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# SOLUBLE CYTOCHROME $b_5$ REDUCTASE FROM HUMAN ERYTHROCYTES*

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SUMMARY

1. An enzyme that catalyzes the reduction of erythrocyte cytochrome $b_5$ has been isolated from the supernatant fraction of erythrocyte hemolysates by chromatography on DEAE-cellulose, Amberlite CG-50, and Bio-Gel P-60.

2. Erythrocyte cytochrome $b_5$ reductase has been shown to contain FAD. Incubation of the reductase in the absence of EDTA results in both the appearance of flavin fluorescence and the loss of enzymatic activity with time.

3. The reductase catalyzes the reduction of erythrocyte cytochrome $b_5$ 50 times faster with NADH than with NADPH. The apparent $K_m$ of NADH was calculated to be $1.6 \cdot 10^{-6}$ M and the turnover number is 1280 moles of cytochrome $b_5$ per min per mole of flavin. The reduction of electron acceptors proceeded in the following decreasing order of rate: $K_3Fe(CN)_6$, 2,6-dichlorophenolindophenol (DCIP), cytochrome $b_5$, methylene blue, cytochrome *c*, $O_2$, oxidized glutathione, and methemoglobin.

4. The FAD prosthetic group, the substrate specificity, and the effect of ionic strength, pH, and EDTA on activity suggest that the reductase is the same enzyme as NADH dehydrogenase I, the enzyme lacking in many cases of congenital methemoglobinemia. The properties of the reductase, including its molecular weight, are very similar to those of the cytochrome $b_5$ reductases solubilized from microsomes and mitochondria of other tissues.

## INTRODUCTION

A number of reductases isolated from red blood cells have been implicated in the reduction of methemoglobin[1-11]. The various NADPH-dependent "methemoglobin reductases"[1-6], however, catalyze the reduction only in the presence of redox agents such as methylene blue and these enzymes do not appear to have a significant role in the process of methemoglobin reduction under normal conditions[12]. Two NADH-dependent methemoglobin reductases, NADH dehydrogenase I and II, have been

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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partially purified from the supernatant fraction of erythrocytes and studied[7–10]. NADH dehydrogenase I (also referred to as NADH diaphorase) can be distinguished from the other erythrocyte reductases by its substrate specificity, chromatographic behavior, and effect of various ions on its activity[4,13]. This reductase is believed to function in methemoglobin reduction because deficiency of the enzyme results in methemoglobinemia[9,14,15]. A similar reductase has been detected in leucocytes and this enzyme has been reported to be deficient in some cases of congenital methemoglobinemia[16].

An NADH-dependent cytochrome $b_5$ reductase has been detected in the microsomal fraction from many tissues and species and a solubilized and enzymatically active form of this enzyme has been purified and thoroughly studied[17–19]. The finding that this flavoprotein rapidly catalyzes the reduction of cytochrome $b_5$, which is found with the reductase in microsomes, suggests that these two proteins are a part of the same electron transport system. Recent findings suggest that these proteins are involved in the fatty acid desaturation system[20,21]. A cytochrome $b_5$ reductase has also been detected in the outer membrane fraction of liver mitochondria[22,23]. This reductase has been solubilized, purified, and shown to be very similar to the microsomal enzyme.

This report describes the isolation and characterization of a soluble, NADH-dependent reductase of human erythrocytes that rapidly catalyzes the reduction of cytochrome $b_5$[24], isolated from human erythrocytes. The results of this study indicate that the enzyme is NADH dehydrogenase I and demonstrate its similarity to solubilized cytochrome $b_5$ reductase from liver microsomes. The isolation of this cytochrome $b_5$ reductase[25] and a description of its enzymatic properties[26] have been presented in abstract form, and its role in a reconstituted methemoglobin reduction system has been reported previously[27].

## EXPERIMENTAL PROCEDURE

### *Materials*

$K_3Fe(CN)_6$ and methylene blue were obtained from Allied Chemicals; NADH, NADPH, oxidized glutathione, 2,6-dichlorophenolindophenol (DCIP), and horse heart cytochrome *c* from Sigma; bovine serum albumin (Fraction V, B grade), catalase, Bio-Gel P-20 and P-60, 100–200 mesh, from Calbiochem; Amberlite CG-50, 200-400 mesh, from Mallinckrodt; and DEAE-cellulose from Fisher. Collodion bags, with porosity less than 5 nm were obtained from Schleicher and Schuell. Dialysis tubing was boiled for 1 h, washed in 1 mM EDTA, and then rinsed in water before use. Human hemiglobin, obtained from Calbiochem, was purified by chromatography on Bio-Gel P-20 before it was used.

*Naja naja* snake venom and FMN were gifts from Dr V. Massey and rat liver microsomal cytochrome $b_5$ a gift from Dr T. R. Tephly. S-protein[28] and erythrocuprein[29] were isolated as described previously. Outdated human blood cells were graciously provided by St. Joseph's Mercy Hospital Blood Bank, Ann Arbor, Mich., The University of Michigan Medical Center Blood Bank, Ann Arbor, Mich., and the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich. Cells were stored and, immediately before lysis, washed as described previously[29].

