CXXXVI. THE ISOLATION FROM BILE OF A PIGMENT HAVING A DIRECT VAN DEN BERGH REACTION.

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THE pigments present in the blood-serum in jaundice were first differentiated by van den Bergh, who found that, whereas all icteric sera coupled with diazobenzene-*p*-sulphonic acid (Ehrlich's diazo-reagent) in the presence of alcohol, only certain of these sera would couple in simple aqueous solution. These diazo-reactions van den Bergh termed "indirect" and "direct" respectively, and it is now established beyond doubt that they are to be ascribed to two forms of bile pigment, the so-called indirect- and direct-reacting pigments. Subsequent work has shown that these pigments differ so much in other respects as to justify the conclusion that they are of distinctly different types; the evidence on this point has been summarised by Hunter [1930].

Careful investigation of the conditions under which bilirubin couples with diazo-reagent and comparison of solutions of this pigment in serum with indirect-reaction sera led us to the conclusion that bilirubin is responsible for the indirect reaction [Griffiths and Kaye, 1930, 1, 2], a view first put forward by Collinson and Fowweather [1926] and later by Roberts [1928] and Hunter [1930].

In regard to the nature of direct-reacting pigment, however, there is still much difference of opinion. The views of earlier workers were presented in our previous papers, so that we shall confine our attention to subsequent publications. Hunter [1930] found that if an alkaline solution of bilirubin is first buffered with disodium phosphate, it will couple with diazo-reagent in a distinctly acid medium. On the basis of this observation he suggests that direct-reacting pigment is sodium hydrogen bilirubinate, a view in accordance with that of Collinson and Fowweather [1926], who regarded the pigment as a salt of bilirubin, probably the ammonium salt. More recently, Fowweather [1932] has prepared the mono- and di-sodium salts of bilirubin, and these, when dissolved in serum, were found to give only delayed diazo-reactions. This finding seems definitely to exclude the hypothesis of Hunter [1930], and of Newman [1928], that the pigment is a sodium salt. In this connection it is of interest to recall that van den Bergh and Muller [1916] observed that a neutral solution of bilirubin would not couple with diazo-reagent. Fowweather was unable to demonstrate conclusively that ammonium bilirubinate gives a direct reaction.

Bilirubin is known to exist in more than one physical form, and Fowweather, after careful consideration of all aspects of the matter, has suggested that indirect-reacting pigment is a salt of another modification, more soluble in water, which tends to change into the indirect type. This author describes a pigment giving a direct reaction which is produced by precipitation of a solution of bilirubin in weak alkali, or alkali-phosphate, by a concentrated solution of a similar alkaline salt. This precipitate, when dissolved in serum, was found to give a typical prompt reaction, but on solution in water the product failed to react. The possibility that such a preparation might influence the reaction by virtue of its alkalinity must be considered in view of the fact that the behaviour of bilirubin with diazo-reagent is governed by the p_H of the medium in which the test is performed. Under the ordinary conditions, when equal parts of serum and reagent are mixed, the p_H of the mixture approximates to 2.4; slight variations in technique do not appreciably alter this value. The pigment which couples at this low p_H is of course the direct pigment; this pigment will in fact couple if the p_H lies below about 5.0. Bilirubin does not couple under these conditions, but it can be made to do so if the serum is so buffered, or made alkaline, that the final p_H after addition of the reagent is approximately 5–6. It seems therefore that the mere observation that coupling occurs in an acid medium is insufficient to exclude bilirubin unless the reaction of the medium is shown to be on the acid side of p_H 5.0. In fact, as Fowweather rightly points out, the observation that by addition of sufficient alkali an indirect-reacting serum can be made direct is valueless if, by such treatment, the serum is rendered more alkaline than direct-reacting serum. There can be no doubt that many products described in the literature as direct-reacting were, in point of fact, bilirubin giving a direct reaction because of the alkalinity of the medium in which it was dissolved. We found that when the p_H of serum containing bilirubin was raised to 10 by addition of NaOH, prompt coupling occurred [Griffiths and Kaye, 1930, 1], but for the reason already given such serum is not identical with direct-reacting serum. No artificially prepared serum showing a direct reaction can be considered as identical with the direct-reacting serum of jaundice unless it is shown to couple with diazo-reagent at $p_H < 2$.

