CLXIII. AN INVESTIGATION OF PROTEIN-FREE URINES GIVING A BIURET-LIKE REACTION.

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(Received April 27th, 1935.)

OCCASIONALLY when urine is added to Benedict's qualitative or to Fehling's solution the treated copper reagent becomes a deeper blue at room temperature. If the urine is superimposed carefully a reddish-purple ring is observed; it is this reaction, and not the violet colour due to a biuret reaction with urine containing protein, that has been studied.

When the fresh urines of healthy and diseased persons are examined carefully by this ring test, it is found that a large minority of both groups give positive reactions of varying intensity. It is important that the urine shall be relatively fresh, for the reaction diminishes in intensity in a few hours and in most cases becomes negative overnight.

This, though not generally known, is an old observation; Ralfe [1883] stated that the urine should be recently passed, for if kept overnight the reaction became negative, a fact which he suggested might be due to putrefactive changes. Ralfe and the older workers of course used Fehling's reagent which tends to give a slightly less marked test than Benedict's solution, but otherwise it is immaterial which of the two is employed; both copper reagents have been used in the present work, but Benedict's much the more frequently. This being understood, for convenience in subsequent description the test will be referred to as the "cold Benedict reaction".

For a long time peptone was believed to be the cause of the urinary "biuret test"; it will be shown that the substance cannot be peptone, and that, as Salkowski [1897] and Stokvis [1897] clearly recognised, the reaction should not be mistaken for a biuret test. The alternative suggestion of the above investigators, that urobilin was responsible, likewise cannot be supported.

Preliminary observations on the "cold Benedict reaction".

That a positive test cannot be due to peptone is shown by the following observations. When a positive urine is heated at 100° for 5 minutes and cooled, the reaction becomes negative. If now a few drops of tincture of iodine are added a reddish-purple ring does appear (see below under "Dybuc test"). Saturating the urine with ammonium sulphate does not precipitate the substance responsible for the above tests.

If the biuret test is performed in the ordinary way (*e.g.* 3 parts urine, 1 part 40 % NaOH and 1 % CuSO₄ drop by drop) it is negative; only when a weaker alkali is employed is the reaction positive (see later). In other words the reaction is not a biuret test in the sense that this is employed for testing for proteins, proteoses or peptones.

That a positive test cannot be due to urobilin is shown by the following observations. Many urines giving positive cold Benedict reactions do not contain urobilin as judged from spectroscopical examination and Schlesinger's test, and conversely many urines which do contain urobilin give negative cold Benedict

reactions. The reddish purple colour of the developed reaction is not extracted by amyl alcohol, benzene, carbon tetrachloride, chloroform, ether, ethyl acetate, toluene or xylene; spectroscopically the solution shows an ill-defined absorption of the green stretching from about its centre up to the E line and not a definite band in the position of that of the copper-urobilin compound. Many positively reacting urines give a negative result with Ehrlich's reagent (*p*-dimethylamino-benzaldehyde) for urobilinogen.

Positive cold Benedict reactions were obtained in some urines of both sexes, of both children and adults, in health and in a miscellaneous group of diseases. Positive results were not obtained in the urines of rabbits, guinea-pigs, rats or cats, though animals' urines have not been extensively studied.

No parallelism was established between the intensity of urinary tests for indican, or uroerythrin and the cold Benedict reaction. Solutions of pure creatine in water gave negative results.

The impression was gained that urine which has been stored in the urinary bladder for a short time only gives stronger reactions than urine which has been stored there for relatively long periods. One of the assistants in the laboratory usually passed urine giving a marked reaction; a specimen collected over a period of say 15 minutes gave a far stronger reaction than one collected over a period of say 2-3 hours; the effect of polyuria due to drinking and the possible influence of meals were carefully excluded.

Substitution of CuSO_4 and Na_2CO_3 solutions for the copper reagent.

The initial observations were made with Fehling's or Benedict's solutions; it was clearly desirable to simplify the reagents essential for the colour development. Neither copper sulphate solutions alone nor alkalis alone produced the colour on addition to the urine; both were necessary, and the colour development was better if the CuSO_4 were added before the alkali. After many experiments the following proportions appeared to be about optimum, *viz.* urine 5 ml., 1 % crystalline CuSO_4 1 ml. and 5 % anhydrous Na_2CO_3 1 ml. On mixing these in the order given a rose or purplish red colour develops in a few seconds. If ammonia solution (sp. gr. 0.880) is employed as alkali, the colour is a deeper blue, but care has to be taken to avoid excess of ammonia. NaOH or KOH likewise can be substituted, but even greater care has to be taken to avoid excess, otherwise, as previously mentioned, the characteristic colour change does not occur at all.

Relation of "cold Benedict reaction" to "Dybbuc test".

