CIX. STUDIES ON BILE PIGMENTS.

II. A NEW TEST FOR BILIRUBIN IN THE URINE AND ITS USE FOR DETECTION OF BILIRUBIN IN NORMAL URINE.

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Tests for bilirubin in the urine are unsatisfactory owing to their lack of sensitivity. Gmelin's test with nitric acid and Rosin's test with iodine, depending on the formation of green biliverdin or blue bilicyanin, are positive only in the presence of high concentrations of pigment and many modifications of these methods do not give much better results. The reason for this failure is the fact that biliverdin or bilicyanin is only a transitional stage of bilirubin oxidation, the final stage being represented by the pink choletelin which is formed more rapidly in the presence of smaller amounts of bilirubin. Yet it is just in cases of slight jaundice that the detection of small amounts of bilirubin is of particular diagnostic importance.

Zins [1923] modified Steensma's method by adsorbing the pigment on $BaSO_4$ and testing the precipitate on the filter-paper with trichloroacetic acid. Cole [1926] described a modification of Huppert's method, adsorbing bilirubin on $BaSO_4$, eluting the pigment with acid alcohol and oxidising it with $KClO_3$. Kuhn [1928] performs the oxidation by an alkaline copper solution and extracts the biliverdin in a layer of alcohol on the top. A spectroscopical test after oxidation of bilirubin to bilicyanin is described by Beccari [1928]. Itallie [1929] devised a modification of Steensma's method, adsorbing the pigment on talc, eluting it with acid alcohol and oxidising it with $NaNO_3$. Recently Godfried [1934] described a test devised by Harrison who adsorbs the pigment on $BaSO_4$ and tests the precipitate on the filter with a drop of Fouché's reagent.

Experimental.

Preliminary experiments showed that bilirubin is most efficiently adsorbed by filtering through a layer of adsorbing material. The urinary pigments being adsorbed on the surface of the layer the bilirubin can then easily be detected with an oxidising reagent. Talc was found to be the most suitable adsorbent and for oxidation Fouché's reagent or nitric acid are equally suitable. The test is performed in the following way:

A Büchner funnel of $3\frac{1}{2}$ cm. diameter is fitted with the ordinary filter of 3 cm. diameter which is wetted with water. 5 ml. of 10% talc suspension in water after shaking well are poured on the filter and, after sucking dry, 5 ml. of urine are poured on the talc layer, which, after sucking off again appears as a yellow or orange disc. One drop of Fouché's reagent[1] or 10% HNO_3, is put in the middle of the talc disc and sucked off. Even traces of bilirubin are indicated immediately by a distinct blue spot. The colour intensity increases for 1–2 hours fading slowly until, usually after 20 hours, only a faint grey is perceptible unless the reaction has been very strong.

[1] Fouché's reagent consists of 25 g. trichloroacetic acid, 10 ml. of $FeCl_3$ 10% and 100 ml. distilled water.

The method of adsorption by filtration has advantages over the usual method in which a precipitate is formed in the urine; the bilirubin remains on the uppermost layer and so produces favourable conditions for the test. The difference between the two methods is illustrated by the following experiment.

5 ml. of 10 % talc suspension are filtered as described above and 5 ml. of urine containing a slight excess of bilirubin are poured on the talc layer. Another portion of 5 ml. of urine is shaken with 1 g. of talc after acidifying slightly with acetic acid and filtered off. The first talc layer will be found much more stained than the second and if a drop of Fouché's reagent is put on both the blue spot on the first filter is much stronger than on the second.

