ARTICLE:

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THE ABSORPTION SPECTRA OF HEMOGLOBIN AND ITS DERIVATIVES IN THE VISIBLE AND NEAR INFRA-RED REGIONS

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Since the pioneering investigations of Vierordt and of Hüfner and his group, numerous reports on the absorption spectra of hemoglobin and its derivatives have appeared in the literature. A detailed summary of these reports is to be found in Heilmeyer's monograph (1). For the most part these investigations have been confined to the visible portion of the spectrum, although in a few cases the observations were extended into the ultraviolet. The near infra-red, however, has been singularly neglected, despite the fact that as early as 1914 Hartridge and Hill (2) published some qualitative results indicating the presence of an interesting oxyhemoglobin band in this region.

In this laboratory, it became necessary to develop a rapid and accurate spectroscopic method for the estimation of carbon monoxide in blood which could be adapted to a simple portable instrument. The visible portion of the spectrum proved unsuitable for this purpose, since the character of the oxyhemoglobin and carbonylhemoglobin bands in this region require the use of narrower spectral regions than can conveniently be isolated in such an instrument. The infra-red spectra were investigated in the hope that more suitable absorption bands might be found.

The spectrophotometric data for the various hemoglobin derivatives which may be found in the literature are characterized by discrepancies with respect to the absolute values of the absorption coefficients, depending upon the hemoglobin preparations examined, the analytical methods used, and the dispersing power of the spectrophotometers employed by the various investigators. Since the precision of any spectrophotometric method of analysis depends upon the accuracy with which the corresponding absorption coefficients are known, a redetermination of these coefficients was undertaken. The objectives of this investigation were to determine as accurately as possible the absorption coefficients of various hemoglobin derivatives in whole hemolyzed human blood and to compare these with constants obtained with pure hemoglobin, in order that the contribution of hemoglobin to the absorption of whole blood might be evaluated. A similar study was made of the spectra of these substances in the near infra-

red region from 7000 to 10,000 Å. The new absorption bands found in this region promise to be extremely useful for analytical work.

According to the Lambert-Beer Law, the specific absorption coefficients of an absorbing material may be defined by the equation,

$$\text{Log}_{10} \frac{I_0}{I} = \alpha c l$$

where I_0 = intensity of incident light
I = " " transmitted light
l = length of light path through solution
c = concentration of absorbing material
α = specific absorption coefficient

This relationship is valid only when monochromatic light is used and provided that the material investigated contains no absorbing impurities. With instruments available at the present time, sufficiently narrow wavelength intervals can be isolated to permit the determination of accurate absorption coefficients. Much greater difficulty has been encountered in preparing pure hemoglobin and in establishing adequate criteria for its purity. The earlier workers (3–7) employed crystalline hemoglobin prepared by alcohol precipitation or other means, and estimated the concentration from the dry weight of the crystals. It is now generally recognized, however, that crystallinity in the case of proteins is no assurance of homogeneity (8); other criteria for purity must be applied.

With the development of precise gasometric methods by Van Slyke and his coworkers, it became possible to determine accurately the concentration of hemoglobin in solution and in whole blood. Using this method of analysis, Newcomer (9) and, more recently, Kennedy (10) determined the absorption of oxyhemoglobin and carbonylhemoglobin in hemolyzed human and dog blood. In each case, however, only the concentration of active hemoglobin (HbO_2) was measured; the contribution of other blood components, including other hemoglobin derivatives, to the total light absorption was not evaluated. The same is true, to a lesser extent, of the work of Drabkin and Austin (11), although these workers demonstrated that absorption constants obtained from washed, hemolyzed erythrocytes are highly reproducible. The effective slit width obtained with the instruments used by Kennedy and by Drabkin and Austin was about 30 Å. in the green and 50 Å. in the yellow. Measurable differences between the absorption curves of these authors and the present writer may be attributable to the fact that in the present paper narrower slits are used, 7 to 12.5 Å., in the visible spectrum. The narrower slit yields the more precisely defined absorption constants.

The first important measurements in the infra-red were made by Merkelbach (12) in 1935. He found oxyhemoglobin to have a broad absorption band with a maximum at about 9100 Å., while carbonylhemoglobin had

practically no absorption in the infra-red. A small portion of the infra-red spectrum has also been described by Sidwell, Munch, Barron, and Hogness (13). Although they report a band for reduced hemoglobin at 7550 Å., their observations did not extend beyond 7700 Å.; thus they failed to observe the oxyhemoglobin band. Carbonylhemoglobin was not examined.