Attention must, however, be called to an experiment carried out by Fowweather in which an ammoniacal solution of bilirubin was precipitated with saturated ammonium carbonate solution and the precipitate washed with a saturated solution of sodium chloride; when dissolved in serum the precipitate gave a prompt reaction. In this case most of the alkali must have been removed during the process of washing and further investigation of this interesting observation is clearly desirable.

On the whole, most workers have taken the view that direct-reacting pigment is either bilirubin which has undergone some intramolecular rearrangement, or that it is a salt or other derivative of bilirubin; the evidence which has been put forward in support of these conceptions is not, however, at all

convincing. Curiously enough, practically all the work which has been carried out on the direct-reacting pigment has been synthetic in character, although van den Bergh originally obtained the direct reaction with bile, in which, presumably, the pigment is present in considerable amount. Possibly the difficulties arising from the extreme instability of the pigment have blocked the analytical approach to the problem of its identity. Our knowledge of the physical properties of the pigments, both direct and indirect, and of icteric sera generally, has been much enriched by the many investigations, but it cannot be said that they have brought us anywhere near a solution of the chemical nature of direct-reacting pigment.

In our earlier work [Griffiths and Kaye, 1930, 1] we found that the direct-reacting pigment is present in dried gall-bladder bile after extraction of the bilirubin with chloroform, *i.e.* it is present in that fraction of the bile which is not soluble in chloroform. The preparations with which we were then working were crude, being contaminated by bile salts, *etc.*, but we were able to show that solutions of this material in serum were identical with the direct-reacting serum of jaundice. As a result of further work we have been successful in the isolation from this crude material of a pigment capable of giving a true direct reaction. Briefly, our procedure has been as follows: the chloroform-insoluble residue of dried bile was dissolved in weakly alkaline solution and precipitated by acetone. The direct-reacting pigment is adsorbed by the precipitate, from which it was obtained by extraction of the dried product with 80 % acetone. Evaporation of the acetone solution left a pigmented residue which gave a powerful colour with diazo-reagent at low p_H . The direct-reacting pigment was found to be precipitated from this acetone-product by addition of acids to its aqueous solution.

EXPERIMENTAL.

Human gall-bladder bile, obtained at autopsy, was used. Bile from this source was found to be very variable in its response to diazo-reagent; only those specimens which showed a good prompt direct reaction were employed. When necessary the bile was preserved for short periods in a well-stoppered bottle in a refrigerator from which light was excluded. Light has a very deleterious effect upon the direct-reacting pigment, and for this reason at all stages of the preparation the material was as far as possible preserved in a dark place.

Isolation of the pigment-bile salt complex.

The first stage in the extraction of the direct-reacting pigment was the isolation of a complex substance consisting of the pigment associated with the salt of a bile-acid. The gall-bladder bile was dried *in vacuo* over sulphuric acid and finely powdered. The powdered bile was treated with alcoholic HCl (conc. HCl, 1 cc.; alcohol, 100 cc.) until the mixture was acid to moist litmus paper. In our earlier work this mixture was made acid to Congo red, but

experience has shown that this degree of acidity is not only unnecessary, but distinctly harmful; direct-reacting pigment becomes increasingly unstable with diminishing p_H , and acidity strong enough to affect Congo red may often ruin the preparation. By experiment it was found that a p_H of 6.5–5.5, as determined by the glass electrode, was sufficient to ensure complete solubility of the bilirubin in chloroform without adversely affecting the direct-reacting pigment. After again drying *in vacuo* the finely powdered material was extracted with dry ether for 1–2 hours in a Soxhlet apparatus, using a 22 × 80 mm. thimble; in this way a good deal of pigmented fatty material was removed. Finally, to remove bilirubin, the material was extracted with dry chloroform in a modified Soxhlet. In the ordinary apparatus the chamber containing the thimble becomes considerably heated when using chloroform, with destructive effect upon the direct-reacting pigment; we were able to overcome this difficulty by slightly modifying the apparatus (Fig. 1). Extraction was continued until traces only of pigment were being taken up by the chloroform (6–9 hours). The chloroform was removed from the sticky residue *in vacuo*, and the material powdered. If it appeared that some parts of the substance had escaped extraction the process was repeated for a further period of time.

