Improved Determination of Cytochrome b5 in Human Erythrocytes

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A sensitive, precise enzymic/spectrophotometric method for determining cytochrome b5 in small amounts of blood is described. Mean values for healthy individuals, ages 20 to 70 years, were 0.26 (SD 0.03) µmol per liter of erythrocytes or 0.87 (SD 0.14) nmol per gram of hemoglobin. We believe the assay is preferable to methods described hitherto, primarily because of its high sensitivity.

Additional Keyphrases: methemoglobin reduction · methemoglobinemias · enzymic methods · spectrophotometry · reference interval

In erythrocytes, cytoplasmic cytochrome b5 may be the electron carrier in the methemoglobin reducing system catalyzed by cytochrome b5 reductase (EC 1.6.2.2), also called "methemoglobin reductase" (1-3). There is also evidence that, under physiological conditions, cytochrome b5 may be a rate-limiting factor of the reductive system (4, 5). A sensitive, precise method for determining cytochrome b5 in erythrocytes is therefore of clinical interest. We describe such a method. In it, cytochrome b5, partly purified from hemolysates on a column of diethylaminoethyl-cellulose, is introduced into a reaction mixture containing methemoglobin, cytochrome b5 reductase, and NADH. Under the conditions chosen, the rate of methemoglobin reduction depends on the cytochrome b5 concentration and is measured spectrophotometrically. The system is standardized with pure cytochrome b5.

Materials, Preparations, Procedures

Materials

NADH (Grade III) was purchased from Sigma Chemical Co., St. Louis, MO. Diethylaminoethyl cellulose (DE-52) was a product of Whatman, Chemical Separation Division. The gel-filtration medium, Ultragel AcA 54, was purchased from LKB, Bromma, Sweden. All other chemicals used were of the purest grade commercially available.

Preparations

Preparation 1: Methemoglobin free of cytochrome b5 and of cytochrome b5 reductase. Lyse 5 mL of packed erythrocytes, previously washed three times with a 155 µM/L solution of NaCl, by adding 5 mL of distilled water and 4 mL of toluene. Shake the mixture, centrifuge, and pass the supernate through Whatman no. 3 filter paper on a Büchner funnel. Apply the clear hemolysate to a 1.5 × 6 cm column of DE-52 previously equilibrated with potassium phosphate buffer (3 mmol/L, pH 7.2). Hemoglobin passes through the column; cytochrome b5 and cytochrome b6 reductase are quantitatively retained. Test the eluted hemolysate for the absence of cytochrome b5 by the method described here, and for the absence of cytochrome b5 reductase by the method of Hegesh et al. (6). Dialyze the purified hemoglobin solution for 1 h vs 1 L of a 15 mmol/L solution of sodium nitrite in citrate buffer (5 mmol/L, pH 6.0), to convert the pigment to methemoglobin, followed by 5 h vs buffer only.

Stored at 4 °C, this preparation can be used for about two weeks.

Methemoglobin concentration (in terms of total Hb concentration) is measured by the method of Cannon (7).

Preparations 2 and 3 are modifications of procedures described by Passon and Hultquist et al. (3, 8, 9), Yubisui et al. (10), and Takeshita et al. (11).

Preparation 2: Purified cytochrome b5 reductase. Wash about 100 g of DE-52 several times with potassium phosphate buffer (5 mmol/L, pH 7.2). Filter the suspension through Whatman no. 3 filter paper, on a Büchner funnel. Mix 600 g of the wet DE-52 cake with 500 mL of hemolysate that has been prepared as described above for preparation 1, using stored, citrated blood. Stir the suspension for 1 h at 4 °C. Under these conditions cytochrome b5 and cytochrome b5 reductase are adsorbed onto the cellulose. Filter the suspension and wash the cellulose on the Büchner funnel with potassium phosphate buffer (3 mmol/L, pH 7.2), until the hemoglobin is washed out. Transfer the DE-52 cake to a beaker, mix with 500 mL of the phosphate buffer containing 60 mmol of KCl and 100 µmol EDTA per liter, and stir the suspension for 1 h at 4 °C. During this interval cytochrome b5 reductase is eluted from the adsorbent. Filter off and refrigerate the DE-52 cake, which contains the cytochrome b5, until used further for the preparation of cytochrome b5. Concentrate the eluate containing the enzyme to 2 to 4 mL in an Amicon ultrafiltration cell, using a YM-10 filter membrane. The specific activity of the preparation will be about 200 U per gram of protein, as determined according to Hegesh et al. (6). Measure protein by the method of Lowry et al. (12). Further purify the concentrated enzyme preparation on a 1.7 × 100 cm column of Ultragel AcA 54 (LKB) previously equilibrated with potassium phosphate buffer (50 mmol/L, pH 7.2) containing 100 µmol of EDTA per liter. Elute fractions with this same buffer, at about 0.7 mL/min. Combine the most-active fractions and concentrate to about 2.0 mL by ultrafiltration on a YM-10 membrane, dialyze vs citrate buffer (5 mmol/L, pH 6.0) containing 100 µmol of EDTA per liter, and store at -20 °C. The final preparation is stable for several months and has a specific activity of approximately 1500 U per gram of protein, and an activity concentration of about 15 000 U/L.

