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Indirect fluorescence determination of lactate and pyruvate in single erythrocytes by capillary electrophoresis

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ABSTRACT

A scheme of using fluorescein as the fluorophore for indirect detection of anions was demonstrated. This system is quite stable at a fluorescein concentration of 100 μM even without any other buffer components. Different injection modes affect the limit of detection (LOD). A LOD of about 20 amol was obtained for lactate under optimal conditions. Lactate and pyruvate in the intracellular fluid of erythrocytes were measured in this manner. The average amounts in a single erythrocyte for lactate and pyruvate are 1.3 and 2.1 fmol, respectively, or a ratio of 1.6 for pyruvate to lactate. Variations of the absolute amounts and the ratios are fairly large among a group of 27 cells examined. This is consistent with the difference of cells in size and composition. Although the migration times changed by up to 20% during a series of runs from the influence of concomitants in the cells, the migration time ratio was maintained around 1.072 with 3% relative standard deviation.

INTRODUCTION

Indirect detection, as a universal detection approach for ions in capillary electrophoresis (CE), is widely used for the determination of both organic and inorganic compounds that do not possess a suitable detection property [1,2]. To a first approximation, this detection scheme is based on charge displacement between the analyte and a background ion. As many interesting compounds do not always possess a physical property suitable for direct detection, indirect detection became a popular scheme in both absorption [3,4] and fluorescence [5,6]. The limit of detection (LOD) for indirect detection is directly proportional to the concentration of the fluorophore used in the running buffer. By lowering the concentration of the background ions without sacrificing the dynamic reserve, the LOD can be further improved. Despite the impressive LOD obtained in ref. 1, further improvement is necessary for the application of this detection mode to the determination of certain components at the single-cell level. Fluorescein is well-known for its high fluorescence efficiency [7], and is conveniently excited by visible light. So far, little work has been done to optimize the conditions necessary for using fluorescein for the indirect detection of anions.

Determining the chemical and biochemical composition of a single cell can help to elucidate the detailed functions of some organisms. Potentially, it can provide useful information for diagnosis of diseases at an early stage. Much work has been done recently regarding the chemical analysis of individual cells because of the promising biomedical applications. Various schemes [8–11] were developed for determination of different components in different kinds of cells. The analysis of single neurons conducted by Olefirowicz and Ewing [12] and Kennedy *et*

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al. [13] showed the applicability of microscale separation to the analysis of individual cells. Other investigations [13–17] demonstrated the quantitative determination of multiple constituents in individual cells coupled with electrochemical detection modes.

Recent work in this group centered around the determination of several components within single erythrocytes [18–20] with both direct and indirect fluorescence detection. The red blood cell is the smallest cell so far subjected to chemical analysis. The successful analysis of such a small entity demonstrated that laser-induced fluorescence (LIF) is by far the most sensitive approach for direct and indirect detection of intracellular components. The indirect detection method described earlier [18,21,22] provided a general approach for the quantitation of intracellular ions normally with undetectable properties. This is accomplished without the tedious steps associated with derivatization. It also avoids the problems of incomplete reaction and of slow kinetics at low analyte concentrations.

Lactate and pyruvate that exist in the red blood cell play an important role in the glycolysis process [23]. The determination of the amounts of each in a single erythrocyte should provide some information about the carbohydrate metabolism, as these are products during the carbohydrate substrate conversion process. Lactate and pyruvate convert to each other via the following reaction cycle:

$$\text{Pyruvate} \xleftrightarrow[\text{LDH}]{\text{NADH}\rightarrow\text{NAD}} \text{Lactate}$$

This cycle also brings about the conversion of nicotinamide adenine dinucleotide (reduced form) (NADH) to nicotinamide adenine dinucleotide (NAD), and is catalyzed by lactate dehydrogenase (LDH). The determination of pyruvate and lactate can further be correlated to the concentrations of NADH and NAD indirectly. Also it is possible to relate these to the activity of LDH. Both lactate and pyruvate do not contain any physical properties amenable for sensitive detection. They are also not easily derivatized.

