酵素法による毛細管血中乳酸測定

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Enzymatic determination of lactate in capillary blood.

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標準的な酸素法による乳酸測定法（Hohora）を改変し、微量血中の乳酸測定を行なった。採血をヘパリンコートした毛細管ビペットで行ない、血液を4容の0.6M過塩素酸（PCA）で除蛋白、PCA抽出液の中和を省略、乳酸脱水素酸素存在時と非存在時の25℃、60分反応後の340nmの吸光度を測定することにより、容易に、通常の分光光度計で（特殊な測定機器を用いず）微量血中の乳酸か測定可能であった。

ABSTRACT

Standard enzymatic method for lactate determination was modified: (1) the blood was deproteinized with 4 volume of 0.6 M perchloric acid (PCA), (2) neutralization of the PCA extract was omitted, and (3) the absorbance at 340 nm was measured only once after 60 min. incubation with and without lactate dehydrogenase. Lactate level could be calculated from the absorbance difference between with and without the enzyme. One reference (any sample incubated without the enzyme) was necessary for one series of measurement. Lactate level up to 15 mmoles/1 could be determined without varying the sample volume. Use of heparin-coated capillary pipet allowed easy and accurate sampling of 45 μl of blood from the earlobe. Lactate level in this volume of the sample could be determined with a standard spectrophotometer.

INTRODUCTION

Blood lactate is considered an indicator of tissue hypoxia caused by exercise or disease. As the indicator, particularly in the study of exercise, lactate in capillary blood may superior to that in venous blood since lactate is removed from the blood by aerobic tissues. Various methods for measurement of lactate have been reported. The principle of the most methods is either (1) spectro photometric or gas chromatographic determination of acetaldehyde to which lactate is converted by sulfuric acid or other oxidants, or (2) measurement of reduced nicotinamide adenine dinucleotide (NADH) formed in couple with enzymatic oxidation of lactate to pyruvate. The former method is sensitive and often used for measurement of lactate in capillary blood. However, it is cumbersome and not specific for 1-lactate. The enzymatic method is specific and relatively simple especially when NADH is directly (i.e., without further
reaction) measured with spectrophotometer. But usually 0.5-1.0 ml of blood in needed. With some modification of the standard enzymatic method (1) and use of heparin-coated capillary pipet, lactate in capillary blood can be determined accurately and simply with a standard spectrophotometer.

**METHODS**

**Sampling**: All subjects (18-35 years old) were healthy university students or staffs. To obtain capillary blood, ear lobe was punctured with a sterilized razor and oozing blood was drawn into a disposable heparin-coated capillary pipet (Microset 44 μl Clay Adams, Parsippany, N.J.). Venous blood was drawn from the antecubital vein in a vacuum tube containing 143 IU heparin-Na or in a syringe through an indwelling Teflon catheter (0.65 × 51mm) with “heparin-lock” (3).

**Reagents**: Nicotinamide adenine dinucleotide (NAD) (Grade I), lactate dehydrogenase (LDH) from rabbit muscle (5 mg/ml) were from Boehringer Mannheim-Yamanouchi (Tokyo). All other chemicals were reagent grade.

**Table 1. Lactate in the blood determined with or without neutralizing the PCA extract.**

<table>
<thead>
<tr>
<th>Sample or H₂O added (20 μl)</th>
<th>with neutralization</th>
<th>without neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J_{A_n} - J_{A_n}</td>
<td>total lactate found* (mmoles/l)</td>
</tr>
<tr>
<td>H₂O</td>
<td>0.103</td>
<td>1.02</td>
</tr>
<tr>
<td>0.025 M lactate</td>
<td>0.151</td>
<td>1.49</td>
</tr>
<tr>
<td>0.05</td>
<td>0.196</td>
<td>1.93</td>
</tr>
<tr>
<td>0.1</td>
<td>0.292</td>
<td>2.88</td>
</tr>
<tr>
<td>0.2</td>
<td>0.504</td>
<td>4.97</td>
</tr>
<tr>
<td>0.4</td>
<td>0.858</td>
<td>8.46</td>
</tr>
<tr>
<td>0.6</td>
<td>1.281</td>
<td>12.63</td>
</tr>
</tbody>
</table>

To 1 ml of heparinized venous blood, 20 μl of lactate solution or H₂O was added and extracted with 4 volumes of 0.6 M PCA. After neutralization of the PCA extract or without it, lactate level was determined with the standard method. J_{A_n}: absorbance difference of the sample, J_{A_b}: absorbance difference of the blank.

