Reliable routine estimation of small amounts of foetal haemoglobin by alkali denaturation

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Several methods have been developed for the estimation of foetal haemoglobin levels by alkali denaturation (Singer, Chernoff, and Singer, 1951; Betke, Marti, and Schlicht, 1959; Kristoffersen, 1961) but there have been increasing demands for a simple method which is sensitive enough to measure reproducibly small elevations of foetal haemoglobin in both inherited and acquired haematological disorders. The method described by Betke *et al* (1959) comes closest to fulfilling these requirements. We describe a modification of this method which is simple and gives highly reproducible results in the lower range of haemoglobin F values.

Method

Red cell lysates can be prepared from 2 ml or more of whole blood collected in any standard anticoagulant. The cells are washed three times with 0.85% sodium chloride, lysed with 2.3 volumes of distilled water and half a volume of carbon tetrachloride, and centrifuged at 3 000 *g* for 30 minutes at room temperature. If the lysate is prepared in this way the final concentration is in the range 8-10 g/100 ml. This is critical for reproducible results.

REAGENTS

1.2 N sodium hydroxide. This should be prepared fresh for each batch of estimations.

Saturated ammonium sulphate.

Drabkin's solution (KCN, 0.05 g; $K_3Fe(CN)_6$, 0.20 g; distilled water, 1 l) (Drabkin and Austin, 1935; Dacie and Lewis, 1968).

TEST PROCEDURE

A solution of cyanmethaemoglobin (approximately 500 mg/100 ml) is prepared by adding 0.6 ml of haemolysate to 10 ml Drabkin's solution. Then 0.2 ml of 1.2 N sodium hydroxide is added to 2.8 ml cyanmethaemoglobin solution. The solutions are mixed immediately by inversion and a stopwatch is started. The reaction is stopped after exactly two minutes by the addition of 2.0 ml of saturated

ammonium sulphate with vigorous mixing. The mixture is allowed to stand for five minutes and then filtered through a double layer of Whatman no. 6 filter paper in a 1½ in. diameter funnel. An undenatured sample for comparison is prepared by mixing 1.6 ml distilled water, 1.4 ml cyanmethaemoglobin solution, and 2.0 ml saturated ammonium sulphate. This solution is diluted 1 in 10 with distilled water and the test and comparison solutions are read at 415 nm on a suitable spectrophotometer.

The whole procedure is carried out at room temperature (18°C-26°C).

CALCULATION

The percentage of alkali-resistant haemoglobin is derived from the following formula:

$$\frac{OD_{415nm} \text{ of test solution} \times 100}{OD_{415nm} \text{ of undenatured solution} \times 20}$$

Comment

REPRODUCIBILITY

Excellent reproducibility was obtained after performing duplicate estimations on 16 separate haemolysates prepared from a blood sample from a normal adult male. The estimations were done in separate batches of eight on different days, with the following results: mean 0.465; standard deviation 0.041; standard error of the mean 0.007; 94% of estimations fell between ± 2 standard deviations. Equal reproducibility was obtained on blood samples with raised levels of foetal haemoglobin.

SENSITIVITY AND ACCURACY

The sensitivity has been increased considerably by reading at 415 nm rather than 540 nm. Probably not all the alkali-resistant material is genuine foetal haemoglobin. Starch gel electrophoretic analysis of an alkali-resistant residue shows, in addition to any haemoglobin F which may be present, many non-haemoglobin components including carbonic anhydrase B. An estimation of alkali-resistant haemoglobin in a sample of pure haemoglobin A prepared by DEAE-Sephadex chromatography gave a value of 0.26%. Like other alkali denaturation techniques this method gives falsely low values when the haemoglobin F level rises above 50%. Thus it is inadequate for really accurate determinations of haemoglobin F level in many cord blood samples.

HAEMOLYSATE CONCENTRATION

Figure 1 demonstrates the effect on the level of 'alkali-resistant haemoglobin' of reducing the haemolysate concentration. This dilution phenomenon is consistent and is independent on the level

Technical methods

Fig. 1 The effect of dilution of the haemolysate on the alkali-resistant haemoglobin value. The three lysates are prepared from the cells of a normal adult, from the cells of a patient with β -thalassaemia trait with a slightly elevated foetal haemoglobin, and pure haemoglobin A prepared by DEAE-Sephadex chromatography. It is clear that concentrations below 5 g/100 ml show falsely high readings.

or even the presence, of foetal haemoglobin. Thus as the haemoglobin concentration with assay mixture falls below 250 mg/100 ml the rate of denaturation of haemoglobin A becomes slower although the pH tends to rise slightly due to the reduced buffering effect at the lower concentration of protein. Errors due to variation in the haemolysate concentration are probably the most important source of inaccurate estimation of low levels of foetal haemoglobin. They can be minimized if the method of preparing the lysate as outlined above is adhered to rigidly, since this will give a concentration between 8 and 10 g/100 ml which provides a final concentration in the assay mixture of 480 to 600 mg/100 ml.

