1973

The Oxygen Equilibrium Properties of Hemerythrin in Solution and in Coelomic Cells of Phascolopsis gouldi

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THE OXYGEN EQUILIBRIUM PROPERTIES OF HEMERYTHRIN IN SOLUTION AND IN COELOMIC CELLS OF *PHASCOLOPSIS GOULDI*

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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by

Maria Kondon

1973

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts

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Approved, November 1973

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ABSTRACT

The respiratory pigment hemerythrin is contained within coelomic cells of the sipunculid Phascolopsis gouldi. The oxygen equilibrium properties including the Hill constant n and p50 of this pigment in coelomic cells and in solution were studied. For cell suspensions, the oxygen affinity of the pigment is moderately high and the shape of the oxygen equilibrium curve is slightly sigmoid. No Bohr effect is observed at pH 7.0, 7.4, and 7.8. For solutions, the oxygen affinity of the pigment is higher than that found for cells at pH 7.4 and pH 7.8. The curve is hyperbolic. A Bohr effect is reported between pH 7.4 and pH 7.0. These differences between the properties of the pigment in cells and in solutions are pronounced and must, therefore, be considered in O_2 equilibrium studies.

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ACKNOWLEDGMENTS

The writer wishes to express her appreciation to Dr. Charlotte P. Mangum, under whose guidance this investigation was conducted, for her patient guidance and criticism throughout the investigation. The author is also indebted to Drs. Robert E. L. Black and Eric L. Bradley for their careful reading and criticism of the manuscript.

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INTRODUCTION

Distribution of the respiratory pigment hemerythrin among animal phyla includes the sipunculids, some priapulids, the brachiopod Lingula and the polychate annelid Magelona (Prosser and Brown, 1961). It is generally accepted that the Sipunculida and Annelida are closely related phylogenetically. In the sipunculid worm Phascolopsis (=Golfingia) gouldi, the pigment for the most part is contained within the coelomic cells which move about within the coelomic cavity (Hyman, 1959). Very small quantities are reported to occur in the tentacular region of the animal (Manwell, 1963).

The habitat of Phascolopsis gouldi is intertidal, lying just below the low tide mark. The animal burrows in the sand by means of its proboscis (Hyman, 1959). The O_2 availability in such an environment is not constant; at low tide, ventilation is presumably impossible and, therefore, the oxygen level in the microhabitat is low, whereas at high tide, ventilation is believed to occur (Mangum, unpublished). A possible function of the pigment hemerythrin is oxygen storage which is possibly more important to an organism in such an environment than oxygen transport (Manwell, 1960b; 1964). Oxygen consumption studies on the Australian sipunculid Dendrostomum cymodoceae reveal that the oxygen carried on the pigment of animals kept under oil is "consumed" after six hours (Edmonds, 1957).

Oxygen equilibrium properties of the coelomic pigment found

in Phascolopsis gouldi have been reported in the literature. According to Kubo (1953) at pH 6.80 and 20° C., the p50 of the pigment in solution is approximately 2.76 mmHg. The Hill constant n is 1.35. Values close to these were also found at pH 6.26 and 8.80. He, therefore, concluded that there is no Bohr effect. Manwell (1963) found that at pH 7.4 and 25° C., the p50 of a solution consisting of electrophoretically "fast" coelomic hemerythrin and "slow" coelomic hemerythrin is approximately 4 mmHg. Marrian (1926) and Love (1957) had each observed that the oxygen affinity of the pigment in solution is rather high. No oxygen equilibrium in interpretable units were reported.

Previously, all studies of oxygen equilibrium of Phascolopsis gouldi were done on solutions of hemerythrin. The properties of the pigment contained within the intact cell are unknown. It is reasonable to assume that intracellular processes may affect the interaction of hemerythrin and oxygen. Therefore, the present study was undertaken to compare oxygen equilibrium properties of isolated hemerythrin in solution and hemerythrin within the intact cell at various pH values, including pH 7.0, found in the low oxygen environment (Sverdrup et al., 1942).

MATERIALS AND METHODS

The organism Phascolopsis gouldi was obtained from Woods Hole, Massachusetts, and kept in the laboratory at 15° C. and 31°/oo salinity. Approximately 3 ml. of coelomic fluid containing gametes and coelomic cells was removed with a 24 gauge hypodermic needle and syringe and centrifuged. This centrifugation separated the suspension into a layer of packed dark red coelomic cells at the bottom, a layer of gametes, and a layer of coelomic fluid lying on top. The coelomic fluid and the gametes were drawn off and discarded. Sea water of 31°/oo salinity was added to the original volume and the suspension was again centrifuged. This procedure was carried out three times, resulting in the collection of red coelomic cells relatively free of gametes.

