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The Light Absorption of Sheep Hemoglobin and Myoglobin Compounds in the Soret region

A Spectrophotometric Method for the Simultaneous Determination of Hemoglobin and Myoglobin

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Myoglobin was first crystallized by Theorell[1] in 1932; its possible physiological function was discussed by Millikan[2] (1939). Its quantitative estimation has been attempted several times. The latest of these is the detailed study of de Duve[3] (1948), who used the differences in the light absorption of the CO compounds of hemoglobin and myoglobin at three well-chosen wave-lengths (575.7 mμ = an isobestic point; 568 and 583.4 mμ where the CO-myoglobin has equal extinction coefficients, while those of the CO-hemoglobin differ widely from each other) for estimating the relative amount of the two compounds in the same solution. For a detailed discussion of the literature see de Duve's article [3].

The present work was made on myoglobin prepared from sheeps' hearts, at the suggestion of Professor H. Theorell. Sheep were selected as experimental animals in order to determine the myoglobin content in normal hearts and during experimental cardiac hypertrophy.

In the present investigation, the absorption of different sheep hemoglobin and myoglobin preparations is studied in the Soret region (between 400—450 mμ) and a method for the possible estimation of hemoglobin and myoglobin in the same solution suggested on the basis of the differences observed.

Fig. 1. The light absorption curves of myoglobin from sheeps' hearts precipitated with different ammonium sulphate saturations. All precipitates are redissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 174 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

○——○ *75— 85 % saturation.*
●——● *85—100 » »*
×——× *85— 90 » »*
△——△ *90—100 » »*

METHODS

Preparation of myoglobin

Sheep myoglobin was prepared as described by Theorell [1, 4]. It was possible to obtain it only in amorphous form, though the pure amorphous preparation contained a small quantity of very irregular crystals. It was found that a 75 % ammonium sulphate saturation always removed all the hemoglobin present in the heart extracts and a further fractionation proved the remaining material to be spectroscopically homogeneous. (This was the case even in those preparations where the hearts were not washed free of blood.) Fig. 1 shows the light absorption curves of a heart extract with which most of the absorption curves in the Soret region were carried out. It is to be seen that, in equal concentrations referred to hematin, the precipitates from 75 % ammonium sulphate saturation onwards give identical absorption curves, proving the homogeneity of the material. (All precipitates were redissolved in *M*/100 phosphate buffer, pH 7.)

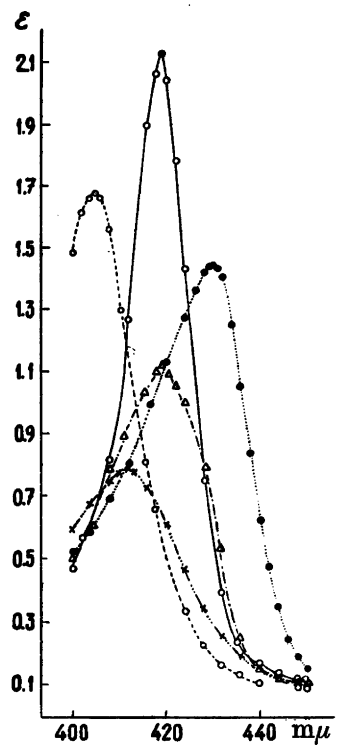


Fig. 2. The light absorption curves of sheep hemoglobin compounds in the Soret region. Hemoglobin is dissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 10.8 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

○— — —○ *Met-compound*
×—···—× *Oxy-* »
△—·—·—△ *CN-* »
○———○ *CO-compound*
●·····● *Reduced compound*

Preparation of hemoglobin

This was done by washing the centrifuged corpuscles of sheep blood (citrated blood) three times with Ringer solution, laking them with distilled water and centrifuging off the cellular residue.

Preparation of the different compounds

All dilutions were made with *M*/100 phosphate buffer, pH 7.

Reduced compounds. Solid $Na_2S_2O_4$ was added to a suitably diluted hemoglobin or myoglobin solution.

Oxy-compounds. Oxygen from a bomb was bubbled through the reduced solution for different times.

CO-compounds. Pure, washed CO was bubbled through a suitably diluted solution for about 5 minutes, solid $Na_2S_2O_4$ added while the bubbling was continued for another 5 minutes. The cuvettes, on the bottom of which a very small amount of $Na_2S_2O_4$ was placed, were filled and the readings taken immediately.

Met-compounds. One drop (about 0.04 ml) of a 0.5 % $K_3Fe(CN)_6$ solution was added to 3 ml of the suitably diluted solutions.

