Single- and Coupled-Enzyme Nylon Tube Reactors for Routine Determination of Pyruvate and Lactate in Serum

P. V. Sundaram and W. Hinsch

We describe the use of a continuous-flow clinical analyzer with an immobilized coupled-enzyme nylon tube reactor and an immobilized single-enzyme nylon tube reactor for routine estimation of lactate and pyruvate in serum. These reactors are incorporated into the flow system of a modified continuous-flow analyzer (Technicon AutoAnalyzer). Results for serum lactate and pyruvate by this method are compared with those by corresponding methods in which the same enzymes are used in solution, either automatically (pyruvate) or manually (lactate) performed. Routine clinical laboratory determinations with use of the coupled-enzyme system lactate dehydrogenase and alanine aminotransferase, co-immobilized in the nylon tube reactor for estimation of lactate, and lactate dehydrogenase reactors for estimation of pyruvate give reliable and reproducible results with high precision at low cost.

Additional Keyphrases: heart disease · infarction · thiamine deficiency · heavy-metal poisoning · therapy with biguanide

Serum lactate, estimated hitherto by use of lactate dehydrogenase (EC 1.1.1.27; LDH) in solution, is becoming increasingly important in clinical investigation and diagnosis of lactate acidosis in diabetic patients undergoing therapy with biguanide (1–4) and in prognosis and diagnosis of acute myocardial infarction complicated by shock (5). The size of an acute infarct can be estimated from results of serum lactate estimation, either alone or in combination with other indicators.

Pyruvate is routinely estimated only in unusual circumstances such as in the diagnosis of severe thiamine deficiency or of heavy-metal poisoning (when blood pyruvate increases after oral intake of glucose, 6). A means for routinely estimating pyruvate may also be needed in certain research.

Pyruvate and lactate in serum may be estimated with use of LDH and the coenzyme NAD or of LDH and NAD+ accompanied by a reagent such as 2,4-dinitrophenylhydrazine to trap the pyruvate formed in the backward reaction.

\[
\text{Pyruvate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+ \]

This is necessary because the reaction equilibrium favors the formation of lactate. An alternative would be to combine LDH with another enzyme such as alanine aminotransferase (EC 2.6.1.2), to trap the pyruvate.

Use of enzymes in solution in routine analysis is often expensive; lessening the amount of enzyme required reduces costs. This is an advantage of immobilized enzymes, as demonstrated recently by Sundaram et al., who estimated serum urea (7) and uric acid (8) with use of nylon tube reactors containing immobilized urease and uricase, and by Garber et al. (9) in the estimation of serum glucose with use of immobilized hexokinase and glucose-6-phosphate dehydrogenase. Hornby et al. (10) crosslinked LDH to hydrolyzed nylon.

This paper describes methods of preparing a LDH reactor3 for the estimation of pyruvate and a co-immobilized LDH-ALT reactor catalyzing the conversion of lactate to pyruvate.

\[
\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NAD}^+ \\
\text{Pyruvate} + \text{glutamate} \xrightarrow{\text{ALT}} 2\text{-oxoglutarate} + \text{alanine} \\
\]

The increase in absorbance at 340 or 334 nm gives a measure of the lactate in the original sample solution.

Materials and Methods

In our preliminary trials we used a peristaltic pump ("varioperpx"; LKB Produkter, Bromma, Sweden) to control the rate of flow of substrate through the immobilized-enzyme nylon tube reactors. For the clinical trials we used a Technicon AutoAnalyzer I connected to an Eppendorf photometer and an Eppendorf plotter, Model 4412.

Materials

"Nylon" tubing (i.e., 1 mm i.d.) was supplied by Portex Ltd., Hythe, Kent, U.K. Rabbit skeletal muscle LDH with a specific activity of 550 kU/g and calf liver ALT of 80 kU/g activity were obtained from Boehringer Mannheim GmbH, 68 Mannheim, G.F.R., as were NAD+ and NADH.

