

Quantitative determination of haemoglobin A₂ using paper electrophoresis

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SYNOPSIS A quantitative paper electrophoresis method for the separation of haemoglobins A₁ and A₂ is described. The possible sources of error and the accuracy of the method have been investigated. The normal range for haemoglobin A₂ by this method is 1.3% to 4.3% of the total haemoglobin concentration. Good clinical correlation has so far been obtained.

With the migration of peoples, some of whom may be carriers of the thalassaemia gene, and the rising standards of medicine which today require the intensive laboratory study of anaemias of unknown aetiology, the necessity for further laboratory assistance in the diagnosis of thalassaemia has become obvious. A considerable amount of work has been carried out since Kunkel and Wallenius (1955), using starch block electrophoresis, first isolated haemoglobin A₂, and showed (Kunkel, Ceppellini, Müller-Eberhard, and Wolf, 1957) that raised values are usually found in thalassaemia minor. Gerald and Diamond (1958) suggested that a raised A₂ level combined with microcytosis are essential criteria before a diagnosis of thalassaemia minor can be established. The disadvantages of the starch block method are that it is relatively time consuming, and the number of samples that can be estimated is correspondingly restricted. Cradock-Watson, Fenton, and Lehmann (1959) demonstrated that good separation of A and A₂ haemoglobin components can be obtained on paper using a buffer containing Tris (trishydroxymethyl aminomethane) and E.D.T.A. acid. The purpose of this paper is to show the results of the combination of the Tris buffer method for the separation of the haemoglobins with the quantitative method for the estimation of proteins using bromphenol blue dye (Wilkinson and Wilkinson, 1960), to describe the modification of techniques, and to assess the reliability of the results.

TECHNICAL METHODS

All blood specimens were collected using di-potassium sequestrene as an anticoagulant. Haemolysates were

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prepared by the distilled water and toluene method of Singer, Chernoff, and Singer (1951). The foetal haemoglobin levels were estimated when required, and the haemolysates were then converted to carbonmonoxy-haemoglobin.

Paper electrophoresis was carried out in vertical tanks (Flynn and de Mayo, 1951), using nine paper strips (Whatmann No. 3 MM) per tank, each measuring 25.5 × 4.0 cm. The shorter paper length of 25.5 cm compared with the 36 cm. of Cradock-Watson *et al.* (1959) gave a higher voltage gradient with better separation under our conditions. Power was supplied from a 250 v. constant voltage power pack, the current being 2 milliamperes per strip applied for 16 hours. The Tris buffer as described by Cradock-Watson *et al.* (1959) was used and adjusted to pH 8.6 with boric acid. On each paper 200 c. mm. of haemolysate was applied with a micropipette and the paper immediately moistened with buffer on both sides to within a quarter of an inch of the point of application. This method of application was found to give the sharpest definition of the bands. After completion of the run, the papers were dried in an oven at 90-100°C and fixed for 10 minutes in an ethanol solution containing 10% mercuric chloride and 10% glacial acetic acid. It was found to be essential to discard the fixative after each use, as prolonged standing with the buffer salts gave rise to some precipitation, which resulted in artificially high values for the smaller components. Accordingly, only small quantities sufficient to moisten the papers were used on each occasion.

After fixation, the papers were dried, washed in two changes of distilled water for 10 minutes, and then thoroughly dried. Staining was then carried out for 10 minutes with an ethanol solution containing 1% bromphenol blue and 1% glacial acetic acid. A solution of 0.5% acetic acid in 25% ethanol was used for washing, as the addition of ethanol to the washing solution was found to give more rapid elution of the excess dye. Three washes of 10 minutes and two of 15 minutes were found to give a stable green colour. Wilkinson and Wilkinson have shown the importance of reaching this stable

point. After drying, the papers were marked and divided at the point of least colour between the two bands. The dye from the A₂ band was eluted with 10 ml. of 1.5% (w/v) sodium carbonate in 50% (v/v) methanol, and that from the main A band with 30 ml. of the same solution. This latter solution was subsequently diluted 11 times to bring the optical density well within the most sensitive region of the instrument. The optical densities of the eluates were measured at 595 m μ in a Unicam S.P. 400 spectrophotometer.