In the present work, the use of fluorescein as the fluorophore for the indirect detection of anions was evaluated. It was then used as the background ion for the determination of pyruvate and lactate in single human red blood cells. The variations in the amounts of pyruvate and lactate from cell to cell were depicted.

EXPERIMENTAL

Instrumentation

The CE system used in this work is similar to that described before [21,22]. For single cell analysis, a 70 cm long, 55 cm to detector, 14 μm I.D. and 350 μm O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) and a high-voltage power supply (Spellman Electronics, Plainview, NY, USA) were used throughout the study. The running voltage was kept at 25 kV. An on-capillary detection window was created by burning off a short section of the polyimide coating. The cell injection end was conditioned by removing a 2-mm section of the polyimide coating for visual monitoring. About 3.2 mW of 330 nm laser light (after separation from the 350 and 360 nm lines) from an argon ion laser (Model Innova 90; Coherent, Palo Alto, CA, USA) was used for excitation. The laser beam was focused onto the detection region by a 1-cm focal length lens and the emitted fluorescence was collected using a 20× microscope objective lens at an angle of 90° to the laser beam. A spatial filter and a 400 nm longpass filter plus an interference filter at 516 nm were used to eliminate the scattered light before imaging onto the photomultiplier tube (PMT). The current produced was converted to voltage by an electrometer. Then, via a 24-bit A/D conversion interface (ChromPerfect Direct, Justice Innovations, Palo Alto, CA, USA), data was collected and stored on an IBM-compatible PC/AT computer.

For evaluation of the fluorescein system, a modified setup was also used. The major change is that a visible argon ion laser (Model 2211-10SL; Cyonics, Uniphase, San Jose, CA, USA) was used. The laser beam was not focused in order to simplify optical alignment. Basically, the original laser beam with a diameter of about 1.5 mm directly irradiates the capillary at the detection window. Since indirect detection involves a fair background concentration of a

fluorophore, the signal level is adequate even though only a few percent of the unfocused laser intensity is used.

Cell preparation and injection

Human erythrocytes were isolated from the fresh plasma of a healthy adult male. Usually 5 ml of plasma were obtained, with heparin and EDTA added to protect the red blood cells from coagulating during storage at 4°C in a refrigerator for as long as 4 days. Before detection of the anions, the red blood cells were separated from the serum by centrifuging, and also were washed by a process similar to that described in ref. 18. A different wash solution (8% glucose, 100 μM fluorescein disodium salt, 2-hydrate with no further adjustments in pH) was used. Multiple washings were performed so that the cells were free from the extracellular ions.

The cells were suspended in the wash solution at a concentration of about 0.1% (v/v). A 50-μl droplet of cell suspension was mixed with an identical volume of running buffer already deposited on the glass microscope slide. An individual red blood cell was selected for injection by manually guiding the capillary orifice close to the cell of interest with the help of the microscope and a 3-dimensional micromanipulator. A vacuum pulse produced by pulling on a syringe connected to the ground end of the airtight buffer vial was applied to draw the cell into the capillary. The whole process was clearly monitored and easily controlled under 100× magnification. It is easy to push out any air bubbles from the capillary that are inadvertently introduced during the injection process by squeezing on the piston of the syringe.

Red blood cells are easily distinguished from other constituents in blood by their number, size and uniform shape. In order to move the capillary orifice to the cell as close as possible, the injection end was etched to form a tip of about 50 μm O.D. with HF using the similar procedure as reported in ref. 17. By using the etched capillary, we can decrease the injected amount of the suspension solution, which was the main interfering component in this detection scheme. Injection of large amounts of suspension solution resulted in instability of the baseline. After a red blood cell was drawn into the capillary, the capillary was immediately moved back into the vial containing the running buffer. After 30 s to allow lysing, the separation begins. In the running buffer, erythrocytes typically lysed in a time shorter than 1 s. The fast lysis is desirable for the determination of intracellular components without extra manipulation. The 30 s waiting period allows the running buffer to mix with the suspension solution and to reach the cell.

