

## CCCXXII. THE APPLICATION TO URINE OF TOLLENS'S NAPHTHORESORCINOL TEST FOR CONJUGATED GLUCURONIDES.

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It is well known that the administration of certain drugs, notably those containing an actual or potential hydroxyl group, results in an increase in the quantity of glucuronic acid excreted in the urine. The increase is due to the formation of a conjugated compound of the drug and glucuronic acid and represents a process of detoxication which is attributed to the liver. Quick [1927] pointed out that we had no direct evidence that the liver is the organ responsible for this detoxication but proof has now been furnished by Hemingway *et al.* [1934]. When the liver is seriously disordered, its failure to eliminate such drugs as conjugation compounds has been made the basis of a test for the detoxicatory efficiency of the liver [Beaumont and Dodds, 1931]. The liver test involves the application of Tollens's naphthoresorcinol reaction to urine and many variations of this have been described in the literature. Tollens's reaction consists essentially in heating glucuronic acid or a conjugated glucuronide with naphthoresorcinol and hydrochloric acid, a blue substance being formed giving a blue-violet solution in ether.

*Discussion of Tollens's reaction applied to urine.* The test, when applied directly to urine, has been found by many to be unsatisfactory owing to the presence of interfering substances. The writer has invariably found a deep red colour, masking any blue or violet, when testing many urine samples. In order to remove the interfering substances, different methods of preliminary treatment of the urine have been proposed.

The use of mercuric acetate described by Roger [1916] does not remove glucose, the presence of which may inhibit the test [Brule *et al.*, 1925; Roger, 1916]. Hence, this method is not applicable to glycosuric urine. Furthermore, when used by the writer, even with urine collected after ingestion of aspirin and free from sugar, a most unsatisfactory red colour was always obtained, making any interpretation impossible.

The urine test described by Beaumont and Dodds [1931] has been found to be quite unsatisfactory. According to Tollens [1914], the addition of basic lead acetate to urine results in complete precipitation of conjugated glucuronides, a fact confirmed by present experiments. It is therefore useless to test the filtrate for the presence of such. Like the mercuric acetate filtrate, the basic lead acetate precipitate yielded an unsatisfactory red colour when Tollens's test was applied to it. Precipitation of urine with zinc acetate and testing both filtrate and precipitate also failed to give a satisfactory test.

Attention was then turned to methods of ether extraction as recommended by Neuberg and Schewket [1912]. However, using urine collected after ingestion of aspirin, the conjugated compound was found to be insoluble in ether. This was confirmed by extracting "aspirin urine" with ether in a Clausen extractor as described by Quick [1924]. The ether extract gave a negative Tollens's test (colourless).

It may be mentioned here that throughout this work aspirin has been used exclusively as a glucuronogenic drug. The conjugated compound found in the urine is perhaps an unusual one, as Quick [1932] has obtained evidence that salicylic acid undergoes double conjugation with glucuronic acid, there being formed one glucoside linkage and one ester linkage.

A new method of treatment was then sought. The principle was suggested by the method of separating glucuronic acid monobenzoate described by Quick [1926]. The interfering substances are first removed from urine by precipitating with lead acetate in slightly acid solution and centrifuging. The glucuronic acid compound is then completely separated from the supernatant fluid by neutralising and precipitating with basic lead acetate. Tollens's reaction is applied to the basic lead acetate precipitate.

By this method glucose is separated, as it is not precipitated by basic lead acetate. Pentoses cannot interfere, as even if precipitated with the glucuronic acid compound, the colour which they give with naphthoresorcinol is insoluble in ether [Tollens, 1914]. The final extraction with ether yields a blue-violet colour, the depth of which is roughly proportional to the amount of glucuronic acid present. The replacement of ether by chloroform or benzene suggested by Neuberg and Saneyoshi [1912] was found to be unsatisfactory.

The actual technique now recommended for the urine test was developed from the following results, applying Tollens's reaction to the different fractions of urine collected for 12 hours after aspirin ingestion.

Table I.

Test fraction		Result of test
Urine untreated	—	Deep red
Urine <i>plus</i> excess lead acetate	Precipitate Supernatant	Pale violet Deep red
Urine <i>plus</i> excess basic lead acetate	Precipitate Supernatant	Deep red Pale red
Urine <i>plus</i> just sufficient lead acetate	Precipitate Supernatant (*)	Colourless Deep red
Supernatant from above (*) <i>plus</i> excess basic lead acetate	Precipitate Supernatant	Deep violet Red-brown

It is seen that both untreated urine and the basic lead acetate precipitate from urine fail to give a satisfactory test. An excess of lead acetate precipitates a little glucuronide, but just sufficient lead acetate avoids this and removes certain interfering substances. The supernatant fluid from this first lead treatment still contains interfering substances, but treatment with basic lead acetate yields a precipitate containing all the glucuronide and giving a satisfactory colour test, whilst the interfering substances remain in the supernatant.

A similar series of results was obtained when 5% glucose was added to the urine, except that the last supernatant yielded a deeper red-brown colour.

#### THE TECHNIQUE OF THE URINE TEST.

##### *Solutions required:*

33% acetic acid.

5% lead acetate.

Approximately *N* sodium hydroxide.

10% basic lead acetate prepared by heating 20 g. tribasic lead acetate with 200 ml. water to boiling. After boiling and stirring for a few minutes, the

solution is allowed to cool and then filtered. A large amount of insoluble matter is discarded.

Hydrochloric acid, 50 % by volume.

1 % pure naphthoresorcinol in absolute alcohol.

