THE STRUCTURES AND OXYGEN EQUILIBRIUM CHARACTERISTICS OF THE TWO HEMOGLOBINS OF THE TEREBELLID POLYCHAETE THELEPUS CRISPUS JOHNSON

by

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INTRODUCTION

Vertebrate hemoglobins are in most cases tetrameric proteins which reversibly bind oxygen in order to transport it from respiratory surfaces to tissues located more deeply within the animal. Each polypeptide chain of vertebrate hemoglobin is of molecular weight ca. 16,000 and contains one heme group. The molecule is carried within erythrocytes which are suspended in the blood plasma and pumped through a well defined vascular system. The structures and functions of annelid hemoglobins are beginning to be understood and seem to be more diverse in nature than those of vertebrate hemoglobins.

Annelid vascular hemoglobins, also called erythrocruorins, are high molecular weight oligomeric proteins which are carried in solution with the extracellular vascular plasma. The erythrocruorins are of molecular weight ca. 3×10^6 (Svedberg, 1933; Svedberg and Eriksson-Quensel, 1934; Chew et al., 1965; Yamaghishi et al., 1966; Rossi-Fanelli et al., 1970; Swaney and Klotz, 1971; Waxman, 1971; Wiechelman and Parkhurst, 1972), and possess a characteristic subunit assembly. It has been demonstrated that most annelid vascular hemoglobin molecules contain twelve major subunits, or submultiples, of molecular weight ca. 250,000 each (Waxman, 1971; Wiechelman and Parkhurst, 1972). However, Chew et al. (1965) indicate a putative one-sixteenth submultiple for the erythrocruorin of Marphysa sanguinea. Electron micrographs indicate

also that the erythrocruorin molecules of several annelid species contain twelve polygonal particles arranged at the vertices of two planar hexagons, one situated above the other, and with a central open space (Roche, 1965; Yamaghishi et al., 1966), or with the central space containing a protein core (Levin, 1963).

Most workers agree that native erythrocruorin molecules from several annelid species contain twelve submultiples, but uncertainty arises as to the quaternary structure of the one-twelfth submultiple. Yamaghishi et al.(1966) interpret the structure of the submultiple of Limnodrilus gotoi vascular hemoglobin as a nonomer with three groups of three protomers comprising the intact submultiple. The erythrocruorin of Lumbricus terrestris is thought either to have a submultiple which is composed of three major subunits which are in turn composed of four monomers each (Rossi-Fanelli et al., 1970), or to have a submultiple which is composed of four major subunits which are in turn made up of four monomers each (Wiechelman and Parkhurst, 1972), and each monomer is thought by both groups of investigators to contain one heme group. It is proposed therefore that Lumbricus terrestris erythrocruorin has a submultiple which is either a dodecamer (Rossi-Fanelli et al., 1970) or a hexadecamer (Wiechelman and Parkhurst, 1972). Swaney and Klotz (1971) postulate that the submultiple of Cirraformia grandis vascular hemoglobin is a dodecamer with each monomer of molecular weight 18,500 containing one prosthetic group.

From electron micrographs of Eumenia crassa erythrocruorin (Levin, 1963), the one-twelfth submultiple is envisaged as a four-sided diamond shaped particle. The submultiple of Arenicola cristata vascular hemo-

globin (Waxman, 1971) is seen as a tetrahedral array of subunits of molecular weight 54,000 each which contain two hemes and four polypeptide chains each, the polypeptide chains being held together by six disulfide linkages. The primary and tertiary structures of erythrocruorin monomers have not yet been determined.

Oxygen equilibrium studies have been conducted on a number of annelid vascular hemoglobins. The results which emerge are extremely diverse as regards the degree of oxygen affinity, presence or absence of the Bohr effect, and the degree of sigmoidicity, if present, of the erythrocruorins from various annelid species. The vascular hemoglobins of two terebellid polychaetes have been examined. Eupolymnia crescentis (Manwell, 1959a) and Pista pacifica (Terwilliger, personal communication) vascular hemoglobins display low oxygen affinities and no Bohr effect over a presumed physiological pH range. Eupolymnia crescentis erythrocruorin displays a hyperbolic oxygen equilibrium curve while that of Pista pacifica is slightly sigmoid.

Although numerous polychaetes contain hemoglobin within coelomic cells (Eisig, 1887; Svedberg, 1933; Manwell, 1960; Dales, 1964, 1967; Ochi, 1969; Hoffmann and Mangum, 1970; Terwilliger and Koppenheffer, 1973), the structures and oxygen equilibrium properties have been determined for those of only a few polychaete species. Glycera dibranchiata coelomic cell hemoglobin consists of both monomeric and polymeric components with at least five polypeptide chains (Hoffmann and Mangum, 1970; Vinogradov et al., 1970; Seamonds et al., 1971a, b). The amino acid sequence of the polypeptide chain of one monomer shares limited homology with the vertebrate hemoglobins and myoglobins (Li and Riggs, 1971;

Imamura et al., 1972). X-ray chrystallographic methods suggest that the tertiary folding of this protein also resembles that found in vertebrate hemoglobin and myoglobin (Padlan and Love, 1968). Glycera goesii and the capitellid polychaete Notomastus latericeus have coelomic cell hemoglobins of molecular weight ca. 34,000 which appear to be dimeric (Svedberg, 1933). The only coelomic cell hemoglobin of a terebellid polychaete which has been investigated is that of Pista pacifica (Terwilliger and Koppenheffer, 1973) which is composed of at least two monomeric components which differ in amino acid composition.

Oxygen equilibrium properties have been described for the coelomic cell hemoglobins of the polychaetes Travisia pupa (Manwell, 1960) and Glycera dibranchiata (Hoffmann and Mangum, 1970; Mangum and Carhart, 1972; Mizukami and Vinogradov, 1972).

This paper will attempt to discuss the purification, structures, and the oxygen equilibrium characteristics of the two hemoglobins of the terebellid polychaete Thelepus crispus Johnson.

MATERIALS AND METHODS

Thelepus crispus Johnson was collected from under intertidal rocks at Cape Blanco and Cape Arago on the Oregon Coast. The worms were identified according to Johnson (1901) and Hartman and Reish (1950) and were either dissected immediately or maintained under running seawater until needed.

Animals were observed and dissected under a dissecting microscope. Samples of vascular and coelomic fluids were placed on a glass slide and observed under a compound microscope with an occular micrometer attachment.

Hematocrit values were obtained using a standard hematocrit centrifuge and heparinized hematocrit tubes. Samples were centrifuged at 3000 RPM for five minutes and then recentrifuged to assure total packing of the coelomocytes.

Eight medium sized worms were minced in a beaker of cold Tris-HCl buffer, pH 7.0, 0.01 M in $MgCl_2$ and spun for 15 minutes at 500 g in a Sorvall RC2-B refrigerated centrifuge. Prior studies concerning the erythrocruorins of other annelid species suggest that magnesium ion is needed in order to maintain the native oligomeric structure of the molecule (Swaney and Klotz, 1971; Terwilliger, personal communication). The resulting supernatant from low-speed centrifugation contained the vascular hemoglobin and the pellet contained coelomocytes, gametes, and debris.

The vascular hemoglobin was fractionated with ammonium sulfate, adding increments to make the solution successively 25, 50, and 60 percent in ammonium sulfate, and centrifuging the sample for fifteen minutes at 10,000 g after each addition. Thelepus crispus vascular hemoglobin precipitates from a solution 55-60 percent in ammonium sulfate.

The vascular hemoglobin pellet was redissolved in an aliquot of the extraction buffer and applied to a 1.8 x 54 cm column of Sepharose 4-B in equilibrium with the same Tris buffer. Hemoglobin which eluted from this Sepharose column was rechromatographed on a 1.8 x 120 cm column of Sepharose 4-B also in equilibrium with the Tris extraction buffer. The long Sepharose 4-B column had been calibrated previously with the following proteins of known molecular weight: Katharina tunicata hemocyanin (m. w. 4.05×10^6, Terwilliger, personal communication), Pista pacifica vascular hemoglobin (m. w. 3.38×10^6, Terwilliger, personal communication), Lumbricus terrestris vascular hemoglobin (m. w. by Sepharose 4-B chromatography 2.5×10^6, Wiechelman and Parkhurst, 1972), and Cancer magister hemocyanin (m. w. main fraction 938,000, Ellerton et al., 1970).

T. crispus erythrocruorin which had eluted from the long Sepharose column was saved for determination of the iron content of this pigment. Erythrocruorin from the short Sepharose column was used in subunit molecular weight determinations and for oxygen equilibrium experiments.

To determine the molecular weight of one possible subunit of the vascular hemoglobin, an aliquot of purified pigment was dialysed in the cold for 24 hours against frequent changes of 0.1 M glycine-NaOH

buffer, pH 10.4, 0.01 M in disodium-ethylenedinitrilotetracetic acid (disodium-EDTA), and then for an additional 24 hours against 0.1 M Tris-HCl buffer, pH 7.0, 0.01 M in disodium-EDTA and applied to a 1.8 x 120 cm column of Sephadex G-200 in equilibrium with the same Tris-EDTA buffer. The column had been calibrated previously with Blue Dextran, Pista pacifica erythrocruorin one-twelfth submultiple (m. w. 280-290,000, Terwilliger, personal communication), bovine catalase (m. w. 230,000), rabbit muscle aldolase (m. w. 160,000), bovine serum albumin (m. w. 68,000), ovalbumin (m. w. 43,000), and sperm whale metmyoglobin (m. w. 17,816). A second aliquot of T. crispus vascular hemoglobin also treated in the previous manner was chromatographed on a 1.8 x 120 cm column of Sephadex G-75 (Superfine) in equilibrium with the same Tris-EDTA buffer. This column had been calibrated with Blue Dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A (m. w. 25,100), sperm whale metmyoglobin, and cytochrome c (m. w. 12,384).

Coelomic cells were lysed in 0.01 M sodium phosphate buffer, pH 7.4, 0.01 M in NaCl, using a ground glass tissue homogenizer. An aliquot of 0.1 M sodium phosphate buffer, pH 7.4, 0.1 M in NaCl was added to the cell lysate and the mixture was centrifuged for 15 minutes at 10,000 g.

Coelomic cell hemoglobin was fractionated with ammonium sulfate by making the solution successively 25, 50, 60, and 90 percent in ammonium sulfate and centrifuging the sample for 15 minutes at 10,000 g after each addition. T. crispus coelomic cell hemoglobin precipitates from a solution 90 percent in ammonium sulfate.

The coelomic hemoglobin pellet was redissolved in the 0.1 M

sodium phosphate-sodium chloride buffer and applied in the oxy- and the carbonmonoxy- forms to a 1.8 x 110 cm column of Sephadex G-75 (Superfine) in equilibrium with the same phosphate buffer. The column had been calibrated previously with Blue Dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A, sperm whale metmyoglobin, and cytochrome c.

Following Sephadex chromatography the coelomic cell hemoglobin was chromatographed through a 1.8 x 33 cm column of diethylaminoethyl (DEAE)-cellulose in equilibrium with 0.01 M NH_4HCO_3 buffer. Peak coelomic hemoglobin fractions were saved for disc gel electrophoresis, sodium dodecyl sulfate (SDS) electrophoresis, and for the determination of the iron content of this pigment.

Disc gel electrophoresis of the coelomic cell hemoglobin was accomplished according to Davis (1964) with and without the stacking gel mentioned in Davis. Hemoglobin samples were electrophoresed in the cyanmet form (Moss and Ingram, 1968). Gels were stained with 1 percent Amido Schwarz in 7 percent acetic acid and destained against frequent changes of 7 percent acetic acid.