Reagents

Pyruvate determination. Solution I: Phosphate buffer (pH

3 Nonstandard abbreviations used: "Immobilized-enzyme nylon tube reactor" is a generic name given to nylon-tube-supported enzymes. For brevity, in this paper the two types of reactors are also referred to as "LDH reactor" and "LDH-ALT reactor." Other abbreviations: LDH, lactate dehydrogenase; ALT, alanine aminotransferase (referred to as GPT in Figs. 4, 5, 6, and 7); EDTA, ethylenediaminetetraacetate; PEI, polyethyleneimine; and nylon-PEI, nylon-polyethyleneimine.
Procedures

Both the LDH reactor and LDH-ALT reactor were made of nylon-polyethyleneimine copolymer according to the original method of Sundaram and Apps (11). The copolymer itself is made as described by Sundaram et al. (8), as follows: A nylon6 tubing (1 mm i.d.) is first O-alkylated by using a 0.1 mol/L solution of triethylxonium tetrafluoroborate made up in dichloromethane and allowed to react for 4 min at room temperature and then quickly washed with ice-cold de-ionized water. This procedure activates the nylon by O-alkylation to produce a nylon-imidate derivative (see Figure 1 in E). The tube is then filled with a 10 g/L dilution of stock solution of PEI (supplied by Serva GmbH, 69 Heidelberg, F.R.G.) in bicarbonate buffer (pH 9.4, 0.1 mol/L) and left at room temperature for 4 h. It is then washed well with de-ionized water and filled with a freshly made 25 g/L solution of glutaraldehyde (a 10-fold dilution of a 250 g/L commercially supplied solution) in bicarbonate buffer (0.1 mol/L, pH 9.4). After 40 min at room temperature, the tube is again washed well with water.

LDH reactor: A 1-m-long nylon-PEI copolymer tube treated with glutaraldehyde is filled with a freshly made solution of LDH (1100 kU/L) in phosphate buffer (pH 7, 0.1 mol/L) containing EDTA, 1 mmol/L, and left at 4 °C for about 20 h. The enzyme solution is then removed and the tube washed thoroughly with 0.1 mol/L NaCl, followed by de-ionized water to remove any adsorbed protein.

LDH-ALT coupled enzyme reactor: This reactor was made by crosslinking both the enzymes to a nylon-PEI tube at the same time. We made reactors consisting of the two enzymes mixed in various ratios according to specific activity and protein dry weight. Performance in terms of activity and stability was evaluated with a LDH-ALT reactor made from an enzyme mixture in the ratios of 1:2 in protein content and 1:7:1 in specific activity.

ALT (spec. acty., 320 U), 0.4 mL, and 0.2 mL of LDH (spec. acty., 550 U), both supplied as a suspension in 3.2 mol/L ammonium sulfate, were separately centrifuged and the pellets combined and dissolved in 2 mL of phosphate buffer (pH 8.0, 0.1 mol/L) containing EDTA 1 mmol/L. This represents a combined concentrated 5% of enzyme protein per liter.

A nylon-PEI tube (1 m long, 1 mm i.d.), pretreated with glutaraldehyde as mentioned above, was filled in a LDH-ALT enzyme mixture and left for about 20 h at 4 °C. The enzyme solution was then removed and the tube washed thoroughly with the coupling buffer, 0.1 mol/L NaCl, and deionized water, and then stored filled with the storage buffer.

Analytical Methods

To find the optimal conditions for routine use, we characterized the pH dependence and kinetic properties of the LDH reactor as follows: 1 mmol/L pyruvate was made up in phosphate buffer (0.1 mol/L) of various pH values containing, per liter, 1 mmol of EDTA and 0.6 mmol of NAD+, and pumped through the reactor at a flow-rate of 0.32 mL/min. From this experiment we determined the pH optimum and the kinetic parameters.

In the case of lactate estimation, the pH optimum for the LDH-ALT reactor was determined by using phosphate buffer (0.1 mol/L) up to pH 8 and bicarbonate buffer (0.1 mol/L) between pH 8 and 10. We used 1 mmol/L lactate, 0.66 mmol/L NAD+, and 9 mmol/L glutamate as substrates in this experiment; again, the flow rate was 0.32 mL/min.

