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Analysis of metabolites of glucose pathways in human erythrocytes by analytical isotachophoresis

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Abstract

The aim of this study was to establish an isotachophoretic (ITP) method for the determination of the main compounds of glycolysis in human erythrocytes in order to analyze the influence of different glucose concentrations (mimicking the situation in diabetes mellitus) on this pathway. Samples for ITP were prepared by isolation of erythrocytes, lysis of the cells by heating in double-distilled water and subsequent ultrafiltration (M_r cut-off: 5000). All the main compounds of glycolysis were characterized by ITP. The influence of different glucose concentrations on the main compounds of the energy metabolism (ATP, ADP, lactate, pyruvate) and 2,3-diphosphoglycerate were analyzed in short- and long-time incubations.

Keywords: Isotachophoresis; Glucose; Adenosine 5′-triphosphate; Adenosine 5′-diphosphate; Lactate; Pyruvate; Diphosphogylcerate; Organophosphorus compounds; Carbohydrates

1. Introduction

Erythrocytes have a life span in blood of about 120 days. They lack organelles like the nucleus and mitochondria, energy is generated almost exclusively by glycolysis [1]. Glucose is taken up in an insulin-independent, saturable, passive way by erythrocytes via the glucose transporter GLUT1 [2]. Inside the cells it is converted to glucose-6-phosphate in the hexokinase reaction. All compounds of glycolysis, from glucose-6-phosphate up to the end product, lactate, can be easily determined by isotachophoresis. The metabolites are listed in Table 1, according to their order in glycolysis. Usually, these compounds are determined photometrically by enzymatic methods [3]. The aim of our project is the determination of the most important compounds of energy metabolism of glucose in erythrocytes in a single run in order to get a metabolic survey about the energy status in different disorders (e.g., in different forms of anaemia, in metabolic disorders like glucose-6-phosphatase deficiency, and in conditions of oxidative stress). In this presentation we investigated the influence of different glucose concentrations (mimicking the situation in diabetes mellitus) and started to analyze the consequences of oxidative stress on glycolysis. For this purpose, a method for the isotachophoretic determination of metabolites of glycolysis was established.

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Table 1
RSHs of the metabolites of glycolysis

Metabolite	RSH[a]
Glucose-6-phosphate	0.26
Fructose-6-phosphate	0.26
Fructose-1,6-diphosphate	0.12
Glyceraldehyde-3-phosphate	0.22
Dihydroxyacetone-phosphate	0.23
2,3-Diphosphoglycerate	0.07
1,3-Diphosphoglycerate	–
3-Phosphoglycerate	0.12
2-Phosphoglycerate	0.12
Phosphoenolpyruvate	0.11
Pyruvate	0.10
Lactate	0.31
ATP	0.14
ADP	0.22
NAD^+	0.67
NADH	0.30
$NADP^+$	0.32
NADPH	0.18

[a] RSH: relative step height; ΔL:T=1.0 (*L*: 0.01 *M* HCl–β-alanine, pH 3.2; T: 0.005 *M* caproic acid–histidine, pH~6.0).

2. Experimental

2.1. Chemicals

Methylhydroxyethylcellulose for preparation of the leading electrolyte (L) was from Roth, Karlsruhe, Germany. The terminating electrolyte (T), caproic acid–histidine, pH~6.0 was from J&M, Aalen, Germany. Metabolites of the glycolytic pathways (listed in Table 1) were from Sigma, Deisenhofen, Germany. All other chemicals were from Merck, Darmstadt, Germany.

2.2. Isolation and incubation of erythrocytes

Erythrocytes were isolated from heparinized whole blood samples by centrifugation (1200 *g*, 3 min) at room temperature. After washing with 0.9% NaCl solution, the erythrocyte pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4; one volume erythrocyte pellet+two volumes PBS). For glucose depletion, erythrocyte suspensions were incubated at 37°C for 1 h, and subsequently centrifuged (1200 *g*, 3 min). The supernatant was removed and the pellet was suspended in PBS+Ca–Mg. Incubations were carried out in Eppendorf cups for different time intervals in these suspensions, supplemented or not with different glucose concentrations.

