The Detection of Galactose in Urine

By F. S. FOWWEATHER

Department of Chemical Pathology, University of Leeds

(Received 22 May 1953)

The recognition of the condition known as galactosaemia or chronic galactosuria which occurs mainly in infants and young children has directed attention to the detection of galactose in urine. Though the condition is rare, an increasing number of cases is being reported, and it is advisable to consider the possibility of its occurrence whenever an illness in an infant is accompanied by the presence of a reducing substance in the urine. Diabetes mellitus, though also rare in infants, must of course also be considered, and therefore any test for galactose must be able to differentiate this sugar quite clearly from glucose.

The use of paper chromatography in the investigation of galactosaemia has been described by Bickel & Hickmans (1952). The glucose and galactose spots on the chromatogram however are very close together, and some experience is needed before galactose can be identified with certainty by this method. Yet the need to decide if a reducing substance found in urine is galactose may arise in a hospital laboratory where the necessary experience or equipment for chromatographic examination is not available. Hence it is desirable to have a reasonably simple, reliable chemical test for the recognition of galactose in urine.

The mucic acid test is positive for lactose and galactose but the usual description of the test recommends the use of 100 ml of the urine under examination. Many of the patients with galactosaemia are infants of not more than a few weeks, and though positive results can be obtained on much less than 100 ml of urine, it is impossible to get satisfactory results on the specimens obtainable from these infants. The concentration of galactose in the urine is not usually large, and often does not exceed 0·5%. This low concentration, as well as small volume, make the application of certain other tests difficult. Recently, when dealing with a case of galactosaemia, my attention was drawn to the work of van der Haar (1917), who claimed that, of seven sugars tested, d-galactose was the only sugar that formed an insoluble c-tolylhydrazone. This fact seemed worth investigation as a possible basis for a test for galactose in urine. The Tollens phloroglucinol reaction is also recommended by certain text-books (Duncan, 1947; Hawk, Oser & Summerson, 1947) as a test for galactosuria, and this test was also examined.

EXPERIMENTAL

(1) The Tollens phloroglucinol reaction. This reaction was not originally put forward as a test for galactose, but for pentoses. As first described by Wheeler & Tollens (1889), it consists in heating the liquid to be tested with an equal vol. of HCl (sp.gr. 1·19) and a small quantity of phloroglucinol. In the presence of pentose (or of glucuronic acid) a beautiful cherry-red colour appears, followed soon by turbidity and precipitate formation. It is clearly stated that galactose and dextrose give no specific colour. Later, Tollens (1896) dealt with the reaction in greater detail, and showed that the cherry-red colour shows definite absorption bands. If the precipitate which forms on cooling and standing is filtered and washed with water and then treated with ethanol, it dissolves to give a violet-red solution which shows the pentose absorption bands as before. According to Tollens, galactose under the same conditions gives first a yellow-brown colour, and glucose a similar one, and the precipitate which subsequently forms is dark violet in the case of galactose, dissolving in ethanol to a red-brown colour showing no absorption band, and yellowish red in the case of glucose dissolving in ethanol to a yellow-red colour which darkens and shows no band. Tollens points out that while detection of pentose in pure solution by means of this reaction is easy, the task so far as urine is concerned is more difficult.

The Tollens test, as described in some text-books, consists in heating equal vol. of HCl and the solution to be tested, to which a little phloroglucinol has been added, in the water bath. The appearance of a red colour indicates galactose, pentose or glucuronic acid. Galactose is differentiated from the other two substances by the fact that its solution in this test shows no absorption bands.

The discrepancy between the description of the reaction by Tollens, and that of some later writers, so far as galactose is concerned needs to be clarified if the test is to be of any value in the investigation of galactosaemia, and experiments were undertaken for this purpose. These experiments have shown that when the Tollens reaction is carried out on pure aqueous solutions of xylose, galactose and glucose, in 2% concentration, a bright cherry-red colour is obtained with xylose and a deep red-brown colour with galactose and glucose, the colour developing much more quickly in the case of galactose than glucose. Ultimately all three show turbidity, followed by definite precipitation, and in the case of glucose the precipitate appears almost before the red-brown colour has clearly shown itself. On filtering and washing the precipitate the galactose precipitate has a more definitely purple colour than the glucose precipitate, but the difference is not great. Both precipitates dissolve in ethanol to give similar deep brownish red solutions.

When the reaction is carried out on solutions of the sugars in urine the results are as follows.
(a) Sugars in 0·5% concentration. On first heating all solutions become orange. The one containing xylose quickly deepens to red, but not quite the bright cherry colour seen with pure solutions. The galactose solution deepens less rapidly to reddish-brown, while glucose solutions still less rapidly to yellowish-brown. The washed precipitate from the xylose solution is purple, that from the galactose purplish, and from glucose, lighter in colour than that from galactose and less in amount.

