ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 77, 478-492 (1958)

Studies on the Oxygen and Carbon Monoxide Equilibria of Human Myoglobin[1]

A. Rossi-Fanelli and E. Antonini

From the Institute of Biological Chemistry University of Rome and National Research Council Unit for Enzyme Studies, Rome, Italy

Received March 10, 1958

Introduction

The reactions of hemoglobin (Hb) and myoglobin (Mb) with gas are, for the particular mechanisms involved, one of the most interesting problems in biochemistry.

In spite of a great number of researches on the gaseous equilibria of Hb from different animal species, Mb has not been extensively investigated from this point of view. The classical work by Theorell (1) with crystallized horse Mb appears to be the only detailed research on the oxygen-dissociation curves of this pigment.

With respect to human Mb, this pigment has recently been obtained in pure crystallized form (2, 3), but only some of its chemical and physicochemical properties have been investigated so far (4, 5).

It seemed therefore a matter of interest to perform a complete study of the oxygen equilibrium of crystallized human Mb.

The study of oxygen equilibrium of Mb offers some technical difficulties related to the nature of the pigment: (*a*) Mb must be available in pure crystallized form and this, in the case of human Mb, is not easy to obtain; (*b*) the crystallized pigment, which is obtained in the ferric form, should be reduced through a system that does not modify the properties of the protein; (*c*) the usual gasometric methods for the determination of the oxygen-dissociation curves of Hb are not very suitable owing to the relatively small quantities of pigment available and to the great speed of oxidation of Mb.

On the basis of these considerations, a convenient spectrophotometric nethod of determining the oxygen equilibrium of Mb was developed.

[1] Aided in part by a grant from the Rockefeller Foundation.

The main advantages of this method are the following. Very simple apparatus is required, and the procedure is rapid and easy to perform. The reduction of the pigment is obtained by an enzymic system that operates in a physiological manner, so that the use of chemical reducing agents is avoided. During the determination of the oxygen-dissociation curve, the pigment is subjected to a very mild treatment and the state of the pigment can be controlled at any time. Moreover, the oxygen-dissociation curve can be determined with very small quantities of pigment and with both concentrated and diluted solutions. The object of this note is to describe this method and to examine, in detail, the equilibria of the reactions of human Mb with O_2 and CO.

Experimental

Apparatus

Small tonometers, suitable for spectrophotometric analysis with the Beckman DU apparatus, were used for the determination of the oxygen-dissociation curves. They consisted in modified Thunberg tubes with the lower part sealed to a 1-cm. pyrex optical cuvette (Fig. 1).

The tube is provided with a mobile glass spacer, 1 × 0.8 × 6 cm., that fits into the cuvette in order to reduce the light path to 0.2 cm. and the volume of the liquid to 1 ml.

Many tubes of the same type, but of different size and volume, were tested: the one shown in the figure and most frequently employed had a total volume, with the spacer inserted, of 16 ml.

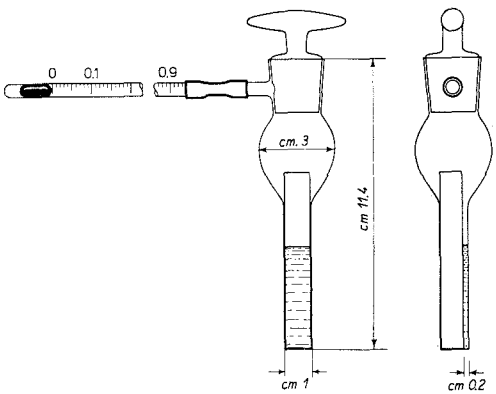

Fig. 1. Modified Thunberg tube used for the determination of the oxygen-dissociation curves.

Preparation of Human Mb

Human Mb was crystallized according to Rossi-Fanelli (6) from skeletal muscles collected in post-mortem examinations from seven cases of different sex and age. Since no appreciable difference has been found in the oxygen equilibrium among the various Mb preparations, the different origin of the material will not be considered in this work.