2.3. Preparation of erythrocyte lysates

Erythrocyte suspensions were centrifuged, washed with 0.9% NaCl solution, and the pellets were finally suspended in twice the volume of double-distilled water and boiled for 2 min. After centrifugation, the supernatant was ultrafiltrated (M_r cut-off: 30 000 followed by 5000; Ultrafree Centrifugal Filter Units, Millipore, Bedford, MA, USA). The final ultrafiltrate was diluted 1:1 with double-distilled water before isotachophoresis (ITP).

2.4. Isotachophoresis

ITP was carried out on an ITA-Chrom EA 101 (J&M) (pre-column: capillary 90 mm×0.8 mm I.D.×1.2 mm O.D.; separation column: 160 mm×0.3 mm I.D.×0.7 mm O.D.). Preseparation was carried out at 200 μA, analysis at 50 μA. Leading electrolyte (L): 0.01 *M* HCl–β-alanine+0.1% (w/v) methylhydroxyethylcellulose, pH 3.21; terminating electrolyte (T): 0.005 *M* caproic acid–histidine, pH 6.0; 30 μl of sample was injected.

2.5. Other methods

Whole blood and erythrocyte suspensions were characterized on the automatic blood cell counter Bayer Advia 120.

Glucose and lactate in erythrocyte suspensions were measured on a blood gas analyzer ABL 700 (Radiometer, Copenhagen, Denmark).

3. Results

3.1. Order and characterization of glucose metabolites by ITP

The order of compounds of glycolysis in erythrocytes is listed in Table 1, and the respective relative step heights (RSHs) obtained by ITP using the L/T system indicated are included. Because of the very similar chemical structure, charge and molecular mass of some of these substances, a clear separation is sometimes not possible. However, the most rel-

evant compounds of the energy metabolism of erythrocytes can be separated, including ATP, ADP, lactate, pyruvate as well as 2,3-diphosphoglycerate.

3.2. Isotachopherograms of erythrocyte lysates, calibration curves, and method validation

Calibration curves for all compounds indicated in Table 1 were established using mixtures of 4–5 pure compounds with clearly different mobilities. These compounds were dissolved in double-distilled water and in erythrocyte lysates. The ions were identified by their RSHs and by adding single compounds to the sample mixtures. The relative standard deviations (RSDs) of the RSHs in lysates of erythrocytes were determined in six different samples ranging from 1.6% (ATP) to 3.6% ($NADP^+$). Fig. 1 shows the isotachopherogram of an erythrocyte lysate before and after addition of a mixture consisting of ATP, NADPH, NADH and lactate. As an example, the calibration curves of ATP in double-distilled water and in an erythrocyte lysate are presented.

In further experiments, the influence of different preparation procedures on stability of the erythrocyte lysates was investigated. The best results were obtained by boiling in double-distilled water for 2 min. Values for ATP, ADP, NAD^+, NADPH, $NADP^+$, lactate, pyruvate and 2,3-diphosphoglycerate after boiling ranged from 92 to 105% compared to the samples before boiling. Only NADH significantly decreased after this procedure (66%).

In order to study the reproducibility of sample preparation, erythrocyte lysates were prepared from six aliquots of the same blood sample. The RSDs of zone lengths from the most relevant ions were 7.8% (ATP) and 4.2% (ADP) (both measured using the UV signal), 7.6% (pyruvate), 8.7% (lactate) and 5.1% (2,3-diphosphoglycerate), indicating a good reproducibility of this multi-steps isolation procedure. All compounds of the samples finally used for isotachophoretic analysis were stable at 4°C for at least 24 h.

3.3. Time-dependent changes of energy metabolism in erythrocytes in the absence or presence of physiological glucose concentrations (100 mg/dl~ 5.5 mM)

Changes in glycolysis of erythrocyte suspensions were measured in the absence and presence of physiological glucose concentrations (5.5 m*M*). Fig. 2 shows the relationship between the glucose consumption and production of lactate, the end product

