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HAEMOGLOBIN A_2

A Comparative Study on the Estimation Using Different Methods.

P. K. Sukumaran* and H. R. Master*

Haemoglobin A_2, a minor component of normal adult haemoglobin has a molecular formula $\alpha_2\delta_2$ and was discovered by Kunkel and Wallenius (1955) and using starch block electrophoresis showed that this minor fraction was normally seen at about 2.5% of the total haemoglobin and that this level was raised in betathalassaemia carriers. The level of haemoglobin A_2 is remarkably constant throughout life in normal people reflecting that the precise quantitative control of haemoglobin synthesis. This fraction is found to be reduced in iron deficiency conditions (Josephson *et al* 1958; Sukumaran *et al* 1960; Wasi *et al* 1968).

Unlike other abnormal haemoglobins, it is not easy to diagnosis beta-thalassaemia trait. This is much more so in alpha-thalassaemia as there are no electrophoretically detectable abnormal haemoglobins associated with both these genes. Beta-thalassaemia being not uncommon in India and it is useful to have estimation of Hb A_2 as one of the diagnostic procedures for thalassaemia trait in the absence of iron deficiency. For the quantitation of Hb A_2, besides the original method of starch block electrophoresis, various investigators have used techniques based on electrophoresis using starch gel (Goldberg and Ross 1960), cellulose acetate (Marengo-Rowe 1965), Filter paper (Black *et al* 1966) and starch gel (Aksoy & Erdem 1965). Besides electrophoresis, chromatography using carboxymethylcellulose (Muller and Pik 1962) and $DEAE_{50}$ (Huisman *et al* 1962) are also used. This minor component can be easily detected by paper electrophoresis and a visual comparison of this was successfully used for detecting thalassaemia trait (Cradock-Watson *et al* 1959). Mean values for normals varies from 2 to 9.9% depending on the technique used for separation and the method of estimation (Black *et al* 1966). It is now accepted that any reliable method used would give a value of about 2.5% and that these values should be reproducible.

For routine work in haematological laboratory or for those interested in population surveys on abnormal haemoglobin including thalassaemia, most of the methods so far mentioned are either too complicated, time consuming or expensive requiring spectrophotometer or a densitometer. Thus there is necessity for a quick and cheap method but not at the cost of accuracy. With this in view an attempt has been made to standardise tolerably accurate method for the estimation of heamoglobin A_2 using paper electrophoresis. This paper presents a comparative data on the estimation of this fraction by electrophoresis using starch block, cellulose acetate and filter paper.

MATERIALS AND METHODS :

Blood samples were washed three times in normal saline to obtain cells free of plasma proteins. Haemoglobin from the packed cells were prepared according to the method of Kunkel *et al* (1957) using carbon tetrachloride instead of toluene. Haemolysates were standardised to have a concentration of 10 gm%. Individuals apparently healthy with normal haematological findings, including normal red cell morphlogy and osmotic fragility were used as normal subjects. Beta thalassaemia trait subjects were obtained

* Present Address : Cancer Research Institute, Tata Memorial Hospital, Parel, Bombay 12.

from either parents or siblings of patients with proved beta-thalassaemia disease and in whom complete laboratory investigation showed this trait. Iron deficiency cases were from those admitted in the nearby hospitals with anaemia, clinically proved to be due to iron deficiency and confirmed by serum iron studies. It must be admitted that number in this group was small.

Starch electrophoresis was done according to the method of Kunkel *et al* (1957) using BDH potato starch which was previously washed four times with buffer before preparing blocks. Veronal buffer pH 8.6 was used both for preparing starch blocks and in the buffer chamber. Starch block was prepared in perpex trays measuring 10″ x 12″ x ½″. Haemoglobin solution in cynmeth-form was applied into a slot evenly distributed and later sealed. The block, thus ready for run, was covered with polythene sheet, to prevent evaporation and kept firm by means of a glass plate, fixed with clamps. Electrophoresis was run overnight at 4°C with about 300 volts across the block. After the run the fractions were scooped out and eluted through a sintered glass funnel. Haemoglobin A fraction was eluted in 100 ml. while Hb A_2 in 10 ml. of barbiturate buffer. Optical density of each fraction was read in Hilger "UVSPEK"-Spectrophotometer at 415 mμ. Values for Hb A_2 were calculated according to the method of Kunkel *et al* (1957). Sepraphore III (Gelman, U.S.A.) membrane was used for cellulose acetate electrophoresis. Tris-borate buffer, pH 8.9, (MarengoRowe 1965) was used in this test. Twenty micro-liters of 10% haemoglobin solution was applied by means of "pulled out" capillary graduated to contain this volume. Electrophoretic run was for about an hour at 0.3 mA/cm after which the fractions were carefully cut out, care being taken not to allow the membrane to dry. Hb A_2 fraction was eluted in 3.0 ml. and Hb A was eluted in 30 ml. of Tris buffer. Elution was allowed to continue in the refrigerater till the membranes were free of haemoglobin and the resultant haemoglobin solution were read in a spectrophotometer at 415 mμ. If eluted in Drabkins solution, reading was taken at 540 mμ, and concentration calculated.