Routine analyses with a Technicon AutoAnalyzer. For both pyruvate and lactate determinations, we connected the sampler, pump, and analytical cartridge of a Technicon AutoAnalyzer I to an Eppendorf spectrophotometer and Eppendorf plotter 4412, used with a fourfold extension of sensitivity for pyruvate and a 10-fold extension of the sensitivity for lactate. The decrease in absorbance (pyruvate) or increase in absorbance (lactate) at 334 nm gave a measure of the concentration of the two metabolites. For both pyruvate and lactate, 40 samples/h with a sample to wash ratio of 2:1 was chosen for an optimal wash-out.

During the reaction the immobilized enzyme reactors were maintained at 25 °C; they were stored at 4 °C, filled with the storage buffer mentioned earlier.

Results and Discussion

In the determination of pyruvate with a LDH reactor, the pH optimum for the reaction was found to be pH 8.0 with an apparent K_m of 340 μmol/L for pyruvate. Sundaram (12, 13) has extensively studied the kinetics of the reaction catalyzed by a LDH reactor in the conversion of pyruvate to lactate and found that the reaction is considerably perturbed by diffusion. This is reflected in the increase in apparent K_m with increase in diffusional control and decrease in flow rate of the substrate. For this reason we studied the flow-rate dependency of turnover of substrate (Figure 1). This flow-rate dependency
curve has a biphasic character. As seen from the micromoles of pyruvate converted per minute, the turnover increases with flow-rate and has not reached a maximum up to 1.35 mL/min, the highest flow-rate we tried. This means that catalysis is still not independent of diffusion. In the flow-diagram adapted in this study, the dialyzed serum flows through the LDH reactor at 1.2 mL/min. Although Figure 1 implies that higher turnover may be achieved at even higher flow-rates, which will also speed up the rate of analysis, this will only be at the expense of an efficient wash-out.

Thus in routine use, when the LDH reactor is used in combination with an AutoAnalyzer, optimum conditions of operation are chosen such that (a) the reactor displays sufficient activity, (b) the apparent $K_m$ is sufficiently high but not too high to cause reactor performance to be crippled by diffusion, and (c) the flow-rate chosen permits analysis at the rate of at least 40 samples per hour. Figure 2 depicts the flow diagram.

Figure 3 shows a regression analysis, comparing the performance of the LDH reactor method for pyruvate estimation with an AutoAnalyzer method in which LDH is used in solution. The flow diagram used in the latter case is the same as in Figure 2 except that the LDH reactor is deleted from the circuit and 10 kU of LDH (spec. acty., 55 kU/g of protein) per liter is added to solution II.

For 125 sera with pyruvate in the concentration range of 30 to 1000 $\mu$mol/L, the correlation coefficient was 0.997, and the equation for the line of regression was $y = 1.081x - 2.5$.

Analysis showed within-day precision (CV) for an 80 $\mu$mol/L pyruvate concentration to be 3.3%; for a 60 $\mu$mol/L pyruvate concentration it was 3.5%.

Fig. 2. Flow-diagram in the analysis of pyruvate using a LDH reactor linked to a modified Technicon AutoAnalyzer I

Fig. 3. Comparison of results of pyruvate estimation by a LDH reactor method with the LDH solution method (automatic)

Fig. 4. Flow-rate dependence of the activity of a LDH-GPT reactor in the estimation of lactate with 1 mol/L lactate, 0.66 mmol/L NAD+, and 9 mmol/L glutamic acid, made up in pH 9.4 bicarbonate buffer (0.1 mol/L) at 22°C

Day-to-day CV was within 5%, based on analyses of at least 20 samples.

Lactate estimation with use of a chemical trapping agent such as 2,4-dinitrophenylhydrazine mixed with the substrate did not work satisfactorily with a LDH reactor, for reasons not clear. This is why we decided to estimate lactate by coupling the reaction to ALT. Routine analyses were performed at pH 9.4, the optimum pH for this coupled enzyme reactor with an apparent $K_m$ of 290 $\mu$mol/L for lactate.