Cyanide-compounds. To the above solutions of the met-compounds, 0.3 ml 0.1 % KCN solution was added.

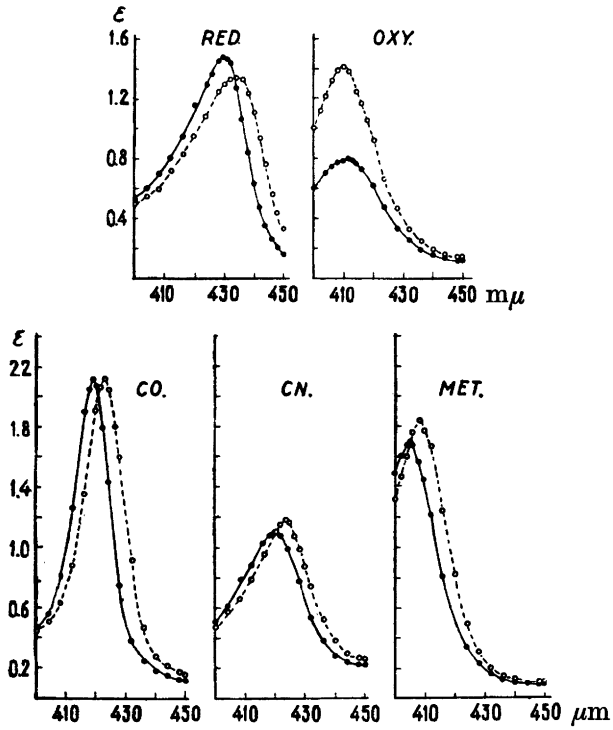


Fig. 3. The light absorption curves of sheep hemoglobin and myoglobin in the Soret region. Both hemoglobin and myoglobin are dissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 10.8 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

●——● *Hemoglobin* ○– – –○ *Myoglobin*

Pyridine-hemochromogen. To 3 ml of a suitably diluted solution 0.3 ml *N*.NaOH was added, then 1 ml pyridine. After mixing, solid $Na_2S_2O_4$ was added in excess, and readings taken immediately. All concentrations were calculated on the basis that a millimolar solution of pyridine protohemochromogen gives an extinction coefficient of 31.0 at 557 mμ.

Apparatus

The Beckmann spectrophotometer was used. The width of the cuvette, *d*, was 1 cm in all cases.

EXPERIMENTAL RESULTS AND DISCUSSION

Fig. 2 shows the light absorption curves of sheep hemoglobin compounds in the Soret region. It is to be noted that not only the wave length where each compound has its absorption maximum is characteristic and different

from the other, but there is also a marked difference in the value of the extinction coefficients. The CO-compound has a much higher peak than any other compound, *e. g.* cyan-ferri-hemoglobin which has its maximum at the same wave-length, shows only half the absorption of the CO-compound. A very striking difference is found between the reduced and oxy compounds. Not only is the peak of the oxy compound shifted to lower wave-lengths, but also its extinction coefficient is about half that of the reduced compound.

Having thus established the differences between the sheep hemoglobin compounds, it seemed interesting to investigate the absorption of similar myoglobin compounds. Fig. 3 shows the absorption curves of sheep hemoglobin and myoglobin in equal concentrations, with regard to hematin. It is seen that the absorption peak of myoglobin is in all cases shifted by a few mμ towards longer wave-lengths as compared to those of the respective hemoglobin compounds. The value of the extinction coefficients is equal in all hemoglobin and myoglobin compounds, with the one exception of the oxy compounds. In this case the light absorption of the oxy-myoglobin compound is about twice as high as that of oxy-hemoglobin, or, more correctly, the bubbling through of oxygen does not decrease the extinction coefficient of the reduced compound in the case of myoglobin, but strongly diminishes it (to about half its value) in the case of hemoglobin. These results are summarized in Table 1.

Table 1. The absorption maxima and the extinction coefficients of sheep hemoglobin and myoglobin compounds.

	Hemoglobin		Myoglobin		Hemoglobin		Myoglobin	
	No.	mμ	No.	mμ	No.	$\varepsilon = \log \frac{I_0}{I} \cdot 10^3$	No.	$\varepsilon = \log \frac{I_0}{I} \cdot 10^3$
Reduced	24	430	9	434	6	1423 $\pm$ 20	6	1323 $\pm$ 37
CO	7	419	7	423	6	2175 $\pm$ 55	6	2172 $\pm$ 54
CN	3	420	2	423	2	1126 $\pm$ 39	2	1204 $\pm$ 43
Oxy	28	412	15	410	7	778 $\pm$ 19	7	1403 $\pm$ 35
Met	2	405	2	408	2	1690 $\pm$ 4	2	1867 $\pm$ 62

All data are calculated for 10.8 μM/*l* hematin.
No. = number of determinations.