The vascular hemoglobin and the coelomic cell hemoglobin were electrophoresed in the presence of sodium dodecyl sulfate (SDS) (Weber and Osborn, 1969) to determine the molecular weight of the coelomic cell hemoglobin and to ascertain the molecular weights of various vascular hemoglobin subunits. Protein standards used in SDS electrophoresis of the erythrocruorin were the following: bovine serum albumin, ovalbumin, chymotrypsinogen A, sperm whale metmyoglobin, bovine hemoglobin (m. w. subunit 15,500), lysozyme (m. w. 14,300), and cytochrome

c. Protein standards used in electrophoresing the coelomic cell hemoglobin were the following: bovine serum albumin, ovalbumin, chymotrypsinogen A, sperm whale metmyoglobin, and lysozyme. Prior to electrophoresing in SDS, all protein standards and hemoglobin samples were first heated to 100° C in 0.01 M sodium phosphate buffer, pH 7.0, 1 percent in SDS, and 1 percent in 2-mercaptoethanol, and were then incubated for two hours at 40° C in the same buffer. Gels were stained with Coomassie Brilliant Blue and were destained against frequent changes of 7.5 percent acetic acid in 5 percent methanol.

The iron content of each pigment was determined as in Cameron (1965) using ferrous ammonium sulfate and sperm whale metmyoglobin as standards. Sperm whale metmyoglobin and purified and lyophilized T. crispus hemoglobins were dried for two days at 40° C prior to iron analyses.

Absorption maxima were determined for each pigment using a Zeiss PMQ-II spectrophotometer. Deoxygenated hemoglobin samples were prepared by adding a small aliquot of sodium dithionite to a hemoglobin solution and evacuating the air from the solution under argon. Carbonmonoxyhemoglobin was prepared by reacting concentrated sulfuric acid with concentrated formic acid and bubbling the liberated carbon monoxide gas through a hemoglobin sample. Cyanmet derivatives were prepared as in Moss and Ingram (1968).

Oxygen equilibria were determined as in Benesch et al. (1965) using the Zeiss PMQ-II spectrophotometer with a constant temperature cell holder and tonometers purchased from Eck and Krebs Company. Wave-

lengths used to determine percent oxygenation for both pigments were 525, 540, 558, and 575 nanometers for dilute hemoglobin samples, and 620 nm for concentrated hemoglobin samples. Prior to oxygen equilibrium experiments the coelomic cell hemoglobin was purified either through Sephadex G-75 or G-100, and the vascular hemoglobin was purified through Sepharose 4-B.

The molar extinction coefficient for oxygenated hemoglobin, 1.53 x 10^4 M^{-1} cm^{-1} per heme at 540 nm (Benesch et al., 1965) was used to determine coelomic cell hemoglobin concentrations. A one percent extinction coefficient at 280 nm was determined for the vascular hemoglobin by weighing out purified and lyophilized vascular hemoglobin into a known volume of 0.1 M Tris-HCl buffer and measuring the absorbance at 280 nm. T. crispus vascular hemoglobin was found to have a one percent extinction coefficient at 280 nm equal to 22.0.

RESULTS

Examinations of several specimens of Thelepus crispus indicate three pairs of filamentous branchiae which arise from anterior segments two, three, and four (see Johnson, 1901 for drawings of these), and small gills associated with the thoracic parapodia. Dissections indicate that the vascular hemoglobin flows through both sets of gills whereas the coelomic cell hemoglobin does not. The body wall is richly vascularized and is seen to pulsate rhythmically as contractions pass from the tail to the head. The body wall of this worm is so thin that gametes can be observed through the wall within the coelomic cavity circulating with the passage of each wave of muscle contraction. It is assumed that the coelomic cells also circulate in this manner.

The coelomic fluid is viscous and milky-pink to red-brown in color and contains nucleated coelomocytes which are about 22 microns in diameter. When coelomic fluid is removed from the coelomic cavity the coelomocytes rapidly aggregate into a loose association of cells. The hematocrit of freshly collected worms is 57.59 $\pm$ 16.97 (S. D.) percent packed coelomocytes (89 trials, 33 worms). Preliminary results fail to demonstrate a seasonal fluctuation in hematocrit values.

The vascular fluid does not contain cells but contains hemoglobin which is in solution with the vascular fluid. The vascular fluid does not clot when extracted from the animal.

Tests indicate worms to contain 0.46 to 0.82 milligrams vascular hemoglobin per gram wet weight worm (mean of 6 trials equals 0.63), and 0.39 to 0.78 milligrams coelomic cell hemoglobin per gram wet weight worm (mean of 6 trials equals 0.62).

Ammonium sulfate fractionated T. crispus vascular hemoglobin chromatographs on Sepharose 4-B as a protein of apparent molecular weight 3.3×10^6 (Figures 1 and 2). Vascular hemoglobin which has been subjected to high pH displays a heme-containing subunit which chromatographs on Sephadex G-200 as a protein of apparent molecular weight 270,000 and a second heme-containing fraction which elutes with sperm whale metmyoglobin on the Sephadex G-200 column (Fig. 3), and has an apparent molecular weight of 16,000 on Sephadex G-75 (Superfine).

SDS electrophoresis of the vascular hemoglobin (9 trials) produces three protein bands which correspond to molecular weights 17,000, 31,000, and 62,000; all $\pm$ 1000. Iron determinations (7 trials) indicate the presence of 0.269 $\pm$ 0.017 percent iron, corresponding to one gram atom iron per 20,760 grams vascular hemoglobin.

Ammonium sulfate fractionated coelomic cell hemoglobin chromatographs on Sephadex G-75 (Superfine) as a protein of apparent molecular weight 17,000 (Fig. 4). A non-heme-containing fraction is eluted in the void volume of the column, and a yellow substance, not shown in Fig. 4 elutes with the salt peak. When coelomic cell hemoglobin under the bar in Fig. 4 was chromatographed on DEAE-cellulose the hemoglobin at first stuck to the column but eventually eluted from the column without a salt gradient. Coelomic cell hemoglobin eluting from the DEAE-cellulose column emerged as a single hemoglobin fraction with

constant 280 to 540 nm absorbance ratios over the entire hemoglobin peak. (Fig. 5).

Polyacrylamide disc gel electrophoresis of purified coelomic cell hemoglobin produces one protein band. When coelomic hemoglobin is electrophoresed in the presence of SDS (8 trials) a single protein band is observed which migrates as a protein of apparent molecular weight 16,000 $\pm$ 1000. Heme determinations (5 trials) indicate the presence of 0.343 $\pm$ 0.013 (S. D.) percent iron, which corresponds to one gram atom iron per 16,280 grams coelomic cell hemoglobin.

Absorption maxima for the vascular and the coelomic cell hemoglobins of T. crispus are listed in Table 1. These values are very similar to wavelength maxima for other hemoglobins (Lemberg and Legge, 1949).

Figure 6 illustrates the oxygen equilibrium curves for both hemoglobins of T. crispus at pH 7.0 and 20° C. As can be seen from Fig. 6, the oxygen affinity of the coelomic cell hemoglobin is much greater than that for the vascular hemoglobin under these conditions.

The coelomic cell hemoglobin of T. crispus displays a sigmoid oxygen equilibrium curve with P_{50} equal to 3.13 $\pm$ 0.02 (S. D.) mm Hg and "n" equal to 1.54 $\pm$ 0.12 at 20° C and pH 7.0 in a 0.1 M Na_2HPO_4-KH_2PO_4 buffer (Fig. 7), and is independent of hemoglobin concentration over the range 3.0×10^{-5} M to 5.6×10^{-4} M. The apparent concentration of coelomic cell hemoglobin in vivo is 1.0×10^{-3} M (16 mg/ml). This pigment lacks a Bohr effect over the pH range 5.45 to 9.25 (Fig. 8). (0.1 M Na_2HPO_4-KH_2PO_4 buffers were used for pH values 5.45 to 8.22, and 0.1 M glycine-NaOH buffers were used for pH values above 8.22). All

buffer solutions were prepared according to the Biochemists' Handbook (Long, 1961).

The effect of NaCl concentration on the oxygen equilibrium of the coelomic cell hemoglobin was examined. It was found that 2.0 M NaCl does not affect the shape or the position of the oxygen equilibrium curve of this pigment at 20° C and pH 7.0 (Fig. 9).

The effect of temperature on the oxygen affinity of the coelomic cell hemoglobin is shown in Fig. 10. As the temperature rises from 10° to 25° C the oxygen affinity of this pigment decreases while the Hill coefficient remains the same. The overall heat of oxygenation calculated from the van't Hoff equation is -8.4 kcals per mole at pH 7.0 over the temperature range 10° to 25° C.

T. crispus vascular hemoglobin displays a pH-dependent sigmoid oxygen equilibrium curve with P_{50} equal to 29.52 $\pm$ 2.55 mm Hg (S. D.) and "n" equal to 1.59 $\pm$ 0.20 at 20° C in a 0.1 M Tris-HCl buffer, pH 7.0, 0.01 M in $MgCl_2$. The shape and position of the oxygen equilibrium curve of the vascular hemoglobin is unaffected by hemoglobin concentration over the range 0.9 mg/ml to 17.6 mg/ml (Fig. 11) at pH 7.0 and 20° C. The concentration of vascular hemoglobin in the blood vessels of this worm is 80-90 mg/ml.

The effect of pH on the oxygen equilibrium of T. crispus vascular hemoglobin is shown in Fig. 12. There is no apparent Bohr effect over the pH range 5.40 to 7.00, but the oxygen affinity and the sigmoidicity increase as the pH is increased from 7.00 to 10.00 at 20° C. The value for Φ (change in log P_{50}/change in pH) for the pH interval 7.00 to 7.50 is -0.40, and ϕ equals -0.49 for the pH interval 7.00 to 8.00 at

20° C. The Hill coefficient is fairly constant over the pH range 5.40 to 7.00, but increases from 1.6 to 3.0 at pH 8.80, and declines somewhat at higher pH values. (0.1 M acetic acid-NaOH buffers were used for pH values 5.40 and 5.60, 0.1 M Na_2HPO_4-KH_2PO_4 buffers were used for pH values 5.80 to 6.80, 0.1 M Tris-HCl buffers were used for the pH interval 7.00 to 9.00, and 0.1 M glycine-NaOH buffers were used for pH values above 9.00. All buffers were prepared with 0.01 M $MgCl_2$).

The effect of NaCl concentration on the oxygen affinity of T. crispus vascular hemoglobin is shown in Fig. 13. As the NaCl concentration increases from 0 to 2.0 M, the P_{50} of the vascular hemoglobin decreases from 29.52 to 11.48 mm Hg and "n" increases from 1.6 to 3.0.

As the temperature increases from 10° to 25° C, the oxygen affinity of the vascular hemoglobin decreases while "n" remains the same. The overall heat of oxygenation for this pigment is -9.3 kcals per mole between 10° and 20° C, and -9.9 kcals per mole between 20° and 25° C (Fig. 14).

DISCUSSION

The terebellid polychaete Thelepus crispus Johnson contains an extracellular vascular hemoglobin and a coelomic cell hemoglobin. The presence of these two pigments within one animal also occurs in the terebellid polychaetes Pista pacifica (Terwilliger and Koppenheffer, 1973), Amphitrite johnstoni (Dales, 1964), Terebella ehrenbergi (Ochi, 1969), Amphitrite robusta and Articama conifera (personal observations), and the opheliid polychaete Travisia pupa (Manwell, 1960). The terebellid polychaetes Eupolymnia crescentis (Manwell, 1959a), Amphitrite ornata (Mangum and Shick, 1972), and Thelepus setosus (Ochi, 1969) contain an extracellular vascular hemoglobin but lack the coelomic cell pigment.

The hemoglobin containing coelomocytes of T. crispus are nucleated and ca. 22 microns in diamter. These cells are larger than human erythrocytes, which are ca. 7 microns in diameter (Houssay et al., 1951), but are similar in size to the coelomocytes of Pista pacifica (Terwilliger and Koppenheffer, 1973), Glycera dibranchiata (Hoffmann and Mangum, 1970), and those of several other polychaete species (Ochi, 1969). The hematocrit of T. crispus coelomic fluid is 58 percent packed coelomocytes, which is greater than the value for Glycera dibranchiata coelomic fluid, which is 26.5 percent packed coelomocytes (Hoffman and Mangum, 1970).

