THE COLORIMETRIC DETERMINATION OF LACTIC ACID IN BIOLOGICAL MATERIAL

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The trend of recent research in the field of cellular and tissue metabolism has emphasized the importance of methods for the precise determination of small amounts of lactic acid. The quantities of lactic acid which are encountered when tissue slices, suspensions, and extracts are used for studies in metabolism are ordinarily below the range of the standard titrimetric lactic acid method of Friedemann, Cotonio, and Shaffer (1), which requires a minimum of about 0.5 mg. of lactic acid for the usual determination in duplicate. Of the various colorimetric methods which have heretofore been available for the determination of small amounts of lactic acid, only those of Mendel and Goldscheider (2) and Miller and Muntz (3) are sufficiently sensitive to be of possible value in this connection.

Both the Mendel-Goldscheider and Miller-Muntz methods depend primarily upon the reaction of Denigès (4), by which lactic acid is converted quantitatively into acetaldehyde on being heated with concentrated sulfuric acid. Mendel and Goldscheider determine the acetaldehyde colorimetrically by virtue of its reaction with veratrole (o-dimethoxybenzene), while Miller and Muntz utilize the reaction between acetaldehyde and p-hydroxydiphenyl which was first described by Eegriwe (5).

The reaction between p-hydroxydiphenyl and acetaldehyde is fundamentally far more sensitive than is the veratrole reaction. Nevertheless, in our study of the applicability of the two reagents to the determination of lactic acid in tissue metabolism preparations we were at first completely unable to obtain as satisfactory results with p-hydroxydiphenyl as had been obtained with a
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modification of the veratrole procedure.\(^1\) The variable results by the Miller-Muntz method, under presumably well controlled conditions, led us to suspect that an unknown factor was involved in the reaction. It was soon found that the reaction between acetaldehyde and \(p\)-hydroxydiphenyl in concentrated sulfuric acid is markedly influenced by the presence of certain inorganic ions, notably those of iron, copper, and cerium. Other ions such as those of lead, mercury, nickel, and cobalt are without effect, and we are thus unable to confirm the finding of Block and Bolling (7) that lead influences the color reaction.

The effect of iron ions on the reaction is quite significant and points toward the explanation for the phenomenon which is offered later. While the presence of neither ferrous ion nor ferric ion alone is of analytical value, a suitable mixture of the two results in a final color which is from 3 to 5 times as intense as when the reaction is carried out in the ordinary way as described by Miller and Muntz, and the specificity of the reaction is also increased. The disadvantage of iron as a promoter of the color reaction lies in the fact that variations in the ratio between ferrous and ferric iron markedly influence color development, as is shown later, and an excessive amount of ferric iron produces a green color with the reagent alone.

The effect of copper is quite different from that of iron. The addition of cuprous ion is not necessary, since added cupric ion alone enhances color development. As the cupric ion concentration is increased, color enhancement increases until it becomes almost as great as that obtained with the optimal mixture of ferrous and ferric iron. Higher concentrations of cupric ion have no further effect, nor does the ion produce a color with the reagent alone. Cerium behaves similarly to copper, but offers no advantages over the latter.

We have therefore devised a colorimetric procedure for the determination of small amounts of lactic acid which incorporates the findings just described. The various steps in the procedure

\(^1\) Barker (6). When this report was presented at New Orleans in March, 1940, the essential details of the \(p\)-hydroxydiphenyl method described here, including the use of copper and of an aqueous alkaline solution of the reagent, were also presented orally but are not included in the published abstract.
include (a) treatment of the protein-free sample by the copper hydroxide-calcium hydroxide procedure of Van Slyke (8) to remove interfering material; (b) conversion of lactic acid to acetaldehyde by heating in concentrated sulfuric acid in the proportion recommended by Mendel and Goldscheider; (c) color development by treatment in the concentrated acid solution with \( p \)-hydroxydiphenyl in the presence of added cupric ions. The \( p \)-hydroxydiphenyl is added as an aqueous alkaline solution rather than in the solid form recommended by Miller and Muntz.2

The procedure described here has been applied with satisfactory results to a large number of samples of whole blood, serum, saliva, tissue filtrates, and tissue hydrolysates. The sensitivity of the procedure is such that color development is ordinarily carried out on a portion of the sample which contains not more than 5 to 10 \( \gamma \) of lactic acid. This represents 1 cc. of a 1:50 or 1:100 dilution of the usual sample. Since the final volume of colored solution under the conditions described here is about 7 cc., it would appear possible to apply the method to much smaller amounts of lactic acid than are specified above, by the suitable use of smaller volumes and microphotoelectric colorimetry.