*Methods*

Protein concentrations were determined by the method of Lowry *et al.*[30] using serum albumin as standard. Fluorescence spectra were observed as described previously[29]. Changes of fluorescence with time were measured with an Aminco–Bowman fluorometer. The concentrations of FMN and FAD were determined fluorometrically using *Naja naja* snake venom to liberate the FMN from the FAD, and using FMN as standard[31].

The activity of the erythrocyte reductase with cytochrome $b_5$ was measured at 25 °C and pH 8.1 on a Gilford recording photometer in a 1.0 ml reaction mixture containing: 0.78 nmole cytochrome $b_5$, 60 nmoles NADH, 0.5–1.0 $\mu$mole EDTA, 50–100 $\mu$moles Tris–HCl, and 0.2–0.6 $\mu$g of a reductase sample with a specific activity of 21.1 $[(\Delta A_{600\,nm}/\text{min per ml})/A_{280\,nm}]$. Increase in absorbance at 424 nm was followed with time. A value of 105 was used for the change in the extinction coefficient ($\Delta\varepsilon_{mM}$) at this wavelength[32]. In most experiments, the cytochrome $b_5$ reducing capacity of the reductase was determined in a coupled reaction with cytochrome *c*[17]. In these experiments, the reaction mixture also contained 35 nmoles of horse heart cytochrome *c* and the increase in absorbance at 550 nm was followed with time. The value of 21 was used for the $\Delta\varepsilon_{mM}$[33]. The dependence of this assay on each component of the system is shown in Table I. Linearity between rate of reduction and the amount of

TABLE I

REQUIREMENTS FOR CYTOCHROME $b_5$ REDUCTION WITH CYTOCHROME *c* AS FINAL ACCEPTOR

| *Constituents* | $\Delta A_{550\,nm}$/10 min |
|---|---|
| Complete system * | 0.120 |
| *Minus* NADH | 0.000 |
| *Minus* reductase | 0.003 |
| *Minus* cytochrome $b_5$ | 0.004 |
| *Minus* cytochrome *c* | 0.000 |
| *Minus* reductase and cytochrome $b_5$ | 0.002 |

* The system is that described under Methods with a 0.1 M Tris–HCl buffer, pH 8.1, containing 0.5 mM EDTA.

reductase is shown in Fig. 1a for assays carried out with 0.78 nmole of cytochrome $b_5$. As can be seen from the plot of rate of cytochrome *c* reduction *vs* the amount of cytochrome $b_5$ (Fig. 2), at concentrations of cytochrome $b_5$ greater than 0.6 $\mu$M the rate of the reaction is independent of the cytochrome $b_5$ concentration. Thus, under these conditions the rate of the overall reaction is dependent on the catalysis of the reduction of cytochrome $b_5$ and independent of the rate of the reaction between reduced cytochrome $b_5$ and oxidized cytochrome *c*. The fact that absorbance changes at 550 nm were due to reduction of ferricytochrome *c* was confirmed by difference spectroscopy. The rate of cytochrome $b_5$ reduction was obtained by subtracting the rate obtained in the absence of cytochrome $b_5$ from the rate for the complete system.

The enzymatic reductions of ferricyanide, methylene blue, cytochrome *c*, glutathione, methemoglobin and S-protein were carried out as described for the direct reduction of cytochrome $b_5$, except that saturating amounts of these electron acceptors were used in place of the cytochrome $b_5$. The reductions of ferricyanide, glutathione,

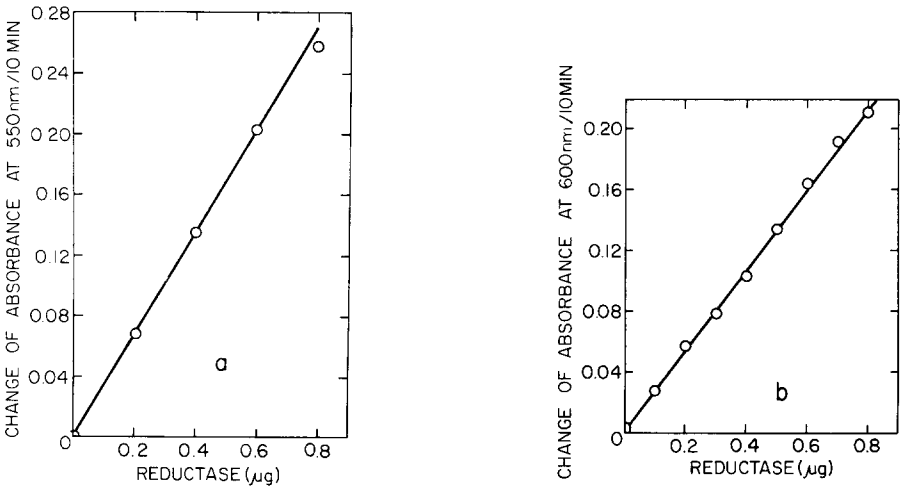


Fig. 1. Dependence of rate of reduction on the concentration of erythrocyte cytochrome $b_5$ reductase. Conditions were those described under Methods except that the buffer was 0.1 M Tris–HCl containing 0.001 M EDTA, pH 8.1. a. Reduction of human erythrocyte cytochrome $b_5$ as assayed by coupling the reaction to cytochrome *c*. b. Reduction of DCIP.