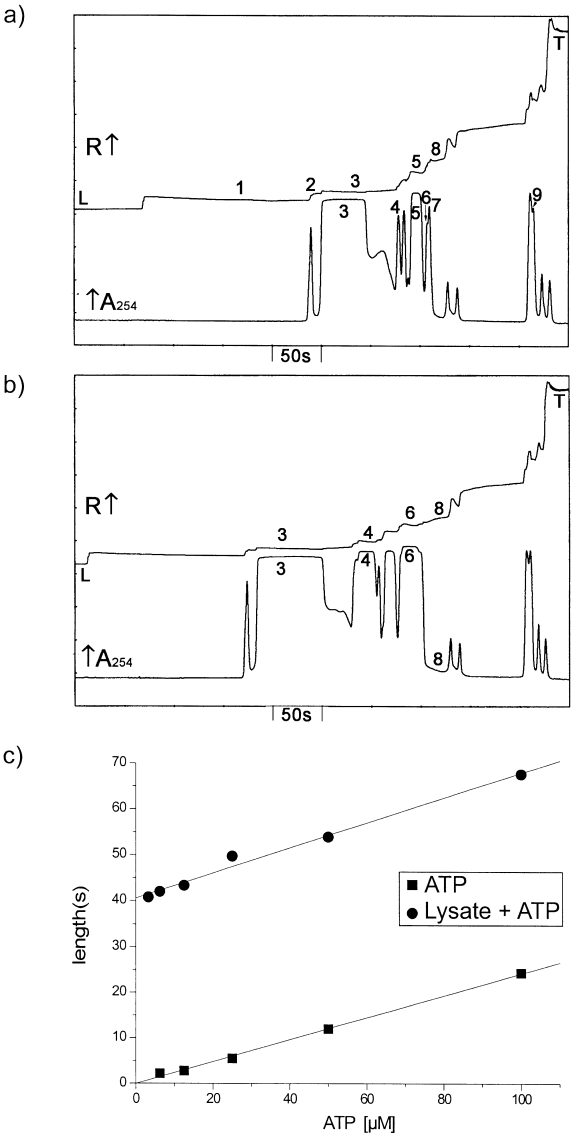

Fig. 1. Isotachopherograms of erythrocyte lysates alone (a) and loaded with ATP(3), NADPH(4), NADH(6) and lactate(8) (b). As an example, the calibration curves of ATP in double-distilled water (r=0.999) and in erythrocyte lysates (r=0.994) are presented (c). A 100-µ*M* concentration of ATP corresponds to $3\cdot10^{-9}$ mol/30 µl samples injected into the ITP system. 1=2,3-Diphosphoglycerate; 2=pyruvate; 3=ATP; 4=NADPH; 5=ADP; 6=NADH; 7=$NADP^+$; 8=lactate; 9=NAD^+.

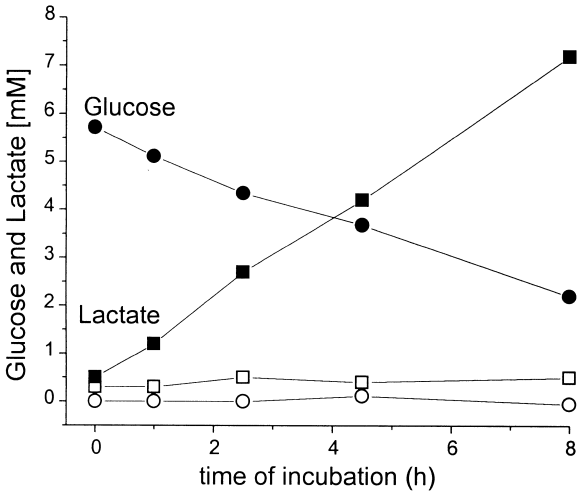

Fig. 2. Time-dependent glucose consumption and lactate production in erythrocyte suspensions in the absence (empty symbols) and presence (filled symbols) of glucose (5.5 m*M*), measured with the blood gas analyzer ABL 700.

