Decolorization on Freezing of Ferricyanide-Cyanide Solution used for Hemoglobin Determinations

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When Drabkin's solution (used for the determination of hemoglobin), which contains $K_3Fe(CN)_6$, KCN, and NaHCO$_3$, is frozen, it loses its greenish-yellow color. The solution remains colorless even after thawing. The reaction, for which there is no satisfactory explanation, involves the reduction of the $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$, with KCN being oxidized to KCNO.

The cyanmethemoglobin procedure was chosen for use in a field study of hemoglobin values among Indian children. The method, which appeared best suited for such work because of the stability of the cyanmethemoglobin (1), should have permitted the transport of the hemoglobin solutions to the laboratory where colorimetric readings could be made with a more constant voltage than would be possible in the field.

During the course of work aimed at elucidating the aberrant results obtained, it was noticed that the reagent used—Drabkin's solution (2, 3)—became colorless on freezing. For the field work, the reagent was measured into plastic vials and stored overnight in the back of a station wagon. Theoretically, this should have been the best way to store the reagent since the tempera-

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Concentrations of the reagents were those listed for Drahkimi's solution (1').

\[ \text{HO} = 100\% \text{ transmission.} \]

The low temperatures may have resulted in the temporary freezing of the Drabkin reagent.

After the first trial, it was observed that a large number of hemoglobin values were lower than the clinical conditions of the children warranted. Verification thereof came from hemoglobin determinations made in a local clinical laboratory. This observation suggested that something was wrong with the hemoglobin procedure as used in the field trial. To check the method, tests were made of the accuracy of the pipets and colorimeter and the stability of the cyanmethemoglobin solution under conditions simulating those that might be encountered in the field trial.

It was in the course of this work, that it was observed that the greenish-yellow Drabkin's reagent became colorless when frozen. There is nothing in the literature on the stability of this reagent, which contains in one liter: \( 61 \times 10^{-8} \) moles \( K_3Fe(CN)_6 \), \( 77 \times 10^{-8} \) moles KCN, and \( 1190 \times 10^{-8} \) moles NaHCO\(_3\). The cyanmethemoglobin, when once formed in this reagent, shows only a 4% loss of color when stored in sealed amber bottles for 3 years (1).

**Study of Decolorization**

**Compounds Involved**

To study the decolorization, solutions were prepared containing \( K_3Fe(CN)_6 \) and either KCN or NaHCO\(_3\) in concentrations equal to those in the original Drabkin's solution. Fifteen milliliters of each solution were put into separate test tubes (20-ml capacity). The tubes were kept in a freezer \((-17^\circ)\) for 18 hr. and then thawed at room temperature. The loss of color was measured by the per cent transmission at 420 m\(\mu\) (Evelyn colorimeter).

The results of the foregoing tests indicated that the solutions were

<table>
<thead>
<tr>
<th>Compounds in solution</th>
<th>Before freezing</th>
<th>After freezing</th>
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<tbody>
<tr>
<td>( K_3Fe(CN)_6 ), KCN, NaHCO(_3)</td>
<td>18</td>
<td>95</td>
</tr>
<tr>
<td>( K_3Fe(CN)_6 ), KCN</td>
<td>18</td>
<td>77</td>
</tr>
<tr>
<td>( K_3Fe(CN)_6 ), NaHCO(_3)</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

Concentrations of the reagents were those listed for Drabkin's solution (1').

\(^1\text{H}_{2}\text{O} = 100\% \text{ transmission.}\)
decolorized only in the presence of cyanide and that the decolorization was greatest in the original reagent containing all three compounds (Table 1). After thawing, the solution remained colorless even when boiled or stored in amber bottles for 2 months.

**Effect of KCN Concentration**

Preliminary tests were carried out with solutions in which the concentration of ferricyanide was kept constant at the level found in Drabkin's solution while the concentration of cyanide was varied.

![Figure 1](https://academic.oup.com/clinchem/article-abstract/10/7/611/5672811)

There was no bicarbonate in these tubes (Fig. 1). Very little decolorization occurred when the ratio of cyanide to ferricyanide was less than 1. With increasing concentrations of cyanide, the color after freezing was reduced but never to the same extent as in the original reagent containing all three compounds. The slight decolorization observed when the ratio of cyanide to ferricyanide was less than 1 probably results from the few hydroxyl ions formed from the hydrolysis of the small amount of CN⁻ present (CN⁻ + H₂O → HCN + OH⁻).

