct-reaction serum, nor with the product used by Davies and Dodds [1927] and by Newman [1928].

The isolation of "direct-reaction" pigment.

The following experiments were carried out on golden-brown bile from the cadaver, which gave a good direct van den Bergh reaction: samples failing in this were not employed. Ox-bile from the slaughter-house was also used.

Hydrochloric acid was added to bile until it was acid to Congo red, a green sludge or precipitate forming; the mixture was shaken with chloroform and allowed to stand overnight; thereafter, the chloroform fraction was separated and evaporated to dryness under coal-gas, as described by Newman [1928]. A green-brown pigment was thus obtained, giving a direct red diazo-reaction when taken up in serum, and a delayed violet reaction with the reagent alone, reactions which were more apparent when no particular care had been taken to prevent admixture of the precipitate with the chloroform fraction. In these respects, the pigment differed from pure bilirubin, although it gave the indirect reaction with alcohol and was soluble in chloroform.

At this stage, the idea of testing the aqueous fraction after extraction with chloroform occurred to us. We found that it gave an immediate violet colour on addition of diazo-reagent; the precipitate being especially active in this respect. Using another specimen of bile, the precipitate was separated and extracted with chloroform in a Soxhlet apparatus until only a trace of pigment entered the chloroform. The chloroform was evaporated, and the dry residue was found to give, when dissolved in serum, the prompt red direct reaction described by other workers, as well as the violet chloroform-alcohol reaction similar to that of bilirubin. The precipitate itself, after extraction with chloroform, possessed the following properties: (1) it dissolved in water, and gave a somewhat delayed violet reaction on addition of reagent; (2) when treated directly with reagent, it developed a violet coloration, which emerged from the precipitate and spread through the solution; (3) it was readily soluble in alcohol, yielding a greenish solution which gave an intense immediate violet colour with the diazo-reagent; (4) in serum, it dissolved to give a prompt red direct reaction, the intensity of the colour depending upon the concentration, and tending more towards red-violet in high concentration and at low p_H ; (5) it was unstable in presence of acids, and readily became oxidised, when it lost its reactivity to the prompt reaction, but retained the ability to react in

presence of alcohol. This appeared to be the clue to the problem. Apparently another pigment is present in bile, and is quite likely to find its way into the bilirubin which is prepared from bile under ordinary conditions.

With the idea of isolating this "direct-reaction" pigment, bile was desiccated *in vacuo*, and then treated with alcoholic hydrochloric acid (about 2 %) until the residue was acid to Congo red. After again drying *in vacuo*, the residue was extracted with chloroform in a Soxhlet apparatus until only a trace of pigment entered the chloroform. If the chloroform were then changed, it could be shown that the small amount of pigment dissolved in the final stages of the extraction had the properties of the "direct-reaction" pigment, and not of bilirubin, the "direct-reaction" pigment being sparingly soluble in the warm chloroform. The extracted residue, almost insoluble in chloroform, was dried *in vacuo*, and then presented the appearance of a greenish-yellow powder having the properties of the precipitate described above. Some specimens of bile were found to be very sticky even after drying *in vacuo*. Such specimens were extracted with ether, to remove fat, etc., before treatment with chloroform. In the first stages of ether-extraction, a small amount of yellow pigment is removed; this does not affect the character of the final product. Earlier attempts at the isolation of the "direct-reaction" pigment were frustrated by its extreme instability in acids and in presence of air; under these conditions, it readily became green, and lost its capacity of giving a direct reaction, though retaining its indirect reaction. The greatest care is necessary to avoid undue exposure to air and excessive acidity, but, once prepared and sealed in a glass tube, it keeps fairly well.

