An Enzymatic-Spectrophotometric Determination of Pyruvic and Lactic Acid in Blood

Methodologic Aspects

J. C. Rosenberg* and B. F. Rush

Investigation of an enzymatic-spectrophotometric determination for lactic and pyruvic acid in blood has confirmed its suitability and reliability. The precision of both methods is high. No variability is encountered in the pyruvic acid analysis. Less than 5% variation is found with the lactic acid analysis. The recovery in both procedures approaches 100%.

MANY clinical and experimental problems concerning anaerobic metabolism and the accumulation of an oxygen debt have recently been studied, using Huckabee’s concept of “excess lactate” (1). Colorimetric methods have most often been used to measure pyruvic and lactic acid in blood (2, 3). The estimation of these two metabolites by an enzymatic analysis has advantages—e.g., specificity and ease of performance—that would make such methods more suitable than determinations based on colorimetry. The following study was undertaken to delineate the linearity, sensitivity, precision, reproducibility, and recovery of an enzymatic-spectrophotometric analysis of pyruvic and lactic acid in whole blood.

Material and Methods

Principle

Lactic acid dehydrogenase (LDH), in combination with the reduced form of nicotinamide adenine dinucleotide (NADH₂), is responsible for the reduction of pyruvic acid to lactic acid; thus

\[ \text{NADH}_2 + \text{pyruvic acid} = \text{NAD}^+ + \text{lactic acid} \]
Although the reaction is reversible, the conversion of pyruvic to lactic acid predominates \((k, 10^{12})\). Thus, the equilibrium is well suited for the analysis of pyruvic acid. For the determination of lactic acid, the equilibrium can be completely displaced toward pyruvic acid in 3 ways: (1) an alkaline medium; (2) an excess of \(\text{NAD}^+\); and (3) removal of the end product of the reaction, pyruvic acid, by a carbonyl-binding substance such as semicarbazide. On the basis of the above stoichiometric relationship of \(\text{NADH}_2\) concentration and lactic and pyruvic acid concentration, and by measuring the change in absorbance of the \(\text{NADH}_2\) at 340 mm\(\mu\) in the reaction mixture, an assay system for these two metabolites can be constructed.

**Reagents**

*Perchloric acid, 3% and 6% (v/v)*

*Pyruvic acid standards* These are prepared by dissolving 110.1 mg. of crystalline sodium pyruvate (Calbiochem) in 1000 ml. of 3% perchloric acid. Solutions of 0.1, 0.04, and 0.02 mmole/L. are prepared with 3% perchloric acid, to establish a calibration curve. The stock standard is kept at 5\(^\circ\).

*Lactic acid standards* These are prepared from a solution of free L(+)-lactic acid (Mann Research Chemicals which contains 41.5 gm. of lactic acid per 100 gm. of solution. A stock standard of 50 mmole/L. is prepared by weighing out 2.17 gm. and bringing the volume to 200 ml. with 3% perchloric acid. Solutions of 1.0 mmole/L., 2.0 mmole/L., and 3.0 mmole/L. are prepared with 3% perchloric acid and used to establish a calibration curve. The stock standard is kept at 5\(^\circ\).

*2N \(\text{NaOH}\)*

*0.02 M nicotinamide adenine dinucleotide* \(\text{NAD}^+\) (Sigma Chemical Company, 98% pure) is prepared daily in sufficient quantities for the number of samples to be analyzed that day. Usually, 266 mg. of \(\text{NAD}^+\) is dissolved in 10 ml. of distilled water and the pH brought from 3.0 to 6.0 by carefully adding 4–5 drops of 2N NaOH. A pH meter is used to monitor the pH. If the \(\text{NAD}^+\) solution is inadvertently made alkaline, it is discarded because \(\text{NAD}^+\) is very unstable in weak alkaline solutions. The final volume is brought to 20 ml.