It is furthermore essential to use an acid-resistant adsorbent; most of the adsorbing substances such as $CaCO_3$, $BaPO_3$, $BaCO_3$, $CaPO_3$, *etc.*, are partly dissolved by acid reagents, so that part of the pigment is dissolved and therefore subjected to a much more vigorous oxidation and transformed rapidly into choletelin. The reaction between acid and adsorbed pigment, on the other hand, is milder and can be better controlled. Thus, by using oxidising agents of different strengths it is possible to produce at will any transitional stage of oxidation between bilirubin and choletelin from yellow through green, blue, violet to pink. Table I gives a survey of these colour reactions with different reagents. As will

Table I. *Tests with drops of different oxidising agents on a talc disc with adsorbed jaundice urine.*

Reagents	Colour of reaction
Fouché's reagent	Blue
HNO_3 95 %	Pink
HNO_3 25 %	Violet
HNO_3 10 %	Blue
HNO_3 5 %	Bluish green
HNO_3 1 %	Green
Trichloroacetic acid 50 %	Greyish white
Trichloroacetic acid 25 %	Grey
Obermeyer's reagent	Brown
$KMnO_4$ *N*/10	Pink with greenish periphery
Iodine *N*/10	Brown with greenish periphery
H_2O_2 90/100 vol.	No colour reaction
H_2O_2 90/100 vol. + $FeCl_2$ 10 %	Pink with sharp blue ring

be seen Fouché's reagent and 10 % HNO_3 are the most suitable for producing a blue bilicyanin reaction. More concentrated HNO_3 as well as $KMnO_4$ and iodine have more vigorous actions, oxidising the bilirubin rapidly to choletelin. H_2O_2 reacts only in conjunction with $FeCl_2$ producing a spot with a centre of pink choletelin surrounded by a circle of blue bilicyanin.

It is interesting to note that the method gives a positive reaction also with normal urine, and as this is not the case with other methods it is necessary to show that this positive test is actually due to bilirubin. The only possible interfering substances would be indole compounds which can be transformed into indigo blue. However, pure indole did not react with Fouché's reagent, and indican only after about 30 min. and neither gave a pink choletelin colour. Furthermore, urines with a large amount of indole as shown by Jolles's test, did not give an increased bilirubin test nor did a positive test appear if before adsorption the urine was treated with strong oxidising agents. The blue reaction in normal urine can therefore be attributed to the presence of bilirubin.

The sensitivity of the test was determined by dilution of pure bilirubin solutions until a faint grey reaction was just observed. Proceeding according to the method of Van den Bergh and Grotepass [1934] the bilirubin was dissolved

finally in diluted aqueous NaOH, diluted alcohol and urine freed from preformed bilirubin by treatment with HNO_3 and subsequent neutralisation. The limiting concentrations varied with the solvents being 6·0, 0·8 and 0·9 parts per million in NaOH, alcohol and urine respectively. These values compare favourably with the value of 70 parts per million in Harrison's test according to Godfried's statement.

The dilution technique may be applied to obtain an approximate figure for the actual concentration of bilirubin in normal urine. From 20 normal urines the limiting dilutions for a positive reaction varied between 1 : 350 and 1 : 480. These figures multiplied by the limiting figure of 0·9 part per million as stated above give a content of roughly 0·3 mg./100 ml., a figure corresponding to the bilirubin content of the serum. Assuming a daily urinary output of $1\frac{1}{2}$ l. the output of bilirubin in normal urine should be about 5 mg. per day. However, these figures cannot be more than approximate.

Summary.

1. A new test for bilirubin in urine has been described consisting in the adsorption of urinary pigments on a layer of talc and the production of blue bilicyanin by oxidation with a drop of Fouché's reagent or 10 % HNO_3.

2. The described method permits for the first time the detection of bilirubin in normal urine.

3. The blue bilicyanin reaction in normal urine has been investigated with regard to the interference of indole compounds and has been found to be specific for bilirubin; excessive amounts of indican react only after about 30 min.

4. The limits of detectability of pure bilirubin dissolved in weak NaOH, diluted alcohol and urine freed from preformed bilirubin are 6·0, 0·8 and 0·9 parts per million respectively.

5. Quantitative estimation by means of a dilution technique gave a bilirubin content of approximately 0·3 mg./100 ml. in normal urine and an output of about 5 mg. bilirubin per day.

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