The oxygen combining capacities of T. crispus hemoglobins,

calculated from hemoglobin content as in Miller (1952) are 0.0006 to 0.0011 ml oxygen per gram wet weight worm for the vascular hemoglobin, and 0.0005 to 0.0010 ml oxygen per gram wet weight worm for the coelomic cell hemoglobin. Dales (1961) indicates that average size specimens of T. crispus utilize oxygen at the rate of 0.06 ml oxygen per gram wet weight per hour at 13° C in seawater which is 80 to 100 percent saturated with oxygen. Preliminary experiments in this laboratory indicate that the uptake of oxygen by this worm decreases sharply as the oxygen tension of the medium decreases. If this were not so the animal would rapidly deplete its supply of oxygen in burrows which are not supplied with large amounts of oxygenated seawater at low tide (personal observation). It is not known if T. crispus shuts off its aerobic respiration at extremely low oxygen tensions as does Glycera dibranchiata (Hoffmann and Mangum, 1970), or if this animal can utilize atmospheric oxygen.

Native Thelepus crispus vascular hemoglobin has an apparent molecular weight of 3.3×10^6 in the oxygenated form and contains 12 submultiples of molecular weight 270,000 each. Similar molecular weight values are reported for the vascular hemoglobins of a number of other annelid species (Svedberg, 1933; Svedberg and Eriksson-Quensel, 1934; Yamaghishi et al., 1966; Rossi-Fanelli et al., 1970; Swaney and Klotz, 1971; Waxman, 1971; Wiechelman and Parkhurst, 1972). The smallest heme-containing subunit of T. crispus vascular hemoglobin appears to be of molecular weight 17,000 by SDS electrophoresis, 16,000 by Sephadex chromatography, and 20,760 by iron content. The protomer of T. crispus vascular hemoglobin is similar in molecular weight to the smallest

subunits of vascular hemoglobin molecules of the following annelid species: Lumbricus terrestris (m. w. subunit 23,000, Rossi-Fanelli et al., 1970; m. w. 17,000, Wiechelman and Parkhurst, 1972), Arenicola marina (m. w. subunit 17,250, Patel and Spencer, 1963), and that of Cirraformia grandis (m. w. subunit 18,500, Swaney and Klotz, 1971). Arenicola cristata hemoglobin has a smallest heme-containing subunit of molecular weight 28,000 which is in turn divisible into two polypeptide chains of molecular weights 13,000 and 14,000 (Waxman, 1971). Limnodrilus gotoi erythrocruorin has a protomer of molecular weight 28,000 (Yamaghishi et al., 1966), and Marphysa sanguinea vascular hemoglobin has a smallest subunit of molecular weight 26,000 (Chew et al., 1965). If the molecular weight of the smallest subunit of T. crispus erythrocruorin is 17,000, there are 16 $\pm$ 1 subunits per one-twelfth submultiple and 192 $\pm$ 12 subunits per native oligomer. Lumbricus terrestris vascular hemoglobin is thought to consist of 144 (Rossi-Fanelli et al., 1970), or 196 (Wiechelman and Parkhurst, 1972) heme-containing subunits per native molecule, and the one-twelfth submultiple is thought to be either a dodecamer or a hexadecamer. Cirraformia grandis erythrocruorin is made up of 162 $\pm$ 24 subunits and is thought to contain a submultiple which is a dodecamer (Swaney and Klotz, 1971). Limnodrilus gotoi vascular hemoglobin contains 108 subunits and each submultiple is thought to be a nonomer (Yamaghishi et al., 1966). Waxman (1971) indicates that Arenicola cristata vascular hemoglobin contains 96 heme groups and 192 polypeptide chains per intact molecule, and as such more closely resembles the structure of chlorocruorin (Guerritore et al., 1965) than the subunit structure of vertebrate hemo-

globin.

Thelepus crispus coelomic cell hemoglobin is monomeric in the oxy- and the carbonmonoxy- forms and has an apparent molecular weight of 16-17,000 on Sephadex chromatography, SDS electrophoresis, and iron analyses. Anion-exchange chromatography and disc gel electrophoresis indicate the presence of only one coelomic cell hemoglobin component. The terebellid polychaete Pista pacifica (Terwilliger and Koppenheffer, 1973) contains coelomic cell hemoglobin which is composed of at least two monomeric components which differ in amino acid composition. Glycera dibranchiata coelomic cell hemoglobin contains two major monomeric components and at least three polypeptide chains comprising the polymeric higher order aggregate (Vinogradov et al., 1970; Seamonds et al., 1971a, b; Imamura et al., 1972). Among annelid coelomic cell hemoglobins which have been examined T. crispus coelomic cell hemoglobin is unique in containing only one hemoglobin component.

Thelepus crispus vascular hemoglobin binds oxygen in a pH-dependent sigmoid oxygen equilibrium curve with P_{50} equal to 29.5 mm Hg and "n" equal to 1.6 at 20° C and pH 7.0 in 0.1 M Tris-HCl buffer, 0.01 M in $MgCl_2$. The value for ϕ is -0.40 for the pH interval 7.0 to 7.5, and -0.49 for the interval 7.0 to 8.0 at 20° C. The vascular hemoglobin of the terebellid polychaete Pista pacifica displays a somewhat higher oxygen affinity with P_{50} equal to 22.2 mm Hg and "n" equal to 1.5 at pH 7.0 and 20° C (Terwilliger, personal communication). P. pacifica erythrocruorin does not show a Bohr effect over the pH range 6.0 to 8.0, but does possess a normal Bohr effect between pH values 8.0 and 10.0, with ϕ equal to -0.40 between pH 8.0 and 9.0. Over the same pH range,

T. crispus vascular hemoglobin displays a ϕ value of -0.50. Vascular hemoglobin from the terebellid polychaete Eupolymnia crescentis (Manwell, 1959a) has a very low oxygen affinity, displays hyperbolic binding, and lacks a Bohr effect. Manwell, however, does not include oxygen binding data for this pigment for pH values higher than 7.08. Mangum and Shick (1972) indicate the pH of vascular fluid from the terebellid polychaete Amphitrite ornata to be 7.4 at 21° C, therefore Manwell's data probably does not include values which would indicate whether or not a Bohr effect exists over physiological pH values.

Thelepus crispus coelomic cell hemoglobin binds oxygen according to a pH-independent sigmoid oxygen equilibrium curve and has a relatively high oxygen affinity. Sigmoidicity, a measure of intermolecular cooperativity, would not be expected in a monomeric hemoglobin. Lamprey hemoglobin, also a monomer, binds oxygen in a sigmoid oxygen equilibrium curve due to aggregation of hemoglobin monomers at low oxygen tensions (Briehl, 1963; Anderson and Gibson, 1971). The polymeric hemoglobin component of Glycera dibranchiata has also been shown to aggregate from a dimer in the oxygenated state to a higher-order aggregate in the deoxy-state (Mizukami and Vinogradov, 1972). Aggregation has not been demonstrated in Thelepus crispus coelomic cell hemoglobins and sigmoidicity in oxygen binding is at present unexplained for this pigment.

T. crispus coelomic cell hemoglobin lacks a Bohr effect over the pH range 5.45 to 9.25. Coelomic cell hemoglobin from the polychaete Travisia pupa (Manwell, 1960) also lacks a Bohr effect, and the monomeric component of Glycera dibranchiata coelomic cell hemoglobin either displays a Bohr effect (Hoffmann and Mangum, 1970), or lacks a Bohr effect

(Mizukami and Vinogradov, 1972). The polymeric hemoglobin component of Glycera dibranchiata coelomic cell hemoglobin lacks a Bohr effect (Hoffmann and Mangum, 1970; Mizukami and Vinogradov, 1972). Lack of the Bohr effect has also been demonstrated in human myoglobin (Rossi-Fanelli et al., 1958), hemoglobin from the sea cucumbers Cucumaria miniata (Manwell, 1959b) and Molpadia oolitica (Terwilliger and Read, 1972), and the radular muscle myoglobin of the gastropod mollusc Buccinum undatum (Terwilliger and Read, 1971).

The oxygen equilibrium of T. crispus vascular hemoglobin is unaffected by hemoglobin concentration over the range 0.5 mg/ml to 17.6 mg/ml. The coelomic cell hemoglobin is likewise unaffected by hemoglobin concentration over the range 3.0×10^{-5} M to 5.6×10^{-4} M. Vascular hemoglobin of Pista pacifica also does not display the concentration effect (Terwilliger, personal communication). Human hemoglobin (Antonini, 1965), lamprey hemoglobin (Briehl, 1963), and Glycera dibranchiata polymeric coelomic cell hemoglobin (Mizukami and Vinogradov, 1972) show a decrease in oxygen affinity as the hemoglobin concentration is increased, due to an oxygenation-linked dissociation in the case of lamprey hemoglobin (Briehl, 1963) and polymeric G. dibranchiata hemoglobin(Mizukami and Vinogradov, 1972). In human hemoglobin the concentration phenomenon is interpreted as an indication of intermolecular interactions which are present in oligomeric hemoglobins but would not be expected in hemoglobins which are monomeric (reviewed in Antonini, 1965). Monomeric human myoglobin (Rossi-Fanelli and Antonini, 1958) and the dimeric radular muscle myoglobin of the gastropod mollusc Buccinum undatum (Terwilliger and Read, 1971) do not display the concentration

effect.

The shape and position of the oxygen equilibrium curve of T. crispus coelomic cell hemoglobin is unaffected by NaCl concentrations up to 2.0 M, while T. crispus vascular hemoglobin displays an increased affinity and an increased Hill coefficient over the same range of NaCl concentrations. Pista pacifica vascular hemoglobin is unaffected by NaCl concentration (Terwilliger, personal communication). Human hemoglobin (Antonini et al., 1962) displays an increase in cooperativity and a decrease in oxygen affinity as the concentration of NaCl is increased, which is explained as a dissociation phenomenon; that is, human hemoglobin tends to dissociate into dimers at high NaCl concentrations. Human myoglobin (Rossi-Fanelli and Anronini, 1958) does not display a change in oxygen affinity or shape of the oxygen equilibrium curve as the NaCl concentration is increased.

Both hemoglobins of T. crispus display a decrease in oxygen affinity without a corresponding change in the shape of the curve, as the temperature is increased. Vertebrate hemoglobins and myoglobins show a decrease in oxygen affinity as the temperature is increased (Antonini, 1965). The heat of oxygenation, H, for the coelomic cell hemoglobin of Thelepus crispus is -8.4 kcals per mole over the temperature range 10° to 25° C. The heat of oxygenation for the vascular hemoglobin of this worm is -9.3 kcals per mole for the range 10-20° C, and -9.9 kcals per mole for the temperature range 20-25° C. Literature values for hemoglobin heats of oxygenation range from that of snake hemoglobin, with H equal to -15.5 kcals per mole (Sullivan, 1967), to that of tuna fish hemoglobin, with H equal to -1.8 kcals per mole (Rossi-Fanelli and Antonini, 1960).

Heats of oxygenation for the two hemoglobins of T. crispus are similar to those for sheep hemoglobin, -8.2 kcals per mole (Roughton et al., 1955), adult and fetal dogfish hemoglobins, -8.8 and -9.3 kcals per mole, respectively (Manwell, 1958), Buccinum undatum and Busycon canaliculatum (both gastropods) radular muscle myoglobins, -8.4 and -8.8 kcals per mole, respectively(Terwilliger and Read, 1971). The heats of oxygenation of T. crispus hemoglobins fall within the range of those of hemoglobins from other animals.