EXPERIMENTAL

Preparation of Purified Hemoglobin—Purified hemoglobin was prepared from calf blood by the method of Altschul, Sidwell, and Hogness (14), involving treatment with aluminum hydroxide gel. This method was selected because hemoglobin solutions so prepared showed, at low O_2 tensions, a higher affinity for oxygen than did any other preparations, including hemoglobin crystallized by the method of Heidelberger. The percentage saturation of hemoglobin with oxygen at low O_2 tension was used by these investigators as a criterion of purity, in accordance with their finding that impurities lowered the percentage saturation.

From 500 cc. of whole calf blood about 300 cc. of a clear red solution are obtained, having about one-half the hemoglobin content of the original blood.

Determination of Purity—The concentration of active hemoglobin was determined on 2.0 cc. samples by the carbon monoxide capacity method of Van Slyke and Hiller (15). Some preparations were also analyzed for methemoglobin by determination of the carbon monoxide capacity after reduction with sodium hydrosulfite. Since the values for total hemoglobin obtained in this way always agreed closely with values calculated from the dry weight of the preparations, this determination was found to be unnecessary. Dry weight determinations were made by evaporating aliquots of the solution to constant weight at 100–105°. From the dry weight the total hemoglobin concentration was calculated, with 66,800 as the molecular weight. This value, calculated by Svedberg and Fahraeus (16) from sedimentation measurements, agrees well with 67,000 calculated by Adair (17) from osmotic pressure data and 66,000 calculated by Morrison and Hisey (18) from the iron content and gas capacity. The following is a typical analysis of a hemoglobin solution purified by the above method. The assumption is made that hemoglobin has four iron-containing groups and that the equivalent weight is one-fourth the molecular weight.

$$\text{Dry weight} = 83.9 \text{ mg. per cc.} = 5.02 \times 10^{-6} \frac{\text{equivalents } HbO_2}{\text{cc.}}$$

$$HbO_2 \text{ concentration from CO capacity} = 4.99 \times 10^{-6} \frac{\text{equivalents}}{\text{cc.}}$$

$$\text{Total Hb concentration from CO capacity} = 5.03 \times 10^{-6} \frac{\text{equivalents}}{\text{cc.}}$$

The sample thus contains only 0.6 per cent of methemoglobin and no other impurities.

The term equivalent is here used to indicate the amount of hemoglobin which contains 1 gm. atom of Fe and combines with 1 gm. molecule of O_2 or CO. 1 equivalent of hemoglobin is assumed to be 66,800/4, or 16,700 gm. A concentration of 1×10^{-6} equivalent is 16.7 mg. of hemoglobin per cc.

Stability of Pure Hemoglobin Preparations—The hemoglobin solutions obtained could be stored in the refrigerator for over a month with no apparent decrease in their carbon monoxide-combining power. The absorption spectrum in the visible region of the spectrum also remained unchanged. After a week or two, however, the preparations began to show evidence of an absorption band at 8200 Å. which was absent in the fresh preparations, and which increased in intensity with the age of the preparation. The position of this band would indicate that the preparations were becoming contaminated with methemoglobin, despite the fact that the Van Slyke analysis showed no decrease in the active hemoglobin content. In practice, preparations were discarded at the first appearance of this band.

Preparation of Solutions—The absorption spectra of oxyhemoglobin (HbO_2) and carbonylhemoglobin (HbCO) were determined with pure calf hemoglobin. For the visible spectrum the stock solution was diluted 1:50. For the infra-red spectrum the stock solution was diluted 1:2. All measurements were made in a cell of length 0.500 cm. The dilutions were made in borate buffer of pH 9.2 to a final buffer concentration of 0.1 M. Carbonylhemoglobin was prepared by equilibrating the diluted solutions in a rotating tonometer through which pure carbon monoxide was passed for 20 to 30 minutes. The absorption cell was then filled with carbon monoxide gas and the solutions transferred directly from the tonometer to the cell without exposure to air.

From hemolyzed human blood, solutions were prepared for the determination of the spectra of reduced hemoglobin (Hb), alkaline and acid methemoglobin (MHb), and metcyanhemoglobin (MHbCN), as well as HbO_2 and HbCO. The hemoglobin concentration was determined on the whole unhemolyzed blood samples by the O_2 capacity method of Sendroy ((15) p. 338). For the determination of the infra-red spectra of HbO_2, HbCO, and IIb, the blood was diluted 1:5 with saponin and borate buffer, pH 9.2, to a final saponin concentration of 0.3 per cent and a final buffer concentration of 0.02 M. A portion of this solution was saturated with CO as described above. A second portion was washed with pure N_2 in a rotating tonometer until the violet color of reduced hemoglobin was produced. The solution