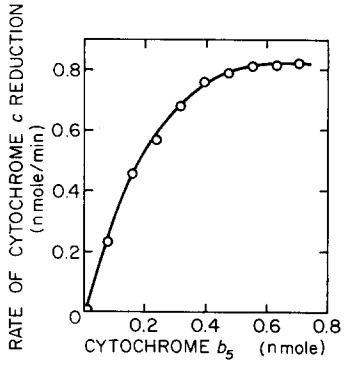


Fig. 2. Dependence of the reductase-catalyzed reduction of cytochrome *c* on the concentration of erythrocyte cytochrome $b_5$. See Methods for details.

methylene blue, molecular oxygen and S-protein were measured by following with time the absorbance of NADH at 340 nm. An $\varepsilon_{mM}$ of 6.22 was used in each instance, except for ferricyanide, in which case an $\varepsilon_{mM}$ of 6.82 was used. This extinction took into consideration the decrease in absorbance at 340 nm which accompanies the reduction of ferricyanide. Methemoglobin reduction was measured by following the formation of oxyhemoglobin at 577 nm using a value of 13.7 for the difference between the $\varepsilon_{mM}$ (per heme) of oxyhemoglobin and methemoglobin. When NADPH was used as the electron donor, saturating amounts were substituted for NADH in the assays described above.

DCIP reduction was carried out with a DCIP concentration of 25 $\mu$M. Reduction was measured by following the decrease in absorbance at 600 nm, assuming an $\varepsilon_{mM}$ of —21. DCIP reductase activity was used as a measure of the enzyme during the isolation procedure. For this purpose, the assay was carried out at pH 8.1 in a 3 ml

solution containing: 150 μmoles Tris–HCl, 1.5 μmoles NADH, 1.5 μmoles EDTA, 0.075 μmole DCIP, and reductase.

*Purification of cytochrome $b_5$ reductase*

Washed, outdated, packed human red blood cells (260 ml) were lysed by forcefully adding 1040 ml of cold water. The pH of the lysate was adjusted to pH 6.0 with HCl and centrifuged at 20000 × *g* for 20 min.

The reductase was removed from the supernatant fraction with DEAE-cellulose by modification of published methods for the isolation of erythrocyte reductases[4,11]. The supernatant fraction was diluted with 1.5 vol. of water and adjusted to pH 7.2 with KOH solution. The protein was charged at a rate of 150 ml/h onto a 2.4 cm × 20 cm DEAE-cellulose column previously equilibrated at 4 °C with 0.001 M buffer of pH 7.2. The column was washed with 1 l of the 0.001 M buffer and then eluted at pH 7.2 with a linear gradient formed with 600 ml of 0.001 M phosphate in the mixing chamber and 600 ml of 0.2 M phosphate–0.5 M KCl in the reservoir. Eluent was passed through the column at 75 ml/h. Fractions were collected in tubes that contained concentrated EDTA to give a final concentration of 1 mM. The DCIP reductase-containing fractions eluted from the column with the linear gradient of buffer (Fig. 3) were pooled, adjusted to pH 6.0 with HCl, and dialyzed for 2 h *vs* 0.05 M phosphate–0.001 M EDTA, pH 6.0.

The reductase was further purified by chromatography on Amberlite CG-50. The solution was applied to a 3.4 cm × 7 cm column of CG-50 that had been equilibrated with 0.05 M phosphate–0.001 M EDTA, pH 6.0. The charged column was first washed with 300 ml of 0.05 M phosphate–0.001 M EDTA buffer and the reductase then eluted with 0.5 M phosphate–0.001 M EDTA, pH 6.0, at a flow rate of 180 ml/h. Fractions with reductase activity were pooled and adjusted to pH 7.2 with KOH solution.

In preparation for gel filtration, the reductase was concentrated by $(NH_4)_2SO_4$ precipitation. $(NH_4)_2SO_4$ was slowly added to 90% saturation (66.2 g/100 ml); the pH was maintained at pH 7.2 during the addition. The precipitate was removed by

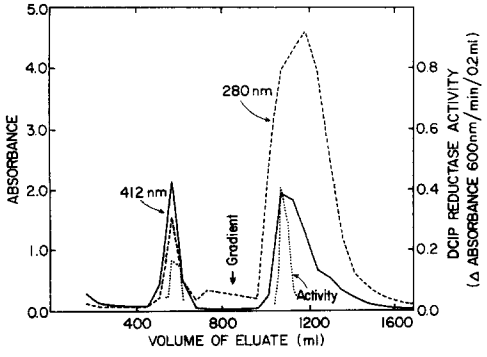


Fig. 3. Purification of erythrocyte cytochrome $b_5$ reductase on DEAE-cellulose at pH 7.2. Fractions of 14 ml were collected. For details see Experimental Procedure.