In Thelepus crispus the presence of a vascular hemoglobin with a low oxygen affinity and a coelomic cell hemoglobin with a high affinity for oxygen suggests the presence of an oxygen transfer system in which oxygen is taken up via the gills into the vascular hemoglobin pool, and then transferred from the vascular hemoglobin to the coelomic cell hemoglobin to be released to the tissues as needed. Such an oxygen transfer system is proposed for the polychaete Travisia pupa (Manwell, 1960). In Travisia oxygen is taken up via the parapodial gills into the vascular hemoglobin, and from there transferred to the coelomic cell hemoglobin, which has a higher oxygen affinity. The myoglobin of Travisia pupa has a higher oxygen affinity than either the vascular or the coelomic cell hemoglobin, and is assumed by Manwell to be the final acceptor in the oxygen transfer system. Thelepus crispus seems to have a body wall thin enough that some oxygen could possibly enter directly through the body wall and into the coelomic hemoglobin pool, thus bypassing the gills and the vascular hemoglobin. The functions of the two hemoglobins of Thelepus crispus are not known, and will be determined only via experiments which deal not only with the physiology, but

also the ecology of the animal. Until such field observations are made, no solid speculation will be offered as regards the functions of the two hemoglobins. Hopefully, these experiments can be undertaken in the future.

Figure 1. Chromatography of ammonium sulfate fractionated Thelepus crispus erythrocruorin on Sepharose 4-B. Column volume: 305 ml. Flow rate: 12 ml/hr. Buffer: 0.1 M Tris-HCl, pH 7.0, 0.01 M in $MgCl_2$. ● , Absorbance 280 nm. ▲ , Absorbance 540 nm. Protein markers: Katharina tunicata hemocyanin (K. t.), Pista pacifica erythrocruorin (P. p.), Lumbricus terrestris erythrocruorin (L. t.), Cancer magister hemocyanin (C. m.).

Figure 1

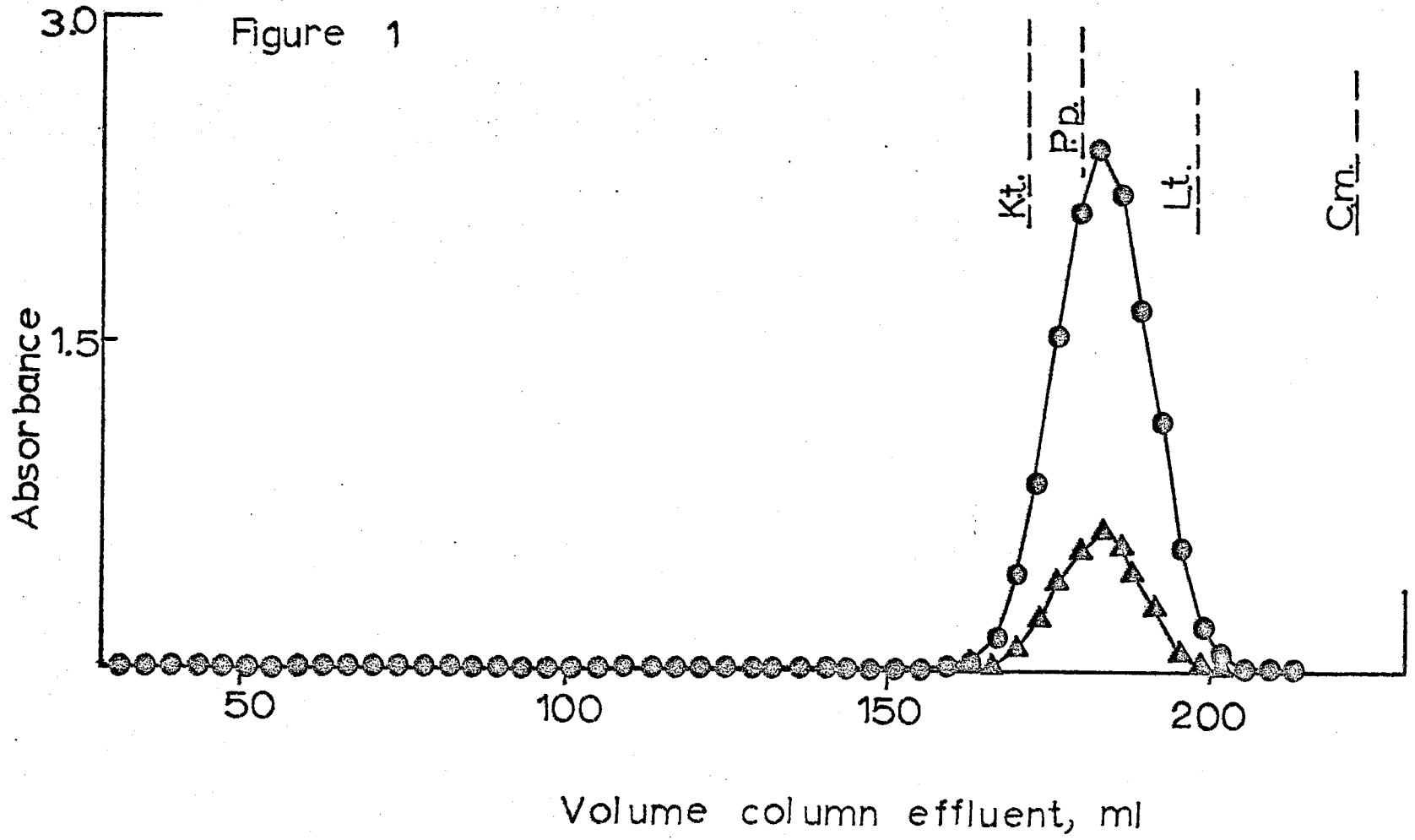
3.0
1.5
Absorbance
Kt.
P.p.
Lt.
Cm.
50
100
150
200
Volume column effluent, ml

Figure 2. Chromatography of ammonium sulfate fractionated Thelepus crispus vascular hemoglobin on Sepharose 4-B, log plot. Data is from Fig. 1. Protein markers are Katharina tunicata hemocyanin (Kt hcy), Pista pacifica vascular hemoglobin (P p hb), Lumbricus terrestris vascular hemoglobin (Lt hb), and Cancer magister hemocyanin (C m hcy). The arrow indicates the point at which Thelepus crispus vascular hemoglobin elutes from the column.

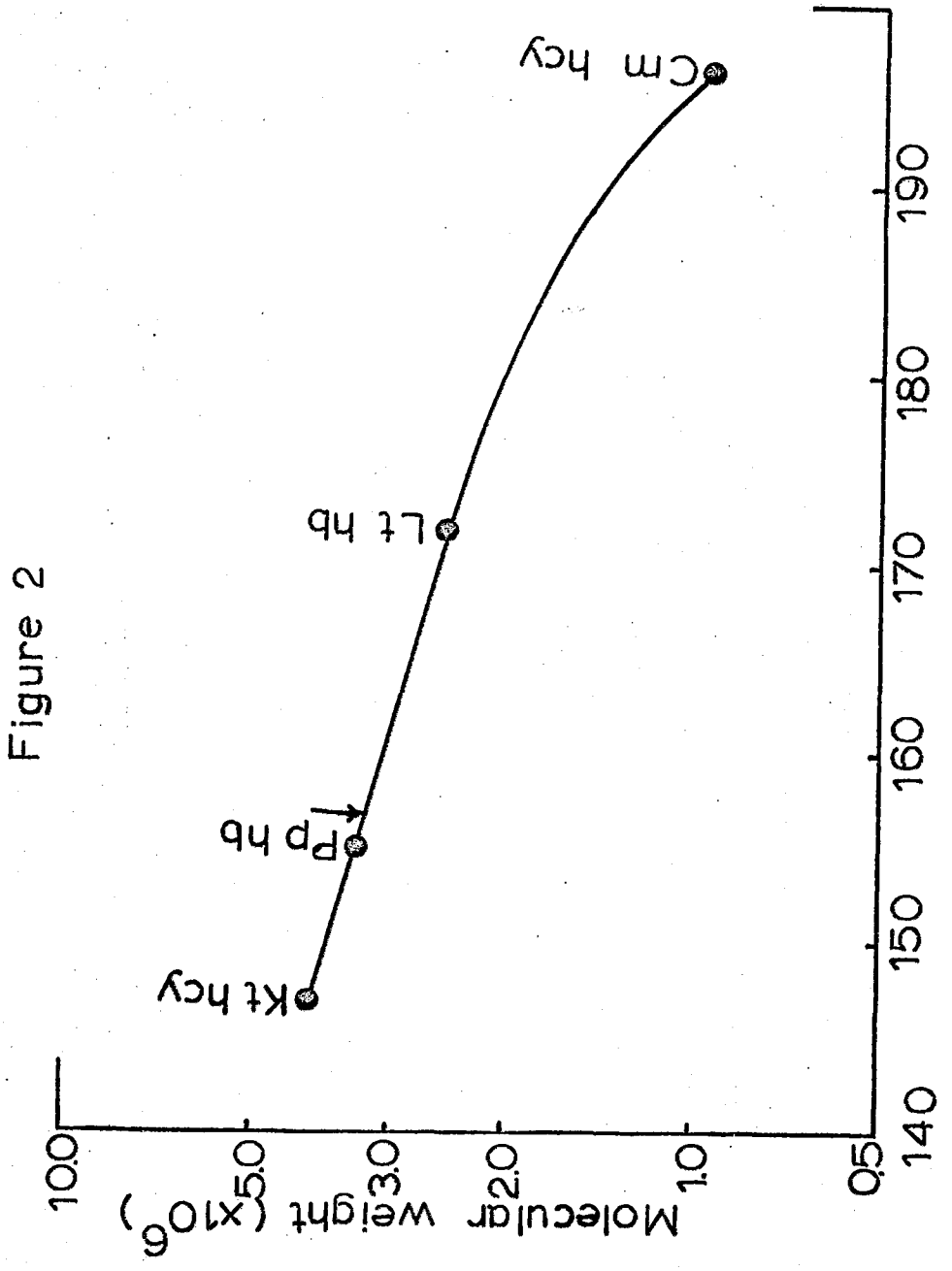
Molecular weight ($\times 10^6$)
10.0
5.0
3.0
2.0
1.0
0.5
140
150
160
170
180
190
Column effluent, ml
Kt hcy
Pp hb
Lt hb
Cm hcy

Figure 2

Figure 3. Chromatography of the submultiple of Thelepus crispus vascular hemoglobin on Sephadex G-200, log plot. Column volume: 305 ml. Flow rate: 11 ml/hr. Buffer: 0.1 M Tris-HCl, pH 7.0, 0.01 M in disodium-EDTA. Protein markers: Pista pacifica vascular hemoglobin one-twelfth submultiple (PPS), bovine catalase (BC), rabbit muscle aldolase (RMA), bovine serum albumin (BSA), ovalbumin (OVA), and sperm whale metmyoglobin (SWM). Thelepus crispus vascular hemoglobin was prepared as in the text. The arrows indicate the points of elution of two major heme-containing protein fractions.