The complete absence of Hb in each preparation was checked by spectrophotometry (7) and paper electrophoresis (5).

As previously reported, human crystallized Mb is electrophoretically inhomogeneous (5). The O_2-dissociation curves of the isolated, pure components (Mb I and Mb II) will be discussed later in the text.

Determination of the O_2-Dissociation Curves

Enzymic Reduction of Ferri-Mb

The reduction of ferri-Mb was obtained as follows (8): 1 μmole Mb^+, 1.5–2 μmoles DPNH, 1×10^{-2} μmole methylene blue (MB), and 100–200 μg. diphosphopyridine nucleotide (DPN) reductase were mixed together in 2–10 ml. of tris-(hydroxymethyl)amino methane buffer, pH 7.45, 0.05 *M*; the formation of MbO_2 was followed spectrophotometrically.

After completion of the reduction, the O_2 uptake of the Mb solution as measured with standard Warburg apparatus was found to be negligible (1–2 μl. O_2/hr./ml.). The solutions of MbO_2 so obtained can be stored at + 2°C. for 3 or 4 days.

The DPN and the MB can be removed by dialysis, but since they do not affect the O_2 equilibrium in the concentrations used, the MbO_2 solutions were generally used for the determinations of the O_2-dissociation curves without further treatment. Tris buffer, 0.05 *M*, pH 7.45, was used in most of the experiments. When necessary, the pH was varied by adding to the stock Mb solution (in Tris, 0.05 *M*, pH 7.45) 0.1 *N* HCl or 0.1 *N* NaOH. In this way the ionic strength of the solutions was almost unchanged. After each determination the pH was measured with a Beckman pH meter.

The use of Tris buffer in the study of the oxygen equilibrium of Mb and Hb seemed suitable because it can be used between pH 7 and 9 and has a small salt effect compared with that of NaCl.[2]

Oxygenation of Mb

One milliliter of the Mb solution is measured into the tonometer cuvette, and the stopper is sealed with silicone. The tonometer is then connected to a water pump and kept at a temperature higher than the tap water in the pump (8–9°).

In some experiments a high vacuum pump was used; in this case deoxygenation can be performed at a much lower temperature. After prolonged (10–15 min.) evacuation of the tonometer, the level of the liquid in the cuvette is measured and the complete deoxygenation controlled spectrophotometrically.

Known amounts of air were then introduced in the tonometer in the following

[2] Unpublished experiments by the authors.

manner: a 1-ml. graduated pipet having a meniscus of mercury was connected to the side arm of the tonometer through a rubber tube; on cautiously opening the tap, the atmospheric air contained in the pipet is sucked into the tube. The volume of air introduced is measured by the displacement of the Hg meniscus in the pipet.

The tonometer was then rotated horizontally at constant temperature for 3–10 min.; in the case of experiments at high temperatures, the equilibrium time must be the shortest possible since in these conditions Mb is rather quickly transformed into Mb^+.

In our experimental conditions, the equilibrium was completed within 2–3 min. After the equilibrium was established, the tonometer was transferred to the Beckman spectrophotometer and readings were taken at the wavelengths chosen.

The procedure was then repeated. In most experiments several points (three or four) of the curve can be obtained; at low pH and high temperature (above 20°), however, only one or two points of the curve can be determined because, as mentioned, Mb readily changes into Mb^+. The tonometer was then opened to air and the optical density for complete oxygenation recorded. The experiments were performed in a room kept at constant temperature (20°), and the humidity and atmospheric pressure were controlled.

The pressure of oxygen in the tonometer corresponding to each introduction of air was calculated by the following formula:

$$P_T = \frac{P_a \times V_0}{V_t} - FX$$

where

P_T = oxygen pressure in the tonometer (at 20°)
P_a = oxygen pressure in air (at 20°)
V_o = volume of air introduced into the tonometer
V_t = volume of the tonometer minus the volume of the Mb solution
X = oxygen bound to MbO_2
F = factor to convert X in p_{O_2}.

