A COLORIMETRIC DETERMINATION OF BLOOD ACETOIN*

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(Received for publication, September 11, 1945)

Most of the methods described for the determination of acetoin and biacetyl are based on the original Lemoigne-van Niel procedure (1, 2), which depends upon the formation of insoluble nickel dimethylglyoxime when biacetyl is treated with hydroxylamine in the presence of a nickel salt; acetoin is similarly determined after oxidation to biacetyl by heating with ferric chloride. In a recent modification Stotz and Raborg (3) have employed a colorimetric determination of the nickel in the precipitate instead of the usual gravimetric analysis, and the sensitivity of the method has thereby been greatly increased. A polarographic method for the determination of acetoin and biacetyl has also been described by Greenberg (4).

The method herein described was developed because none of the previous methods was sufficiently sensitive for our purpose; its chief advantages are simplicity and sensitivity. It is based on the color reaction originally discovered by Voges and Proskauer (5), and later shown by Harden and Norris (6, 7) to be due to the reaction between biacetyl and a guanidino group in the presence of alkali. Attempts to increase the sensitivity of this reaction led to the addition of creatine by O’Meara (8) and α-naphthol by Barritt (9). While the original Voges-Proskauer reaction could not be developed into a satisfactory quantitative method, the Barritt modification was found to be suitable. In this laboratory it was first tried by W. E. Knox, and a similar method has recently been described by Eggleton et al. (10). The chemical reactions involved in the development of the red color have been studied (10-12) but not fully elucidated.

Details of the procedure for color development and the method for determining acetoin in blood are described.

**Colorimetric Determination of Acetoin and Biacetyl**

*Reagents—*

0.5 per cent creatine; 1 gm. of creatine dissolved in 200 cc. of water.

5 per cent α-naphthol; 1 gm. of powdered colorless α-naphthol (redistilled under nitrogen) dissolved in 20 cc. of 2.5 N NaOH. The solution is prepared immediately before using.

*Method—*To 5 cc. of solution containing between 1 and 12 γ of acetoin or

* This study was supported by a grant from the Nutrition Foundation, Inc.
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Determination of Blood Acetoin

Biacetyl are added consecutively 1 cc. of 0.5 per cent creatine and 1 cc. of the 5 per cent α-naphthol solution. The latter reagent should not be prepared until after the creatine has been added to the test solution, and it is used as soon as the α-naphthol dissolves. The color is allowed to develop at room temperature for exactly 10 minutes when biacetyl is being determined, and 1 hour for the determination of acetoin. The amount of color is then read in an Evelyn photoelectric colorimeter with a 540 filter; a reagent blank prepared simultaneously with 5 cc. of water is used to adjust the galvanometer to 100, and a standard solution containing 5 γ of acetoin or biacetyl can be included in each series of analysis.

The colors obtained by this method were reproducible but somewhat unstable. The maximum color was obtained with biacetyl between 5 and 10 minutes after the addition of the alkaline α-naphthol; fading was barely perceptible on the galvanometer at 15 minutes, and it amounted to a 10 per cent error at 30 minutes. The color obtained with acetoin was more stable; the maximum intensity was reached in 60 minutes after the addition of the alkaline α-naphthol; fading could not be detected at 75 minutes, and was barely perceptible at 90 minutes. The absorption spectra of the colors obtained with biacetyl and acetoin were quite similar. The peak of absorption occurred at 530 mµ, and both curves were relatively flat between 520 and 540 mµ.

Maximum color was obtained with creatine, but other compounds containing the guanidino group (arginine, creatinine, guanidine carbonate, methylguanidine sulfate) gave similar colors in the presence of biacetyl, α-naphthol, and alkali. α-Naphthol could not be replaced advantageously by any one of a large number of substances tested, and it was without effect if added to the test solution of creatine and biacetyl after the alkali.

It was found convenient, but not essential, to dissolve the α-naphthol in the alkali under a stream of nitrogen, since the reagent became colored readily in air. Only freshly prepared reagent, containing no more than a faint yellow color, was used. Less consistent results were obtained when the α-naphthol was dissolved in 95 per cent alcohol and added separately from the alkali.

Standard Curves—A standard solution of biacetyl containing approximately 1 mg. per cc. was prepared from the fraction boiling sharply at 88°. A similar solution of acetoin was prepared from the crystalline polymer that was equally well purified by thorough washing with anhydrous ether or by fractional distillation. Both standards remained unchanged for several months at 6°, after which the biacetyl solution deteriorated.