Whilst working in Prof. Snapper's clinic in Amsterdam the following reaction was frequently performed by several of the pupils, including the writer, during routine chemical examination of urines. The cause of the reaction was not known, and the test was nicknamed "Dybbuc" (Hebrew for ghost or evil spirit). To about 5 ml. of urine 1 drop of ammonia solution (sp. gr. 0.880) is added, followed by tincture of iodine drop by drop until the colour is definitely reddish. One half treated with a solution of a zinc salt (*e.g.* 1 drop of 1 % ZnSO_4) becomes slightly more red. The other half treated with dilute CuSO_4 solution (*e.g.* 1 drop of 5 %) becomes reddish violet.

It naturally occurred to the writer that possibly the substance responsible for the cold Benedict reaction was the same as, or closely related to, the substance responsible for the "Dybbuc" test. Stokvis [1897] observed that the addition of iodine improved the colour obtained by treating urine with dilute solutions of KOH and CuSO_4 .

In several hundred tests on the urines of more than 20 individuals a positive cold Benedict reaction has always been accompanied by a positive Dybbuc test. When urine which, initially positive, gives a negative cold Benedict reaction owing to keeping, heating at 100° , treatment with a small proportion of adsorbent charcoal or saturation with ammonium sulphate, it nevertheless still gives a positive "Dybbuc" test. On the other hand the cold Benedict reaction can again be made positive by adding a little iodine solution either before or after the addition of the urine to the Benedict's solution. This suggests that the substance responsible for the cold Benedict reaction may be a precursor of the substance responsible for the "Dybbuc" test.

In the hope of deciding whether the iodine merely causes oxidation other oxidising agents were substituted. Negative results were obtained employing in varying proportions solutions of each of the following: H_2O_2 , NaNO_2 , KMnO_4 , KClO_3 , FeCl_3 and Cl_2 . Positive results could be obtained, but with less ease than with iodine, by using bromine water or potassium persulphate. It therefore looks as though it is not purely a question of simple oxidation.

The three tests, the cold Benedict reaction, treatment of the urine with CuSO_4 and Na_2CO_3 , and the "Dybbuc" test were all used at the different stages in the subsequent attempt to decide the nature of the substance responsible for the colour development. The pigment is essentially red when extracted, the purple or violet tints in tests on urine being due to excess of CuSO_4 . Further it is believed that the red pigments of the three tests are the same or closely allied; certainly in each case the same ill-defined absorption of the half of the green nearest the blue end of the spectrum is observed, and the red colour is unstable, fading slowly on standing, immediately upon acidifying or heating. The red pigment can be separated and concentrated by saturating the urine with ammonium sulphate and extracting with ethyl alcohol (e.g. 1/5 volume).

Further observations on the substance, X, responsible for the above tests.

X is destroyed by strong acids, and by H_2S in a few minutes. Strong alkali destroys it slowly, especially on heating. The other properties observed for X closely resemble those reported in the literature for substances in the urochrome group and may be summarised as follows.

(1) X is not extracted from urine by amyl alcohol, benzene, chloroform, ether or light petroleum.

(2) When a positively reacting urine is concentrated *in vacuo* below 50° to a syrup, both urochrome and X are extracted by ethyl alcohol. X therefore belongs to the "Extraktivstoffe" of Berzelius [Dombrowski, 1907-08, 1, 2].

(3) X is not adsorbed on the precipitate formed by treating urine with lead acetate at an acid reaction. Urochrome behaves similarly [Weisz, 1911; 1920, 1, 2; 1922; 1923; 1930]. $(\text{NH}_4)_2\text{SO}_4$, Na_2CO_3 or NaCl should be used for subsequent removal of the lead before testing the filtrate for X. The main bulk of the filtrate is precipitated by NH_4OH or NaOH because both urochrome [Weisz, 1920, 1, 2; 1930] and X are adsorbed on the alkaline lead precipitate. Both may be separated from the precipitate by adding the latter to a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, followed by alcohol, when they pass into the alcohol layer; the strong alkali however soon destroys X.

(4) Saturation of the urine with ammonium sulphate precipitates urobilin which is removed by filtration. On treating the filtrate with ethyl alcohol urochrome is extracted [Garrod, 1894] and likewise X. The alcoholic extract is washed with saturated ammonium sulphate and evaporated to dryness *in vacuo*.

On extracting the residue with ethyl alcohol both urochrome [Garrod, 1894] and X are removed.

(5) Addition of mercuric acetate to the urine gives a precipitate on which urochrome [Thudichum, 1864; Garrod, 1894] and X are adsorbed. Both may be removed from the mercury precipitate in the same way as from the $\text{Pb}(\text{OH})_2$ precipitate (see above).

(6) The urine is concentrated to a small volume *in vacuo* and dialysed. Neither urochrome [Fischer and Zerweck, 1924] nor X passes the membrane.

(7) Saturation with $(\text{NH}_4)_2\text{SO}_4$ followed by extraction with butyl alcohol removes urochrome [Drabkin, 1930, 1, 2] and X.