*0.2 M Glycine-semicarbazide buffer* This is also prepared daily by dissolving 1.50 gm. of glycine (General Biochemicals, NRC grade) and 2.2 gm. of semicarbazide hydrochloride (Fisher Scientific Co.) in 80 ml. of distilled water. The pH of the solution is brought to 10.0 with 2N NaOH (about 10 ml.) using a pH meter and a magnetic stirrer. The solution is then diluted to 100 ml.
1.1 M Potassium phosphate-potassium hydroxide The solution is prepared by adding 18.5 gm. of K$_2$HPO$_4$ (Fisher Scientific Co.) to 90 ml. of distilled water and then adding 10 ml. of 50% (w/v) KOH. This solution is kept at 5°.

0.1 M phosphate buffer, pH 7.5 The buffer is prepared by mixing 84.1 ml. of 0.1 M Na$_2$HPO$_4$ and 15.9 ml. of 0.1 M KH$_2$PO$_4$.

Reduced nicotinamide adenine dinucleotide The NADH$_2$ (Sigma Chemical Company; 95% pure) is stored at 5°. It is protected from light and moisture by keeping the vial in an air-tight and water-tight bag which also contains anhydrous calcium chloride particles. Immediately prior to use, a sufficient quantity of NADH$_2$ is weighed out and added to the 0.1 M phosphate buffer to make a concentration of 1 mg. NADH$_2$ per 1.0 ml. of phosphate buffer.

Lactic acid dehydrogenase LDH (Sigma Chemical Company) is obtained from rabbit muscle as a crystalline suspension in ammonium sulfate. The enzyme concentration, usually supplied by the company, contains 65 mg. of protein per milliliter (1 mg. will convert approximately 60 mmoles of NADH$_2$ to NAD$^+$ at pH 7.5 and 37°. The enzyme is diluted to a concentration of 2 mg. of protein per milliliter with distilled water (for lactic acid analysis) or to a concentration of 5 mg./ml. with 0.1 M phosphate buffer (for pyruvic acid analysis) within 72 hr. of being used. It is stored at 5° at all times.

Procedure

Collection of Blood

Ground-glass stoppered, 12-ml. graduated centrifuge tubes containing 6 ml. of 6% perchloric acid are brought to 5°. Six milliliters of blood is obtained without stasis and allowed to flow directly into the centrifuge tube containing perchloric acid. The tubes are immediately shaken and centrifuged for 10 min. at 3000 r.p.m. The total volume and the volume of the supernate are noted and recorded. Occasionally the supernate has to be recentrifuged.

Pyruvic Acid

Three milliliters of the protein-free supernatant fluid, standards, and 3% perchloric acid as a blank are transferred to test tubes to which 1.0 ml. of the K$_2$HPO$_4$-KOH solution is added. The mixture is placed in an ice bath for 15 min. to allow the potassium perchlorate to precipitate. Two milliliters of the clear supernatant fluid is decanted while still cold and placed in 1-cm. square cuvets which are suited for use
with an ultraviolet photometer (Calbiometer*) designed to measure the changes in absorbance of the nicotinamide adenine dinucleotides at 340 m\(\mu\). The supernatant fluid is allowed to warm to room temperature by standing for 15–20 min. Phosphate buffer (0.7 ml.) and the buffered NADH\(_2\) solution (0.2 ml.) are then added to the cuvet, and the absorbance determined and recorded as \(R_1\). The LDH preparation (0.1 ml.) containing 5 mg./ml. of protein in 0.1 M phosphate buffer is added to the cuvet. A second reading is obtained 1 min. later (\(R_2\)).

**Lactic Acid**

The determination is carried out in 1-cm.-square cuvets suited for use with the Calbiometer. Added to the cuvets are 2.8 ml. of the glycine-semicarbazide solution, 0.6 ml. of the NADT solution, and 0.2 ml. of the sample, standard, or blank. A blank, consisting of 0.2 ml. of 3% perchloric acid, and standards are included with every determination. The tubes are inverted 5 times after being covered with Parafilm, and the absorbance determined (\(R_1\)). LDH (0.4 ml.) is added to the cuvet, and the mixture is placed in a water bath at 40°. After 1 hr. of incubation, the absorbance is again determined (\(R_2\)).