As stated above, the "direct-reaction" pigment from bile forms a coloured compound on addition of pure reagent; the p_H of this reagent is approximately 1.2. In this respect, the pigment differs entirely from bilirubin, which gives no reaction with the pure reagent, but reacts most promptly (in absence of alcohol) in alkalinised serum at a final p_H of 6.0, almost ceasing to give a direct reaction at p_H 5.0. Moreover, we have found that bile exhibiting a direct reaction will continue to react at a p_H so low as 1.3, as will also direct-reaction icteric serum. Higher acidity than this merely delays the appearance of the reaction-colour. The "direct-reaction" pigment, therefore, in contrast to bilirubin, is capable of coupling at a very low p_H .

Oxidation of "direct-reaction" pigment.

"Direct-reaction" pigment isolated from bile loses its power of giving a direct diazo-reaction after standing in air for several hours, the loss in reactivity being accompanied by a change in colour from greenish-yellow to bright green. The product continues, however, to give a prompt reaction in alcohol for a considerable time. Since it has been stated that biliverdin gives a diazo-reaction in presence of alcohol, it seemed possible that the "direct-reaction" pigment changed gradually into biliverdin. Van den Bergh [1923] stated that biliverdin gives no diazo-reaction. Davies and Dodds [1927] claimed that

it gives a direct reaction only after 12 hours, but that it gives an immediate indirect reaction. Newman [1928] has confirmed its very delayed direct reaction. We prepared biliverdin by passing a stream of oxygen for one hour through a warm solution of Merck's bilirubin in $N/2$ sodium hydroxide. The pigment, which was now green, was precipitated with hydrochloric acid, filtered off, washed and dried as described by Städeler [1909]. It was found to be insoluble in water, and almost so in cold chloroform, but freely soluble in alcohol, in which solvent it did not begin to give a diazo-reaction for $2\frac{1}{2}$ hours, and then only feebly. Since the green oxidation product of the "direct-reaction" pigment is soluble in water and yields an immediate and intense diazo-reaction in alcohol, it cannot be biliverdin.

DISCUSSION.

In the first place we feel convinced that the very conflicting views concerning the nature of the pigments in the blood in jaundice are directly attributable to the fact that investigators have not taken the precaution to work with pure bilirubin. Having established beyond doubt that pure bilirubin is incapable of reproducing the properties of the pigment in direct-reaction serum, we were forced to conclude that another pigment, differing in certain respects from bilirubin, must be present in such sera. We have described at some length the isolation of this pigment (probably in an impure form) from bile, and the various respects in which it differs from bilirubin. The "direct-reaction" pigment, as we have called it, has the necessary physico-chemical properties to justify the conclusion that its existence in the serum in certain cases of jaundice is responsible for the direct diazo-reaction encountered in such cases. This pigment, as well as its oxidation product, gives the indirect diazo-reaction as does bilirubin. It will be obvious that, if bilirubin be not carefully purified, it may quite easily contain some of this "direct-reaction" pigment or its oxidation product, and it seems reasonable to suppose that this has often been a source of error.

It has been observed by Grünenburg [1922] and confirmed by Andrewes [1924], Roberts [1928] and by Newman [1928] that the pigment of direct-reaction icteric serum is not soluble in chloroform even after acidification, whereas the pigment of indirect-reaction icteric serum is soluble in chloroform even in the absence of acid. This difference in solubility is capable of explanation, since, as we have described, "direct-reaction" pigment prepared from bile is almost insoluble in cold chloroform, whereas bilirubin is fairly soluble in this solvent.

Van den Bergh [1923] observed that the pigment in direct-reaction serum is much more susceptible to oxidation than is the pigment of indirect-reaction serum. We have remarked the instability of "direct-reaction" pigment in air, and serum containing this pigment in solution loses its reactivity fairly rapidly on standing in contact with air.

At the p_H of the blood bilirubin appears to exist as a free acid in colloidal

state and does not react with diazo-reagent. Before coupling can occur the p_H of the serum must be high, and the final p_H between 5.0 and 7.0. Bilirubin couples in alcohol, or in a mixture of chloroform and alcohol, in which it is soluble, almost independently of the final p_H of the medium, which may be as low as 1.7; low p_H values merely delay the development of the colour.

