STEADY-STATE DEPENDENCE OF RATE OF REDUCTION OF METHEMOGLOBIN ON ITS CONCENTRATION IN INTACT HUMAN ERYTHROCYTES

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The rate of the reduction of methemoglobin in intact human erythrocytes by the methemoglobin reductase system is determined from the rate of pyruvate accumulation under quasisteady-state conditions. Different levels of methemoglobin (from normal to 100%) were produced by the addition of different concentrations of sodium nitrite (from 0 to 8 mM), and steady-state levels of methemoglobin were maintained for several hours by introducing a solution of nitrate into the suspension at a rate of 2.8 mmoles/h/liter of erythrocytes. The dependence of the rate of pyruvate accumulation on the steady-state methemoglobin concentration in the range from 0 to 100% was linear and was the same in erythrocytes from all the donors investigated. The maximum rate of pyruvate accumulation at 100% methemoglobin was ~500 μmoles/h/liter of erythrocytes. It was found that the addition of nitrite in a concentration causing the formation of 100% methemoglobin increases the rate of evolution of CO_2 by the erythrocytes to a value constituting ~40% of the maximum possible rate of the pentose phosphate pathway.

The reduction of methemoglobin in human erythrocytes has been studied fairly thoroughly. It is believed that under physiological conditions NADH-dependent methemoglobin reductase, which uses the NADH formed in the glycolytic reaction catalyzed by glyceraldehyde phosphate dehydrogenase [1, 2], is the main pathway of the reduction of methemoglobin. Under physiological conditions the content of methemoglobin is less than 1%, however, it is not known how well stabilized this value is. To answer this question, as well as for a description of the functioning of the methemoglobin reduction system in intact erythrocytes, one has to know the steady-state dependence of the rate of the reduction of methemoglobin on its concentration.

However, in an experimental measurement of this dependence, which we shall call the regulatory characteristic of the methemoglobin reducing system from here on, two problems arise. The first is to establish different steady-state methemoglobin levels ranging from 0 to 100%. The second is the need for a direct measurement of the rate of methemoglobin reduction in intact erythrocytes under steady-state conditions. However, with certain assumptions this rate can be determined indirectly. It can be assumed that under normal conditions methemoglobin is almost completely reduced by NADH-dependent methemoglobin reductase [1]. Under quasi-steady-state conditions (when the derivatives with respect to time of all the intermediate metabolites of glycolysis are equal to zero) it can be seen from the scheme and equations presented in Fig. 1 that the rate of methemoglobin reduction is twice as high as the rate of pyruvate production. Earlier [3] it was actually shown experimentally that the amount of methemoglobin reduced and of pyruvate formed are linked by a coefficient of 2. Thus, by measuring the rate of pyruvate accumulation, one can determine the rate of methemoglobin reduction.

METHODS

Blood from healthy donors in preservative 7b, stored in a refrigerator for no more than 20 h, was used. The erythrocytes were washed three times with Tyrode's solution. They were divided into several portions, each containing 1 ml of washed erythrocytes and 9 ml of Tyrode's solution and different concentrations of sodium nitrite: 0, 0.73, 1.46, 2.90, and

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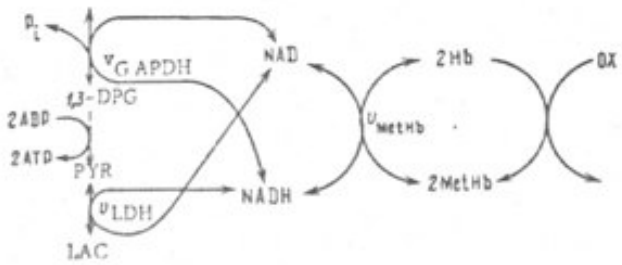