**Calculations**

**Pyruvic Acid**

The net decrease in absorbance of NADH\(_2\) which results from the reduction of pyruvic acid to lactic acid is obtained from the following formula:

\[
A = 0.97R_1 - R_2
\]

The reagent blank does not change significantly in the pyruvic acid determination and need not be considered. After the determination of \(A\), the amount of pyruvic acid per 100 ml. of blood is determined by the simple calculation for concentration.

**Lactic Acid**

The net absorbance of the NADH\(_2\) formed as a result of the enzymatic dehydrogenation of lactic acid to pyruvic acid is obtained from the following formula:

\[
A = (R_2 - 0.9R_1) - (B_2 - 0.9B_1)
\]

Where \(A\) indicates net absorbance; \(R_1\), the first reading of the sample; \(R_2\), the second reading of the sample, after incubation with LDH; \(B_1\), the first reading of the blank; and \(B_2\), the second reading of the blank, after incubation with LDH.

*Calbiochem, Los Angeles, Calif.
After the determination of $A$, the amount of lactic acid per 100 ml. is determined by the simple calculation for concentration.

**Results**

**Pyruvic Acid**

**Linearity and Sensitivity**

Figure 1 illustrates the linear relationship between pyruvic acid concentration and absorbance at 340 m$\mu$ as determined by the method described herein. If concentrations greater than 1.0 mmole/L are anticipated, the deproteinized sample should be diluted with 3% perchloric acid. Blood pyruvic acid concentrations as low as 0.01 mmole/L. can be easily measured.

**Precision and Reproducibility**

Virtually no variation was observed in aqueous solutions or deproteinized venous blood samples (Tables 1 and 2). Unlike the lactic acid determination, the blank did not absorb at 340 m$\mu$. A 6% difference in

![Graph: Standard curve for pyruvic acid determination.](image)

<table>
<thead>
<tr>
<th>Concentration (mmole/L)</th>
<th>Mean absorbance*</th>
<th>Standard deviation</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.100</td>
<td>0.252</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.090</td>
<td>0.236</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.048</td>
<td>0.122</td>
<td>±0.003</td>
<td>1.4</td>
</tr>
<tr>
<td>0.040</td>
<td>0.098</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.020</td>
<td>0.049</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blank</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Of 6 replicates for all concentrations of pyruvic acid; of 12 replicates for blank.
Table 2. Precision of Pyruvic Acid Determination in Venous Blood

<table>
<thead>
<tr>
<th></th>
<th>Exp. A</th>
<th>Exp. B</th>
<th>Exp. C</th>
<th>Exp. D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean concentration (mmole/L.)</td>
<td>0.063</td>
<td>0.070</td>
<td>0.071</td>
<td>0.060</td>
</tr>
<tr>
<td>No. of replicates</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0</td>
<td>0</td>
<td>±0.0009</td>
<td>0</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

concentration was found when the same blood sample was analyzed 4 days apart.

Recovery

Two pooled canine blood samples were collected with perchloric acid. To each of several replicates, 0.100 mmole of lactic acid was added to the supernate (Table 3). The mean recovery was 100 ± 5%.

Lactic Acid

Linearity and Sensitivity

In order to determine the optimal range of lactic acid concentrations which could be measured by this method, aqueous lactic acid standard solutions were prepared ranging from 0.02 to 10 mmole/L. Concentrations greater than 3.0 mmole/L. had absorbances greater than 1.0 and could not be reliably determined. Concentrations less than 0.2 mmole/L. were too close to the blank to be significant. Over a range of concentrations from 0.2 to 3.0 mmole/L. an acceptable calibration curve was obtained (Fig. 2).