**EXPERIMENTAL**

**Reagents—**
20 per cent solution of CuSO\(_4\)·5H\(_2\)O.
4 per cent solution of CuSO\(_4\)·5H\(_2\)O.
Solid calcium hydroxide, c.p., powdered.
Sulfuric acid, concentrated, sp. gr. 1.84.
1.5 per cent solution of \( p \)-hydroxydiphenyl in 0.5 per cent NaOH.

**Removal of Protein—** The solution undergoing analysis must be free from protein. Protein may be removed by any of the common procedures in which trichloroacetic acid, tungstic acid, zinc hydroxide, or cadmium hydroxide is employed. This was established by comparative analyses on blood samples which had been deproteinized by various procedures. In one such series of analyses a blood sample which contained 21.8 mg. per cent of lactic

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2 While this article was in preparation, the report of Koenemann (9) appeared, in which the use of an aqueous alkaline solution of \( p \)-hydroxydiphenyl for the Miller-Muntz procedure was proposed.
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Acid by the titrimetric Friedemann method gave colorimetric values of 21.7, 21.1, and 21.9 mg. per cent after deproteinization by trichloroacetic acid, tungstic acid, and zinc hydroxide respectively. Lactic acid added to the samples before precipitation of the proteins, in amounts equivalent to from 5 to 20 mg. per cent, was recovered quantitatively in the filtrate from all three precipitants. Similar results have been obtained with cadmium hydroxide. The precipitated protein should be removed by centrifugation rather than filtration, to avoid possible contamination from the filter paper.

Treatment with Copper and Calcium—A portion of the protein-free fluid is treated with 20 per cent copper sulfate solution and solid calcium hydroxide in the proportion recommended by Van Slyke (8). A convenient procedure is as follows: 1.0 to 5.0 cc. of protein-free filtrate, containing from 20 to 100 γ of lactic acid (i.e., 2.0 cc. of a 1:10 blood filtrate, from a resting subject), are added to 1 cc. of 20 per cent copper sulfate solution and made to a total volume of 10.0 cc. with water. Approximately 1 gm. of powdered calcium hydroxide is added and the mixture immediately shaken vigorously. The mixture is allowed to stand at room temperature for at least half an hour, with occasional shaking, and then centrifuged. The supernatant fluid is used for the final color development as described below. In removing the aliquot for analysis, care should be taken to avoid including any of the solid material which is usually present in the surface film. The aliquot is pipetted out from beneath the surface film, and the outside of the pipette is then wiped clean.

With certain types of material, the treatment with copper and calcium described above has been found suitable for the removal not only of glucose and other interfering substances but also of protein, thus eliminating the need for a separate deproteinization. For instance, a lactate solution containing 5 per cent of protein and 200 mg. per cent of glucose gave the same results on a direct 1:100 dilution of the original sample by the copper-calcium treatment as were obtained by the usual 1:10 deproteinization and subsequent 1:10 dilution through the copper-calcium procedure. Saliva has been analyzed satisfactorily by this method, as have acidified tissue extracts and acid tissue hydrolysates. The method is not applicable to whole blood, however, since there is a substance in blood corpuscles which interferes with the lactic acid determina-
tion and which is not removed by the copper-calcium treatment alone but is readily removed by any of the common methods of deproteinization. The nature of this substance is under investigation.3

Excessively acid or alkaline solutions should be approximately neutralized before treatment with copper and calcium. If the bright blue color of cupric hydroxide is not obtained when the mixture is shaken after the addition of calcium hydroxide, more calcium hydroxide must be added. It is also possible to carry out the copper-calcium procedure on a 1:5 or even a 4:5 dilution of the protein-free filtrate, but the amounts of copper sulfate solution and calcium hydroxide should not be changed.

