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EFFECTS OF HEMOLYSATE CONCENTRATION, IONIC STRENGTH AND CYTOCHROME b_5 CONCENTRATION ON THE RATE OF METHEMOGLOBIN REDUCTION IN HEMOLYSATES OF HUMAN ERYTHROCYTES

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Summary

An assay for determining the rate of methemoglobin reduction in hemolysates of human erythrocytes has been developed. The rates obtained by this assay, when corrected for dilution, are comparable to those obtained with intact cells. Increased ionic strength inhibits the reaction, whereas EDTA increases the rate of reduction. The rate with NADPH as electron donor is 65—70% of the rate with NADH. Added cytochrome b_5 stimulates the reaction. The assay has been used to examine erythrocytes from two methemoglobinemic sisters and their asymptomatic mother. Hemolysates of the two patients have both decreased dichlorophenolindophenol reductase activity and decreased ability to reduce methemoglobin. Hemolysates from the heterozygous mother have intermediate dichlorophenolindophenol reductase activity and intermediate methemoglobin reduction ability. The data presented in this paper indicate that the concentrations of cytochrome b_5 and cytochrome b_5 reductase determine the rate of methemoglobin reduction in hemolysates.

Introduction

From 0.5 to 3% of the hemoglobin of a normal adult human is oxidized to methemoglobin each day [1], yet the normal steady-state level of methemoglobin in circulating red cells is less than 1% of the total hemoglobin. This low steady-state level is maintained by an erythrocyte methemoglobin reduction system comprised of a reductase and a soluble cytochrome b_5. The cytosolic

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

NADH reductase has been purified and characterized [2—5], and a defect of this enzyme has been shown to be the basis for congenital methemoglobinemia [6]. The water-soluble cytochrome b_5 of the cytosol of erythrocytes has been purified, characterized, and shown to stimulate markedly methemoglobin reduction in a reconstituted system [3,7—10].

Studies of the kinetics of methemoglobin reduction have been carried out with intact erythrocytes [11,12] and with the purified proteins (see ref. 13 for a review). It has generally been accepted that methemoglobin reduction ceases upon lysis of the red cell [11,13,14]. However, very slow rates of reduction have been reported in hemolysates by some investigators [15—20]. Addition of nicotinamide to hemolysates allows methemoglobin reduction to occur [16,19], presumably by inhibiting the degradation of pyridine nucleotides, and thus keeping NADH available for the NADH-methemoglobin reduction system [21]. Also, reduction has been reported when an excess of NADH was used [17,18,20].

This paper presents an assay for measuring methemoglobin reduction in hemolysates using a high concentration of NADH. This assay has been used to measure the reduction in hemolysates of normal and methemoglobinemic individuals, and to examine the effects of added cytochrome b_5, NADPH, salts and EDTA on the rate of reduction.

Experimental Procedure

Materials

Fresh blood samples from normal controls and from two methemoglobinemic patients and their mother were obtained and stored in acid-citrate-dextrose solution A (2.45 g glucose, 2.20 g trisodium citrate and 0.80 g citric acid in 100 ml water). Outdated blood was donated by The University of Michigan Medical Center Blood Bank, and was stored in citrate-phosphate-dextrose (3.20 g citric acid, 25.8 g sodium citrate, 25 g glucose and 2.18 g $NaH_2PO_4 \cdot H_2O$ in 1 l water). The outdated blood was used within 1 week of the expiration date.

NADH, NADPH and DCIP were purchased from Sigma Chemical Co. Human erythrocyte cytochrome b_5 was purified according to the procedure of Hultquist et al. [22]. Bovine liver microsomal cytochrome b_5 was solubilized with bovine liver lysosomal cathepsin D and purified (Peters, C.L. and Hultquist, D.E., unpublished results).