Injection of standard samples

The standard samples were injected hydrodynamically at a height of 20 cm relative to the ground end. The hydrodynamic injection mode is comparable to and consistent with the cell injection process. Also, it is not biased for ions with different mobilities and the injected amount is easier to control based on the Poiseuille equation.

Reagents

Sodium pyruvate was obtained from Fluka (Buchs, Switzerland). Sodium lactate was purchased from Sigma (St. Louis, MO, USA). Fluorescein disodium salt, 2-hydrate ($C_{20}H_{10}Na_2O_5 \cdot 2H_2O$, M_r 412.3) was purchased from Eastman Kodak (Rochester, NY, USA). Other chemicals were obtained from Fisher Chemical (Fair Lawn, NJ, USA).

Solutions

Running buffer was made with 1% glucose (W), 100 μM fluorescein disodium salt, 2-hydrate, and 500 μM Tris with pH 8.5 without further adjustment. The standards were dissolved in the running buffer. For the evaluation of fluorescein, different running buffer solutions were used as described below. All solutions were prepared in deionized water and were filtered with a 0.22-μm filter before using.

RESULTS AND DISCUSSION

Evaluation of fluorescein performance

Since fluorescein is a highly efficient fluorophore, we expect that even at low concentration a stable baseline can still be obtained for small capillaries. For a new bare capillary and buffer solutions without cetyltrimethylammonium bro-

mide (CTAB), a positive system peak comes out very early at the running conditions tested. After that the baseline becomes quite stable and clean. When using a DB-1 capillary (J&W Scientific, Folsom, CA, USA) the system peak shows up much later, as the capillary coating and the low concentration of CTAB (20 μM) lead to a slow electroosmotic flow (EOF) rate. The system is also quite stable and clean. The relationship between anion migration and EOF is different for these two conditions. In first case, the anions migrate upstream. The EOF rate is greater than the electrophoretic rate so that the anions are carried to the grounded buffer vial. The more mobile anions will come out later. However, for the second case, the capillary walls are positively charged, leading to EOF and anion migration in the same direction. The more mobile anions will come out earlier. Both systems are suitable for the detection of common anions.

Fig. 1 shows that 8 anions were nicely separated in a DB-1 column, except that nitrate and iodide coeluted. Corresponding concentrations and migration times are listed in Table I. A dynamic reserve of >400 is easily obtained when using fluorescein as the fluorophore at this low concentration. This is a promising feature for the detection of anions. However, the LOD in indirect detection is not simply determined by the concentration of the background fluorophore

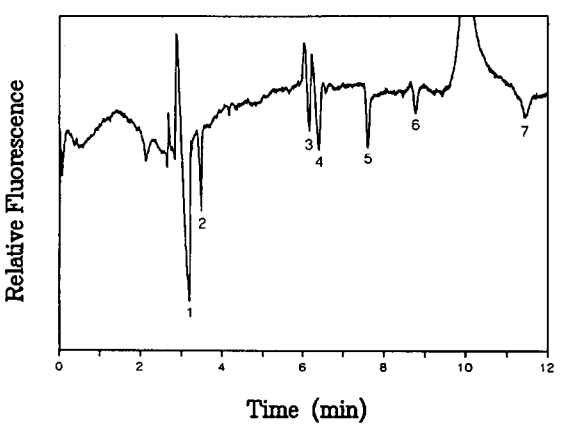

Fig. 1. Electropherogram of anion separation. Concentrations and migration times are listed in Table I. 50-μm DB-1 capillary, 76 cm long and 44 cm to detector. Running voltage: 30 kV. Injection: 30 kV, 0.5 s. Buffer: 100 μM fluorescein and 20 μM CTAB.