**Color Development**—Transfer 1.0 cc. of the supernatant fluid from the copper-calcium treatment, containing between 2 and 10 \( \gamma \) of lactic acid, to a fairly wide test-tube (18 to 23 mm. inside diameter) and add 0.05 cc. of 4 per cent copper sulfate solution. Run in from a burette exactly 6.0 cc. of concentrated sulfuric acid, mixing the contents of the tube while the acid is being added. It is not necessary to keep the mixture cold. With adequate mixing the temperature of the solution does not rise above 70-80\(^\circ\). After the acid has been added, place the tube upright in a boiling water bath and allow it to remain for 5 minutes. Remove and place in cold water to cool to below 20\(^\circ\).

When the contents of the tube are sufficiently cool (but not before), add from a pipette exactly 0.1 cc. of the alkaline solution of \( p \)-hydroxydiphenyl. Disperse the precipitated reagent as quickly and uniformly as possible through the solution, and place the tube in a beaker of water at 30\(^\circ\). Allow to stand for 30 minutes (longer does no harm), then place the tube in boiling water for 90 seconds, remove, and cool in cold water to room temperature.

It is good practice to redisperse the precipitated reagent through the solution by gentle shaking at least once during the 30 minute incubation period. The 90 second heating in boiling water dissolves excess reagent, leaving a clear solution.

**Color Measurement**—Transfer the colored solution to a suitable container and read in a photoelectric colorimeter, using a filter which has a peak transmission at about 560 m\( \mu \). This filter was

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3 This material has now been identified as acetaldehyde (16).
selected on the basis of the spectrophotometric data of Block and Bolling (7), and has proved completely satisfactory. Because of the composite nature of the color (i.e., appreciable light absorption in various portions of the spectrum), it is important that a filter with a spectral band not over 50 to 60 m\(\upmu\) wide be used. Both the Rubicon No. 565 and the Klett No. 56 filters fulfil this condition.

For the initial setting of the colorimeter, either sulfuric acid alone or the reagent blank may be used. The latter procedure corrects the unknown reading for the value of the reagent blank, while the former does not. An alternative procedure for correcting for the value of the reagent blank is described in detail below.

Calculation of Results—The lactate concentration of the unknown may be obtained from the colorimeter reading by reference to a previously prepared calibration curve relating colorimeter readings and known concentrations of lactate. If such a curve is to be used, the standard lactate solutions should be put through the copper-calcium procedure before the color development is run. It is also obvious that the analytical conditions must be rigorously controlled with respect to every detail if future analyses are to be related to a previously prepared calibration curve.

A more convenient procedure for obtaining results is to make use of the strict adherence to Beer's law by the analytical system under the conditions described above. With the Evelyn (10) colorimeter (and also others equipped with a 0 to 100 linear scale and employing an equivalent depth of solution), a straight line is obtained over a concentration range from 0 to about 5 \(\gamma\) of lactate per cc. if instead of plotting the galvanometer reading \(G\) against lactate concentration, the value of \(2 - \log G\) is used. This line passes through the origin when a reagent blank tube is used for adjusting the colorimeter to the 100 reading, and the \(2 - \log G\) values then become directly proportional to the lactate concentration. The proportionality factor is readily established by careful analysis of known lactate solutions, and once it is known, the lactate concentration of an unknown solution is obtained by multiplying its \(2 - \log G\) value by the proportionality factor.

With the Summerson (11) colorimeter, the logarithmic scale makes the calculation of results somewhat simpler, since the scale
readings themselves are directly proportional to concentration for systems which adhere to Beer's law. With the reagent blank tube set at 0, the scale reading for a standard lactate solution divided into the lactate concentration gives the proportionality factor directly.

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Range of proportionality between color intensity and concentration of lactic acid, as obtained with the Evelyn (EV) and Klett-Summerson (K-S) photoelectric colorimeters. For the Evelyn calorimeter, color intensity is proportional to $2 - \log G$, where $G$ is the galvanometer reading. The reason for the differences in slope and range between the two colorimeters is given in the text.