* Corrected for dilution caused by neutralization.

**Table 2. Absorbance at 340 nm after incubation with or without LDH.**

<table>
<thead>
<tr>
<th>LDH</th>
<th>Sample (n=40) (mean±SD)</th>
<th>0.6 M PCA (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>0.273 ± 0.004</td>
<td>0.258, 0.257</td>
</tr>
<tr>
<td>(+)</td>
<td>0.401 ± 0.034</td>
<td>0.255, 0.268</td>
</tr>
</tbody>
</table>

PCA extract (not neutralized) of blood from 40 subjects (18-24 years old, 36 males and 4 females) or 0.6 M PCA was mixed with hydrazine-glycine buffer and NAD and then incubated with (+) or without (-) adding LDH for 60 min. at 25°C. Not all subjects had been at complete rest before sampling.
Apparatus: Double beam spectrophotometer with a recorder (Hitachi 557, Tokvo) or single beam spectrophotometer with vacuum-operated sampling system (Hitachi 100-26, Tokyo) was used. The temperature of the cuvette holder was controlled with a circulation bath (Yamato BH-61, Tokvo) equipped with a cooling system (Yamato BD-11, Tokyo). The enzyme and the sample extracted with perchloric acid (PCA) was pipetted with Pipetman P-20 and P-200 (Gilson Villers-le-Bel, respectively).

Procedures: Forty-five µl of blood was mixed vigorously with 180 µl of ice-cold 0.6 M PCA in a small polystyrene test tube immediately after obtaining. After standing in ice for 10 min., supernatant was obtained with centrifugation at 1000 g and 4°C for 15 min. When neutralization of the PCA extract was done, 2.5 M K₂CO₃ was added and precipitated percholate was removed by centrifugation at 4°C.

One hundred µl of the deproteinized sample was mixed in a small glass test tube or cuvette with 1.0 ml of hydrazine-glucose buffer, pH 9.5, which was prepared following Hohorst (1) and whose temperature was made 25°C, and then 100 µl of 30 mM NAD. After adding 6 µl of LDH, the mixture was incubated at 25°C in water bath and the absorbance at 340 nm was read with a spectro-photometer after 60 min., or the changes of the absorbance was monitored with a recorder keeping the temperature of the cuvette holder at 25°C. In some experiments, the volume of the sample and the reagents was doubled.

Calculation: Concentration of lactate (C, moles/l) was calculated with the following equation (2):

\[ C = \frac{dA}{e \times d} \times \frac{V}{v} \times F \]

where \( dA \) is the absorbance difference, \( e \) the extinction coefficient for NADH (6.3 \times 10^3), \( d \) the light path length (10 mm), \( V \) mixture volume (1.206), \( v \) the sample volume (0.1 ml), \( F \) dilution factor (4.848, Footnote 1). Therefore, \( C = dA \times 0.00928 \) moles/l or \( dA \times 9.28 \) mmoles/l.

RESULTS AND DISCUSSION

Clear supernatant was always obtained after centrifugation of the mixture of the blood and 4 volumes of 0.6 M PCA (Footnote 2). When less volume of PCA, especially 2 volumes or less, was used, centrifugation or extraction had occasionally to be repeated. Thus, extraction of the sample with 4 volumes of 0.6 M PCA appears appropriate because both too much dilution of the sample and the reagents can be avoided.