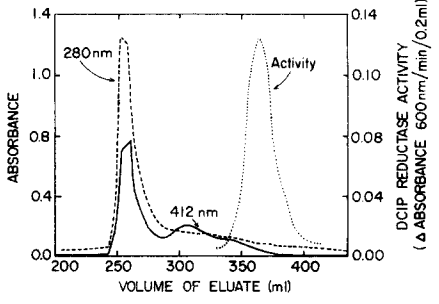


Fig. 4. Purification of erythrocyte cytochrome $b_5$ reductase on Bio-Gel P-60. Fractions of 2.46 ml were collected. For details see Experimental Procedure.

centrifugation, suspended in a minimum volume (28 ml) of 0.05 M phosphate–0.001 M EDTA, pH 7.2, and dialyzed against this buffer for 6 h. The dialyzed fraction was concentrated to 6 ml by ultrafiltration on a collodion membrane and applied to a 3.4 cm × 106.4 cm P-60 column that had previously equilibrated with the phosphate–EDTA buffer. The column was eluted with this buffer at a flow rate of 12 ml/h (Fig. 4).

In order to obtain a more highly purified reductase preparation for enzymatic studies, the most pure fractions of the Bio-Gel P-60 eluates from two reductase preparations were pooled and subjected again to chromatography on Bio-Gel P-60. Concentration by ultrafiltration and chromatography were carried out as described above.

## RESULTS

The purification of erythrocyte cytochrome $b_5$ reductase is summarized in Table II. Rechromatography of the most pure fractions from two preparations gave a sample with a specific activity of 21 $[(\Delta A_{600\,nm}/\text{min per ml enzyme})/A_{280\,nm}]$. Because of the presence in red blood cell hemolysates of other reductases which catalyze the reduction of DCIP, the overall yield and recovery can not be calculated. The visible absorbance spectrum of the purified sample indicates that the reductase is contaminated by a small amount of hemoglobin.

TABLE II

PURIFICATION OF ERYTHROCYTE CYTOCHROME $b_5$ REDUCTASE

| *Fraction* | *Total protein* ($A_{280\,nm} \times ml$) | *Total activity* [($\Delta A/min$ *per ml*) × *ml*] | *% of original activity* | *Specific activity* $\left(\frac{\Delta A/min\,per\,ml}{A_{280\,nm}}\right)$ |
|---|---|---|---|---|
| Hemolysate | 113 400 | 792 | 100 | 0.00701 |
| DEAE-cellulose eluate | 462 | 109 | 13.8 | 0.237 |
| CG-50 eluate after concentration | 39.6 | 23.2 | 2.9 | 0.587 |
| Bio-Gel P-60 eluate | 2.42 | 16.7 | 2.1 | 6.90 |

The intact protein showed little fluorescence. However, the supernatant fraction resulting from treatment of the sample with trichloroacetic acid showed fluorescence typical of flavin and the action of phosphodiesterase resulted in a 10-fold increase in fluorescence intensity. Assuming the prosthetic group to be FAD, the concentration of total flavin in the purified sample of erythrocyte cytochrome $b_5$ reductase was calculated to be $1.42 \cdot 10^{-10}$ mole/ml. Protein analysis of this sample showed a protein concentration of 0.041 mg/ml, which is equivalent to 280000 g protein per mole of flavin. Gel filtration of the reductase indicates a molecular weight of less than 40 000.

Erythrocyte cytochrome $b_5$ reductase is stable at pH 7.5 or 8.1 when stored at −20 °C in Tris–HCl buffer containing 0.5 mM EDTA and, in this solution, shows no measurable loss of activity over a 6 h period at 4 °C if the protein concentration is no lower than 0.02 mg/ml. However, in more dilute solution a significant loss of activity does occur. Thus, the same sample diluted to 0.002 mg protein/ml with the same

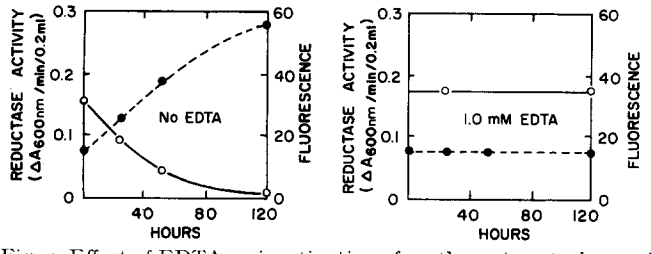


Fig. 5. Effect of EDTA on inactivation of erythrocyte cytochrome $b_5$ reductase and the appearance of flavin fluorescence. Samples were incubated at 4 °C in 0.04 M potassium phosphate buffer, pH 7.2. DCIP reductase activity was determined as described in Experimental Procedure. Fluorescence emission was measured at 530 nm with activation at 455 nm. ———, activity; – – – –, fluorescence.