The range of proportionality for both the Evelyn and Klett-Summerson photoelectric colorimeters is shown in Fig. 1. The data were obtained by the analysis of solutions of known lactate content, prepared from both lithium lactate and zinc lactate. For the Evelyn colorimeter the data are expressed in terms of the $2 - \log G$ values, while for the Klett-Summerson colorimeter the scale readings are plotted directly. Both curves have been corrected for the reagent blank values.
The difference in slope between the two curves is due to the use of narrower test-tubes in the Klett-Summerson instrument than are used in the Evelyn instrument. The range of satisfactory proportionality is therefore greater for the former colorimeter, but the sensitivity in terms of scale divisions per microgram of lactate is correspondingly less. For a particular colorimeter and color filter, the slope of the curve, and hence the proportionality factor, is reasonably constant. Any serious deviation from previous values should be reinvestigated.

The desirability of calculating results with the photoelectric calorimeter in terms of the reading of a simultaneously prepared standard instead of by reference to a previously prepared calibration curve has been discussed elsewhere (11). In the present instance, this may be done by running a standard and a reagent blank along with each series of unknowns, and calculating the proportionality factor as described above. An alternative procedure, which has been much practiced and which does not make the accuracy of the results depend upon the precision with which the relatively small reagent blank may be established, is to omit the reagent blank determination and to run two standards, at different levels, in each series of unknowns. The difference in reading between the two standards enables the proportionality factor to be calculated, and from this and the reading of either standard the "apparent blank" is obtained. An example of this calculation is given below. The data are in terms of the readings on the Klett-Summerson colorimeter, but the principle is equally applicable to instruments with which the $2 - \log G$ value must be used.

Example

| Reading of unknown | 170 |
| " 2γ standard | 95 |
| " 4" | 175 |

Therefore $2γ = 80$ scale divisions

and $4 = 40$

"Apparent blank" = 95 - (2 × 40) = 15

Unknown reading minus blank = 170 - 15 = 155

Lactate concentration of unknown = 155/40 = 3.88γ

Proof of Method—The three commonly accepted criteria of an analytical procedure are that the values obtained correspond well
with those obtained by other procedures, that added material is completely recovered, and that the procedure has a high degree of specificity for the substance in question. Evidence bearing on these points is presented below.

Fig. 2 shows a comparison on a variety of samples between the colorimetric method described here and the titrimetric method of Friedemann, Cotonio, and Shaffer (1). The agreement is within ±5 per cent in general, with a few values falling in the ±10 per cent range. It should be pointed out, however, that in our experience the latter is the range of reliability of the titrimetric procedure, and may not entirely represent a discrepancy between the amount of lactic acid as determined by the two methods.

The inset in Fig. 2 shows a similar comparison between the
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colorimetric method and the manometric method of Warburg (12), as applied to the determination of the lactic acid production by tumor tissue in the presence of glucose under anaerobic conditions. The quantities of lactic acid concerned here were too small for analysis by the titrimetric method. By the manometric method acid production is measured in terms of the decomposition of bicarbonate, and the method is not specific for lactic acid. However, Negelein (cf. Warburg (12)) has shown that the only acid which comes into question under these conditions is lactic acid.

The recovery of lactate added to a variety of analytical samples is shown in Table I. Quantitative recovery of amounts from 4 to 200 mg. per cent was obtained, even when the added lactate was as little as one-fourth of that already present. This fully covers the concentration range encountered under most conditions, but undoubtedly even higher values can be handled satisfactorily by proper dilution.

### Table I
Recovery of Added Lactic Acid

<table>
<thead>
<tr>
<th>Tissue filtrate</th>
<th>No. of analyses</th>
<th>Lactic acid added</th>
<th>Lactic acid originally present</th>
<th>Lactic acid recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>20.0</td>
<td>25</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>50.0</td>
<td>25</td>
<td>50.9</td>
</tr>
<tr>
<td>Heart</td>
<td>4</td>
<td>50.0</td>
<td>10-35</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>75.0</td>
<td>10-35</td>
<td>74.3</td>
</tr>
<tr>
<td>Entire rat carcass</td>
<td>3</td>
<td>50.0</td>
<td>150-200</td>
<td>50.7</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>8</td>
<td>100.0</td>
<td>150-200</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>200.0</td>
<td>150-200</td>
<td>207.5</td>
</tr>
<tr>
<td>Blood, dog</td>
<td>3</td>
<td>4.0</td>
<td>25</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.0</td>
<td>10</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.0</td>
<td>10-15</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.0</td>
<td>60</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20.0</td>
<td>10-15</td>
<td>18.9</td>
</tr>
<tr>
<td>Blood, cat</td>
<td>3</td>
<td>5.0</td>
<td>20</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.0</td>
<td>15</td>
<td>9.7</td>
</tr>
</tbody>
</table>