Cell suspensions of the coelomic cells containing hemerythrin were prepared at pH 7.0, 7.4, and 7.8 using a borate buffer (0.05 M H_3BO_3-NaOH) in Molluscan (Marine) Ringer's solution prepared as outlined in Formulae and Methods V of The Marine Biological Chemical Room. Added to 0.9 ml. of coelomic cell suspension was 9.1 ml. of buffer. The 10 ml. suspension was placed in a 625.0 ml. tonometer stoppered with a three-way stopcock. Evacuation was carried out for 2- to 3-minutes and N_2 gas (99.9 percent purity, extra dry; Matheson Gas Company) was injected into the tonometer through a serum stopper until a slight excess in pressure was obtained. The stopcock was then opened and

the excess gas released. The tonometer was placed in a water bath (15° C.) where it was rotated for 15 minutes. This procedure was repeated several times until the color of the suspension changed from violet-red (oxygenated state) to yellow-white (deoxygenated state). Upon deoxygenation, 0.2 ml. sample was removed with a 24 gauge hypodermic needle through the serum stopper and drawn into an extractor syringe. By use of the Scholander microvolumetric gas analysis technique described by Hoffmann and Mangum (1970), the volume of O_2 was determined. The same analysis was performed after injections of the following quantities of water-saturated air: 1.0 ml., 2.0 ml., 4.0 ml., 8.0 ml., and an additional 8.0 ml. Following the injection and prior to the analysis, the suspension was allowed to equilibrate for 15- to 20-minutes as the tonometer rotated in the water bath. Finally, the suspension was exposed to the atmosphere, allowed to equilibrate, and its O_2 content analyzed. A low oxygen blank was prepared by bubbling N_2 through distilled water. This blank measurement estimates the volume of O_2 present in the reagents being used and is, therefore, subtracted from each of the readings obtained. pH was measured before and after each experiment; no changes in pH occurred.

Solutions of hemerythrin were prepared at pH 7.0, 7.4, and 7.8 using a phosphate buffer (1/15 M KH_2PO_4-Na_2HPO_4). The pH of the buffer was measured at 15° C. (Radiometer Blood Micro System BMS 1). Approximately 0.7 ml. of buffer was added to 0.3 ml. of red coelomic cells. The cells were lysed and centrifuged. The centrifuged

solution of buffer and coelomic cell inclusions was added to a polyacrylamide gel filtration column, 30 centimeter in length, consisting of Cal Bio-Gel P-100 supported with glass beads. The molecular weight of Phascolopsis gouldi hemerythrin is 107,000 (Klotz and Keresztes-Nagy, 1963). This filtration ensured the collection of a hemerythrin solution relatively free of other cellular inclusions, which may have an effect on the oxygen equilibrium properties. The pinkish solution was collected from the column and buffer was added to the 10 ml. mark. The 10 ml. solution was then placed in the tonometer. Deoxygenation proceeded as described previously. The solution was allowed to equilibrate for 15 minutes at each deoxygenation step. An optical density measurement was made using a Bausch and Lomb Spectronic 20 Colorimeter when the color of the solution had become clear. The wavelength was set at 467 mμ, used by Kubo (1953). Deoxygenation was continued until three consistent O.D. readings were made. Subsequent O.D. readings were made after injections of the following quantities of water-saturated air: 2.0 ml., 4.0 ml., 8.0 ml. and an additional 8.0 ml. of air. Prior to each measurement, the solution was allowed to equilibrate for 20 minutes, as the tonometer rotated in the water bath. A reading was made 10 minutes after injection of air, then in successive 5-minute intervals until three consecutive readings were consistent. pH was measured before and after each experiment; no changes occurred.

On the average, six experiments for cell suspensions and six for solutions were made at each pH. The percent saturation Y of the pigment and the corresponding oxygen partial pressure were calculated.

Y/100-Y and oxygen partial pressure were plotted according to Barcroft and Hill (1910). The Hill equation constants $\underline{n}$ and p50 were determined. p50 represents the oxygen partial pressure at which 50 percent of the pigment is oxygenated and is, therefore, an indication of the oxygen affinity of the pigment. The Hill constant $\underline{n}$ is determined as the slope of the line of the Hill plot and is an indication of the degree of molecular interaction. The data for each pH have been combined since the results of separate linear regression analyses are homogenous ($p > .05$, F test).

Oxygen carrying capacity of the coelomic fluid was determined using the Scholander microvolumetric gas analysis technique (Hoffman and Mangum, 1970). Coelomic fluid taken from four animals was allowed to equilibrate with the atmosphere while constantly mixed for 25 minutes.

RESULTS

Oxygen Equilibrium Curves of Cell Suspensions

Oxygen equilibrium of coelomic cell hemerythrin appears in Tables I and II and Figures 1A, 1B, and 1C. The coefficients of determination (r^2) are much lower for cell suspensions than for solutions of hemerythrin.

Two phases of the oxygen equilibrium curves of cell suspensions are observed and linear regression analysis has been performed on each (Table I, Figures 1A, 1B, and 1C). The slope n of the major phase is significantly steeper ($p < .05$) than that of the other phase. The overlapping of 95 percent confidence intervals (L_1 and L_2) is the criterion used. The slope of the first phase is extremely flat. There is no significant variation in this slope at pH 7.0, 7.4, and 7.8. The values approximate 0.06 (Table I). The change in slope occurs before the p50 is reached. For plots of the second phase of the equilibrium curves, the value n is greater than 1.0 at pH 7.0, where n is 1.0660, and at pH 7.8, where n is 1.500. At pH 7.4, n is 0.9338, very close to 1.0 (Table I). Again, there is no significant variation in n at the three pHs.