For dilute Mb solutions there is no need to introduce the correction for the O_2 in combination with Mb. Since the volume of the liquid is very small, the O_2 physically dissolved can be neglected.

In order to maintain the proportionality between the O_2 pressure and the O_2 concentration, the O_2 pressure in the tonometer was calculated for 20° as well as for the higher and lower temperatures.

Spectrophotometry

The percentage of MbO_2 was calculated from the following formula:

$$MbO_2\,\% = \frac{D_{obs} - D_{Mb}}{D_{MbO_2} - D_{Mb}} \times 100$$

where

D_{obs} = optical density at the p_{O_2} under examination
D_{Mb} = optical density at complete deoxygenation
D_{MbO_2} = optical density at complete oxygenation.

The spectrophotometric readings were taken, for the usual concentration of the pigment (between 0.2 and 1%) and 2 mm. light path, at 460, 510, 540, 560, and 580 mμ; for higher Mb concentrations the readings were taken at 600 and 800 mμ; for lower concentrations at 410 and 430 mμ.

The reading at 510 mμ (isosbestic point between Mb and MbO_2) is taken in order to control the possible formation of Mb^+ and denaturation of the pigment.

The formula was applied to the readings obtained from the different wavelengths, and the mean of the values obtained was used for the plot of the O_2-dissociation curve.

Results

Shape of the O_2-Dissociation Curve

The O_2-dissociation curve of human Mb was found to be hyperbolic in all the experimental conditions examined. Such curves follow the Hill equation

$$Y = \frac{K(p_{O_2})^n}{1 + K(p_{O_2})^n}$$

with a value of $n = 1$. The mean value of n experimentally determined from about 50 dissociation curves was 1.03 (0.95–1.1). The equilibrium constant, K, of the reaction can be then expressed as

$$K = \frac{MbO_2}{Mb \times p_{O_2}}$$

where Mb and MbO_2 denote concentrations, and p_{O_2} oxygen pressure in mm. Hg.

The Effect of Temperature on the Dissociation Curves

Figure 2 and Table I show the effect of temperature on the dissociation curves of human Mb in 0.05 *M* Tris buffer, pH 7.45.

From 10° to 40°, the curves obtained have the same shape and present a marked increase in $p_{\frac{1}{2}}$ with increasing temperature.

Figure 3 shows that, for the range of temperature examined (10°–40°), a linear relation exists between log K and $1/T$.

On application of the van't Hoff equation (9), it is possible to calculate

indirectly the over-all heat of the reaction Mb + O_2 (gas) = MbO_2, viz.

$$\Delta H = -\frac{Rd \ln K}{d(1/T)}$$

where ΔH is the heat of reaction; K the equilibrium constant; T the absolute temperature; R the gas constant.

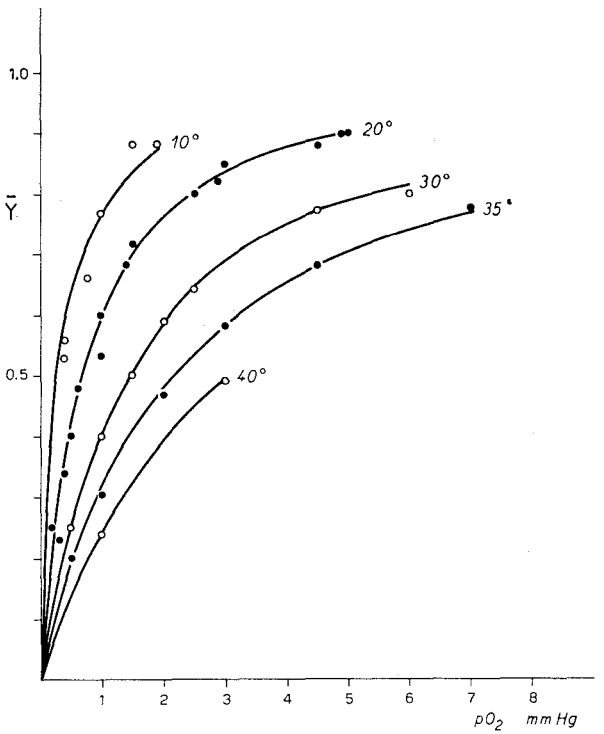

FIG. 2. Oxygen-dissociation curves of human Mb at different temperatures. Tris buffer, 0.05 M, pH 7.45; Mb 2 × 10^{-4} M. $\bar{Y}$ = fractional saturation with oxygen.