TABLE I

CONCENTRATIONS AND MIGRATION TIMES FOR THE SEPARATION SHOWN IN FIG. 1

No.	Anion	Concentration (μM)	Migration time (s)
1	Nitrate	47.2	191.6
	Iodide	15.1	191.6
2	Chlorate	17.8	210.1
3	Pyruvate	34.5	371.6
4	Acetate	23.3	385.4
5	Benzoate	20.1	460.4
6	Lactate	18.8	533.1
7	Glutamate	16.3	698.1

and the dynamic reserve. The displacement ratio is also an important factor that affects the LOD. The problems with fluorescein are the relatively large M_r and the double charge at pH 8.5, which may degrade the LOD. From the pyruvate peak area and peak height, the estimated displacement ratio is 0.2 to 0.08, which is dependent on the buffer components and the capillary size. The buffer components can introduce additional interaction between the analytes and the fluorescein ions. The effect of capillary size is not clear. Generally, simpler systems (using only 100 μM fluorescein as the running buffer) and smaller capillaries give larger displacement ratios.

Table II lists the LOD for different experimental conditions. Certain conditions provide improvements in LOD compared to that reported previously [1,22]. Even though different anions were tested, the LODs are comparable because the M_r values are similar and the charge is the same. The lowest LOD was obtained for electromigration injection in bare fused-silica column. A possible reason is that the total injection period is much shorter than hydrodynamic injection when injecting the same volume of sample. Therefore the baseline disturbance is smaller in magnitude. However, for electromigration injection in bare columns, the separation is not as good as that obtained for hydrodynamic injection. The LODs listed in Table II confirm the applicability to the detection of pyruvate and lactate in single red blood cells.

TABLE II

ABSOLUTE LOD (10^{-16} mol) UNDER DIFFERENT OPERATING CONDITIONS

Conditions: 1 = buffer: 100 μM fluorescein and 20 μM CTAB, pH 6.3; capillary; DB-1, 350 μm O.D., 50 μm I.D.; length 76 cm and 44 cm to the detector; voltage: 30 kV; injection: 30 kV, 0.5 s; laser: unfocused 488 nm beam; sample was dissolved in running buffer. 2 = Buffer: 100 μM fluorescein, 1% glucose and 500 μM Tris, pH 8.5; capillary: bare fused-silica, 362 μm O.D., 14 μm I.D., length 70 cm and 55 cm to the detector; voltage: 25 kV; injection: gravity 20 cm, 30 s; laser: focused 330 nm beam; sample was dissolved in 8% glucose and 100 μM fluorescein. 3 = As 2 except that the sample was dissolved in the running buffer. 4 = As 3 except that the running buffer did not contain glucose. 5 = As 4 except that injection was conducted by electromigration at 25 kV, 0.5 s. 6 = Buffer: 250 μM sodium salicylate, pH 6.0. Other conditions as in 4.

	Conditions					
	1	2	3	4	5	6
Pyruvate	4.49	1.58	1.49	0.68	0.32	1.79
Lactate	[a]	1.35	1.23	0.88	0.20	1.05
Glutamate	7.51	[a]	[a]	1.36	0.34	1.40

[a] Not measured.

Determination of intracellular pyruvate and lactate in a single erythrocyte

Bare fused-silica capillaries (14 μm I.D.) were used because this size is suitable for injection of cells with a diameter of 8–9 μm and a volume of 53–87 fl [24]. There is only a small dilution effect after the lysis of the cell and optical alignment is not very tricky. Also, it allows a reasonable vacuum injection time (<15 s) for introducing one cell into the capillary without drawing in too much extracellular fluid. The separation efficiency and detection sensitivity are still well maintained for both fluorescence detection [21] and electrochemical detection [25]. The results shown in Table II provide support for selecting small bore capillaries for single-cell studies.

LIF [19,26] provides the high sensitivity needed for small amounts of sample when the analyte possesses natural fluorescence. Unfortunately, most ionic components existing within a single erythrocyte do not have appreciable natural fluorescence except proteins, DNA and some amino acids. *In vivo* derivatization [18,27] is one of the possibilities for detecting certain compounds. Nevertheless, the tedious process and possible contamination during treatment are formidable challenges for applying that to such a tiny organism as the red blood cell. Some kind of a specific biosensor is an alternative. The high selectivity however limits its applicability for multiple component analysis. The indirect detection approach [1,18] proves to be practical for determining the ionic analytes inside a cell. Even though it is far from being a perfect method, especially near the LOD due to instability of the baseline and system peak interference, the advantages of easy operation and suitability for monitoring compounds without specific functionality make this a unique technique for the analysis of single cells.

