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Measurement of Glycolytic Rates in Cell Suspensions Using a Recording pH Meter *

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A method for measuring continuously glycolytic rates in cell suspensions, using a recording pH meter, is described. Under the described conditions the method is very exact, sensitive and reproducible. The method can be applied to different cells and different conditions of assay calibrating in each case the pH range, cell concentration range and the ratio of delta protons to delta lactic acid.

The study of metabolic control requires, in many instances, kinetic methods able to measure rapid transients in metabolic rates. One chief example of this requirement is the study of the CRABTREE (1), PASTEUR (8) and CHANCE (2) effects in cell glycolysis where rapid transients in glycolysis and respiration occur in a very short time after the addition of glucose to cells or the exhaustion of oxygen (5). The oxygen electrode (4) represents an available method to record continuously respiration in cell suspensions. However, a method for recording glycolytic rates continously is not available, and it would be highly desirable for the study of the metabolic control between glycolisis and respiration (6).

In this report we describe a method to measure glycolytic rates continously in cell suspensions through the use of a recording pH meter. This method has been applied successfully to the study of glycolytic metabolic control (5) in combination with a recording oxygen electrode.

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Materials and Methods

Cells. Ehrlich ascites cells were used throughout all the study. The cells were collected from mice bearing the ascites tumor, seven days after inoculation and were freed of blood and washed as described by CHANCE and HESS (3). A stock cell suspension of 400×10^6 cells/ml was prepared for adding aliquots to the chamber of the pH meter. The cells were counted in a hemocytometer.

Recording pH Meter. A recording pH meter, composed of a Radiometer 71 and a Honeywell Electronic 194 recorder, was used throughout all the study. This pH meter gives a 500 mv output per unit of pH and has full scale zero correction. The pH meter in the expanded scale can be set to record less than 0.1 units pH per full scale of the recorder. The electrode was a combined radiometer GK2311C which shows enough sensibility and stability. The electrode is immersed in a chamber (3 ml volume) made of lucite and provided with a stirring bar which is subjected to continuous agitation with a magnetic stirrer.

Assay Medium. The assay medium was composed of 6.16 mM KCl, 1.65 mM $Na_2H_2PO_4$, 9.35 mM Na_2HPO_4 and 9 g/l of NaCl. It was carefully adjusted to pH 7.30 and used at room temperature. Glucose (10 mM) was added to start glycolysis. Distilled water and 50 % ethanol were used to wash the chamber between determinations.

Lactic Acid Assay. At fixed times, samples of the cell incubation medium were taken out of the chamber, treated with 12 % perchloric acid in a ice bath, neutralized with 6N KCO_3 and centrifugated. The lactic acid content was assayed in the supernatant, as described by HOHORST (7).

Recording Oxygen Electrode. The chamber was provided with a Clark-type oxygen electrode, connected to an electronic system, constructed by Instech Laboratories (Philadelphia) whose output was fed into a graphic recorder. One hundred percent oxygen was calibrated with the medium, in the absence of cells and zero percent oxygen was calibrated adding dithionite to the medium. The concentration of molecular oxygen in the medium was estimated as 0.25 μmol per ml. The speed of the recording paper was adjusted to 10 mm per minute equal to the recording speed of the recording pH meter to synchronize the recording of oxygen uptake with the recording of the glycolytic rate.

Results

When Ehrlich ascites tumor cells are glycolyzed in a slightly buffered medium, lactic acid difuses from the cells to the medium of suspension, lowering its pH. The rate of acidification is proportional to the rate of glycolysis of the cells, is also proportional to the number of cells per ml within certain ranges and depends on the buffering capacity of the medium and on the initial pH of the medium.

In preliminary experiments, different low-buffered mediums, suitable for glycolysis, were tested. The medium described by CHANCE and HESS (3), yields maximum glycolytic rates and is enough poorly buffered to permit sensitive pH recordings. As the glycolityc rate of cells depends on the pH of the medium, the pH dependency of glycolysis was studied. It was found that above pH 7.4 and below pH 6.9, the glycolytic rate varies extensively with minor pH changes. However, between pH 7.3 and pH 7.0, the rate of glycolisis is almost constant (fig. 1).

