Congenital Methemoglobinemia
Methemoglobin Reductase Deficiency

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Shortly after the first white settlers crossed through the Cumberland Gap into the western foothills of the Appalachians there arose in one kinship several particularly swarthy offspring characterized by deep bluish skin coloration. These folks—the Blue Fugates—apparently suffered no ill effects of this condition and indeed numerous off-spring were born some of which also had this peculiar cyanotic appearance. The tale of an Indian princess being an ancestor in this family tree is more likely due to the resemblance of the skin color to the Shawnees of the "dark and bloody ground," i.e., Kentucky.

In time the condition yielded to the advances of biomedical science and the exact biochemical lesion associated with these dark-skinned folks was determined. While being much more accurate and precise such knowledge is not nearly so exciting.

The deep color in these people has been shown to be due to methemoglobin (MeHb). When the ferrous (+2) atom of the hemoglobin molecule is converted (oxidized) to the ferric (+3) state by the addition of the OH group the resulting molecule is called MeHb. This process of MeHb production is occurring continually. Reducing enzymes continually convert methemoglobin to the physiologically useful hemoglobin which is capable of transporting oxygen and carbon dioxide. These two counter mechanisms result in a steady state of blood MeHb of about one percent or less of the total hemoglobin concentration.

In general two large classes of methemoglobinemia are known i.e. the congenital and acquired varieties. The acquired variety is caused by the ingestion, inhalation or absorption through the skin of a great variety of drugs and chemicals. Chemicals include inorganic as well as organic compounds. The nitrates and nitrates are causal particularly in infants.

Congenital or familial methemoglobinemia is due to an insufficiency or decompensation of the normal biochemical process (an rbc enzyme function) which ordinarily keep methemoglobin levels at very low levels. This is a function of the red cell enzyme, methemoglobin reductase, at times called diaphorase. It is an NADH dependent enzyme. A deficiency of this enzyme results in a build-up of methemoglobin in the affected individual.

Recently Bloom and Zarkowsky have investigated several unrelated patients with methemoglobin reductase deficiency.1 Three distinct and different types of enzyme deficiency were reported. These included a complete absence of detectable enzyme,
decreased quantities of presumably normal enzyme and two cases characterized by decreased quantities of structural variants. Although their work has not been confirmed in other laboratories, the findings are consistent with studies done on other enzyme abnormalities.

Finally, methemoglobinemia may be due to a structural abnormality of the hemoglobin molecule. Several varieties of this type have been studied and characterized. Clinically these forms are similar to the biochemical forms (vide supra) but do not exhibit the characteristic absorption spectra of the above forms. In several (e.g., Hb M_Boston) a tyrosine residue is substituted for a histidine in the hemoglobin chain in an area adjacent to the heme iron contact. This results in a decreased ability of the ferric iron to be converted to the ferrous valence state. In another variety, Hb M_Milwaukee a glutamyl residue replaces the normal valine in the heme contact area of the $\beta$ peptide chain and methemoglobinemia results. In Hb M_Saskatoon tyrosine replaces histidine in the beta hemoglobin chain. Thus, a variety of M hemoglobins have been characterized and presumably others will be discovered.4

The common denominator of all of these is the substitution of an amino acid with an active side chain (e.g., tyrosine or glutamic acid) in an area of the folded $\alpha$ or $\beta$ peptide chain of hemoglobin which is adjacent to (and hence can influence the reactivity of) the heme iron. The reaction of the amino acid side chain forms a stable complex with iron and thus makes the heme iron biochemically inactive for the normal transportation of oxygen to the tissues.

**Chemical Testing for Methemoglobin**

**Specimen**

Heparinized, oxylated or other anticoagulated erythrocytes are suitable for this determination. One-tenth ml of whole blood is sufficient.