Methods

Methemoglobin was prepared from outdated, packed, human erythrocytes, which had been washed three times at 4°C in 5 vols. of 0.9% NaCl, spinning each time for 15 min at $480 \times g$. The buffy coat was removed after the first spin. The cells were incubated in 1% $NaNO_2$ in 0.9% NaCl for 1 h at room temperature to convert the hemoglobin to methemoglobin and then washed five times in 5 vols. of 0.9% NaCl to remove the excess $NaNO_2$. Methemoglobin was purified from these cells by the procedure of Antonini and Brunori [23] for the purification of hemoglobin. The purified methemoglobin showed no contamination by reductase.

Reductase was quantitated using DCIP as an artificial electron acceptor [6].

Measurement of methemoglobin reduction in hemolysates. Methemoglobin reduction was assayed by a procedure that is similar to the method of Betke et al. [17]. Packed cells were washed three times in 10 vols. of phosphate-buffered saline solution (9 vols. of 0.9% NaCl plus 1 vol. of 0.1 M KH_2PO_4, pH 7.4). One volume of 1% $NaNO_2$ in phosphate-buffered saline solution was added to the washed cells, and the cell suspension was allowed to stand at room temperature for 10 min to convert the hemoglobin to methemoglobin. The cells were then washed five times in 10 vols. of phosphate-buffered saline solution to remove the excess nitrite. The volume of packed cells was measured, and the appropriate volume of water was added to lyse the cells. The resulting hemolysate was centrifuged for 10 min at $2725 \times g$ to remove the stroma. The supernatant fraction was removed and used in the incubation mixture. To each assay tube was added 1 ml of stroma-free hemolysate and salts, EDTA, or cytochrome b_5 as indicated. NADH (or NADPH, where indicated) was added to a final concentration of 4 mM to start the reaction. The total reaction volume was 1.2 ml and the pH was 7.2.

The reaction mixtures were incubated with shaking at 37°C. At appropriate time intervals, 10-μl aliquots were removed, diluted 1 : 101 with water, centrifuged to remove any remaining stroma, and the absorbance at 577 nm was recorded. To convert change of absorbance per h to μmol heme reduced per h, a $\Delta\epsilon_{mM}$ at 577 nm of 13.7 was used for the conversion of methemoglobin to oxyhemoglobin [7]. To convert initial A_{577nm} to initial methemoglobin concentration, an ϵ_{mM} at 577 nm of 3.6 was used [24].

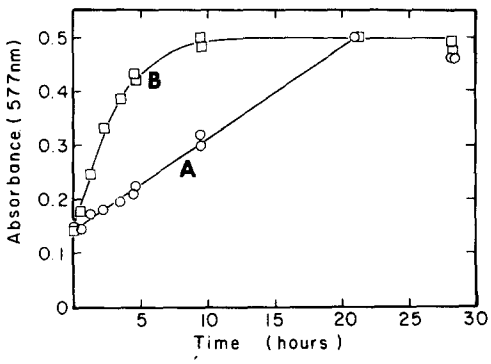
Results and Discussion

Rate of methemoglobin reduction with NADH

The time course of the reduction of methemoglobin by NADH in a hemolysate made by a 1 : 4 dilution is given in Fig. 1 (curve A). The slope is equivalent to a velocity of 0.125 μmol heme reduced $\cdot h^{-1} \cdot ml^{-1}$. No reduction occurred when NADH was omitted. In the absence of hemolysate, purified methemoglobin (50 mg/1.2 ml) was reduced slowly by NADH (0.016 μmol heme $\cdot h^{-1} \cdot ml^{-1}$); direct reduction of methemoglobin by NADH has been previously reported [25]. In the complete system the reaction proceeds linearly until nearly all of the methemoglobin has been converted to oxyhemoglobin. In contrast, Gruener and Cohen [26] reported sigmoidal curves when activity was plotted versus methemoglobin concentration. However, the ferricyanide present in their assays could have accelerated and possibly changed the mechanism of the methemoglobin reduction [19].