At pH 7.0, 7.4, and 7.8, the coordinate points of pO_2 and Y/100-Y at which the change in slope occurs are not significantly different (Table II). The pO_2s are similar and approximate 0.95 mmHg;

TABLE I

REGRESSION ANALYSIS OF O_2 EQUILIBRIUM CURVES OF CELL SUSPENSIONS

(± 95% CONFIDENCE INTERVALS)

pH	N	Hill constant $\underline{n}$	$L_1{}_{\underline{n}}$	$L_2{}_{\underline{n}}$	r^2	p50 (mmHg)	$L_1{}_{p50}$	$L_2{}_{p50}$
First Phase								
7.0	18	0.0322	-0.0090	0.0733	0.1465			
7.4	21	0.0777	0.0276	0.1278	0.3570			
7.8	20	0.0831	0.0402	0.1259	0.4798			
Second Phase								
7.0	22	1.0660	0.8746	1.2575	0.8709	1.80	1.50	2.20
7.4	18	0.9338	0.4961	1.3715	0.5611	2.00	1.35	2.80
7.8	18	1.5000	1.0757	1.9242	0.7783	1.85	1.50	2.25

TABLE II

pO_2 AND Y/100-Y COORDINATE POINTS AT WHICH CHANGE IN SLOPE OF CELL SUSPENSION CURVES OCCURS (± 95% CONFIDENCE INTERVALS)

pH	pO_2 (mmHg)	$L_1{}_{pO_2}$	$L_2{}_{pO_2}$	Y/100-Y	$L_1{}_{Y/100-Y}$	$L_2{}_{Y/100-Y}$
7.0	0.83	0.70	0.95	0.44	0.32	0.58
7.4	0.70	0.48	0.95	0.37	0.25	0.52
7.8	1.30	0.95	1.60	0.57	0.41	0.79

Figures 1A, 1B, and 1C

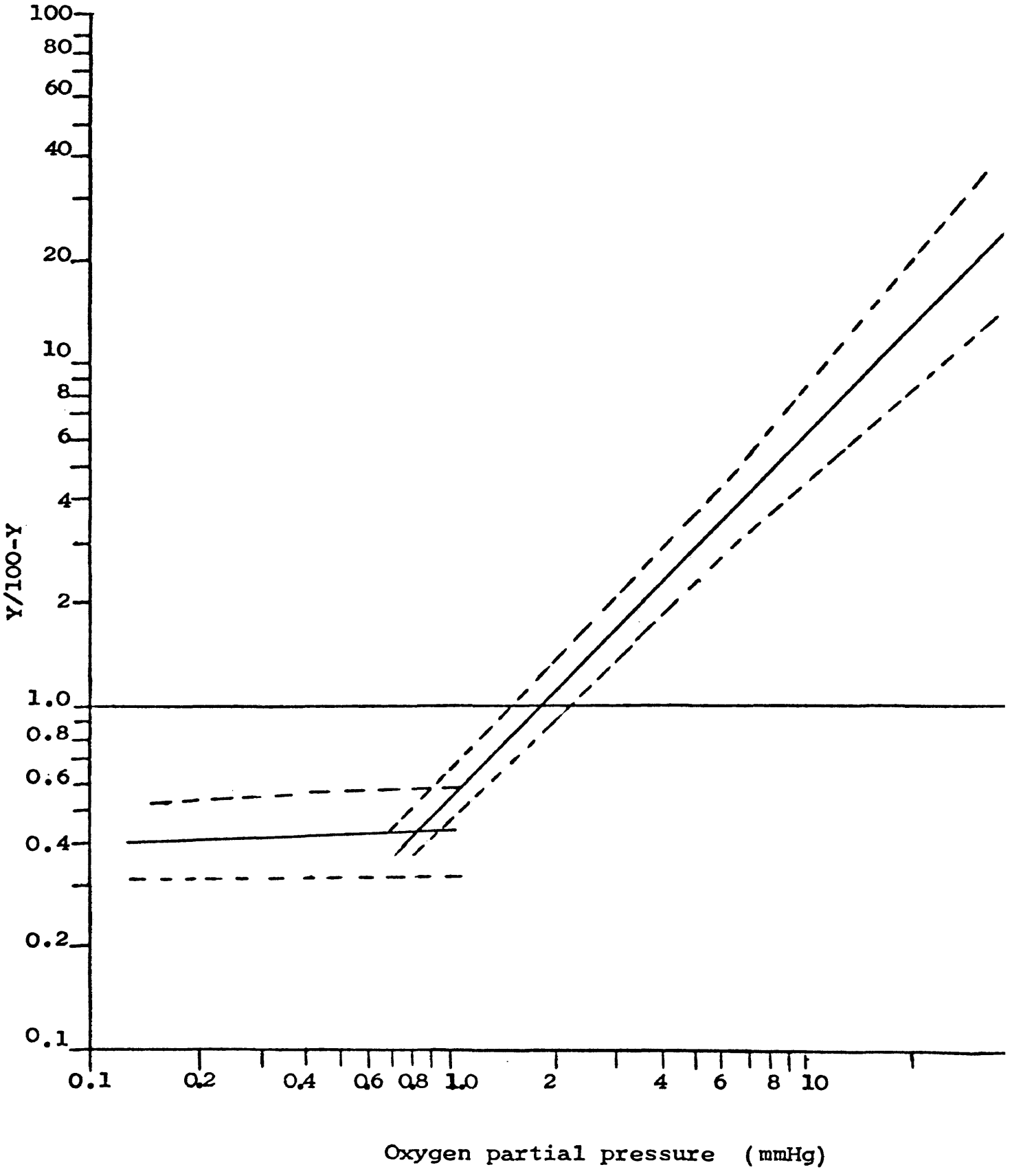
Regression lines of O_2 equilibrium curves of cell suspensions at 15° C. 95 percent confidence belts appear as broken lines. Y is the percent saturation.