Preparation 3: Purified cytochrome b5. Wash the wet cake of DE-52 remaining after elution of the enzyme (see preparation 2) with 500 mL of potassium phosphate buffer (5 mmol/L, pH 7.2) containing 120 mmol of KCl per liter. This removes additional hemoglobin, cytochrome b5 reductase, and other, unidentified substances. Elute the firmly adsorbed cytochrome b5 with 500 mL of a 500 mmol/L solution of KCl in the same buffer by stirring for 1 h at 4 °C. Separate it by filtration and concentrate to approximately 20 mL by using an Amicon UM-2 membrane. Dialyze the concentrate vs phosphate buffer (5 mmol/L, pH 7.2) for 10 h and further purify on a 3 × 20 cm column of DE-52 previously equilibrated with this phosphate buffer. Develop the column with

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the equilibration buffer to which is added 250 mmol of KCl per liter. Combine the most-active fractions (activity measured by the method described here), concentrate to 2–4 mL (Amicon UM-2), and load onto a 1.7 × 100 cm column of Ultrogel AcA 54, equilibrated with phosphate buffer (50 mmol/L, pH 7.2). Elute with this same buffer. Combine the active fractions, dilute with four volumes of distilled water, and apply to a 1.6 × 6 cm column of DE-52, equilibrated with phosphate buffer (5 mmol/L, pH 7.2). Develop the small column by use of a linear gradient, starting with the equilibration buffer and going up to 350 mmol of KCl per liter, in the same buffer. Examine the most-active fractions spectrophotometrically and use those with an A413 nm/A280 nm ratio >3.5 (3, 12) for further purification. Dilute the combined fractions with four volumes of distilled water and chromatograph a second time on a smaller (0.7 × 6 cm) DE-52 column. A reddish-brown band of cytochrome b5 can be observed at the top of the column. Elute it with less than 1 mL of pH 7.2 phosphate buffer containing 250 mmol of KCl per liter. Dialyze the preparation for 5 h vs the citrate buffer and store at −20 °C. It contains approximately 20 nmol of cytochrome b5. Use the following criteria for the purity of the preparation:

* The A413 nm/A280 nm ratio should be higher than 4.
* The electrophoresis (13) on 15% polyacrylamide gel should reveal only one protein band of cytochrome b5.
* The absorption spectra of the oxidized and reduced forms of the cytochrome b5 preparation should coincide with that of the purest preparations described to date (3); the oxidized form has a Soret band peak at 413 ± 0.5 nm; (b) the dithionite-reduced form has peaks at 423, 527, and 556 ± 0.5 nm; (c) the difference spectrum of the reduced minus oxidized forms has a trough at 409 nm and peaks at 424, 527, and 55 ± 0.5 nm.

**Assay Procedure**

**Preparation of sample.** Wash the erythrocytes of 4–5 mL heparinised or citrated blood of known hemoglobin concentration and hematocrit value three times with 155 mmol/L NaCl and separate by centrifugation at 600 × g. Prepare hemolysate as described in "preparation 1." Determine the hemoglobin concentration of the clear hemolysate. Apply a measured volume, about 2 mL, of hemolysate to a 0.7 × 6 cm column of DE-52 equilibrated with potassium phosphate buffer (5 mmol/L, pH 7.2). Wash the column consecutively with 15 mL of the equilibration buffer and 15 mL of the same buffer containing 120 mmol of KCl per liter. Elute the cytochrome b5 with 10 mL of the 5 mmol/L phosphate buffer containing 250 mmol of KCl per liter. Dialyze the eluate vs 2 L of the citrate buffer for 5 h at 4 °C. Concentrate it to about 1.0–1.5 mL in an Amicon ultrafiltration cell, Model 8010, using a UM-2 membrane. Transfer the concentrated eluate quantitatively to a small tube with the aid of a Pasteur pipet and measure its volume. Use 50 μL of the eluate for cytochrome b5 determination. The eluate can be stored at −20 °C for several months without loss of activity.