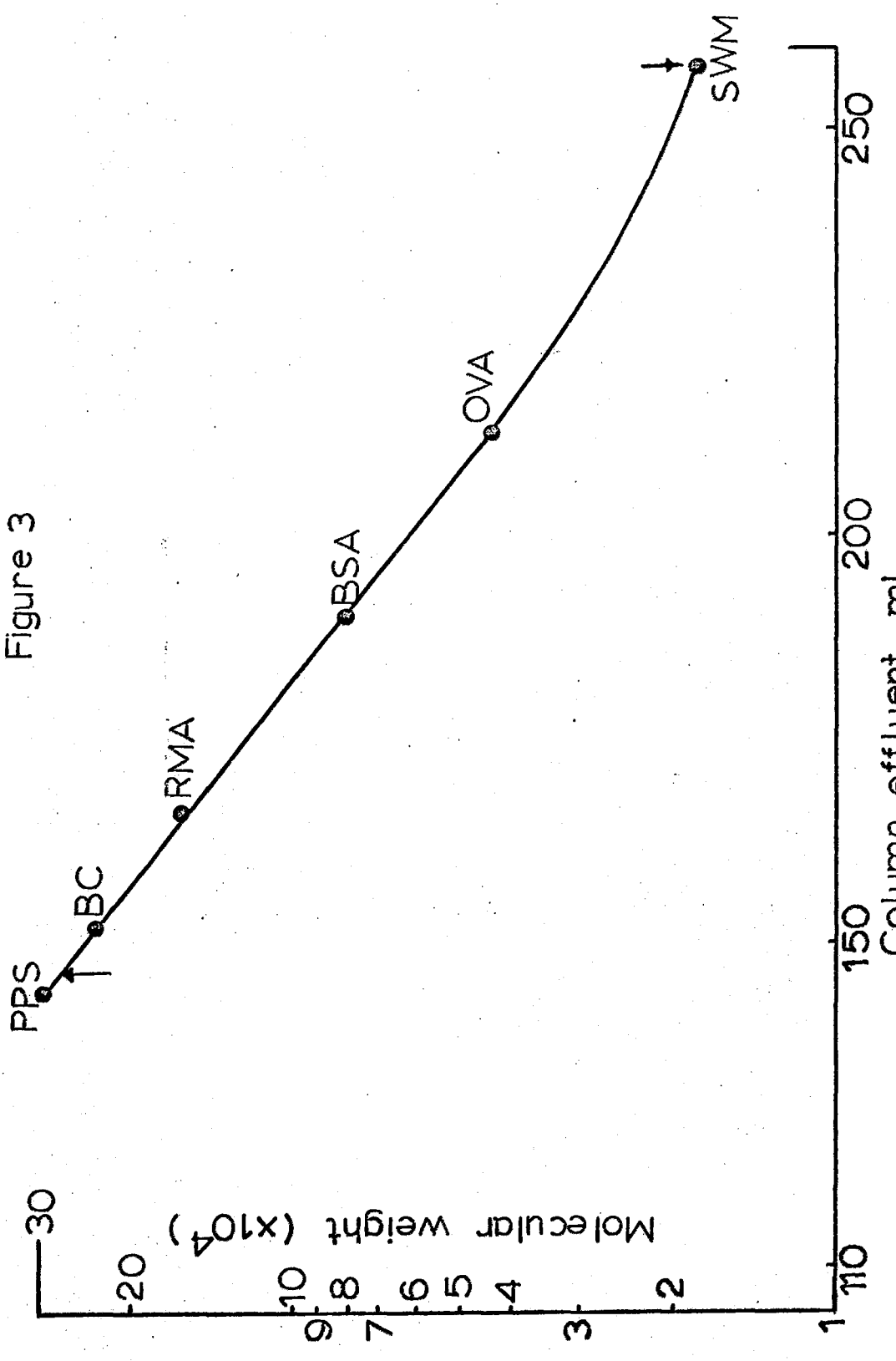
Molecular weight (x10⁴)
30
20
10
9
8
7
6
5
4
3
2
1
110
150
200
250
Column effluent, ml
PPS
BC
RMA
BSA
OVA
SWM

Figure 3

Figure 4. Chromatography of ammonium sulfate fractionated Thelepus crispus coelomic cell hemoglobin on Sephadex G-75 (Superfine). Column volume: 280 ml. Flow rate: 11 ml/hr. Buffer: 0.1 M sodium phosphate, pH 7.4, 0.1 M in NaCl. ○ , Absorbance 280 nm. △ , Absorbance 540 nm. Protein calibrants: Blue Dextran (b d), bovine serum albumin (bsa), ovalbumin (ova), chymotrypsinogen A (chy), sperm whale metmyoglobin (swm), and cytochrome c (cyt c).

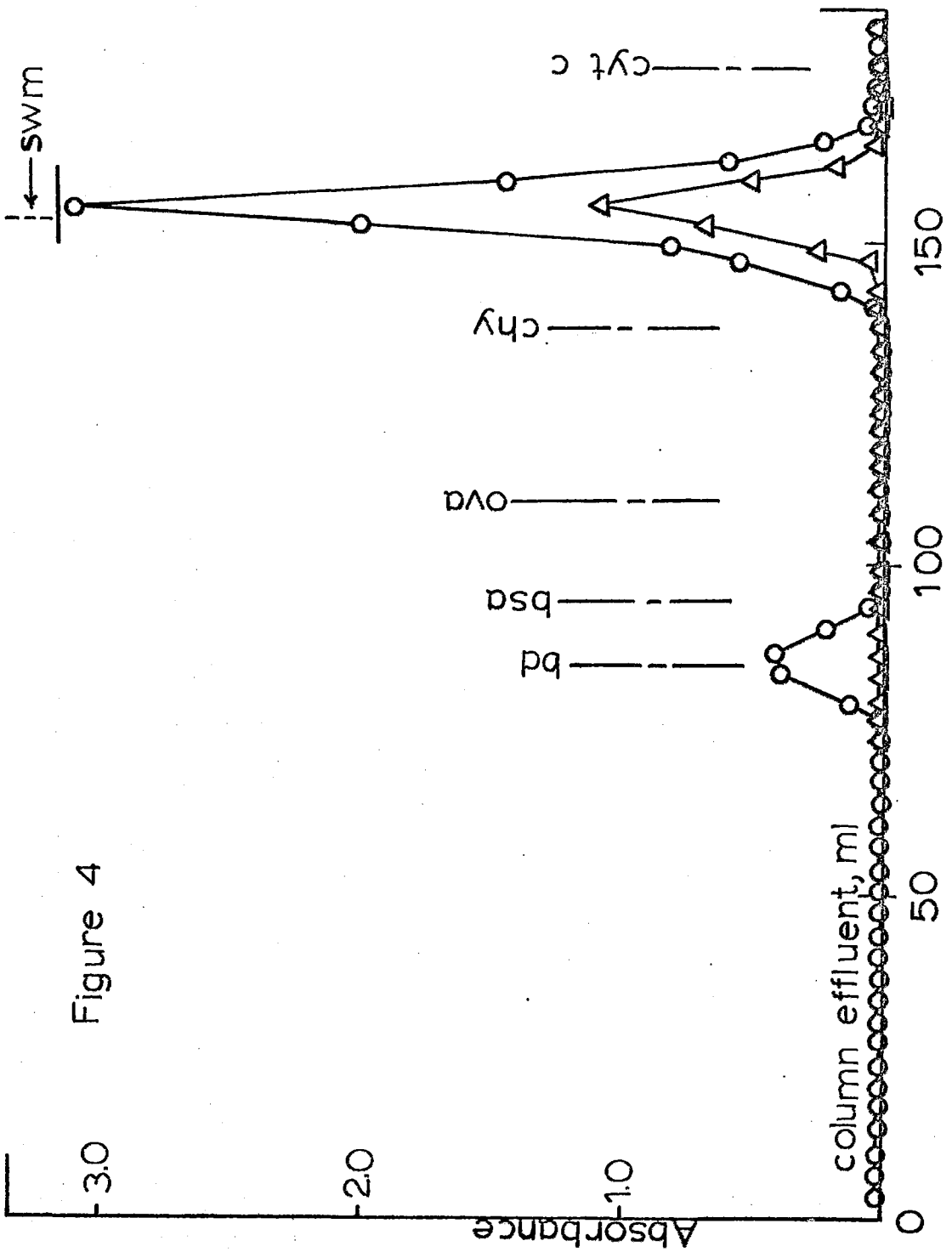
Absorbance
3.0
2.0
1.0
0
column effluent, ml
50
100
150
bd
bsa
ova
chy
cyt c
swm

Figure 4

Figure 5. Chromatography of the material under the bar in Fig. 4 on DEAE-cellulose. Column volume: 75 ml. Flow rate: 18 ml/hr. Buffer: 0.01 M NH_4HCO_3. ● , Absorbance 280 nm. ▲, Absorbance 540 nm.

Figure 5

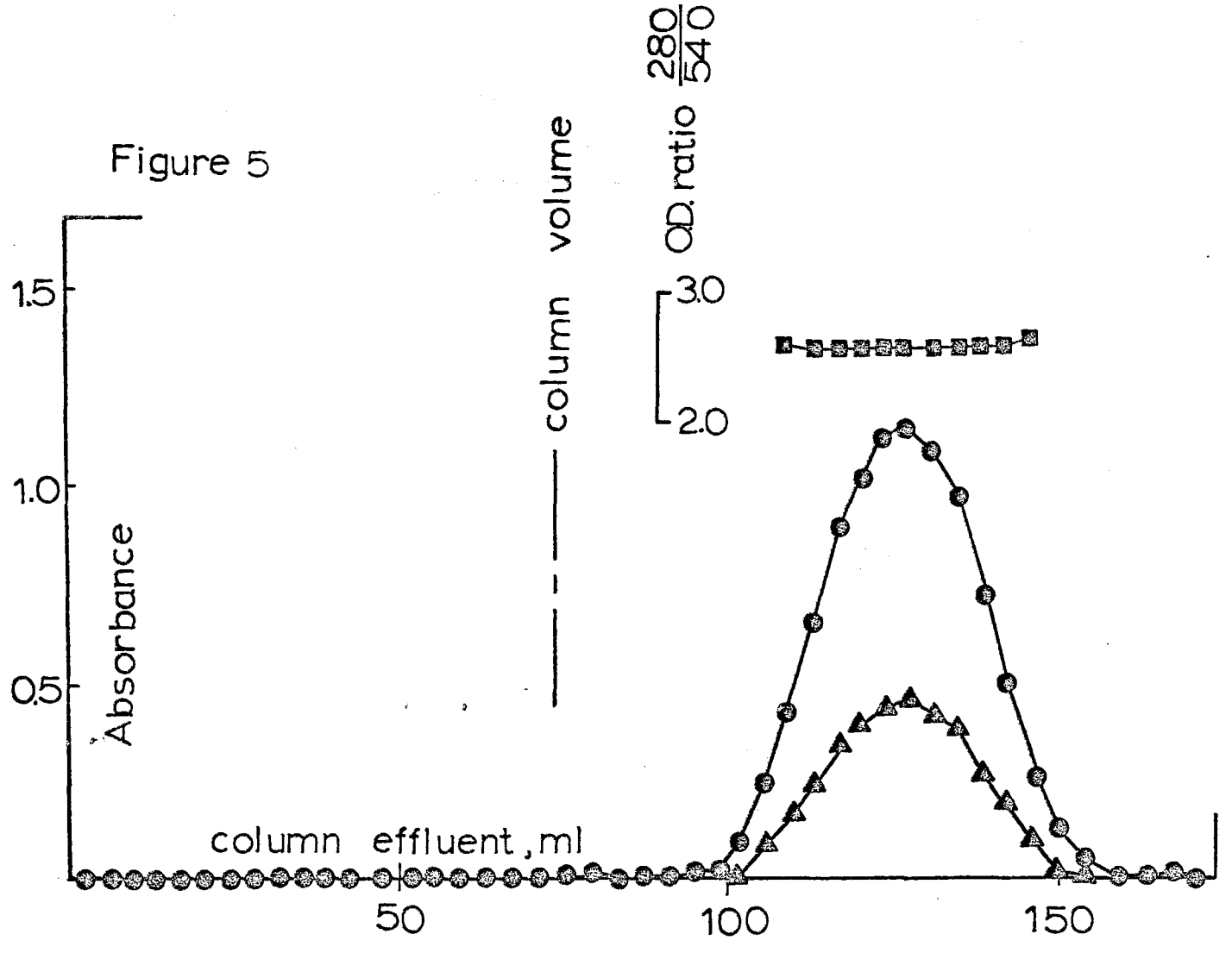
Absorbance
1.5
1.0
0.5
column volume
O.D. ratio 280/540
3.0
2.0
column effluent, ml
50
100
150

Figure 6. Oxygen equilibrium curves for the two hemoglobins of Thelepus crispus. ● , the coelomic cell hemoglobin. ▲ , the vascular hemoglobin. Buffers: 0.1 M Tris-HCl, pH 7.0, 0.01 M in $MgCl_2$ for the vascular hemoglobin; 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.0 for the coelomic cell hemoglobin. Temperature: 20° C. Hemoglobin concentrations: 5×10^{-5} M for the coelomic cell hemoglobin, 1.0 mg/ml for the vascular hemoglobin.