The agreement between the colorimetric and manometric methods is just as definite as with the titrimetric method.
The results of specificity tests on a considerable number of organic compounds are summarized in Tables II and III. For these tests, a solution of the substance containing 25 \( \gamma \) per cc. was examined with regard to both the production of a significant color under the analytical conditions and interference with the determination of lactate. This concentration is from 5 to 10 times greater than that of lactate in the usual aliquot taken for color development, and is equivalent to a concentration of 250 mg. per cent at a 1:100 dilution.

The compounds were selected from the point of view not only of their possible occurrence in biological fluids but also of their use as experimental variables in tissue metabolism studies. The data of Tables II and III demonstrate that the complete analytical

<p>| Table II |</p>
<table>
<thead>
<tr>
<th>Substances Which Do Not Interfere with Analysis for Lactic Acid under Conditions Specified in Text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
</tr>
<tr>
<td>Acetone</td>
</tr>
<tr>
<td>Adenine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Allantoin</td>
</tr>
<tr>
<td>( \alpha )-Aminobutyric acid</td>
</tr>
<tr>
<td>Arabinose</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>Asparagine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Caffeine</td>
</tr>
<tr>
<td>Carnosine</td>
</tr>
<tr>
<td>Choline</td>
</tr>
<tr>
<td>Citric acid</td>
</tr>
<tr>
<td>Creatine</td>
</tr>
<tr>
<td>Creatinine</td>
</tr>
<tr>
<td>Crotonic acid</td>
</tr>
<tr>
<td>Cystine</td>
</tr>
<tr>
<td>Diphosphoglyceric acid</td>
</tr>
<tr>
<td>Dulcitol</td>
</tr>
<tr>
<td>Ergothioneine</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
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procedure is highly specific for lactic acid, if the copper-calcium treatment is routinely included. Of the compounds which are listed as not interfering with the method, in Table II, many give significant amounts of color if the copper-calcium treatment is omitted, but all are adequately removed by this treatment under the conditions specified. It must be mentioned, however, that

<p>| Interfering Substances Which Copper-Calcium Treatment Will Remove Only at Low Concentration or Will Not Remove at All |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Color obtained</th>
<th>Maximum allowable amount for complete removal, γ per cc. final solution</th>
<th>Color yielded by 1 γ in terms of γ of lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removed at low concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>Similar to lactate</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>&quot; &quot; &quot;</td>
<td>10</td>
<td>0.005</td>
</tr>
<tr>
<td>p-Hydroxyphenyllactic acid</td>
<td>&quot; &quot; &quot;</td>
<td>15</td>
<td>0.005</td>
</tr>
<tr>
<td>Malic acid</td>
<td>&quot; &quot; &quot;</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>&quot; &quot; &quot;</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>&quot; &quot; &quot;</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>Not removed at all</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Similar to lactate</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td>Acrolein</td>
<td>&quot; &quot; &quot;</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>&quot; &quot; &quot;</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>&quot; &quot; &quot;</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Djenkolic acid</td>
<td>Blue-green</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-Hydroxybutyric acid</td>
<td>Blue-gray</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>(See text)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the copper-calcium treatment appears to depend for its effectiveness in some instances on a process of adsorption rather than precipitation, and the completeness of removal at higher concentrations than that specified should be established by experiment.

This is illustrated by the data of Table III, which lists those substances which the copper-calcium treatment will remove completely at low concentrations only, or will not remove at all. For
substances in the former class, the limiting concentration for complete removal is given, as well as the color value in terms of lactate. From these data the extent of possible interference under particular experimental conditions may be evaluated.

Those interfering substances which are not removed at all by the copper-calcium treatment are, with the exception of methionine and propylene glycol, readily distinguishable from lactate in that they either react directly with \( p \)-hydroxydiphenyl in the cold (which lactate does not) or they yield a final color which is distinctly different from the characteristic violet of the acetaldehyde reaction. The question of their occurrence in an analytical sample may therefore be readily answered. No interference of this type has ever been noted in biological samples except in the case of the substance present in red blood cells which was mentioned above and which was detected because of its ability to yield a color in the cold.