The resulting yellowish-brown powder should give a prompt and marked reaction with diazo-reagent; if it fails in this it is useless to proceed and the specimen must be discarded.

The product after extraction with chloroform consisted of the direct-reacting pigment admixed with bile salts, mucin, *etc.*; separation of the pigment from this material proved to be a matter of great difficulty. We found, however, that addition of acetone to an aqueous alkaline solution of the material resulted in the formation of a precipitate, on which, to a very large extent, the direct pigment was adsorbed. We therefore proceeded as follows. The powder was rubbed up with water in a mortar, using about 40 cc. for every 10 g. of material. The dark-brown viscid mixture was made alkaline to

Fig. 1.

litmus with a few drops of *N* NaOH, transferred to a flask, and precipitated by addition, with shaking, of four times its volume of acetone (distilled from CaCl_2). The mixture was shaken vigorously and filtered immediately. The precipitate was taken up in sufficient water to form a cream, and was reprecipitated with another 4 volumes of acetone. The precipitated material was filtered off, washed with acetone, and dried *in vacuo*. The filtrates from these operations, containing mainly bile salts and some pigment, were discarded. The dry acetone-precipitate had an amorphous, sometimes fibrous, appearance, and varied in colour from pale golden-brown to chocolate. It contained N, 7–8 % and P, 1–1.5 %, and was readily soluble in water yielding a solution having a powerful direct diazo-reaction. In the case of strong solutions the colour was masked by a precipitate produced by the reagent, but when this precipitate was filtered off, an intensely purple filtrate resulted, and much azo-pigment could be washed away with water from the residue on the filter-paper. It appeared from this that the substance was split by the reagent, probably because of the acidity of the latter. This was confirmed by the observation that an aqueous solution of the material was precipitated by the reagent acid alone. An equal volume of van den Bergh's solution A was added to a solution of the substance precipitated by acetone, and after being allowed to stand for some minutes, the mixture was filtered. Addition of reagent to the feebly coloured filtrate gave only a very weak reaction, whereas the precipitate reacted strongly with the reagent. The precipitate rapidly lost its reactivity on being left exposed to the air.

The material precipitated by acetone consisted in the main of pigment and mucin. By experiment it was found that the pigment was best extracted from the dry powder by cold 80 % acetone. The material was finely powdered, rubbed up with successive quantities of about 20 cc. of 80 % acetone (80 cc. anhydrous acetone made up to 100 cc. with water) and allowed to stand for 5–10 minutes after each treatment, when the supernatant fluid was decanted on to a filter. After about 10 washings in this manner very little pigment was taken up by the acetone. This operation was carried out by artificial light. Without delay, the acetone was removed from the dark reddish-brown filtrate under reduced pressure in an atmosphere of coal-gas, the containing flask and its contents being warmed to about 60°. When, on account of frothing, further distillation was impossible, any precipitated fatty material was filtered off and the residue evaporated to dryness over sulphuric acid *in vacuo*. The yield, which was variable, amounted to 1–3 % of the chloroform-extracted bile. This preparation will be referred to as the acetone-product.

The nature of the acetone-product.

The dry residue was a reddish-brown, scaly, amorphous and hygroscopic substance, extremely soluble in water, moderately so in alcohol; when powdered it appeared bright orange in colour. Heated on platinum foil, the substance left a very small infusible alkaline ash. Its aqueous solution was

lemon-yellow or orange-red according to concentration, and gave Hay's test for bile-acids. Solutions in normal serum gave an excellent prompt direct diazo-reaction, and were strictly comparable with direct-reacting serum. The intensity of the diazo-reaction of this product was most striking; weak aqueous solutions, containing as little as 1-2 mg./100 cc., gave an immediate purple colour, at approximately p_H 1.5, on addition of an equal volume of reagent. With more concentrated solutions the diazo-reagent produced a precipitate which obscured the colour of the azo-compound, but filtration resulted in an intensely purple filtrate. If allowed to stand for some time after addition of the reagent and then filtered, a small green residue remained on the filter-paper; this was considered to correspond to the oxidation product already described [Griffiths and Kaye, 1930, 1].