We might expect that many peaks would be present in the electropherograms if the amounts of typical anions within the red blood cell were higher than the LOD obtained in this work (Table II). In actual fact most of them are at concentrations well below the LOD. The potentially troublesome components were proteins if they could displace enough fluorescein to produce peaks. At the pH and the low concentration of buffer used in this system, proteins interact strongly with the capillary wall and adsorb there. Also, generally the large biomolecules have poor displacement ratios leading to poor LOD [28]. This is in contrast to operation at pH 10 with native fluorescence detection [29]. Actually, for the low absolute amounts of proteins in a cell, they could not produce significant interferences except for changing the EO flowrate due to adsorption. According to Table II, only pyruvate (*ca.* 1.29 fmol) and lactate (*ca.* 0.78 fmol) [30] are at detectable levels in a single erythrocyte.

Preliminary results showed that the high concentration of glucose used in the cell suspension solution caused an unstable baseline. The disturbance was so strong that no recognizable peak was obtained. To reduce the disturbance, 1% glucose was added into the running buffer. Also, the capillary was equilibrated for 24 h at the same voltage before the analysis of single cells. The system became quite stable and could toler-

ate the injection of a high concentration of glucose. Furthermore, the mixing of an equivolume of running buffer with the cell suspension on the microscope slide before injection of a cell decreased the actual concentration of glucose to about 4% and reduced the disturbance. Examination under a microscope shows that red blood cells in 4% glucose can last up to 6 h with no obvious hemolysis. Naturally, leaving cells in the glucose solution for a long time can promote the leakage of intracellular components. Our experiments are performed immediately after washing of the cells to protect pyruvate and lactate from leaking out.

Fig. 2 shows the electropherograms for injection of the running buffer (A), cell suspension solution (B), standards (C), a single cell (D) and a lysed-cell suspension (E). The clean background in (A) and (B) demonstrates a desirable condition for the detection of anions. Considering the detectable components in the cell as well as the consistency of migration times for the standard samples and the cell, one can attribute the two peaks in (D) and (E) to lactate and pyruvate. The stability of the baseline for single cell analysis is dependent on the amount (volume) of suspension solution drawn into the capillary. Sometimes a system peak appeared at the position of the lactate peak, which affects the precise quantitation of intracellular lactate. Therefore, minimizing the volume of the suspension solution injected is crucial for the determination of lactate. With the HF-etched fine tip, the injection orifice can be more easily moved to approach the cell of interest without pushing it away. The other approaches used for controlling the excess injection volume are to use a slightly more concentrated cell suspension and to apply the vacuum pulse gradually after the orifice is near the selected cell.

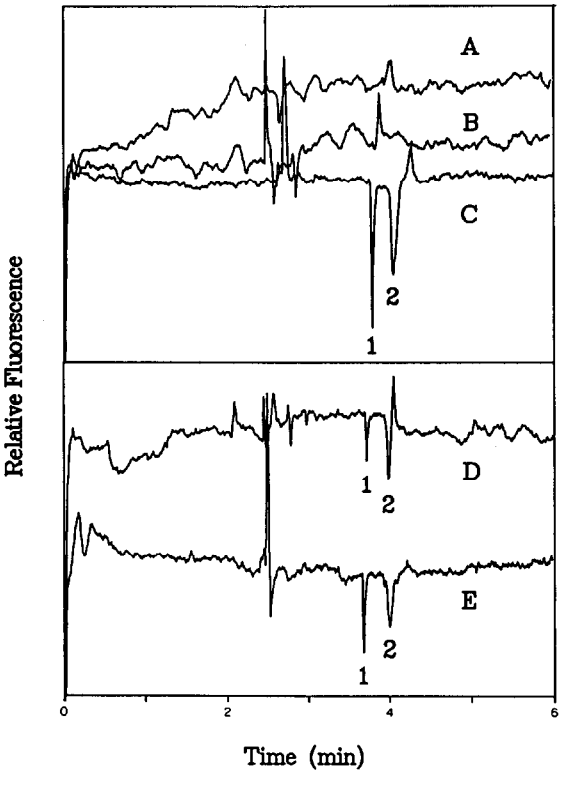

Fig. 2. Electropherograms for the analysis of the running buffer (A), cell suspension solution (B), standards (C), a single red blood cell (D) and a lysed-cell suspension (E). Peaks 1 and 2 refer to lactate and pyruvate, respectively.

