AN ASSAY METHOD FOR FERRICHROME

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(Received for publication, July 6, 1953)

The isolation, chemical nature, and biological activity of the new iron compound ferrichrome have been reported elsewhere (1-3). Biochemical interest in ferrichrome centers, at present, around (a) its growth factor activity for Pilobolus and (b) its possible relationship to cytochrome c formation in Ustilago sphaerogena.

A suitable assay method for ferrichrome has been much desired as an aid to studying the production of this new compound by living cells. One possible technique is a Pilobolus assay in which the weight of the mycelial pad would serve as the index of growth. However, Pilobolus responds to both hemin (4) and coprogen, the new iron compound of the Lederle group (5, 6).

The present report describes a chemical assay for ferrichrome based on a cyanide blocking of the autoxidation of the reduced compound. When dilute, neutral, buffered solutions of ferrichrome are treated with sodium hydrosulfite, the broad absorption band at 425 mµ vanishes. On aeration this band reappears. However, in the presence of cyanide, the spectrum does not reappear. The difference in light absorption between the oxidized form and the reduced, cyanide-treated form is used to calculate the ferrichrome concentration of the sample.

EXPERIMENTAL

Method

Preparation of Sample—The procedure used in extracting ferrichrome from natural material will depend on the source. We have routinely used extraction at pH 10 for removal of ferrichrome from the cells of U. sphaerogena. In any case the extract is brought to pH 7.0 with either dilute sodium hydroxide or phosphoric acid and then saturated with solid ammonium sulfate. The sample is then filtered, transferred to a separatory funnel, and extracted with 0.2 volume of benzyl alcohol. The benzyl alcohol extract is placed in a clean separatory funnel and to it are added 1 volume of distilled water and 10 volumes of ethyl ether. The aqueous phase is removed and the benzyl alcohol-ether phase washed with 0.1 volume of

1 In a personal communication to the author, Dr. A. G. Lochhead of the Department of Agriculture, Ottawa, Canada, has stated that ferrichrome acts as a growth factor for Arthrobacter terrigenes.
distilled water. The aqueous phase and washing are combined, evaporated to dryness under reduced pressure at 25°, and dissolved in 0.1 M phosphate buffer, pH 7.0.

The ferrichrome concentration of this solution should be 30 to 60 γ per ml.

Reagents—

Phosphate buffer. 0.05 mole of KH₂PO₄ and 0.05 mole of K₂HPO₄ dissolved in 1.0 liter of distilled water and adjusted to pH 7.0 with dilute KOH.

Cyanide solution. 200 mg. of KCN dissolved in 100 ml. of the above buffer. Store in a glass-stoppered bottle.

Hydrosulfite solution. 500 mg. of sodium hydrosulfite (Lykopon²) dissolved in 100 ml. of the above buffer. Store in a glass-stoppered bottle.

Procedure—The following reactions are carried out in 1.0 × 10 cm. Pyrex test-tubes.

A 1.0 ml. aliquot of the sample is placed in each of two test-tubes, Tubes A and B. A standard ferrichrome sample is included in a duplicate set of tubes, C and D. The ferrichrome, 30 to 60 γ, should be dissolved in the same phosphate buffer. To Tubes A and C are added 1.4 ml. of cyanide solution, and the volume in all four tubes is then made up to 2.9 ml. by addition of buffer. Finally, 0.1 ml. of hydrosulfite solution is added to all tubes and the contents very gently stirred with a glass rod.

After 10 minutes to allow for full reduction, a stream of air is bubbled through all the tubes for 5 minutes. The solutions are now poured into cuvettes and the optical densities recorded at 425 mμ against a phosphate buffer blank. A Beckman model DUR spectrophotometer with 1.00 cm. cells was used.

Calculations—The optical density difference of Tube D less Tube C corresponds to a known concentration of ferrichrome per ml. This figure is used in computing the ferrichrome concentration, from the optical density difference between Tubes B and A, for the unknown sample. Tube C serves as a reagent blank and gives an optical density of only about 0.004. The latter is contributed mostly by the cyanide solution.

Recovery Experiments

Dilute aqueous solutions of ferrichrome obey Beer's law, at least over the optical density range 0.050 to 0.150 in a 1.00 cm. cell.

