XXII. BILIRUBIN AND THE VAN DEN BERGH REACTION.

BY FRANK SCOTT FOWWEATHER.

*From the Department of Pathology and Bacteriology,
University of Leeds.*

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INTRODUCTION.

THE difference between the bile pigment which gives the "direct" van den Bergh reaction and that which gives only the "indirect" reaction has been the subject of much investigation, and many attempts have been made to explain it; none of them however appears to be entirely satisfactory.

Davies and Dodds [1927] have reviewed this work up to the end of 1926, at the same time offering a contribution of their own in which they suggest that the pigment of blood-sera giving the direct reaction is bilirubin, while that of sera giving only the indirect reaction is an oxidation product of bilirubin. Roberts [1928] put forward the view that bilirubin in the free colloidal condition was the pigment of indirect sera, while in direct sera the bilirubin was in combination with some substance the nature of which is as yet undetermined. Newman [1928] opposed the view that the pigment of indirect sera was free bilirubin and claimed that the pigment of direct sera was sodium hydrogen bilirubinate. M'Gowan [1930] considered that the pigments of direct and indirect sera were identical, the types of van den Bergh reaction being dependent on the alkali reserve of the blood; indirect reactions were obtained when the alkali reserve was diminished. Snider and Reinhold [1930] claimed that the type of van den Bergh reaction was dependent on the concentration of the pigment which is present, direct reactions being associated with high concentrations of bilirubin, indirect reactions with low concentrations. They also called attention to the accelerating effect of alkali, which has been noticed by other workers. Griffiths and Kaye [1930] agreed with Roberts in ascribing the indirect reaction (in sera giving this reaction alone) to free bilirubin, but they stated that "direct-reaction pigment" was not bilirubin or one of the ordinary salts of bilirubin. It should be added that the three papers last mentioned appeared after the commencement of the work about to be described.

Collinson and Fowweather [1926] put forward the view that the pigment of indirect sera was free bilirubin, while the pigment of direct sera was a salt of bilirubin, probably the ammonium salt. It will thus be seen that our views on

the nature of the indirect pigment have been supported by Roberts and by Griffiths and Kaye, while our view regarding the direct pigment, so far as it considers this to be a salt of bilirubin, is supported by Newman though he is not in agreement with us as to the probable nature of this salt.

Many of the experiments described by some of the workers quoted above appear to support the view that the difference between the pigments concerned depends on whether we are dealing with a free acid or a salt. Newman, for example, showed that the addition of alkali in proper amounts to indirect sera would convert the reaction to a direct one, while the addition of acid would convert a direct to an indirect one. Experiments of this nature however do not prove the point conclusively, for while it may be accepted that a bile-pigment-containing serum to which alkali has been added probably contains a salt of bilirubin, the serum will be more alkaline than a natural serum which gives a similar result with the van den Bergh reagent. The conditions under which the reaction takes place are decidedly different in the two cases, and therefore the fact that identical results are obtained hardly allows us to assume definitely that the pigments responsible for these results must be identical, though a similarity and possibly an identity is suggested. Another objection to experiments of this type is that they do not, in all probability, represent what actually occurs in the body. The two types of pigment, according to the theory most generally accepted at the present time, do not alter after their introduction into the blood; their production and any change that occurs from one type to another take place in certain organs of the body and not within the blood stream.

It appeared to us therefore that more valuable evidence would be obtained by preparing and isolating in the solid state certain salts of bilirubin and noting what type of reaction was obtained when small amounts of these were added to normal serum.

METHODS.

The bilirubin we have used has been prepared from human gallstones. These were finely ground and extracted with chloroform to remove all cholesterol. The residue was well washed with water and then treated with dilute hydrochloric acid, a small amount of sodium sulphite being added to minimise oxidation. The resulting product after washing and drying was again extracted with chloroform. The chloroform extract, as it became concentrated, began to deposit brick-red crystals of bilirubin, and after a quantity of these had been formed the extract was filtered, and the crystals were washed with a little cold chloroform.

The filtrate and washings were treated with excess of light petroleum, which caused the precipitation of a yellowish brown amorphous solid. This was also filtered and washed with light petroleum.

Most of the experiments to be described were performed with the red,

crystalline bilirubin; a few, however, as will be indicated later, were carried out on the precipitated product.

The van den Bergh reaction was carried out only in the direct form, reagent being added to serum in the proportion of 0.4 cc. of reagent to 0.5 cc. of serum.

EXPERIMENTAL.

Preparation of certain salts of bilirubin, and their reactions when added to serum.

The preparation and analysis of the mono-ammonium salt are given by Küster, Reihling and Schmiedel [1914]. The product obtained is dark brown in colour, very soluble in water, but also soluble in cold chloroform; it only gave a delayed reaction in serum, in agreement with Grünenberg's [1922] observation that the pigment of sera which give a prompt direct reaction is not extracted by chloroform.

An attempt was next made to prepare a monosodium salt by a method analogous to that used for the ammonium salt. A weighed quantity of bilirubin was suspended in methyl alcohol and the theoretical quantity of 0.1 *N* sodium ethoxide was added. Only a portion of the bilirubin went into solution, and so a further equivalent of sodium ethoxide was added. After shaking, and rubbing the coarser particles, the whole of the solid originally present dissolved. The solution was precipitated with ether and the precipitate was filtered off and dried. (Found: Na 7.42 %; the disodium salt requires 7.32 %.)

This, product when dissolved in serum only gave a delayed reaction.