Nevertheless, the carefully manipulated injection process is still not good enough to make every run informative. Some other factors can make the system unstable, *e.g.* shaking when moving the capillary in and out of the buffer vial, etc. Several electropherograms for cell separation are shown in Fig. 3. Variations among these trials are presumably due to the individual cell volumes and actual compositional differences. Runs 11 and 23 show other detectable components, which denote unusual cells. According to the analysis of whole blood extracts [31], the chemical composition of normal cells are dramatically different from those of abnormal cells. The common feature for these runs are the peaks for pyruvate and lactate.

In total, 32 cells were analyzed consecutively. Due to unpredictable disturbances, five runs were affected by serious baseline jumps and shifts. Therefore, those five data files were eliminated from further processing to quantify the individual components. A significant feature is that the individual cells are very different from each other with regard to the contents of pyruvate and lactate, as shown in Fig. 4. This is consistent with earlier observations [18,19]. One of the reasons for the large variability among these results may be that the red blood cells have significant differences in content, since volume

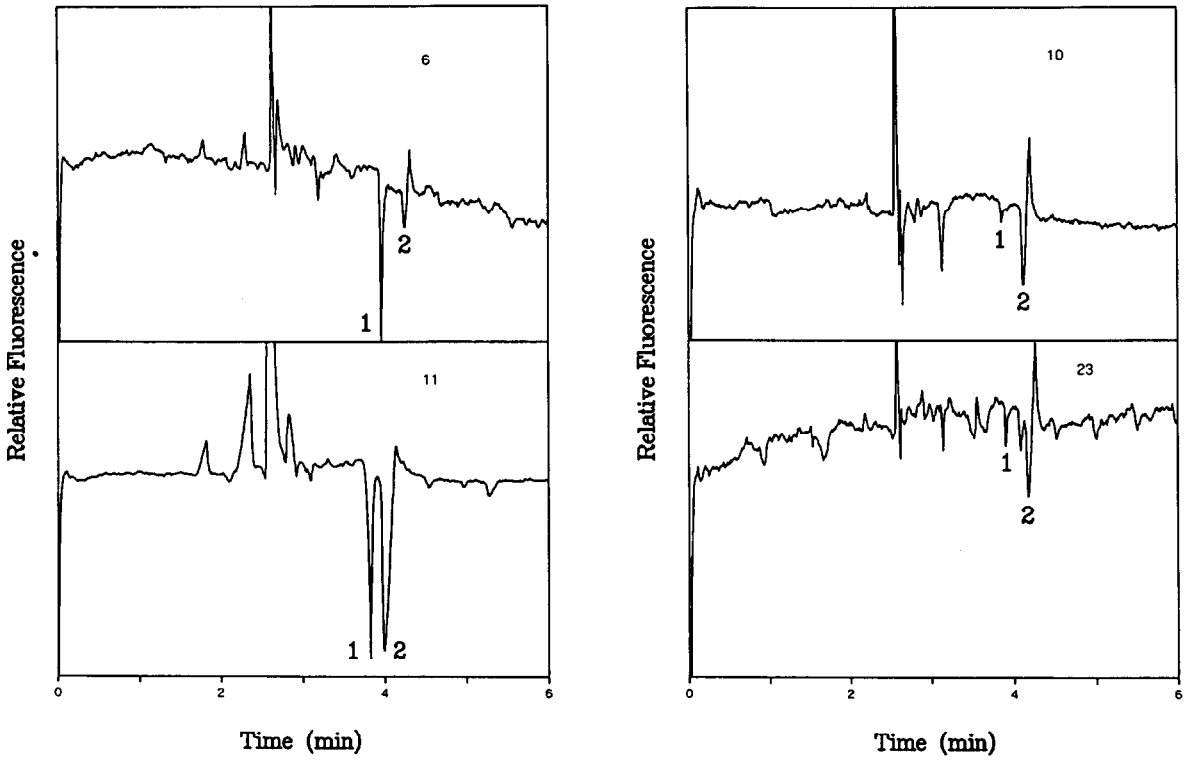

Fig. 3. Electropherograms for the analysis of individual red blood cells. Numbers (6, 11, 10, 23) refer to chronological order of the experiments. Peaks 1 and 2 refer to lactate and pyruvate, respectively.

and age variations can lead to compositional differences. In fact, lactate and pyruvate reflect enzyme activity that can be very different among cells. Another possibility is associated with the stability and accuracy of the indirect detection [3]. Owing to the low concentration of fluorescein used here, the reproducibility is