Suitable dilutions were prepared from each of these standards, so that 5

1 The acetoin and biacetyl were obtained from the Lucidel Corporation, Buffalo, New York.
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cc. contained from 1 to 12 γ of acetoin or biacetyl. Colors were developed in a series of such dilutions and read against the reagent blank in the photo-electric colorimeter after 10 minutes for the biacetyl determination and 1 hour for acetoin. The standard curves for both acetoin and biacetyl, shown in Fig. 1, were linear for amounts of acetoin and biacetyl below 5 to 6 γ, but departed slightly from this relationship with larger amounts.

Oxidation of Acetoin to Biacetyl

Reagents—

FeSO₄·7 H₂O (solid).

50 per cent FeCl₃·6 H₂O; 500 gm. of FeCl₃·6 H₂O dissolved in water and diluted to 1 liter.

10 N H₂SO₄; 280 cc. of concentrated H₂SO₄ diluted to 1 liter.

Fig. 1. The relationship of color intensity at 540 mμ to the amount of biacetyl or acetoin tested.

When acetoin cannot be determined directly because of interfering substances, a preliminary oxidation to biacetyl and distillation of the latter can be employed; smaller amounts can also be determined by this procedure, since the biacetyl can be concentrated during the distillation. The determination of total acetoin plus biacetyl in mixtures of the two likewise depends upon this preliminary oxidation.

The usual method of carrying out this oxidation with ferric chloride gave 85 to 90 per cent of the theoretical amount of biacetyl; this has been the common experience (13-15). By using a mixture of ferric chloride and ferrous sulfate, as suggested by Kniphorst and Kruisheer (15), the yields were substantially improved, and the following method was adopted.

The oxidizing reagent is prepared by dissolving 10 gm. of FeSO₄·7 H₂O in 30 cc. of 50 per cent FeCl₃, 2 cc. of this solution and 1 cc. of 10 N H₂SO₄.
are added to 7 cc. of the acetoin-containing solution. The tube is stoppered lightly with a clean rubber stopper and placed in a boiling water bath for 30 seconds to relieve excessive pressure before the stopper is inserted tightly. After heating for 30 minutes in a boiling water bath, the tube is cooled and the contents rinsed into a small distillation flask described by Stotz (16). A glass bead is added and the solution distilled carefully to avoid obtaining any trace of iron salt in the distillate by bumping or spraying. 5 cc. of distillate are collected in a 10 cc. graduated cylinder, and the contents made up to 5.5 or 10 cc., depending upon the amount of biacetyl expected. The color reaction for biacetyl is carried out with 5 cc. of this solution or an aliquot diluted to 5 cc.

### Table I

<table>
<thead>
<tr>
<th>Acetoin oxidized</th>
<th>Biacetyl in aliquot analyzed</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered</td>
<td>Theory</td>
</tr>
<tr>
<td>γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.82</td>
<td>1.78</td>
</tr>
<tr>
<td>4</td>
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<td>7.34</td>
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<td>4.83</td>
<td>4.89</td>
</tr>
<tr>
<td>35</td>
<td>6.67</td>
<td>6.84</td>
</tr>
<tr>
<td>50</td>
<td>4.60</td>
<td>4.89</td>
</tr>
<tr>
<td>80</td>
<td>7.50</td>
<td>7.83</td>
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<tr>
<td>100</td>
<td>9.35</td>
<td>9.78</td>
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<tr>
<td>140</td>
<td>5.30</td>
<td>5.48</td>
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<tr>
<td>250</td>
<td>4.87</td>
<td>4.89</td>
</tr>
<tr>
<td>500</td>
<td>4.83</td>
<td>4.89</td>
</tr>
</tbody>
</table>

The recovery of biacetyl from the oxidation of acetoin by this method is shown in Table I. 98 per cent of the theoretical amount was obtained from 2 to 500 γ of acetoin; larger amounts were not tested.

**Specificity**—The original Voges-Proskauer reaction is nearly specific for acetoin and biacetyl. The related 5-carbon ketol gives a somewhat similar color, while the 6-carbon ketol gives a light olive-green color within the usual time limit employed (17). In the test herein described, approximately 90 γ of methylglyoxal gave as much color as 1 γ of biacetyl, while 1 mg. of glyoxal gave no color. 1 mg. of benzil and benzoin in alcohol gave a slight color equivalent to approximately 1 γ of biacetyl and 0.5 γ of acetoin respectively.
The nickel dimethylglyoxime precipitation is probably more specific for biacetyl than this color reaction. 1.8 mg. of methylglyoxal gave no precipitate in the procedure described by Stotz and Raborg (3). The introduction of α-naphthol into the reaction also increases the possibility of obtaining colors from unrelated substances that react with the α-naphthol alone. Such interference, as yet unknown, could readily be detected by omitting the creatine from the test.