A further attempt was made to prepare the mono-salt. Bilirubin was again suspended in methyl alcohol, and one equivalent of sodium ethoxide was added. Some bilirubin went into solution immediately, but after a few minutes there appeared to be a deposition of solid from the solution, suggesting that the di-salt was first formed but later reacted with some of the free bilirubin present, to form the mono-salt. The mixture was left in an ice-chest overnight and next morning much of the solid seemed to have disappeared. The solution was filtered from a small amount of residue which appeared to be free bilirubin. The filtrate was treated with ether and the resulting precipitate isolated and dried. (Found: Na 3.88 %; monosodium bilirubinate requires 3.80 %.)

This, too, when dissolved in serum gave a delayed reaction.

In another preparation a considerable excess of sodium ethoxide over that required to form the di-salt was used. Again only a delayed reaction was obtained when the resulting product was added to serum.

In a further series of experiments water was used in place of methyl alcohol. A weighed quantity of bilirubin was suspended in water and one equivalent of 0.1 *N* sodium hydroxide added. Again much of the bilirubin failed to dissolve, and neither the remaining solid nor the filtrate from it, nor a mixture of the two, gave a prompt reaction when added to serum. Two equivalents of alkali caused all the bilirubin to dissolve, but addition of the solution

to serum gave the same result as before; addition of further equivalents, one at a time until ten equivalents had been added, produced the same result.

Experiments were next made to ascertain the effect of p_H on the preparation. A series of twelve solutions was prepared from B.D.H. universal buffer, starting with a p_H of 7.0 and advancing by 0.5 up to 12.5. To a little solid bilirubin 3–4 cc. of the solution were added, and the mixture was well shaken and allowed to stand for half an hour at room temperature. On inspection, no solution of the bilirubin was apparent at p_H 7.0 to 10.5. Partial solution was evident at p_H 11.0, and complete solution at p_H 11.5 to 12.5. After centrifuging, the supernatant fluid was practically colourless with solutions of p_H 7.0 to 8.0, slightly but increasingly yellow at p_H 8.5 to 10.5, but deep red at p_H 11.0 and upwards. When 0.1 cc. of the supernatant fluid in each case was added to 0.5 cc. of serum and the van den Bergh reagent added, very little change was produced with solutions at p_H 7.0 to 10.5, whereas with the remainder some colour production occurred on the addition of the first few drops of reagent, but full colour development was very slow. This series of experiments was then repeated, the mixtures being kept at 37° for 3 hours. This modification however also failed to produce a product which would give a prompt direct reaction when added to serum.

Preparation of a direct-reacting pigment from bilirubin.

Bilirubin is readily soluble in the cold in dilute solutions of sodium and potassium carbonates, and when one or two drops of such solutions are added to 0.5 cc. of serum the resulting mixture gives a biphasic reaction. In such cases the conditions are similar to those of Newman's experiments, in which the serum mixture was decidedly more alkaline than a natural serum. With bicarbonates the same objection does not hold. Bilirubin is not soluble in the cold in solutions of either NaHCO_3 or KHCO_3 , but dissolves on warming, and when the solutions are added to serum biphasic reactions are obtained. Other salts which in aqueous solutions show an alkaline reaction were next tried, *e.g.* K_2SO_3 , Na_2SO_3 , Na_2HPO_4 and K_3PO_4 . The nearest approach to a prompt direct reaction was obtained with K_3PO_4 and further experiments were made with it. In a moderately strong solution bilirubin is not completely soluble. Hence the mixture was centrifuged and 1 drop of the supernatant fluid (in which rapid oxidation of the pigment on standing was noticed) was added to serum. This gave a reaction in which colour development began rapidly and reached its maximum in about 5 minutes. The deposit left after removal of this fluid gave a much slower reaction. A stronger solution of K_3PO_4 was next tried, but in this bilirubin was insoluble; in a dilute solution bilirubin dissolved completely, but the solution only gave a delayed reaction when added to serum. It was then found that if to a complete solution of bilirubin in dilute K_3PO_4 a strong solution of the salt is added, the pigment is almost completely precipitated. Such a mixture was centrifuged and a little of the precipitate was added to serum, in which it was readily soluble. The serum was then found to give a typical

prompt reaction. If, however, the precipitate were first dissolved in water and some of this solution added to serum, no prompt reaction was obtained. The precipitate itself appears to be insoluble in cold chloroform; in aqueous solutions it oxidises rapidly.

Having obtained a method for the preparation of a direct-reacting pigment from bilirubin, various factors in the method were examined separately and the following results obtained.

(a) It is not necessary to dissolve the bilirubin in a weak solution of the salt whose concentrated solution is the precipitating agent. Any of the alkaline substances capable of bringing bilirubin into aqueous solution may be used for the preparation, including the caustic alkalis. A convenient method is to suspend the bilirubin in water and add just sufficient dilute ammonia to bring about complete solution of the pigment.

(b) K_3PO_4 is not the only precipitating agent which can produce the desired result. Most of the alkaline phosphates can be used, *e.g.* K_2HPO_4 , Na_2HPO_4 , $(NH_4)_3PO_4$. It was found too that ammonium carbonate was quite as good in this respect as any of the phosphates. Sodium bicarbonate was not quite so good, *i.e.* the reaction obtained with the precipitate was slightly delayed. Potassium acetate was not so good as sodium bicarbonate, while sodium sulphite was useless.