TABLE I

Oxygen Equilibrium of Human Mb at Different Temperatures

Tris buffer, 0.05 M, pH 7.45; Mb 2 × 10^{-4} M.

Temperature, °C.	10	20	30	35	40
Log K	+0.49	+0.14	−0.18	−0.32	−0.48

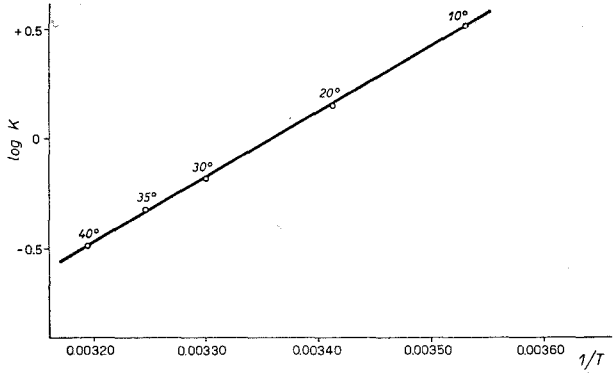

FIG. 3. Influence of temperature on the oxygen equilibrium of human Mb.

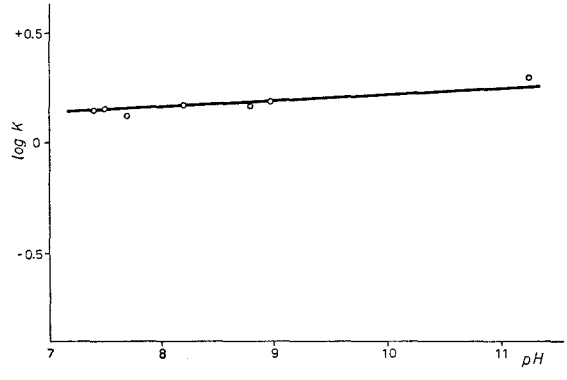

FIG. 4. Influence of pH on the oxygen equilibrium of human Mb. 20°C.; Mb 2×10^{-4} *M*.

The average value of ΔH calculated from the data obtained was -13.1 kcal.[3] The heat so calculated is that of the combination of 1 g. mole O_2 in the gaseous phase with Mb in the liquid phase; it therefore includes the heat of solution of O_2.

[3] For 10–20°, $\Delta H = -13.3$ kcal.
20–30°, -13.0 kcal.
30–35°, -12.0 kcal.
35–40°, -14.1 kcal.
Mean $= -13.1 \pm 0.6$ kcal.
From the slope of the line drawn in Fig. 3, $\Delta H = -13.0$ kcal.

Effect of pH

The effect of hydrogen-ion concentration on the oxygen equilibrium of human Mb in Tris buffer of ionic strength 0.05 at 20°C. is shown in Fig. 4 and Table II.

It can be seen that from pH 7.4 to 11.3 the Bohr effect is almost absent. At lower pH values the accurate determination of the equilibrium constant is difficult since Mb readily changes into Mb^+; however, no significant difference in the oxygen affinity was found above pH 5.5.

Effect of the Ionic Strength

The effect was studied at 20° with Mb solutions in Tris 0.05 *M*, the ionic strength of which was increased by adding NaCl.

As shown in Fig. 5, even very high concentrations of NaCl do not affect the oxygen affinity.

