The whole-cell kinetic metabolic model of the pH regulation mechanisms in human erythrocytes

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Introduction

Monitoring intracellular pH has importance in understanding intracellular metabolism and functions (Chao et al., 2018; Michl et al., 2019; Huang et al., 2020; Doyen et al., 2022). Protein-protein, protein-ligand, protein-membrane interactions are highly dependent on the pH value, so pH is an important factor affecting the functioning of macromolecules and a whole-cell. At the cellular level, maintenance of cytosolic pH (intracellular pH, pHi) within a narrow range is important for many processes, including biochemical reactions, functions of transporters, channels, receptors, structural proteins, and regulatory molecules (Boron, 2010; Occhipinti & Boron, 2015; Shartau et al., 2016; Calvetti et al., 2020). The pH gradients determine the direction and time of macromolecular interactions. In this context, several molecular mechanisms evolved for solving the problem of pH regulation by transporting acid-base equivalents across membranes (Hsu, 2018; Lee & Hong, 2020; Li et al., 2021; Michenkova et al., 2021; Doyen et al., 2022).

Erythrocytes are special cells because they carry out the transport of HCO₃⁻ ions to enhance the transfer of CO₂ from the systemic circulation capillaries to the pulmonary capillaries (Boron, 2010; Jennings, 2021). These cells play a fundamental role in regulating the acid-base balance of extracellular fluids. The role of carbonic anhydrase (CA) and hemoglobin in the processes of pH stabilization is shown and analyzed. The physiological role of the metabolon between band 3 protein (AE1), CA, aquaporin and hemoglobin in maintaining pH homeostasis in the conditions of in vitro experiments is discussed.

Keywords: carbonic anhydrase; anion exchanger AE1; gas exchange; Jacobs-Stewart cycle; bicarbonate; COPASI.
tion in buffer solutions of various compositions. These procedures change the intracellular pH (Meyrman & Hornblower, 1991) and consequently the activity of cellular reactions that are pH dependent. However, few people pay attention to this in such studies. In this connection, difficulties arise with the reproducibility of the results and their interpretation. The transmembrane flux of any substance could affect the transmembrane gradients of all others, creating complex interdependencies that cannot be understood by intuition alone. And although the role of erythrocytes in the regulation of plasma pH has been widely studied (Swietach et al., 2010), much less is known about the dynamics of hydrogen ions inside the erythrocytes themselves.

The dynamics of pH cannot simply be described as a combination of transporter rates and enzyme activities because their activities are linked to other metabolic reactions, even if they do not directly affect pH. Furthermore, since protonation reactions are numerous within the cell, it is also imperative that cellular chemistry is fully considered as it affects the availability of substrates for transporters and the protonation and deprotonation of groups that act as buffers (Doyen, 2022). Even at the level of a single cell, perturbations in acid-base reactions, diffusion and transport are so complex that they cannot be understood without a quantitative model (Boron, 2010; Occhipinti & Boron, 2015). Due to the complexity and diversity of the processes underlying pH regulation, mathematical modeling and metabolome analysis are powerful tools for analyzing complex, dynamic, and large-scale systems (Occhipinti & Boron, 2015; Doyen et al., 2022). The construction of mathematical and computational models can provide valuable information about the mechanisms of intracellular regulation, pH in particular. This prompts researchers to develop mathematical models of acid-base homeostasis (Geers & Gross, 2000; Rees et al., 2010; Al-Samir et al., 2013; O'Neill & Robbins, 2017, Cherif et al., 2020; Doyen et al., 2022). Mathematical models of blood acid-base chemistry, based on the equations of the laws of active masses and mass balance, have found application as diagnostic tools in intensive care (Cherif et al., 2020; Leyoldt et al., 2020). However, some of them are focused on the study of gas exchange between erythrocytes and blood plasma, others include a small number of reactions involved in the Jacobs-Stewart cycle and do not consider the relationships with other reactions that may affect them. Some models are difficult to apply to the analysis of experimental data.

As computational methods improve, it becomes possible to model the functioning and relationships of many metabolic reactions. The combination of computational approaches and a huge stock of existing (and future) experimental data has the potential to reach a true understanding of the molecular mechanisms (Nishino et al., 2013; Dotsenko, 2016; Jennings, 2021) of processes, as well as the principles of their regulation by various physical or chemical factors, including pH and temperature.

Thus, the goal of the work was to create a mathematical metabolic model of erythrocytes, which combines cellular metabolism with acid-base processes and gas exchange. The model provides the ability to quantify the current value of intracellular pH, based on a small set of experimental data, such as the composition of the extracellular medium and the initial value of intracellular pH. So far, the model has been used to analyze the mechanisms of pH regulation in human erythrocytes in vitro. Along with this, the model allows one: a) to predict the course of processes and the content of metabolites in the cell depending on the value of intracellular pH in in vitro experiments, b) to analyze experimental data more accurately and qualitatively, c) to save time and money by performing a smaller number of experimental studies.

