Comp. Biochem. Physiol., Vol. 61A, pp. 19 to 22
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0300-9629/78/0801-0019$02.00/0

SOME STUDIES ON THE OXYGEN AFFINITY OF HAEMOGLOBIN FROM THE DUGONG

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(Received 22 December 1977)

Abstract—1. The P_{50} of dugong haemoglobin at pH 7.4 and 30°C in 0.1 M sodium phosphate buffer and 0.1 g % concentration was found to be 6.2 mm Hg.

2. The Bohr shift in the pH range 6.5–7.0 was −0.48. The value of the Hill coefficient was maximally 1.85.

3. The heat of combination, for the oxygen–haemoglobin binding reaction was found to be 77.2 kjoules/°K per mole.

4. The results are interpreted as suggesting significant subunit dissociation of dugong haemoglobin at low concentrations.

INTRODUCTION

The order Sirenia today consists of only four species in two genera, all of which are considered vulnerable to extinction (*ICUN Red Data Book*, 1976). There are three species of manatees, genus *Trichechus*, and a single species of dugong, *Dugong dugon* (Müller). Sirenians are unique in that they are the only living aquatic mammalian herbivores. The dugong is of particular interest since, unlike the manatees which spend significant periods in fresh water, it is strictly marine.

Few physiological studies have been performed on Sirenians, particularly the dugong which has only rarely been maintained in captivity (Jones, 1967). Information on the dugong which is pertinent to future physiological research is summarized by Marsh *et al.* (1978). Observations on dugong blood appear at present to be limited to a single measurement of oxygen capacity (Lenfant, 1969). Because of this lack of data, and the uncertainty of obtaining additional specimen material, we present here a brief report of some of the properties of haemoglobin obtained from the dugong.

METHODS

(a) *Blood collection*

Whole blood was taken by syringe from the heart of a freshly killed mature male dugong of body length 2.39 m, and added to a heparinized bottle. The dugong had been drowned after being speared for food by Aboriginal dugong hunters at Mornington Island in the Gulf of Carpentaria. Despite the presence of heparin the blood commenced to clot almost immediately, and as a result oxygen binding studies were restricted to a purified sample of the haemoglobin and could not be performed on whole red cells. The blood sample was transported and stored in ice until the isolation of a sample of haemoglobin.

(b) *Preparation of haemoglobin*

The clot was removed and the remaining red cells were washed 5 times with 0.9% saline and packed by centrifugation at 1500 rev/min after each wash. Haemolysis was achieved by the addition of 1.5 vols of distilled water and 0.5 vols of toluene. The resulting mixture was agitated vigorously and centrifuged at 1500 rev/min for 10 min. The toluene layer and cell debris which collected at the interface was removed with a Pasteur pipette. The solution was dialysed against phosphate buffer (0.1 M; pH 7.4) for 24 hr. The haemoglobin solution was further purified by chromatography on a column of Sephadex G-150 (2.6 × 90 cm). The same buffer was used to elute the haemoglobin which was collected using an L.K.B. Ultrorac 7000 fraction collector. The entire procedure was carried out in a cold room at 4°C, and the haemoglobin solution so obtained was stored at −20°C until use.

(c) *Visible spectra of the dugong haemoglobin*

Haemoglobin solution (20 ml) was redialysed against distilled water, until no more phosphate could be detected in the dialysate (five changes over 8 hr). The solution was reconcentrated to a final volume of approx 12 ml by filtration under moderate pressure using N_2 gas. A 10 ml sample of this solution was placed in a preweighed flask and freeze dried using a Dynavac freeze drying unit. The remaining 2 ml was reserved for the spectral measurements. The lyophilized product was finally warmed in a vacuum oven (40°C) over P_2O_5 for 4 hr before being allowed to cool to room temperature and finally reweighed. The remaining sample was suitably diluted with a known amount of distilled water and the absorption spectrum of the oxyhaemoglobin solution was measured. The deoxy absorption spectrum was also measured on the same sample after the addition of a few grains of sodium dithionite using a filled and stoppered spectrophotometer cuvette.

(d) *Measurement of oxygen–haemoglobin equilibria*

The technique used was basically that of Imai *et al.* (1970), and depends upon the simultaneous recording of the oxygen partial pressure of a solution of haemoglobin and the corresponding absorbance. From a knowledge of the absorbance spectra of the oxy and deoxy forms a wavelength of 560 nm was selected as the monitoring wavelength. All of the equilibrium curves were measured using 0.1% w/v haemoglobin solution in 0.1 M sodium phosphate buffer over the pH range 6.0–7.4, and a temperature range of 20–30°C. As a check on the accuracy of the apparatus, samples of human haemoglobin were concurrently measured and values obtained for the P_{50} and n (from the Hill plot) for fresh human haemoglobin were compared

