SPECTROPHOTOMETRIC DETERMINATION OF MINUTE AMOUNTS OF HEMOGLOBIN IN HUMAN BLOOD SERUM

MUNEO OKABE AND SUSUMU SHIBATA

Department of Hygiene and Department of Clinical Pathology, Yamaguchi Medical School, Ube

(Received August 8, 1957)

In the course of our spectroscopic study of human blood serum[1] an urgent need of the estimation of a minute amount of oxyhemoglobin was felt. The sera separated from coagulated blood were frequently tinged with hemoglobin in spite of meticulous precautions taken in the withdrawal of blood. Exact appraisal of visual spectrum was impossible for the hemolysed sera, because even the least amount of hemoglobin caused a deformation of the absorption curve of the serum, particularly in the near-ultraviolet region.

A correction of absorbance for the contaminated hemoglobin was therefore necessary, and for this correction the hemoglobin dissolved in serum had to be determined exactly.

After unsuccessful trials with a reported mehod[2] an attempt was made to develop our own procedure. Assuming that oxyhemoglobin obeys Lambert-Beer's law,a theoretical equation was derived for the calculation of oxyhemoglobin concentration from the absorbances at 650, 600 and 575 mμ. Recovery tests were done by adding known minute amounts of oxyhemoglobin to non-hemolyzed sera.

The results of our experiment revealed that the conformity between the actually measured and the theoretically calculated values of hemoglobin concentration was not very exact. However, the difficulty was overcome by the construction of a scale which enabled the reading of real hemoglobin concentration from the calculated hemoglobin concentration. The procedure thus developed gave satisfactory results, and will be presented in this paper.

METHOD

The serum separated from clotted blood (approximately 4ml) by centrifugation (3000 RPM for 10 minutes) is diluted two-fold with an equal volume of

岡部宗雄, 柴田 進

Sφrensen's phosphate buffer solution (pH 7.4)[3]. The diluted serum is measured for the absorbances E_{650}, E_{600} and E_{575} at 650, 600 and 575 mμ, respectively, in a cell of 10.0 mm optical path with a Beckman spectrophotometer (Shimadzu QB 50), using Sφrensen's buffer solution (pH 7.4) as a blank. The aperture of the spectrophotometer is adjusted to 0.03 mm so that the effective band widths listed in Table I may be obtained. Tungsten lamp is used as light source. The theoretical hemoglobin concentration in the serum C_H (mg/dl) is calculated from the equation

$$C_H = \frac{E_{575} - 1.5E_{600} + 0.5E_{650}}{E_{h575}^*} \quad \cdots\cdots\cdots\cdots (1),$$

where E_{h575}^*[(4)] is the specific extinction coefficient (the absorbance of 1 mg/dl oxyhemoglobin solution in a cell of 10.0 mm optical path) of oxyhemoglobin at 575 mμ. Using the scale in Figure 1 the true hemoglobin concentration in diluted serum C_h (mg/dl) is read from C_H. The hemoglobin concentration of the original serum is then given as $2 \times C_h$ mg/dl.

TABLE I

Effective band width of measurement

wavelength (mμ)	effective band width (mμ)
650	1.29
600	1.05
575	0.93

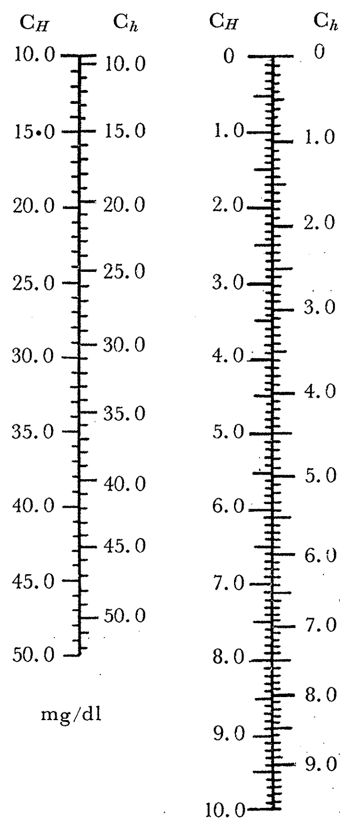

Fig. 1. The scale for reading C_h from C_H.

DISCUSSION

The visual spectrum of non-hemolyzed blood serum depends on its turbidity,

* In our measurement of the absorbances of oxyhemoglobin E_{h575} was 9.46×10^{-3}.

and its content of protein, bilirubin and carotenoids, which absorb the visual light. According to *Morton* and *Stubbs*[5] the absorbance of non-hemolyzed serum exhibits a linear relationship with the increase in wave length of the light within the range from 650 mμ to 575 mμ, whichever of these factors on which the absorbance is dependent may be predominant. In the hemolyzed serum the hemoglobin is present as oxygenated form, i. e. oxyhemoglobin, which gives rise to two peaks M and N at 575 mμ and 540 mμ, respectively, as shown in Figure 2. If the oxyhemoglobin in serum obeys *Lambert-Beer's* law[6], the magnitude of