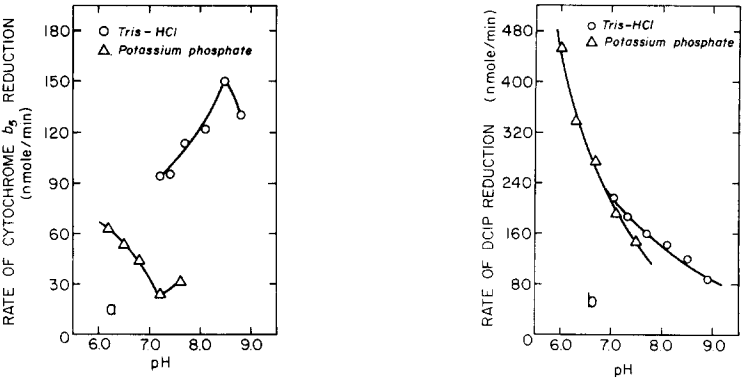


Fig. 6. pH–activity curve for erythrocyte cytochrome $b_5$ reductase. ○, Tris–HCl buffers; △, potassium phosphate buffers. Rates were adjusted to correspond to 1.0 ml enzyme. For details see Experimental Procedure. a. Reduction of human erythrocyte cytochrome $b_5$. Rates were determined by coupling the reaction to cytochrome *c*. Buffers were 0.12 M with 0.5 mM EDTA. b. Reduction of DCIP. Buffers were 0.1 M with 1 mM EDTA.

Tris–HCl–EDTA buffer lost 32 % activity after 6 h at 4 °C. In the absence of EDTA, the loss of activity from even concentrated solutions of the reductase occurs and this is accompanied by the appearance of fluorescence (Fig. 5). Like the fluorescence of free flavin, this fluorescence showed an emission maximum at 530 nm when activated at 380 or 470 nm and showed activation maxima at 378 and 457 nm when emission at 540 nm was measured.

The dependence of DCIP reduction and cytochrome $b_5$ reduction on the concentration of reductase is shown in Fig. 1. The pH–activity curve of the reductase for these two electron acceptors is shown in Fig. 6. Effect of the concentration of Tris–HCl and KCl on the catalysis of the two reductions is shown in Fig. 7. Whereas both $Tris^+$ and $K^+$ inhibit the reduction of cytochrome $b_5$, only the $K^+$ inhibits the reduction of DCIP. The inhibition by $Tris^+$ and $K^+$ appears to be largely responsible for the unusual pH–activity curves shown in Fig. 6. Catalysis of DCIP reduction is unaltered by EDTA concentrations up to 10 mM, but EDTA concentrations of 2, 4 and 10 mM inhibit cytochrome $b_5$ reduction 6 %, 17 % and 32 %, respectively.

The substrate specificity of the erythrocyte cytochrome $b_5$ reductase is shown in Table III. Of the electron acceptors studied, only cytochrome $b_5$, DCIP, and ferri-

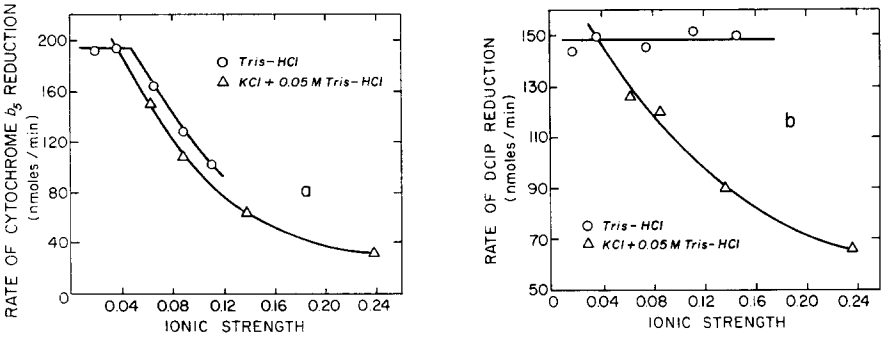


Fig. 7. Effect of Tris and $K^+$ on the activity of erythrocyte cytochrome $b_5$ reductase at pH 8.1. Rates were adjusted to correspond to 1.0 ml enzyme. For details see Experimental Procedure. ○, varying concentrations of Tris–HCl; △, varying concentrations of KCl in the presence of 0.05 M Tris–HCl. a. Reduction of human erythrocyte cytochrome $b_5$ as assayed by the coupled reaction with cytochrome *c*. Buffer contained 0.5 mM EDTA. b. Reduction of DCIP. Buffer contained 1 mM EDTA.

TABLE III

SUBSTRATE SPECIFICITY OF ERYTHROCYTE CYTOCHROME $b_5$ REDUCTASE

Rates are compared on a per electron basis. Conditions are those described in Methods. The buffer was 0.05 M Tris–HCl, pH 8.1, containing 0.5 mM EDTA.

| *Electron acceptor* | *Relative rate with NADH* | *Rate with NADPH / Rate with NADH* |
|---|---|---|
| $K_3Fe(CN)_6$ | 8.1 | 0.002 |
| DCIP | 2.45 | 0.017 |
| Human erythrocyte cytochrome $b_5$ * | 1.00 | 0.019 |
| Bovine erythrocyte cytochrome $b_5$ * | 0.35 | 0.011 |
| Methylene blue | 0.073 | 1.5 |
| Cytochrome *c* | 0.012 | 1.6 |
| $O_2$ | 0.0041 | 3.7 |
| Glutathione | 0.0025 | 2.5 |
| Methemoglobin | 0.0005 | — |

* Assay was performed by coupling the reaction to cytochrome *c*.

cyanide were rapidly reduced. With these electron acceptors the reductase is highly specific for NADH. Cytochrome $b_5$ from rabbit erythrocytes, rabbit reticulocytes, and rat liver microsomes likewise served well as electron acceptors for the reductase.