Figure 4 shows the relation between flow-rate and activity for the LDH-ALT reactor. In contrast to the pyruvate estimation with the LDH reactor in the present case, the activity–flow-rate profile is very different in that lactate conversion increases linearly up to 0.5 mL/min and then rapidly attains a maximum at 0.9 mL/min and becomes constant. Thus above 0.9 mL/min the reaction is independent of flow rate and diffusion. The fact that this happens at a relatively low flow rate is not surprising, because in this coupled-enzyme system pyruvate production is in the unfavorable direction of the reaction catalyzed by LDH, the amount of the intermediate pyruvate available for the production of alanine and 2-oxoglutarate is low and this controls the system. In other words, with increased production of pyruvate the flow rate at which the reaction attains a plateau should be higher because increase in flow-rate increases turbulence and enhances catalysis.

Fig. 5. Flow-diagram in the analysis of lactate using a LDH-GPT reactor linked to a modified Technicon AutoAnalyzer I
Dialyzed serum flows through the LDH-ALT reactor at 0.52 mL/min in routine analysis, a speed at which the wash-out was satisfactory. The flow diagram is shown in Figure 5.

The continuous-flow LDH-ALT reactor method was compared to the fully enzymic (LDH-ALT) test pack method performed manually without deproteinization (Boehringer Mannheim GmbH, G.F.R.). Correlation between the two methods was very good (r = 0.993), the equation for the line of regression being y = 0.97x – 0.04 in the range of 1 to 10 mmol/L lactate (Figure 6).

Results of precision analysis were satisfactory. Within-day precision (CV) for a 5.9 mmol/L lactate concentration was 3.1%; for 8.6 mmol/L lactate concentration it was 2.4%.

Day-to-day precision (CV) for a 2.5 mmol/L lactate was 4.9% and for 4.9 mmol/L lactate it was 4.3%.

Washout between a sample of 10 and 2 mmol/L amounted to 0.2 mmol/L, or 10%, for 20 such sample pairs.

The kinetics of the coupled enzyme system are puzzling, because a plot of rate vs. substrate concentration always looks biphasic, the change in slope occurring at about 4 mmol of lactate per liter when the concentrations of glutamate and NAD+ were varied within a wide range. Even a large excess of both these substrates does not eliminate this feature. We are investigating this problem further.

As recently pointed out by Daka and Laidler (14), even a relatively simple reaction, the conversion of pyruvate to lactate by an LDH reactor, is evidently very complicated and appears as yet intractable to theoretical analysis. Sufficient to say that a reaction that involves three substrates, an intermediate, and three products (LDH-ALT reactor) is bound to have strict kinetic limitations. In spite of these problems, operational performance as shown by the correlation in regression analysis indicates that the immobilized coupled enzyme reactor meets the rigorous requirements of routine clinical laboratory use.

Operational and storage stability. The LDH-reactor is found to be very stable; it showed no significant loss in activity after use for 700 tests, and we think it will probably last for several thousand tests. After a reactor had been stored for 18 months at 4 °C it still maintained considerable activity and was ready for use in the assay.

The LDH-ALT reactor was less stable, and we found this to be due to the aminotransferase. Nevertheless, the reactor maintained enough activity for at least four weeks, during which about 2000 tests were performed (Figure 7). Initial loss of activity during the first few days was probably due to the slow loss of minute amounts of enzymic protein more strongly adsorbed to the tube, which was not removed by the initial washing.

We conclude that immobilized-enzyme nylon tube reactors, made with LDH and ALT and integrated into the flow system of a continuous-flow analyzer, will be an invaluable addition to a clinical laboratory, especially since the preparation, storage, and use of these reactors are simple, as demonstrated in this report.

This project was in part supported by a grant to Dr. P. V. Sundaram from DFVLR (Deutsche Forschungs- und Versuchsanstalt für Luft- und Raumfahrt). We thank Mrs. R. Wassermann and Mr. H. O. Polmann for excellent technical assistance.

References