1A pH 7.0

1B pH 7.4

1C pH 7.8

Figure 1 A.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Y/100-Y
0.1
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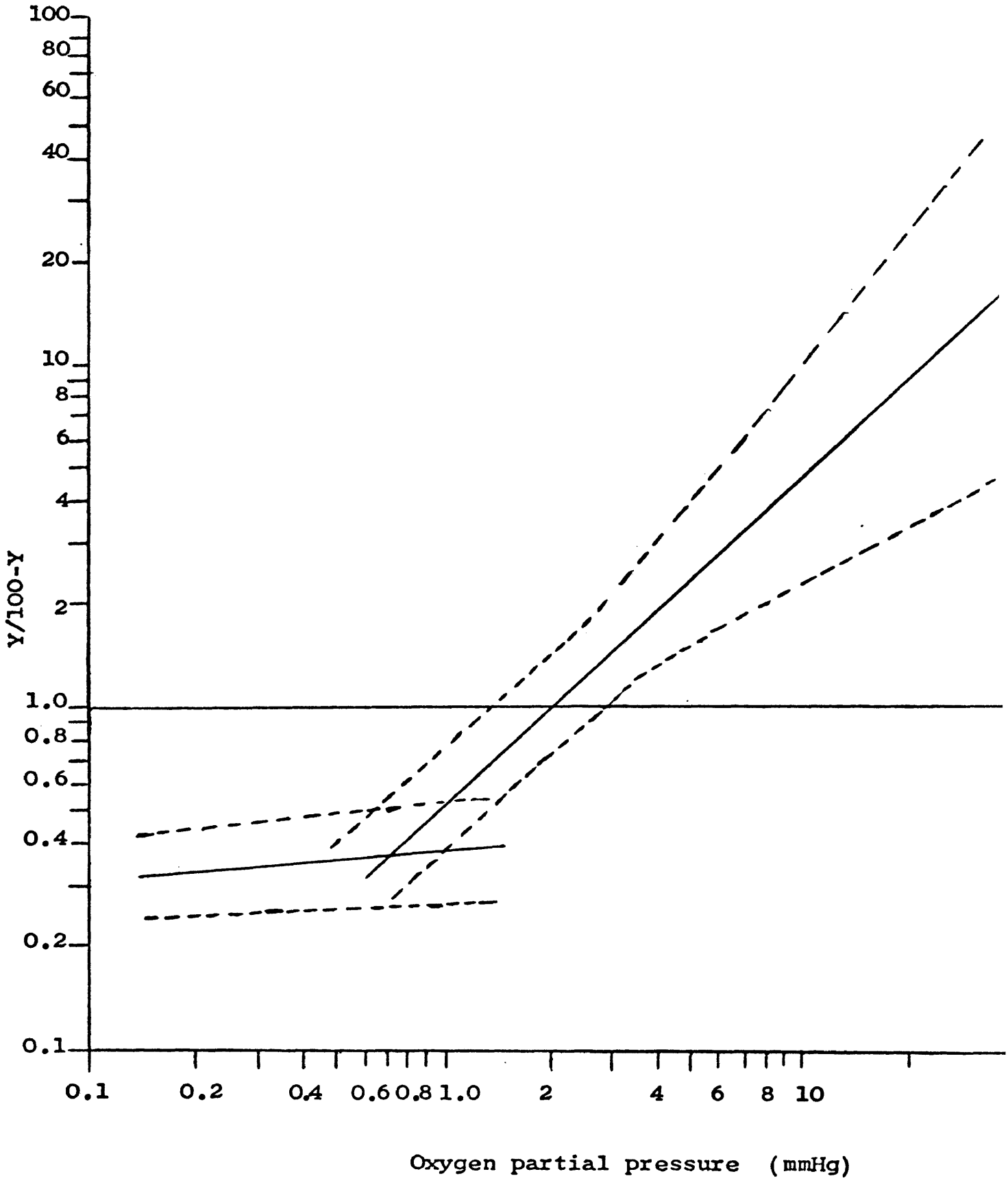
10
Oxygen partial pressure (mmHg)