Table 3. Recovery of Pyruvic Acid from Venous Blood

<table>
<thead>
<tr>
<th>Amount added (mmole)</th>
<th>Amount recovered (mmole)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENT A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.105</td>
<td>105</td>
</tr>
<tr>
<td>0.10</td>
<td>0.103</td>
<td>103</td>
</tr>
<tr>
<td>0.10</td>
<td>0.099</td>
<td>99</td>
</tr>
<tr>
<td>0.10</td>
<td>0.103</td>
<td>103</td>
</tr>
<tr>
<td>0.10</td>
<td>0.105</td>
<td>105</td>
</tr>
<tr>
<td>EXPERIMENT B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.107</td>
<td>107</td>
</tr>
<tr>
<td>0.10</td>
<td>0.099</td>
<td>99</td>
</tr>
<tr>
<td>0.10</td>
<td>0.097</td>
<td>97</td>
</tr>
<tr>
<td>0.10</td>
<td>0.096</td>
<td>96</td>
</tr>
<tr>
<td>0.10</td>
<td>0.094</td>
<td>94</td>
</tr>
<tr>
<td>0.10</td>
<td>0.095</td>
<td>95</td>
</tr>
<tr>
<td>MEAN AND S.D.</td>
<td></td>
<td>100.3 ± 5</td>
</tr>
</tbody>
</table>
Precision and Reproducibility

The coefficient of variation among 10 replicates of aqueous solutions containing 1.03 mmole/L, 1.72 mmole/L, and 2.58 mmole/L, was 4.4%, 3.6%, and 1.4% respectively (Table 4). The coefficient of variation of the blank was 14%. Two experiments on the same pooled canine venous blood sample were carried out 4 days apart (Table 5). The coefficient of variation was 2.6% and 3.4%. A 9% difference was found between the 2 values obtained.

Recovery

A pooled canine venous blood sample was collected with perchloric acid. To each of 10 replicates of the supernate, 0.258 mmole of lactic acid was added. The mean recovery was 97.2 ± 3% (Table 6).

Discussion

Enzymatic analyses, particularly those systems linked to the coenzymes NAD⁺-NADH₂, have increased in number and popularity be-

![Fig. 2. Standard curve for lactic acid determination.](image)

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**Table 4. Precision of Lactic Acid Determination in Aqueous Solutions**

<table>
<thead>
<tr>
<th>Concentration (mmole/L)</th>
<th>Mean absorbance*</th>
<th>Standard deviation</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.58</td>
<td>0.704</td>
<td>±0.010</td>
<td>1.4</td>
</tr>
<tr>
<td>1.72</td>
<td>0.491</td>
<td>±0.018</td>
<td>3.6</td>
</tr>
<tr>
<td>1.03</td>
<td>0.291</td>
<td>±0.013</td>
<td>4.4</td>
</tr>
<tr>
<td>Blank</td>
<td>0.072</td>
<td>±0.010</td>
<td>14</td>
</tr>
</tbody>
</table>

*Of 6 replicates.
cause of the simplicity and specificity of such determinations (4). Warburg and Christian first described the reactions upon which the pyruvic and lactic-acid determinations are based and also demonstrated the favorable spectral characteristics of the nicotinamide adenine dinucleotides (5). Prior to this, cumbersome titration methods combined with the use of crude enzyme preparations had been used to measure lactic acid (6).

The feasibility of measuring pyruvic acid enzymatically was first shown by Kubowitz and Ott in 1943 (7). Although there had been previous reports in German of procedures suitable for the determination of pyruvic acid in blood, the paper of Segal et al. in 1956 made this information available in English and clearly demonstrated the practica-
bility of an enzymatic determination for pyruvic acid (8). The reliability of the method is confirmed by our studies. As many as 30 samples may be processed simultaneously. The only drawback we have found is the need to repeat determinations for high concentrations. We, therefore, routinely dilute the deproteinized sample with 3% perchloric acid in a ratio of 1:1. We have rarely found this to decrease the concentration below the sensitivity of the method.

Hess's investigations with the enzymatic analysis for pyruvic acid (9) led him to devise one for lactic acid which was an improvement over the one described a year previously by Pfleiderer and Dose (10). Reports on investigations of these methods were also confined to the German literature until recently, when Olson lucidly described the advantages of an enzymatic analysis for lactic acid (11).

Most of the previously described enzymatic methods for the determination of pyruvic and lactic acid in blood have utilized the absorbivity of \( \text{NADH}_2 \) to calculate the concentration of these metabolites. We have preferred to utilize standard curves and determine the absorbance of standard solutions of pyruvic and lactic acid whenever unknown samples are analyzed.

References