of glycolysis, at 37°C in erythrocyte suspensions during an 8-h incubation time. After 8 h, 3.5 mmol glucose/l were consumed, and 6.7 mmol/l lactate were formed, indicating an almost complete transformation of glucose to lactate (1 mol glucose produces 2 mol lactate in glycolysis). In the absence of glucose, the lactate production was, of course, extremely small. Simultaneously, the compounds of glycolysis were measured in erythrocyte lysates by isotachophoresis. Changes in glycolytic parameters are demonstrated in Fig. 3: the upper diagram shows the isotachopherogram at t_0. As an example, the situation after 8 h incubation time is demonstrated in the lower diagrams (left: in the absence of glucose; right: in the presence of 5.5 m*M* glucose). There is a significant decrease of ATP, especially in the absence of glucose. Furthermore, 2,3-diphosphoglycerate, a particular metabolite of glycolysis in erythrocytes which regulates binding and release of oxygen on hemoglobin, is drastically reduced. Lactate production increases strongly; however, the amount of lactate still present in erythrocytes (as measured by ITP) is much smaller than in whole erythrocyte suspensions (Fig. 2) since the major part of lactate is rapidly released. Fig. 4 shows quantitatively the time-dependent changes of ATP, ADP, lactate, pyruvate and 2,3-diphosphoglycerate in erythrocytes in the presence and absence of glucose.

3.4. Changes of ATP levels in erythrocytes after a 20-h incubation time in the presence of different glucose concentrations

In these experiments, the influence of low (2.25 m*M*), physiologically normal (5.33 m*M*) and high (15.5 m*M*) glucose concentrations on ATP levels in erythrocytes were investigated. These glucose concentrations reflect the clinical situation of hypo-, normo- and hyperglycaemia, respectively. Table 2 shows that glycolysis is strictly enhanced in normoglycaemic situations compared to hypoglycaemic conditions, whereas only small differences are seen between normo- and hyperglycaemic conditions. This is due to the characteristics of the flux of glucose ($J_{glucose}$) into erythrocytes with $J_{max}=1.0\cdot 10^{-6}$ m*M* cm s^{-1} and $K_m=0.5$ m*M*. Remaining ATP levels after 20 h incubation time, however, were similar in hypo- compared to normoglycaemic conditions, whereas higher ATP levels were observed in the presence of elevated glucose concentrations.

4. Discussion

The aim of our study was to establish a method for determination of main metabolites of glycolysis in erythrocytes by isotachophoresis in order to obtain a metabolic survey in a single run. Our first interest was to use this method for determination of the influence of different glucose concentrations (mimicking the situation in diabetes mellitus) and of oxidative stress. For erythrocyte lysis, we proved different methods and found finally that boiling in water for 2 min, followed by ultrafiltration, was most suitable for subsequent isotachophoretic analysis. This process did not destroy ATP, ADP, pyruvate, lactate and 2,3-diphosphoglycerate, the compounds that were estimated quantitatively (Fig. 4). A similar method was described some years ago [4,5] for determination of the energy status of erythrocytes under conditions of blood banking. Although several of the many low-molecular-mass compounds of glycolysis cannot be separated under the conditions

