A method for collection of urine from infants onto squares of filter paper was described as part of a program for early detection of phenylketonuria (1). These urine papers have proved extremely versatile as an aid to testing urine for a number of substances that are excreted as a result of certain metabolic defects, all easily detectable by simple means. Phenylpyruvic acid, phenylalanine, and o-hydroxyphenylacetic acid are excretory products characteristic of phenylketonuria (2, 3). A reducing sugar, galactose, is a significant finding in urine of patients with galactose disease (4). Sucrose has been reported in urine of several mentally retarded children (5)—apparently the result of a defect in the enzymatic system responsible for hydrolysis of the disaccharide. Chondroitin sulfuric acid has been found in urine of children with Hurler’s syndrome (gargoylism) (6, 7).

METHODS

COLLECTION OF URINE

Place a two-inch square of absorbent filter paper in diaper to become wet, or dip in urine; allow to dry. If urine seems very pale and dilute, dip a second time and dry again. Identification marks with lead pencil will not interfere in any of the tests described below.

TEST FOR PHENYLPYRUVIC ACID

Note odor of dry urine. In phenylketonuric urine very small
amounts of phenylpyruvic acid give a distinctive aromatic odor. Place a small drop of 10% ferric chloride solution (10 Gm. FeCl₃·10H₂O/100 ml.) on the urine paper (about 1 cm. diameter), using a small glass rod. Observe while wet, while drying, and when dry. Papers containing phenylpyruvic acid will turn green within a few seconds. The green color will fade to yellow-green as the spot of reagent dries, but the edges of the spot remain blue-green for several days. With negative tests the spot is clear yellow when wet (compare with blank paper); when dry the edges are slightly darker yellow than the center. Concentrated urines may produce a brownish color. Aspirin and its metabolites in urine produce a purple color; diacetic acid gives a red color. Urines from children with certain types of liver disturbances may give a greenish color with ferric chloride. After ingestion of chlorpromazine there may also be a green color with ferric chloride. In none of these instances, however, is there any unusual odor associated with the dry urine specimen. The remainder of the filter paper showing a positive or doubtful reaction for phenylpyruvic acid should be used for elution and chromatographic identification of phenylalanine and o-hydroxyphenylacetic acid.

**TEST FOR SUGAR (I)**

To prepare the reagent, weigh 8.5 Gm. phthalic anhydride; add 50 ml. water, 25 ml. ethyl alcohol, 425 ml. n-butyl alcohol, and 5.0 ml. aniline. Cut a half-inch square from the dry urine paper. Using the tip of a small glass rod apply a drop of aniline-phthalate reagent. Heat 10 minutes at 90-100°. Positive reactions are tan to dark brown against a white background. Traces of sugar give a yellowish discoloration and are not uncommon. Glucose, galactose, fructose, and lactose produce similar brown colors; pentoses give pink to reddish colors. p-Hydroxyphenylpyruvic acid in urine has given false positive reactions. The positive specimens should be retained for elution and identification of sugars.

**TEST FOR SUCROSE (II)**

Cut half-inch square as for sugar in I. Add a small drop of 50% acetic acid. Heat 10 minutes at 100° to hydrolyse sucrose. Repeat test described in I: add a small drop of aniline phthalate reagent and heat an additional 10 minutes. A positive test for sugar (tan or brown) on the second square, but not the first, indicates sucrose.
TEST FOR PROTEIN

Prepare indicator solution by dissolving 100 mg. of bromoresol green, sodium salt, in 100 ml. of 95% ethyl alcohol. Cut half-inch square. Dip in indicator solution. Wash in 1% acetic acid. Urines containing protein give a blue to blue-green color; all others are yellow.

TEST FOR CHONDROITIN SULFURIC ACID

To prepare the reagent dissolve 1.0 Gm. toluidine blue in 400 ml. acetone and 100 ml. water. Cut a half-inch square from the urine paper. Dip in toluidine blue reagent for about 45 seconds, drain. Wash in two separate portions of 10% acetic acid. Urine specimens containing chondroitin sulfuric acid give a purplish color; negative specimens are light blue. Protein does not give a positive reaction.

