Classroom Demonstration of a Spot Test for Phenylpyruvic Acid and Its Relationship to Phenylketonuria

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Classroom Demonstration of a Spot Test for Phenylpyruvic Acid and Its Relationship to Phenylketonuria

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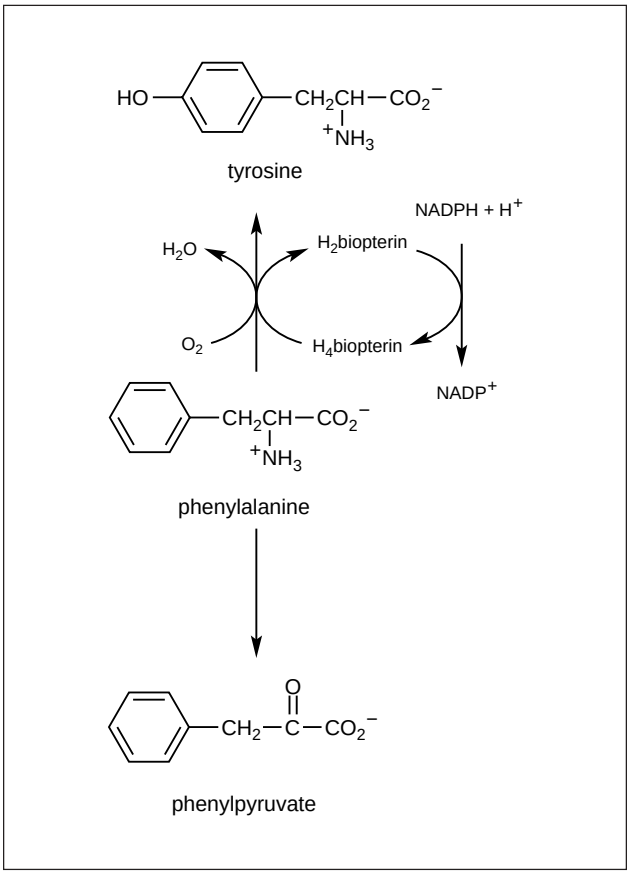
Classical phenylketonuria (PKU) is caused by a lack of activity in the enzyme phenylalanine hydroxylase (phenylalanine 4-monooxygenase), leading to elevated concentrations of phenylalanine in the blood *(1)*. This enzyme uses O_2 and tetrahydrobiopterin to hydroxylate phenylalanine at the 4-position of the aromatic ring, producing tyrosine, dihydrobiopterin, and water (Scheme I). Defects in enzymes that form or recycle the biopterin cosubstrate also produce hyperphenylalanemia *(2)*.



Scheme I. Reaction pathway from phenylalanine to tyrosine and phenylpyruvate.

If untreated, PKU leads to severe mental retardation, possibly caused by a number of factors *(3)*. Neonatal screening can detect the presence of excess concentrations of phenylalanine or its metabolites. Moreover, when infants with PKU are put on a diet that restricts the intake of phenylalanine, they experience dramatically less severe mental retardation. However, the intake of phenylalanine cannot be halted altogether since it is an essential amino acid. Treating protein hydrolyzates with charcoal removes all of the aromatic amino acids, which can then be added back in controlled quantities *(3)*.

Early methods of screening for PKU relied upon the appearance of urinary phenylpyruvate (Scheme I), which is produced by transamination of phenylalanine *(4)*. Although normal urine does not contain phenylpyruvate, the concentration of phenylpyruvate is as high as 4.5 mM in the urine of phenylketonurics *(3)*. Phenylpyruvate gives rise to phenylacetate via oxidative decarboxylation, and the mousy odor of the latter compound is also indicative of PKU *(5)*. Modern neonatal screening for PKU most commonly uses the Guthrie method to test for phenylalanine in the blood, as the Guthrie test is less prone to false negatives than assays based on the detection of phenylpyruvate with ferric ions.

The reaction of phenylpyruvic acid with ferric chloride produces a deep green-colored complex. One may determine the quantity of phenylpyruvic acid by reading the absorption at 620 nm *(6)*. Early PKU screening utilized strips of cellulose impregnated with ferric chloride and acetic acid *(4)*. In addition to ferric ammonium sulfate and cyclohexylsulfamic acid, one commercial product (Phenistix) used magnesium ions as a masking agent to minimize the interference as a result of urinary phosphate that would otherwise react with ferric ions *(7)*.