The first graph of figure 1 shows the production of lactate by Ehrlich ascites cells for 15 minutes of incubation. The cells were incubated in the chamber of

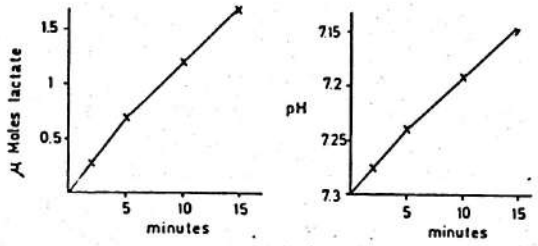

Fig. 1. *Correlation between lactate rate production and acidification.*
First Graph: Increasing of lactate production after glucose addition with time of incubation of 94 × 10: Ehrlich ascites cells in a 3 ml chamber. Lactate was determined in the supernatant of samples taken from the chamber at the indicated times. *Second Graph:* Acidification of the medium produced by the cells of the experiment of the first graph. The acidification was measured by the recording pH electrode inserted in the chamber.

the recording pH meter in 3 ml of assay medium (pH 7.3) containing 10 mM glucose. Glycolysis started with the addition of the cells (94 × 10^6 cells) and the pH decrease was recorded continuously for 15 minutes. Samples were taken at 2, 5, 10 and 15 minutes for determination of lactic acid. It can be observed that the glycolityc rate is essentially constant during the first 15 minutes, but in the critical first five minutes the glycolysis is slightly more active until reaching a steady state. In these fifteen minutes, the pH of the assay medium decreased essentially from 7.30 to 7.15, as can be seen in the second graph of figure 1, but in the initial five minutes, there is more active rate of acidification, corresponding to the glycolytic rate. For this experiment, it can be calculated that there was a change of 0.009 units pH in the chamber for the apparition of 100 nmoles of lactate. The glycolytic rate can be expressed as 1.33 nmoles lactate/min/10^6 cells as in terms of lactic acid production or as 0.0011 units pH decrease/min/10 × 10^6 cells. After the fifteen minutes of incubation, the pH decrease and the lactic acid production were not linear.

Figure 2 shows the relationship between lactic acid production or the acidification with the number of cells glycolyzed. The upper graph shows that there is a linear increase of lactic acid production with increasing number of cells in the chamber. The linearity disappears above 60 × 10^6 cells in the chamber. The lower graph of the figure shows the data from the recording of p Hin the same experiment. Again, the loss of linearity after 60× 10^6 cells per chamber, can be appreciated. From this experiment, it can be again calculated that there is a 0.009 units decrease in pH for each 100 nmoles lactic acid which appear

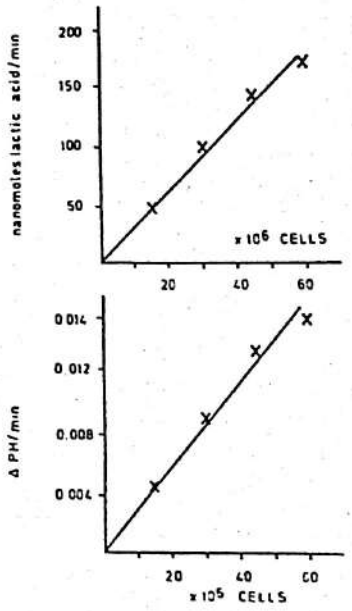

Fig. 2. *Correlation between lactate and pH at different cell concentrations.*
Upper Graph: Rate of lactic acid production per minute in the 3 ml chamber caused by cell suspensions of different concentrations. Cell concentrations are expressed in abscissas, per 3 ml chamber. *Lower Graph:* pH decrease per minute in the 3 ml chamber caused by cell suspensions of different cell concentrations. Cell concentrations are expressed in abscissas per 3 ml chamber.

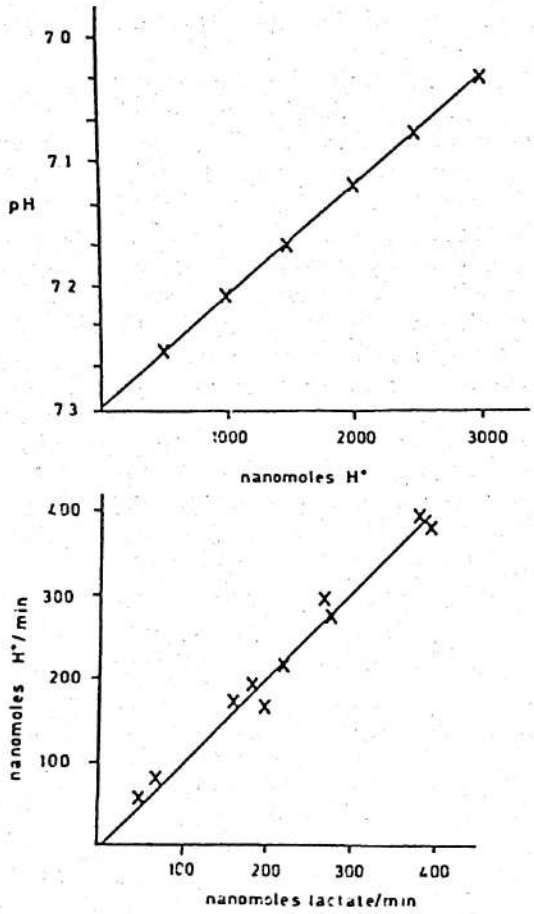

Fig. 3. *Correlation of glycolytic rate with acidification and pH.*
Upper Graph: Decrease of pH related to the addition of increasing concentration of HCl to 3 ml of assay medium. *Lower Graph:* Correlation between the glycolytic rates of cell suspensions of different cell concentrations which are expressed in nmoles produced per minute or in nmoles H^+ produced per minute.