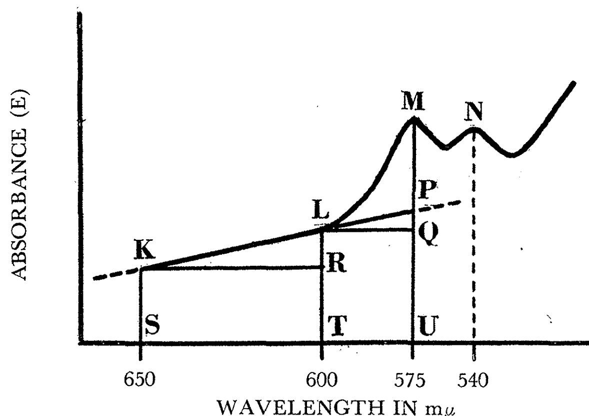

Fig. 2. The diagram for computation of C_H.
C_H: estimated amount of HbO_2 in hemolyzed serum (diluted twofold)
KLMU: measured absorption curve of hemolyzed serum.
KLP: normal absorption curve of serum (non-hemolyzed)
MP: $C_H E_{h575}$
PQ: $\frac{LQ.LR}{KR} = \frac{(600-575)(E_{600}-E_{650})}{650-600} = 0.5(E_{600}-E_{650})$
$QU = LT = E_{600}$

the protrusions M and N will mirror the concentration of hemoglobin in the serum. Since the light absorption of the serum without hemoglobin is supposed to form a straight line KLP within the range from650 mμ to 575 mμ, as *Morton* and *Stubbs*[5] have claimed, the magnitude of protrusion M at 575 mμ is represented by MP. The scrutiny of Figure 2 will lead to the following equation.

$$E_{575} = MU = MP + PQ + QU = C_H.E_{h575} + \frac{(600-575)}{(650-600)}(E_{600} - E_{650}) + E_{600}$$

$$\therefore \quad E_{575} = C_H E_{h575} + 1.5E_{600} - 0.5E_{650}$$

This will be transformed into

$$C_H = \frac{E_{575} - 1.5E_{600} + 0.5E_{650}}{E_{h575}}$$

which is identical with the theoretical equation (1) mentioned above. Recovery

test was carried out by adding known amounts of oxyhemoglobin to non-hemolyzed sera in order to check the reliability of equation (1). The oxyhemoglobin was prepared as follows:– About 4 ml of venous blood was withdrawn from a healthy person, introduced into oxalated tubes* A and B, 2 ml to each, and shaken to prevent coagulation. The blood in tube A was completely oxygenated with a tonometer to be measured (in duplicate) for its oxygen capacity (vol. %). Hemoglobin concentration (C_h mg/dl) was calculated from the average oxygen capacity multiplying it by 1000/1.36.**

The blood in tube B was transferred into a centrifuge tube, centrifuged (3000 RPM for 10 minutes) and the supernatant plasma thus obtained was carefully aspirated to get the packed cells without any loss of erythrocytes. 10 ml of physiological saline added to the packed cells, and the mixture was stirred. It was centrifuged (3000 RPM for 10 minutes) and the supernatant was discarded. The erythrocytes were washed five times repeatedly in a similar way before the supernatant (or washing solution) gave no longer positive protein reaction against sulfosalicylic acid. The packed erythrocytes were made up to the original volume of the blood with 0.1 g/dl sodium carbonate solution and mixed to get complete hemolysis. The hemolyzed solution had C_h mg/dl of hemoglobin. It was diluted N times with 0.1 g/dl sodium carbonate solution to prepare the oxyhemoglobin solutions of different concentrations. Its hemoglobin concentration was C_h/N mg per dl. An aliquot of 0.5 ml of the oxyhemoglobin solution was added to 1.5 ml of non-hemolyzed serum so that artificially hemolyzed sera of the hemoglobin concentration ranging from 0 to 50 mg/dl might be obtained. The sera were diluted two-fold with Sørensen's phosphate buffer (pH 7.4). Their E_{650}, E_{600} and E_{575} were measured with a Beckman's spectrophotometer to get the theoretical hemoglobin concentration C_H of the sera. The result of the recovery test is illustrated in Figure 3, in which C_H represents the hemoglobin concentration of the oxyhemoglobin added sera calculated by equation (1), while C_h refers to the true or known oxyhemoglobin concentration of the sera. The correlation of C_H to C_h appears at first glance to make a straight line which passes through the origin of the coordinates with an inclination of 45°. It is therefore thought that the assumption made for the derivation of equation (1) was correct. However, the scrutiny reveals that C_H-C_h line is not exactly straight. It forms a delicately curved line which changes gradually its inclination

* Oxalated tubes contain an amount of Heller-Paul's ammonium oxalate potassium oxalate mixture which is sufficient to prevent coagulation of about 2ml of blood.