"Direct-reaction" pigment, however, reacts under conditions which differ widely from those of bilirubin. Being water-soluble at reactions more acid than that of blood-serum, it couples quite easily even at p_H 1.0, the rate of reaction and the colour of the diazo-compound changing with increasing p_H . As the p_H becomes low the appearance of the colour is more delayed, and the tint becomes more violet. Unlike bilirubin, too, it develops in serum an immediate direct red reaction, of great intensity, and with a tendency to a final violet-red tint.

As a result of these investigations we feel justified in supposing that serum giving a direct diazo-reaction contains the water-soluble, chloroform-insoluble pigment which we have found to be present in fresh cadaver bile. We have compared the properties of serum in which this "direct-reaction" pigment has been dissolved with normal direct-reaction sera from cases of jaundice, and the identity of the two classes of sera has entirely confirmed this view. These observations will be described in a subsequent communication. All icteric sera give an indirect van den Bergh reaction; this follows from the fact

Table II. *Properties of bilirubin and "direct-reaction" pigment.*

Bile	
Bilirubin	"Direct-reaction" pigment (probably impure)
Red-brown in colour	? Greenish yellow colour
Insoluble in water	Soluble in water and in alcohol; very slightly soluble in chloroform
Forms water-soluble alkaline salts	Soluble in serum
Soluble in chloroform and alcohol; forms colloidal suspension in serum	Unstable in air, especially in the presence of acids
Stable in air in the dry state	In reagent alone, a delayed purple diazo-reaction
No coupling with diazo-reagent alone. Diazo-coupling occurs in buffered alkaline phosphate, alcohol and chloroform-alcohol mixture. In case of alkaline solution of bilirubin final p_H after addition of reagent must be between approx. 5.0 and 7.0	Immediate and intense red direct diazo-reaction in water or in serum
In serum, no diazo-coupling, or at the most a slight red-brown colour after standing 20-30 minutes	Range of p_H after addition of reagent over which coupling occurs approx. 1.0-5.0
In serum it is not adsorbed on to protein precipitated by alcohol	In serum it is adsorbed largely on to the protein precipitated by alcohol
Oxidation of alkaline solution ↓ Biliverdin	Oxidation ↓ A green pigment
Green in colour	Soluble in water, readily so in alcohol, somewhat unstable in air
Insoluble in water and chloroform. Soluble in alcohol	No direct diazo-reaction
No direct diazo-reaction. Very delayed diazo-reaction in alcohol	Immediate and intense purple diazo-reaction in the presence of alcohol

that both "direct-reaction" pigment and bilirubin give indirect reactions. In those sera, however, which do not give a direct, but only an indirect reaction, the pigment present is apparently bilirubin. The presence of bilirubin may be confirmed by testing the degree of chloroform-solubility of the pigment; when this is high much bilirubin would seem to be present.

The properties of bilirubin, of the "direct-reaction" pigment, and of their respective oxidation products are summarised in Table II. It seems probable that "direct-reaction" pigment is closely related to bilirubin but at the present time we have no knowledge of its chemical nature.

SUMMARY.

1. Pure bilirubin, when suspended in blood-serum, does not give a prompt direct van den Bergh reaction; serum containing bilirubin does, however, give the indirect reaction.

2. Fresh bile contains a pigment which, when taken up in serum, yields a prompt direct reaction. The term "direct-reaction pigment" has been applied to this substance, and its isolation from bile in an impure state is described.

3. "Direct-reaction pigment" is not bilirubin or one of the ordinary salts of bilirubin; it differs from bilirubin in its physico-chemical properties and in respect of the conditions under which it will couple with diazo-reagent.

4. It is concluded that "direct-reaction" pigment of bile is present in those icteric sera which give a direct van den Bergh reaction.

We wish to thank Professor Hugh MacLean, at whose suggestion this research was carried out, for his helpful advice and criticism and for facilities placed at our disposal. One of us (W. J. G.) desires gratefully to acknowledge assistance received from the Medical Research Council.

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