in the chamber. The glycolytic rate is 3.3 nmoles per minute per 10^6 cells or 0.0033 units pH decrease/min/10×10^6 cells. In experiment of figure 1, the glycolytic rate was lower than in this experiment, due to excess of cells. The calibration of the pH meter with HCl, is shown in the upper graph of figure 3. There is linear pH decrease with increasing concentration of HCl in the medium. There is a change of 0.009 units of pH for each 100 nmoles H^+ in the chamber. In the lower graph of figure 3, there is the correspondence between glycolytic rates expressed in lactate per minute or H^+ production per minute in 10 different cell preparations of different cell concentrations. From this experiment, it is calculated that the ratio Δ^+/Δlactate = n, is the unity. This value of n permits the glycolytic rates expressed in H^+ production per minute to be calculated directly as lactate per minute.

An example of continuous determination of glycolytic rates by pH recording, is shown in figure 4: time procedures from left to right and the time calibration is shown between brackets) (2 min). The oxygen concentration diminishes from up to down and the oxygen concentration calibration is shown between brackest (140 natoms). The pH calibrations

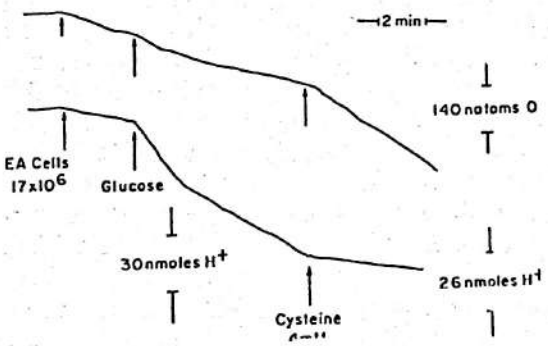

Fig. 4. *Recording of the oxygen consumption with the recording oxygen electrode (upper trace) and the rate of glycolysis with the recordlng pH electrode (lower trace) done simultaneously in the same cell suspension which, at different times, is supplemented with glucose and cysteine.*
Time proceeds from left to right in both recordings. The concentration of oxygen in the medium diminishes from up to down in the upper recording and pH decreases from up to down. A higher downward inclination of the traces means higher rates of respiration or glycolysis, respectively. The inserts show the time, pH and oxygen calibration.

are shown, also, between brackets as hydrogenous concentration (26 nmoles H^+) and 30 nmoles H^+. The second calibration was different due to the buffering capacity of the added cysteine. The pH and the oxygen recordings were obtained simultaneously from the same cell suspension in the chamber, using two separate recorders, connected to the pH electrode and oxygen electrode with which the chamber was provided. In this experiment, the upper trace is a measurement of cell respiration with the recording oxygen electrode and the lower trace is the measurement of the glycolytic rate with the recording pH meter (5). After the addition of the cells, there is a slow rate of respiration and glycolysis with endogenous substrate. The addition of glucose gives a very rapid burst of respiration (Chance effect) and a transient large increase in glycolysis due to ADP formation. Two minutes after the glucose addition, the respiration becomes inhibited (Crabtree effect) and the glycolysis becomes stable. The addition of cysteine, an inhibitor of pyruvate kinase, produces an inhibition of the glycolytic rate and a consequent stimulation of respiration, due to competition for ADP favorable to mitochondria (5). The continuous recording of glycolysis shows four different glycolytic rates recorded in less than fourteen minutes; a performance that is impossible to achieve with sampling and determination of lactic acid.

Discussion

Under the described conditions of pH, temperature, cell concentrations and assay medium, the described method represents a reliable, exact, very sensitive and reproducible method of continuously determining glycolytic rates in cell suspensions. This kinetic method represents a great advantage over conventional sampling methods when the measurement of rapid metabolic transients is required. The method can be applied to other cells and conditions of assay calibrating in each case the pH range, the cell concentration range and the $\Delta H^+/\Delta$lactic ratio.

When this method is used to measure the effect of several additions of substrates, cofactors or inhibitors on the glycolytic rate, a new calibration with H^+ ions should be done after new addition. This calibration is necessary to account for the possible increase in buffer capacity after each new addition. Another important point is that the additions must be carefully adjusted at the mean pH of the medium in order to introduce a minimal pH change.

Resumen

Se describe un método para medir continuamente glicolisis en suspensiones celulares mediante el uso de un peachímetro registrador. En las condiciones descritas de pH, temperatura, concentración celular y medio de ensayo, el método es muy exacto, sensible y reproducible. Este método se puede aplicar a diferentes tipos de células y a diferentes condiciones de ensayo, calibrando en cada caso el pH, la concentración celular y la razón del incremento de protones al incremento de ácido láctico.

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