TABLE II

Oxygen Equilibrium of Human Mb at Different pH's

20°C.; $I = 0.05$; Mb 2×10^{-4} *M*.

pH	7.4	7.45	7.7	8.2	8.8	9.0	11.3
Log *K*	0.14	0.15	0.12	0.16	0.16	0.18	0.28

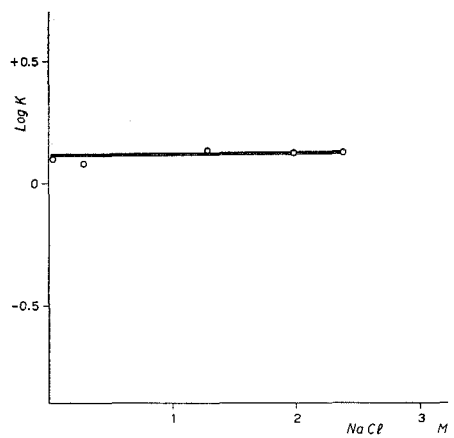

FIG. 5. Oxygen equilibrium of human Mb at different salt concentrations. 20°C.; pH 7.45; Mb 2×10^{-4} *M*.

Effect of Mb Concentration

The oxygen-dissociation curves of human Mb have been determined for concentrations of the pigment from 0.2×10^{-4} *M* to 0.45×10^{-3} *M*. The Mb concentration in the muscles lies within these ranges. Figure 6 shows that no difference can be demonstrated in the oxygen-dissociation curves for the different Mb concentrations.

Oxygen-Dissociation Curves of Mb in Strong Urea Solutions

Considering the important results obtained by Wyman (9) from the study of the O_2 equilibrium of Hb in strong urea solutions, it seemed interesting to examine the effect of urea on the reaction of Mb with oxygen. In the case of Mb, no splitting in subunits can be produced by urea, and therefore only the direct effect of urea on the oxygenation reaction should be present.

As shown in Table III, urea has no appreciable influence on the oxy-

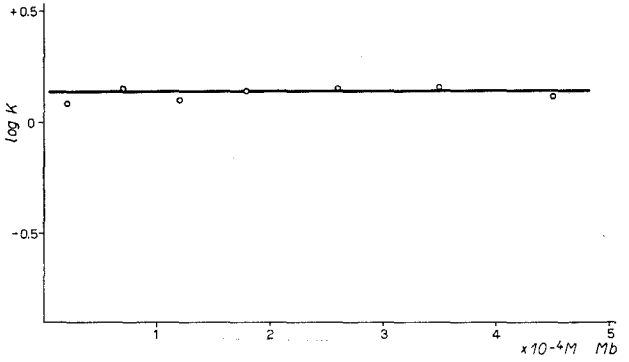

FIG. 6. Oxygen equilibrium of human Mb for different concentrations of the pigment. 20°C.; Tris buffer, 0.05 *M*, pH 7.45.

TABLE III

Oxygen Equilibrium of Human Mb in Strong Urea Solutions

Mb 2×10^{-4} *M* in Tris buffer, 0.1 *M*, pH 7.45.

	Temperature	Log *K*
	°C.	
Untreated Mb (control)	30	−0.18
Mb in 5 *M* urea	30	−0.15
Mb in 5 *M* urea	20	+0.10

gen-dissociation curves of human Mb. This finding gives a still greater significance to the results obtained by Wyman on Hb.

Oxygen-Dissociation Curves of Reconstituted Mb

In previous research (10) we reported the reversible splitting of human Mb and the resynthesis of the pigment with protoheme and deuteroheme (with H substituted for $CH{=}CH_2$ groups in the 2 and 4 positions of the porphyrin). The oxygen equilibria of reconstituted proto- and deutero-Mb are summarized in Figs. 7, 8, and 9.