(b) Sugars in 1% concentration. All first become orange-red. The xylose solution quickly becomes bright red and then dark red and soon the precipitate appears. The galactose and glucose solutions become red-brown, the galactose more rapidly than the glucose. The washed precipitates are similar to those in the previous experiment.

(c) Sugars in 2% concentration. All quickly become orange-red. The xylose solution changes quickly to red at the top and this colour spreads rapidly downwards, and deepens to a port-wine colour before becoming opaque. The galactose and glucose solutions deepen to dark red-brown, the glucose solution as before changing more slowly than the galactose. Soon xylose and galactose solutions look very similar and the glucose solution closely resembles them a little later. The washed precipitate from the xylose solution is almost black and in coarse lumps; that from galactose is very dark purple, and from glucose, a slightly lighter purple. The xylose precipitate dissolves in ethanol to give a purple solution, while the other precipitates give deep red-brown solutions; the glucose precipitate appears to be more soluble than the galactose precipitate.

In general, the colour produced when the Tollens reaction is performed on glucose in urine appears more slowly and in the lower concentrations can be seen to be less deep in shade than that produced from galactose in urine in the same concentration. If, however, the reaction is performed on a urine containing glucose, and also on a urine containing galactose in a lower concentration, the results may well be identical. The Tollens reaction is therefore of no value in differentiating galactose from glucose in urine if carried out on single specimens. To anyone with experience of the test a correct decision as to whether a given urine contains glucose or galactose will often be possible if the amount of sugar in the urine under examination is first determined and the Tollens reaction then carried out simultaneously on this urine and two controls consisting of sugar-free urine of very similar colour to the test specimen, to one part of which is added galactose, and to the other glucose, so as to give approximately the same sugar concentration as in the test specimen.

(2) The o-tolylhydrazone of D-galactose. If, as claimed by van der Haar, D-galactose is the only sugar to form an insoluble o-tolylhydrazone, it seems at first sight strange that this reaction has not been used as a basis for the identification of galactose in urine. The instructions given by van der Haar for the preparation of the hydrazone are as follows:

Heat on the water-bath for 30 min. a solution of 1 part of D-galactose in 1 part of water, with 1 part of o-tolylhydrazine in 20 parts of ethanol. The hydrazone which separates is dried at the pump after 24 hr., washed with ethanol and ether, and then recrystallized from 95% ethanol. After successive washings with ethanol, water, ethanol and ether, it is dried, and then has m.p. 176°. This suggests that the galactose should be in high concentration; it utilizes o-tolylhydrazine base which is not easy to obtain, is easily decomposed on exposure to air and needs to be kept in a sealed vessel; and the hydrazone after recrystallization is finally identified by its melting point. All these features are sufficiently good reasons why the preparation has been neglected as a test for galactose in urine.

Accordingly, experiments were made to see if the hydrazone could be prepared from dilute solutions of galactose using o-tolylhydrazine hydrochloride, which is readily available and keeps indefinitely, and if the identity of the hydrazone could be assumed without the necessity for purification and determining the melting point. Experiments on aqueous solutions showed:

(i) o-Tolylhydrazine hydrochloride and sodium acetate can be used successfully instead of the free base, and the quantity of sodium acetate can be varied within wide limits.

(ii) The best results are obtained when the hydrochloride and galactose are present in equal amounts by weight.

(iii) Using 1 ml. galactose solution, the appropriate amount of hydrazine hydrochloride in 5% solution and 10 ml. ethanol, spontaneous crystallization of hydrazone occurs after heating and evaporating off the ethanol, cooling the residue and adding one drop of cold water, when the galactose solution contains 1% or more of the sugar.

When the galactose is in 0·25–0·5% concentration, slow crystallization, seen through the microscope, can be seen when a drop of the residual liquid is poured on to a microscope slide. The crystals appear as rounded yellow spots with needles projecting all round the edge (‘cartwheels’).

Experiments were next made with galactose in urine. With 1% galactose, crystallization occurs almost immediately in some cases; in others it is observed a few minutes after a drop of the residual fluid is placed on a microscope slide. In a specimen of urine containing 0·8% galactose obtained during the performance of a galactose tolerance test, crystals appeared about 5 min. after putting a drop of residual fluid on a microscope slide, but in general it is felt best not to use concentrations below 1%.

The nature of the crystals was confirmed by carrying out the preparation on 10 ml. of urine containing 0·5g. galactose, using the same technique. This gave ample material for recrystallization according to van der Haar’s instructions, and the purified product had m.p. 174–175°.

