CCLXX. CLINICAL TESTS FOR BILIRUBIN IN URINE.

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THE usual tests for bilirubinuria in routine clinical work are relatively insensitive; that this has long been recognised is shown by the various attempts to introduce more sensitive tests from time to time, but none of these has been widely employed.

The object of this paper is to review once more the available methods, and to describe a simple yet very sensitive test which, in the opinion of the writer, could with advantage be generally adopted in clinical work.

In order to compare the relative sensitivity of the different tests on a quantitative basis, it was necessary in the first instance to be able to estimate the amount of bilirubin in the urines examined. For this purpose the diazo-method [Hunter, 1930] has been modified to yield a satisfactory quantitative procedure.

A new simple qualitative clinical test.

I am indebted to Dr G. A. Harrison for the description of this test which has been used in his laboratory for several months. It consists in the oxidation by Fouchet's reagent of bilirubin adsorbed on a barium precipitate and is performed as follows.

A test-tube is half filled with urine (about 10 ml.) and half the volume of 10 % barium chloride solution is added. The contents are mixed and filtered. After the fluid has passed through, the paper is spread on another dry piece of filter-paper and 1 or 2 drops of Fouchet's reagent are added to the precipitate. A green or blue colour indicates bilirubin. This test will be referred to as "Harrison's test."

Fouchet's reagent consists of

Trichloroacetic acid	...	...	25 g.
Distilled water ...	...	...	100 ml.
10 % ferric chloride...	...	...	10 ml.

The idea of oxidising a bilirubin-barium precipitate spread on a filter-paper is not new, for nitric acid was employed many years ago [Zeehuisen, 1895], but with nitric acid there is more risk of over-oxidation and there is apt to be a greater personal factor in deciding whether a green or a blue colour has developed or not. Similarly Obermayer's reagent ($FeCl_3$ 1 g., concentrated HCl 500 ml.) can be added to the bilirubin-barium precipitate, but it is less satisfactory, because it is more coloured and contains relatively less $FeCl_3$ and more acid than Fouchet's reagent.

In the writer's experience false positive reactions in Harrison's test have not occurred. When the reaction was positive, at least one of the other methods employed was also positive. It is true that there is some adsorption of indican

on the barium precipitate, when indicanuria is intense, as shown by the writer's observation that water used to wash such a precipitate gives a positive test for indican by Jaffé's or Obermayer's method, but the amount adsorbed has never been sufficient to give a false positive reaction for bilirubin.

Outline of older methods for detection of bilirubinuria.

In detecting bilirubin in urine three principles are involved. The first, detection by the simple spectroscope is not of much practical value clinically, for only in concentrated solution does bilirubin show even an ill-defined general absorption of the blue, and there are no absorption bands.

The second is the formation of azobilirubin in acid solution, *i.e.* an application to urine of the test described by van den Bergh for blood [Hunter, 1930]. The writer performs this test in the following way. The urine, if not naturally acid, must be acidified with acetic acid. To 10 ml. of acid urine 4 ml. of 10 % barium chloride solution are added, and the mixture is centrifuged. The supernatant fluid is poured off and the precipitate washed with about 10 ml. of distilled water. After centrifuging again and pouring off the supernatant fluid 0·5 ml. of freshly prepared diazo-reagent is added to the precipitate and well mixed. Then 2 ml. of absolute alcohol and 0·3 ml. of a 6 % solution of Na_2HPO_4, $12H_2O$ are added. If bilirubin is present, azobilirubin is formed and the mixture turns red. That the red colour is due to azobilirubin can be shown by adding 1 or 2 drops of concentrated HCl, when the colour turns to a reddish blue. Hunter recommended 5 ml. of urine and 2 ml. of 10 % barium chloride. The larger quantities were used to make the test more sensitive. It is essential in performing Hunter's test, that all the reagents be measured accurately. The method can be employed as a spot test as follows. To about 10 ml. of acid urine 5 ml. of 10 % barium chloride solution are added. The contents are mixed and filtered. After the fluid has passed through, the paper is spread on another dry piece of filter-paper, and 1 drop of diazo-reagent, 4 drops of absolute alcohol and 1 drop of Na_2HPO_4, $12H_2O$ solution are added. A red colour is obtained when bilirubin is present. This test will be referred to as the "diazo-spot test." Hunter's test and the diazo-spot test are specific for bilirubin. The spot test gives a good colour with small amounts of bilirubin, but if much is present the brown colour of unchanged bilirubin masks the red colour of the azobilirubin.