When the bicarbonate was included, the decolorization was greater than in the series described above and again was dependent upon the ratio of cyanide to ferricyanide (Fig. 1). When the cyanide concentration was lower than that in the original Drabkin's solution (cyanide:ferricyanide=1.26), there was less decolorization. With higher concentrations, the decolorization produced by freezing was approximately the same or less than that in the original solution.
Effect of pH

The pH of the complete Drabkin’s solution was 8.8 before freezing and 8.4 after thawing. In the absence of bicarbonate, the decolorization was associated with a reduction from pH 9.7 before freezing to pH 6 or 7 after freezing.

To determine whether the original pH of the solution had any effect on the reaction, buffered solutions were prepared containing the ferricyanide and cyanide in concentrations equal to those in the original Drabkin’s solution. No single buffer spanned the desired pH range, so a number of buffers were used; the buffers were present at a concentration of 0.01M. The nature of the buffer influenced the extent of decolorization produced by freezing. This effect may have been a reflection of the ionic strengths of the buffers.

Decolorization was found to occur to only a limited extent below pH 7 (Table 2). The degree of decolorization was influenced to a certain degree by the type of ions present. At pH 8, the ferricyanide decolorization (expressed as per cent transmission) was as follows: in the presence of Tris, 96; phosphate, 90; and borate, 44.

Related to this was the work of Grube (4), who was apparently the
only other individual to study the nonphotochemical decomposition of K$_3$Fe(CN)$_6$ in the presence of KCN and alkali. He mixed 100 ml. 0.5M K$_3$Fe(CN)$_6$, 60 ml. 2N KOH, and 25 ml. 2N KCN. This solution was heated to 90°. Over a 70-min. period, samples were removed for Fe(CN)$_6^{3-}$ analyses by an unstated method. His results indicated that approximately $\frac{1}{2}$ the K$_3$Fe(CN)$_6$ was decomposed during the course of the experiment.

Since we observed that Drabkin’s solution decolorized at room temperature when 0.1 or 1.0 N KOH replaced the NaHCO$_3$, it appeared that the reaction in the highly concentrated and very alkaline solution used by Grube (4) should proceed in the absence of heat. When a solution was prepared according to Grube’s directions and left at room temperature, the rate of decomposition of the K$_3$Fe(CN)$_6$ was the same as that which he reported (Fig. 2). In our study, the concentration of K$_3$Fe(CN)$_6$ was estimated by measuring the absorbance at 420 m$\mu$ after the solution was diluted 1 to 1000.

**Development of Equation for Reaction**

The absorption curve (Cary recording spectrophotometer, Model 14*) of the Drabkin’s reagent, unfrozen and after freezing and thawing, suggested that the ferricyanide was converted to ferrocyanide. The absorption curve for the reaction product agrees with that of ferrocyanide reported by the American Cyanamid Company (5) and with that of a 61 × $10^{-5}$M K$_4$Fe(CN)$_6$ solution.

When it was realized that cyanide is a reducing agent in an alkaline solution (6), the amount of this ion remaining in the decolorized solu-

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tion was determined. The decolorized solution was slightly acidified (H₂SO₄) and placed in a water bath at 60°. Hydrogen was bubbled through the solution and the evolved HCN collected in dilute NaOH. According to McAlpine and Soule (6), the ferro- and ferricyanides are not decomposed in an acid solution at temperatures below 80°. Cyanide was determined by titrating the solution with AgNO₃ after the addition of NH₄OH and KI. When the decolorized Drabkin’s solution was analyzed, only 51% of the original cyanide could be recovered. The data indicated that for each mole of ferricyanide which was reduced, 0.5 mole of cyanide disappeared. These facts suggested that the reaction could be explained by the equation:

\[
2\text{Fe(CN)}₆^3⁻ + \text{CN}⁻ + 20\text{H}^⁺ \rightarrow \text{CNO}⁻ + \text{H}_2\text{O} + 2\text{Fe(CN)}₆^4⁻
\]

On the basis of this equation, the theoretical recovery of cyanide should have been 60%. The low recovery was probably due to hydrolysis of the cyanide, with the formation of hydrogen cyanide and its subsequent volatilization. This equation accounts for the absence of any decolorization when the solution becomes acid and bears out the fact that the bicarbonate only maintained the alkalinity of the solution.