Caustic alkalis themselves may be used as precipitating agents. Thus the addition of concentrated sodium hydroxide to bilirubin dissolved in a dilute solution of the same reagent brings about precipitation of the direct-reacting pigment.

In strong ammonia bilirubin is at first soluble, but soon a gelatinous precipitate forms which shows direct-reacting properties.

(c) Neutral or acid salts cause precipitation of a pigment in the same way as do the alkaline salts just mentioned, but the precipitate obtained is not a direct-reacting pigment.

It thus appears that a direct-reacting pigment is obtained as a precipitate when concentrated solutions of alkalis or of certain alkaline salts are added to solutions of bilirubin in dilute alkalis or in dilute solutions of these alkaline salts.

The isolation of this direct-reacting pigment as a pure, dry solid has not yet been accomplished; the tests of the product have been made with the wet solid left behind after centrifuging and removing as far as possible the clear supernatant layer. The solid when precipitated is somewhat gelatinous though it is easily separated by centrifuging; filtration is very slow, and during filtration and drying certain changes occur, so that the resulting dry solid fails to give a prompt reaction when dissolved in serum. A dried preparation containing the direct-reacting pigment has however been prepared, and will be referred to later.

It was shown by the following experiment that the ability to give the prompt reaction still remains after removal of the precipitating solution.

Bilirubin, dissolved in a little ammonia, was treated with saturated ammonium carbonate solution. The precipitate was centrifuged and washed in the centrifuge-tube with saturated sodium chloride solution. The wet solid still gave a prompt reaction when added to serum. Filtration was attempted, but after being on the filter for some time a portion of the solid, still moist, only gave a delayed reaction when added to serum.

Experiments with Griffiths and Kaye's direct-reacting pigment.

At this stage of the investigation Griffiths and Kaye's communication appeared. In this the isolation of a direct-reacting pigment (in an impure state) from bile is described, and the question as to the possible identity of this pigment with the one prepared from bilirubin by the above method seemed to be of considerable importance. From the treatment accorded to the pigments in each case, identity seemed extremely unlikely. The method of preparation of the direct-reacting pigment from bilirubin makes it certain that the product is a salt of some kind, whereas the isolation of the direct-reacting pigment from bile by the method of Griffiths and Kaye, in which the bile is made acid to Congo red with hydrochloric acid, suggests that the extracted pigment is a free acid. In the latter method, however, it appeared possible that some salt present in the bile might be occluded by the thick, slimy precipitate which at once forms on addition of hydrochloric acid, and so escape further action of the acid and be responsible for the direct-reacting properties of the final product. It was therefore decided to ascertain if a direct-reacting salt could be obtained from bile if treatment by hydrochloric acid is omitted.

A quantity of human bile was evaporated to dryness *in vacuo*, and the sticky residue was washed with light petroleum to remove fatty matter. The solid was then ground up and extracted with ether in the apparatus used for extracting cholesterol from blood [Myers and Wardell, 1918]. On evaporation of the ether a small amount of yellow solid was left, which, on addition to serum gave a prompt reaction. The residue from the ether extraction was then extracted with chloroform. Much more pigment was extracted by the chloroform than by ether and, after evaporating off the chloroform in a current of coal-gas, a considerable quantity of a greenish brown solid remained. On addition of a small amount of this solid to serum an excellent prompt reaction was obtained. The solid after ignition left a soluble, fusible alkaline ash which contained sodium. A solution of the solid in water, when added to serum, also gave a prompt reaction, but not quite so good as that obtained with the solid itself.

The method of isolation of this direct-reacting pigment without the use of any acid, its solubility in water, and the nature of the ash, give strong grounds for supposing that the product is a sodium salt. On treatment of its aqueous solution with hydrochloric acid a precipitate is formed.

The result of this experiment does not conclusively prove that Griffiths and Kaye's direct-reacting pigment is also a sodium salt.

A fresh specimen of bile was diluted with two or three volumes of water and the well-stirred mixture made acid to Congo red with hydrochloric acid. The precipitate was washed with water and then dried *in vacuo*. It was then ground and washed with light petroleum and extracted first with ether and then with chloroform.

The ether extract after evaporation left a yellowish brown solid which was insoluble in water but soluble in serum, in which it gave a prompt reaction. The chloroform extract yielded a greenish solid which showed the same solubility and reaction as the ether extract. On igniting the ether extract no ash whatever remained, and from the chloroform extract only a very slight trace of ash was left. No ammonia was present, so that it was thought that the substance must be a free acid. Its general behaviour was similar to that of the red bilirubin extracted from gallstones, but with the outstanding difference that it was soluble in serum and when so dissolved gave a prompt reaction. It was also found to be soluble in the cold in sodium bicarbonate solution. The ether-extracted portion also resembled gallstone bilirubin in colour, except that it was somewhat yellower.

The results so far obtained show that not one but two direct-reacting pigments may be isolated from bile, their probable relationship being that of free acid to sodium salt.

It should be added that not all specimens of bile yield direct-reacting pigments when treated by the methods outlined above. Only fresh, golden bile can be relied on to give these results; old or very dark coloured specimens generally yield pigments which on addition to serum give biphasic or delayed reactions. It is evident therefore that the direct-reacting pigments are somewhat unstable. The loss of direct-reacting property is not entirely due to oxidation though this certainly occurs. It must be due in part to a change to a more stable, indirect-reacting pigment.