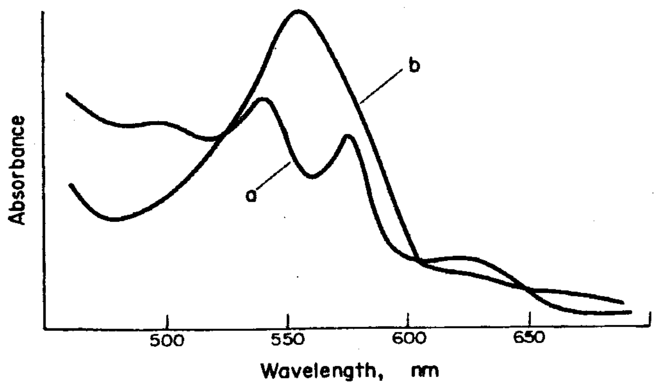

Fig. 1. Visible absorption spectra of (a) deoxyhaemoglobin and (b) oxyhaemoglobin.

with published values (Imai, 1968; Imai *et al.*, 1970) and shown to agree closely.

Deoxygenation of the sample in the cell was achieved by blowing a stream of high purity nitrogen gas gently over the surface of the haemoglobin solution while vigorous stirring was maintained. In order to minimize errors due to the evaporation of the solution under these conditions the nitrogen stream was first saturated with water vapour at the same temperature as the haemoglobin solution. The haemoglobin solution was judged deoxygenated when the recorded pO_2 of the solution remained steady at zero and the haemoglobin spectrum showed a single peak with no inflection at 560 nm. When complete deoxygenation was achieved (approx 40–60 min), the nitrogen flow was turned off and the resulting slow diffusion of air back into the cell reoxygenated the haemoglobin. Reoxygenation (during which the absorbance and pO_2 measurements were recorded) took approx 30 min in the apparatus. Because the total time for the experiments was up to 90 min the absorption spectra were routinely examined before and after the measurements in order to assess the extent of any methemoglobin formation during the experiment (Van Assendelft & Zijstra, 1975).

RESULTS

(*a*) *Absorption spectra of oxy- and deoxyhaemoglobin*

The visible spectra of oxyhaemoglobin and deoxyhaemoglobin are shown in Fig. 1, and the calculated extinction coefficients are displayed in Table 1. An examination of these spectra reveals three major features which are absent from the spectra of human haemoglobin. First, the oxyhaemoglobin spectrum exhibits two extra peaks at 492 and 626 nm. Second, the oxyhaemoglobin peak at 537 nm is significantly higher than that at 572 nm. Third, the major deoxyhaemoglobin peak at 553 nm is particularly pronounced.

(*b*) *Oxygen–haemoglobin equilibria*

The series of curves determined over the pH range 6–7.4 is shown in Fig. 2. The Hill plot for dugong haemoglobin at pH 7.0 and 30°C which is shown in Fig. 3 is typical of all the Hill plots drawn for this haemoglobin. The P_{50} and *n* values, determined from the oxyhaemoglobin equilibrium curves and the Hill plots, respectively, are shown in Table 2.

DISCUSSION

The oxygen–haemoglobin equilibria reported here have some unusual features. First, the curves do not show the pronounced sigmoidality of human haemoglobin under the same conditions. An observation quantified by a mean value of *n*, the slope of the Hill plot, of 1.7 compared to a value of almost 3 for human haemoglobin. Second, the oxygen affinities as measured by the P_{50} are rather high, for example at pH 7.4 and 30°C, dugong haemoglobin has a P_{50} of 6.2 mm Hg, while human haemoglobin under the same experimental conditions and measured in the same apparatus gave a value of 10.2 mm Hg. Third, the Bohr effect, defined as $\phi = \Delta\log P_{50}/\Delta\text{pH}$, is not

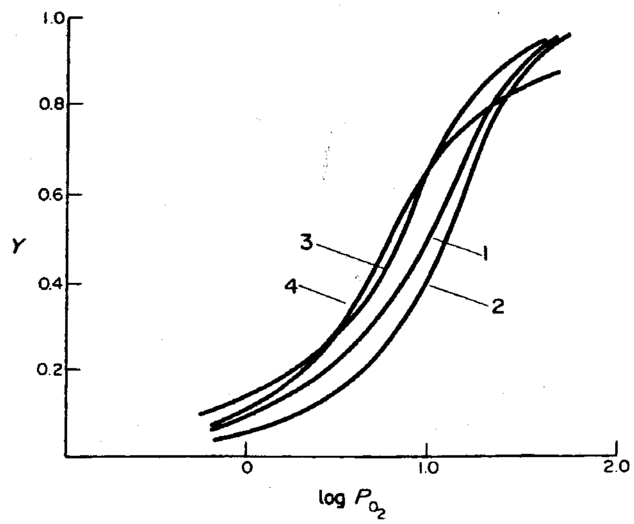

Fig. 2. Effect of pH on the oxygen–haemoglobin equilibrium curve. Haemoglobin 0.1% in 0.1 M phosphate buffer at 30°C. Y is the fraction oxygenated. Curves 1–4 obtained at pH 6.0, 6.5, 7.0, 7.4, respectively.