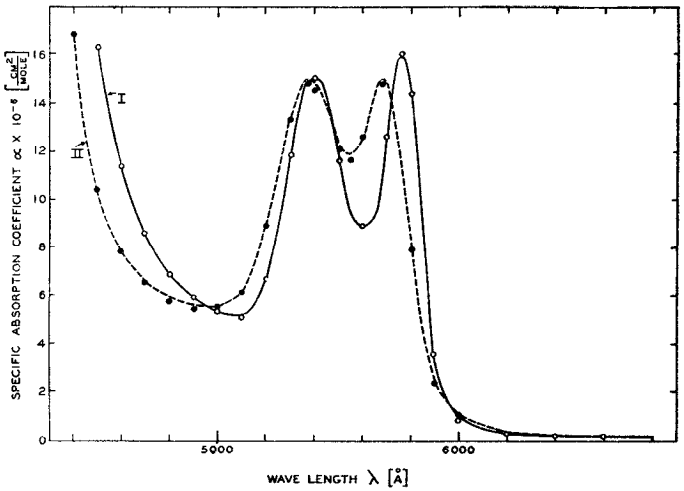

FIG. 1. Absorption spectra of HbO_2 and HbCO in the visible region. Curves I and II represent absorption constants obtained from pure calf hemoglobin for HbO_2 and HbCO, respectively. ○ and ● represent constants for HbO_2 and HbCO, respectively in hemolyzed human blood.

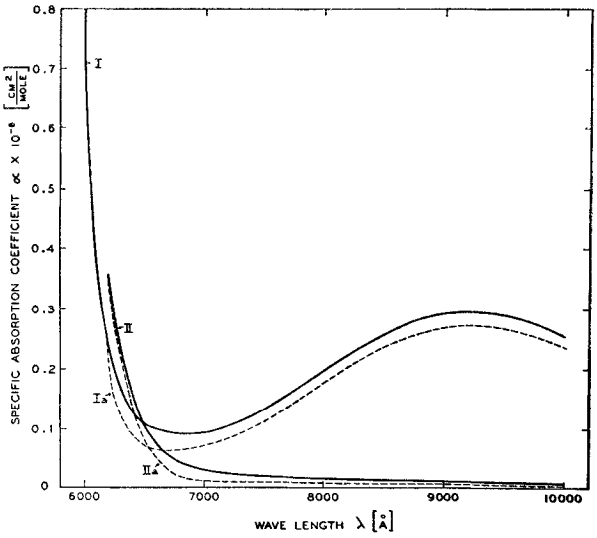

FIG. 2. Absorption spectra of HbO_2 and HbCO in the infra-red region. Curves I and Ia represent HbO_2 in hemolyzed human blood and pure calf hemoglobin, respectively. Curves II and IIa represent HbCO in hemolyzed human blood and pure calf hemoglobin, respectively.

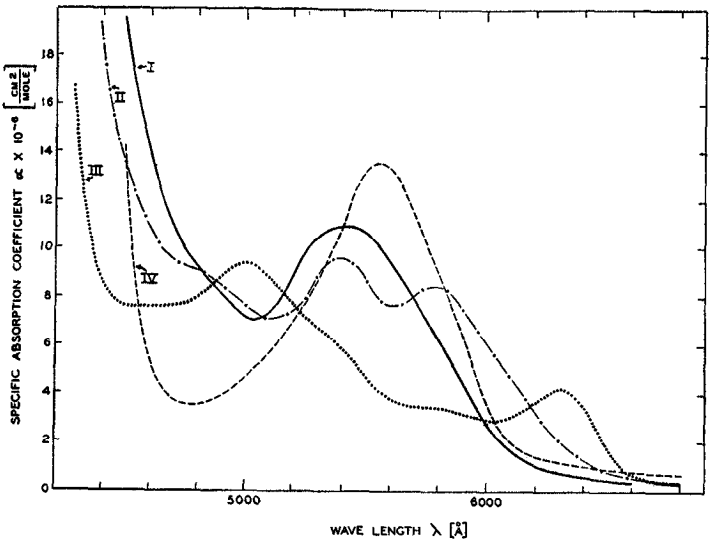

FIG. 3. Absorption spectra of Hb, MHb, and MHbCN in hemolyzed human blood in the visible region. Curve I represents MHbCN; Curve II, MHb at pH 9.18 to 9.20; Curve III, MHb at pH 6.29 to 6.51; Curve IV, Hb.