The dependence of reductions on the concentration of NADH is shown in reciprocal plots in Fig. 8, for arbitrary concentrations of cytochrome $b_5$ and DCIP. The apparent $K_m$ of NADH for the reduction of cytochrome $b_5$ was calculated to be $1.6 \cdot 10^{-6}$ M at 25 °C in pH 8.1 buffer. The $V$ of this reaction was $2.06 \cdot 10^{-4}$ M cyto-cytochrome $b_5$/min per ml enzyme. From the flavin content of this preparation, the turnover number was calculated to be 1280 moles of cytochrome $b_5$ per min per mole flavin. For the reduction of DCIP the apparent $K_m$ of NADH was calculated to be $4.8 \cdot 10^{-7}$ M and, at this concentration of DCIP, the $V$ was $2.3 \cdot 10^{-4}$ M/min per ml enzyme. The stoichiometry of the reaction was found to be 1.08 mole NADH oxidized/mole DCIP reduced.

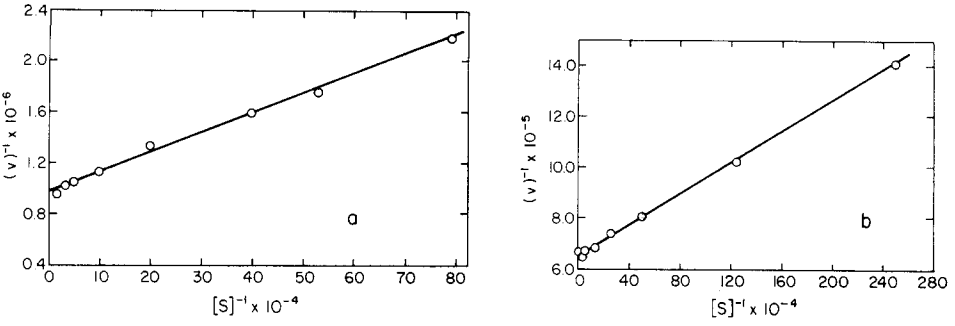


Fig. 8. Double-reciprocal plots of rate of reduction *vs* NADH concentrations. [*S*] is the molar concentration of NADH and *v* is the rate of reduction in moles/l per min for the reactions carried out at 25 °C in 0.05 M Tris–HCl buffer, pH 8.1, containing 0.5 mM EDTA. a. Cytochrome $b_5$ reduction. b. DCIP reduction.

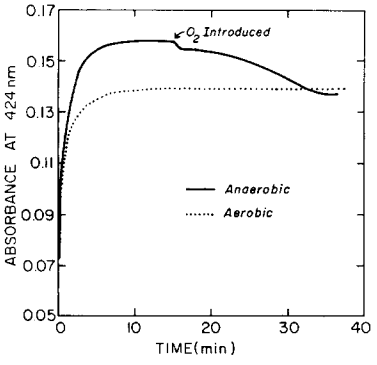


Fig. 9. Autoxidation of erythrocyte cytochrome $b_5$. Anaerobic and aerobic enzymatic reduction of cytochrome $b_5$ was carried out at pH 8.1 in the absence of cytochrome *c* as described in Experimental Procedure. The buffer was 0.05 M Tris–HCl containing 0.5 mM EDTA. Air was introduced into the anaerobic cuvette at time of 15 min as indicated.

The enzymatic reduction of cytochrome $b_5$ in aerobic and anaerobic conditions is shown in Fig. 9. Relative to the reduction achieved with sodium dithionite, enzymatic reduction gave 83 % reduction in the presence of air and 96 % reduction under anaerobic conditions. When air was introduced into the anaerobic reaction mixture, partial oxidation of the ferrocytochrome $b_5$ occurred. When the reaction was run with limiting amounts of NADH, the ferrocytochrome $b_5$ was completely reoxidized after the introduction of air.

The reduction of ferricytochrome *c* by the reductase–cytochrome $b_5$ system was inhibited by neither catalase nor superoxide dismutase.

## DISCUSSION

The soluble, NADH-dependent reductase which we have isolated from hemolysates of human erythrocytes efficiently catalyzes the reduction of soluble cytochrome $b_5$ from human, bovine, and rabbit red cells; solubilized cytochrome $b_5$ from rat

liver microsomes; DCIP; and ferricyanide. For these electron acceptors, the rate with NADPH is very slow relative to the rate with NADH. The lack of pyridine nucleotide specificity with the less efficient electron acceptors—methylene blue, cytochrome *c*, molecular oxygen, glutathione, and methemoglobin—may indicate the presence of a very small amount of a second activity.

There is no indication that the heme-containing component of the reductase preparation is involved in the enzymatic activity. However, the concomitant loss of activity and appearance of flavin fluorescence upon storage of the enzyme in the absence of EDTA indicate that flavin is an essential component of the reductase. The 10-fold increase in fluorescence intensity which resulted from treatment of the dissociated flavin with phosphodiesterase is characteristic of the release of FMN from FAD[31], suggesting that the prosthetic group of the reductase is FAD.