**Qualitative Test**

If the laboratory has a double beam recording spectrophotometer which measures in the visible range (specifically between 500 and 700 nm) the unknown sample can be conveniently screened for the presence of the characteristic MeHb absorption spectra. Make a 1 to 150 dilution of the blood by adding 0.02 ml of well-mixed blood to 3 ml of distilled water in a spectrophotometer cuvette. After hemolysis, solution and mixing of the test specimen, the unknown specimen is placed into the sample holder and a distilled water blank is placed in the blank holder. An absorption spectrum between 500 and 700 nm is examined for the presence of a significant shoulder in the region of 620 to 635 nm (figure 1). If such a peak is present it may be due to MeHb or possibly sulfhemoglobin. A drop of 10 percent sodium cyanide solution is added to the cuvette. After mixing, a second absorption spectrum is made. If the peak was due to MeHb it is obliterated by cyanide. The chemical reaction which occurs is the second stage of the well-known Drabkin's reaction, i.e., methemoglobin is converted to cyanmethemoglobin. If the 635 nm peak was due to sulfhemoglobin (a rare occurrence) it will not be changed by the addition of cyanide. Levels of McHb even less than 3 percent are detectable using this screening procedure.

**QUANTITATIVE TEST**

**Reagents**

1. **Stock Phosphate Buffer.** (0.1 M) Dissolve 17.91 grams of Na$_2$HPO$_4$·12 H$_2$O in 500 ml of distilled water. Similarly, dissolve 13.6 grams of KH$_2$PO$_4$ in 1000 ml of distilled water. Mix 400 ml of Na$_2$HPO$_4$ solution with 600 ml of KH$_2$PO$_4$ solution. Check the pH and adjust to a pH of 6.6 with the remaining solutions, if necessary.

2. **Working Phosphate Buffer.** Dilute
166 ml of the above phosphate buffer to 1000 ml with distilled water.

3. Potassium Ferricyanide (5 percent). Dissolve 5 grams of potassium ferricyanide (reagent grade) in about 90 ml of distilled water. Dilute to 100 ml.

4. Sodium Cyanide (10 percent). (Poisonous) Dissolve 5 grams of sodium cyanide (reagent grade) in 45 ml of distilled water and dilute to 50 ml.

5. Acetic Acid (12 percent).

6. Neutralized Sodium Cyanide (Poisonous) In a hood, add 1.0 ml of 12 percent acetic acid to 1.0 ml of 10 percent sodium cyanide. Mix well. This solution is stable for one hour.

**Preparation of Intracellular Methemoglobin**
Since cases of methemoglobin are quite unusual, methemoglobin can be prepared using the method as outlined in the first steps of the methemoglobin reductase procedure.

**Determination of Calibration Factor**
1. Determine hemoglobin content of a normal blood sample using the cyanmethemoglobin procedure.

2. Transfer 0.1 ml of well-mixed blood into a cuvette containing 9.9 ml phosphate buffer (0.016 M) and 0.1 ml of potassium ferricyanide solution (5 percent). If smaller cuvettes are used, appropriately decrease the volumes of reagent retaining similar proportions.

3. Mix and allow to stand for two minutes.

4. Read optical density (ODa) at 635 nm against blank consisting of 10.0 ml phosphate buffer (0.016 M) and 0.1 ml potassium ferricyanide (5 percent).

5. Add one drop of neutralized sodium cyanide solution to the sample and the blank.

6. Mix and allow to stand for two minutes.

7. Read optical density (ODb) at 635 nm of the sample against the blank.

8. Calibration factor (F) = Hemoglobin (grams percent)  
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   \frac{\text{Hemoglobin (grams percent)}}{(\text{OD}_a - \text{OD}_b)}
   \]

Substitute this factor into formula for calculating MeHb.
CONGENITAL METHEMOGLOBINEMIA

PROCEDURE

1. Transfer 0.1 ml of blood (oxylated or capillary) to a test tube containing 10 ml of 0.016 M phosphate buffer.
2. Mix and allow to stand for ten minutes.
3. Measure optical density of above solution at 635 nm against water. (OD₁)
4. Add one drop of neutralized sodium cyanide to the entire 10 ml sample. Mix.
5. After two minutes, measure optical density again against water. (OD₂)

Calculation

MeHb (g per dl) = F (OD₁ - OD₂)
Percent MeHb = \( \frac{\text{MeHb (g per dl)}}{\text{Hb (g per dl)}} \)

QUANTITATIVE TEST FOR METHEMOGLOBIN REDUCTASE

If initial studies indicate the presence of congenital methemoglobinemia, the measurement of methemoglobin reductase (diaphorase 1.6.99.) may be indicated. The following procedure is modified from that given by Scott.⁶

SPECIMEN

A one ml heparinized specimen of whole blood is suitable for this determination.