The product exhibited marked instability, especially when in the acid state; the pigment thrown down by acids rapidly became green and lost its power to react with diazo-reagent. Aqueous solutions, which at first were lemon-yellow in colour, rapidly became decolorised on exposure to light and ceased to give a diazo-reaction; preservation in a dark place greatly delayed this change.

Acids generally caused precipitation of strong solutions of the product in water when the p_H was brought below about 3.0, whereby practically all the pigment was removed from solution. The precipitated pigment was soluble in alkalis and contained nitrogen and traces of phosphorus.

As it appeared that the pigment represented a small fraction only of the acetone-product, experiments were made in order to determine the nature of the rest of the material. A fairly strong solution of the acetone-product in water was treated with dilute HCl until precipitation was complete, and after filtering NaOH was added to the filtrate until it was only faintly acid to litmus. The neutralised fluid was evaporated to dryness on a water-bath. A pinkish-white, hygroscopic residue was obtained, readily soluble in water to yield a solution which gave Hay's and Pettenkofer's tests; much of the substance was therefore a bile salt. Microscopically the residue, apart from sodium chloride, appeared to consist only of colourless crystals of roughly hexagonal shape.

The relationship between the pigment and this bile salt was not further investigated at this stage, but it seems probable that the association of these bodies was merely accidental, although it is remarkable that, whereas the greater part of the bile salts is removed during the initial precipitation of the bile-residue with acetone, this fraction constantly escaped elimination. The action of acids, which presumably decompose an alkaline salt of the direct-reacting pigment with resulting precipitation of the pigment, leaving the bile-acid in solution, appeared to be reversible; when by addition of alkali the change was reversed, the pigment re-dissolved, and on evaporation to dryness *in vacuo* no significant alteration in the character of the product could be detected.

Isolation of the direct-reacting pigment.

As the pigment appeared to be readily separated by addition of weak acids to its aqueous solution, this method was adopted for its isolation. Care was necessary, however, in view of the harmful influence of strong acidity, and acetic acid was at first employed. The powdered material was treated with glacial, or with alcoholic, acetic acid, dried *in vacuo*, and the dried material extracted with chloroform in order to remove any traces of bilirubin. The amount of chloroform-soluble material was found to vary considerably in different preparations; some showed practically none, others a fair amount. Although some of this pigment was undoubtedly bilirubin, often much was the direct-reacting pigment, whose solubility in chloroform seemed to vary from preparation to preparation. After extraction with chloroform the products, when dissolved in weak alkali, were highly reactive towards the diazo-reagent, giving a true direct reaction. The material varied in colour from orange-red to dark greenish-brown, probably because of the presence of some oxidation products; total nitrogen (micro-Kjeldahl) estimations on six different samples gave values ranging from 3.7 to 5.5 %, and the preparations could not, therefore, be regarded as of uniform composition.

Better results were obtained by precipitation of an alkaline solution of the acetone-product with dilute mineral acid. The acetone-product readily dissolved in water to give a deep orange-red solution of p_H 7-8. Addition of $N/10$ HCl dropwise resulted in a gradual change of colour to a more greenish hue, and ultimately to definite greenish turbidity or actual precipitation at p_H 3.6-4.0. When precipitation did not occur it was readily induced by addition of a little solid sodium chloride to the solution. The greenish pigment, which appeared reddish-brown by transmitted light, was centrifuged, and the supernatant yellowish-green fluid removed. The precipitate was dissolved in $N/100$ NaOH, and the golden-brown solution treated with just enough $N/10$ HCl to re-precipitate the pigment. It was observed that the pigment separated at the same p_H as in the first case; it is interesting to note that bilirubin is precipitated from its alkaline solutions at p_H 6.8. The precipitate was centrifuged, washed 2 or 3 times with $N/100$ HCl, and dried *in vacuo*.