In the above experiments, though extractions have been made with both ether and chloroform, it is not considered that there is any essential difference between the two extracts. In both cases the pigments are accompanied by other substances extracted from the bile. The amount of material obtained with chloroform is always greater than with ether, but with chloroform more oxidation of the pigment occurs owing to the higher temperature of extraction, and this no doubt accounts for the differences in colour which have been noted.

The experiments described have shown that the direct-reacting pigment prepared from bilirubin is not identical with Griffiths and Kaye's pigment, since the latter is not a salt. There remains the possibility that it is identical with the direct-reacting pigment obtained from bile without acidification, and attention was next directed to this point.

Between the two pigments under consideration there is one important difference, namely, that the pigment prepared from bilirubin loses its direct-reacting properties if dissolved in water, but the same is not true, at any rate to the same extent, of the material isolated from bile. But the latter contains

other substances from bile besides the pigment, and even this has some tendency to change to a non-direct-reacting form, as shown by the fact that old specimens of bile tend to yield a product without direct-reacting properties. The difference in stability is relative, and it appears possible that the greater stability of the bile product may be due to the effect of the substances accompanying the pigment. An attempt was therefore made to separate the latter from the other substances present. Concentrated sodium hydroxide solution (in which the direct-reacting pigment from bilirubin is insoluble) was added to an aqueous solution of the direct-reacting product from bile until a precipitate was formed. On centrifuging a pellicle of brown pigment resulted, lying on top of a practically colourless solution. This pigment gave a prompt reaction when dissolved in serum, but if previously dissolved in water and its solution added to serum only a delayed reaction was obtained, *i.e.* it behaved like the pigment from bilirubin. This result suggests either that the pigment from bile is identical with that from bilirubin but is stabilised by other substances accompanying it and which have been separated from it by the procedure adopted in this experiment, or that the two pigments are closely related and that the pigment from bile is converted to a form identical with that from bilirubin by the treatment described.

Bile salts are among the substances present in the direct-reacting product from bile, and experiments were made to determine the stabilising effect of these on the direct-reacting pigment prepared from bilirubin. In one of these bilirubin, suspended in water, was dissolved by the aid of a little ammonia, and sodium glycocholate was added. The resulting solution was not precipitated by strong ammonium carbonate solution, and did not give a prompt reaction when added to serum. When a little more ammonia was added, however, it did give a prompt reaction. In the presence of bile salt, therefore, the direct-reacting pigment is formed by a sufficient concentration of alkali, without precipitation of the pigment. The solution was evaporated to dryness *in vacuo*, but the resulting solid was without direct-reacting properties.

In another experiment bilirubin in dilute potassium hydroxide was precipitated with tripotassium phosphate and the precipitate separated by centrifuging. The supernatant fluid was poured off and replaced by a solution of sodium glycocholate, in which the precipitate dissolved. The solution, however, did not give a prompt reaction until a little more tripotassium phosphate had been added. After doing this the solution was added to an equal volume of serum and the mixture evaporated to dryness *in vacuo*. The dried residue was yellowish-red, and when added to serum gave a prompt reaction. When dissolved in water it was alkaline to litmus and when treated with chloroform in the extraction apparatus very little pigment was extracted by the solvent. This solid represents the first successful attempt to obtain a direct-reacting pigment prepared from bilirubin in the solid state, but it differs in its alkaline reaction and solubility in chloroform from the direct-reacting material obtained from bile.

In another case the direct-reacting pigment was prepared and centrifuged in the usual way and then dissolved in about 10 cc. of serum. It was then divided into two parts. The first part was evaporated *in vacuo*. To the second sodium glycocholate was added, and then it was also evaporated. The solid from the first part, when dissolved in serum, gave a reaction which was fairly rapid, but not prompt, if it was in fair concentration, but much less rapid if in dilute solution. The solid from the second part gave a prompt reaction in fairly concentrated solution, less prompt when dilute.

Here there does seem to be an increased stability, so far as direct-reacting properties are concerned, in the presence of bile salts, but neither in this nor in many other experiments with bile salts was a product obtained having the same stability as that obtained from bile.

The identity of the pigment from bilirubin with that isolated from bile has therefore not so far been proved, but the evidence suggests at any rate that it is highly probable. Bile salts may not be the sole or even principal stabilising agents, and other substances in the bile extract which might act in this way have not so far been examined.

Küster's two forms of "Bilirubinammonium."

The simplest and most satisfactory explanation of the facts so far recounted is that bilirubin may exist in two forms one of which, in serum, gives a prompt reaction and the other does not. Each of these free acid forms may give rise to salts which behave towards the van den Bergh reagent in the same way as the acids from which they are derived. This explanation assumes that the direct-reacting pigment from bilirubin and that obtained from bile are identical, and that in the preparation of the former we have succeeded in preparing from bilirubin of one form the sodium salt of the other form.

A search through the literature on bilirubin showed that the idea of multiple forms of bilirubin and its salts is not new, for Küster [1915, 1917] arrived at a similar view on totally different grounds. In particular Küster [1917] described the preparation of two forms of ammonium salt, and from these he obtained different forms of free bilirubin. In view of the results which have already been described, it seemed to be of special interest to examine the behaviour of Küster's ammonium salts, when dissolved in serum, towards the van den Bergh reagent. For their preparation bilirubin is suspended in methyl alcohol and dry ammonia gas passed into the liquid. Next, instead of precipitating the ammonium salt at once with ether, as in the earlier method, the solution is placed in a cooling mixture and a portion of the pigment crystallises out. This is Küster's crystalline "Bilirubinammonium." The filtrate from it is then treated with ether and the precipitate produced is the second ammonium salt, Küster's ether-precipitated "Bilirubinammonium." Küster used bilirubin obtained from ox gallstones.

