Automated determination of red cell methaemoglobin reductase activity by a continuous-flow system for screening hereditary methaemoglobinaemia

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SUMMARY A flow diagram for the automated determination of ferricyanide reductase activity in red blood cells was prepared in the modules from AutoAnalyzer AA I (Technicon Instruments Inc). Ferricyanide reductase assay can be substituted for assay of cytochrome b_5 reductase (EC 1.6.2.2), which plays a major role in reducing methaemoglobin in erythrocytes, and is defective specifically in the erythrocytes of patients with hereditary methaemoglobinaemia.

The effective sampling rate of the analysis is 30/h, and less than 0.05 ml of whole blood is required. Interference of haemoglobin with absorption by potassium ferricyanide at 420 nm is effectively excluded by dialysis. This automated method was compared with the accepted diaphorase method, and it distinguished clearly the ferricyanide reductase activity of cord bloods from that of adult bloods. The activity of the blood from a patient with hereditary methaemoglobinaemia was only residual.

It is suggested that the method is useful as a mass screening test for hereditary methaemoglobin-aemia.

The deficiency of erythrocyte NADH-methaemoglobin reductase has been documented in many other patients with congenital methaemoglobinaemia and reviewed by Hsieh and Jaffe (1975). Recently, many investigators have considered NADH-cytochrome b_5 reductase to be the major enzyme catalysing the reduction of methaemoglobin *in vivo* and hereditary methaemoglobinaemia to be due to an abnormality of this enzyme (Hultquist and Passon, 1971; Sugita *et al.*, 1971; Kuma and Inomata, 1972). In general, the patients encounter little difficulty although they have slate-grey cyanosis. However, Dine (1956) found congenital methaemoglobinaemia with mental retardation. Recently, it has been evident that a small but significant number have mental retardation, and the early detection of hereditary methaemoglobinaemia in the newborn has been of great value (Fialkow *et al.*, 1965; Leroux *et al.*, 1975).

Methaemoglobin reductase activity in red blood cells is generally measured as 'diaphorase' by using

the dye 2,6-dichlorophenolindophenol (DCIP) as an electron acceptor. However, diaphorase is resolved into various fractions by ion-exchange chromatography, and only one of these fractions shows the activity of cytochrome b_5 reductase. For this reason it is most satisfactory to measure the cytochrome b_5 reductase activity for the specific diagnosis of hereditary methaemoglobinaemia. With regard to the assay of cytochrome b_5 reductase, it is difficult and time-consuming to prepare cytochrome b_5 as a terminal electron acceptor from tissue sources. We reported previously the ferricyanide reductase assay substituted for the cytochrome b_5 reductase assay, which had the advantage of using a chemical reagent as substrate electron acceptor and specificity for cytochrome b_5 reductase activity, but involved excluding haemoglobin from the haemolysate using a CM-cellulose column I (Tanishima *et al.*, 1978).

The purpose of this report is to describe exclusion of interference by haemoglobin with the absorption by potassium ferricyanide at 420 nm using a continuous-flow dialysing system, and to establish a simple and rapid automated assay for screening hereditary methaemoglobinaemia.

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Fig. 1 Flow diagram of manifold in AutoAnalyzer AA I for methaemoglobin reductase assay using potassium ferricyanide as an electron acceptor (ferricyanide reductase assay).

Material and methods

BLOOD SAMPLES

Normal human blood samples were obtained from healthy volunteer students and university officials. Cord blood specimens were from the Department of Obstetrics and Gynaecology, Kanazawa University Hospital. Blood samples for the screening test came from the Central Clinical Laboratory of the same hospital. The blood samples from a patient with hereditary methaemoglobinaemia were kindly provided by Dr Hirano (Hirano *et al.*, 1976), of the Department of Internal Medicine, Medical School of Nagoya Health Science University.

AUTOMATED METHOD FOR MEASUREMENT OF FERRICYANIDE REDUCTASE ACTIVITY

A flow diagram for the automated determination of ferricyanide reductase activity was prepared in the modules of the AutoAnalyzer AA I (Technicon Instruments Inc) (Fig. 1). Volumes of 0.05 ml of whole blood oxidised with 5 μ l of 0.2 mol/l sodium nitrite are haemolysed with 0.45 ml volumes of distilled water and placed in sample cups. The haemolysate is mixed with phosphate buffer, potassium ferricyanide, and NADH in the stream. The mixed sample (donor) stream enters one plate of the dialyser in a 37°C water bath. The recipient stream of water flowing through the other plate receives the diffusible potassium ferricyanide from the sample stream, which remains unreduced after the reaction. This stream is then analysed by the

spectrophotometric method at 420 nm for potassium ferricyanide. An effective sampling rate is 30/h.

Reagents are as follows: 0.5 mol/l sodium phosphate buffer at pH 7.5; 15 mmol/l potassium ferricyanide; 10 mmol/l NADH; all in solution containing 0.3 ml Brij-35/l.