The product was a greenish amorphous powder, practically insoluble in water, but readily soluble in alkalis and in alcohol, in which it yielded a greenish-yellow solution, which rapidly darkened on standing. Solubility in alcohol was not always complete. In chloroform the solubility was variable, some samples showing practically none, others a fair amount; it was noticed that it was particularly those samples showing incomplete solubility in alcohol which also showed chloroform-solubility, which was thought to be due to bilirubin. Bilirubin being practically insoluble in alcohol, the pigment was treated with this solvent and filtered; a small brown residue on the filter-paper was found to be soluble in chloroform. After evaporation of the alcohol *in vacuo*, the dark-green amorphous residue was practically insoluble in water

and in chloroform, but readily dissolved in alkalis to yield a golden- or reddish-brown solution. When dissolved in a little $N/100$ NaOH, the solution, after dilution with water, gave an immediate and strong reaction with diazo-reagent at p_H as low as 1-2. In this connection we must remark the very unstable nature of the pigment, especially in the presence of free acid; although the majority of the products of the action of acid on the acetone-product gave a good van den Bergh reaction, occasionally a product was obtained which did not appear to react promptly. In such cases it was observed that the reaction mixture was clouded by a fine green precipitate; when this was filtered off, the filtrate was found to be highly coloured by the azo-compound. The green material, the amount of which increased with the age of the specimen when it was exposed to air and light, is an oxidation product, and is not reactive towards the diazo-reagent. The pigment contained no phosphorus, evolved pyrrole when distilled with zinc dust, and rapidly lost its power to give a diazo-reaction on exposure to air and light.

The following analytical data were obtained for preparations from different samples of bile (mol. wt. by Rast's method, N by micro-Kjeldahl): (a) m.p. 145-150°, mol. wt. 721, N, 4.6 %; (b) m.p. 142-150°, mol. wt. 688, N, 3.9 %; (c) m.p. 145-150°, mol. wt. 696, N, 4.5 %; (d), (e), (f) and (g) N, 3.4, 4.3, 4.3, 4.7 % respectively. The probable error in the nitrogen determinations is large on account of the small amounts of substance available for analysis. Micro-analysis of samples (a) and (c) showed: (a) C, 60.4; H, 7.71; N, 4.35 %; (c) C, 64.0; H, 8.48 %; $C_{32}H_{50}O_{11}N_2$ requires C, 60.2; H, 7.89; N, 4.39 %. Further investigation at this stage was precluded by the small amounts of material available. It is obvious that there are discrepancies in these results greater than the probable experimental error, which is not surprising in view of the instability of the pigment, the small amounts obtained, and the fact that some degree of oxidation is unavoidable.

In the absence of more precise data, which must await the investigation of larger quantities of the product, the present results indicate the general nature of the pigment; we feel that there is ample justification for supposing that we were, in fact, dealing with a chemical entity. The low N content is of interest; the N in the samples analysed averaged 4.3 % whereas bilirubin ($C_{33}H_{36}O_6N_4$) contains 9.6 %; this finding alone shows that we were dealing with something other than a modified form of bilirubin. So far as we are aware, there is one other blood-pigment derivative only which is known definitely to contain two N atoms in the molecule, namely, bilirubinic acid ($C_{17}H_{24}O_3N_2$), a reduction product of bilirubin [Fischer and Röse, 1912]. Bilirubin and related derivatives of haemoglobin which have been sufficiently studied contain four N atoms, probably in the form of four pyrrole residues, but in the case of bilirubinic acid two pyrrole groups only remain attached to one another. It seems not unlikely, therefore, that the direct-reacting pigment is similar in nature to bilirubinic acid.

The pigments present in the blood-serum in jaundice have come to be

named after the type of van den Bergh reaction which they give, *i.e.* direct- and indirect-reacting pigments; these terms are clumsy and do not fit in with the nomenclature of the bile pigments. Harrison [1930] has suggested that the pigments should be designated according to their origin in the body by addition of a prefix to the word bilirubin. Thus the indirect-reacting pigment, which is associated with jaundice of the haemolytic type, takes the name of *haemobilirubin*; direct-reacting pigment, which is formed from bilirubin in the liver and excreted along with the bile, Harrison calls *cholebilirubin*. These names are in every way suitable and should, we feel, be brought into common use.

SUMMARY.

The isolation from gall-bladder bile of a pigment having a direct van den Bergh reaction is described. This pigment, for which the name of cholebilirubin is recommended, is shown to exhibit those properties known to be associated with the direct-reacting pigment of obstructive jaundice. Cholebilirubin is not a modification of bilirubin, but a separate and distinct pigment with certain features in common with the other bile pigments; analysis, which must be regarded as preliminary in nature, is in favour of the formula $C_{32}H_{50}O_{11}N_2$.

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