Figure 1 B.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Y/100-Y
0.1
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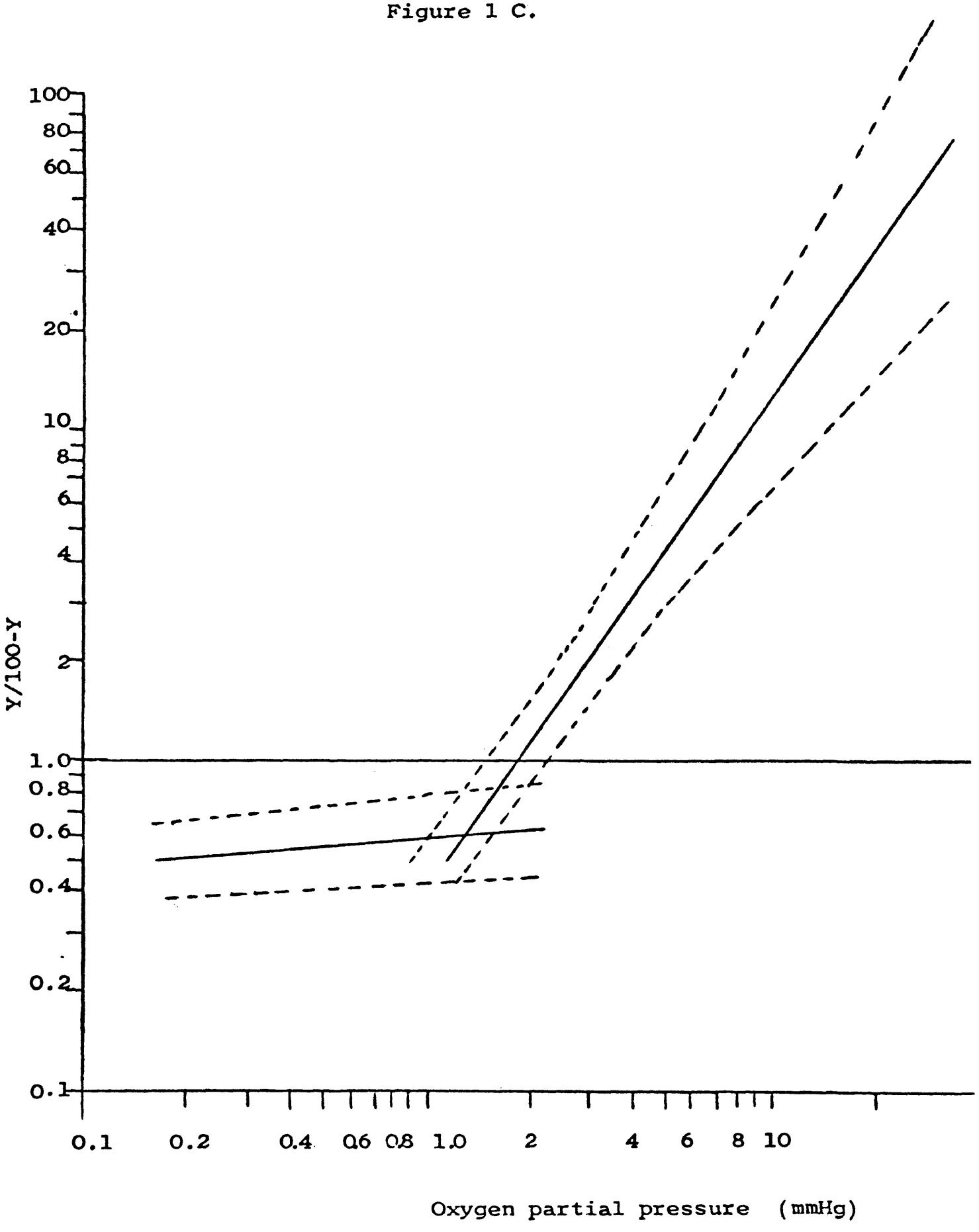
Oxygen partial pressure (mmHg)

Figure 1 C.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Oxygen partial pressure (mmHg)

the Y/100-Y values are also similar and approximate 0.46.

The fairly high O_2 affinity of this pigment is evidenced by the low p50s (Table II, Figures 1A, 1B, and 1C). At pH 7.0, 7.4, and 7.8, the p50s are 1.80, 2.00, and 1.85 mmHg, respectively. No change in p50 is accompanied by a change in pH (Table II, Figure 2).

Oxygen Equilibrium Curves of Solutions

The slopes of the oxygen equilibrium curves of solutions at pH 7.0, 7.4, and 7.8 are fairly close to 1.0 and are not significantly different (Table III, Figure 3). At pH 7.0, n is 0.9750. At pH 7.4, n is 1.0187. At pH 7.8, n is 1.0226. These n values do not differ significantly from those reported for cell suspensions at pH 7.0 and 7.4. Only at pH 7.8 does n for cells differ from those reported for solutions.