Estimation of Hb A_2 using filter paper was carried out using a vertical chamber and Whatman No. 3 (chromatographic grade) filter paper. Electrode chambers contained veronal buffer pH 8.6 and Tris buffer, pH 8.9 according to the formula of Cradock Watson *et al* (1959) diluted 1 in 5 was used for wetting the paper. On strips (3½″ wide) soaked in the diluted buffer and blotted between filter paper to remove excess, were applied about 100 1 of haemoglobin solution of 10% concentration and run over-night at 2 mA per strip. After the run, strips were removed, fraction A_2, separated very well, (see Fig. 1) was cut out

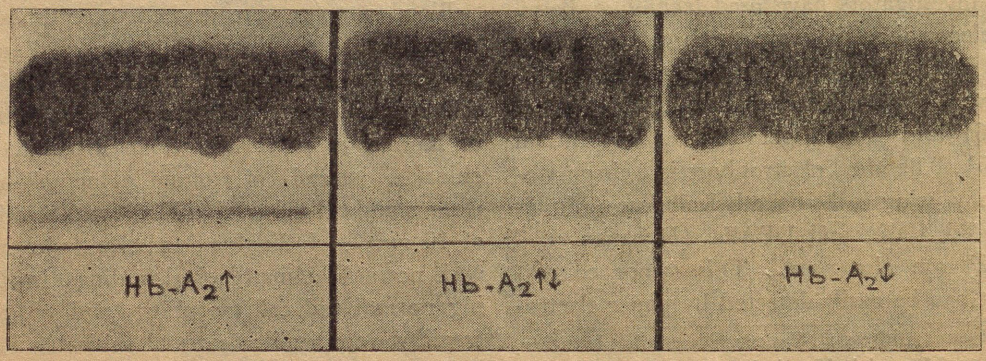

Fig. 1

TABLE I

Mean Values of Haemoglobin A_2 in Normal, Beta thalassaemia trait and Iron deficiency using different methods :-

Subjects	Paper Mean	N	Cellulose Acetate Mean	N	Starch Block Mean	N
Normal	2.31 ± 0.03	100	2.04 ± 0.03	25	2.04 ± 0.06	20
Thalassaemia trait	4.82 ± 0.06	120	4.39 ± 0.10	23	4.46 ± 0.04	11
Iron Deficiency	1.32 ± 0.04	25	1.00 ± 0.03	5	0.95 ± 0.02	5

N = Number of subjects.

These values were statistically tested and no significant differences were seen. These results are shown in Table II.

TABLE II

Differences of Mean Values

	Normal			Beta-thalassaemia trait		
Pairs	t	D.F.	P	t	D.F.	P
Paper vs. Starch block	0.41	118	.6<P<.7	0.16	129	.8<P<.9
Paper vs. Cellulose acetate	0.49	123	.6<P<.7	0.26	141	.7<P<.8

eluted in 3 ml. and Hb A in 30 ml. of veronal buffer. It is of advantage to use haemoglobin in cyanmeth-form if the samples are to be stored. Elute was collected by filtration and read in a Klett-Summerson photoelectric colorimeter using green filter. Readings were also taken on spectrophotometer at 540 mμ. Data used for comparison were those obtained using Klett-Summerson readings. The formula for calculation is as follows :

$$\text{Haemoglobin } A_2 = \frac{\text{Reading for Hb } A_2}{(\text{Reading for Hb A} \times 10) + \text{Reading for Hb } A_2} \times 100$$

RESULTS :

Values obtained on normal individuals (100 in all) were compared to those obtained for beta-thalassaemia trait (120 in all) and 20 subjects of iron deficiency. Table 1 shows mean values of Hb A_2 in normal, beta-thalassaemia trait and iron deficiency subjects using three different methods.

DISCUSSION :

It is seen from the Table I that the mean values for normal haemoglobin A_2 in normal subjects, using paper, cellulose accetate and starch block electrophoresis were 2.31 ±0.03, 2.04 ± 0.03 and 2.04 + 0.06 respectively, and the values are comparable. Percentage of haemoglobin A_2 in beta-thalassaemia trait showed a mean value of 4.82 ± 0.06 by paper; 4.39 ± 0.01 by cellulose acetate and 4.46 ± 0.04 by starch block electrophoresis, while values for iron deficiency cases are significantly lower by the three methods. This is of very great importance as superimposed iron deficiency state in beta-thalassaemia trait might mask the real level of Hb A_2 and this exhibits low values. There-fore estimation of Hb A_2 alone may not be sufficient for detecting beta-thalassaemia trait in a population when iron deficiency coexists.

In order to find out the reproducability of values by paper electrophoresis, six con-

secutive determinations of haemoglobin A_2 levels on the same samples were carried out, which gave a mean value of 2.06 (S.E. 0.02) in normal subjects and a mean value of 4.82 (S. E. 0.03) in beta-thalassaemia trait.

Estimation of haemoglobin A_2 by paper method described above is reproducible, simple in technique, accurate and inexpensive. It is also seen that normal and high values, by this method, are easily distinguishable.

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