(8) Amyl alcohol extraction of acid urine removes substances of the urobilin group, but neither urochrome [Weisz, 1930] nor X.

(9) Butyl alcohol extracts urochrome [Drabkin, 1930, 1, 2] and X from acidified fresh urine. Drabkin washed his butyl alcoholic extracts and concentrated them *in vacuo*. In the writer's opinion this changes the nature of the urochrome for the following reasons: on standing the colour of the concentrated extract deepens considerably as Drabkin also noted; the final pigment appears to be no longer soluble in water but may be made so by simple washing with ether, which however must be carefully done for, as noted also by Drabkin, too long a contact with ether results in changes in the solubility of the pigment. The writer is doubtful whether unchanged urochrome is soluble in anhydrous butyl alcohol, though "urochrome" prepared by Drabkin's method is.

Butyl alcoholic extracts of X similarly washed and concentrated *in vacuo* yield material which no longer gives the tests for X.

(10) Alcoholic solutions of urochrome, prepared as described in either (2) or (4), yield a precipitate of pigment on addition of ether, but this "urochrome" precipitate has changed chemically for it is either insoluble or only partially soluble in ethyl alcohol [Garrod, 1894]. Likewise alcoholic solutions of X, similarly prepared, after treatment with ether no longer give the tests for X; neither the precipitate, nor the filtrate nor the two combined give a positive reaction.

(11) Charcoal adsorbs urochrome from urine [Hohlweg, 1908; Salomonsen, 1908; Mancini, 1908; Klemperer, 1903; 1904] and also X. Both can be extracted from the charcoal by 10 % NH_4OH in 50 % ethyl alcohol; mere traces are extracted by ethyl alcohol or methyl alcohol alone. Alternate washings with large volumes of acetone and of water remove urochrome partially and X completely.

Attempts to isolate X.

After the above study of its properties, the following procedure was employed in attempts to isolate X. The quantities of reagents given below are for 1000 ml. portions of urine.

The acid urine was treated with 250 ml. of saturated normal lead acetate and filtered. Lead was removed from the filtrate by excess of saturated ammonium sulphate. The second filtrate was shaken with about 50 g. of charcoal for 5 minutes. The precipitate was collected on a Büchner funnel and washed with large volumes of distilled water (filtrate and washings contained no X) and was then washed alternately five times with about 100 ml. portions each of acetone and of water, about 1000 ml. in all. The aqueous acetone solution was refiltered and concentrated to a small volume (about 20 ml.) below 40° under reduced pressure. The concentrate was submitted to one of the two following procedures.

(a) When the concentrate was submitted to chromatographic analysis in a column of CaCO_3 , adsorption of pigment occurred in one layer only, and this

layer gave strong reactions for X. In a series of experiments the deeper the pigmentation the stronger were these reactions. BaCO_3 and kaolin give less satisfactory adsorption.

(b) The concentrate was taken to dryness in a vacuum desiccator over CaCl_2 . The yellow-brown residue was repeatedly submitted to prolonged extraction with anhydrous butyl alcohol in which a portion slowly dissolved. The butyl alcoholic extract gave with water, NH_4OH and iodine a beautiful red colour, and the usual subsequent colour changes with zinc and copper salts. The insoluble residue on solution in water gave negative tests for X. All attempts to concentrate the butyl alcoholic extract, or to separate X from it, resulted in partial or complete destruction of X.

The above procedure has been carried out on more than ten occasions. It has also been tried on urines which initially gave negative tests for X, when the final butyl alcoholic extract, if faintly yellow gave definite but weak reactions for X, but if colourless gave negative reactions.

It looks therefore as though X is either a pigment of the urochrome group or a substance associated with urochromes throughout the process of separation.

Opinions differ as to whether urochrome is a chemical entity or embraces a group of unknown substances [Weisz, 1920, 1, 2]. In the literature a number of pigments have been described which are supposed to be identical with urochrome because they are not precipitated by saturation with ammonium sulphate, and after such treatment are extracted from the filtrate by ethyl alcohol. The author has prepared "urochrome" from faeces [Garrod, 1903], "lactochrome" from milk [Palmer and Coolidge, 1914] and "protochrome" from peptone [Pelkan, 1920]. All these preparations gave negative tests for X.

SUMMARY.

1. The biuret-like reaction in protein-free urines which for many years has been regarded as due either to peptones or to urobilin has been further studied.
2. In agreement with certain authorities it is recognised that the reaction is not a biuret test in the usual sense.
3. It is shown that the reaction is not due to peptones or urobilin.
4. It is concluded that the substance responsible for the reaction is either a pigment of the urochrome group, or is associated with urochromes throughout the processes of separation.

I am greatly indebted to Dr G. A. Harrison for suggesting this investigation in which he himself had made a few preliminary observations, for the facilities provided in his laboratory and for his help in writing this paper. The work was undertaken with the aid of the Prof. Stokvis' funds, University of Amsterdam, and with a grant from the Cassel Foundation.

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