In the present work the crystalline bilirubin from human gallstones was first used. The crystalline ammonium salt prepared from it when added to

serum gave a delayed reaction, while the ether-precipitated salt gave a reaction which was biphasic. A preparation of the ammonium salts was then made from the precipitated bilirubin described in our method for the isolation of bilirubin from gallstones. In this case the quantity of crystalline ammonium salt obtained was extremely small, and it gave a delayed reaction. Fractional precipitation with ether was carried out on the mother-liquor and three fractions of precipitated salt were obtained. All gave a biphasic reaction, but the last fraction gave the reaction which was most rapid and most nearly approached a prompt one.

The above was repeated, using sodium salts prepared by suspending bilirubin in methyl alcohol and adding sodium ethoxide until the bilirubin dissolved. When crystalline bilirubin was used, the crystalline sodium salt gave a delayed reaction; of two separate fractions of ether-precipitated salt, the first gave a delayed reaction while the second gave a biphasic reaction of fair rapidity. With precipitated bilirubin the quantity of crystalline sodium salt obtained was negligible; of two fractions of ether-precipitated salt the first gave a delayed reaction, the second a biphasic one.

These results show that in addition to the differences pointed out by Küster between his two salts of bilirubin, there is also some difference in their behaviour towards the van den Bergh reagent, but the difference is not so sharp as between the two salts we have previously dealt with. This does not entirely disprove an essential identity between the ether-precipitated salt of Küster, which gives biphasic reactions, and the prompt-reacting pigment prepared from bilirubin, or that isolated from bile. In the first place it could hardly be expected that the method of separation used by Küster would give pure products unless carried out under very carefully controlled conditions; the ether-precipitated product probably contains a certain amount of the more slowly reacting form of salt, so that the reaction obtained would not be so rapid as would be the case if complete separation from the crystalline salt had been attained. Further, it is possible that Küster's precipitated salt may be a prompt-reacting pigment when first formed, but that in the absence of stabilising agents, such as alkaline salts or certain constituents of bile, changes occur which result in some loss of direct-reacting properties.

Crystalline and precipitated bilirubin.

In view of the differences which have been noted above between the products obtained from crystalline bilirubin and those obtained from precipitated bilirubin, as well as differences which appear in the statements of different workers with respect to certain properties of the bilirubin they had employed, we were led to make a further examination of these two substances. Difference in colour is certainly evident, but this of itself is of little importance. But there are other differences which are of significance. Thus, the crystalline form is decidedly less soluble in chloroform than the precipitated form. The former is insoluble in serum and in cold sodium bicarbonate solution, while the latter is

soluble in both. These differences are precisely those noted by Küster in what he claimed to be different modifications of bilirubin, isolated respectively from his two ammonium salts, and the two substances we have been dealing with are probably identical with Küster's two modifications. We have further found that the two products differ in their behaviour towards the van den Bergh reagent. If crystalline bilirubin is suspended in serum, the mixture only reacts very slowly with the reagent, whereas a solution of the precipitated form in serum reacts much more rapidly, giving a result which, though not prompt, might in most cases be justifiably described as biphasic.

With regard to the possible identity between the precipitated bilirubin and the prompt-reacting free bilirubin obtained from bile, we have to apply the same considerations as those we have just used in discussing the relationship between Küster's ether-precipitated salt and the bilirubin salt from bile.

The ammonia content of bile.

Küster showed that his ammonium salts lose their ammonia when they are heated with chloroform or methyl alcohol, and also when subjected to a vacuum. In the isolation of direct-reacting pigment from bile, the conditions have been such, therefore, that if the pigment had originally been present as ammonium salt it is probable that it would not appear as such in the final product; the fact that the latter appears to contain a sodium salt may be due to replacement of ammonium by sodium at some stage of the process which has been employed.

Direct-reacting pigments from bilirubin, whether prepared as potassium, sodium or ammonium salts, have so far shown nothing in their general behaviour or in their behaviour towards the van den Bergh reagent to distinguish them from one another, so that there has been nothing in our previous results which definitely disproves the suggestion of Collinson and Fowweather that the direct-reacting pigment may be an ammonium salt.

One of the reasons which caused these workers to make this suggestion was the loss of direct-reacting properties which occurs when sera from cases of obstructive jaundice are heated, or kept. Since, in their view, the difference between the direct-reacting and indirect-reacting pigments was that the one was a salt of bilirubin and the other the corresponding free acid, this loss of direct-reacting properties appeared to indicate a change from salt to free acid, and such a change is most likely to occur if the salt is an ammonium salt.

The present work however, since it proves that indirect-reacting salts exist, as well as direct-reacting salts, shows that it is not necessary to assume that a change to free acid must occur when direct-reacting properties are lost, and that therefore this loss is of no value as evidence in favour of the existence of the direct-reacting pigment as ammonium salt.

Another fact which influenced Collinson and Fowweather was that they found that bile, which contains much more pigment than blood, also contains

much more ammonia, but beyond establishing this fact in a general way by comparative tests, they did not obtain actual figures.

It was thought advisable to return to this point and make quantitative determinations of the ammonia in bile. The method employed was that used by Nash and Benedict [1921] for ammonia in blood, except that the amount of ammonia present allowed of its determination by titration instead of by nesslerisation. Eight determinations were made, seven on human gall-bladder bile obtained from the *post-mortem* room, and one on dog's bile obtained from the animal within half an hour of death. The results are as follows.