There is significant variation between p50 found at pH 7.0 and that found at pH 7.4 and 7.8. No significant difference in p50 is noted between pH 7.4 and 7.8. This decrease in O_2 affinity is thought to be an example of the Bohr effect. This result contrasts with what was reported for cell suspensions. In general, p50s found for solutions differ significantly from p50s found for cells at pH 7.0, 7.4, and 7.8. p50s for cells tend to be higher than for solutions. An exception occurs at pH 7.0 where p50 for solutions is similar to that found for cells.

The effect of the state of hemerythrin, that is, whether the pigment is in solution or in the intact cell, and pH on the oxygen affinity of hemerythrin is greater than the effect on the Hill constant n.

Figure 2

p50 versus pH for cell suspensions and solutions.

Figure 2.

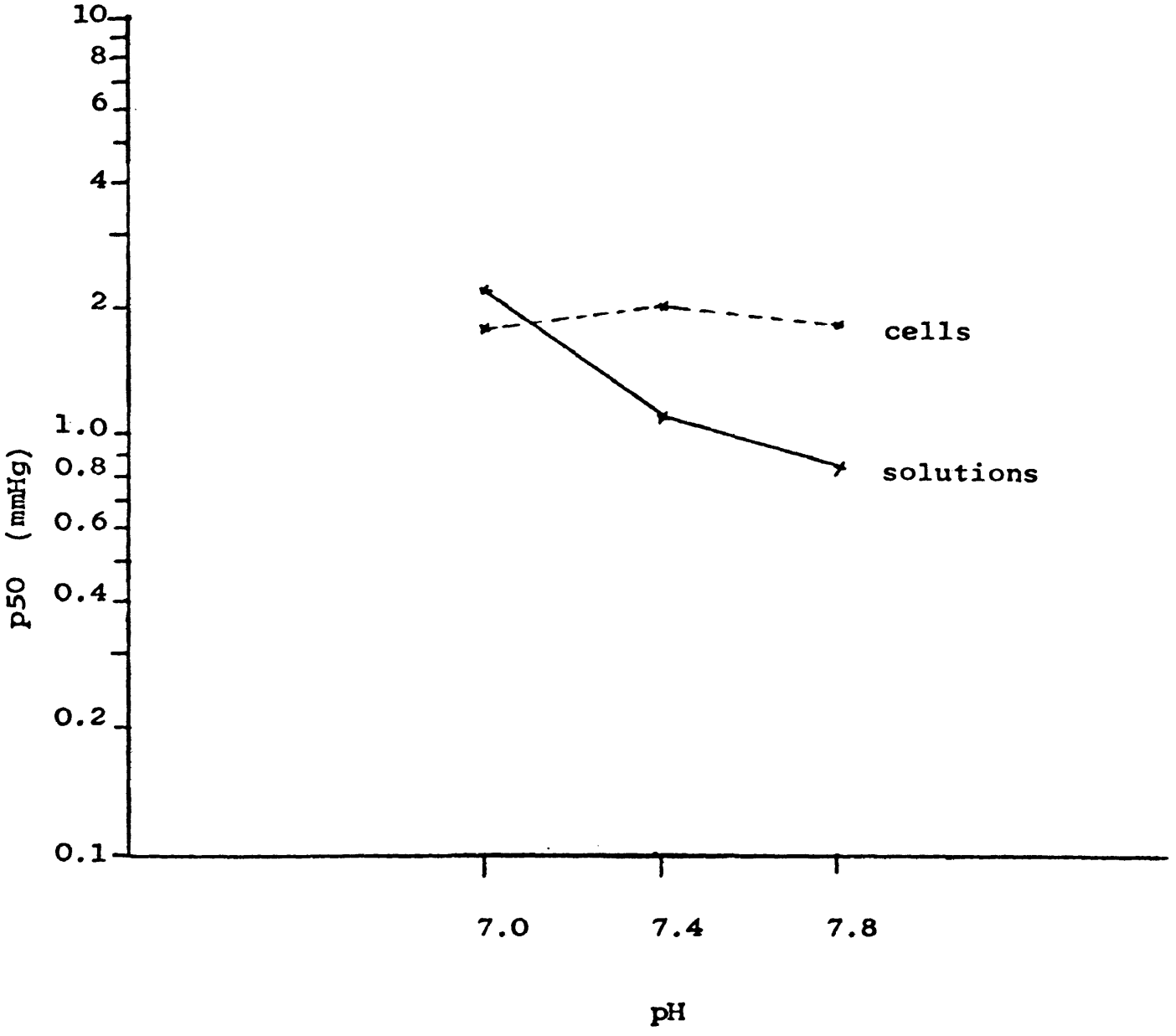


TABLE III

REGRESSION ANALYSIS OF O_2 EQUILIBRIUM CURVES OF SOLUTIONS

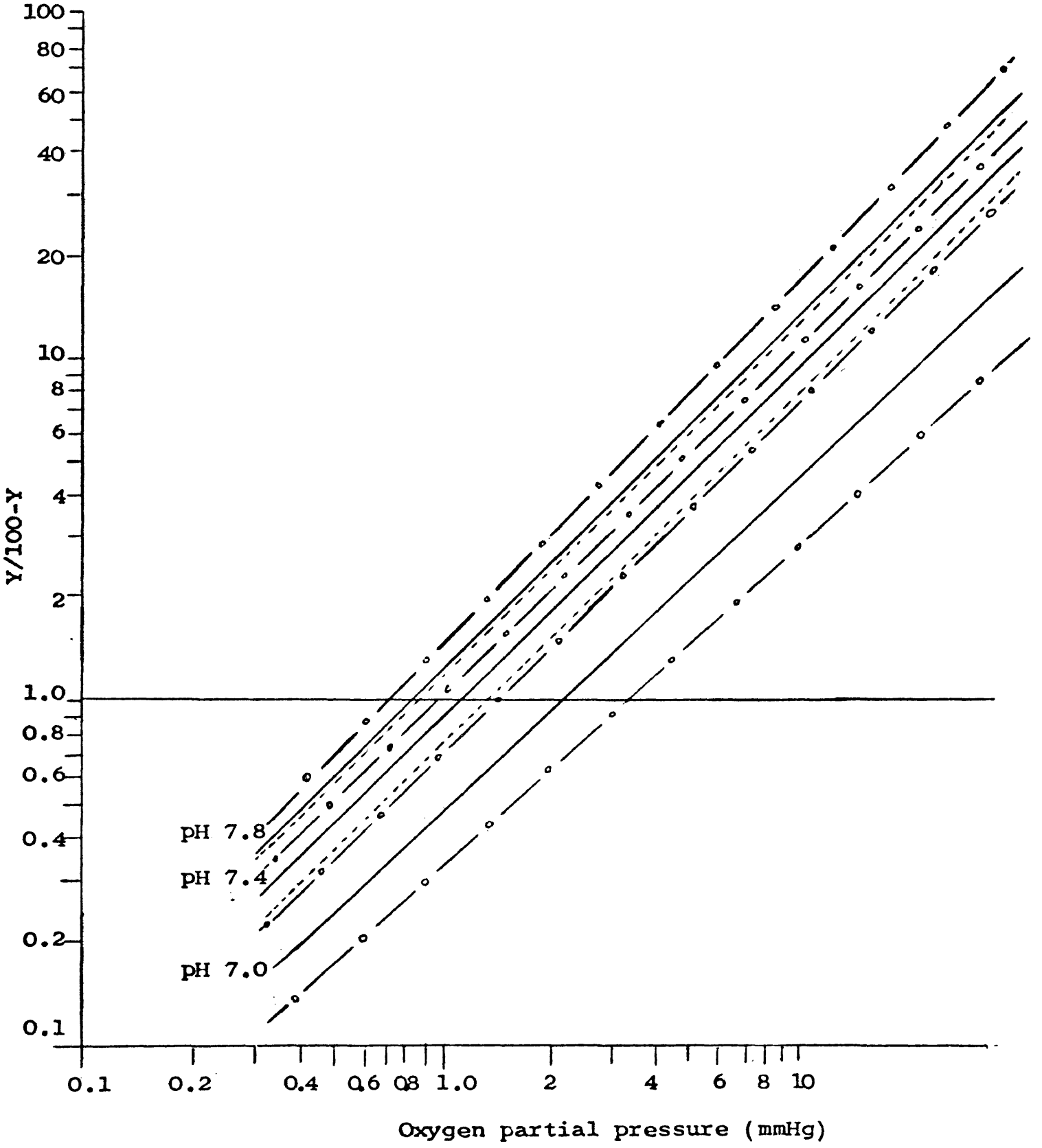
(± 95% CONFIDENCE INTERVALS)

pH	N	Hill constant $\underline{n}$	$L_1{}_{\underline{n}}$	$L_2{}_{\underline{n}}$	r^2	p50 (mmHg)	$L_1{}_{p50}$	$L_2{}_{p50}$
7.0	30	0.9750	0.9072	1.0428	0.9687	2.20	1.45	3.20
7.4	35	1.0187	0.9845	1.0528	0.9911	1.10	0.95	1.35
7.8	30	1.0226	0.9927	1.0526	0.9943	0.85	0.73	0.98

Figure 3

Regression lines of O_2 equilibrium curves of solutions at 15° C. and pH 7.0, 7.4, and 7.8; 95 percent confidence belts appear as broken lines; Y is the percent saturation.

Figure 3.