Materials and methods

Mathematical model and modeling methods. Model creation and all numerical calculations based on the mathematical model of human erythrocyte metabolism were carried out using the simulation environment COPASI 4.36. The model includes two compartments, 85 reactions and 99 metabolites. The reactions included in the model are shown in Table 1, the metabolic scheme reproduced in the model is shown in Figure 1. The model is based on kinetic equations. The published model of human erythrocyte metabolism from BioModels Database (www.ebi.ac.uk/biometric/Model/3595052398/Files) was used as the basic model (Mulquiney & Buch, 1999). The basic model consists of the main metabolic pathways of human erythrocytes (53 reactions), such as glycolysis, the pentose phosphate pathway, some membrane transport systems of intermediate products, reactions of the interaction between hemoglobin and metabolites. In this model, certain enzymatic reactions of glycolysis are controlled by the enzymes hexokinase (HK), phosphofructokinase (PFK), glyceraldehyde phosphate dehydrogenase (GA3PDH), pyruvate kinase (PK), lactate dehydrogenase (LDH), 2,3-diphosphoglycerate (2,3-DPG) shunt reactions are presented as pH-dependent kinetics. In addition, we added the reactions of the interaction of band 3 protein with ligand forms of Hb and glycolytic enzymes, such as PFK, aldolase (ALD), GAPDH and LDG. The kinetics of these interactions are described in Nishino et al. (2013). The effects of inorganic phosphates and adenosine nucleotides, which can serve as allosteric effectors of glycolysis, were included in the model, as in recent mathematical models of erythrocytes (Kanoshita, 2007; Nishino et al., 2013).

The created model includes two compartments: intracellular and extracellular, which allows one to study the influence of the composition of the external environment (primarily the content of gases) on the processes taking place in the cell. The extracellular space was treated as a well-mixed compartment of infinite size, and the unmixed layers around the cells were not considered.

The transition of hemoglobin between oxygenated (R-) and deoxygenated states (T-state) depending on pH, temperature and 2,3-BPG level is described as in Nishino et al., 2013) using the following ratios:

\[
S_{\text{HbO}} = \frac{[HbO]}{[Hb]} = \frac{K_{\text{HbO}}}{[O]} + 1,
\]

where \(S_{\text{HbO2}}\) is the degree of saturation of hemoglobin with oxygen.

\[
K_i = \frac{10^{10 [CO_2]}}{10^{10 [CO_2]} + \left(\frac{[CO_2]}{[CO_2]}\right)^2 \cdot \left(\frac{2.3 - BPG}{2.3 - BPG}\right)^3 \cdot \left(T - T^c\right)^4},
\]

\[
[O_i] = 10^{-8} \left[3.07 - 0.057(T - 37) + 0.0002(T - 37)^2\right],
\]

\[
[O_2] = 0.94 \left[3.07 - 0.057(T - 37) + 0.0002(T - 37)^2\right] - 0.65 \cdot P_{O_2}\text{sat}.
\]

\[
n = -6.775 + 2.0372 \cdot pH - 0.1235 \cdot pH^2,
\]

\[
n = 0.008765 + 0.00086 \cdot P_{O_2}\text{sat} + 6.3 \cdot 10^{-7} \cdot P^2,
\]

\[
T = 2.583 + 28.6978 \cdot [2.3 - BPG] + 917.69 \cdot [2.3 - BPG],
\]

\[
a = 1.6914 + 0.0618 \cdot T + 0.00048 \cdot T^2.
\]

The development of the pH homeostasis cycle. Carbon dioxide: The content of intracellular CO2 consists of the formation or consumption of CO2 via a) intracellular and extracellular hydration-dehydration reactions, b) CO2 exchange with the incubation medium, and c) the reaction of CO2 with hemoglobin.

The hydration-dehydration of CO2 occurs according to the reaction:

\[
CO_2 + H_2O \xrightleftharpoons{\text{HCO}_3^-}{\text{K}} H^+ + \text{HCO}_3^-.
\]

It was believed that in the extracellular environment, CO2 hydrates non-enzymatically, and inside cells it metabolizes by carbonic anhydrase (CA). The kinetics of the hydration process was described by the equation:

\[
v = a_k [CO_2] K_{\text{K}} [H^+] [\text{HCO}_3^-].
\]
where $a$ is a parameter that determines the reaction activation level with the participation of CA. $k_0 = 0.137 \, \text{s}^{-1}$, $k_1 = 57.5 \, \text{s}^{-1}$, $K = 0.35 \, \text{mM}$ (Bidani et al., 1978; Geers & Gros, 2000). $a = 1$ is for a non-enzymatic process, $a = 1000$ is for the intracellular reaction catalyzed by CA (established in the simulation process).

b) The flux $\text{CO}_2$ was described by the reverse process $\text{CO}_2^- \rightarrow \text{CO}_2^-$, in work (Geers et al., 2000; Al-Samir et al., 2013; Michenkova et al., 2021). The rate of the process was described by the equation: $v = \frac{k_1}{k_2} [\text{CO}_2^-]_{in} - [\text{CO}_2^-]_{out}$, where $k_1 = k_2 = 0.93 \times 10^{-5} \, \text{s}^{-1}$ (set during simulation).

c) Binding of $\text{CO}_2$ to oxy-, deoxyhemoglobin was described by the reaction:

$$n \text{CO}_2 + \text{Hb} \cdot \text{NH}_4 \xrightarrow{k_1} \text{Hb} \cdot \text{NHCOO}^- + H^+.$$ 