**Determination of cytochrome b5.** The 1-mL reaction mixture contains 500 μL of a second citrate buffer (10 mmol/L, pH 5.8), 50 μL of disodium EDTA (10 mmol/L), 25 μL of purified methemoglobin (1 mmol/L, preparation 1), 25 μL of NADH (10 mmol/L), 200 μU of purified cytochrome b5 reductase (preparation 2), 50 μL of sample, and distilled water to 1.0 mL. Prepare a blank, substituting buffer for sample. The pH of the reaction mixture is 6.0–6.1. The reaction, which is carried out at 30 °C, is started by adding NADH to the sample and blank cuvettes at zero time. Record the change in absorbance at 575 nm vs the blank for at least 15 min. Standardize the method with pure cytochrome b5 (preparation 3).

**Standardization.** The purity of the cytochrome b5 used for standardization is verified and its concentration determined spectrophotometrically (see preparation 3). A millimolar absorptivity of 105 mmol L⁻¹ cm⁻¹ for the difference in absorption of the reduced (by sodium dithionite) and oxidized forms of cytochrome b5 at 424 nm is used to establish cytochrome b5 concentration of the standard (14). Figure 1 illustrates a typical standardization curve, showing the dependence of a net rate of methemoglobin reduction on cytochrome b5 concentration.

**Calculation.** Calculate the amount of cytochrome b5 in the blood sample as follows: (nmol cytochrome b5 in sample × mL of eluate)/(mL sample used × g Hb loaded onto the column) = nmol cytochrome b5/g of hemoglobin. The result can be expressed also in micromoles of cytochrome b5 per liter of erythrocytes as calculated from information on the hemoglobin concentration and the hematocrit of the blood being analyzed.

**Results**

**Optimal Assay Conditions**

**Methemoglobin concentration.** We chose a final concentration of purified methemoglobin of 25 μmol/L in the reaction mixture. At this comparatively low concentration (A575 nm approximately 0.55) optical interferences are avoided. It was shown that in the reaction mixture methemoglobin is present in excess (Figure 2).

**Effect of cytochrome b5 reductase.** Figure 3 illustrates that methemoglobin reduction is activated by cytochrome b5 reductase even in the absence of cytochrome b5 (Figure 3, I) and that the rate of reduction depends on the enzyme concentration. However, the addition of cytochrome b5 (5 nmol/L) greatly accelerates the reaction. (Figure 3, III, Figure 1). As shown in Figure 3, the net activity (II, total activity minus blank activity, III – I) vs enzyme activity (blank) declines at high enzyme concentrations. We chose 200 U of enzyme per liter of reaction mixture for the standard assay, to spare enzyme without significantly affecting sensitivity.

**pH.** Figure 4 demonstrates a pH optimum at 6.0–6.1.

**Buffer.** Citrate buffer at concentrations >5 mmol/L has an inhibitory effect on the rate of methemoglobin reduction.

**Temperature.** Activity increases with temperature. How-
Fig. 2. Effect of methemoglobin concentration on the rate of its reduction
Conditions as described under “assay procedure,” except that different concentrations of purified methemoglobin were used. 50 μL of buffer containing 5 pmol of pure cytochrome b5 was substituted for sample.

Fig. 3. Effect of cytochrome b5 reductase concentration on methemoglobin reduction
Conditions as described under “assay procedure,” except that increasing amounts of enzyme were added to the reaction mixture. Measurements were made against a reference cuvette in which cytochrome b5 and enzyme were replaced by buffer. I, blank; II, buffer substituted for sample (measures methemoglobin reduction by enzyme alone); III, complete system (b), 50 μL of buffer containing 5 pmol of pure cytochrome b5 was used as sample; II (c), net activity of cytochrome b5 (calculated): III – I = II.

Fig. 4. Relation between rate of methemoglobin reduction and pH
Conditions as described under “assay procedure,” except that buffers and pHs were altered. 7 pmol of pure cytochrome b5 were used instead of sample; (b) 5 μmol/L sodium citrate buffer; (c) 5 μmol/L citrate-phosphate buffer; (d) 5 μmol/L Tris HCl buffer.