Storage of the red blood cells in acid-citrate-dextrose solution A for 5 weeks resulted in only a 4% decrease in the rate of methemoglobin reduction. Consequently, all of the experiments presented in this paper, except those performed with the methemoglobinemic blood, were done with outdated blood. Our finding contrasts with the results of Ioppolo et al. [27], who found a greater loss of activity upon blood storage when methemoglobin reduction was measured in intact erythrocytes with glucose as the substrate.

EDTA stimulated the reduction of methemoglobin in hemolysates (Fig. 2). EDTA stimulation of methemoglobin reduction catalyzed by purified reductase



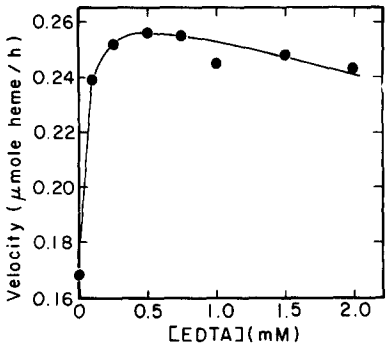


Fig. 1. Time course of methemoglobin reduction in the presence and absence of added cytochrome b_5. The hemolysate was obtained by a 1 : 4 dilution of the packed cells with water. A, no added cytochrome b_5; B, 0.32 nmol human erythrocyte cytochrome b_5 added.

Fig. 2. The effect of EDTA concentration on the rate of methemoglobin reduction in a hemolysate prepared by a 1 : 4 dilution of the packed cells with water.

has been observed previously [28,29], and EDTA has been added to buffers during purification of the reductase to stabilize the enzyme [2,4].

Effects of dilution and ionic strength

Added salt resulted in inhibition of methemoglobin reduction (Fig. 3). When the ionic strength of added salt was 0.1 M, the average inhibition was 50% in a hemolysate made by a 1 : 4 dilution of the packed cells with water. Reproducibility with a single blood sample was good, but different blood samples gave variations in inhibition ranging from 40 to 60%. The curves for added KCl, $MgCl_2$, $MnCl_2$, NaCl and Na_2SO_4 are indistinguishable, demonstrating that inhibition is an ionic strength effect, and not the effect of a particular ion. Inhibition is maximal at added ionic strength above 0.1 M (total ionic strength of approx. 0.14 M).

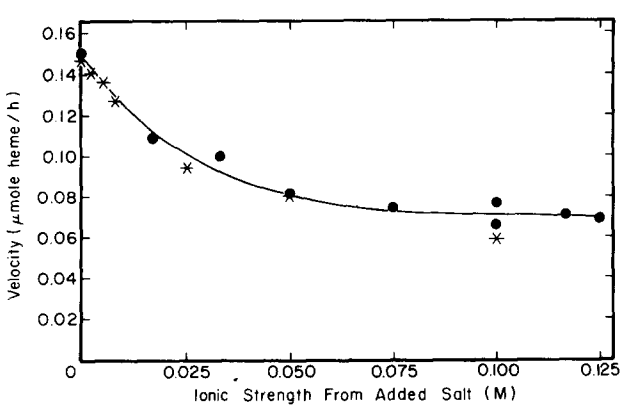


Fig. 3. The effect of added KCl and $MgCl_2$ on the rate of methemoglobin reduction in a hemolysate prepared by a 1 : 4 dilution. The ionic strength values shown are those of the added KCl (●——●) of $MgCl_2$ (*——*).

Methemoglobin reduction in intact mouse erythrocytes has been reported to be inhibited by Cl^- [30]. Moreover, the catalysis of DCIP [2,4] and cytochrome b_5 [4] reduction by the purified NADH-reductase has also been shown to be inhibited by high ionic strength. Thus our observed effect on methemoglobin reduction in hemolysates may be due to an effect on the reductase. Increasing ionic strength may decrease V or may alter the K_m of the reductase for NADH or for cytochrome b_5. These explanations are consistent with the preliminary data of Hultquist and Passon [7], which showed that ionic strength had no effect on the rate of electron transfer between cytochrome b_5 and methemoglobin.