Case	Time after death hrs.	Description of bile	Ammonia mg. per 100 cc.
1 (human)	29	Fairly thin, dark brown	30.4
2 "	12	Thick, almost black	7.85
3 "	13	Thin, dark brown	3.83
4 "	3	Fairly thin, golden	2.11
5 "	6	Thick, brown	7.66
6 "	5	Thin, golden	2.87
7 "	34	Fairly thick, dark brown	32.7
8 (dog)	$\frac{1}{2}$	Thin, brown	1.03

The ammonia content of human blood is less than 0.3 mg. per 100 cc.

The above series of estimations on bile is small, and is simply intended to give a general impression as to the amount of ammonia in bile compared with that in blood. It is known that the ammonia content of blood increases if the specimen is allowed to stand. The above figures for bile suggest that here too a similar increase may occur, since the highest figures are obtained in the cases in which the greatest interval of time has elapsed between death and analysis of the specimen. Probably only the lower figures in the Table approximate to the actual ammonia content of fresh bile, but considering these alone, there seems to be sufficient evidence that bile contains more ammonia than blood.

The approximate average of the three lowest figures for human bile is 3 mg., and this, if present as ammonium bilirubinate, would correspond to 0.103 g. of bilirubin.

Many of the analyses of bile which are to be found in the literature do not give separate figures for bile pigment, but give a single figure for mucin and pigments. Of twelve such analyses relating to human gall-bladder bile, collected by Rosenbloom [1913], the highest figure for mucin and pigments is 4.44 % (by weight), and the lowest 0.23 %. The mean of the twelve values is 2.23 %. Evans [1930] quotes an analysis by Czyhlarz *et al.* in which mucin and pigments together amount to 1.80 %, mucin being responsible for 1.64 % and pigment for 0.16 %, *i.e.* the figure for pigment constitutes one-eleventh of the total of mucin and pigment. On this basis the analyses quoted by Rosenbloom would give an average value of 0.2 % for pigment, with considerable divergence in individual analyses on either side of this value. The figure for bilirubin, *viz.* 0.103 %, which has been calculated as equivalent to the mean of the three lowest figures for ammonia in the results above, may therefore be reasonably

taken to fall within the range shown by actual analyses of bile, and is quite close to the figure quoted by Evans. This is not taken to be a proof, or even a confirmation, of the view that the natural salt of bilirubin present in bile and showing direct-reacting properties, is the ammonium salt, but it does show that what we know of the ammonia and pigment content of bile is not inconsistent with this view. The possibility therefore that the natural direct-reacting pigment may be an ammonium salt still remains until we have further information to prove or disprove it. Simultaneous determinations of ammonia and bilirubin in bile, or in sera from cases of obstructive jaundice, might reasonably be expected to give further information on this point.

DISCUSSION.

The results just reported establish beyond reasonable doubt the fact that free bilirubin may exist in more than one form, and that corresponding to these different forms are different series of salts, and the recognition of this fact explains the conflicting claims which have been made by different workers as to certain important properties of bilirubin. Newman, for example, states that his bilirubin dissolved in serum and gave a prompt reaction, but his bilirubin was obtained from bile by a process very similar to that by which Griffiths and Kaye obtained their direct-reacting pigment, and it is evident that he was dealing with the direct-reacting, serum-soluble form. Collinson and Fowweather on the other hand have claimed that bilirubin is insoluble in serum and when suspended in serum gives a delayed reaction. Their product however was the crystalline bilirubin obtained from gallstones, and it is thus easy to see why they differed from Newman. In general, much conflicting evidence on the bilirubin problem can be explained when it is realised that where different results are claimed it is due to the fact that different forms of bilirubin have been used.

In the light of the present findings the explanation of the van den Bergh reaction appears to be as follows.

The pigment of sera from non-obstructive cases of jaundice, which only give a delayed reaction, is bilirubin in the more stable, slowly reacting form, which we shall refer to as bilirubin A. Much of the evidence previously obtained suggests that this is in the free acid form, but in view of the fact that its salts are also non-direct-reacting, and soluble in chloroform, the possibility of its being present as a salt requires further consideration and investigation. The pigment in sera from cases of obstructive jaundice, and in bile, is a salt of the less stable, more soluble, prompt-reacting form, which we shall call the B form. Bilirubin B and its salts have a great tendency to change into the A form, and this accounts for the loss of direct-reacting properties observed in old specimens of bile, or in obstructive sera which have been heated or allowed to stand for some time. Whether the salt is the ammonium salt or the sodium salt is not yet clear; this question has already been discussed in dealing with the ammonia content of bile.

From what has been said of the properties and mode of formation of the

direct-reacting salts, it will be evident that if the pigment of natural obstructive jaundice sera is such a salt, states of acidosis will have an unfavourable effect on the formation or the stability of such a pigment. This probably explains why M'Gowan found some relation between the alkali reserve of the blood and the type of reaction. We have also shown that the stability of the direct-reacting pigment, as such, is apparently dependent on other material derived from bile. Hence in slight degrees of obstructive jaundice it is probable that the amount of stabilising substance passing into the blood along with the pigment is often insufficient to maintain the latter in its prompt-reacting form. There will thus be some relation between the concentration of pigment and type of reaction, but we do not agree with Snider and Reinhold that concentration is the only factor concerned. No doubt the general view that the nature of the pigment in icteric sera is dependent on the nature and mechanism of the jaundice is true, but if it is recognised that it is also dependent on such factors as the alkali reserve of the blood, and the concentration of certain stabilising substances, it will be realised that these latter factors may sometimes obscure the original relationship between type of pigment and nature of jaundice. This explains why sera are occasionally met with whose reactions are not in agreement with the nature of the jaundice which is present, and so have led to some disappointment in the application of the van den Bergh reaction as a clinical test and aid to diagnosis.

