THE EFFECT OF VARYING CONCENTRATIONS OF OXYHEMOGLOBIN ON ITS LIGHT ABSORPTION

BY G. B. RAY AND H. A. BLAIR

(From the Departments of Physiology, School of Medicine, Western Reserve University, Cleveland, and the Long Island College of Medicine, Brooklyn)

(Received for publication, June 20, 1935)

The validity of any method for determining the concentration of a colored substance by measuring its absorption of monochromatic light depends upon the ability of that substance to obey certain physical laws. In our discussion of the spectrophotometric determination of oxyhemoglobin and certain of its derivatives (Ray, Blair, and Thomas, 1932) it was assumed that these substances obeyed such fundamental laws, since the relationships required for the determination had never been questioned, although the method had been in use for over 40 years. The particular laws essential in this determination are the laws of Lambert and of Beer. Lambert's law states that light is absorbed by a homogeneous medium in such a way that absorption is directly proportional to the intensity of the light at any point under consideration. Thus,

\[ I = I_0e^{-kn} = I_010^{-En} \]

where \( I_0 \) is the intensity of the incident light and \( I \) the intensity after penetration of a medium the thickness of which equals \( n \). This relationship may be written as

\[ E = -\frac{1}{n} \log_{10} T \]

where the quantity \( E \) is the extinction coefficient and \( T \) the fraction of the impinging light emerging from the solution. In the case of solutions, it is convenient to use the extinction coefficient per unit concentration, since the light absorption varies directly with concentration as well as with the light intensity. This fact
is expressed by Beer's law which may be stated as follows: $E = \alpha C$, or, the extinction coefficient per unit length varies as the concentration. Therefore, for any given compound, $E = C/A$ where $A$ is a constant which equals the number of gm. per cc. of pigment necessary to absorb 90 per cent of the impinging light. $A$ is usually derived from the reciprocal of the slope of a line representing the relationship of $C$ to $E$. It is obvious, therefore, that if the relationship of $C$ to $E$ is not constant, the method cannot be considered reliable except for a restricted concentration. In the case of many colored solutions it is known that Beer's law is not obeyed, e.g. hemocyanin (Svedberg and Heyroth, 1929, a). This is equivalent to the statement that in certain cases each dissolved molecule does not absorb light in the same manner at all concentrations, or that these molecules do not retain the same form upon dilution or concentration. The importance of this relationship for the spectrophotometric determination of oxyhemoglobin was first recognized by Hüfner (1877-78) in the course of his development of the spectrophotometer as an instrument for the study of blood pigments. Shortly following Hüfner's original observations von Noorden (1880), working under Hüfner's direction, noted that absorption increased upon dilution. Later Hüfner (1893), using more precise apparatus, showed the law to be valid within the probable error of measurement at the wavelengths ordinarily used in the spectrophotometric analysis of hemoglobin.

Hüfner's observations were made on both oxyhemoglobin and carboxyhemoglobin but extended over a rather restricted range of concentrations, namely 0.8 to 1.7 gm. per cent in the case of oxyhemoglobin and from 0.8 to 2.0 gm. per cent in the case of carboxyhemoglobin. While these concentrations are those one ordinarily uses in spectrophotometric measurements, it is not infrequent for one to use much more dilute blood especially when the supply is limited. Criticism has also been advanced against Hüfner's spectrophotometric work by Kennedy (1927), whose results indicate that Hüfner's apparatus must have had a relatively wide entrance slit and consequently a poor resolution. Hence the measurements were made over a wide band at a point where the curve indicates absorption with respect to wave-length is changing rapidly. The results of this condition have been
discussed in our previous paper. Later work carried out by Butterfield (1912), unfortunately upon the Hüfner type of instrument, shows a similar error as indicated by the fact that the value of $E_{540}/E_{560} = 1.58$, while the majority of recent observers using narrow slits have found this ratio to be about 1.62. It is, therefore, obviously desirable that if the spectrophotometer is to be used as a method of hemoglobin determination, the obedience of this pigment to Beer's law must be demonstrated over as wide a range as possible. The present paper deals with such a study of Beer's law and hemoglobin carried out over a maximal range at wave-lengths ordinarily used, i.e. 540 μμ and 560 μμ and also over a wave-length permitting much higher concentrations (650 μμ).

**Methods**

The observations in this series of determinations were made upon two types of instruments. The first experiments, chiefly concerned with dog hemoglobin, were made upon the Keuffel and Esser color analyzer (Keuffel, 1925). Later experiments which were extended to ox, sheep, and human oxyhemoglobin, were made upon the Bausch and Lomb spectrophotometer. In all cases the apparatus was so adjusted as to give a maximal definition as suggested by Kennedy (1927). The hemoglobin was separated and purified according to the method described by Adair and coworkers (1921). It was either dialyzed against distilled water or a buffer solution. In the case of dog hemoglobin, when dialyzed against distilled water, the concentration of the hemoglobin was sufficiently high to produce a massive precipitation when the process had removed most of the salts. This precipitate was filtered off and dissolved in a buffer solution. In other experiments the pigment was brought into equilibrium with the buffer solution during the process of dialyzing. Every effort was made to keep the inactivation of hemoglobin as low as possible and in no case was a solution of hemoglobin used when the degree of inactivation (calculated as methemoglobin) was over 5 per cent.