The most striking difference between hemoglobin and myoglobin, therefore, was the difference in the value of the extinction coefficients of the oxy compounds. It was found in 20 experiments that the extinction coefficient on the top of the Soret bands of the oxy-hemoglobin compound was 53 $\pm$

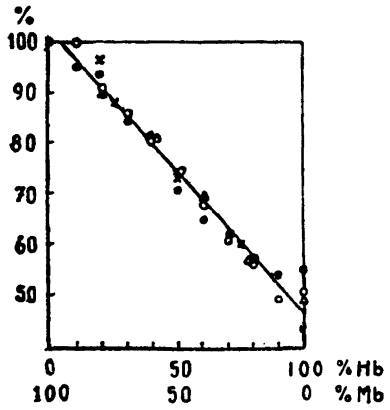


Fig. 4. Abscissa: The percentual hemoglobin and myoglobin content of a solution.

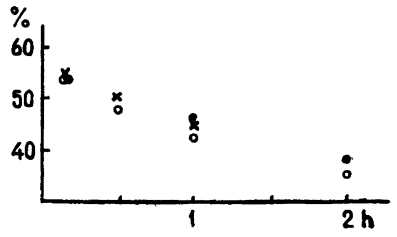
Ordinate: $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}} \times 100.$

Dilution with M/100 phosphate buffer, pH 7.

1.9 % of the reduced compound, whereas the absorption of the oxy-myoglobin was 105 $\pm$ 1.1 % of the reduced myoglobin in 16 experiments. Thus it seemed possible to estimate the amount of myoglobin and hemoglobin present in a given solution by determining the absorption maxima of its reduced and oxidised forms, thus finding the percentual decrease in the absorption of the oxy compound. Evidently, the more hemoglobin the solution contained, the greater would be this decrease and vice versa. Solutions of pure sheep hemoglobin and myoglobin were, therefore, prepared, suitably diluted with *M* 100 phosphate buffer, pH 7, and mixed in different proportions. The absorption of each mixture was determined in the reduced form, and again after bubbling through oxygen for 15 minutes. The decrease in the height of the absorption was calculated and the amount of hemoglobin and myoglobin present in that particular mixture established by the pyridine hemochromogen method. The results thus obtained are represented in Fig. 4, where the abscissa shows the percentual proportion of hemoglobin and myoglobin, the ordinate the percentual decrease in absorption of the oxy-form as compared to that of the reduced form, of the mixture in question. The different signs show estimations with different solutions. It is seen from this figure that a straight line is thus obtained. For example, if the extinction coefficient of a suitably diluted mixture after oxygenation is 80 % of that of the reduced form, the mixture will contain 40 % hemoglobin and 60 % myoglobin.

A few experiments were carried out to see how the time of oxygenation and the concentration of the solution would influence the absorption of the oxy compound. It was found that in the case of myoglobin neither its concentration, nor the time of oxygenation had any marked influence on the absorption of the oxy-compound, this being in all cases 104—106 % of that

Fig. 5. The influence of the time of oxygenation on the value $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}} \times 100$ *of sheep hemoglobin.*
Abscissa: Time of oxygenation.
Ordinate: $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}}$



of the reduced form. In the case of hemoglobin, however, the absorption of the oxy compound decreased with the time of the oxygenation (Fig. 5). Also the concentration of the hemoglobin seemed to have some influence, the absorption of the oxy compound showing a tendency to decrease more slowly in the case of more concentrated solutions. This, however, was more irregular than the influence of time. In view of these findings, the above factors were carefully standardised. The oxygen was allowed to bubble through the solution at such a rate that the individual bubbles were just countable and yet did not flow together in a stream. The time of oxygenation was exactly 15 minutes in all cases. The dilution of the solutions was selected in such a way that the maximal absorption of the reduced compound should give an extinction value of about 1.2—1.5. The data shown in Fig. 4 were obtained by using all these precautions.

A few experiments were carried out in order to estimate the myoglobin and hemoglobin contents of heart extracts. The heart muscle, after dissecting it free of connective tissue and fat, and after washing it with Ringer solution in 2 of the experiments, was homogenised in a Turmix blendor with about the same amount of distilled water, and left in the ice-chest overnight. 0.25

Table 2. The percentual proportion of hemoglobin and myoglobin in sheep heart extracts.