Figure 6

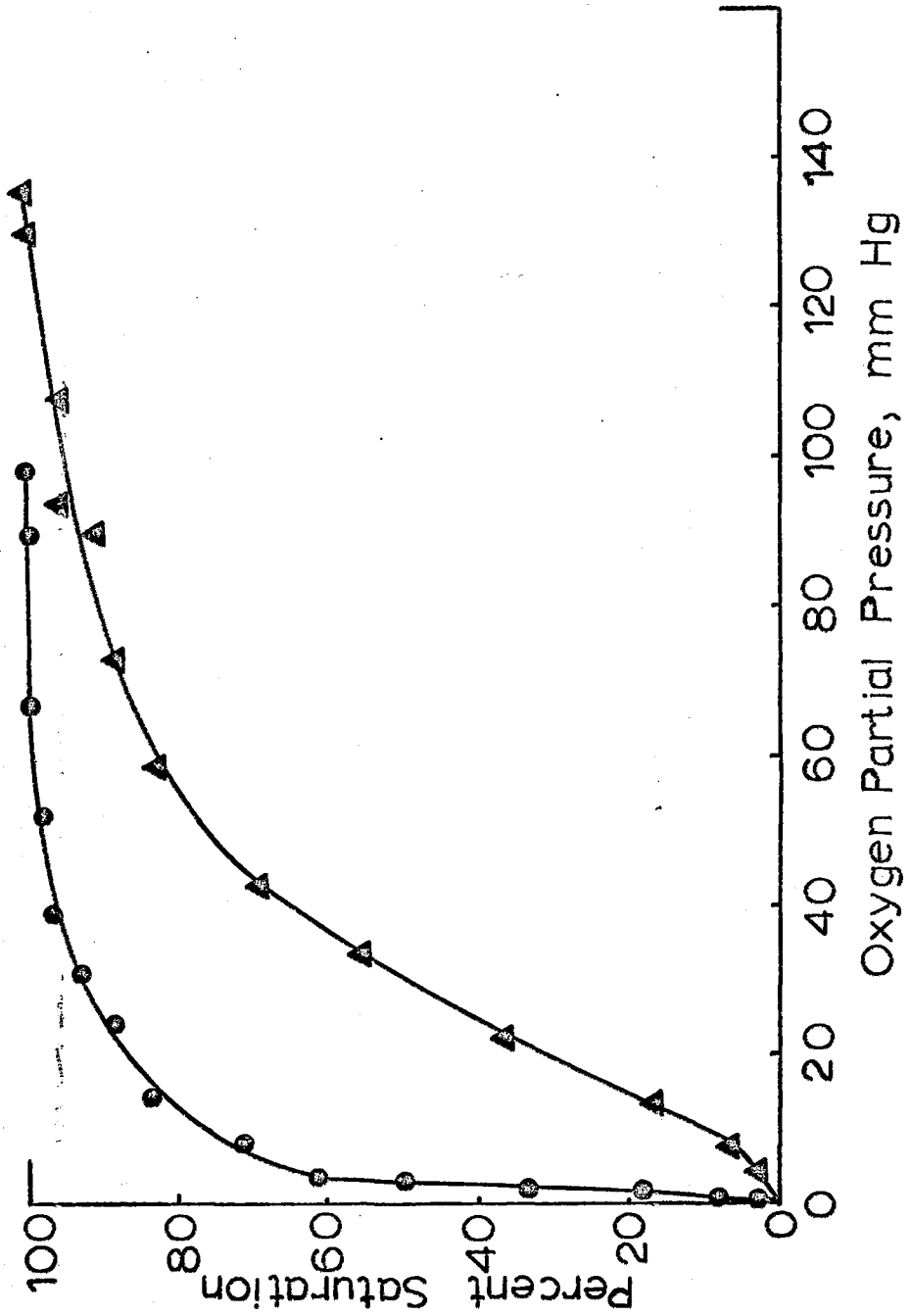
Percent Saturation
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Oxygen Partial Pressure, mm Hg

Figure 7. Effect of hemoglobin concentration on the oxygen equilibrium of Thelepus crispus coelomic cell hemoglobin. Buffer: 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.0. Temperature: 20° C. Hemoglobin concentrations: ● , 3.0×10^{-5} M; ○ , 1.8×10^{-4} M; ■ , 2.9×10^{-4} M; △ , 3.9×10^{-4} M; ▲ , 5.6×10^{-4} M.

Figure 7

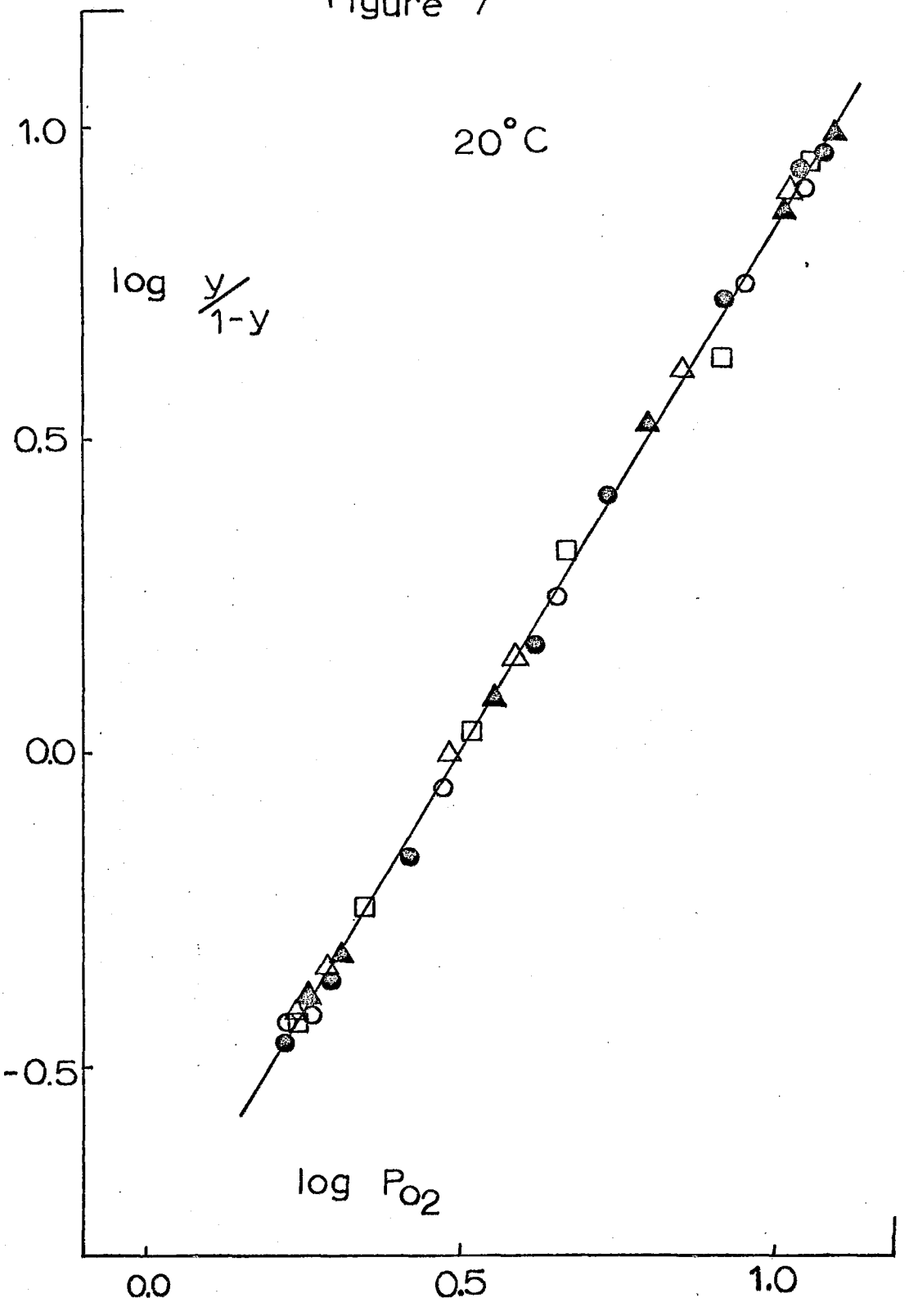

Figure 8. Effect of pH on the oxygen affinity of Thelepus crispus coelomic cell hemoglobin at 20° C. Hemoglobin concentration: 5 x 10^{-5} M. Buffers: 0.1 M Na_2HPO_4 for pH values 5.45 to 8.22; 0.1 M glycine-NaOH for pH values above 8.22.

Figure 8

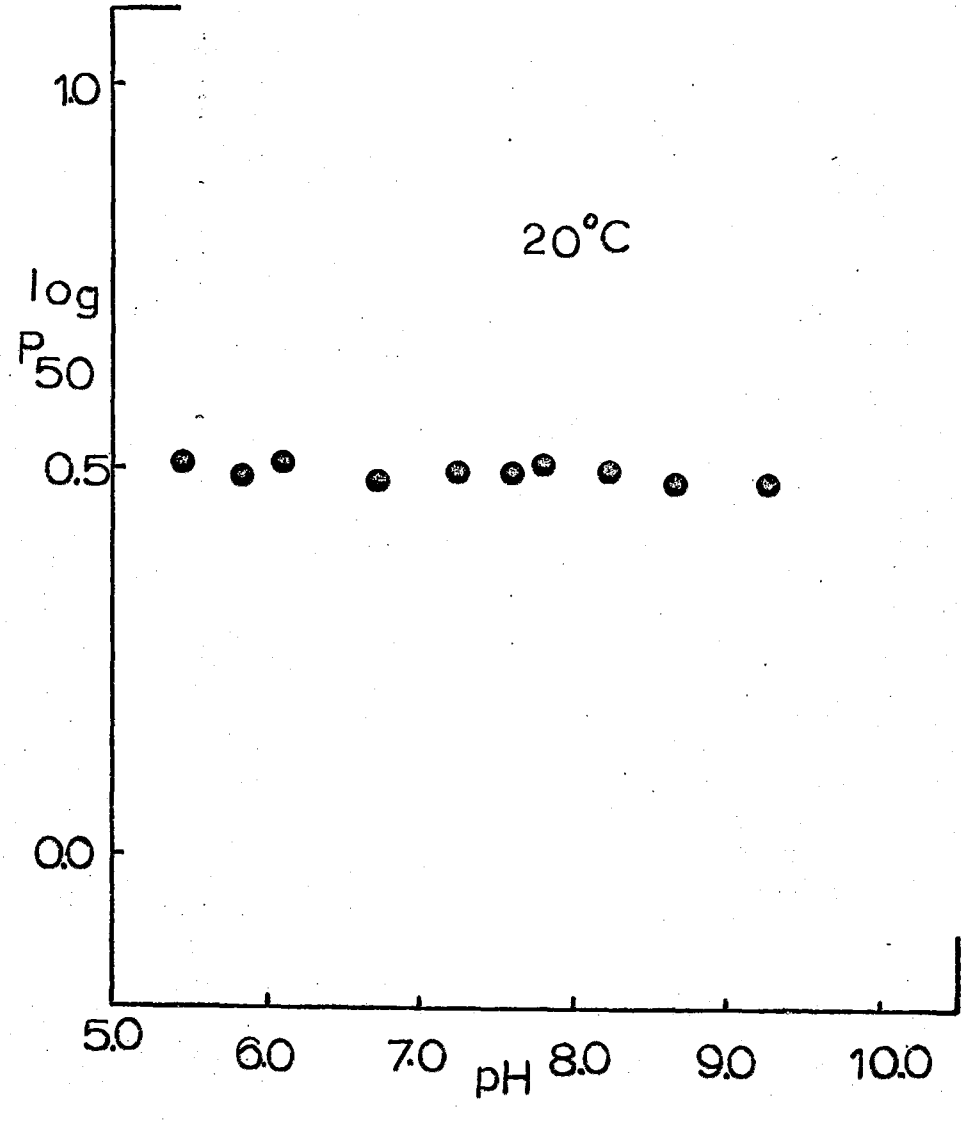

Figure 9. Effect of NaCl on the oxygen affinity of Thelepus crispus coelomic cell hemoglobin. Buffer: 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.0. Temperature: 20° C. Hemoglobin concentration: 5×10^{-5} M.