The ferricyanide reductase activity was expressed as international units (IU) per ml of erythrocytes according to the following equation:

$$\frac{E}{1020} \times \frac{V}{t} \times \frac{10^5}{Ht \times v}$$

where E = the difference in absorption between ferricyanide and distilled water at 420 nm, V = volume of reaction mixture = 1.6 ml, t = reaction time = 2 minutes, Ht = haematocrit value (%), v = volume of blood sample = 0.01 ml, 1020 = molar extinction coefficient of potassium ferricyanide at 420 nm.

The limit of resolution for the difference between total and unreacted substrates (Δ substrate) was 15 nmol per minute, when a diluted solution of the purified enzyme was assayed with the AutoAnalyzer instrumentation using potassium ferricyanide as an electron acceptor (Δ OD was 0.01 in spectrophotometry).

DETERMINATION OF HAEMATOCRIT VALUE

The haematocrit value was determined according to the microhaematocrit method using the Haematocrit centrifuge RC-24BN (Tomy Seiko Co. Ltd) at 11 000 rpm for 5 minutes.

MANUAL METHODS FOR MEASURING REDUCTASE ACTIVITY

NADH-ferricyanide reductase activity of a partially purified enzyme from normal red cells and NADH-diaphorase activity of a haemolysate using 2,6-dichlorophenolindophenol as an electron acceptor were assayed according to the methods reported previously by Tanishima *et al.* (1978). In this report, linearity, effect of pH and kinetics of ferricyanide reductase activity were studied.

PURIFIED CYTOCHROME b_5 REDUCTASE

Cytochrome b_5 reductase for use in fundamental studies and quality control was purified from normal human red cell haemolysate by DE-32 column chromatography as reported previously (Tanishima *et al.*, 1978).

Results

STUDIES ON FUNDAMENTAL PROBLEMS

Linearity for ferricyanide reductase activity in both the manual reference method and the automated test method were studied by assaying the enzyme purified from normal red cell haemolysate (Fig. 2).

Fig. 2 Standard curves for purified cytochrome b_5 reductase activity by AutoAnalyzer and manual ferricyanide reductase assay methods.

The enzyme was purified from normal human red blood cells by DE-32 column chromatography. Dilutions were performed with phosphate buffer. The assays were carried out by both the manual (reference) method and the automated (test) method described in the text: IU = international units per ml of diluted solution of enzyme; ●—● manual assay; ○—○ AutoAnalyzer assay.

The reduction rate of potassium ferricyanide by the enzyme with NADH ν concentrations of the enzyme was linear in both methods. The time course for the enzyme reaction is shown in Figure 3. In the manual

Fig. 3 Time course in ferricyanide reductase assay. The reduction of potassium ferricyanide by the purified cytochrome b_5 reductase (1 mg per 1.5 ml of reaction mixture as protein) was recorded by Hitachi Model 200-20 recording spectrophotometer at 420 nm for 30 minutes by the manual method, and was tested in the AutoAnalyzer system described in the text, respectively: ΔE = differences of absorbance for corresponding time; ●—● manual method; ○—○ AutoAnalyzer method.

reference method, the initial rate of reduction of potassium ferricyanide, as an electron acceptor, was essentially linear with time for 7 minutes. In the AutoAnalyzer test method, the same result was obtained by increasing the number of reaction-mixing coils in the heating bath. From these results, consumption of substrate is about $0.35 \mu\text{mol}$ in 7 minutes in 1.5 ml of reaction mixture containing $1.5 \mu\text{mol}$ of potassium ferricyanide, and the reaction rate is linear over this change in substrate concentration.

MEASUREMENT OF ENZYME ACTIVITY IN NORMAL RED CELL HAEMOLYSATE BY TEST METHOD

Figure 4 shows the relationship between the concentration of haemoglobin in the haemolysate to be placed in the sample cup and ferricyanide reductase activity in the test method. The linear relationship was observed up to 30 mg/dl of the final concentration of haemoglobin.

A recovery test was performed on the haemolysate with 20 mg/dl of haemoglobin. When the purified enzyme, which had approximately the same activity as the haemolysate, was added to the haemolysate,

Fig. 4 Ferricyanide reductase activity in normal red cell haemolysate by AutoAnalyzer method: IU = international units per ml of diluted haemolysate.

recovery of ferricyanide reductase activity in the test method was 92%.

PRECISION

Ten samples from each of three normal human adults were tested on the same working days. The mean ferricyanide reductase activity in the test method, standard deviation (SD), and coefficient of variation (CV) in percent are as follows:

3.75 ± 0.13 international units (IU) per ml of erythrocytes, 3.5%

3.46 ± 0.11 international units (IU) per ml of erythrocytes, 3.2%

3.98 ± 0.21 international units (IU) per ml of erythrocytes, 5.3%

With respect to reproducibility day to day, the heparinised whole blood samples from three normal adults were stored at 4°C. Volumes of 0.05 ml were haemolysed with 0.45 ml volumes of distilled water and placed in sample cups for each day's assay. The automated assay of these samples was carried out 10 times during two working months. The mean $\pm$ SD was 3.45 ± 0.11 , 3.68 ± 0.14 , and 4.11 ± 0.18 (IU per ml of erythrocytes), and CV was 3.2%, 3.8%, and 4.4%, respectively.