RESULTS

ELECTROPHORETIC SEPARATION OF HAEMOGLOBIN A₂

Fig. 1 shows typical electrophoretic patterns obtained from both normal and thalassaemia minor haemolysates.

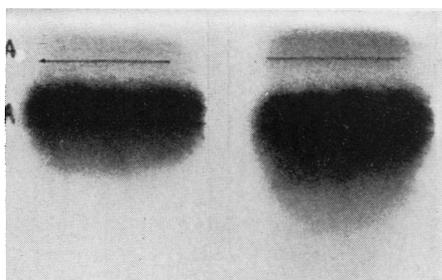


FIG. 1. Typical electrophoretic patterns obtained from the blood of normal and thalassaemia minor patients.

LINEARITY OF DYE UPTAKE By serial dilutions of the dye it was demonstrated that Beer's law was obeyed over the range of densities in use, and that the density readings were well within the maximal sensitivity range of the instrument. Wilkinson and Wilkinson have demonstrated that this linearity may not always hold, particularly at higher density values.

Standard curves for haemoglobins A and A₂ were constructed by subjecting 20 c.mm. samples of varying concentrations of haemoglobin solutions to electrophoresis. The concentration of the solutions under test ranged from 1 to 19 g. of haemoglobin per 100 ml. of haemolysate. Blanks were also tested and the values deducted from the readings of the other components. These results are shown in Figs. 2 and 3, and it can be seen that departure from linearity of dye uptake by the haemoglobin A occurs at concentrations above 7 g. of haemoglobin per 100 ml. of haemolysate. As the A₂ band usually comprises less than 10% of the haemoglobin A band, linearity would be anticipated throughout the range of solutions applied, and that above 7 g. of haemoglobin per 100 ml. of haemolysate, a relative

increase in the percentage haemoglobin A₂ would occur. After this experiment all haemolysates applied have ranged from 4.5 g. to 7 g. of haemoglobin per 100 ml.

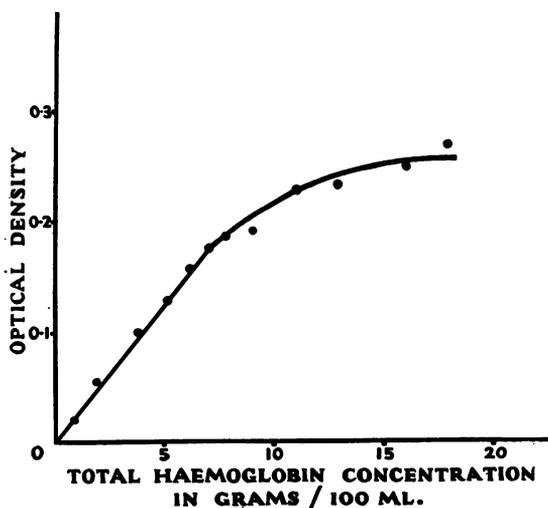


FIG. 2. The optical densities of the diluted dye eluates obtained from the A haemoglobin band, plotted against the original haemoglobin content of the haemolysates which were subjected to electrophoresis. Linearity can be seen to fall off at concentrations in excess of 7 g. of haemoglobin per 100 ml.

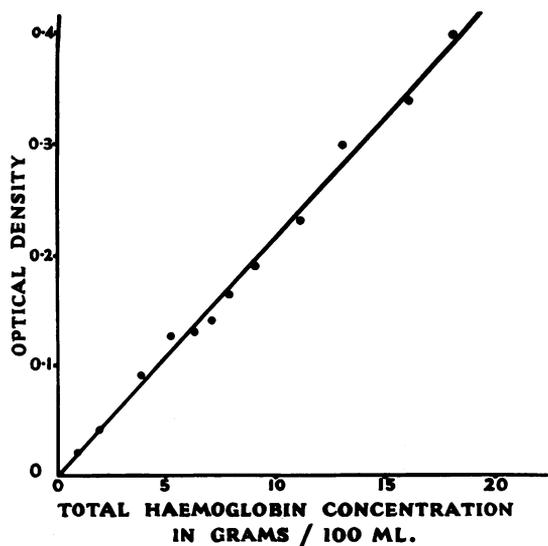


FIG. 3. The optical densities of the diluted dye eluates obtained from the A₂ haemoglobin band, plotted against the original haemoglobin content of the haemolysates which were subjected to electrophoresis. Linearity holds throughout the range of concentrations used.