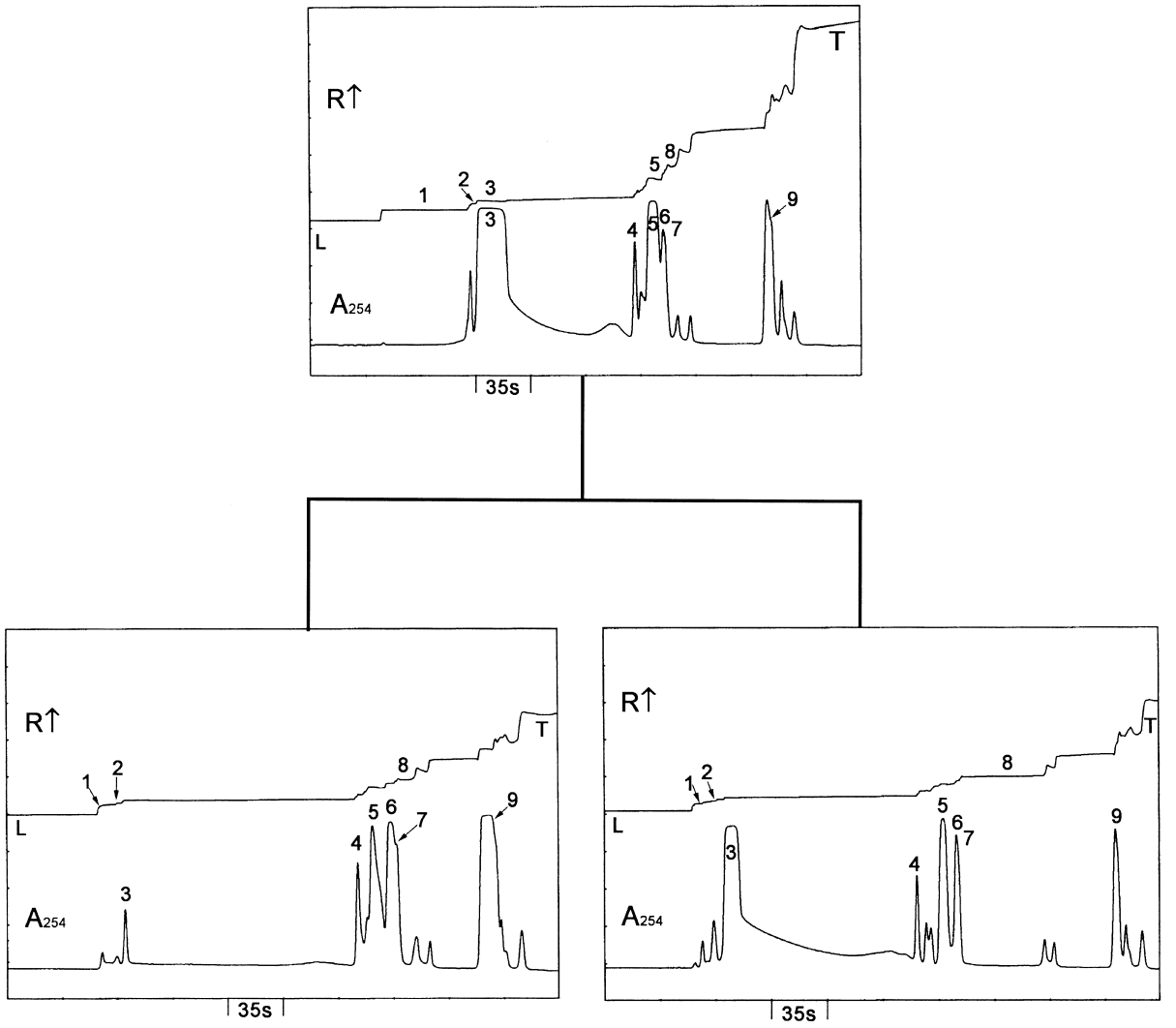

Fig. 3. Changes in metabolites of glycolysis during an 8-h incubation period in the absence and presence of 5.5 m*M* glucose. Upper diagram: isotachopherogram at t_0; lower part: left diagram: isotachopherogram in the absence of glucose at $t_{8\ h}$; right diagram: isotachopherogram in the presence of 5.5 m*M* glucose at $t_{8\ h}$. Numbers relate to the same substances as in Fig. 1.

chosen, the determination of most compounds of interest is possible, among them ATP/ADP, 2,3-diphosphoglycerate, pyruvate, lactate, as well as NAD^+ and NADPH. NADH and $NADP^+$ form a mixed zone, and further steps would be necessary for their separation. The most important compound of the energy metabolism, ATP (ADP) can be determined very well. An influence of the glucose concentration on these parameters could be demonstrated.

In addition, we started to investigate the influence of oxidative stress on the glycolytic pathway in erythrocytes. In contrast to the dependency of ATP levels from the glucose concentration as shown above, almost no influence of H_2O_2 in concentrations as high as 5 m*M* on the ATP levels and other parameters of the isotachopherograms could be detected, irrespectively, on the presence or absence of glucose (data not shown). This may be due to the fact that erythrocytes contain an excellent defence