** Hematocrit (Ht) was determined by Wintrobe's method[7]. The hemoglobin concentration C_h was checked by calculating the mean corpscular hemoglobin concentration (MCC, %)

$$MCC = \frac{100\ C_h}{Ht \times 1000} = \frac{C_h}{10Ht}$$

which varies from 32 to 36 in the normal person.

from 47.5° to 43° as it diverges from the origin. The scale in Figure 1 is a clearer demonstration of the relation of C_H to C_h. In this figure the discrepancy between the calculated and the true hemoglobin concentration is apparent.

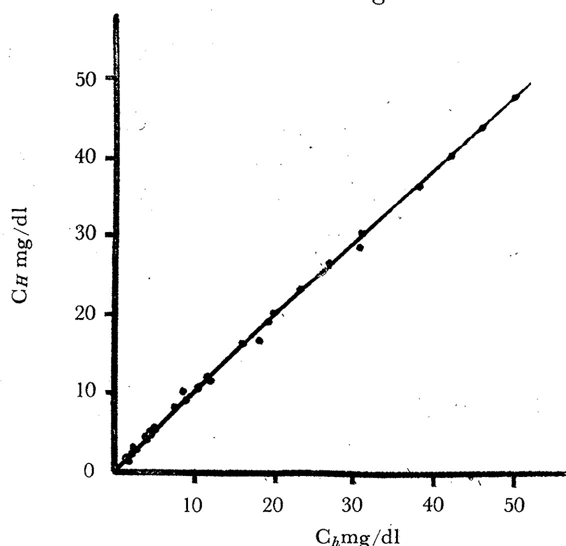

Fig. 3. The correlation betwen C_H and C_h.
C_H: estimated amount of HbO_2 in hemolyzed serum (diluted twofold, mg/dl)
C_h: real amount of HbO_2 in hemolyzed serum (diluted twofold, mg/dl)

The validity of the assumption was accordingly not absolute.

The cause of the discrepancy is entirely unknown, but the scale in Figure 1 will enable us to read the real amount of hemoglobin in hemolyzed serum (C_h) from the hemoglobin concentration obtaineb by the measurement of the absorbances E_{650}, E_{600} and E_{575} (C_H), because the scale is based on the recovery experiment.

Inasmuch as, in this procedure, the serum is diluted with an equal volume of phoshate buffer before it is subjected to spectrophotometry, the C_h read from the scale have to be doubled in order to obtain the hemoglobin concentration of the orginal serum.

Recently, a spectrophotometric method for the estimation of oxyhemoglobin and bilirubin in plasma was developed dy *Shinowara*[2]. In his procedure the plasma was diluted with phosphate buffer and the absorbances at 575, 560 and 450 mμ were measured to get the oxyhemoglobin concentration from the theoretically derived equation.

Our method is different from the Shinowara's in the principle. The equation (1) in our procedure was derived from the idea that turbidity is more responsible than bilirubin for the absorbance of serum at 575 mμ. The absorbances at 650 and 600 mμ represent the degree of serum turbidity.

SUMMARY AND CONCLUSION

A spectrophotometric determination of minute amounts of oxyhemoglobin (less than 50 mg/dl) in serum has been presented. The procedure is summarized as follows: After diluting with an equal volume of Sϕrensen's phosphate buffer solution (pH 7.4), the serum is subjected to spectrophotometry for the mesurement of absorbances at 650, 600 and 575 mμ. From the determined absorbances C_H is calculated using an equation (1) which was derived with the assumption that *Lambert-Beer*'s law and *Morton-Stubb*'s principle are valid for the serum contaminated with hemoglobin. C_H is converted into C_h by a C_H–C_h scale which is shown in Figure 1. The oxyhemoglobin concentration of the serum is given as $2 \times C_h$ mg/dl.

Grateful acknowledgements are due to Dr. Goro Kawabata (Department of Physiology) and Dr. Wataru Mizuta (Department of Clinical Pathology) for carrying out the measurement of oxygen capacity of the blood.

REFERENCES

1) OKABE, M.: Spectrochemical Studies of Sera in Various Human Conditions. Report. 1. The Absorption Spectrum of Normal Human Sera in Visual Spectral Region. *Annual Report of the Research Institute of Industrial Medicine,* Yamaguchi Med. Sch., **4**: 63–65, 1956.

2) SHINOWARA. G. Y.: Spectrophotometric Studies on Blood Serum and Plasma. *A. J. Clin. Path.* **24**: 496–507, 1954.

3) YOSHIMURA, H.: *Theory and Practice for pH Measurement.* Maruzen (Tokyo), 1942.

4) OKABE,M. and SHIBATA, S.: Specific Extinction Coefficient of Human Oxyhemoglobin in the Visual Spectral Region. *Medicine and Biology.*, **39**: 62–66, 1956.

5) YAGI, K.: *The Newest Method for Vitamin Measurement.* Ishiyaku-Shuppan Co. (Tokyo), 1954. (cited in p. 146–149)

6) HEILMEYER. L.: *Spectrophotometry in Medicine,* translated by A. Jordan & tippeell, T. L. Adam Hilger (London), 1943.

7) CARTWRIGHT, G. E.: *Diagnostic Laboratory Hematology*, Grune & Stratton (New York, London), 1954.