not very good. Up to 40% deviation is observed sometimes, while typical deviations are below 20%. Another possible cause is the stability of the column after several hours of running. From the result of standard samples examined after 32 cell analyses, the peak area and height of four injections yielded a 20% deviation compared to the same standard samples injected before the analysis of cells. The deviation was still within the individual precisions for standard injections. This indicates that the low concentration buffer and the adsorbed material do not damage the capillary. Also, no obvious decreasing or increasing trend was observed in Fig. 4. The average intracellular contents of pyruvate and lactate are found to be 2.1 fmol and 1.3 fmol, respectively (a ratio of 1.6 for pyruvate to lactate). These values are different from the literature values of 1.29 and 0.78 fmol (a ratio of 1.6) for pyruvate and lactate, respectively. The ratio of pyruvate to lactate for each cell is shown in Fig. 5. A considerable variation was observed even though this is a cell-size independent quantity. This is further evidence that metabolism and enzyme activity can be very different in individual cells.

The migration time is always a useful marker for identifying the analyte in capillary electrophoresis. The migration times for pyruvate and lactate are shown in Fig. 6 for each cell injection. We note there is no significant change with run number except for the first several runs, where there is a change of up to 20%. A longer equilibration time between runs leads to a small-

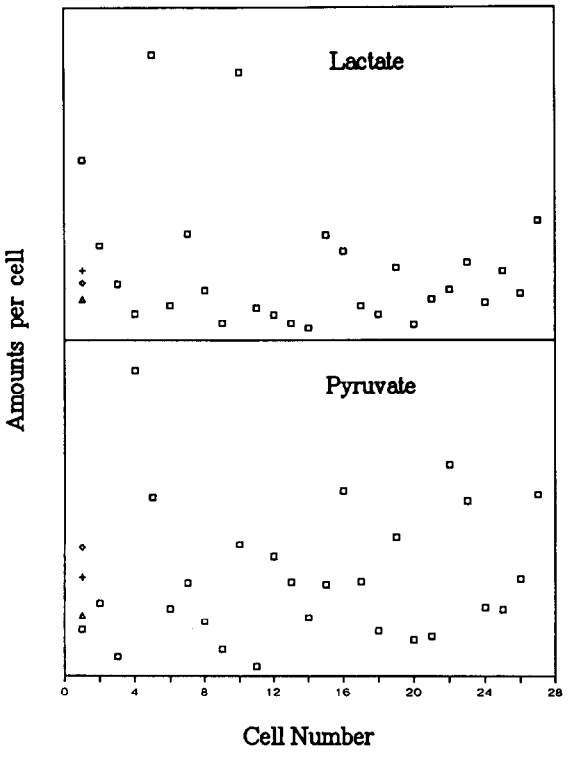

Fig. 4. Distributions of signals from lactate and pyruvate in individual human red blood cells over a series of 27 trials. Zero is indicated by the x-axis in each plot. □ = Amount in individual cells; △ = literature value; + = average for all 27 cells; ◇ = amount measured from a lysed-cell suspension.

