Spectrophotometric Determination of Hb M_{Iwate} in the Hemolysate of Hereditary Nigremia*

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(Received January 31, 1963)

Hemoglobin M disease[1-9] has been reported to be a heterozygous condition with hemoglobin (Hb) A. The hemoglobin concentration and proportion of Hb M in the blood of patients with Hb M disease have not been measured with accuracy[2,8] because of the dark black color of this Hb exhibits peculiar absorption spectrum. Therefore, the orthodox hemoglobinometry for oxy-Hb and cyanmet-Hb forms[10,11] are inapplicable to the blood material of Hb M disease. Hb M_{Iwate} being no exception, we have recently devised a method for the determination of total hemoglobin concentration and proportion of abnormal hemoglobin in the hemolysate of Hb M_{Iwate} disease. The following principle on which this method is based may also apply to the determination of other Hb M variants.

The hemolysate containing Hb M is first electrophoresed to obtain a purified Hb M solution. The hemoglobin concentration of this solution is determined colorimetrically after changing hemoglobin to globin by Teale's method[12]. Then, same concentrations of Hb A and Hb M are subjected to absorption spectroscopy at the range of visible wave length. The absorption spectrums of the two are compared to determine the isobestic wave lengths. When they are known, the total hemoglobin is easily calculated with a fair degree of accuracy by measuring the absorbance of the hemolysate at these wave lengths. Calculation of the proportion of Hb M in a hemolysate is also possible with the same single specimen if the absorbance is measured at wave lengths where the absorbances of the two differ considerably.

This paper describes a spectrophorometric method for the total hemoglobin concentration and proportion of Hb M_{Iwate} in a single blood specimen with suggestion of its applicability to other Hb M variants.

Experimental

1) Principle: To determine the isobestic points, globin concentrations of Hb A

* This investigation was supported in part by a PHS research grant RG-9469 from the Division of General Medical Sciences, U. S. Public Health Service. This is a copy of the article which is to be published in Acta Haematologica Japonica.

and Hb M_{Iwate} solutions having an identical absorbance at 540 mμ (isotranslucent), are determined with the aid of biuret reagent. The theoretical absorption spectrum of Hb M_{Iwate} which has the same Hb concentration as that of Hb A is drawn from the original absorption spectrum of Hb M_{Iwate} solution and compared with that of Hb A.

The isobestic points which are defined as the intersections, or the points in common, of the two continuous absorption curves of equal concentration of the pigments, are obtained on the figure. At such isobestic wave lengths, equal concentrations of the two pigments have a same extinction coefficient. Hence, any given concentration of total Hb regardless of the proportion of Hb M_{Iwate} present, could be calculated by applying the absorbance of the hemolysate to the calibration curve which is made from Hb A solution.

When the absorbances (E_{iso}) of a hemolysate at an isobestic wave length is given, the proportion of Hb M_{Iwate} (r; $0 \leqq r \leqq 1$) is calculated by measuring again the absorbance (E_d) at another wave length where there is a wide difference in absorbance between Hb M_{Iwate} and Hb A.

The value of E_{iso} should be the sum of the values for the proportion of Hb M_{Iwate} ($E_{iso} \times r$) and of Hb A ($E_{iso} \times (1-r)$). Hence,

$$E_{iso} = E_{iso} \times r + E_{iso} \times (1 - r) \quad \cdots\cdots(1)$$

If the multiplication factors m and a are given from purified solutions for Hb M_{Iwate} and Hb A, respectively, for the conversion of absorbance at isobestic wave length to that at another wave length, E_d is expressed as

$$E_d = E_{iso} \times r \times m + E_{iso} \times (1 - r) \times a \quad \cdots\cdots(2)$$

Therefore, the proportion of Hb M_{Iwate} (r) is calculated by the following equation:

$$r = \frac{\frac{E_d}{E_{iso}} - a}{m - a} \quad \cdots\cdots(3)$$

2) Preparation of Hb M_{Iwate} and Hb A solutions and the determination of their ratios in an isotranslucent condition at 540 mμ: The hemolysate of hereditary nigremia was subjected to agar gel electrophoresis (in larger scale) at a neutral pH (pH; 7.0).[13,14] Purified Hb M_{Iwate} and Hb A solutions could be prepared by eluting each fraction from agar with the aid of partially wetted starch granules with a buffer (pH; 7.0).[14] Both hemoglobin solutions were adjusted to an isotranslucent state with optical density of 1.500 at 540 mμ using a Beckman spectrophotometer (cuvette, 1 cm optical path, effective band width, 1 mμ).