The rate of the reaction was described Geers & Gros (2000):

$$v_{\text{oxyHb}} = \frac{k_1}{k_2} \cdot \{\text{oxyHb}\} \cdot z_0 - \{\text{carbHb}\},$$ 

where $z_0 = \frac{2\langle[\text{CO}_2^-]_{in}\rangle}{4\left([\text{CO}_2^-]_{in} + \left[\frac{H^+}{K_{\text{eq, oxy}}} + \frac{H^+}{K_{\text{eq, deoxy}}}\right] \right)},$ 

$k_{\text{eq, oxy}} = 90 \, M^{-1} \cdot s^{-1}$, 

$k_{\text{eq, deoxy}} = 21 \, M^{-1} \cdot s^{-1}$, 

$K_{\text{eq}} = \text{dissociation constant of the certain form of hemoglobin}$

Therefore, the Cl– flux across the erythrocyte membrane is equal and opposite to that for $\text{HCO}_3^-$ (Bidani et al., 1978).

The rate of the reaction was described Geers & Gros (2000):

$$H^+ + \text{Hb} \cdot \text{NH}_4 \xrightarrow{k_1} \text{Hb} \cdot \text{NHCOO}^- + H^+.$$

The rate of the process was described: 

$$v = k_1 \cdot \{\text{Hb}\} \cdot [\text{NH}_4] - [\text{NH}_4] \cdot [\text{Hb}],$$

where $k_1 = k_2 = 0.93 \times 10^{-5} \, \text{s}^{-1}$ (set during simulation).

Bicarbonate ion. The mass balance of bicarbonate ions in each compartment is determined by the hydration-dehydration reaction rate expression, and the flux of $\text{HCO}_3^-$ through the erythrocyte membrane. The model considered the exchange flux of $\text{HCO}_3^-$ with the participation of band 3 protein (AE1), which acts as an anion exchanger.

The transport of $\text{HCO}_3^-$ through the AE1 transporter was described by the reverse process $\text{HCO}_3^-$ out $\xrightarrow{k_1} \text{HCO}_3^-$ in.$$

The exchange of $\text{HCO}_3^-$ for $\text{Cl}$ was described in the paper in terms of passive diffusion along an electrochemical gradient:

$$v = k_1 \cdot [\text{HCO}_3^-]_{in} - k_2 \cdot \left[\frac{(1+10^{N_{s, -h_0}} \cdot r)}{1+10^{N_{s, -h_0}} \cdot p_{KA}} \cdot [\text{HCO}_3^-]_{in}\right],$$

where $k_1 = k_2 = 6.9 \times 10^{-6} \, \text{s}^{-1}$ (established in the simulation process), $p_{KA} = 6.3$, $r$ -- the Donnan ratio, $r = \left[\frac{[\text{HCO}_3^-]_{in}}{[\text{HCO}_3^-]_{out}}\right] = 0.69$. The $\text{HCO}_3^-$ transfer of across the cell membrane approaches the intracellular concentration of $\text{HCO}_3^-$, that is not equal to the extracellular concentration, but is in Donnan equilibrium (Geers & Gros, 2000; Nishino et al., 2013). The condition of maintaining of the Donnan distribution for $\text{HCO}_3^-$ is described in the simulation process.

Chloride ion. The $\text{Cl}$ flux ions was not considered in this version of the model. The concentration of chloride ions in the environment and in erythrocytes changes only with the $\text{Cl}$ flux across the cell membrane in exchange for $\text{HCO}_3^-$. Therefore, the $\text{Cl}$ flux across the erythrocyte membrane is equal and opposite to that for $\text{HCO}_3^-$. $\text{H}^+$ changes in cells occur due to hydration-dehydration of $\text{CO}_2$ association-dissociation of hemoglobin and carbamate, oxygenation-deoxygenation of hemoglobin (the last two processes occur only intracellularly). When 1 mol of $\text{CO}_2$ is hydrated, 1 mol of $\text{H}^+$ is formed. According to the literature, the moles of $\text{H}^+$ released per mole of $\text{CO}_2$ bound by hemoglobin (QH2-carbamate) range from 1 to 2 (Bidani et al., 1978). Oxygenated hemoglobin is a stronger acid than deoxygenated hemoglobin, also $\text{H}^+$ are released from hemoglobin during oxygenation. According to data (Bidani et al., 1978), at normal levels of 2,3-DPG, 0.7 mol of $\text{H}^+$ is released per mol of HbO2, formed under normal physiological conditions. Thus, the actual change in free intracellular $\text{H}^+$ is due to the balance between its production and consumption and the action of intracellular buffers, mainly hemoglobin.

Buffer cellular reactions. Buffering of protons by hemoglobin occurs according to the reactions:

$$bH^+ + \text{Hb} \cdot \text{NH}_4 \xrightarrow{k_1} \text{Hb} \cdot \text{NHCOO}^- + H^+,$$

$$bH^+ + \text{HbO}_2 \cdot \text{NH}_4 \xrightarrow{k_1} \text{HbO}_2 \cdot \text{NH}^- + H^+.$$
from plasma by centrifugation and washed three times with Na-phosphate buffer (0.015 M, pH 7.4) (buffer solution 1) containing 0.15 M NaCl (cells a). To obtain erythrocytes with a reduced initial value of intracellular pH (cells b), additional washing with Na-phosphate buffer (0.015 M, pH 7.4) was used (Meryman & Hornblower, 1991).