Propylene glycol is indistinguishable from lactate except for its relative color intensity per microgram, as given in Table III. As far as we know, the presence of significant amounts of propylene glycol in biological material is not to be expected. If it has been added to the system as an experimental variable, it will interfere to the extent indicated by its relative color value.

The effect of methionine on the analytical procedure is quite unusual. In the presence of small amounts of free methionine, lactate (and acetaldehyde) solutions give definitely less color than expected. The decrement in color is proportional within certain limits to the amount of methionine present; amounts of the same order of magnitude as the lactate present are readily detected, and larger amounts lead to complete inhibition of color formation. The nature of this interference is still under investigation, but it appears at the present time to be due to a reaction in concentrated sulfuric acid between methionine (or decomposition products) and acetaldehyde, to produce a substance which does not react with \( p \)-hydroxydiphenyl. The reaction appears to be highly specific for methionine, and is at present being used as the basis of a method for the microdetermination of methionine. The details of this method will appear shortly.

From the point of view of lactic acid determination, interference by methionine is not regarded as significant under ordinary condi-
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tions, since the occurrence of free methionine in a sample in
amounts equivalent to lactate would be rather unusual. Furthermore, neither the titrimetric nor the manometric lactic acid
method is interfered with in a similar manner by methionine,
and the degree of correlation between these methods and the
colorimetric method which has already been shown in Fig. 2
indicates that the colorimetric method gives accurate results
under all ordinary conditions.

DISCUSSION

Analytical Precautions—Few precautions with regard to the
reagents appear to be necessary. The sulfuric acid should be
of reagent quality; acid from a number of different sources
has proved equally satisfactory. The acid should be protected
from contamination by organic matter while standing in the
laboratory, and since the amount of acid must be measured exactly
during the analysis, it is best dispensed from a burette. It is
convenient to arrange the burette so that it may be filled directly
from the acid bottle by suction, or a burette with an attached
reservoir may be used. All stop-cocks which come in contact
with acid must be free from grease, so that a "greaseless" stop-
cock or an ordinary one lubricated by a small amount of the acid
itself must be used.

The Eastman Kodak Company grade of p-hydroxydiphenyl
may be used directly without further purification by recrystal-
lation. One recrystallization from alcohol or acetone removes
material which gives a small amount of color in alkali, but no
significant differences in analytical performance have been noted
between the purified and unpurified material. The reagent
solution is prepared by dissolving the dry material in 5 per cent or
10 per cent sodium hydroxide by stirring and warming and then
diluting to a final alkali concentration of 0.5 per cent. The solu-
tion keeps indefinitely at room temperature in a stoppered brown
bottle, and is conveniently dispensed from a pipette attached to
the stopper and known to deliver 2 drops per 0.1 cc.

The U.S.P. or C.P. grades of calcium hydroxide are equally satis-
factory for use in the preliminary copper-calcium procedure.

We do not find it necessary to cool the solution during the addi-
tion of the sulfuric acid, nor are stoppered tubes required during
the heating period which converts lactate to acetaldehyde. The time of heating at this stage is not critical, identical results being obtained with heating periods of from 3 to 10 minutes. Longer heating than 10 minutes may lead to slight losses. The 4 per cent copper sulfate solution is added to the system before heating merely as a matter of convenience, since it does not affect the conversion of lactate to acetaldehyde in any way.

The color reaction is not significantly affected by a slight excess of either the reagent or of copper ions. Color development is complete in 30 minutes at about 30°, and longer standing at this stage does not affect the results. The curve relating temperature and color intensity is almost flat between 20-30°, temperatures outside of this range giving less color. This is particularly true of temperatures above 35°, owing to rapid destruction of the reagent. It is therefore important that the solution be well cooled after the 5 minute heating period and before the addition of the reagent, as otherwise low results will be obtained. The color of the mixture during the 30 minute incubation period is usually a blue-violet, and the appearance of a red-violet color at this stage, coupled with the disappearance of the precipitated reagent, is indicative of too high a temperature.