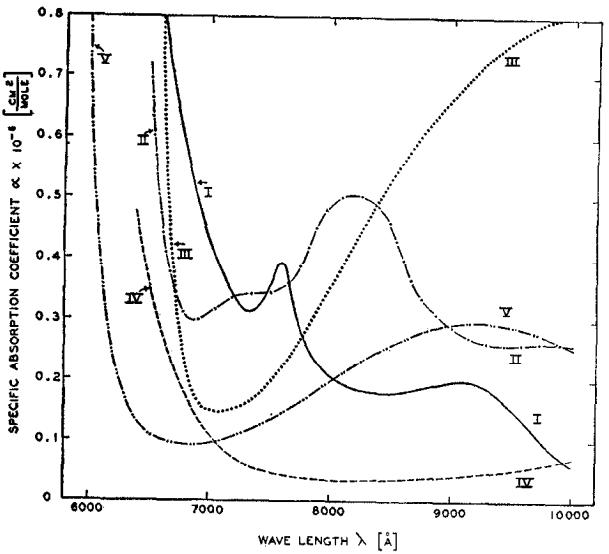

FIG. 4. Absorption spectra of Hb, MHb, and MHbCN in hemolyzed human blood in the infra-red region. Curve I represents Hb; Curve II, MHb at pH 8.88 to 8.92; Curve III, MHb at pH 6.30 to 6.72; Curve IV, MHbCN; Curve V, HbO_2.

was then transferred directly from the tonometer into an absorption cell filled with pure N_2 and containing a trace of dry $Na_2S_2O_4$ to insure complete reduction. For the visible spectra the blood diluted 1:5 was further diluted with 24 volumes of 0.01 M borate buffer to a final dilution of 1:125. From this solution, HbCO and Hb were prepared as above.

For the preparation of acid and alkaline methemoglobin, blood was hemolyzed with saponin and treated with a 6-fold excess of $K_3Fe(CN)_6$. The mixture was then buffered with either phosphate or borate buffer and diluted with water to 5 times the volume of the original blood sample. The saponin concentration was then 0.3 per cent and the buffer concentration was 0.05 M. For the visible spectra 1 cc. of each of these solutions was further diluted to 25 cc. with 0.02 M buffer. The pH of each solution was measured with the glass electrode and is indicated in Table I. From the borate-buffered solutions MHbCN was prepared by the addition of a small amount of solid KCN.

Visible and Infra-Red Absorption Spectra—The absorption measurements were made on an automatic recording spectrophotometer constructed in this laboratory by F. S. Brackett and J. B. H. Kuper. This instrument is essentially similar in construction to the one described by them in 1940 (19), but extending into the infra-red to 10,000 Å. The effective slit widths at various wave-lengths are as follows:

Wave-length, Å.....	4000	5000	6000	7000	8000	9000	10,000
Slit width, Å.....	7	7	7	12.5	25	70	140

The values of incident and transmitted light intensity were read from curves obtained on photographic paper. The concentration of hemoglobin was determined as described above and the values of the specific absorption coefficients calculated from the relation

$$\alpha = \frac{\log_{10} \frac{I_0}{I}}{cl}$$

c is given in equivalents per cc. and l in cm. The units of α are then sq. cm. per equivalent.

The absorption spectra are plotted in Figs. 1 to 4.

In each instance, the constants are the average values obtained from at least two hemoglobin preparations or blood samples. In Table I are summarized the values of the absorption constants at the maxima and minima, showing the spread of the determinations.

TABLE I

Absorption Constants for Hemoglobin Derivatives

Source	Substance	No. of samples	Wavelength	Specific absorption constants, $\alpha \times 10^{-6}\left(\frac{\text{sq. cm.}}{\text{mole}}\right)$		
				Average	High	Low
			Å.			
Pure calf hemoglobin	HbO_2	3	5100	5.19	5.28	5.11
			5400	15.0	15.1	14.8
			5600	8.88	9.06	8.76
			5765	15.9	16.0	15.8
		3	6800	0.064	0.069	0.061
			9200	0.274	0.279	0.268
" " "	HbCO	4	5000	5.50	5.54	5.46
			5375	15.0	15.2	14.9
			5550	11.9	12.0	11.8
			5680	15.0	15.2	14.9
		3	9200	0.004	0.005	0.004
Hemolyzed human blood	HbO_2	4	5100	5.07	5.12	5.00
			5400	15.0	15.2	14.8
			5600	8.87	9.03	8.72
			5765	16.0	16.3	15.8
		4	7000	0.093	0.097	0.089
			9200	0.296	0.299	0.292
" " "	HbCO	4	5000	5.44	5.34	5.56
			5375	14.8	15.0	14.6
			5550	11.7	11.9	11.5
			5680	14.8	15.1	14.6
		4	9200	0.010	0.011	0.010
" " "	Hb	2	4800	3.50	3.52	3.49
			5550	13.6	13.8	13.5
		3	7310	0.310	0.336	0.302
			7600	0.395	0.417	0.376
			8400	0.179	0.190	0.169
			9000	0.198	0.206	0.190
" " "	MHbCN	3	5040	7.03	7.11	6.95
			5400	11.0	11.4	10.8
		3	8000	0.033	0.036	0.039
" " "	MHb	3*	5100	7.18	7.32	7.06
			5400	9.68	9.78	9.50
			5600	7.68	7.76	7.56
			5770	8.51	8.61	8.37
		2†	6850	0.297	0.306	0.288
			7200	0.336	0.341	0.331
			8175	0.525	0.526	0.524
			9400	0.259	0.263	0.255
" " "	MHb	3‡	4700	7.72	7.93	7.45
			5000	9.47	9.65	9.23
			6000	3.01	3.09	2.88
			6200	3.68	3.75	3.58
		3§	7000	0.151	0.158	0.139
			9800	0.794	0.804	0.788