From these data it is evident that the oxygen equilibrium of recon-

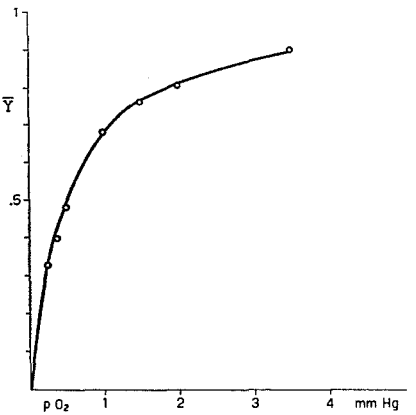

FIG. 7. Oxygen-dissociation curve of reconstituted deutero-Mb. 30°C.; Tris buffer, 0.1 *M*, pH 7.4; deutero-Mb 1×10^{-4} *M*. $\bar{Y}$ = fractional saturation with oxygen.

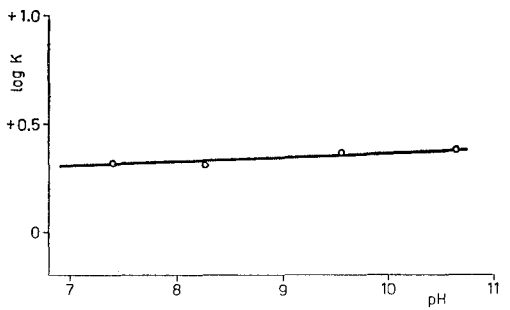

FIG. 8. Oxygen equilibrium of deutero-Mb at different pH's. 30°C.; deutero-Mb 0.5×10^{-4} *M*; $I = 0.1$.

stituted proto-Mb is identical to that of the natural pigment and that deutero-Mb has a much greater oxygen affinity than proto-Mb while the shape of the dissociation curve, the temperature, and the pH effects are the same.

Oxygen-Dissociation Curves of Mb I and Mb II

Our previous research (5) demonstrated that crystallized human Mb is inhomogeneous and that three distinct components (Mb I, Mb II, and Mb III) can be detected by electrophoresis. The relative percentages of the three components in the crystallized pigment are as follows: Mb I 70–80 %, Mb II 10–20 %, Mb III 5–7 %.

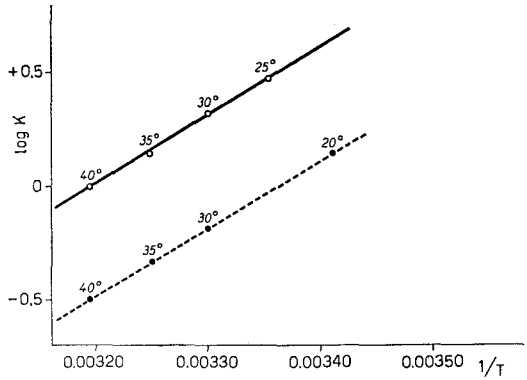

FIG. 9. Oxygen equilibrium of reconstituted proto-Mb – –●– – and deutero-Mb –○– at different temperatures. Tris buffer, 0.1 *M*, pH 7.40; proto-Mb 1.5 × 10^{-4} *M*, deutero-Mb 0.5–1 × 10^{-4} *M*.

TABLE IV

Oxygen Equilibrium of Mb I and Mb II at Different Temperatures

Borate buffer pH 8; $I = 0.05$; Mb I 0.5 × 10^{-4} *M*, Mb II 0.4 × 10^{-4} *M*. The oxygen dissociation curves of both components follow the Hill equation with $n = 1$.

Temperature	Log *K*	
	Mb I	Mb II
°C.		
20	+0.18	+0.19
25	+0.03	+0.04
30	−0.13	−0.11
35	−0.28	−0.23
40	−0.47	−0.42

For a correct evaluation of the results reported above on the oxygen equilibrium of crystallized human Mb, it was necessary to determine whether the oxygen-dissociation curves of the Mb components were different from those obtained from the crystallized Mb.[4]

For this purpose Mb I and Mb II were isolated by electrophoresis on starch gel, and the O_2-dissociation curves of the pure homogeneous components were determined.[5]

The results of these experiments are summarized in Table IV. It can be seen that the Mb components are almost identical to each other as the oxygen equilibrium is concerned.