Heating the solution for 90 seconds, after color development is complete, is for the purpose of clarifying the solution by destroying excess reagent, and stabilizing the color. Heating from 1 to 2 minutes at this stage does not affect results, but longer heating changes the spectral characteristics of the color, making it unsuitable for measurement at 560 m. After the heated solution has been cooled, the color is quite stable for some time, a decrease of only about 5 per cent being noticeable after 3 hours at room temperature.

A major source of error appears to be contamination of the sample by lactic acid itself, from the skin surfaces. It should be remembered that the standard solutions contain only a few parts per million of lactic acid. If a test-tube of distilled water is closed with the palm of the hand and shaken vigorously for a few minutes, the resulting solution will contain about as much lactate as such a standard. Duplicate analyses on a given copper-calcium filtrate are expected to agree within 2 per cent or less. When deviations greater than this have been obtained, and the analyses
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repeated, the results have invariably indicated the presence of excess lactate as the source of error. Precautions against this type of contamination include the use of glass-stoppered vessels for aqueous solutions which are to be mixed or shaken, or the mouth of the vessel may be closed with a small square of Parafilm (fresh surface down) held in place by the finger tip. For wiping off the outside of pipettes, individual pieces of fresh cleansing tissue are recommended, and glassware should not be drained in contact with surfaces which are apt to be contaminated by dust or perspiration. These precautions may appear to be extreme, but experience has demonstrated their value.

Other possible sources of error include inadequate mixing of the water and the viscous sulfuric acid during the analysis, and the presence of traces of chromic acid (from cleaning mixture) on the glassware. Glassware which has been cleaned with chromic acid should be passed through an alkaline wash solution (soapy water, calgonite) before being rinsed and dried. With regard to adequate mixing, the use of wide test-tubes as specified will aid in this respect. Particular attention should be paid to mixing well after the addition of the p-hydroxydiphenyl, since this substance is quite insoluble in the concentrated acid mixture and must be uniformly dispersed if quantitative results are expected.

Effect of Inorganic Ions on Color Development—The effect of inorganic ions on the color reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid has been studied in the light of the observation recorded above that, while neither ferrous nor ferric iron alone is of appreciable analytical value, a suitable mixture of the two enhances color development significantly. Furthermore, the amounts of iron or copper which are necessary for optimal color development are several hundred times greater than the amount of acetaldehyde present and approach the order of magnitude of the p-hydroxydiphenyl concentration. These facts indicate that the effect of added ions is primarily on the p-hydroxydiphenyl itself, and that the action involves oxidation-reduction equilibria of some kind.

We therefore carried out some measurements of the E.M.F. of the p-hydroxydiphenyl-sulfuric acid mixture against a platinum electrode in the presence of those ions which we found to be of significance. The results obtained are illustrated by the data...
of Fig. 3. The left-hand section of Fig. 3 gives data on the relative color intensity and observed E.M.F. in the presence of varying proportions of ferrous and ferric ions. The right-hand section shows the influence of a single type of added ion, either ferrous, ferric, or cupric.

![Fig. 3](image)

**Fig. 3.** The influence of added inorganic ions on the intensity of color obtained in the lactic acid method, and on the E.M.F. as measured against a platinum electrode. The left-hand chart gives data for mixtures of ferrous and ferric ions, while the right-hand chart refers to ferrous, ferric, or cupric ions alone. The symbol G refers to the appearance of a green color rather than the characteristic violet of the acetaldehyde reaction.

The electrometric measurements were made with a smooth platinum electrode, a mercurous sulfate-saturated potassium sulfate half-cell as reference electrode, and a vacuum tube voltmeter. The system investigated consisted of 6 parts of concentrated sulfuric acid and 1 part of water containing a few micrograms of acetaldehyde (prepared from lactate by previous treat-
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ment with hot concentrated sulfuric acid) and the inorganic ion or ions to be studied, together with 1.5 mg. of p-hydroxydiphenyl in suspension. In one series of measurements the p-hydroxydiphenyl was omitted (curve labeled "without reagent" in Fig. 3). Liquid junction with the reference electrode was effected through a layer of saturated potassium sulfate solution over a restricted portion of the surface of the sulfuric acid-acetaldehyde mixture.