Figure 9

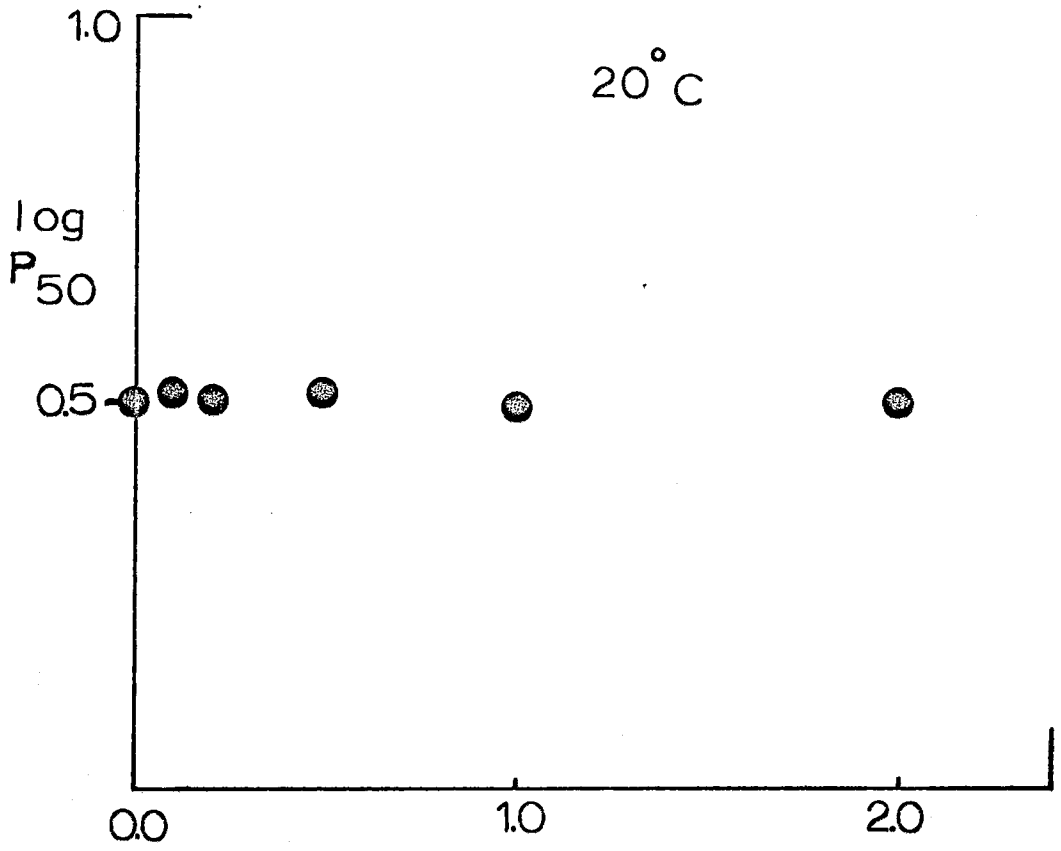

Figure 10. Effect of temperature on the oxygen affinity of Thelepus crispus coelomic cell hemoglobin. Temperatures: 10-25° C. Buffer: 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.0. Hemoglobin concentration: 5×10^{-5} M.

Figure 10

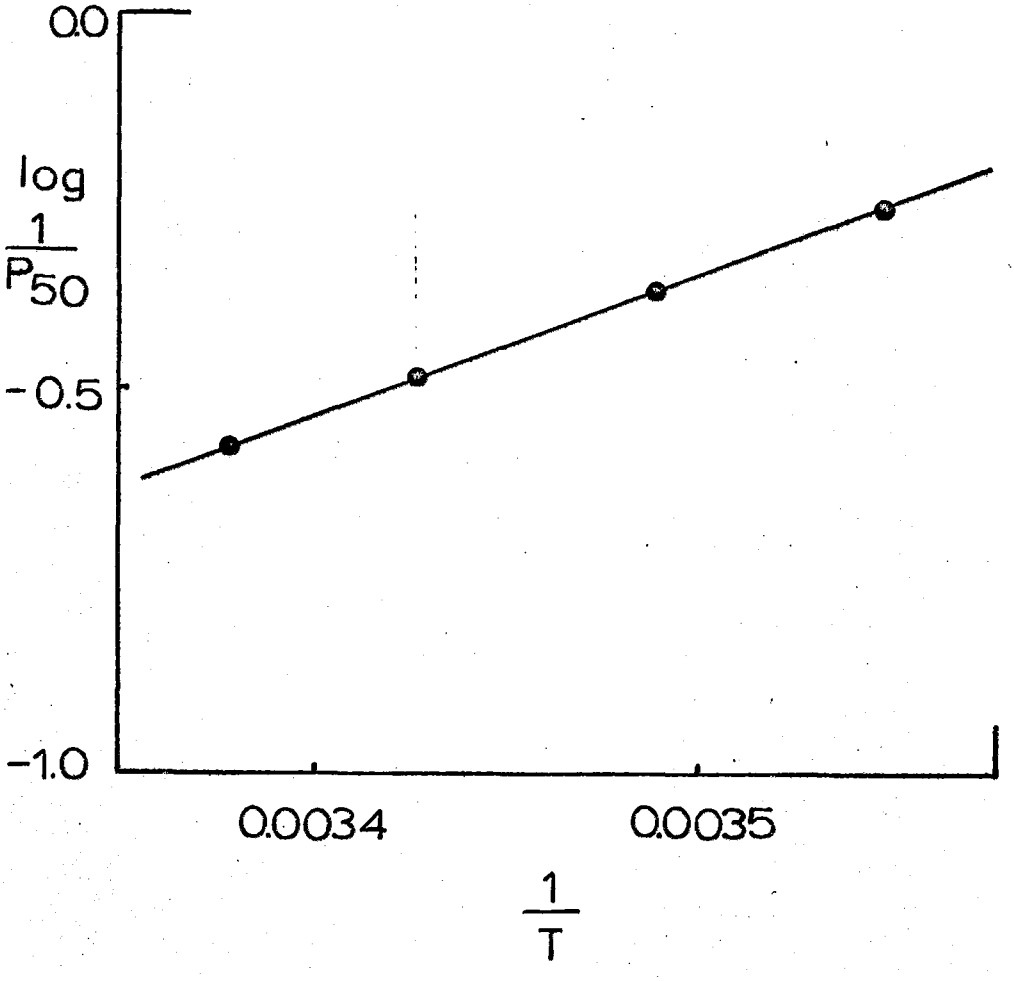

Figure 11. Effect of hemoglobin concentration on the oxygen equilibrium of Thelepus crispus vascular hemoglobin. Temperature: 20° C. Buffer: 0.1 M Tris-HCl, pH 7.0, 0.01 M in $MgCl_2$. Hemoglobin concentrations: ● , 0.9 mg/ml; ○ , 6.0 mg/ml; □ , 9.8 mg/ml; △ , 14.9 mg/ml; ▲ , 17.6 mg/ml.

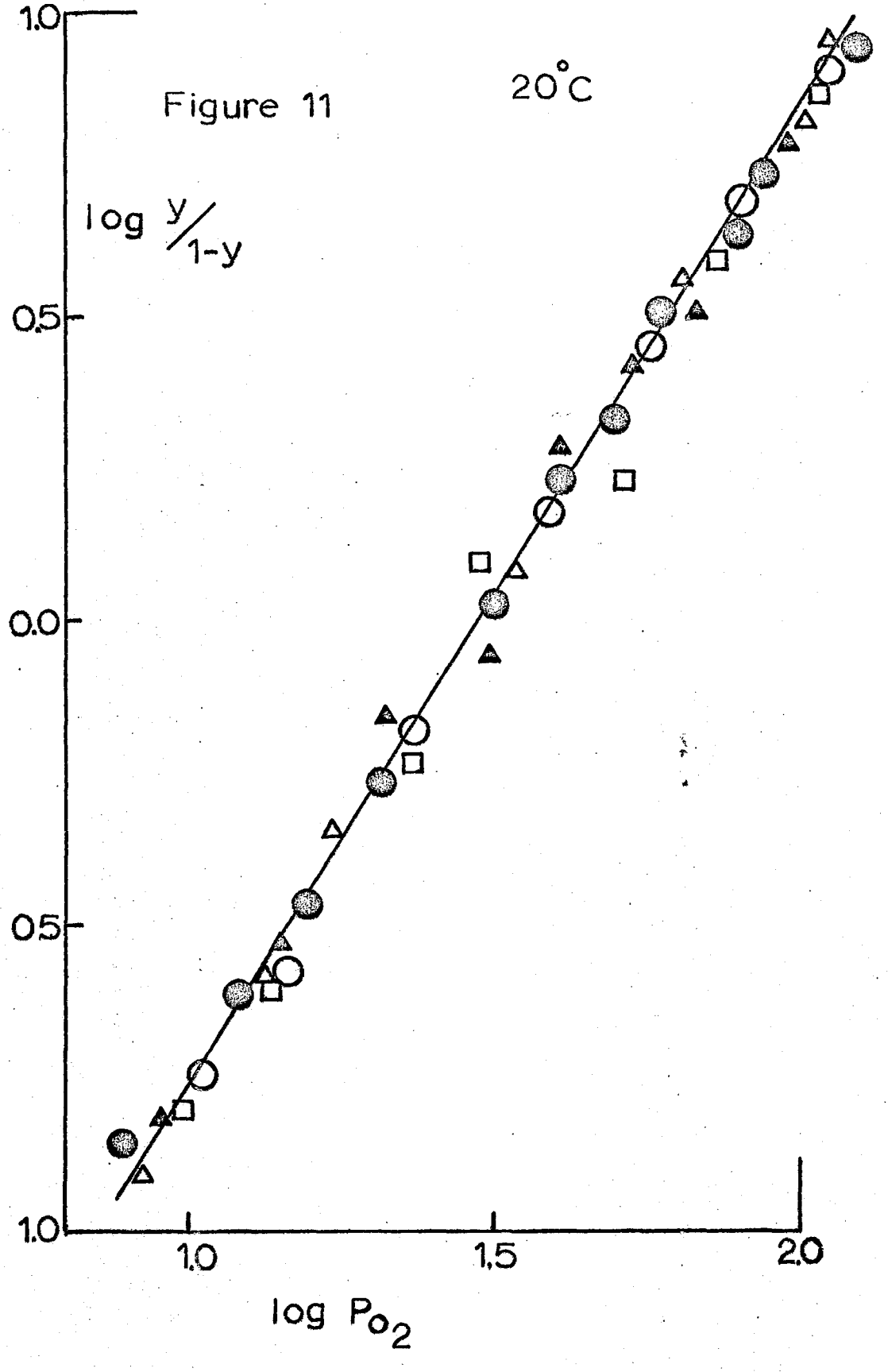

Figure 11

Figure 12. Effect of pH on the oxygen affinity of Thelepus crispus vascular hemoglobin. Temperature: 20° C. Hemoglobin concentration: 0.9 mg/ml. Buffers: 0.1 M acetic acid-NaOH for pH values 5.40 and 5.60, 0.1 M Na_2HPO_4-KH_2PO_4 for the pH range 5.80 to 6.80, 0.1 M Tris-HCl for the pH range 7.00 to 9.00, and 0.1 M glycine-NaOH for pH values above 9.00. 0.01 M $MgCl_2$ was included in all buffers.