$$\frac{d}{dt}[NAD] = -v_{GAPDH} + v_{LDH} + \frac{1}{2} v_{MetHb} = 0$$

$$v_{MetHb} = 2(v_{GAPDH} - v_{LDH}) = 2\frac{d}{dt}[PYR] \equiv 2v_{PYR}$$

Fig. 1. Stoichiometric scheme of reduction of methemoglobin. GAP) Glyceraldehyde phosphate, 1,3-DPG) diphosphoglycerophosphate, PYR) pyruvate, LAC) lactate, Hb) hemoglobin, MetHb) methemoglobin, OX) oxidizing agent, v_{GAPDH}) rate of glyceraldehyde phosphate dehydrogenase reaction, v_{LDH}) rate of lactate formation, v_{MetHb}) rate of MetHb reductase reaction, v_{PYR}) rate of pyruvate formation. The directions coinciding with the normal course of glycolysis were taken as the positive directions.

7.25 mM, and incubated at 37°C without mixing for 1 h. The suspension was then centrifuged for 10 min at 1000g and 8 ml of the supernatant was suctioned off. A suspension concentrated in this way was incubated at 37° with mixing. A 0.1 M solution of nitrate was introduced at a rate of 28 µl/h after the beginning of incubation into the series containing 0.73, 1.46, and 2.9 mM sodium nitrite.

The flow through the pentose phosphate pathway was determined from the rate of evolution of CO_2 by a previously described method [4]. A suspension of erythrocytes (30 ml) with a hematocrit of ∿50% was incubated at 37° with mixing at a constant pH (in different experiments the pH lay in the range of 7.46-7.67). The rate of CO_2 evolution without additions ("physiological"), after the addition of 8 mM nitrite, and after the introduction of 200 µl of 0.6 M tert-butyl hydroperoxide (the maximum rate of the pentose phosphate pathway) was determined.

Samples for a determination of the pyruvate concentration began to be taken 2 h after the beginning of incubation at intervals of 30 min. A sample with a volume of 200 µl was fixed in 800 µl of 5% perchloric acid, the precipitate was removed by centrifugation (5 min, 1000g), and the supernatant was neutralized to pH 7-8 with a saturated solution of K_2CO_3. The pyruvate concentration was determined by the standard enzymatic method [5]. For a determination of the percentage content of methemoglobin, the optical density in the hemolysate (25 µl of the suspension was hemolyzed in 2.5 ml of 5 mM phosphate buffer, pH 7.7) was measured at 2 wavelengths: λ_1 = 524.8 nm and λ_2 = 566.7 nm on a DW-2a spectrophotometer (Aminco, USA). The relative concentration of methemoglobin (h) was computed from the equation:

$$h = \frac{\varepsilon_2^{2+} - \frac{D_2}{D_1} \cdot \varepsilon_1^{2+}}{\varepsilon_2^{2+} - \varepsilon_2^{3+}} \cdot 100\%,$$

where D_1 and D_2 are the optical densities at wavelengths λ_1 and λ_2, and ε are the millimolar extinction coefficients: ε_2^{2+} = 9.86; ε_2^{3+} = 5.12; ε_1^{2+} = 7.80 [6].

RESULTS

After the addition of nitrite the methemoglobin concentration begins to increase rapidly and reaches a maximum after 2-5 h (Fig. 2). A slow decrease in the methemoglobin concentration is found upon longer incubation. This evidently is due to the fact that active reduction of methemoglobin is being carried out in the erythrocytes and the nitrite concentration decreases