In the obtained erythrocyte paste, the total hemoglobin content was introduced into the medium of the same buffer solution without glucose content. The hemoglobin content in the studied suspensions was at the level of 3.00 ± 0.18 mg/mL. рНi was studied in hemolysates of erythrocytes before the start of the experiment and then every 20 min during incubation in buffer solution 1. During the experiment, the system under study was in contact with ambient air. After certain time intervals, 2 mL of erythrocyte suspension was taken into a test tube and subjected to centrifugation at 3000 rpm for 1 min. The supernatant was carefully aspirated, the cells were lysed by adding 1 mL of de-ionized water. The pH measurement was performed using a laboratory ionomer using a combined electrode (ESK-10614/7) (Nishino et al., 2013). The experiments were carried out at a temperature of 25 °C in three to five replicates (n).

The results of measuring pH in erythrocytes that were washed in buffer medium 1 and then incubated in this medium for 3 hours are shown in Figure 2. The рНi change in erythrocytes with their initial value 7.12 ± 0.05 is shown in Figure 2a. The рНi change in erythrocytes with their initial value 7.05 ± 0.04 is shown in Figure 2b. It is evident that manipulation with cells violates the stationary pH value to varying degrees, but over time, restoration to equilibrium value takes place.

The results were analyzed in the Statistica 8.0 program (StatSoft Inc., USA). Experimental data are presented as x ± SE (x is the mean, SE is standard error of mean).

**Results**

The initial values of intracellular concentrations of CO2 and HCO3⁻ were obtained using the parameter optimization procedure. Values were in case (a) 6.2 ± 1.7 and 231.1 ± 68.7 µM, and in case (b) 42.6 ± 8.7 and 298.9 ± 47.7 µM respectively. The content of dissolved CO2 and HCO3⁻ was considered fixed at the level of 8.38 and 168.8 µM (calculated according to the Henderson-Hasselbach equation). It was believed that the extracellular medium pH did not change and was 7.4.

![Fig. 1. Pathways reaction involved in the model: adapted from Nishino et al. (2013); abbreviations for metabolites: 13DPG, 1,3-biphosphoglycerate, 23DPG, 2,3-diphosphoglycerate, DHAP, dihydroxyacetone phosphate, ERY4P, erythrose 4-phosphate, F6P, fructose-6-phosphate, FDP, fructose diphosphate, G6P, glucose-6-phosphate, GA3P, glyceraldehydes-3-phosphate, GL6P, 6-phosphogluconate lactone, GLC, glucose, GO6P 6-phosphogluconate, GSH, reduced glutathione, GSSG, oxidized glutathione, LAC, lactate, P2G, 2-phosphoglycolate, P3G, 3-phosphoglycerate, PK, pyruvate kinase, PEP, phosphoenolpyruvate, PYR, pyruvate, R5P, ribose-5-phosphate, RL5P, ribulose-5-phosphate, SED7P, sedoheptulose 7-phosphate, XYL5P, xylose-5-phosphate; abbreviations for reactions: AdylK, adenylate kinase (EC 2.7.4.3), ALD, aldolase (EC 4.1.2.13), DPGase, diphosphoglycerate phosphatase (EC 3.1.3.13), DPGM, diphosphoglycerate mutase (EC 5.4.2.4), EN, enolase (EC 4.2.1.11), G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.1.1.44), GSSGR, glutathione-disulfide reductase (EC 1.8.1.7), HK, hexokinase (EC 2.7.1.1), LDH, lactate dehydrogenase (EC 1.1.1.27), MetHbRed, methemoglobin reductase, MT, PFK, phosphofructokinase (EC 2.7.1.11), PGI, glucose-6-phosphate isomerase (EC 5.3.1.9), PGK, phosphoglycerate kinase (EC 2.7.2.3), PGLase, phosphogluconolactonase (EC 3.1.1.13), PGM, phosphoglycerate mutase (EC 5.4.2.1), PK, pyruvate kinase (EC 2.7.1.40), PRM, phosphoribomutase (EC 5.4.2.7), RPI, ribose-5-phosphate isomerase (EC 5.3.1.6), TA, transaldolase (EC 2.2.1.2), TK, transketolase (EC 2.2.1.1), TPI, triosephosphate isomerase (EC 5.3.1.1), XPL, ribulose phosphate epimerase (EC 5.3.1.1)

The results of measuring pH in erythrocytes that were washed in buffer medium 1 and then incubated in this medium for 3 hours are shown in Figure 2. The pH change in erythrocytes with their initial value 7.12 ± 0.05 is shown in Figure 2a. The pH change in erythrocytes with their initial value 7.05 ± 0.04 is shown in Figure 2b. It is evident that manipulation with cells violates the stationary pH value to varying degrees, but over time, restoration to equilibrium value takes place. The nature of the pH change during the first hour of incubation is different and depends on the CO2 content in the cells. The solid line is the pH dependence obtained during the model’s parameter optimization (parameter estimation) followed by the solution of the model’s differential equations (time course procedure). The model predicts a sharp increase in pH in the condition a, and a sharp decrease in condition b within 1 s from the start of the experiment. The following Figures 3–7 show the information obtained during the simulation.
The coincidence of experimental and calculated values of pH allows us to claim that the simulation results reflect real processes in cells.