One milliliter of each hemoglobin solution was treated with Teale's technique[12]

to remove heme*. The amount of globin (μ for Hb M_{Iwate} and α for Hb A) was measured colorimetrically by redissolving the precipitated globin into 1.0 ml of 10 % NaOH and adding 2.0 ml of Gornall's reagent.[15] Both μ and α are to be proportional to their respective Hb concentrations. And since they come from isotranslucent, purified hemoglobin solutions with identical optical density of 1.500 at 540 mμ, the optical density quotient (q) of isotranslucent Hb M_{Iwate} solution to that of equal concentration of Hb A solution is

$$q = 1.500/\mu \div 1.500/\alpha = \alpha/\mu$$

When the q value for Hb M_{Iwate} was calculated, theoretical absorption spectrum of Hb M_{Iwate} having the same hemoglobin concentration as Hb A could be drawn from the original absorption spectrum by dividing the corresponding absorbance at each wave length by q.

Isobestic wave lengths were obtained from the intersection points of the two curves in the figure.

Results and discussions

Repeated determination of optical density quotient, q, gave the values of 1.24, 1.27, 1.25 with the average of 1.25. This value indicates that Hb M_{Iwate} has a lower optical density as compared with that of Hb A. This finding is important in the gross judgement of Hb M_{Iwate} solution, because it tends to be estimated higher due to its dark color.

There are two other methods for the determination of q.

1) Hb A and Hb M_{Iwate} solutions are treated with pyridine to produce pyridine-hemochrome pigment. The densities of the color are compared colorimetrically after extraction with ether,[16] 2) Solutions of Hb A and Hb M_{Iwate} are applied separately on a same sheet of filter paper which is stained with bromphenol blue. The blue spots on the paper are then eluted with a dilute alkaline solution and compared colorimetrically.[17] When studied, both of these methods proved to be unreliable because in the former the pigment had a tendency to lose color with time passage and, in the latter, results varied with the washing process especially when the globin content was low.

Absorption spectrums for equal concentrations of Hb M_{Iwate} and Hb A are shown in figure 1. The isobestic wave length were 457, 528, 554, 567 and 582 mμ. In figure 1, the point at which there is a large difference in absorbance between the

*1 Five per cent aqueous solution of potassium ferricyanide (0.01 ml) was added to hemoglobin solutions (1.0 ml) in Visking tubes, allowed to stand for 30 to 60 minutes. The Visking tubes were sealed tightly, and dialyzed overnight against running water to remove ferricyanide and buffer constituents. Teale's technique was applied after this preliminary treatment.

two curves is 615 mμ. At this wave length, the optical density of Hb A is practically zero, while a fairly high optical density of Hb M_{Iwate} is seen making an inflection curve.

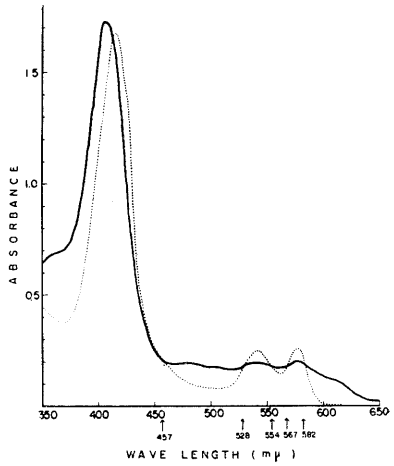

Figure 1. Absorption curve of the purified Hb M_{Iwate} (O_2 Hb type) solution in comparison with that of Hb A solution of the same concentration. Solid line: Hb M_{Iwate}. Broken line: Hb A. Arrows indicate the isobestic points.