COLLECTION AND STORAGE

The following anticoagulants have been examined and found to produce no difference in alkaline-resistant haemoglobin values; disodium ethylenediamine tetra-acetic acid (EDTA), acid citrate dextrose, lithium heparin, and ammonium oxalate. There was no alteration in the level after storage of whole blood at 4°C for one week or as haemolysate at 4°C or -20°C for up to two weeks. Longer storage at -20°C resulted in a reduction in alkali-resistant haemoglobin values by about 15% of original value.

METHAEMOGLOBIN AND CARBONMONOXYHAEMOGLOBIN

A great advantage of using the cyanmethaemoglobin derivative is that oxhaemoglobin, methaemoglobin, and carbonmonoxyhaemoglobin are all readily converted to the cyanmethaemoglobin state (Drabkin and Austin, 1935). Thus the accumulation of methae-

Fig. 2 The distribution of alkali-resistant haemoglobin values in normal male and female populations.

moglobin does not affect the level of alkali-resistant haemoglobin. The poor reproducibility experienced by Beaven *et al* (1960) when using cyanmethaemoglobin has not been encountered.

NORMAL VALUES

Estimations of 75 normal non-pregnant females and 24 normal males between 18 and 45 years are shown in Figure 2. An upper limit of 0.9% has been taken as normal because the distribution becomes discontinuous above this level.

References

- Beaven, G. H., Ellis, M. J., and White, J. C. (1960). Studies on human foetal haemoglobin. I. Detection and estimation. *Brit. J. Haemat.*, **6**, 1-22.
- Betke, K., Marti, H. R., and Schlicht, I. (1959). Estimation of small percentages of foetal haemoglobin. *Nature (Lond.)*, **184**, 1877-1878.
- Chernoff, A. (1953). Immunologic studies of hemoglobins. II. Quantitative precipitin test using anti fetal hemoglobin sera. *Blood*, **8**, 413-421.
- Christensen, J. (1968). Haemoglobin F: serological conditions and occurrence in normal adults and normal pregnant and post-partum women with particular reference to foeto-maternal haemorrhage. *Dan. med. Bull.*, **15**, 166-174.
- Dacie, J. V., and Lewis, S. M. (1968). *Practical Haematology*, 4th ed. p. 37, Churchill, London.
- Drabkin, D. L., and Austin, J. H. (1935). Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin, and sulphemoglobin. *J. biol. Chem.*, **112**, 51-65.
- Kristoffersen, K. (1961). An improved method for the estimation of small quantities of alkali-resistant hemoglobin in blood. *Scand. J. clin. Lab. Invest.*, **13**, 402-409.
- Schroeder, W. A., Huisman, T. J. H., Shelton, J. R., and Wilson, J. B. (1970). An improved method for quantitative determination of human fetal hemoglobin. *Analyt. Biochem.*, **35**, 235-243.
- Singer, K., Chernoff, A. I., and Singer, L. (1951). Studies on abnormal hemoglobins. I. Their demonstration in sickle cell anaemia and other hematologic disorders by means of alkali denaturation. *Blood*, **6**, 413-428.

A technique for demonstrating fibrinolysis by cutaneous bacteria

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Substances which convert plasminogen to plasmin, ie, activators of plasminogen, derive from various sources. They were first demonstrated in filtrates of haemolytic streptococci (Tillett and Garner, 1933) and since have been found in almost all fluids and organs of the body (Fearnley, 1965) and have been demonstrated in culture filtrates of some strains of staphylococci (Elek, 1959). Substances collected from the skin surface have also been shown to have fibrinolytic activity (Dawber, Nishioka, and Ryan, 1971) and it has been suggested that this activity together with the clot-promoting effect of surface lipids, is important in reconstituting the skin surface following injury.

Dawber and his colleagues (1971) failed to find fibrinolytic activity among the bacteria of the normal flora of the skin. As some strains of *Staphylococcus aureus* have long been known to produce staphylokinase (Elek, 1959) it was decided to study skin isolates of these organisms and the closely linked groups, *Staph. epidermidis* and *Micrococcus* species. The investigation was later extended to include isolates of the cutaneous diphtheroids.

Materials and Methods

Fibrinolytic activity of bacteria has previously been demonstrated and measured using the fibrin plate technique (Astrup and Müllertz, 1952) in which a solution of fibrinogen is clotted with thrombin in a Petri dish and a small amount of the substance to be assayed placed on the fibrin so prepared. The plate is then incubated for a set period, usually about 20 hours, and lysis is shown as a clear zone which gives a measure of the fibrinolytic activity.

This technique is not ideal for testing large numbers of strains of bacteria and it was therefore modified and the tests performed in 50 × 6 mm test tubes. In each sterile test tube were placed 0.5 ml of fibrinogen (1% in veronal buffer, pH 7.4), 0.02 ml of 0.1M calcium chloride, and 1 drop of peptone water. All solutions were sterilized by filtration before use. A large inoculum taken from an agar plate culture of the appropriate bacterial isolate