When un-neutralized PCA extract was added to the buffer, pH lowered by 0.15 or less. Besides, when the lactate level was measured with or without neutralizing the PCA extract of the blood to which various amount of lactate had been added, there was no difference in the lactate level found or its recovery (Table 1). These indicate that neutralization can be omitted in the present experimental condition. Hence, somewhat cumbersome procedure (and some dilution of the sample) can be eliminated.

In the standard enzymatic method (1), absorbances before \( (A₁) \) and after \( (A₂) \) the incubation with LDH are obtained and lactate level calculated from the difference \( (dA = A₂ - A₁) \). Namely, the absorbance of each sample has to be measured at least twice. In the present method, when the samples were incubated without adding the enzyme, there was no difference in the absorbance among the samples of different lactate level (Fig. 1) or among the samples obtained from different persons (Table 2). And the absorbances were constant within 60 min. after addition of LDH (Fig. 1). Therefore, \( dA \) can be obtained from the difference of post-
incubation (60 min.) absorbance between with and without the enzyme. And only one reference (any sample incubated without LDC) is needed (Footnote 3). This means that measurement of the absorbance has to be done only once. It reduces not only the time necessary for the measurement but makes the application to a spectrophotometer with automatic sampling system much easier than the standard method. Moreover, the absorbance difference between with and without LDH may be a better index than pre- and post-incubation difference because the absorbance increased slightly even in the absence of LDH. We measured the blank (0.6 M PCA, instead of the sample, was added) also, but the ΔA of the blank was little and may be negligible.

Lactate level up to 15 mmoles/l could be determined without further dilution of the sample (data not shown). Therefore, in most cases in the present method, there will be no need to

![Figure 1](image1.png)

Figure 1: Various amount of lactate was added (A and A': 0 μmole, B: 1 μmole, C: 4 μmoles, D: 8 μmoles, E and E': 12 μmoles) to heparinized venous blood and extracted with 0.6 M PCA, and changes of the absorbance of the reaction mixture at 340 nm was recorded. In F and F', 0.6 M PCA was mixed instead of the deproteinized sample. LDH was added to A, B, C, D, E, and F at 0 min.. No LDH was added to A', E' and F'.

![Figure 2](image2.png)

Figure 2: Venous and capillary blood was obtained simultaneously from bilateral antecubital veins and left ear lobe. The subject lying supine on the examining table performed left forearm exercise on the hand ergometer (60 contractions/min. for 5 min. with approximately 20% of maximal power). No exercise was done with the right. ●: blood from the left antecubital vein. ○: blood from the right antecubital vein. ▲: capillary blood from the left ear lobe.
vary the volume of the sample which may have very high lactate content.

There was no difference in lactate level between caipillary and venous blood at rest (Footnote 4). However, when exercised, the level could differ remarkably as in the typical case shown in Fig. 2. Thus, in the study of exercise, caipillary blood lactate can be a better indicator of tissue hypoxia than venous blood lactate since changes of lactate may be larger in caipillary blood than in venous blood from non-exercising part while venous blood lactate may be greatly influenced by the exercise performed by the part of the body from which the sample is obtained.

With the use of heparin-coated caipillary pipet, no coagulation of the blood occurred and accurate sampling of caipillary blood was possible. Sampling from the same subject could be repeated without redoing the puncture for at least 1 hour because blood oozed out when the clotted blood on the wound was removed with ethanol-soaked cotton, wiped with sterilized gauze and the ear lobe pushed with fingers.

TEXT FOOTNOTES

1. Specific gravity of blood is 1.06 and water content 80 %. Therefore, the dilution factor is $(45 \times 1.06 \times 0.8) + 180 \div 45 = 4.848$

2. This supernatant could be stored at 4°C for at least 3 days.

3. When one cuvette or automatic sampling system is used, the absorbance of this reference should be measured first to avoid possible contamination of LDH from other samples.

4. The lactate in caipillary and venous blood obtained from 19 subjects (20-35 years old) who had been at rest (sitting quietly on a bench) for more than 30 min. was $0.78 \pm 0.23$ and $0.79 \pm 0.20$ mmoles/1 (mean ± SD), respectively.

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REFERENCES