TABLE I—*Concluded*

* pH = 9.18, 9.20, 9.12.
† pH = 8.88, 8.92.
‡ pH = 6.29, 6.32, 6.51.
§ pH = 6.72, 6.30, 6.44.

DISCUSSION

It is shown in Fig. 1 that there is no perceptible difference in the visible region between the absorption spectra obtained from hemolyzed human blood and pure calf hemoglobin. It may therefore be concluded that other blood constituents make a negligible contribution to the light absorption of hemolyzed blood in this region. The results also bear out the previous findings of Drabkin and Austin (11) and others with regard to the spectroscopic identity of the hemoglobins of various mammalian species.

In the infra-red region, where the absorption of hemoglobin is much less intense, whole blood absorbs appreciably more than does pure hemoglobin (see Fig. 2). This difference may be attributed to the absorption of light by other blood constituents and to scattering of light by suspended material such as lipids and cell fragments. At the high dilutions used for the visible measurements the contribution of these materials is negligible. It is noteworthy that no special precautions were taken in the collection of the blood samples. The individuals were not required to fast before the venipunctures were made, the only limitation being that no samples were taken for several hours after lunch.

The broad absorption band of oxyhemoglobin in the infra-red, together with the almost complete lack of absorption by carbonylhemoglobin, makes this region ideally suited for the determination of the CO content of blood. In any spectral interval beyond about 7500 Å., the presence of HbCO will produce a marked decrease in the total absorption. The total hemoglobin concentration may be determined, independent of the presence or absence of CO, in the neighborhood of the isobestic point at 4965 Å. The concentration of HbO_2 may be calculated from the infra-red absorption and the concentration of CO computed by subtracting HbO_2 from total hemoglobin.

In order to eliminate the effect of reduced hemoglobin, which will usually be present in the samples diluted 1:5 if blood is collected by venipuncture, the spectral region in the infra-red may be so selected that the oxyhemoglobin and reduced hemoglobin have the same absorption. An examination of Fig. 4 will show that the isobestic point for these substances lies at about 8000 Å.

The two measurements described above are sufficient if only two hemoglobin derivatives, HbO_2 and HbCO, are present. For this purpose Hb and HbO_2 may be considered identical, since they will absorb alike in the infra-red and Hb will be converted to HbO_2 at the high dilutions required

for the determination at 4965 Å. If, however, a third component, such as MHb, is present, a third measurement is necessary for the evaluation of the HbCO, HbO_2, and MHb concentrations. The required data may be obtained from the absorption at a third spectral interval, such as that around 6400 Å., or by repeating the absorption measurement at 8000 Å. after the sample is saturated with CO.

In this laboratory we have developed a portable photoelectric instrument for the determination of carbon monoxide in blood which is based on the principles outlined above. With this device, the carbon monoxide content of human blood can be rapidly and conveniently determined with an error of less than 1 per cent HbCO. The details of construction and operation of this instrument will be described in a subsequent publication.

SUMMARY

The visible and infra-red absorption spectra of oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin, methemoglobin, and metcyanhemoglobin have been determined. Several new bands in the infra-red are described.

The absorption spectra of oxyhemoglobin and carbonylhemoglobin in hemolyzed human blood are identical with those obtained from pure calf hemoglobin in the visible region of the spectrum. In the infra-red the whole hemolyzed blood has a somewhat higher absorption.

A method of evaluating the absolute purity of purified hemoglobin preparations is described.

A simple spectrophotometric method for determining the CO and methemoglobin contents of blood is indicated.

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