The flux through CA in cells a and b is shown in Figure 3. In cells a, after their introduction into the incubation medium, the flux through CA varied from –120.9 µM/s (CA consumed HCO$_3^-$ and H$^+$ and produced CO$_2$) to –0.096 µM/s. After 1.3 s, the flux changed its sign and reached the level of 1.2 µM/s, after which it rapidly decreased (Fig. 3a). After 2 min, the flow through the CA reached the level of 0.060 µM/s and further decreased very slowly. In cells b, the initial flow through CA was 1.3 µM/s. The calculated values of the flows through CA are much lower, but the nature of the changes is similar to condition a (Fig. 3b). After 60 min of the experiment, the magnitudes of currents through CA in cells a and b did not differ.

The results of modeling the dynamics of CO$_2$ and HCO$_3^-$ content are shown in Figure 4. In the case of a cells, the initial value of CO$_2$ in the cells is 2.15 µM and HCO$_3^-$ 227.56 µM. In 1.3 s, CA reduces HCO$_3^-$ to 184 µM (Fig. 4a), while the content of CO$_2$ increases only by 2.32 µM. In b cells, the initial content of CO$_2$ was 33 µM and HCO$_3^-$ 245.85 µM. Due to the high concentration of CO$_2$, CA converts CO$_2$ to HCO$_3^-$, as a result of which its concentration decreases for 7 minutes (Fig. 4b) and the content of HCO$_3^-$ increases. After 60 minutes of the experiment, the predicted values of the content of CO$_2$ and HCO$_3^-$ in cells a and b differed slightly, and for 180 minutes there was an increase in the intracellular content of both CO$_2$ and HCO$_3^-$.

Fluxes of CO$_2$ and HCO$_3^-$ across the channels and exchanger AE1 are shown in Figure 5. The fluxes' sign is showing its direction (according to how the process is described in the model (Table 1)). A positive flux of CO$_2$ means its outflow from the cell, a positive flux of HCO$_3^-$ means an influx of HCO$_3^-$ into the cell from the outside. In cells a, during 1.3 s, CO$_2$ and HCO$_3^-$ enter, during the same period, the fluxes into the cell decrease, and after 1.3 s, the outward fluxes of CO$_2$ and HCO$_3^-$ become dominant. From the beginning of the experiment, CO$_2$ and HCO$_3^-$ fluxes outward from cells b.

The total H$^+$ fluxes of hemoglobin-involved buffering processes are shown in Figure 6. Figure 7 shows changes in the content of carbhemoglobin in cells. In a cells for 1.3 s it is oxyhemoglobin which is the donor of H$^+$ for CA (data not shown) which during this time interval converts HCO$_3^-$ into CO$_2$. The processes of H$^+$ binding are significantly reduced (Fig. 6a) and a significant amount of the formed CO$_2$ binds to hemoglobin (Fig. 7a). After 1.3 s CA begins to catalyze the CO$_2$ hydration reaction, which leads to acidification of the internal environment (Fig. 2a). An increase in H$^+$ content shifts the carboxamination reaction towards dissociation, the level of carboxymygoglobin in decreases, and CO$_2$ increases. This could be explained by higher flux across CA in the 1–50 s time interval in cells a. CO$_2$ is hydrated by CA and removed from the cell.

In cells b, there is an excess of CO$_2$, which is immediately converted by CA into HCO$_3^-$ and H$^+$. Subsequently there is a need to bind H$, which is indicated by an increase in the flux across the buffer systems. After 25 minutes, H$^+$ binding fluxes in both cell types become the same and then tend to decrease. In cells b, the level of carboxyhemoglobin slowly gradually increases (Fig. 7b).
the scheme in Figure 8c. A detailed discussion of these mechanisms is provided in the Discussion section.

Discussion

In the cytosol, pH is both one of the most controllable and one of the most difficult parameters to control. The pH of the medium determines the degree of protonation of acid-base groups, which are especially abundant in macromolecular aggregates. Since enzymes and cellular metabolites exhibit a strong dependence on pH, modifying the protonation of key residues can profoundly affect primarily the surface charge of macromolecules. Thus, pH regulation is based on an intricate interaction between the large number of protonated groups in biological molecules, pKa values, expression parameters, stability, kinetics, and affinity of pH regulation systems (Bouret et al., 2014). The regulation of pH depends on the opposite actions of enzymes and transporters in the plasma membrane, capable of both increasing and decreasing pH (Occhipinti & Boron, 2015; Occhipinti & Boron, 2019). In the absence of good mathematical models, it is very difficult to discern the relative contributions of the many simultaneous and interconnected processes responsible for pH changes in a single living cell.