The explanation offered by Küster to account for the different forms of bilirubin is that in this substance we have an example of keto-enol tautomerism. He considers bilirubin A to be a keto-form, while bilirubin B is an enol modification. He also calls attention to the possibility that between these two forms intermediate products may exist, and this is to be borne in mind as a possible explanation of our failure to establish definitely an identity between certain substances we have dealt with.

Küster offers graphic representation of his views, based on his formula for bilirubin as a tripyrrylmethane compound. The "enol" form is represented in two ways, each showing two hydroxyl groups and the "keto"-forms are also represented by two formulae, each showing one hydroxyl and one keto-group. Küster's formula for bilirubin has been opposed by H. Fischer, who represents bilirubin as a tetrapyrrolethylene [see Fischer and Postowsky, 1926], and assigns to it a formula which allows of different forms as a result of stereoisomerism as well as indicating the possibility of tautomerism of keto-enol type. Fischer's formula is the one most generally accepted.

Little is known concerning the actual mechanism of the coupling between bilirubin and diazo-compounds. Pröscher [1900] isolated what he believed to be a mono-azo-compound, as a result of coupling bilirubin with diazotised aminoacetophenone. But he assumed a formula $C_{16}H_{18}O_3N_2$ for bilirubin, and if his results are re-calculated on the basis of the modern formula $C_{33}H_{36}O_6N_4$, it is seen that he was really dealing with a bis-azo-compound. Orndorf and Teeple [1905] showed that when bilirubin couples both mono- and

bis-azo-compounds are formed. Fischer and Barrenscheen [1921] confirmed these findings, but did not arrive at any definite conclusion as to the nature of the coupling which occurred. Küster's formula does not appear to indicate how the coupling might take place according to the usual conceptions of the formation of azo-colouring matters, but a modification of Fischer's formula, along the lines suggested by an earlier form given by Fischer and Röse [1914] would provide two unsaturated side-chains $\text{CH}_2\text{:CH}$, which form part of a butadiene type of structure. Meyer [1919] showed that typical azo-derivatives could be obtained by coupling suitable diazo-compounds with butadienes. Hence we should have in this modified Fischer's formula an indication of how the coupling might take place, and give mono- or bis-azo products according as one or both side-chains took part in the reaction. The difference in rate of coupling shown by the two forms of bilirubin might be explained on the grounds that the presence of the OH group in the enol form might favour rapid and complete coupling in much the same way as does a phenol group in aromatic compounds.

The fact that both forms of bilirubin couple rapidly when dissolved in alcohol (as in the indirect van den Bergh reaction) is also not inconsistent with the assumption of a keto-enol tautomerism, for there are many cases of such tautomerism in which it has been shown that solution in alcohol favours the change towards the enol modification. It has been noticed in the present work that crystalline bilirubin when dissolved in alcohol oxidises much more rapidly than when dissolved in chloroform, suggesting that in the former solvent a greater amount of the more reactive, more readily oxidisable form is present than in the latter solvent.

The mechanism of the process by which free bilirubin A is converted into salts of bilirubin B remains to be considered. The process requires in the first place solution of the bilirubin in alkali. This would be expected to favour a change from keto- to enol-form. The precipitation of the prompt-reacting form from this solution by the addition of concentrated solutions of alkaline salts might be explained on the grounds that the latter, while offering a sufficiently alkaline reaction to maintain the enol form, also exert a salting out effect on this form, so that it appears as a precipitate. The successive changes are:

When the precipitate is dissolved in water, the absence of alkali allows rapid change again towards the keto-modification, so that when the solution is added to serum no prompt reaction is obtained. If the solid itself is added to serum there will also be a tendency to change to keto-form, but the presence of bicarbonate in serum is likely to have a retarding effect on this change; serum-proteins too may have a similar influence, so that we get a prompt reaction for some time, but that finally a change occurs in which most of the pigment passes into the keto-form is seen in the fact that both natural and artificial sera ultimately lose the capacity to give prompt reactions.

Along these lines it appears possible to explain most of the experimental results, but when we consider the formation of the prompt-reacting pigment within the body we meet with certain difficulties. If this is assumed to be the same as the process we have just dealt with, the solution of the free bilirubin A would require a degree of alkalinity which, in view of what we know of the reaction of body fluids and tissues, seems impossibly high, for no appreciable solution of free bilirubin A takes place in the cold below a p_H of 10.5, and at 37° the alkalinity required is only slightly less. The rest of the process, namely change of salts of A to salts of B, is not difficult to understand. We have seen that certain substances in bile appear to have the property of stabilising the B forms. These will be present in the cells of the liver, in which the change from indirect to direct pigment is believed to take place, and it seems probable that besides stabilising B forms (possibly by forming loose combinations with them) they may also have considerable influence in favouring their production. But the whole change might conceivably occur as follows:

Free bilirubin A $\rightarrow$ free bilirubin B $\rightarrow$ salts of bilirubin B.