Readings on each solution were begun immediately after the p-hydroxydiphenyl was added and the liquid junction effected, and were taken at intervals until constancy within 1 millivolt was obtained. This ordinarily required from 10 to 30 minutes, depending upon the experimental conditions. Since equilibrium was reached almost immediately in the absence of p-hydroxydiphenyl, it is concluded that the potentials measured are determined to a certain extent by the rate of solution of the insoluble reagent. Despite this variable factor, readings under a given set of conditions were readily reproducible from day to day, even with regard to the rate of attainment of equilibrium. The least stable system electrometrically was that containing p-hydroxydiphenyl without added ions, the potentials in this case having varying initial values and rarely reaching even approximate constancy. The uncertainty with regard to this point in Fig. 3 is indicated by the broken sections of the curves in the right-hand portion of the figure.

The electrometric data are plotted in terms of the observed E.M.F. of the system rather than by reference to the normal hydrogen electrode, since neither the electrode reaction nor the liquid junction potential or pH of the solution is known. The difficulties associated with the precise interpretation of potentiometric measurement in concentrated acid solution have been pointed out by Michaelis, Schubert, and Grunick (13).

Nevertheless, the data present certain significant points. We note a particular correlation between the observed values of the E.M.F. and the relative color intensity. Maximum color intensity with ferrous-ferric ion mixtures corresponds to an E.M.F. of between -10 and -30 millivolts, and it is when this zone is reached by the addition of cupric ion that color intensity in the presence of this ion corresponds to that obtained with ferrous-ferric ion mixtures. Neither ferrous nor ferric ion alone gives an E.M.F. falling within this zone, and the color intensity under these condi-
tions is proportionately low. In the absence of added ions, the E.M.F., while quite variable, as stated above, always passes through the zone in question, and this is probably why any color at all is obtained under these conditions.

The data also demonstrate why copper is superior to iron for color enhancement. In the presence of variable amounts of cupric ion the E.M.F. is relatively stable within the zone associated with maximal color values, even though the color obtained with copper is not quite as great as when an optimal mixture of ferrous and ferric iron is used, so that a slight excess or deficit of cupric ion is without significance. For the ferrous-ferric mixture, on the other hand, the problem of how to maintain the proper ratio between the two ions becomes of importance, and an excess of ferric ion will produce a green color rather than the characteristic violet of the acetaldehyde reaction.

We postulate therefore an oxidation-reduction equilibrium of some kind between p-hydroxydiphenyl and certain inorganic ions, with the color reaction between this reagent and acetaldehyde markedly dependent upon the maintenance of the proper equilibrium conditions. We plan a further study of this phenomenon, particularly with regard to defining more precisely the nature of the reactions involved, since the effect of inorganic ions noted here appears to be but one instance of a general condition. For instance, Dische and Laszlo (14) have found that cupric ions promote color development between hydroquinone and acetaldehyde in concentrated sulfuric acid; Winkler (15) enhances color development in the Hopkins-Cole reaction for tryptophane by the addition of cupric ions; and we have noted that the Hopkins-Cole reaction is likewise susceptible to color enhancement in the presence of a suitable mixture of ferrous and ferric ions.

SUMMARY

A method for the colorimetric determination of lactic acid in biological material is described, in which lactic acid is converted into acetaldehyde by treatment with concentrated sulfuric acid, and the acetaldehyde determined by its color reaction with p-hydroxydiphenyl in the presence of cupric ions. The color is read in a photoelectric colorimeter with a filter having a peak transmission at 560 mμ.
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The method is applicable to a variety of biological material, after deproteinization by any of the standard procedures. Comparative analyses by other methods, as well as specificity studies on a large number of pure compounds, indicate that the procedure has a high degree of specificity for lactic acid. The sensitivity of the method is such that color development is carried out on a portion of sample containing not over 5 to 10 γ of lactic acid per cc., with differences of less than 0.1 γ per cc. readily detectable.

An electrometric study of the reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid indicates that this reaction is dependent upon the establishment of an oxidation-reduction equilibrium of some kind, and that optimal conditions for color development are only obtained in the presence of certain inorganic ions. Data are presented showing the relation between color development and the E.M.F. against a platinum electrode in the presence of these ions.

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THE COLORIMETRIC DETERMINATION OF LACTIC ACID IN BIOLOGICAL MATERIAL
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