Table 1
Some reactions in erythrocyte metabolism included in the model and corresponding catalyzing enzymes (Arrow type, → or ↔ indicates irreversible or reversible reactions)

| Reactions | Enzymes | Effector |
|-----------|---------|---------|
| Glc + MgATP → G6P + MgADP | HK | 23DPG, GDP, GSH |
| Glc6P → F6P | PGI | ATP, Mg, 23DPG |
| F6P + ATP → FDP + ADP | PFK | AMP, Phos |
| FDP + GA3P + DHAP | ALD | 23-DPG, Mg23DPG |
| DHAP + GA3P | TPI | — |
| GA3P + NADP → D13PG + NADH | GAPDH | pH |
| D13PG + ADP ↔ P3G + ATP | PGK | — |
| P3G → P2G | PGM | — |
| P2G → PEP | EN | Mg |
| PEP + ATP → PYR + ATP | PK | ATP, F6DP |
| PYR + NADH + LAC + NAD+ | LDG | pH |
| LAC → LACext | transport | pH |
| Phos → Phosext | transport | pH |

2,3-bisphosphoglycerate shunt

D13PG → D23PG
D23PG → P3G

Pentose phosphate pathway

G6P + NADP → Glu6P + NADPH | Glu6Pase | MgATP, 23DPG |
Glu6P + G6P → RL5P + NADPH | Glu6PDH | — |
RL5P + XYL5P | XPI | — |
XYL5P + R5P + SED7P + GA3P | TK-1 | — |
SED7P + GA3P → F6P + ERY4P | TA | — |
XYL5P + ERY4P + F6P + GA3P | TK | — |
GSSG + NADPH + GSH + NADP | GSSGR | — |
GSH → GSSG | GSHox | — |
MgATP + MgADP + Phos | ATPase | — |
NADH → NAD | non-glycolytic NADH consumption process |

Binding of metabolites to hemoglobin

deoxyHb + 13DPG ↔ deoxyHb 13DPG | — | pH |
deoxyHb + 23DPG ↔ deoxyHb 23DPG | — | pH |
deoxyHb + ATP ↔ deoxyHb ATP | — | pH |
deoxyHb + ADP ↔ deoxyHb ADP | — | pH |
deoxyHb + FDP ↔ deoxyHb FDP | — | pH |
deoxyHb + MgATP + deoxyHb MgATP | — | pH |
deoxyHb + H ↔ H deoxyHb | — | pH |
deoxyHb + CO2 + deoxyHbCO2 + H+ | — | pH |
oxylHb + 13DPG ↔ oxylHb 13DPG | — | pH |
oxylHb + 23DPG ↔ oxylHb 23DPG | — | pH |
oxylHb + ATP ↔ oxylHb ATP | — | pH |
oxylHb + ADP ↔ oxylHb ADP | — | pH |
oxylHb + FDP ↔ oxylHb FDP | — | pH |
oxylHb + MgATP + oxylHb MgATP | — | pH |
oxylHb + H ↔ H oxylHb | — | pH |
oxylHb + CO2 + oxylHbCO2 + H+ | — | pH |

Hemoglobin transition

deoxyHb + O2 ↔ oxylHb | — | pH, CO2, T |

In this study, a metabolic model of erythrocytes is presented, which makes it possible to quantitatively evaluate acid-base transformations in erythrocytes.
erythrocytes as a response to the action of the external environment. The model of human erythrocyte metabolism (Mulquiney & Kuchel, 1999) was chosen as the basis, consisting of two metabolic cycles—glycolysis and the pentose phosphate pathway. The advantage of this model is that the rate of most reactions is described as a function of pH. To this model, we add metabolic processes involving hemoglobin, namely, interactions with cell metabolites, binding to band 3 protein, oxygenation processes, the Jacobs-Stewart cycle, using the currently known kinetic equations and their parameters (Bidani et al., 1978; Geers & Gros, 2000; Kinoshita et al., 2007; Nishino et al., 2013). The combination of all these processes in one model is crucial, since hemoglobin, 2,3-DPG and other cellular metabolites directly or indirectly affect pH. The procedure of optimization parameters search (parameter estimation) followed by the solution of differential equations of the model (time course procedure) was used to predict the behaviour of all measured and non-measurable variables over time. At this stage, the model is used for the analysis of experimental data on changes in the pH of erythrocytes during their incubation in a buffer solution and the study of the mechanisms of pH regulation in cells.

According to the simulation results, regulation of pH in erythrocytes placed in a buffer medium takes place with the participation of two types of processes—fast (passing in 1.3 s) and slow. As it was established, CA is responsible for fast processes. The driving force behind the rapid process is the difference between the CO$_2$ concentration and the value of processes—fast (passing in 1.3 s) and slow. As it was established, CA is placed in a buffer medium takes place with the participation of two types of cells.

In cells a, the difference between these indicators is

$$k_1[H^+] \cdot [HCO_3^+] / K - k_2$$

19.56 µM. Equilibrium is restored in the reaction

$$CO_2 + H_2O \xrightleftharpoons{K} HCO_3^- + H^+ \text{ in 1.3 s.}$$

The flux across carbonic anhydrase at t = 0.1 s is ~120.9 µM/s (CA consumes HCO$_3^-$ and H$^+$ and produces CO$_2$), at t = 1.3 s the flow through CA is already ~0.096 µM/s (Fig. 3a). During this time

$$[CO_2] \xrightleftharpoons{K} [H^+] \cdot [HCO_3^+] / K - k_2$$

According to the simulation results, the high speed of this reaction is ensured by hemoglobin, which supplies the greater part of the consumed H$^+$ (Fig. 6a, 8a). At the same time, the content of CO$_2$ increases only from 2.16 to 2.31 µM. The payment for equilibrium is a rapid rise in pH.