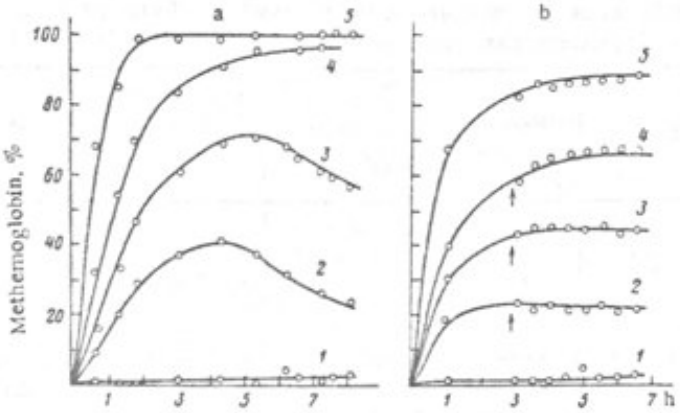

Fig. 2. Dependence of methemoglobin concentration on time: a) after single addition of sodium nitrite; b) upon introduction of nitrite solution into erythrocyte suspension 3 h after single addition of nitrite. The rate of the addition of an 0.1 M solution and nitrite was 28 µl/h to 3 ml of an erythrocyte suspension with a hematocrit of 40%. The arrows indicate the times of the beginning of the addition and the series into which the addition was made. The sodium nitrite concentration after a single addition at the initial time constituted (mM): 1) 0; 2) 0.73; 3) 1.46; 4) 2.9; 5) 7.25.

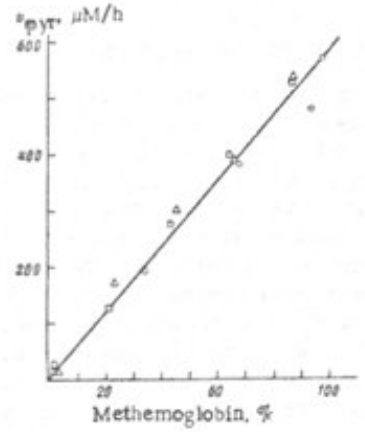

Fig. 3. Dependence of rate of pyruvate accumulation (µmoles/h/liter of erythrocytes) on steady-state level of methemoglobin. The results obtained on erythrocytes from three donors are presented.

with time, since nitrate is reduced during the oxidation of hemoglobin [7]. To obtain a steady-state level of methemoglobin, nitrite was introduced into the cells containing the erythrocyte suspensions at a rate of 28 µl/h. The volume of the addition was selected so as to provide minimal dilution of the suspension, and the nitrite concentration (0.1 M) needed to maintain a fixed level of methemoglobin was selected in preliminary experiments. Figure 2b shows that steady-state levels of methemoglobin can be maintained for a long time in this way.

The rates of pyruvate accumulation proved to be proportional to the corresponding steady-state methemoglobin concentrations in the range of concentrations from 0 to 100% in all the experiments conducted. Figure 3 presents the dependence of the rate of pyruvate formation

TABLE 1. Influence of Methemoglobin Level on Rate of CO_2 Evolution by Erythrocytes (nmoles/h/liter of erythrocytes)

pH	Nitrite, mM	Change in amount of methemoglobin, %	Rate of CO_2 evolution in presence of nitrite ($v^{CO_2}_{+nitrite}$)	Maximum rate of CO_2 evol. ($v^{CO_2}_{max}$)	$\frac{v^{CO_2}_{+nitrite}}{v^{CO_2}_{max}}$, %
7.62	9.2	12→65	0.85	2.24	38
7.46	7.7	20→80	1.89	4.89	38.7
7.67	8.2	10→70	0.71	1.85	38.4

on the steady-state methemoglobin concentration obtained on erythrocytes from three donors. It is seen that the dependence is linear over the whole range; the data obtained on erythrocytes from different donors lie on one straight line. The maximum rate of pyruvate formation in the case of completely oxidized hemoglobin constituted 500 μmoles/h/liter of erythrocytes in all the experiments conducted.

In one experiment the characteristic curve of the methemoglobin reducing system was measured in the presence of 50 mM lactate. In this case the shape of the curve did not change, while the slope increased slightly within the limits of the variations among the samples from different donors.

It was found in an investigation of the influence of the methemoglobin level on the pentose phosphate pathway that the addition of nitrite causes a sharp increase in the rate of evolution of CO_2 by the erythrocytes (Table 1). It is interesting that in all cases the rate of CO_2 evolution was ~38% of the maximum flow rate through the pentose phosphate pathway, determined upon the complete oxidation of glutathione in the presence of tert-butyl hydroperoxide.