Figure 12

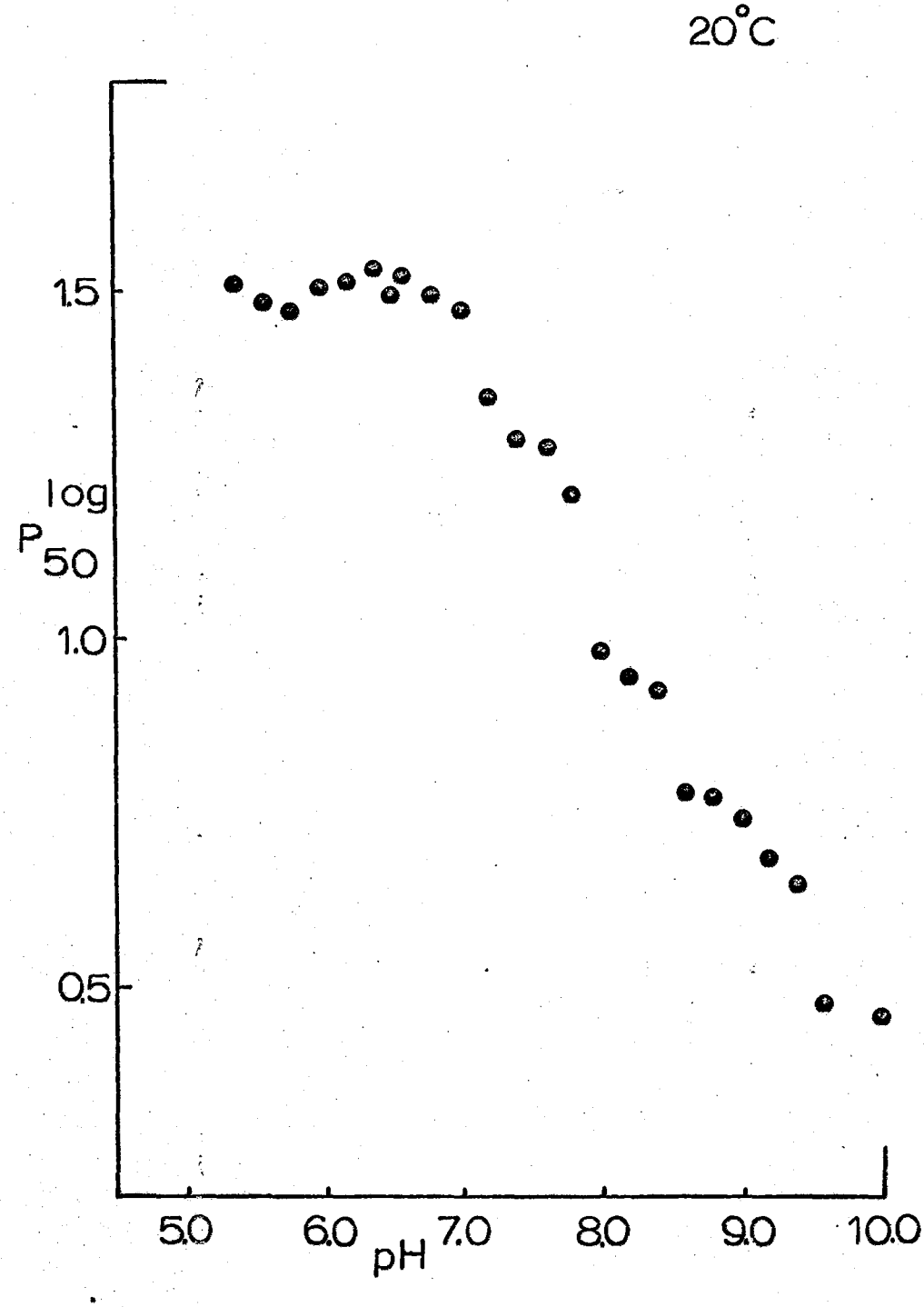

Figure 13. Effect of NaCl concentration on the oxygen affinity of Thelepus crispus vascular hemoglobin. Temperature: 20° C. Hemoglobin concentration: 0.9 mg/ml. Buffer: 0.1 M Tris-HCl, pH 7.0.

Figure 13

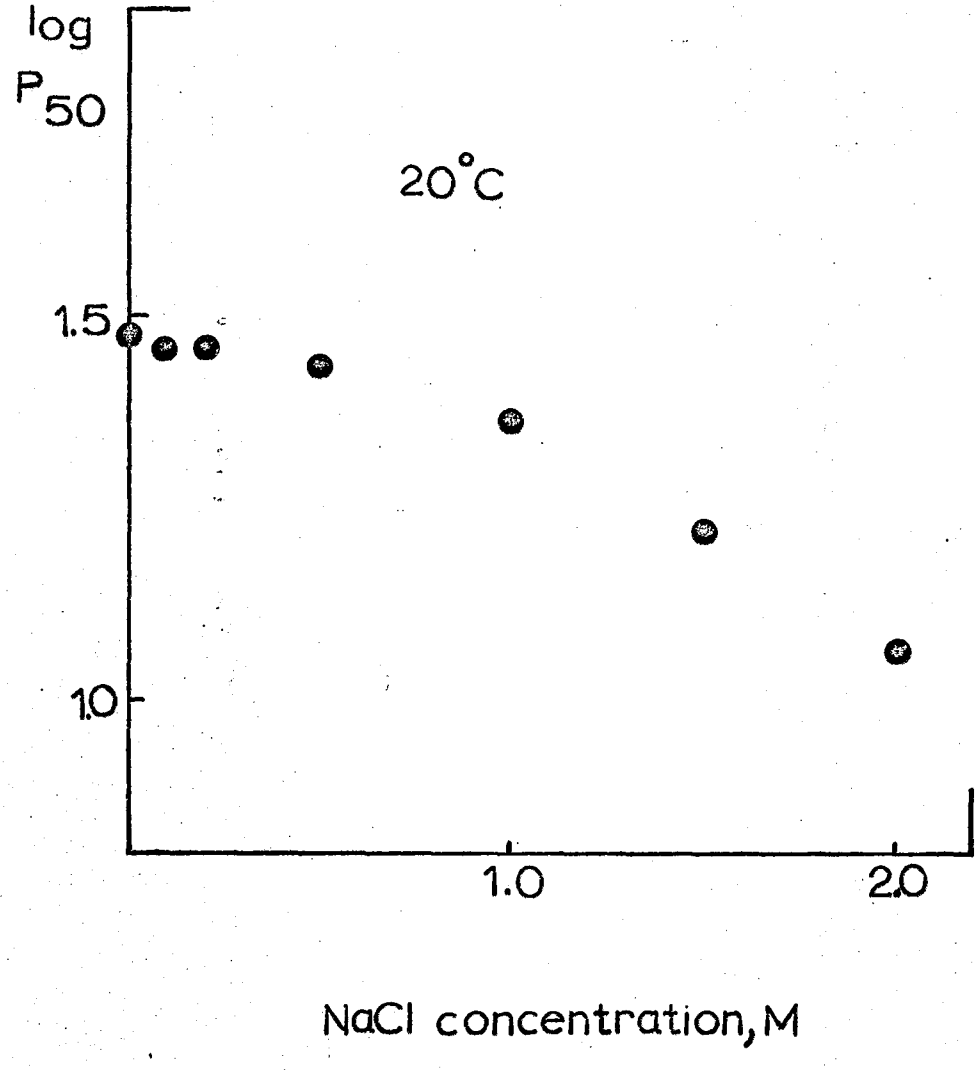

Figure 14. Effect of temperature on the oxygen affinity of *Thelepus crispus* vascular hemoglobin. Temperature range: 10-25° C. Buffer: 0.1 M Tris-HCl, pH 7.0, 0.01 M in $MgCl_2$.

Figure 14

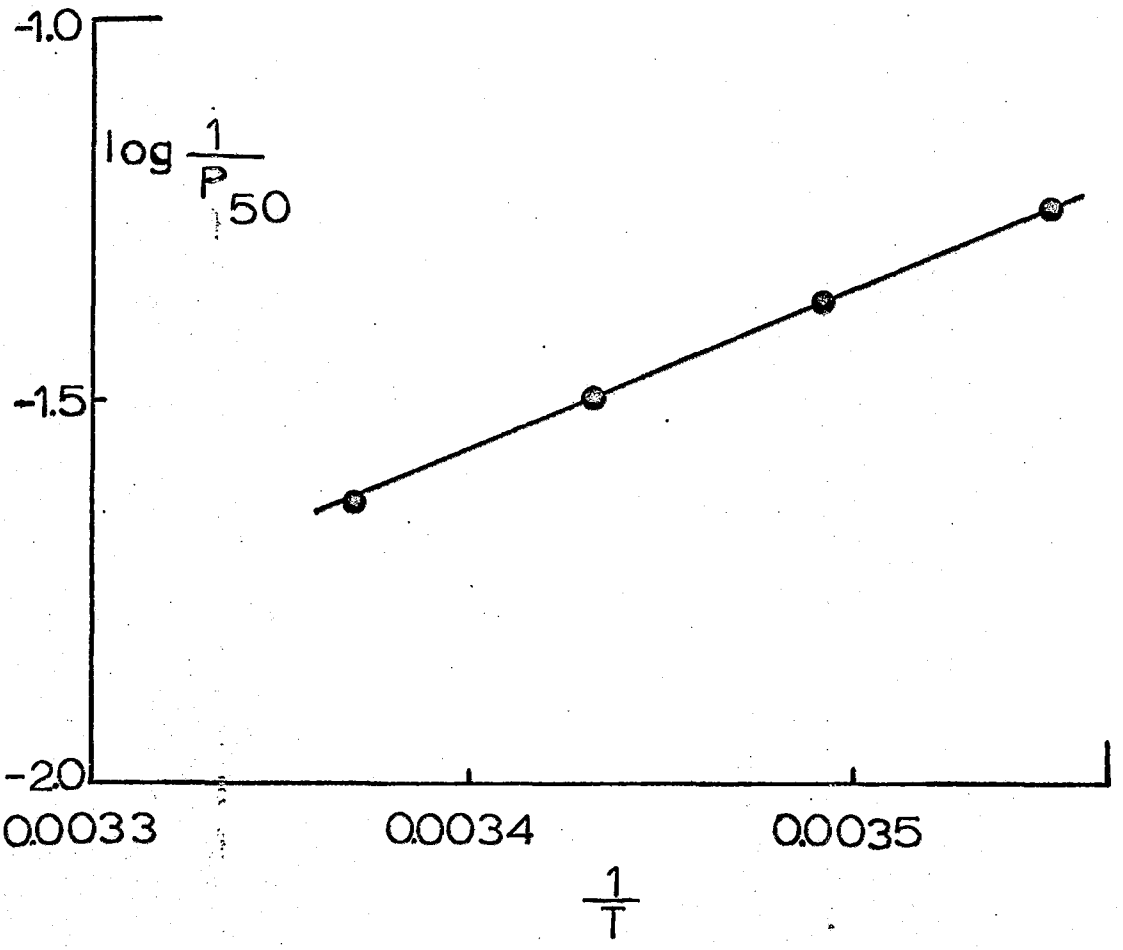

TABLE I

Absorption Maxima of Thelepus crispus Hemoglobins

coelomic cell hemoglobin

	λ_{max}^{α} (nm)	λ_{max}^{β} (nm)	Soret λ_{max} (nm)
OxyHb	577	542	417
*DeoxyHb	560		430
COHb	570	539	422
CyanmetHb		537	420

vascular hemoglobin

	λ_{max}^{α} (nm)	λ_{max}^{β} (nm)	Soret λ_{max} (nm)
OxyHb	576	541	416
*DeoxyHb	555		430
COHb	568	538	420
CyanmetHb		537	421

*Deoxygenated with sodium dithionite under argon.

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