In the case of cells b, the content of CO$_2$ and the value of

$$k_3[H^+] \cdot [HCO_3^+] / K - k_4$$

The modeling results indicate the absence of fast processes (Fig. 3b), the flow through the CA is slow. In this case, CA catalyzes the CO$_2$ hydration reaction. Excess H$^+$ is mainly bound by deoxyhemoglobin (Fig. 6b). Calculations predict a decrease in pH, but this decrease is less than 0.1 pH.

According to the simulation results, after 1.3 s the equilibrium establishing processes for H$^+$ start. The equilibrium condition for H$^+$ is the Donnan condition, meaning H$^+$ must be transferred into the cell. In the case of cells b, the opposite is true. The Jacobs-Stewart cycle ensures slow processes of establishing equilibrium (Swietchak et al., 2010; Jennings, 2013). The speed of this cycle is clearly limited by the slow hydration-dehydration reactions, with the participation of CA and the transport of CO$_2$ and HCO$_3^-$. The relative deficiency of H$^+$ inside the cells leads to intracellular hydration of CO$_2$ (Fig. 3a, dependence of the flow through CA in the positive region). The formed excess HCO$_3^-$ diffuses from erythrocytes through band 3 dimers (AE1) or dimerized dimers (tetramers) along the electrochemical potential gradient in exchange for chloride (reverse chloride shift) (Reithmeier et al., 2016; Jennings, 2021). The decrease in pH, which is recorded experimentally (Fig. 2a), may be explained by the fact that since along with HCO$_3^-$, H$^+$ is also formed. An increase in the level of H$^+$ causes the dissociation of carbonichemoglobin, and already here the excess CO$_2$ is removed through gas channels (Fig. 8b). In general, the processes that allow restoring the level of intracellular pH as shown in Figures 8b, 8c.

Carbonic anhydrase is the most efficient enzyme known to date, with a kat or turnover number (~6 × 10$^5$/s) that is 10 times faster than the rate of CO$_2$/HCO$_3^-$ exchange carried out by AE1 (~5 × 10$^4$/s) (Boone et al., 2014; Hsu, 2018; Kalli & Reithmeier, 2022). Since human erythrocytes have an identical number of CAII and AE1 molecules (~10$^4$ molecules/erythrocyte), the efficiency of CO$_2$/HCO$_3^-$ conversion with the participation of CA is about an order of magnitude higher than the rate of CO$_2$/HCO$_3^-$ – exchange flow with the participation of AE1. It is known that CA is present in erythrocytes in two isoforms: low-affinity, high-capacity enzyme (CAI) and high-affinity, low-capacity isoenzyme (CAII). It is suggested that cytosolic carbonic anhydrase II (CAII) of erythrocytes is highly associated with AE1, forming a “metabolon complex” that significantly enhances the transport activity of AE1 (Boron, 2010; Johnson & Casey, 2011; Al-Sarrar et al., 2013; Occhipinti & Boron, 2015; Hsu, 2018). Recently, an aquaporin (AQPI) has also been shown to spatially associate with CAII and transport H$_2$O for CAII-mediated catalysis (Vilas et al., 2015; Hsu, 2018; Michenkov et al., 2021). Nevertheless, the formation of metabolon seems doubtful to some authors (Al-Sarrar et al., 2013).

The results obtained while modeling indicate that the rapid conversion of HCO$_3^-$ to CO$_2$ with the participation of CA is ensured simultaneously by the hemoglobin participation processes. The binding of hemoglobin to the band protein is well established (Chu et al., 2016; Kosmachevskaya et al., 2019; Giardina, 2022). Deoxygenated hemoglobin (deoxyHb) preferentially binds to the N-terminal cytoplasmic domain of band 3 protein. The catalytic dehydration reaction, which consumes protons to form CO$_2$, can only proceed under the conditions of availability of a sufficient number of protons. Protons bound to hemoglobin and the delivery of protons from the interior of the cell to the membrane region by diffusion of H$^+$ becomes the limiting step. The same applies to the binding of CO$_2$, which is formed in significant quantities during the conversion of HCO$_3^-$ to CO$_2$. Hemoglobin binds CO$_2$ and releases H$^+$. From this point of view, colocalization of CA with hemoglobin and aquaporin could maximize the rate of dehydration and transport of HCO$_3^-$ and CO$_2$. Metabolon formation can be viewed as a temporally extended CAII mechanism that is built on transient interactions between four proteins: AQPI, AE1, CAII, and deoxyHb.

One of the oldest mechanisms that provide a quick response to external influences is the ability of the molecules to reversibly bind to membrane components (Kosmachevskaya et al., 2019). In erythrocytes, an example of feedback is the O$_2$-dependent association of hemoglobin with band 3 protein, which regulates the assembly of a complex of glycolytic enzymes on the erythrocyte membrane when the glucose level changes, switches the metabolism between the pentose phosphate pathway and glycolysis (Kosmachevskaya et al., 2019; Issaia et al., 2021). However, interactions with AE1 inhibit, rather than stimulate, the activity of glycolytic enzymes. Boron (2010) suggests that in the case of large intracellular gradients for Na$^+$, HCO$_3^-$, H$^+$ ions, the distribution of CAII throughout the cytoplasm helps to quickly dissipate the ion gradient and thereby enhance particle transport. Here we see the need for further development of the model, modeling of CAII binding to AE1, and detailed analysis of the role of membrane-bound and cytoplasmic hemoglobin in the work of CAI.