Relative Affinity for Oxygen and Carbon Monoxide

For the reversible reaction

$$MbO_2 + CO \rightleftharpoons MbCO + O_2$$

the equilibrium constant

$$K = \frac{(MbCO) \times p_{O_2}}{(MbO_2) \times p_{CO}}$$

was determined by spectrophotometric analysis of Mb solutions equilibrated with known mixtures of O_2 and CO.

For this purpose the same tonometer used for the determination of the oxygen-dissociation curves was employed.

One milliliter of the stock MbO_2 solution, in Tris buffer, 0.05 *M*, pH 7.45, was pipetted into the tonometer, and the optical density for oxygenated Mb was recorded; the tonometer was then evacuated and filled with a mixture of air and CO in known proportions. After equilibration at constant temperature for 15–20 min., readings were taken at 575 and 582 mμ; the solution was then saturated with CO, and the optical density at the same wavelengths was measured.

The per cent of MbCO was calculated by the following formula:

$$MbCO\ \% = \frac{D_m - D_{MbO_2}}{D_{MbCO} - D_{MbO_2}} \times 100$$

where D_m is the difference between the readings at 575 and 582 mμ for the solution after equilibration, D_{MbO_2} is the difference (corrected for the change in concentration) between the readings at the same wave-

[4] The Mb components I and II have the same absorption spectra.

[5] The detailed experiments have been reported elsewhere (11).

TABLE V

Relative Affinity of Human Mb for Oxygen and Carbon Monoxide

Temp., 20°C.; Tris buffer, 0.05 M, pH 7.45; Mb 2×10^{-4} M.

Determination	$\frac{(MbCO)}{(MbO_2)}$	$\frac{p_{O_2}}{p_{CO}}$	K
1	1	40	40
2	0.46	86	39
3	0.43	86	37

lengths for oxygenated Mb, and D_{MbCO} is the same difference after saturation with CO.

The values of K obtained at 20° and pH 7.45 are reported in Table V.

Relation between log K and "Span"

Anson *et al.* (12) observed for many Hb's a linear relation between the logarithm of the partition constant K and the "span" in angstrom units of the bands of the oxy and carbomonoxy compounds: $y = \log K/$"span"

As a result of a number of determinations, the "span" for human Mb was found to be 33 A.; the value of y calculated from the "span" and the partition constant was 0.048. Theorell (14) for horse Hb found $y = 0.045$.

Discussion

The reported data give a complete picture of the equilibrium between human Mb and oxygen.

Small but significant differences can be observed between our data and those obtained by Theorell on horse Mb that might be attributed either to the different pigment investigated or to the different methods used.

The $p_{\frac{1}{2}}$ for human Mb at 37° and pH 7.4 calculated from our data is 2.75 mm Hg. This value, compared with that for human Hb in physiological conditions ($p_{\frac{1}{2}} \simeq 25$ mm. Hg) is in agreement with the hypothesis of the function of Mb as a storage of O_2 and as a link between the oxygen carrier (Hb) and the oxidizing systems in the muscle.

The over-all heat of oxygenation of human Mb has been found to be -13.1 kcal. This value is lower than that obtained by Theorell (-17.5 kcal.) and is similar to the heat of oxygenation for the 1° heme in Hb according to Roughton (13).

The oxygen equilibrium of human Mb is not affected by the hydrogen-ion concentration, by the ionic strength or by the concentration of the pigment; this behavior differs from that of Hb and reflects the deep structural and functional differences between the two pigments.

The study of the O_2 equilibrium of the components that are present in crystallized Mb (and in muscle extracts) demonstrates that Mb I and Mb II have almost identical O_2-dissociation curves: The heterogeneity of Mb should therefore be regarded as a problem concerning the chemical structure rather than the functional properties of the pigments.

The results obtained with reconstituted proto- and deutero-Mb emphasize the importance of the vinyl side chains of protoheme in the reaction of Mb with O_2.

The values for the partition constant between O_2 and CO for human Mb are in agreement with those reported for other animal species (14, 15).

Acknowledgment

The authors wish to thank Mr. Renato Giuffrè for skilled technical assistance.