Fig. 4 shows the effect of concentration of the hemolysate on the rate of methemoglobin reduction, in the presence and absence of 0.5 mM EDTA. In both cases, the velocity increases linearly with the hemolysate concentration. The rate of methemoglobin reduction, extrapolated to the concentration of the components of an intact cell, is 1.08 μmol heme reduced $\cdot$ h^{-1} $\cdot$ ml packed $cells^{-1}$. This is very similar to the value of approx. 1 μmol heme $\cdot$ h^{-1} $\cdot$ ml^{-1} found by Gibson [11] and by Keitt et al. [12] in intact erythrocytes with glucose as the substrate.

In the two plots in Fig. 4, both protein concentration and ionic strength of the hemolysate change proportionately. Fig. 5 shows the relationship between the rate of methemoglobin reduction and the concentration of hemolysate when the ionic strength is constant. Since the rate of reduction does not increase linearly with the concentration of protein at constant ionic strength, the rate appears to be dependent on the concentration of more than one component.

Effect of added cytochrome b_5

Cytochrome b_5 stimulated the reduction of methemoglobin in a hemolysate

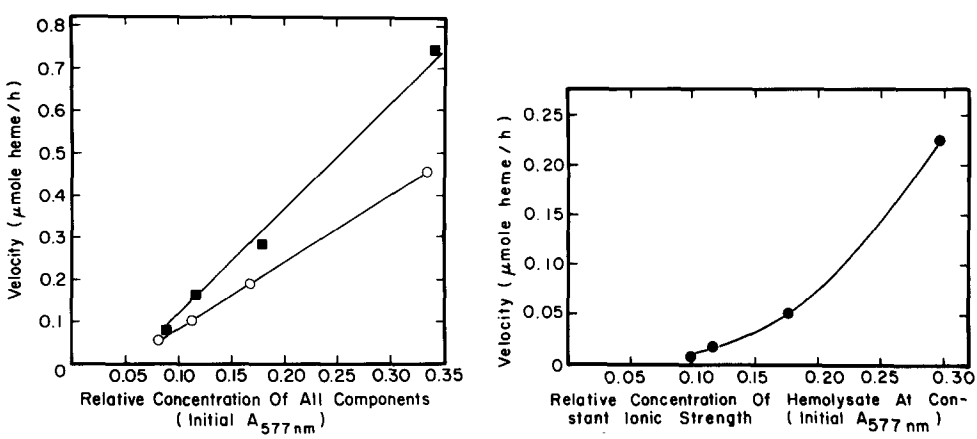


Fig. 4. The effect of hemolysate concentration on the rate of methemoglobin reduction. ■———■, with 0.5 mM EDTA; ○———○, no added EDTA.

Fig. 5. The effect of hemolysate concentration on the rate of methemoglobin reduction at constant ioni strength. NaCl was added to each tube to raise the total ionic strength to 0.15 M.

(Fig. 1, curve B). The rate is not constant with time, as it is in the absence of added cytochrome b_5, suggesting that the reduction has become dependent on the concentration of methemoglobin. Fig. 6 shows the effect of different concentrations of added cytochrome b_5 on the velocity. Identical results were obtained with solubilized microsomal cytochrome b_5. This stimulation is parallel to the stimulation in a purified system found by Hultquist and Passon [7], and supports the hypotheses that the reduction of methemoglobin proceeds through cytochrome b_5 and that the rate is dependent on the concentration of cytochrome b_5.

Effect of NADPH

When reduction was performed using NADPH instead of NADH, the rate of methemoglobin reduction was found to be 65—70% of the rate with NADH. The addition of 0.32 nmol of solubilized microsomal cytochrome b_5 increases the rate of this reduction 3.3-fold, a stimulation which is similar to that seen with NADH. In contrast, the NADH-specific reductase has only a low activity with NADPH [2—4]. The relatively high activity observed in the hemolysate with NADPH may be due to the involvement of the erythrocyte NADPH-methemoglobin reductase [31] or may be catalyzed by the NADH-specific reductase after electrons have been transferred from NADPH to NADH by some unknown process.