DISCUSSION OF RESULTS

Four pathways of the reduction of methemoglobin in erythrocytes are known from the literature: 1) by NADH-dependent methemoglobin reductase, 2) by ascorbic acid, which, in turn, is reduced from dehydroascorbic acid using the reduced glutathione, 3) directly by glutathione, 4) by NADPH dehydrogenase, which reduces methemoglobin only in the presence of methylene blue [1]. In the first pathway the NADH produced in glycolysis, while in the other three pathways the NADPH produced in the pentose phosphate pathway, is used as the source of electrons. The dependence that we measured reflects only the reduction of methemoglobin using NADH. Since the addition of nitrite causes a significant increase in the flow through the pentose phosphate pathway, the possibility of the reduction of methemoglobin using NADPH, i.e., the other three pathways, must be considered. According to the literature data, the rate of the direct reduction of methemoglobin by glutathione is very low [1], but without the addition of ascorbic acid and methylene blue from without the rate on the 2nd and 4th pathways does not exceed 16% of the rate of NADH-dependent methemoglobin reductase [1]. It is known that during the oxidation of hemoglobin by nitrite various radicals and active compounds are formed, O_2^-, NO, H_2O_2 [7], so that the activation of the pentose phosphate pathway most likely involves the consumption of NADPH and reduced glutathione in these oxidative processes.

The conditions of the measurement of the regulatory characteristic of the methemoglobin reducing system differ from the conditions under which an erythrocyte exists in the blood stream: There, a fixed concentration of pyruvate and lactate is maintained, while during incubation, first, these concentrations increase with time and, second, the pyruvate concentration depends heavily on the amount of methemoglobin. Therefore the shape of a characteristic curve measured *in vitro* can differ from that existing in the organism. To verify this possibility, an experiment was conducted with the addition of lactate. If it is assumed that the LDH reaction is close to equilibrium [8], the NADH/NAD ratio can be computed from the pyruvate/lactate ratio. Figure 4 presents the results of such a calculation for one of the experiments (curve 1). In the organism, where the pyruvate/lactate ratio is constant, the NADH/NAD ratio is also constant and constitutes, according to data obtained earlier [10], $1.04 \cdot 10^{-3}$; this value is indicated in Fig. 4 by a dotted line. Curve 2 is the experiment with the addition of lactate. It is seen that in this case at all the methemoglobin concentrations the curve passes above the values of the NADH/NAD ratio realized in the blood stream.

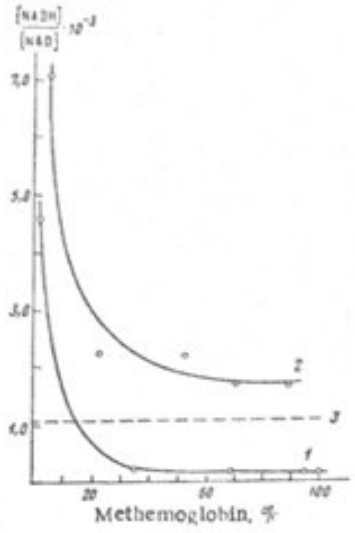

Fig. 4. Dependence of [NADH]/[NAD] ratio on steady-state level of methemoglobin, calculated from data on pyruvate and lactate accumulation, assuming equilibrium of the lactate dehydrogenase reaction. Value of equilibrium constant $5.45 \cdot 10^4$ [9]. 1) Experiment without addition, 2) with addition of lactate; 3) value of [NADH]/[NAD] ratio in blood stream according to data obtained earlier [7].

The NADH/NAD ratios obtained in experiments with and without the addition of lactate lie on both sides of the physiological value of this ratio. The shape of the characteristic curve of the methemoglobin reducing system hardly differs in these two experimental setups. Hence it can be concluded that the given characteristic curve takes the same form *in vivo*.

By comparing the stabilizing properties of the methemoglobin reducing system with other metabolic systems of the erythrocyte (the Embden-Meyerhof pathway, the pentose phosphate pathway), it can be concluded that the level of reduced hemoglobin is not well stabilized in erythrocytes, in contrast to the stabilization of the levels of ATP and reduced glutathione.

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