Summary

1. A new spectrophotometric method of determining the oxygen-dissociation curves of Mb (and Hb) has been developed.

2. The oxygen equilibrium of human crystallized Mb has been studied by this method. The effect of temperature, pH, ionic strength, and concentration of the pigment has been examined.

3. The oxygen-dissociation curves of reconstituted Mb's and of Mb components Mb I and Mb II have also been investigated.

4. The partition constant $K = (\mathrm{MbCO}) \times p_{O_2}/(\mathrm{MbO_2}) \times p_{CO}$, for human crystallized Mb has been determined.

References

1. Theorell, H., *Biochem. Z.* **268,** 73 (1934).
2. Theorell, H., and De Duve, C., *Arch. Biochem.* **12,** 113 (1947).
3. Rossi-Fanelli, A., and Perri, G. C., *Boll. soc. ital. biol. sper.* **23,** 119 (1947).
4. Rossi-Fanelli, A., Cavallini, D., and De Marco, C., *Biochim. et Biophys. Acta* **17,** 377 (1955).
5. Rossi-Fanelli, A., and Antonini, E., *Arch. Biochem. Biophys.* **65,** 587 (1956).
6. Rossi-Fanelli, A., "Haemoglobin," p. 115. Butterworths Sci. Publ., London, 1949.
7. De Duve, C., *Acta Chem. Scand.* **2,** 264 (1948).

8. ROSSI-FANELLI, A., ANTONINI, E., AND MONDOVI, B., *Arch. Biochem. Biophys.* **68,** 341 (1957).
9. WYMAN, J., *Advances in Protein Chem.* **4,** 410 (1948).
10. ROSSI-FANELLI, A., AND ANTONINI, E., *Arch. Biochem. Biophys.* **72,** 243 (1957).
11. ROSSI-FANELLI, A., AND ANTONINI, E., *Experientia* **12,** 477 (1957).
12. ANSON, M. L., BARCROFT, J., MIRSKY, A. E., AND DIMMA, S., *Proc. Roy. Soc.* (London) **B97,** 61 (1925).
13. ROUGHTON, F. J. W., *quoted by* GEORGE, P., *in* "Currents in Biochemical Research" (Green D. E., ed.), p. 365. Interscience Publ., New York, 1956.
14. THEORELL, H., *Biochem. Z.* **268,** 64 (1934).
15. ROCHE, J., *Comp. rend. soc. biol.* **195,** 501 (1932).

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 77, 493–509 (1958)

Studies on Pig Liver Microsomes. I. Enzymic and Pigment Composition of Different Microsomal Fractions[1]

David Garfinkel[2]

From the Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania

Received January 6, 1958

Introduction

Since the demonstration of Strittmatter and Ball (1) that a characteristic cytochrome (b_5) is present in microsomes, much work has been done on the electron-transport behavior of microsomes. This includes both kinetic studies on intact microsomes[3] and isolation of enzymes, including the cytochrome itself (2, 3) and various reductases (4, 5). Thus far there has been little work on how electron-transporting components are organized in microsomes. This communication is concerned with the chemical and enzymic composition of pig liver microsomes, their appearance in the electron microscope and some data as to their organization, much of which was obtained in the course of enzyme preparations (particularly preparation of cytochrome b_5). A more detailed picture may be obtained by following the effects of digestion on a number of enzymes and related substances.[4]

Methods and Materials

Pig liver microsomes were prepared as previously described (3). Almost all the data here reported were obtained from microsomes prepared from pig livers which had been perfused with mammalian Ringer's solution. No precipitant such as $(NH_4)_2SO_4$ (2) or acid (4) was used in the preparations; these precipitants may have an appreciable effect on the microsomal structure (5).

[1] A preliminary report of part of this work was presented at the meeting of the Biophysical Society, Cambridge, Mass., Feb. 5, 1958.

[2] Public Health Service Research Fellow of the National Cancer Institute.

[3] Klingenberg, M., in preparation.

[4] Garfinkel, D., in preparation.