According to Treadwell and Hall (7), the cyanate could be determined by measuring the CO₂ after acidification (CNO⁻ + H⁺ + H₂O → CO₂ + NH₃). To obviate the problem that would result from the NaHCO₃ in the original solution, the NH₃, rather than the CO₂ resulting from the CNO⁻, was estimated by Nesslerization. Theoretically, 3.1 × 10⁻⁴ moles of NH₃ should be formed from the cyanate. Actual analysis of the decolorized solution agreed with this.

**Concentration of Reagents in Drabkin’s Solution During Freezing**

A proposed explanation for the decolorization of the Drabkin’s solution on freezing involved the concentration of the reagents by the removal of water on freezing. To test this, the lower half of a tube of Drabkin’s solution was frozen by immersion in an ice-salt mixture. The concentration of ferricyanide in the supernatant, unfrozen liquid was not changed, as shown by absorption at 420 mμ, even though the frozen part was decolorized. This suggests that the concentration of the reagents in the unfrozen solution was not increased.

**Other Observations**

The degree of decolorization of K₃Fe(CN)₆ in some experiments has
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varied from tube to tube. A number of variables, such as the volume of solution in the tube, the diameter of the tube, the speed of freezing, and the length of time the solution remained frozen prior to thawing offered no satisfactory explanation for the variation in decolorization. However, when volumes of 100 ml. or more were frozen, the loss of color was always less than that secured with small volumes.

The citrate-buffered (pH 3) solution of $K_3Fe(CN)_6$ and KCN (in the concentration of the original Drabkin’s solution) was green both when frozen and after thawing. About 3 days after thawing the frozen solution, a bluish color appeared in the tubes. After a week’s storage at room temperature, the solution was completely blue. The tubes containing citrate that had never been frozen also became blue on longer storage at room temperature. The blue substance in these tubes partially precipitated. Similar reactions occurred in the tubes containing either phosphate (pH 2) or $H_2SO_4$ (pH 2) in addition to the $K_3Fe(CN)_6$ and KCN. It is likely that Turnbull’s blue—$Fe(FeFe(CN)_6)_2$—was formed in these solutions, but a more complete study of this phenomenon was not made.

Discussion

The decolorization of the Drabkin’s solution on freezing involves the reduction of the $K_3Fe(CN)_6$ by the cyanide ion in an alkaline medium. There is a possibility that the selective removal of water, with a consequent concentration of the reagents, may partly explain this phenomenon. Another possibility is that freezing removes the shell of water around the ferricyanide molecule, which then becomes more susceptible to reduction. An attempt was made to remove the hydrating molecules from the ferricyanide by means of absolute ethanol. This was done by mixing 1 part Drabkin’s solution with 9 parts absolute ethanol. There was no change in color other than that which was attributable to the dilution of the sample.

Unfortunately, none of the observations made of the decolorization of the Drabkin’s solution provided an explanation for the low hemoglobin values in the children surveyed during the field study. While working in the field, it was frequently noticed that the reagent measured out for the individual determinations was paler than it should be. This volume of reagent (10 ml.) had been stored overnight in small bottles at temperatures which approximated freezing. Originally, it
was believed that the loss of color was responsible for the low values. However, this was not the case, as shown by determinations of hemoglobin with solutions containing varying proportions of decolorized (as a result of freezing) and fresh Drabkin's solution. Under these circumstances, the hemoglobin values for the blood from each subject were the same regardless of the percentage of decolorized solution in the reagent. This was true even when the readings were made after the solutions had been stored at room temperature for 6 days.

The alkalinity of the decolorized solution was still sufficient to form oxyhemoglobin when blood was added thereto. The oxyhemoglobin was gradually converted to cyanmethemoglobin, with the conversion being completed in 5 hr.

There are two possible explanations for the low hemoglobin values secured for the samples collected on the field trip: (1) The conditions in the laboratory may not have duplicated those existing in the field. (2) There may be something peculiar about the stability of the hemoglobin in children's blood. (All of the field work was done with youngsters below the age of 13 years, while all the laboratory work was done with blood from adults.)

Our data indicate that the Drabkin reagent for hemoglobin determinations should be protected from freezing. By keeping the solution fluid at all times there will be less risk of inaccurate results due to reagent decomposition.

References