**Blood Acetoin**

*Method of Determination*—A 1:5 tungstic acid filtrate of blood is prepared by adding 2 volumes of water, 1 volume of blood, 1 volume of 10 per cent sodium tungstate, and 1 volume of \( \frac{2}{3} \) N H\(_2\)SO\(_4\); the precipitate is centrifuged off. An aliquot of the supernatant is added to solid NaCl (1 gm. per 3 cc.) and distilled from a Claisen flask practically to dryness in vacuo (150 to 200 mm. of Hg). The distillate is oxidized as previously described by adding approximately 2 cc. of the FeCl\(_3\)-FeSO\(_4\) mixture and 1 cc. of 10 N H\(_2\)SO\(_4\) to each 7 cc. of distillate, and heating in a stoppered tube for 30 minutes. The solution is then redistilled, and the biacetyl is collected in the first 5 cc. of distillate and estimated colorimetrically.

The recoveries of added acetoin obtained by this procedure are shown in Table II. For the accurate analysis of the amount of acetoin occurring in normal blood it is necessary to use the maximum amount of filtrate that can

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**Table II**

<table>
<thead>
<tr>
<th>Acetoin added</th>
<th>1 cc. blood per cent</th>
<th>5 cc. blood per cent</th>
<th>25 cc. blood per cent</th>
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<tbody>
<tr>
<td>1</td>
<td>101</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>92</td>
<td></td>
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<tr>
<td>3</td>
<td>105</td>
<td>96</td>
<td></td>
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<tr>
<td>5</td>
<td>102</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>10</td>
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<tr>
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<td>101</td>
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</tr>
<tr>
<td>250</td>
<td>97</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>95</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

The acetoin was added to the water with which the blood was laked during the preparation of a protein-free filtrate. The filtrate was analyzed for acetoin by the method described. The percentage recovery includes a correction for the 0.012 to 0.015 mg. per cent of acetoin found in the blood to which the acetoin was added.
be obtained from a relatively large sample of blood, e.g. 15 to 25 cc. When the blood acetoin is elevated slightly, a satisfactory analysis can be run on the filtrate obtained from 5 cc. of blood.

In developing this method for the determination of acetoin in blood, various procedures were tested for the completeness of recovery of added acetoin, and were accordingly developed further or discarded. Direct color development with a tungstic acid or zinc hydroxide filtrate of blood could not be employed because of the appearance of a yellow-brown color during the reaction. When the blood filtrate was oxidized directly with the iron salts previously described and the biacetyl obtained in the distillate was determined colorimetrically, low recoveries were obtained. This was due primarily to the presence of urea in the blood filtrate; urea reacted with the biacetyl, resulting from the oxidation of acetoin, to form a diureide (18, 19).

In order to avoid this type of interference by urea (and to a lesser extent by uric acid and allantoin) the acetoin was first distilled from the filtrate and subsequently oxidized to biacetyl for the purpose of concentrating the latter.

**Artifacts in Acetoin Analyses**—During the studies incidental to developing the method for determining blood acetoin, it was observed that relatively large amounts of biacetyl could be obtained from a blood filtrate by subjecting the latter to an acid hydrolysis prior to oxidation with the FeCl₃-FeSO₄ mixture. Most of this newly formed biacetyl was found to be derived from glucose, and was unrelated to the preexisting blood acetoin. Although the existence of a bound form of acetoin in blood cannot be denied categorically, its existence in appreciable amounts is unlikely, since neither the blood filtrate nor glucose gave rise to any large amount of biacetyl when the oxidizing mixture of FeCl₃-FeSO₄ was present throughout the hydrolysis.

Table III shows the amount of biacetyl formed from crystalline glucose.