The significant electron transfer reactions involving erythrocyte cytochrome $b_5$ reductase and cytochrome $b_5$ are summarized in Fig. 10. The reduction of ferricytochrome *c* by the cytochrome $b_5$–cytochrome $b_5$ reductase system, like the reduction of methemoglobin by this system[27], is not inhibited by catalase nor superoxide dismutase and thus does not involve unbound $H_2O_2$ or superoxide anion.

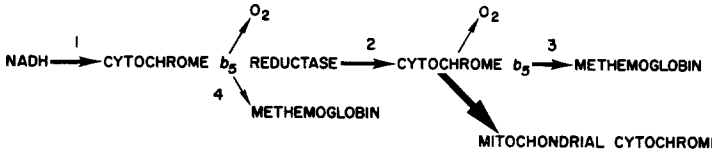


Fig. 10. Electron transfer reactions involving cytochrome $b_5$ reductase and cytochrome $b_5$ from erythrocytes. The heaviness of the arrows indicates the relative rates of the electron transfers.

The erythrocyte cytochrome $b_5$ reductase is distinguished from stromal cytochrome *c* reductase[34], glutathione reductase[35], dihydrofolate reductase[36], the NADPH-dependent "methemoglobin reductases"[1–6], and the other erythrocyte reductases which have been characterized, by its molecular weight, specificity for NADH, specificity toward electron acceptors, and inhibition by KCl.

The results of this study suggest that erythrocyte cytochrome $b_5$ reductase is NADH dehydrogenase I, the enzyme which has been shown to be necessary for normal reduction of methemoglobin in red cells. Erythrocyte cytochrome $b_5$ reductase possesses an FAD prosthetic group and NADH dehydrogenase I appears to be an FAD-containing protein[7] (although the work of Sugita *et al.*[10] fails to support this view). Erythrocyte cytochrome $b_5$ reductase shows great similarity to NADH dehydrogenase I[7,11,13,4] in terms of substrate specificity, dependency of activity on pH, inhibition by KCl, and lack of inhibition by 0.01 M EDTA. Moreover, we have shown that NADH dehydrogenase I, isolated by the method of Scott *et al.*[4], rapidly catalyzes the reduction of both liver microsomal and erythrocyte cytochrome $b_5$ and that catalysis of methemoglobin reduction by this enzyme preparation is stimulated by erythrocyte cytochrome $b_5$ in a manner similar to that which we reported for erythrocyte cytochrome $b_5$ reductase[27]. The disparity between the $K_m$ for NADH (with DCIP as electron acceptor) in the present study ($4.8 \cdot 10^{-7}$ M) as compared to that reported for NADH dehydrogenase I ($7.0 \cdot 10^{-6}$ M) might result from differences in pH, composition of the buffers, and concentration of electron acceptor.

The properties of the erythrocyte cytochrome $b_5$ reductase are also similar to those reported for cytochrome $b_5$ reductase from microsomes[17–19] and from the outer membrane of mitochondria[22,23] of other tissues. Thus, the erythrocyte and microsomal enzymes are similar in terms of FAD prosthetic group, specificity toward pyridine nucleotides and electron acceptors, pH dependency, inhibition by EDTA, and the apparent $K_m$ of NADH. However, the microsomal enzyme does show a considerably greater turnover number for the reaction between NADH and cytochrome $b_5$ than does the erythrocyte enzyme.

The redox systems of erythrocytes, leucocytes, and the membranes of microsomes, Golgi vesicles, mitochondria (outer membrane), and nuclei appear to have a great deal in common. The erythrocyte cytochrome $b_5$ reductase is similar to the NADH-dependent flavoproteins of these other systems. Likewise, erythrocyte cytochrome $b_5$[24] is similar to cytochrome $b_5$ isolated from microsomes, Golgi membranes, mitochondrial outer membranes, nuclear membranes, and leucocytes. In addition, erythrocytes, like most of the other systems, appear to contain either a native or modified form of hemeprotein P-450. However, the physical state of the redox proteins of the erythrocyte differs from that of the redox proteins in these other systems. Thus, in microsomes, cytochrome $b_5$ reductase, cytochrome $b_5$, and hemeprotein P-450 are all an integral part of the membranous fraction and are solubilized only after treatment with protease, detergent, or lysosomes[37]. In contrast, very mild methods for the lysis of red blood cells yield cytochrome $b_5$ reductase and cytochrome $b_5$ in soluble form, and hemeprotein P-420 loosely associated with the stromal fraction[28].

The similarity of these soluble proteins with the corresponding proteins solubilized from subcellular organelles of other tissues suggests that the soluble erythrocyte proteins are derived from particulate fractions present in precursors of the erythrocyte. Our finding, that greater amounts of the soluble cytochrome $b_5$ are present in a cell population high in reticulocytes, is in agreement with such a hypothesis. Since most of the subcellular organelles of the mammalian red cell disappear prior to the reticulocyte stage, a protein solubilized from the endoplasmic reticulum or other membrane fraction would have had less opportunity to degrade in a reticulocyte than in an erythrocyte.