Some tendency towards equilibrium between the two forms of free bilirubin, even in the solid state, is indicated by Küster, who states that if either form is stored as solid for some time a certain amount of the other form begins to appear. In the state in which the indirect pigment exists in the body, changes may be expected to occur more rapidly than in the dry, solid form, and may perhaps be accelerated by those substances in the liver cells which stabilise the B modification when it is formed. Moreover free bilirubin B is soluble in serum. Hence solution and formation of the B salt may, by this process, require no greater alkalinity than that of blood.

But there is another possibility: bilirubin, when formed from haemoglobin, may first appear in the B form. This would dissolve in the blood as a salt of this form, but in the absence of stabilising substances might change rapidly to the A form of salt. Passage through the liver cells would have the effect of reconvertng it to the B form and stabilising it as such. On this view, the indirect pigment would be a salt of bilirubin A, and not the free acid—a possibility which has already been referred to.

If this view is correct, we should not expect bilirubin to appear as an ammonium salt. The liver is the only site in which the formation of an ammonium salt is at all likely to occur. If a salt is formed elsewhere it will probably be the sodium salt, and we should not expect the sodium to be subsequently replaced by ammonium, either in the liver or elsewhere. Hence, if it can be shown that the direct-reacting pigment is an ammonium salt, it will follow that the liver is the site in which salt formation first takes place.

The recognition of the fact that bilirubin may exist in more than one form thus raises many questions as to the structural difference between these forms, the mode of change from one form to another and the mechanism of the coupling with diazo-salts. None of these questions can be regarded as settled,

and, while possible explanations have been suggested, the incompleteness of the evidence and the need for further work are fully recognised.

The original object of the present work, however, was to throw some light on the problem of the van den Bergh reaction, and the recognition of the existence of more than one form of bilirubin undoubtedly clears up many of the discrepancies and contradictions in recent work on this subject. In that, we believe, lies sufficient justification for the publication of these results at the present stage.

SUMMARY.

1. Red bilirubin, obtained from gallstones by crystallisation from chloroform solution, is insoluble in serum and gives a delayed van den Bergh reaction when suspended in serum. The pigment obtained by precipitation from chloroform solution after removal of the crystalline bilirubin is soluble in serum and gives a much more prompt reaction than the crystalline pigment.

2. Certain salts which have been prepared from crystalline bilirubin also fail to give a prompt van den Bergh reaction.

3. A pigment isolated from bile after acidification is soluble in serum, in which it gives a prompt reaction. A pigment having all the characteristics of a sodium salt, isolated from bile without acidification, also gives a prompt reaction when dissolved in serum. It is concluded that these pigments are related to one another as free acid and salt.

4. A method has been described by which salts giving a prompt reaction when dissolved in serum may be prepared from crystalline bilirubin.

5. It is concluded from the foregoing that bilirubin may exist in two different forms, each giving rise to a separate series of salts.

6. Attention is called to the work of Küster, who reached similar conclusions along different lines, and the relation between Küster's different forms and those dealt with in this work is considered.

7. An explanation of the van den Bergh reaction is offered in which it is considered that the indirect pigment is bilirubin of one form, but whether as free acid or salt is not yet clear, while the direct pigment is a salt of the other form. Recent work on the van den Bergh reaction has been examined in the light of this view.

8. The ammonia content of bile has been determined and the results examined as to their bearing on the possibility that the direct pigment in bile may be an ammonium salt.

9. Küster's views, which regard the two forms of bilirubin as keto- and enol-modifications, have been stated and considered with reference to the structural formulae for bilirubin proposed by Küster and Fischer respectively.

10. The mechanism of the coupling of bilirubin with diazo-salts has been considered in relation to the theory of the keto-enol tautomerism of bilirubin.

11. The mode of transformation of bilirubin from one form to another *in vitro* and *in vivo* has been discussed.

REFERENCES.

- Collinson and Fowweather (1926). *Brit. Med. J.* i, 1081.
Davies and Dodds (1927). *Brit. J. Exp. Path.* **8**, 316.
Evans (1930). Starling's Principles of human physiology, 5th ed. p. 582.
(Churchill, London.)
Fischer and Barrenscheen (1921). *Z. physiol. Chem.* **115**, 94.
—— and Postowsky (1926). *Z. physiol. Chem.* **152**, 300.
—— and Röse (1914). *Z. physiol. Chem.* **89**, 255.
Griffiths and Kaye (1930). *Biochem. J.* **24**, 1400.
Grünenberg (1922). *Verhand. Kongr. inn. Med.* **34**, 112.
Küster (1915). *Z. physiol. Chem.* **94**, 136.
—— (1917). *Z. physiol. Chem.* **99**, 86.
—— Reihling and Schmiedel (1914). *Z. physiol. Chem.* **91**, 58.
Meyer (1919). *Ber. deutsch. chem. Ges.* **52** B, 1468.
M'Gowan (1930). *Edin. Med. J.* **37**, 28.
Myers and Wardell (1918). *J. Biol. Chem.* **36**, 147.
Nash and Benedict (1921). *J. Biol. Chem.* **48**, 463.
Newman (1928). *Brit. J. Exp. Path.* **9**, 112.
Orndorf and Teeple (1905). *Amer. Chem. J.* **32**, 215.
Pröscher (1900). *Z. physiol. Chem.* **29**, 411.
Roberts (1928). *Brit. J. Exp. Path.* **9**, 107.
Rosenbloom (1913). *J. Biol. Chem.* **14**, 241.
Snider and Reinhold (1930). *Amer. J. Med. Sci.* **180**, 248.