Table 1. The calculated extinction coefficients for a 1 g/l solution of dugong haemoglobin

Wavelength	Oxyhaemoglobin	Deoxyhaemoglobin
500	0.36	0.57
520	0.50	0.53
540	0.64	0.76
560	0.42	0.86
580	0.47	0.60
600	0.20	0.24
620	0.19	0.16
640	0.16	0.13
660	0.07	0.10

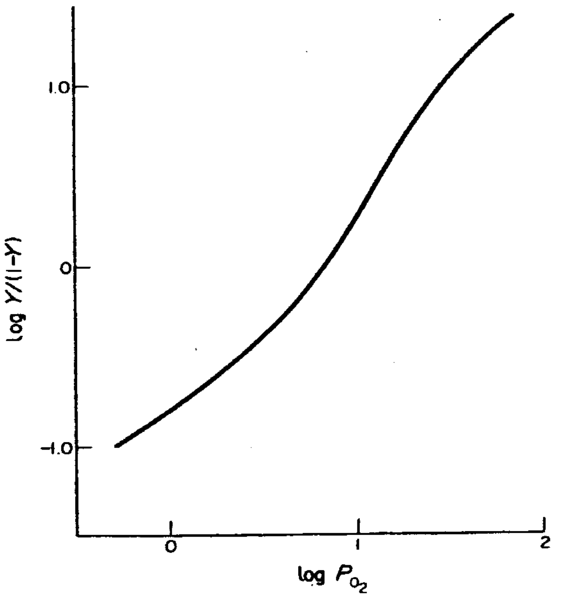

Fig. 3. Hill plot of oxygen–haemoglobin equilibrium curve of dugong haemoglobin at pH 7.0 and 30°C.

very marked, with ϕ values of −0.13 being obtained for the pH range 7.4–7.0 at 20°C. Human haemoglobin measured under the same conditions gave a ϕ value of −0.49.

In view of the modest diving ability of the dugong it seems unlikely that there has been any profound modification of the blood–oxygen dissociation curve; however, it is possible that there could be a shift of the blood–oxygen equilibrium to the left of that for human blood. Such a shift would be in keeping with the known blood–oxygen equilibrium characteristics of the Florida manatee, *Trichechus manatus latirostris*, which are considerably left shifted (White *et al.*, 1976), and with the observations of Turek *et al.* (1973), who have pointed out that a left-displaced oxyhaemoglobin equilibrium may actually favour oxygen release in conditions of acute hypoxia. Thus the low P_{50} of the dugong haemoglobin may reflect such a left shift of the blood–oxygen equilibrium. More difficult to explain is the low value for n, the Hill coefficient. A sample of similarly treated human haemoglobin in the same apparatus gave a value for the Hill coefficient of 3.0, and thus the possibility of a systematic apparatus error can be ruled out. It is well known that mammalian haemoglobin generally will tend to dissociate into dimer and eventually monomer forms under certain conditions, generally of low haemoglobin concentration, high salt concentration and low pH (Field & O'Brien, 1955; Rossi-Fanelli *et al.*, 1961; Briehl, 1970). For human haemoglobin at least, dilution does not cause significant dissociation at concentrations of 0.1% (Wyman, 1964). Wold (1971) has summarized the binding characteristics of different haemoglobin derivatives and states that the values for n are 1, 2.6 and 2.8 for the haemoglobin monomer, dimer and tetramer, respectively. Thus the recorded value for dugong haemoglobin of 1.7 can be interpreted as suggesting the possibility of an equilibrium between monomer, dimer and tetramer occurring to an appreciable extent at this concentration. The purification of the haemoglobin solution on Sephadex G-150 produced a single haemoglobin band which was presumably a system of monomer, dimer and tetramer in rapid equilibrium. Field & Ogston (1955) have shown that a homogeneous band results from the migration of haemoglobin solution despite the presence of several components in rapid equilibrium with each other. Thus the chromatography could not have resolved the dugong haemoglobin into its constituent subunits. At no stage during its preparation was the haemoglobin subjected to high salt concentration or low pH, and thus if the haemoglobin is indeed partly dissociated it must be an intrinsic property of dugong haemoglobin which deserves further investigation. It is possible that during adaptation to its tropical diving existence, the haemoglobin of the dugong has undergone some structural modifications which allow subunit dissociation more readily in dilute solution. This would not produce any physiological disadvantages since the concentration of haemoglobin is normally high inside the erythrocyte. The small observed Bohr effect for the dugong haemoglobin may also be explained if the subunit equilibrium is displaced towards the monomer.

Table 2. Values of P_{50}, n and ϕ determined from the oxygen equilibrium curves of dugong haemoglobin. All values were obtained using 0.1% haemoglobin solution in 0.1 M phosphate buffer at 30°C

pH	log P_{50}	n	ϕ
7.4	0.79	1.45	
			−0.13
7.0	0.84	1.73	
			−0.48
6.5	1.08	1.85	
			+0.20
6.0	0.99	1.82	

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