The multiplication factors, a for Hb A, m for Hb M_{Iwate}, to convert the optical density at an isobestic wave length to that of 615 mμ (E_{615}) were calculated as in Table 1. The optical densities of the hemolysate from patients with Hb M_{Iwate} were measured at these isobestic points and 615 mμ, and the proportion of Hb M_{Iwate} in the hemolysate was readily calculated from equation 3 as in Table 2.

Table 1: The multiplication factors (E_{615}/E_{iso}) a for Hb A and m for Hb M_{Iwate}

wave length (mμ)	m	a
457	0.429	0.0250
528	0.518	0.0305
554	0.542	0.0326
567	0.503	0.0294
582	0.476	0.0270

Table 2: Optical densities and the proportions of Hb M_{Iwate} in a hemolysate

Wave length (mμ)	optical density	Hb M_{Iwate} (%)
457	0.327	30.2
528	0.268	29.7
554	0.261	29.7
567	0.281	29.7
582	0.305	29.1
615	0.048	—

The proportions of Hb M_{Iwate} thus calculated were very consistent, showing almost identical values at 528, 554 and 567 mμ. Since the two absorption curves

cross with steep slopes at the isobestic points of 465 and 582 mμ, the optical density will change appreciably even with a slight deviation from the true isobestic wave length in practical measurement. Therefore, slightly high or low percentages of Hb M_{Iwate} will result even if a narrow absorption band width was effected. The most reliable isobestic wave lengths for measurement were, therefore, thought to be 528, 554 and 567 mμ.

Hb M_{Iwate} content in two other patients with hereditary nigremia were measured by the aforementioned method and the values were 26.4 and 28.1 per cent. Hb mixtures with various proportions of Hb M_{Iwate} were prepared to determine the applicability of equation 3. The effectiveness of this equation was confirmed in the entire range of Hb M_{Iwate} proportion as seen from the closeness of the theoretical and observed values (Table 3).

Table 3: The proportion of Hb M_{Iwate} in various Hb mixtures

theoretical	determined
(%)	(%)
12.5	11.8
25.0	26.0
37.5	37.8
50.0	50.2

Several methods have been reported in the past for the determination of total Hb concentration and proportion of Hb M in Hb M disease.[2,8] However, they have a theoretical drawback in that absorption spectrums of cyanmet Hb and met-Hb forms of Hb M are different from those of Hb A.

The absorbance at 280 mμ which is thought to derive from the globin moiety was almost completely proportional to those at isobestic wave lengths for Hb A and Hb M_{Iwate}. This finding may, therefore, be applicable to all other Hb M variants in the determination of total Hb and proportion of Hb M.

Conclusion

A method has been devised to determine the total Hb and proportion of Hb M_{Iwate} spectrophotometrically.

In this technique, the hemolysate of hereditary nigremia is diluted with Tris-EDTA-Borate buffer of pH 7.0 to adjust the optical density at wave length 567 mμ around 0.5 and exact optical density at 567 and 615 mμ are measured.

1) Total Hb concentration: The optical density of the hemolysate at 567 mμ (isobestic wave length with Hb A) is converted to Hb concentration (g/dl) from the calibration curve which is prepared with Hb A beforehand. True hemoglobin concentration of the hemolysate is calculated after multiplying the sample Hb concentration by a dilution factor.

2) The proportion (r) of Hb M_{Iwate} is

$$r = \frac{E_{615}/E_{567} - 0.0294}{0.5030 - 0.0294} \times 100 \quad (\%)^{2*}$$

*2 For E_{iso}=567 mμ values of m and a are read from Table 1 as 0.5030 and 0.0294, respectively.

This is easily calculated by inserting the measured absorbances of E_{615} and E_{567} into this equation.

3) The percentages of Hb M_{Iwate} in the blood of three patients in two families were 29.7, 26.4 and 28.1 per cent.

4) Absorbance at 280 mμ is still useful for the determination of Hb concentration regardless of the proportion of Hb A and Hb M.

5) Possible application of this principle to the same purpose for other Hb M variants has been suggested.

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