ACCURACY An assessment of reproducibility was made by running at least three samples from each of two normal controls and two cases of thalassaemia on five successive days. Running, staining, and elution were all carried out in the routine manner, and the percentage A_2 of the total haemoglobin estimated. The figures obtained are presented in Table I.

TABLE I

VALUES FOR A_2 AS PERCENTAGE OF TOTAL HAEMOGLOBIN CONTENT FROM TWO NORMAL AND TWO THALASSAEMIC SUBJECTS WITH MULTIPLE ESTIMATIONS ON FIVE SUCCESSIVE DAYS

| Type of Subject | Total No. of Estimations | Mean Value for % A_2 | Same Day | | Different Days | |
|-----------------|--------------------------|------------------------|----------|---------|----------------|---------|
| | | | S.D. | % Error | S.D. | % Error |
| Normal | 69 | 3.3 | 0.28 | 8 | 0.39 | 12 |
| Thalassaemic | 30 | 6.4 | | 4 | | 6 |

It can be seen from these data that the percentage error did not vary greatly between the normal and the thalassaemic patients, but that there was a significantly greater variation between runs on different days than between repeated estimations in the same run. No evidence could be found of a systematic day effect common to all the patients, and the extra variability between runs must therefore be attributable to variations in the degree of separation between the A and A_2 components and possibly to some variation in the cutting between the two bands.

In the evaluation of these results it should be borne in mind that the absolute quantity of the A_2 component is very small, being of the order of 50 μg . of protein. It can be deduced from the standard deviation on repeated runs that two-thirds of all results should be within one standard deviation, i.e., $\pm 0.4\%$ of the true value.

NORMAL RANGES Haemolysates from pregnant patients of northern European, Greek, and Italian extraction have been examined. In those patients classified as normal no evidence has been found of any haemoglobinopathy. The 24 patients, in whom the diagnosis of thalassaemia minor was made,

TABLE II
NORMAL AND ABNORMAL VALUES FOR PERCENTAGE A_2 HAEMOGLOBIN

| Type of Case | Total No. | Quantity of A_2 as Percentage of Total Haemoglobin | | |
|--|-----------|--|---------|------|
| | | Mean | Range | S.D. |
| Normal pregnant patients of northern European origin | 86 | 3.2 | 1.4-4.3 | 0.61 |
| Normal pregnant patients of Mediterranean origin | 115 | 3.2 | 1.4-4.3 | 0.64 |
| Thalassaemia minor | 24 | 7.0 | 5.1-8.6 | 1.20 |

include nine pregnant patients and 13 non-pregnant patients. Further details of these cases will be published at a later stage of these investigations. The values for these normal and abnormal groups of patients are given in Table II, and it can be seen that there is a significant separation between the normal and abnormal values ($P=0.001$).

DISCUSSION

At present it would appear that a raised A_2 value is the most specific and sensitive single indicator of the thalassaemic trait; and although not pathognomonic in itself, raised values taken in conjunction with other peripheral blood findings may be regarded as virtually diagnostic. A requirement therefore exists for the development of a relatively simple and mass reproducible, yet reasonably accurate, test for the estimation of A_2 values. It appears that the described techniques amply fulfil the two former criteria, and that with carefully standardized conditions the accuracy is adequate. The major part of the experimental error probably derives from variations in the site of division between the two bands, but it can be anticipated that repeated estimation of any values falling close to the upper range of normal, should eliminate the possibility of false results.

An advantage of dilution of the two components to similar optical densities is that they are within the range of linearity for the optical density of the dye. It is therefore possible to use the optical densities for calculations and to avoid the necessity for conversion into dye units.

The values for the normal range appear to be slightly higher than those obtained by Kunkel and Wallenius (1957) who found a mean value of 2.5 while estimating normals by the starch block method. The separation, however, between the normal and abnormal groups appears to be quite distinct.

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