Fig. 5. Relation between the rate of methemoglobin reduction and volume of sample extract
Conditions as described under “assay procedure,” except that different volumes of sample extract were used.

The mean value was 0.26 (SD 0.03) μmol per liter of erythrocytes, or 0.87 (SD 0.14) nmol per gram of hemoglobin. No sex-related difference was observed. The values were essentially normally distributed about the mean value.

Discussion
Several methods have been described (3, 11, 16) for determination of cytochrome b5 in erythrocytes. The simplest is that of Takeshita et al. (11). However, in that assay the reference (blank) cuvette, in contrast to the sample and standard, does not contain externally added cytochrome b5 reductase. In our hands, when a blank containing all constituents except cytochrome b5 was prepared (with purified methemoglobin), the rates of methemoglobin reduction in sample and blank cuvettes were practically indistinguishable. The assay of Passon et al. (3) is based on measurement of the difference spectrum for the oxidized and the enzymically reduced forms of cytochrome b5 in a partly purified extract from erythrocytes. The low concentration of this cytochrome in the extract and the relatively high

Evaluation of the Method
The precision of the method was evaluated by 20 independent determinations on a single blood specimen during three days. The standard deviation was ±0.02 μmol of cytochrome b5 per liter of erythrocytes (CV 6.2%).

Analytical recovery of 0.2 nmol of purified cytochrome b5 added to 1 mL of hemolysate was 95%. The sensitivity of the assay is around 0.5 μmol per liter of reaction mixture.

Reference Values
Cytochrome b5 was determined in fresh blood samples from 66 clinically healthy individuals, ages 20 to 70 years.
content of colored impurities results in a distortion of the absorption spectrum, which impairs the accuracy of the
determination. The modifications suggested by Abe and
Sugita (16) marginally improve the accuracy of the method
but complicate the procedure.

In our method both standard and sample are introduced
into the same reconstituted, optimized methemoglobin re-
duction system. We follow the rate of the cytochrome b5-
dependent reaction rather than measuring the amount of
cytochrome b5, and this is responsible for the high sensitiv-
ity and precision. We estimate that our assay is approxi-
mately 200 times more sensitive than assays based on the
measurement of the differential spectrum (3, 16). It is
unlikely that other electron carriers with a cytochrome b5-
like activity could be present in our sample extract, so we
consider our assay to be specific for cytochrome b5.

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References
1. Hultquist DE, Passon PG. Catalysis of methemoglobin reduction
by erythrocyte cytochrome b5 and cytochrome b6 reductase. Nature
2. Sugita Y, Nomura S, Yonesama Y. Purification of reduced
pyridine nucleotide dehydrogenase from human erythrocytes and
methemoglobin reduction by the enzyme. J Biol Chem 246, 6072–
6078 (1971).
3. Passon PG, Reed DW, Hultquist DE. Soluble cytochrome b5 from
4. Sannes LR, Hultquist DE. Effect of hemolysate concentration,
ionic strength and cytochrome b5 concentration on the rate of
methemoglobin reduction in hemolysate of human erythrocytes.
5. Schwartz JM, Reiss AL, Jaffe ER. Hereditary methemoglobinemia
with deficiency of NADH cytochrome b5 reductase. In The
Metabolic Basis of Inherited Disease, 6th ed., JB Stanbury, JB
pp 1654–1663.
6. Hegesh E, Calmanovici N, Avron M. New method for determin-
ing ferrihemoglobin reductase (NADH-methemoglobin reductase)
7. Cannan RK. Proposal for a certified standard for use in hemoglo-
8. Passon PG, Hultquist DE. Soluble cytochrome b5 reductase from
9. Hultquist DE, Dean RT, Duglas RH. Homogeneous cytochrome
b5 from human erythrocytes. Biochem Biophys Res Commun 60,
reductase in human erythrocytes. Biochem Biophys Res Commun
11. Takeshita M, Yubisui T, Tanishima K, et al. A simple enzymat-
ic microdetermination of cytochrome b5 in erythrocytes. Anal
Biochem 107, 305–310 (1980).
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein
measurement with the Folin phenol reagent. J Biol Chem 193, 265–
275 (1951).
13. Davis B. Disc electrophoresis II. Method and application to
14. Hultquist DE. Methemoglobin reduction system of erythro-
pp 463–473.
15. Kuma F, Inomata H. Studies on methemoglobin reductase II. J
16. Abe K, Sugita Y. Properties of cytochrome b5 and methemoglo-
(1979).