The third principle involved is the oxidation of bilirubin to green biliverdin or blue cholecyanin, avoiding over-oxidation to yellow choletelin. A great number of tests have been described depending on this principle, but in this investigation only the following have been considered, *viz.* the well-known Gmelin's nitric acid test, the iodine ring test and the modification of the Huppert-Salkowski test, described by Schippers and Steensma. In the last test to 10 ml. of urine 10 drops of 20 % Na_2CO_3 and 20 drops of 20 % $CaCl_2$ are added, mixed and filtered. The precipitate is washed with distilled water. If it is colourless the test is considered negative. If it is coloured the precipitate is dissolved in 3 ml. of alcohol containing 3–5 vols. % of concentrated hydrochloric acid. One drop of 0·5 % $NaNO_2$ is added and if bilirubin is present a green colour is obtained.

Reference has already been made to Harrison's test and Zeehuisen's method.

As described above the bilirubin from the urine can be adsorbed on the barium precipitate or on $CaCO_3$, but adsorption on $CaCO_3$ is not complete. This statement is easily verified by applying Harrison's test or the diazo-spot test to the filtrates after precipitating with barium or calcium respectively; to the

filtrate after barium precipitation a few drops of ammonium sulphate solution are added, and the tests are applied to this second precipitate; to the filtrate after calcium precipitation a few ml. of the barium chloride solution are added and ammonium sulphate solution if necessary; with small or moderate degrees of bilirubinuria positive results may be obtained with the latter but not with the former, but with intense bilirubinuria adsorption on the barium precipitate also may occasionally be incomplete.

The quantitative estimation of bilirubin in urine.

Hunter's diazo method was modified as follows:

If not already acid, the urine is slightly acidified with a few drops of 33 % acetic acid. In a series of four centrifuge-tubes are placed respectively 10·0, 5·0, 2·5 and 1 ml. of urine, followed by 0·0, 5·0, 7·5 and 9 ml. of water. To each tube are added 10 drops of 3 % acetic acid, 4 ml. of 10 % $BaCl_2$ and a few drops of saturated ammonium sulphate solution. The contents are mixed and centrifuged. The supernatant fluids are decanted. The precipitates are washed twice with 10 ml. of water to which 10 drops of 3 % acetic acid have been added. If the supernatant fluids are turbid a few drops of $BaCl_2$ and of $(NH_4)_2SO_4$ solutions are added, and the tubes are again centrifuged. Finally each precipitate is stirred with 2 ml. of absolute alcohol, 1 ml. of saturated $(NH_4)_2SO_4$ is added and the contents are very thoroughly shaken. After adding 1 ml. of 6 % $Na_2HPO_4, 12H_2O$ and 0·5 ml. of freshly prepared diazo-reagent the contents are mixed and allowed to stand for a few minutes until the colour develops completely. Then 1 ml. of chloroform is added and well mixed. After an interval of a few minutes the tubes are thoroughly centrifuged (10 minutes). The lower layer contains all or almost all the azobilirubin, provided the urine was sufficiently diluted (see below), and its volume is 2 ml. or very nearly so. It is removed with a capillary pipette, placed in a tube and its colour compared with that of standard cobalt solutions in tubes of identical bore. The unknown, if necessary, is diluted with 67 % (V/V) alcohol, but if, when comparing the unknown with the 1 unit cobalt standard, the addition of more than 2·5 volumes of the alcohol is necessary, a "chloroform extract" prepared from a smaller amount of urine must be selected; otherwise the result will not be quantitative.