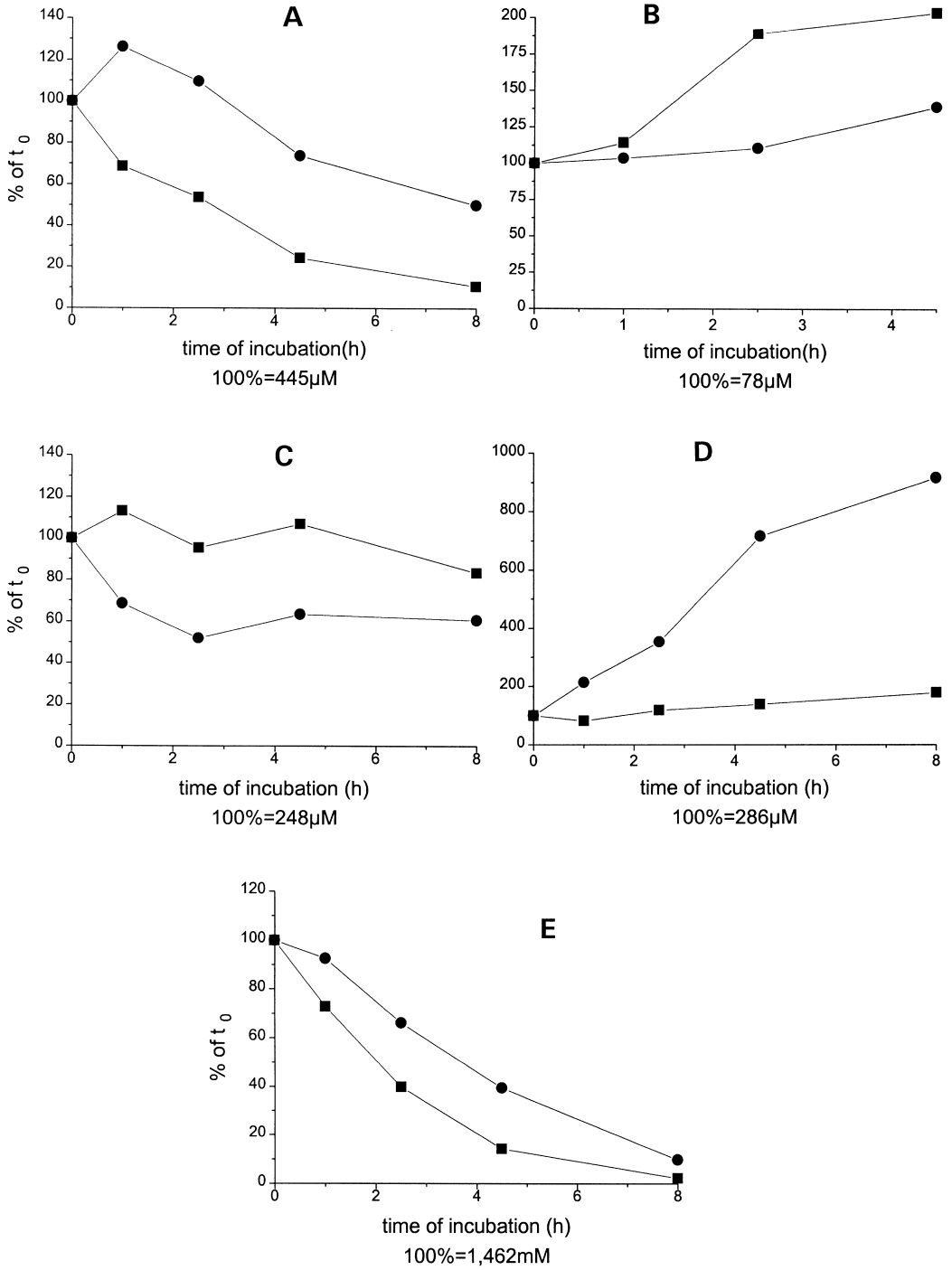

Fig. 4. Time-dependent changes of parameters of the glucose metabolism in erythrocytes during incubation in the absence [■] and presence [●] of 5.5 m*M* glucose. (A) ATP; (B) pyruvate; (C) ADP; (D) lactate; (E) 2,3-diphosphoglycerate.

Table 2
ATP levels in erythrocytes after 20 h incubation time with different glucose concentrations

Glucose (m*M*)[a]	Glucose (consumed) (m*M*)	Lactate (produced) (m*M*)	ATP (remaining)[b] (%)
0	0	0.3	12.6
2.25 (41 mg/dl)	1.48 (66%)	2.8	14.4
5.33 (97 mg/dl)	2.36 (44%)	4.6	14.8
15.5 (281 mg/dl)	2.81 (18%)	5.1	20.1

[a] Concentrations in erythrocyte suspensions measured by ABL 700.
[b] Measured by isotachophoresis in erythrocyte lysates. ATP concentration at t_0 is set as 100%.

system against oxidative stress, in which catalase obviously plays a much more important role than the gluthation system [6].

Acknowledgements

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