Results with a methemoglobinemic family

Methemoglobin reduction and DCIP reduction were assayed in hemolysates of erythrocytes of two methemoglobinemic patients and their mother. As expected [6], assays for DCIP reductase activities demonstrated that the daughters were deficient in reductase and the mother was heterozygous for the trait (Table I). The homozygotes showed approx. 26% of the normal ability to reduce methemoglobin, and the heterozygote showed 66% of the normal activity. These findings are similar to those reported in intact cells from homozygotes and heterozygotes [13]. A hemolysate made by mixing normal and

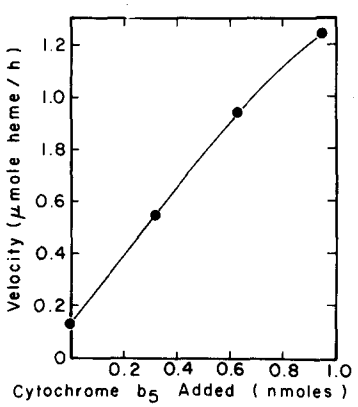


Fig. 6. The effect of added human erythrocyte cytochrome b_5 on the rate of reduction of methemoglobin in a hemolysate made by a 1 : 4 dilution of the packed cells.

TABLE I

REDUCTASE ACTIVITY IN MEMBERS OF A METHEMOGLOBINEMIC FAMILY

All activities reported are relative to the activity of the hemolysate from a normal individual under the same conditions. See Experimental Procedure for details of the assays. All DCIP reduction activities are the average of two determinations.

Sample	DCIP reduction	Methemoglobin reduction	Methemoglobin reduction in presence of 0.1 M KCl
Control	100	100 [a]	100 [a]
Mother	50	66 [b]	70 [b]
Daughter 1	10	27 [c]	34 [c]
Daughter 2	10	22 [b]	33 [b]
Daughter 2 and control [d]	43	69 [b]	74 [b]

[a] Average of three determinations using two different samples of fresh blood from a normal individual.
[b] Average of two determinations.
[c] Single determination.
[d] A mixture of equal volumes of hemolysates from a control sample and from daughter 2.

homozygous blood gave intermediate rates of reduction. Methemoglobin reduction in homozygous and heterozygous hemolysates was inhibited to the same degree as the controls when KCl was added to increase the ionic strength by 0.1 M.

Discussion of rate-limiting components

The data presented in this paper suggest that both reductase and cytochrome b_5 concentrations determine the rate of methemoglobin reduction in an erythrocyte. Kanazawa et al. [32] found no correlation between reductase activity and methemoglobin reduction rates in erythrocytes of normal adults, suggesting that some other factor must also be involved in determining the rate. This factor may be cytochrome b_5, which we have shown stimulates methemoglobin reduction in normal hemolysates.

Sugita et al. [3] found that the rate of methemoglobin reduction is approximately equal to the rate of cytochrome b_5 reduction by the reductase when using purified proteins. They also found that the K_m of the reductase for cytochrome b_5 is $7.1 \cdot 10^{-6}$ M, which is approx. 10-fold greater than the concentrations of cytochrome b_5 used in this paper to stimulate the reaction. Therefore, it appears that cytochrome b_5 has not saturated the cytochrome b_5 reductase. An increase in the concentration of either protein would then be expected to stimulate the rate of methemoglobin reduction. When cytochrome b_5 is added to the hemolysate, the rate becomes dependent on the methemoglobin concentration; under these conditions, the reduction of methemoglobin by cytochrome b_5 may no longer proceed faster than the reduction of cytochrome b_5. Alternatively, a tertiary complex of reductase, cytochrome b_5, and methemoglobin may be formed, and increasing the cytochrome b_5 concentration may alter the K_m of this complex for methemoglobin.

The rate of methemoglobin reduction in hemolysates thus depends on the concentrations of both cytochrome b_5 and cytochrome b_5 reductase, suggesting that the overall rate is dependent on the concentration of the cytochrome b_5-cytochrome b_5 reductase complex.

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