The "cobalt standard" was prepared by dissolving 2·161 g. of anhydrous cobaltous sulphate in water and diluting to 100 ml.; this corresponds to 1 unit or 0·5 mg. of bilirubin per 100 ml. More dilute standards were also prepared corresponding to 0·8, 0·6, 0·5, 0·4, 0·2 and 0·1 unit.

If the 2 ml. of "chloroform extract" are prepared from A ml. of urine and the colour of the extract matches that of a cobalt standard corresponding to R units, then the original urine would contain

$$\frac{2}{A}\times R \text{ units, or } \frac{2}{A}\times R\times 0{\cdot}5=\frac{R}{A} \text{ mg. per 100 ml.}$$

Hunter's test as originally described is unsuitable for quantitative work because of the risks of incomplete conversion of the adsorbed bilirubin into azobilirubin, of incomplete extraction of azobilirubin from the barium precipitate and of interference in the colorimetric determination by other yellow pigments also adsorbed on the barium precipitate. Hunter [1930] described a modification of his own test which avoids partially the interference by other pigments, but which is not quantitative, as he himself notes. The writer is satisfied that the modifications described above have succeeded in overcoming the defects mentioned.

The relative sensitivity of the different tests.

A series of urines was tested for bilirubin by the methods previously described, the urines being diluted with normal urine to varying degrees when necessary. Finally the actual bilirubin content of the original urine was determined by the quantitative method outlined above.

The following is an illustrative protocol of the tests on one urine and on appropriate dilutions thereof.

Parts of patient's urine	50	40	30	20	10	8	6	5	4	3	2	0
Parts of normal urine	0	10	20	30	40	42	44	45	46	47	48	50
Test employed:												
Iodine ring	+	Doubtful	0	0	0	0	0	0	0	0	0	0
Nitric acid	+	+	Trace	0	0	0	0	0	0	0	0	0
Huppert-Salkowski (Schippers-Steensma)	+	+	+	Trace	0	0	0	0	0	0	0	0
Hunter's	+	+	+	+	+	+	+	Trace	0	0	0	0
Zeehuisen's	+	+	+	+	+	Trace	0	0	0	0	0	0
Harrison's	+	+	+	+	+	+	+	+	Trace	Doubtful	0	0
Diazo-spot	+	+	+	+	+	+	+	+	+	Trace	0	0

It is clear that Hunter's test and Harrison's test and the diazo-spot test are far more sensitive than the others. For practical clinical purposes Hunter's test and the diazo-spot test are too complicated, because so many reagents have to be used, and the diazo-reagent has to be prepared daily. Harrison's test is the simplest and even more sensitive than Hunter's test.

Previous investigators have tried to answer the question of the sensitivity of the tests by adding "pure" bilirubin to urine, of which several dilutions were made. Thus Schippers [1908, 1, 2] states that the Huppert-Salkowski (Schippers-Steensma) test is positive if the concentration of the bilirubin is at least 1 in 700,000. Rabinowitch [1932] states that Hunter's test is more sensitive for bilirubin when in solution in water than when dissolved in urine. In urine he was able to detect 1 in 660,000, whereas the corresponding figure in the present investigation was 1 in 10,000,000.

The purity of the preparations of bilirubin obviously influences the results quoted from the literature, but probably the improved technique utilised in the present work is the main cause of the greater sensitivity now reported.

The following table summarises the range of results obtained on a series of 30 different urines and shows the minimum amount of bilirubin which can be detected by the various methods. It therefore shows the relative sensitivity of the different tests.

Name of test	Minimum detectable mg. per 100 ml. of urine
Iodine ring	0·07 –0·3
Gmelin's nitric acid	0·04 –0·08
Huppert-Salkowski (Schippers-Steensma)	0·02 –0·04
Hunter's	0·01
Zeehuisen's	0·01 –0·02
Harrison's	0·003–0·008
Diazo-spot	0·002–0·008

SUMMARY.

1. A new and simple qualitative test for bilirubinuria is described, which should be useful clinically.

2. Hunter's diazo-test has been modified to yield quantitative results.

3. The minimum quantities of bilirubin detectable by the several methods have been determined.

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