During the search optimization of the model parameters (Parameter Estimation), the diffusion rate constant 0.93 × 10$^{-3}$ cm$^2$ s$^{-1}$ was obtained, which is two orders of magnitude higher than that proposed in the literature for CO$_2$ diffusion. If we consider that ~60% of CO$_2$ flow into or out of erythrocytes occurs through the gas channel AQPI (Hsu, 2018; Michenkov et al., 2021), then the use of this constant is more appropriate. The rest of the CO$_2$ flux likely occurs through another gas channel, RhAG, or direct diffusion across the lipid bilayer.

An interesting point that was shown during the simulation is a short-term increase/decrease in pH during the operation of the CA. This fact was also pointed out by other authors (Boron, 2010; Occhipinti & Boron, 2019). Thus, in experiments with oocytes (Musa-Azz et al., 2014) it was shown that cytosolic CA II increases the rate of change of both intracellular pH and pH near the outer surface of the cell when CO$_2$ enters. Evidence for the formation of cytosolic H$^+$ gradients has been found in other cells (Johnson & Casey, 2011). The combination of fast AE1-mediated HCO$_3^-$ transport and slow H$^+$ diffusion creates conditions for the formation of H$^+$ microdomains that develop around AE1. Thus, proteins near
AE1 will be exposed to a different pH than proteins further away from AE1. In turn, this can lead to differential regulation of pH-sensitive processes localized in the environment of pH-regulatory transporters. CA II localizes to the cytosolic C-terminus of AE1 (De Rosa et al., 2007; Johnson & Casey, 2011), the site of H⁺ production or consumption is also localized to the surface of AE1, contributing to the formation of the H⁺ microdomain. The N-terminus of AE1 contains binding sites for glycolytic enzymes (Chu et al., 2016; Issaian et al., 2021), whose activity depends on pH. It is logical to hypothesize that enzymes may localize to the surface of AE1 to undergo pH regulation by H⁺ microdomains. Experimental data on the effect of pH on the glycolytic and pentose phosphate pathways in erythrocytes are given in Huang et al. (2021). This is another argument for the feasibility of forming metabolon CA, AOPI from AE1.

Since the protons produced by bicarbonate formation are released in the immediate vicinity of band 3, the Bohr release of oxygen occurs most rapidly from hemoglobin in the same region. Thus, the structural organization of the band 3 macrocomplex appears to be well formed to fulfill this special requirement, providing short pathways for the movement of oxygen, carbon dioxide, protons, and bicarbonates necessary for efficient oxygen/carbon dioxide exchange in the capillaries, which would not occur if these processes were not physically connected (De Rosa et al., 2007).

Hemoglobin is an example of a molecule that is subject to modulation mechanisms that optimize its functional behaviour according to specific physiological requirements. Hemoglobin binds to band 3 protein due to the insertion of the anionic AE1 segment into the cationic central cavity formed by the central β-chains, which is also the 2.3-DPG binding site (De Rosa et al., 2007; Kosmachevskaya et al., 2019). AE1 stabilizes the formation of a β2-α2-α2-α2-β2-β2-β2 group into an anionic group with the addition of a relatively small mass – (Fig. 4, 5).

Carbamate formation and hemoglobin protonation are easily reversible reactions, which is a useful property for any biological regulation system (Lorimer, 1983). The buffer properties of hemoglobin are carried out by the imidazole group of histidine residues, which has a pKa of approximately 6.8, which provides effective buffering at physiological pH. The amount of CO₂ bound as a carbamate to hemoglobin in erythrocytes depends on the oxygen saturation of hemoglobin and the concentration of 2,3-DPG and the concentration of H⁺ (Blake & Cann, 2022). However, the formation of a carbamate on a protein converts a neutral or cationic group into an anionic group with the addition of a relatively small mass equivalent to a 4 to 5 Å diameter sphere. This creates the possibility of electrostatic interactions within the protein that can further stabilize the carbamate. The formation of the carbamate as well as the T conformation of hemoglobin can lead to subtle conformational changes with profound biological consequences (Lorimer, 1983).

**Conclusions**

The developed model is a tool for the quantitative analysis of biochemical interactions important for the regulation of intracellular pH. The model does not consider metabolic cycles separately from one another, but, on the contrary, the Jacobs-Stewart metabolic cycle is integrated into a complex network of reactions, which allows one to consider most of the interrelationships between reactions. The model consists of 85 reactions, the rate of which is described based on exact kinetic equations. The result of the model's calculations is time dependences of reaction flows and metabolite concentrations, that allow one to reproduce the behaviour of the metabolic system after a disturbance in vitro and to establish the mechanisms of recovery or approach to stationary states. The paper shows that regulation of pH in erythrocytes placed in a buffer medium takes place with the participation of two types of processes – fast (takes place in 1.3 s) and slow. Fast processes are aimed at restoring the intracellular balance between CO₂ and HCO₃⁻, slow processes are aimed at establishing the balance of H⁺ between the cell and the extracellular environment. The roles of carbonic anhydrase (CA) and hemoglobin in the processes of pH stabilization are shown and analyzed. The physiological role of the metabolon between band 3 protein (AE1), CA, aquaporin and hemoglobin in maintaining pH homeostasis in the conditions of in vitro experiments is discussed.

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