<table>
<thead>
<tr>
<th>Heating prior to oxidation</th>
<th>1.2 N H₂SO₄</th>
<th>2.2 N H₂SO₄</th>
<th>4.2 N H₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>γ</td>
<td>γ</td>
<td>γ</td>
</tr>
<tr>
<td>0.5</td>
<td>0.44</td>
<td>0.97</td>
<td>3.14</td>
</tr>
<tr>
<td>1</td>
<td>0.82</td>
<td>1.87</td>
<td>4.35</td>
</tr>
<tr>
<td>2</td>
<td>1.63</td>
<td>3.89</td>
<td>8.30</td>
</tr>
</tbody>
</table>

9 mg. of glucose in approximately 15 cc. of the indicated acid were heated in a stoppered tube in boiling water for the indicated time. After cooling and adding 4 cc. of the FeCl₃-FeSO₄ solution, the tube was restopped and heated an additional 30 minutes. The biacetyl was then distilled and determined colorimetrically.
under various conditions of acid hydrolysis and subsequent oxidation with the iron salts. The identity of the biacetyl formed from glucose was established by the following experiments. (a) No color was obtained in the biacetyl analysis if either creatine or α-naphthol was omitted from the reaction (the small amounts of biacetyl tested could not give a Voges-Proskauer reaction in the absence of α-naphthol). (b) 750 γ of biacetyl were obtained by heating 1 gm. of glucose in 50 cc. of 10 N H₂SO₄ for 6 hours, diluting, and oxidizing with the FeCl₃-FeSO₄ solution; the biacetyl was concentrated to a 5 cc. volume by two distillations, and a copious red precipitate characteristic of nickel dimethylglyoxime was obtained when this solution was treated with hydroxylamine in the presence of a nickel salt (3). (c) 960 γ of biacetyl were similarly obtained after heating 1 gm. of glucose in 200 cc. of 4 N H₂SO₄ for 6½ hours, and the concentrated solution of biacetyl was allowed to react with 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitate was centrifuged, washed with water and alcohol, and recrystallized from nitrobenzene. The crystals were identical with the bis-2,4-dinitrophenylhydrazone of biacetyl in appearance and melting point (315°), and showed no depression on mixing with an authentic sample.

Pyruvate and lactate gave rise to small amounts of biacetyl when they were treated by the procedure used in oxidizing acetoin to biacetyl. A solution of 100 mg. of sodium pyruvate in 7 cc. of water, 1 cc. of 10 N H₂SO₄, and 2 cc. of the FeCl₃-FeSO₄ mixture was heated in a stoppered tube for 1 hour and then distilled; 75 γ of biacetyl were obtained in the distillate, and its identity substantiated by conversion to the red precipitate characteristic of nickel dimethylglyoxime. A similar treatment of 100 mg. of lactic acid yielded 2.4 γ of biacetyl, while 100 mg. of alanine gave none.

The preliminary distillation of acetoin from the blood filtrate was necessary in order to eliminate interference by substances that combined with biacetyl, as well as those that gave rise to it during the chemical reactions incidental to its determination. We cannot be certain that the small amount of acetoin found normally in blood was not an artifact due to the distillation of some other substance that gave biacetyl when treated with acid FeCl₃-FeSO₄. That the chromogenic material obtained from blood was actually biacetyl was indicated by the absence of color when creatine or α-naphthol was omitted from the test, and the formation of the characteristic red precipitate when the biacetyl obtained from 680 cc. of beef blood was treated with hydroxylamine and a nickel salt. Enrichment of the blood with 10 mg. per cent of sodium pyruvate, 10 mg. per cent of acetaldehyde, 300 mg. per cent of glucose, or 200 mg. per cent of alcohol did not alter the amount of biacetyl obtained in the analysis.

It is probable from these studies that the major part of the acetoin previously identified in normal blood (20) was actually formed from some other
constituent, such as pyruvate, during the chemical reactions involved in the isolation procedure. The relatively large amount of acetoin previously reported (21) in normal blood is undoubtedly an artifact for the same reason.

Normal Blood Acetoin—The acetoin concentration in freshly drawn blood from the rat, dog, cat, fasted rabbit, pig, and cow varied from 0.005 to 0.015 mg. per cent, with most of the values falling between 0.007 and 0.012 mg. per cent. Pigeon blood averaged about 0.03 mg. per cent. The concentration of acetoin in beef plasma was approximately 80 per cent of that observed in whole blood; when the acetoin concentration was elevated to 0.365 mg. per cent in rat blood by the administration of acetaldehyde, the plasma contained 0.413 mg. per cent.

SUMMARY

A method has been described for the colorimetric determination of acetoin in blood. Possible interference from several constituents of normal blood was described.

The normal blood concentration of acetoin in most of the species studied was approximately 0.01 mg. per cent.

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