Results with other sugars in urine. In the concentrations used, spontaneous crystallization on cooling the residue and adding a drop of cold water did not occur. A drop of the residual fluid was placed on a microscope slide without cover slip, to allow spontaneous evaporation of any residual ethanol. After observation under low power for 5–10 min. a cover slip was added, and examination made under high power where necessary.

2% glucose. Many globules; some needles were beginning to appear after 45 min. from placing coverslip. As they increased during the next 2–3 hr. they were seen to be long and somewhat loosely arranged in sheaves.

2% fructose. Some globules; needles began to appear about 20 min. after placing cover-slip, and slowly increased afterwards.

2% lactose. A few needle-like crystals seen within globules after about 45 min. After 3 hr., one group of atypical needles was seen.

2% D-xylene, D-arabinose, L-arabinose. Many globules, but no needles after being on slide for over 5 hr.; some crystals of sodium acetate appeared. Under the same conditions, with 2% galactose, there is immediate formation of
a yellow precipitate when the drop of water is added to the residue after evaporation.

1% glucose. A few groups of crystals, long and feathery, were seen after 4 hr.

1% fructose. A few long needle crystals seen in about 45 min.; these are not typical sheaves nor 'cartwheels'.

1% lactose. No crystals seen after 4 hr. In addition to galactose, therefore, fructose, and to a less extent, glucose, can give needle crystals under the conditions described, but the crystals are different in form, and much more slowly formed than those from galactose.

To determine the nature of the crystals from glucose and fructose, 0.5 g. of each sugar in 2.5 ml. water was heated with 0.5 g. of the hydrazine hydrochloride, sodium acetate and 2.5 ml. ethanol. The ethanol was evaporated off as usual and some crystallization soon occurred, and was increased on adding water. The crystals were filtered, washed with ethanol and water, and then recrystallized from ethanol and water, and washed with water, ethanol and ether, and dried. Their melting point in each case was 202° which is the melting point of the o-tolyllosazone.

The experiments confirm van der Haar's claim that of the sugars examined only galactose gives an insoluble hydrazone. This is readily obtained from relatively low concentrations of galactose in urine. Fructose and somewhat more slowly, glucose, under similar conditions, will give some crystals of the osazone, but these are different in form and slower in appearing than the galactose hydrazone crystals, and the appearance of crystals as described, within a short time of the completion of the preparation is a good indication of the presence of galactose in the urine examined.

**Recommended procedure for the hydrazone test.** Determine the concentration of the sugar in the urine (e.g. by a micro modification of the quantitative Benedict method). If the concentration is less than 1%, concentrate the urine by evaporation until 1% concentration is reached. Place 1 ml. of urine in a test tube (150 x 16 mm.), add 0.2 ml. of a freshly prepared 5% solution of the hydrazine hydrochloride. Add a few small crystals of sodium acetate (20-30 mg.). Mix well to dissolve the acetate and then add 10 ml. of ethanol. The mixture becomes opalescent. Place the test tube in a water bath at 90° and 100°. The opalescence quickly changes to a flocculent precipitate which is then filtered off while hot into a test tube containing one or two small pieces of porous pot. Put the tube in water at about 90°. When nearly all the ethanol has evaporated, raise the water to boiling to complete the evaporation, and then cool the small quantity of liquid that remains in cold running water. Add one drop of distilled water, mix and pour a drop on to a microscope slide.

If galactose is present in concentration appreciably greater than 1%, crystals of the hydrazone will already have been formed, and be visible under the microscope as radiating needles. If the concentration does not much exceed 1% crystals may not form immediately; the drop may be observed under low power without cover slip for a few minutes and crystals may be seen to form near the edge of the drop. After 2-3 min. put on a cover slip. Soon opaque yellow spots or streaks will be visible to the naked eye and under the microscope these will be seen to consist of hydrazone crystals in 'cartwheel' arrangement (tightly packed circles with edges of needles just visible round the rim).

If the sugar is glucose, opaque yellow spots or streaks will not form; after 2-3 hr. needle crystals of osazone may be seen, but these will be longer and in looser arrangement than the galactose hydrazone, and generally have a feathery appearance.

Fructose will also yield osazone crystals, usually more rapidly than glucose.

## SUMMARY

1. In view of the need for a simple test for the detection of galactose in urine, when investigating possible cases of galactosaemia or chronic galactosaemia, the Tollens phloroglucinol reaction, and the claim by van der Haar that galactose is the only sugar which gives an insoluble hydrazone with o-tolylhydrazine have been examined.

2. The Tollens reaction, as described in many text-books, is of no value as a test for galactose in urine, as the results given by glucose are very similar; the test may have some value if carried out with appropriate controls.

3. It has been shown that of the sugars likely to be found in urine, galactose is the only one which gives an insoluble hydrazone with o-tolylhydrazine.

4. A simple test for the detection of galactose in small quantities of urine, based on this property, has been described.

## REFERENCES