The recovery of the optical density at 425 mμ in the standard ferrichrome samples is not exactly quantitative, but it does approach 95 per cent.

² Central Scientific Company, San Francisco, California.

* The pipette used here should have a cotton plug at the mouth.
For approximate work the standard ferrichrome can be omitted and an optical density difference of 0.039 between the untreated and cyanide-treated tubes may be assumed to equal 10 \( \gamma \) per ml. (1).

The validity of the assay described in this paper was checked by making a recovery of crystalline ferrichrome added to an extract of \( U. sphaerogena \) (prepared as above). Also, samples prepared in the above manner, i.e. with benzyl alcohol extraction, would be expected to be contaminated with flavin compounds (7), and it was thus necessary to check for their possible interference. Table I shows the results of recovery experiments in which a known quantity of ferrichrome was added both to an extract of \( U. sphaerogena \) and to a solution of riboflavin. In each case a quantitative recovery of ferrichrome was obtained.

TABLE I
Recovery of Ferrichrome from \( U. sphaerogena \) Extract and from Riboflavin Solutions

<table>
<thead>
<tr>
<th>Ferrichrome</th>
<th>( U. sphaerogena ) extract*</th>
<th>Riboflavin</th>
<th>Ferrichrome recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )</td>
<td>( \text{ml.} )</td>
<td>( \gamma )</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.5</td>
<td>30.1</td>
<td>100</td>
</tr>
<tr>
<td>45</td>
<td>1.0</td>
<td>45.0</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>0.5</td>
<td>60.3</td>
<td>101</td>
</tr>
<tr>
<td>30</td>
<td>6.7</td>
<td>31.1</td>
<td>104</td>
</tr>
<tr>
<td>45</td>
<td>4.5</td>
<td>45.2</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>2.2</td>
<td>58.8</td>
<td>98</td>
</tr>
</tbody>
</table>

* See the text.

The sensitivity of the method depends on the magnitude of the ferrichrome extinction coefficient at 425 \( \text{m} \mu \). Less than 10 \( \gamma \) in a volume of 3.0 ml. in the 1.00 cm. cell will give too small an optical density change for accurate work; i.e., less than 0.013. Table I shows that the assay is reproducible within \( \pm 4 \) per cent.

DISCUSSION

The partition of ferrichrome in the solvent system saturated ammonium sulfate-benzyl alcohol is such that, at the low concentrations of ferrichrome normally encountered, a single extraction with benzyl alcohol leaves an almost undetectable quantity of the desired compound in the ammonium sulfate phase.

Lower cyanide concentrations were unable to bind the ferrichrome spectrum completely. Also, a neutral pH is preferred for the reaction. At low pH the ferrichrome was instantly reduced by the hydrosulfite, but
reoxidation, even in the absence of cyanide, was very slow. At high pH the reduction was sluggish and cyanide did not trap the spectrum in the reduced state. When the reaction mixture was made 1 per cent with respect to both NaCl and (NH₄)₂SO₄, quantitative recoveries of ferrichrome were still realized.

The strong Soret band of porphyrin compounds might interfere with this test. However, these substances would probably be eliminated in the preparation of the sample. Metalloporphyrin proteins would not be extracted by the benzyl alcohol and, similarly, the free metalloporphyrins and porphyrins would probably not be removed with the ferrichrome from the ether-benzyl alcohol phase.

Interference from unknown substances is a possibility which should not be excluded. An unequivocal proof for the presence of ferrichrome would entail isolation of the crystalline substance. However, it should be pointed out that in order to influence this reaction the contaminant must (a) survive the purification procedure, (b) suffer a spectral alteration at 425 mµ on treatment with hydrosulfite, the latter being reversible by aeration, or (c) remain trapped in the spectrally altered state in the presence of cyanide.

So far as the author is aware, ferrichrome is the only known substance which will satisfy these requirements.

**SUMMARY**

A chemical assay for ferrichrome has been developed. The method depends upon the irreversible loss of the characteristic ferrichrome spectrum in the presence of both cyanide and hydrosulfite.

**BIBLIOGRAPHY**

AN ASSAY METHOD FOR FERRICHROME
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J. Biol. Chem. 1953, 205:643-646.

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