Whereas microsomal cytochrome $b_5$ reductase and cytochrome $b_5$ appear to function in desaturation of fatty acids, the soluble reductase and cytochrome of erythrocytes appear to function in the reduction of methemoglobin by the pathway involving reactions 1, 2, and 3 of Fig. 10[27]. If particulate forms of these proteins are found in immature red blood cells, it will be of great interest to determine whether such forms function in either fatty acid desaturation or methemoglobin reduction. If a particulate form of cytochrome $b_5$ reductase is present in erythrocyte precursors, it will also be of great interest to determine whether it, like the soluble reductase, is lacking in cells from patients with congenital methemoglobinemia.

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REFERENCES

1 M. Kiese, C. Schneider and H.-D. Waller, *Arch. Exp. Pathol. Pharmakol.*, 231 (1957) 158.
2 F. M. Huennekens, R. W. Caffrey, R. E. Basford and B. W. Gabrio, *J. Biol. Chem.*, 227 (1957) 261.
3 E. Shrago and A. B. Falcone, *Biochim. Biophys. Acta*, 67 (1963) 147.
4 E. M. Scott, I. W. Duncan and V. Ekstrand, *J. Biol. Chem.*, 240 (1965) 481.
5 C. Shimizu and F. Matsuura, *Agric. Biol. Chem.*, 32 (1968) 587.
6 D. Niethammer and F. M. Huennekens, *Arch. Biochem. Biophys.*, 146 (1971) 564.
7 E. M. Scott and J. C. McGraw, *J. Biol. Chem.*, 237 (1962) 249.
8 E. M. Scott, *Clin. Chim. Acta*, 23 (1969) 495.
9 E. M. Scott and I. V. Griffith, *Biochim. Biophys. Acta*, 34 (1959) 584.
10 Y. Sugita, S. Nomura and Y. Yoneyama, *J. Biol. Chem.*, 246 (1971) 6072.
11 E. Hegesh and M. Avron, *Biochim. Biophys. Acta*, 146 (1967) 397.
12 M. D. Sass, C. J. Caruso and D. R. Axelrod, *Clin. Chim. Acta*, 24 (1969) 77.
13 E. M. Scott, *Biochem. Biophys. Res. Commun.*, 9 (1962) 59.
14 E. M. Scott, in E. Beutler, *Hereditary Disorders of Erythrocyte Metabolism*, Grune and Stratton, New York, 1968, p. 102.
15 E. M. Scott, *J. Clin. Invest.*, 39 (1960) 1176.
16 J. C. Kaplan, A. Hanzlickova-Leroux and J. C. Dreyfus, *C. R. Acad. Sci. Paris*, 270, Ser. D (1970) 2223.
17 P. Strittmatter and S. F. Velick, *J. Biol. Chem.*, 221 (1956) 277.
18 P. Strittmatter and S. F. Velick, *J. Biol. Chem.*, 228 (1957) 785.
19 P. Strittmatter, in P. Boyer, H. Lardy and K. Myrbäck, *The Enzymes*, Vol. 8, Academic Press, New York, 1963, p. 113.
20 P. W. Holloway, *Biochemistry*, 10 (1971) 1556.
21 N. Oshino, Y. Imai and R. Sato, *J. Biochem. Tokyo*, 69 (1971) 155.
22 H. R. Mahler, I. Raw, R. Molinari and D. do Amaral, *J. Biol. Chem.*, 233 (1958) 230.
23 G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, *J. Cell Biol.*, 32 (1967) 415.
24 P. G. Passon, D. W. Reed and D. E. Hultquist, *Biochim. Biophys. Acta*, 275 (1972) 51.
25 D. E. Hultquist, D. W. Reed and P. G. Passon, *Fed. Proc.*, 28 (1969) 862.
26 D. E. Hultquist and P. G. Passon, *162nd Meet. Am. Chem. Soc., Washington, D.C.*, (1971) Biol.-172.
27 D. E. Hultquist and P. G. Passon, *Nature*, 229 (1971) 252.
28 D. E. Hultquist, D. W. Reed, P. G. Passon and W. E. Andrews, *Biochim. Biophys. Acta*, 229 (1971) 33.
29 D. W. Reed, P. G. Passon and D. E. Hultquist, *J. Biol. Chem.*, 245 (1970) 2954.
30 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
31 H. B. Burch, O. A. Bessey and O. H. Lowry, *J. Biol. Chem.*, 175 (1948) 457.
32 P. Strittmatter and S. F. Velick, *J. Biol. Chem.*, 221 (1956) 253.
33 V. Massey, *Biochim. Biophys. Acta*, 34 (1959) 255.
34 I. Zamudio and M. Canessa, *Biochim. Biophys. Acta*, 120 (1966) 165.
35 G. E. J. Staal, J. Visser and C. Veeger, *Biochim. Biophys. Acta*, 185 (1969) 39.
36 J. R. Bertino, A. R. Cashmore and B. L. Hillcoat, *Cancer Res.*, 30 (1970) 2372.
37 S. Takesue and T. Omura, *Biochem. Biophys. Res. Commun.*, 30 (1968) 723.