Extract	Hemoglobin				Myoglobin			
1*	16,	14			84,	86		
2**	26,	30,	30,	24	74,	70,	70,	76
3	37,	40			63,	60		
4	38,	42			62,	58		
5	38,	42,	38,	38	62,	58,	62,	62
6	38,	32,	34,	30	62,	68,	66,	70

* Heart washed thoroughly free of blood with Ringer through the aorta.

** Heart washed incompletely free of blood.

3—6 Unwashed heart muscle.

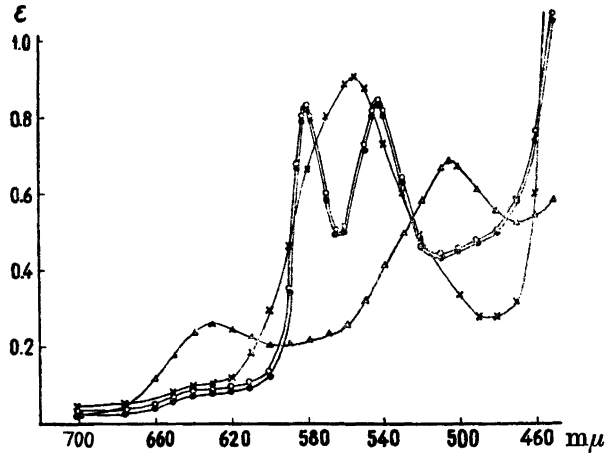


Fig. 6. The light absorption curve of met-, oxy-, and reduced sheep myoglobin. All dilutions are made with M/100 phosphate buffer, pH 7. The data plotted were obtained on solutions containing 67 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

△——△ *The original met myoglobin solution*
×——× *After reduction with solid* $Na_2S_2O_4$
○——○ *After oxygenation for 10 minutes*
●——● *After oxygenation for 60 minutes*

volume % of a 20 % lead acetate solution was then added, the precipitate centrifuged off and the excess lead removed by the addition of solid Na_2HPO_4. (The pH was controlled during all these operations as described by Theorell [1].) A small quantity of these extracts was suitably diluted with *M*/100 phosphate buffer, pH 7. Solid $Na_2S_2O_4$ was added and the extinction of the reduced form determined in a part of it, while oxygen was bubbled through the other part for 15 minutes, then its extinction read. The maximal extinction of the oxy-compound, expressed in percent of that of the reduced compound, was then calculated, and the corresponding percentual hemoglobin content read from the line in Fig. 4. These values are found in Table 2. The parallel determinations mean parallels made on the same extracts. There was no attempt to make determinations on the same heart muscle, extracted differently. As no care was taken to extract the myoglobin quantitatively, the data have not been re-calculated in gs. Thus it is not stated f. i. how many gs of myoglobin 1 kg of wet or dry heart muscle contained, only what percentage of the total hemin content of a particular extract was found in the form of myoglobin and hemoglobin respectively.

In order to check the purity of the oxy-compounds, the absorption curve was determined in the visible region. The myoglobin as prepared from sheep heart, was a met-compound; after reduction and oxygenation the oxy-compound was formed, which was, however, probably not quite free from the met-form. Fig. 6 illustrates these results. It is seen from this figure that a small elevation is present around 630 mμ, characteristic of the met-compound. As no such elevation was seen in the oxy-hemoglobin, the method here described warrants certain precautions. It is impossible to state definitely at present what part of the phenomenon — *i. e.* that the absorption of oxy-myoglobin is unchanged as compared to that of the reduced form — is caused by the small contamination by the met-form not present in hemoglobin. As, however, the contamination with the met-form must — as seen from Fig. 6 — be very small, and as extracted myoglobin used by other authors, was probably never entirely free from it, we feel justified in calling attention to the very big differences found in the Soret bands of oxy-myoglobin and oxy-hemoglobin. Further work is, however, needed to ascertain the general validity of the method here described.

SUMMARY

1. The absorption of sheep myoglobin and hemoglobin in the Soret region is determined.

2. The absorption maxima of the CO, reduced, CN, and Met compounds of myoglobin are shifted with 2—4 mμ to longer wave-lengths, as compared with those of hemoglobin.

3. The absorption maximum of oxy-hemoglobin is about half of that of oxy-myoglobin, reduced myoglobin and reduced hemoglobin.

4. The difference between the extinction coefficients of oxy-myoglobin and oxy-hemoglobin, as compared to that of their respective reduced forms, is suggested as a possible method for estimating simultaneously hemoglobin and myoglobin in the same solution.

5. Some possible sources of error in this estimation are discussed.

6. A few determinations in sheep heart extracts (washed and unwashed) are described.

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