REAGENTS

1. Buffered Saline. Add one volume of 0.1 M KH₂PO₄ solution (Cf. Quantitative MeHb Testing) to nine volumes of 0.9 percent sodium chloride solution.
2. Sodium Nitrite (1 percent). Dissolve 100 mg of sodium nitrite crystals in 10 ml of buffered saline solution. Prepare fresh daily.
3. Tris Buffer (1 M). Dissolve 1.2 grams of Tris (hydroxymethyl) aminomethane in 10 ml of distilled water.
4. Ethylenediamine Tetra Acetic Acid (EDTA) (0.10 M). Dissolve 29 mg of EDTA in 10 ml of distilled water.
5. 2-6 Dichlorobenzenoneindophenol, Sodium Salt (0.0012 M). Dissolve 4 mg of 2-6 dichlorobenzenoneindophenol (Eastman 3463) in 10 ml of distilled water.
6. NADH (0.01 M). Dissolve 35 mg of NADH (Sigma D5755) in 5 ml of distilled water.

PROCEDURE

1. The blood is centrifuged, and the plasma removed. The cells are washed 3 times in equal amounts of cold phosphate buffered saline.
2. The washed cells are then incubated at room temperature for 20 minutes with an equal amount of 1 percent sodium nitrite solution. The nitrite solution is removed, and the cells were washed 5 times with phosphate buffered saline. (These cells may be used to set up known solutions for chemical quantitation of MeHb.)
3. Add 0.1 ml of packed cells to 1.9 ml of distilled water. Then dilute 0.2 ml of the hemolysate to 3 ml with distilled water.
4. Measure the optical density at 600 nm against a distilled water blank. Adjust the solution appropriately to give a constant optical density of approximately 0.13.
5. Reaction vessels are prepared by adding 0.05 ml of 1 M Tris, 0.1 ml of 0.01 M EDTA, 0.05 ml of 0.0012 M 2-6 dichlorobenzenoneindophenol (Eastman), and 1 ml of hemolysate. Add 7.5 ml of distilled water. Substi-
tute one ml of distilled water for hemolysate in the blank. To a 3 ml aliquot of reactants add 0.02 ml of NADH.

6. Determine serial optical densities of the solution at 3 minute intervals for 30 and at 45 and 60 minutes. The change in optical density (ΔOD) multiplied by 1,000 is plotted on arithmetic graph paper. Methemoglobin reductase activity is expressed as change in optical density per minute.

Normal levels for methemoglobin reductase range from 90 to 120 units. Individuals with methemoglobinemia due to a deficiency have levels below 70 units.

Discussion

About two hundred cases of methemoglobinemia due to methemoglobin reductase deficiency have been studied and reported in the medical literature. Thus this condition is quite uncommon. This is in contrast to acquired methemoglobin caused by toxic chemicals or drugs which is seen not infrequently. Infants are especially susceptible apparently due to a relative deficiency of this enzyme.

Treatment of congenital methemoglobinemia is of little value and usually is undertaken only to corroborate the diagnosis. Thus methemoglobin reductase deficiency is relieved by the intravenous administration of methylene blue (1 to 2 mg per kg). Methemoglobinemia due to hemoglobin M is not reversed by methylene blue administration. However treatment of the acquired form especially following large doses of a toxic agent is indicated and indeed may be lifesaving in certain cases. The adequacy of therapy is judged by the reduction of MeHb concentrations to levels approaching the normal.

References