*Procedure.* The urine is mixed and rendered slightly acid with acetic acid. A 5 ml. portion is pipetted into each of four 10 ml. capacity centrifuge-tubes. To the tubes respectively, 0.25, 0.5, 0.75 and 1.0 ml. lead acetate are added, the contents mixed and centrifuged. To each, one drop lead acetate is added and the tube selected in which precipitation is seen to be just complete. If no tube is completely precipitated, a further 1.0 ml. lead acetate is added to each and the procedure repeated. Complete precipitation is thus effected but an appreciable excess of lead acetate is avoided. In some cases it may be necessary to add a further 1.0 ml. lead acetate to each and to repeat the procedure again.

The supernatant fluid is poured from the selected tube into another centrifuge-tube and sodium hydroxide added dropwise until the first permanent precipitate of lead hydroxide is observed. Then 3.0 ml. basic lead acetate are added, the contents mixed and centrifuged. To ensure complete precipitation, one drop more of the reagent is added and if necessary a further quantity of basic lead acetate is added and the process is repeated until an excess is present and precipitation is complete. The supernatant fluid is poured away and the deposit washed on the centrifuge by thoroughly stirring with 5 ml. water and separating.

The precipitate is transferred to a large test-tube using two 5 ml. portions of dilute hydrochloric acid (1 : 1) from a pipette. 0.5 ml. naphthoresorcinol solution is added and mixed. The tube is heated in boiling water for 5 min., then cooled in running water, 10 ml. ether are added, well shaken and allowed to separate. The ether layer is removed to a clean test-tube and examined by transmitted white light. The colour of the ether solution is noted at once, as the colour fades on standing.

*Results of the test applied to urine.* •

The test described was applied to a number of urine specimens and the results are summarised in Table II.

Table II.

	No. of specimens	Result
Normal urine	10	Faint pink to pale violet
Urine after aspirin ingestion	7	Pale violet to deep violet
Blank with reagents	—	Colourless

It was found that the drug is almost wholly eliminated by normal persons in 12 hours after administration.

Attempts were then made to apply the test quantitatively. For this purpose calcium glucuronate was prepared by the method of Kiliani [1921] and 0.1098 g. dissolved in 100 ml. water, so that 1 ml. contained 1 mg. glucuronic acid. Aliquot portions of this solution were heated with naphthoresorcinol and hydrochloric acid to determine the limit of sensitivity of the test and the colour proportionality.

When read in the colorimeter, exact proportionality was not found, though the colour intensity was roughly proportional to the amount of glucuronic acid present.

Table III.

Glucuronic acid mg.	Colour produced
2.0	Very deep blue
1.0	Deep blue
0.5	Pale blue
0.1	Colourless

Aliquot portions of the calcium glucuronate solution were then added to 5 ml. portions of normal urine and the complete test including double lead precipitation carried out.

Table IV.

Glucuronic acid added to 5 ml. urine mg.	Colour produced
1.0	Deep violet
0.75	Violet
0.5	Very pale violet
0.25	Brownish
Nil	Faint pink

It is seen that the test is sensitive to 0.5 mg. glucuronic acid present in 5 ml. urine. The colours did not match well with those obtained from a solution of pure calcium glucuronate. This, together with the lack of true colour proportionality and the unavoidable errors involved in ether extraction, rendered attempts at a quantitative method unprofitable. However, an estimate of the amount of glucuronic acid present in a sample of urine may be made by testing different decreasing amounts of urine until the quantity which just gives a positive test is determined; this quantity of urine will contain about 0.5 mg. glucuronic acid.

It is suggested that this test applied to 24-hourly urine specimens would yield an index of the excretion of naturally formed phenolic toxic substances. Obviously all drugs capable of conjugating with glucuronic acid must be eliminated at the time of the test. The reaction would then indicate the extent of excretion of conjugated compounds of phenol, cresol, indole and skatole and might therefore suggest the extent of absorption of intestinal putrefaction products of this nature, provided that the liver were not seriously disordered. A number of 24-hourly normal specimens have been tested and found to give negative or only faintly positive results, using 5 ml. test portions.

*The test for the detoxicatory efficiency of the liver.*

The principle of the test is the detection of an increase in the amount of conjugated glucuronide excreted after the administration of some toxic drug. Many drugs have been used by various workers, *e.g.* aspirin, sodium salicylate, naphthol, chloral hydrate or camphor by the mouth, or camphor in olive oil injected. Of these aspirin was chosen, as its use is attended with least ill effects. It was found to be quite satisfactory in producing an increase in glucuronide excretion in normal subjects. A dose of 15 grains by the mouth was used.

Urine is collected during the day for 12 hours before the aspirin is given and preserved with a little toluene. The drug is then administered and a further 12 hours' urine collected during the night. The volumes are noted and the naphthoresorcinol test is carried out simultaneously on the two specimens. If the liver has responded to the drug, a much more intense violet colour will be given by the second urine specimen than by the first.

It has been stated by Brule *et al.* [1925] that the presence of urotropin in the urine inhibits the reaction. Thus all other drugs should be excluded during the test.

Much evidence has previously been put forward that a seriously disordered liver fails to carry out its detoxicating function by conjugating toxic substances with glucuronic acid. However, it appears that the naphthoresorcinol test has hitherto been not altogether reliable. It is suggested therefore that the liver test should be re-investigated in cases of liver disease, especially since recent experiments have definitely shown that the liver is responsible for such detoxicatory reactions.

Liver test results obtained from several normal subjects by the method described have shown a prompt and definite increase in the urinary glucuronic acid, following aspirin ingestion.

#### SUMMARY.

Methods are discussed for the application to urine of Tollens's naphthoresorcinol test for conjugated glucuronides. An improved technique for the urine test is described. It is suggested that the test might be used to indicate increased absorption of intestinal putrefaction products. Using aspirin as a glucuronogenic drug, a method of testing the detoxicatory efficiency of the liver is given.

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