CONFIRMING TESTS

To confirm the preliminary findings on the paper spot tests, paper chromatographic procedures are useful for separating and identifying the urinary constituents. The details of the determination of phenylalanine on paper chromatograms have been described (8). Modifications were made to permit the use of urine dried on paper.

TEST FOR PHENYLALANINE

If there is a questionable or positive test for phenylpyruvic acid, the presence of large amounts of phenylalanine in the urine specimen will confirm the diagnosis of phenylketonuria. Moore and Boylen (9) have described a simple method for eluting substances from a test strip to paper chromatograms. From the paper containing urine a rectangle is cut one inch long and one-half inch wide. This area contains approximately 0.050 ml. urine. One end is cut to form a point. The rectangular strip is placed between two glass microscope slides (Fig. 1). Since the urine paper should not be in direct contact with the water used for eluting, a thin wick of filter paper is inserted under the edge of the urine strip between the microscope slides. The assembly is fastened together tightly with steel clips and placed in a 50-ml. beaker containing water. Figure 1 shows the assembly without water to make clear the placement of the paper between the microscope slides. The beaker is raised slightly above the level of the paper onto which the urine is to be eluted. The water rises by capillary action up the wick and flows down by gravity to the point. The point is placed in contact with a spot marked on the chromatogram.
For phenylalanine the spot should be one inch from the bottom of a sheet of Whatman No. 1 filter paper, 11 inches in height. When the spot is approximately 15 mm. in diameter the point must be removed while the chromatogram is dried. The point is then replaced for additional applications of urine. Twenty minutes is sufficient for the urine transfer.

A solvent mixture composed of 80 ml. n-butyl alcohol, 20 ml. glacial acetic acid, and 20 ml. of water gives a satisfactory separation of phenylalanine from other urinary amino acids. After resolution in the solvent mixture for a period of 14-16 hours (overnight) the chromatogram is dried and developed by spraying with 0.2% ninhydrin in
butyl alcohol. The chromatogram should be heated after spraying, to develop the color. Phenylalanine appears as a blue spot six inches (Rₜ .60) from the point of application; leucine, a purple spot, appears at Rₜ .72. In phenylketonuria the blue phenylalanine spot is large and intense; other amino acids are present in normal concentration.

**TEST FOR o-HYDROXYPHENYLACETIC ACID**

o-Hydroxyphenylacetic acid has been found in urine specimens from untreated phenylketonurics (3), but it cannot be detected in normal urines without the use of extraction procedures and concentration. Prepare the urine strip for elution, as described for phenylalanine. Transfer the urine to a chromatogram of the same dimensions and place overnight in a solvent containing 80 ml. isopropyl alcohol, 10 ml. concentrated ammonium hydroxide (59% or 15 N), and 10 ml. water. Spray the finished chromatogram with diazotized sulfanilic acid-sodium carbonate reagent. o-Hydroxyphenylacetic acid appears as an orange spot against a light yellow background at Rₜ .75.

**TO DISTINGUISH BETWEEN GALACTOSE, GLUCOSE, AND PENTOSE**

Transfer urine to chromatogram as described above. Place the urine spot one inch from the bottom of a sheet of Whatman No. 4 filter paper 14 inches in height. (This paper allows the solvent to rise more rapidly.) On the same chromatogram, one inch apart, place separate spots of glucose, galactose, fructose, lactose, and ribose. Add 5 μl. of solutions of each sugar containing 5 mg./ml. These sugars can be separated using a solvent composed of 80 ml. n-butyl alcohol, 80 ml. pyridine, and 40 ml. water (10). The container must be air-tight to prevent evaporation of the solvent during the slightly longer period, 18 to 20 hours, required for resolution of sugars on the 14-inch sheet. Spray the dried chromatogram with aniline-phthalate reagent and heat for 10 minutes. The Rₜ values will vary somewhat from sheet to sheet. Using the Rₜ of glucose as reference, the relative positions are as follows: ribose, 122; fructose, 107; glucose, 100; galactose, 88; lactose, 63.

**SUMMARY**

Procedures have been described for testing for phenylpyruvic acid, sugars, protein, and chondroitin sulfuric acid in urine dried on filter paper. Additional paper chromatographic methods for identification of phenylalanine, o-hydroxyphenylacetic acid, glucose, and galactose have been described.
REFERENCES