The essence of this test can be conveyed with the simple demonstration below. Students with a strong analytical or clinical interest may benefit from a discussion of the potential interference from urinary phosphate that the first advanced demonstration illustrates. The interference is mimicked with the inclusion of phosphoric acid. The masking effect of magnesium ions can also be shown; however, the color is not as intense as in the absence of both magnesium and phosphate. The three advanced demonstrations are unrelated to each other, so the instructor may chose to do any combination of them.

Materials

$FeCl_3$ (MW = 162.22 g/mol): Prepare a 5% stock solution. This solution may be made up a few days ahead of time but degrades over the course of a few weeks. This solution can be as dilute as 1% and still function.

Phenylpyruvic acid (CAS #156-06-9; Aldrich catalog #28,695-8; MW = 164.16 g/mol): Prepare a stock solution that is 2.3 mg/mL (14 mM) in ethanol. This solution may be stored in the freezer for years.

Simple Demonstration

Dilute 200 μL of the phenylpyruvic acid stock solution with 2.5 mL of deionized water in a test tube. The concentration of phenylpyruvic acid in the dilute solution is 1.0 mM. Add 200 μL of ferric chloride to the diluted phenylpyruvic acid. A dark green color that arises from the iron–phenylpyruvic acid complex is immediately apparent, but the color is not indefinitely stable *(6)*.

Advanced Demonstrations

Masking an Interference

After completing the simple demonstration, dilute 200 μL of the phenylpyruvic acid stock solution with 2.00 mL of 25 mM H_3PO_4 and 500 μL of water. Add 200 μL of ferric chloride. The color will be a very pale green, or colorless if 1% ferric chloride is used. Then do the same demonstration replacing the water with 500 μL of 2 M $MgCl_2$. The color will be a pale green, not as green as in the absence of phosphoric acid but stronger than in the presence of phosphoric acid in the absence of magnesium ions. Calcium chloride works approximately as well. Phosphoric acid (pH ~ 2) was chosen to approximate the acidity of the Phenistix strips.

Negative Controls

A 2.3 mM solution of tyrosine (Fisher; prepared by gentle warming) and 1 mM sodium pyruvate in water (Sigma) both give pale yellow solutions when mixed with ferric chloride. The pale yellow color may be from the residual intense yellow of ferric chloride.

Screening for a Different Metabolite

A solution of salicylic acid that is 18 mM in ethanol may be diluted and combined with ferric chloride as in the simple demonstration to produce an intense purple color. Salicylic acid is a potential interference in the PKU test, but this part of the demonstration also illustrates the use of the Phenistix strips to test for salicylate intoxication, presumably arising from the ingestion of aspirin *(8)*.

Discussion

This demonstration is part of one of the lectures on amino acid catabolism. The reaction catalyzed by phenylalanine hydroxylase is discussed in the context of being both a degradation pathway of phenylalanine and a salvage pathway to tyrosine. This leads immediately into describing PKU and performing the demonstration. The author has used this demonstration repeatedly in a senior undergraduate biochemistry course, and the demonstration appears to engage the students' attention. When a small sample of students was given the opportunity to answer an exam question on one of several inborn errors of metabolism, all chose to discuss PKU. This demonstration is also suitable for organic or biochemistry classes frequently taken by nursing students.

After the demonstration, students may be probed with questions based upon what the course has emphasized, the particular interests of the students, or the branch of chemistry to which the instructor wishes forge a link. A question on why a person suffering from PKU should avoid diet soft drinks may prompt students to recall that many such drinks contain aspartame (Nutrasweet), which is a dipeptide consisting of aspartate and phenylalanine. The instructor can ask students how a foodstuff devoid of phenylalanine might be prepared chemically, and the instructor can discuss charcoal filtration at this point. This can serve as a reminder to students that proteins may be hydrolyzed completely to their constituent amino acids. If genetics has been emphasized previously, students may also be asked to speculate on the inheritance pattern of PKU, which is autosomal recessive *(5)*.

In the advanced demonstrations it may be worthwhile to point out that phosphate would form a strong complex with ferric ions, which would keep the complex of ferric ions and phenylpyruvic acid from forming. This part of the demonstration makes the point that analyzing complex body fluids is much more difficult than analyzing simple solutions. Magnesium ions bind with the phosphate ions to allow ferric ion and phenylpyruvic acid to form the green-colored complex. The salicylic acid portion of the demonstration, which produces a purple color, may be used to illustrate the idea that unexpected results may have important interpretations. Instructors whose students have already taken inorganic chemistry may wish to point out that the green or purple color arises because charge-transfer complexes have been formed.

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