New Cholesterol Reagent

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A study was carried out on a new cholesterol reagent which was reported to be a saturated solution of ferrous sulfate in glacial acetic acid. This solution was shown to be unstable and it is suggested that the reactive state of the metal involved is that of the more stable trivalent ferric iron.

Recently a new reagent was proposed for the determination of cholesterol (1, 2). This reagent was purported to be saturated ferrous sulfate in glacial acetic acid. Since it has been reported that ferrous sulfate is qualitatively insoluble in anhydrous acetic acid (3, 4) and that ferrous iron is "subject to pronounced air oxidation in glacial acetic acid" (5), it was felt that several factors should be investigated concerning the reagent. These factors are as follows:

1. The solubility of ferrous sulfate in glacial acetic acid under the described conditions (2)
2. The stability of this ferrous ion in the same system
3. The solubility of ferric sulfate in glacial acetic acid under identical conditions, since this compound is suspected of being the active agent in this reaction
4. The reaction spectra for cholesterol with both ferrous and ferric sulfate solutions

Method and Results

Solubility Study
Several grams of both ferrous sulfate and ferric sulfate were shaken vigorously to saturate glacial acetic acid. The fine suspensions were then allowed to settle overnight, and the clear supernatant fluids were carefully decanted off for testing.

A portion of each of these solutions was tested for its iron content.

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The determination used for iron was the bathophenanthroline sulfonate reaction (6), where 1 ml of glacial acetic acid was added to the standards used for comparison in order to compensate for the presence of this acid in the sample. Only trace amounts of iron were found in each of the cholesterol reagents. The concentrations were 3.1 \mu g./ml for the ferric solution and 3.3 \mu g./ml for the ferrous solution. This would seem to confirm the finding that ferrous and ferric sulfate are both quite insoluble in glacial acetic acid (3, 4).

**Spectral Study**

It was found desirable to test the reaction for cholesterol with both iron solutions retaining the volume relationship reported by Searcy (1, 2). The freshly decanted solutions were used in the test. Six milliliters of each reagent were added to 200 \mu g. of cholesterol in a 0.1-ml volume of acetic acid. Two milliliters of sulfuric acid were pipetted into the solutions, which were mixed thoroughly and then allowed to come to room temperature. The colored solutions were then scanned to obtain their spectra by the use of a Beckman DK-2 automatic ratio-recording spectrophotometer. A control in which glacial acetic acid was used in place of the color reagents was included. The spectrum of the control (in the absence of iron) is shown as Curve A in Fig. 1, while Curves B and C represent the spectra for cholesterol after reaction with the ferrous and ferric iron reagents, respectively. It can be seen that Curve C which has been shifted up slightly to allow better delineation, could easily be superimposed on Curve B. This indicates that there is no reaction difference between the two solutions in this experiment and raises the question of whether there is a difference between the two solutions.
Stability Study

In order to determine whether ferrous iron was stable in glacial acetic acid, the reagent solution was tested for its ferrous ion content by eliminating the addition of a reducing agent to the buffered bathophenanthroline sulfonate sample system. The results showed that in less than 2 days, the ferrous ion content of the reagent had decreased to a very small fraction of the original total amount. These findings are shown in Table 1 and indicate quite clearly that if the criterion of stability is the oxidation state of the reagent, as it must be here, then this is indeed an unstable reagent and poses the likelihood that ferric iron is really the active form in this color reaction.

The following points are inferred then from the findings reported here:

1. Ferrous sulfate not only is quite insoluble, but is quite unstable in glacial acetic acid, and is easily oxidized to the more stable trivalent form.

2. Ferric iron appears to be the reacting form in the cholesterol reaction reported recently in which saturated ferrous sulfate in glacial acetic acid was claimed to be a new cholesterol color reagent (1, 2).

Table 1. Stability Study for Ferrous Iron in Glacial Acetic Acid

<table>
<thead>
<tr>
<th>Reagent in Bath. acetic acid solution</th>
<th>Total iron presence (µg/mL)</th>
<th>Fe²⁺ presence (µg/mL)</th>
<th>% Fe²⁺</th>
<th>Days after decanting</th>
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<tbody>
<tr>
<td>Fe₂(SO₄)₃</td>
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<td>0.0</td>
<td>....</td>
<td>Fresh</td>
</tr>
<tr>
<td>FeSO₄</td>
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<td>0.7</td>
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<td>0.5</td>
<td>15.2</td>
<td>1</td>
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<tr>
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<td>0.3</td>
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<tr>
<td>FeSO₄</td>
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<td>0.1</td>
<td>3.0</td>
<td>3</td>
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References