COMPARISON WITH CONVENTIONAL DIAPHORASE METHOD

Twenty-three samples of heparinised whole blood from normal adults and 14 samples of cord blood were assayed by the conventional diaphorase method (manual) and the automated test method. The results are shown in Figure 5.

It can be seen in the results from both the automated method and the diaphorase method that the values of activity in cord blood are significantly lower than those in adult blood. Only a little overlapping of the values in cord blood with those in adult blood is observed for ferricyanide reductase activity in the test method. In the diaphorase system, however, a certain overlapping between the values from these two blood groups is seen.

The mean ferricyanide reductase activity of blood samples from 23 normal adults was 4.0 ± 0.63 IU per ml of erythrocytes, and that from 14 cord bloods was 1.95 ± 0.43 IU per ml of erythrocytes. In the blood sample from a patient with hereditary methaemoglobinaemia, the ferricyanide reductase activity was 0.56 IU per ml of erythrocytes.

APPLICATION TO SCREENING

Ferricyanide reductase activities of 1466 human blood samples from our hospital were analysed by our automated test method. Figure 6 shows the distribution of the values. From the linearity in plotting cumulative frequency on probability paper, it was evident that these values of ferricyanide reductase activities were quasi-Gaussian.

Discussion

Since Gibson (1948) and Scott and Griffith (1959) reported a congenital deficiency of an enzyme catalysing the reduction of methaemoglobin in erythrocytes, it has been an important problem to determine the methaemoglobin reductase activity for the detection of this disease. Various methods have been reported for determining reductase activity, for example, the diaphorase method by Scott (1960), the cytochrome b_5 reductase method by Hultquist and Passon (1971), and the ferrihaemoglobin reductase method by Hegesh *et al.* (1968). The ferricyanide reductase method previously described by us (Tanishima *et al.*, 1978) not only correlated well with accepted methods but also met the requirement of a simple and specific test for the diagnosis of hereditary methaemoglobinaemia, because of its easy application to routine work in the laboratory without any special reagents or expensive equipment.

Results by the automated method offer more rapid analysis and mass screening of the enzyme deficiency in hereditary methaemoglobinaemia. The screening of hereditary methaemoglobinaemia is important for the early detection of the disease and its prevention in infants. Previous observations record that a small but significant proportion of the cases of congenital enzymopenic methaemoglobinaemia is associated with a progressive neurological disorder with mental retardation causing death at a

Fig. 5 Comparison of methaemoglobin reductase activity of cord and adult blood by AutoAnalyzer ferricyanide reductase assay with that by manual diaphorase assay. The activity was measured for the same individuals at the same time by both methods: SD = standard deviation; IU = international units per ml of erythrocytes.

Fig. 6 Frequency distribution of ferricyanide reductase activities of red cell haemolysate from 1466 samples by AutoAnalyzer. IU = international units per ml of erythrocytes.

young age (Leroux *et al.*, 1975). The detection of heterozygous carriers is a problem because this

inheritance is considered to be a simple recessive trait. We used cord blood to simulate heterozygotes. We believe we are justified in this because heterozygous carriers have methaemoglobin reductase levels about half those of normals (Scott, 1960), and also red cells from cord blood are thought to be weak in their ability to reduce methaemoglobin because of the lower reductase activity in such cells—about half normal levels (Hegesh *et al.*, 1968; Ross, 1963; Vetrella *et al.*, 1971; Kanazawa *et al.*, 1968).

Kaplan *et al.* (1970) reported a simple spot screening test for the fast detection of red cell NADH-diaphorase deficiency. Their results obtained with heterozygous subjects were not clearcut, and they do not recommend this test for the detection of heterozygotes. The suitability of our method for the detection of the heterozygous trait is suggested by its ability to discriminate better than the standard diaphorase method between cord blood and blood from adults (Fig. 5). The use of the AutoAnalyzer is also an advantage of the method and contributes

in speed, convenience, and elimination of interference by haemoglobin in spectrophotometry.

The population screened in this investigation ranged from 19 to 71 years of age and few of these subjects had low ferricyanide reductase activity. Two whose blood showed the lowest enzyme activity have, respectively, hypertension treated with trichlor-methiazide (Fluitran), hydralazine (Apresolin), and methoserpidine (Decaserpyl) (59 years old), and hepatoma with pulmonary metastasis (71 years old). However, any possible relation between these diseases or therapy and a lower methaemoglobin reductase activity in red blood cells is obscure.

In contrast with the result obtained from adult blood, the detection of the heterozygote in children remains a problem. Detection of the heterozygote in cord and infant blood is very difficult but may become possible by increasing the sensitivity of the method. It should be further investigated in order to measure the reduction rate by the enzyme at 340 nm, the maximum absorption wavelength for NADH, in place of 420 nm for potassium ferricyanide.

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