**Results**

Fig. 1 represents an extension of the original Hüfner experiment on oxyhemoglobin over the widest range of concentrations pos-
sible with the apparatus available. This extends from a concentration where the transmission was only 6 per cent to a degree of dilution where 100 cc. of the solution appeared but faintly straw-colored. The actual concentrations varied from 0.002 to 0.2 mM (0.013 to 1.3 gm. per cent). In all cases the mM concentration has been calculated on the basis of Adair's (1925)
figure for the molecular weight of hemoglobin, i.e. 66,800. The absorption is reduced to a solution thickness of 1 cm. The hydrogen ion concentration of the solutions used varied from pH 6.5 to 11.0. It is evident from this chart that Beer’s law is obeyed and as previously noted by Kennedy (1927) is independent of hydrogen ion concentration over the range studied. It is interesting to note also that the value of $A$ at 540 μμ agrees definitely with the value used in our previous paper. We find the reciprocal of the slope of this line to be 0.001104, agreeing very closely with the figure 0.0011 given by Davis and Sheard (1927). The ratio of the slope of the line for wave-length 540 μμ to that of 560 μμ is 1.62, again an excellent agreement with our previously determined figure of 1.619. These experiments represent observations made well beyond the normal concentrations dealt with when normal bloods that have been diluted from 100 to 500 times are used. The maximal concentration, none the less, is very low. It appeared to us to be of considerable interest to extend the range by making observations at some wave-length where absorption is very low, thus permitting the use of high concentrations. Such observations carry a particular significance, since Adair (1929) has shown that variations occur in the neighborhood of 4 gm. per cent. If these changes are the result of molecular alterations incidental to dilution or concentration, additional evidence should be supplied by means of spectrophotometric observations. In order to obtain this high transmission we arbitrarily made readings at 650 μμ. Fig. 2, Curves A and A’, presents the results obtained at this wave-length on a series of hemoglobin solutions varying in hydrogen ion concentration from pH 6.5 to 11.0. These readings indicated a marked variation in each individual curve but for the purpose of clarity in the present discussion the experimental points for any single sample of hemoglobin have been multiplied by a constant in order that the results may be presented as a single line and the analysis of the individual variations has been left for the future. This line shows a very interesting characteristic. It will be noted that in the neighborhood of 0.6 mm the observed Curve A’ diverges from Curve A, predicted from Beer’s law, in a very definite manner. The point of divergence is of particular interest since 0.6 mm corresponds to 4 gm. per cent. Curves B and B’ have been
Fig. 2. Variation in absorption with concentration at 650 μm compared with the variation in activity with concentration (from Adair (1929)).
plotted from Adair's results (1929) which indicate an increase in activity (Curve B') from the predicted activity (Curve B) based upon readings made below 4 per cent. The point of divergence, it will be seen, occurs at the same concentration as in the spectrophotometric results. This similarity is striking.

The phenomenon of a decrease from the predicted in the case of light absorption by a molecule may mean an intramolecular change as a result of which certain bindings, of an energy corresponding to the energy of the radiation absorbed at this wavelength, are weakened as mentioned above or it may be that certain bindings entirely disappear as a result of a reversible molecular disintegration. This latter concept has been demonstrated in the case of hemocyanin by Svedberg and Heyroth (1929, a) who noted that the hemocyanin from Limulus polyphemus undergoes a breakdown with changes in concentration and in consequence does not obey Beer's law. These same authors (1929, b) have also shown that the pigment from Helix shows changes in viscosity and diffusion with a change in concentration of such a nature as to indicate an increasing number of particles in solution. The increased activity of hemoglobin beyond that predicted, as shown by Adair, is so definitely associated with a loss in light absorption, that it seems indicative that we are dealing in this case with results similar to those obtained by Svedberg and Heyroth. In any event, through our choice of 650 μμ as the wavelength at which to study absorption, we found a radiant energy which indicates an alteration in the hemoglobin molecules when the solution is concentrated. At the present state of our experimental work it is quite impossible to say whether these results indicate an actual breakdown of the Hb4O8 to some fractionate type of molecule such as Hb3O6 or Hb2O4 or into the single component HbO2. The fact that the line appears to be a very definite curve would lead one to suspect that mixtures of all four fractions exist at one time in varying proportions and that only at some concentration too high for this mode of study would one reach a state where the entire solution consisted of HbO2 alone.

SUMMARY

Oxyhemoglobin when studied over the widest possible range of concentration at the wave-lengths ordinarily used in the study
Beer's Law and Oxyhemoglobin

of hemoglobin, namely 540 μμ and 560 μμ, shows an excellent obedience to Beer's law, i.e. $E = \frac{C}{A}$. This relationship is independent of hydrogen ion concentration or salts in solution. Since the results were obtained over thicknesses ranging from 1 to 10 cm., it is also obvious that Lambert's law is obeyed.

On the other hand, if a long range of concentrations is studied at a wave-length of high transmission, i.e. 650 μμ, peculiar effects are observed. Beer's law is not obeyed; the measured absorption diverges from the predicted at 4 gm. per cent (0.6 mm), indicating molecular changes starting at about this concentration.

A possible basis for the similarity between Adair's and Svedberg's results and the spectrophotometric is suggested.

The authors wish to acknowledge the technical assistance of Dr. C. I. Thomas and Miss M. M. Taylor.

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