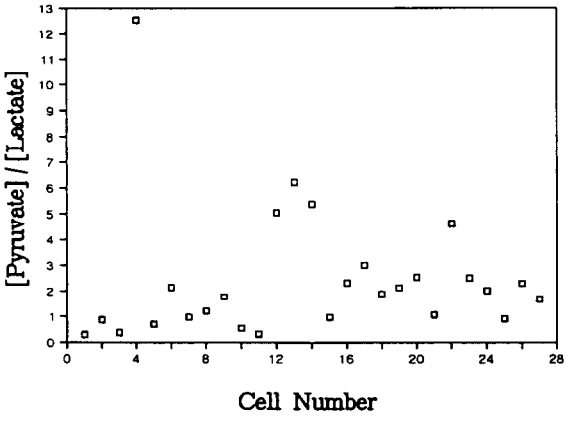

Fig. 5. Ratio of pyruvate to lactate in individual red blood cells for 27 trials.

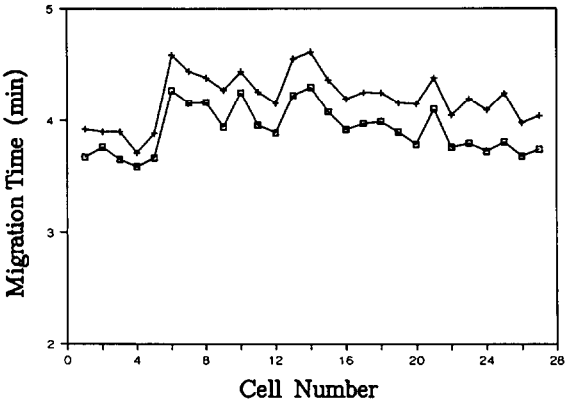

Fig. 6. Changes in migration times over the entire experiment. □ = Lactate; + = pyruvate.

er change in the migration times. We conclude that the change in migration is mainly caused by changing ζ potential due to the injection of cells, because the running voltage and the buffer ionic strength are kept constant for the entire set of runs. Fortunately, the system peak can be used as an internal standard for migration time calibration and for identifying the sample peaks. The ratios of the migration times for pyruvate and lactate are almost constant, as shown in Fig. 7, with an average of 1.072 and a relative standard deviation of only 3%. For standard samples, the ratio is 1.078. This provides positive evidence for identification of the two peaks as pyruvate and lactate.

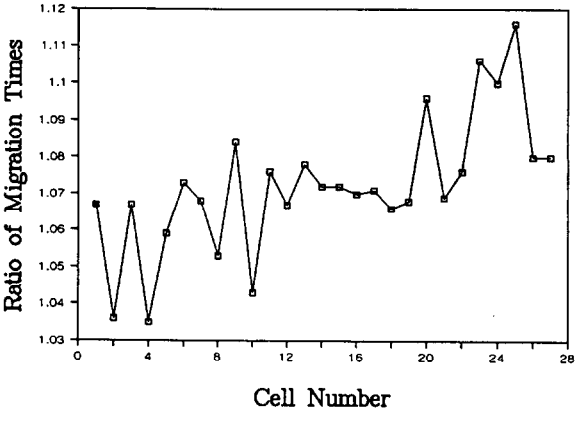

Fig. 7. Ratios of migration times for pyruvate to lactate for 27 runs.

CONCLUSIONS

We have demonstrated the advantages of using fluorescein as the background ion for the indirect detection of anions. The system shows good stability at 100 μM fluorescein. LOD of 20 amol for lactate was achieved. For the best performance in indirect detection, good equilibration is necessary before running the samples. Fluorescein can also be excited by visible laser sources, paving the way for a more compact and a less costly instrument.

The separation and determination of intracellular anions show an important application of this detection approach in the study of mammalian cells. Variations observed from cell to cell should be useful in understanding the biological functions of different components in cells, and perhaps can help to determine whether cells are normal. This scheme might allow the determination of NADH, NAD or LDH by measuring the amounts of pyruvate and lactate, because these are the products of catalysis by those enzymes. This kind of correlation requires very accurate determinations of pyruvate and lactate. Further improvements in stability and reproducibility for this system would thus be necessary to reach this goal.

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