100
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40
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8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.1
Y/100-Y
pH 7.8
pH 7.4
pH 7.0
0.1
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
Oxygen partial pressure (mmHg)

DISCUSSION

When comparing the oxygen equilibrium properties of Phascolopsis gouldi with those of other sipunculids and intertidal organisms, the effects of temperature as well as pH on these properties must be considered. A definite effect of temperature on the oxygen equilibrium properties of hemerythrin of Phascolopsis gouldi has been known for some time (Marrian, 1926). A decrease in temperature produces a decrease in p50. The p50s presented in this study were obtained at 15° C., and, in general, are lower than those reported in the literature at temperatures of approximately 20° C. In addition to the studies on Phascolopsis gouldi previously mentioned, other hemerythrins have been investigated. Solutions of coelomic hemerythrin of Siphonides ingens at 18° C. and pH 8.70, 7.60, 7.10, and 6.03 have p50 values of 4-5 mmHg. In this organism, most of the gas exchange occurs through the cuticle as in Phascolopsis gouldi (Manwell, 1960a). Studies of solutions of Dendrostomum pyroides hemerythrin showed that at 25° C. and pH 7.5, p50 is 4-5 mmHg and n is 1.01. The physical properties such as amino acid composition and molecular weight of Dendrostomum pyroides are very similar to those of Phascolopsis gouldi hemerythrin (Ferrell and Kitto, 1970).

The Hill constant n is found to be fairly close to 1.0 for solutions of Phascolopsis gouldi hemerythrin at pH 7.0, 7.4, and 7.8 and for cell suspensions at pH 7.4. For cell suspensions at pH 7.0

and 7.8, n is only slightly greater than 1.0. Through spectrophotometric titration, n of Phascolopsis gouldi hemerythrin was found to be 1.16 at 25° C. and pH 6.5-7.5 (Keresztes-Nagy and Klotz, 1957). This evidence implies there is a small degree of interaction between the oxygen binding sites on the molecule.

The p50 values for solutions of hemerythrin in the present study show a decrease in oxygen affinity of the pigment between pH 7.4 and 7.0. In previous studies of Phascolopsis gouldi and other sipunculids, the absence of a Bohr effect has been reported. Electrophoretic mobility studies as well indicate no Bohr effect in hemerythrin of Phascolopsis gouldi (Keresztes-Nagy and Klotz, 1957). An additional example is the absence of a Bohr effect in Sipunculus nudus reported by Florkin (1933). The pH value of 7.0 at which the Bohr effect was found to occur in the present study had not been previously tested.

In contrast to the Bohr effect observed for solutions of Phascolopsis gouldi hemerythrin, no such effect is observed for suspensions of cells containing hemerythrin at pH 7.0, 7.4, and 7.8. An absence of Bohr effect implies there is no decrease in oxygen affinity of the respiratory pigment with a decrease in pH. Therefore, under low oxygen conditions, oxygen is not released from the pigment at a greater rate. Instead, a possible means of survival may be through a decrease in oxygen consumption rate as is reported for Dendrostomum cymodoceae. The oxygen available on the pigment supports the organism Dendrostomum cymodoceae for approximately six hours (Edmonds, 1957).

From the results presented here, it may be concluded that the

major function of Phascolopsis gouldi hemerythrin is not one of oxygen transport, but one of oxygen storage. The fairly high oxygen affinity and very slightly sigmoid-shaped curve have been generally reported for intertidal organisms. During periods of low tide when the oxygen level in the microhabitat becomes quite low, the organism is ensured oxygen. Oxygen equilibrium studies on the whole animal would give a more accurate description than that reported here of the role of hemerythrin in relation to the organism's environment.

The oxygen equilibrium properties of Phascolopsis gouldi hemerythrin in cell suspensions differ from those in solutions. The change in slope occurring in the cells does not occur in solutions. This change in slope has not been previously reported for Phascolopsis gouldi. A much more pronounced change than this has been observed for solutions of brachiopod hemerythrin at very high pH values (Manwell, 1964). A similar diphasic nature was ascribed to oxygen equilibrium curves of some sipunculid hemerythrin solution at very low pO_2s. The explanation suggested for such a phenomenon is that under very low pO_2s, there is little chance for more than one oxygen molecule to bind to one hemerythrin molecule (Manwell, 1964).

The p50 values at pH 7.4 and 7.8 are somewhat higher in cells than in solutions. At pH 7.4, the p50 for solutions is 1 mmHg and for cells is 2 mmHg. Differences greater than this are reported for Dendrostomum zostericulum; for which p50 of coelomic hemerythrin in solution is 3.5 mmHg and the p50 of coelomic hemerythrin in cell suspensions is 4-5 mmHg; and the p50 of vascular hemerythrin in

solution is 15 mmHg and the p50 of the vascular type in cell suspensions is 40-50 mmHg (Manwell, 1960a). The observation of differences between human erythrocyte and hemoglobin study led to the discovery of the substance 2,3-DPG, which facilitates the release of O_2 from the hemoglobin molecule (Benesch and Benesch, 1969). The present study demonstrates that, indeed, Phascolopsis gouldi hemerythrin behaves differently within cell suspensions as compared with solutions. Therefore, it may be concluded that some intracellular processes affect the interaction of oxygen and this pigment.

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