SOME ASPECTS OF THE RESPIRATORY PHYSIOLOGY OF POLYCHAETE BODY FLUIDS - A STUDY IN ADAPTATION

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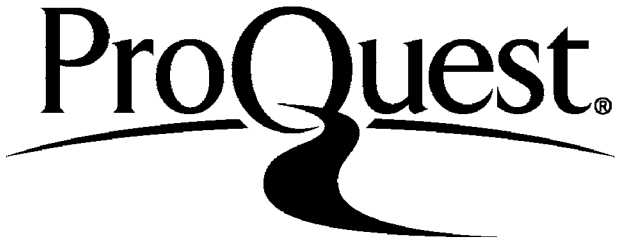

ABSTRACT

This thesis comprises a study of the physiological characteristics of the haemoglobins of several worms in relation to their habitats and life-styles.

A method for measuring oxygen-equilibrium in microlitre quantities of whole blood is described. Equilibrium data were determined for the vascular bloods of the polychaetes Cirriformia tentaculata, Terebella lapidaria, Arenicola marina, and Neoamphitrite figulus. Coelomic haemoglobins were studied in T.lapidaria, Notomastus latericeus, Capitella capitata and the echiuroid Thalassema neptuni. These bloods differed in oxygen affinity (p50) and the shape of the equilibrium curve (N).

Magelona is unique in the annelids in having haemerythrin and some physico-chemical properties are compared with the sipunculid pigment in Golfingia elongata.

Some physico-chemical properties of chlorocruorin, a pigment peculiar to polychaetes, were obtained from Sabella penicillus, Mercierella enigmatica, and Pherusa plumosa and their properties compared.

Assumptions of in vivo pH values were critically examined and large intra- and interspecific differences were measured, some being outside the pH range used in Bohr shift studies.

Carbonic anhydrase kinetics of Arenicola marina blood were compared with a bovine preparation. Inhibition with acetazolamide suggested participation in the removal of metabolic CO_2. The enzyme was looked for in several other polychaete tissues using a micromethod developed for this study.

The possibility of a storage function of a coelomic haemoglobin was examined in T.lapidaria. Aerial respiration rates in CO_2-free and 2.5% CO_2 atmospheres at several temperatures were determined. The use of CO_2 buffers is thought to be an innovation.

The effect of temperature on the p50's of T.lapidaria, A.marina, and Neanthes virens haemoglobins was analysed according to the van't Hoff integration. No molecular adaptations reducing temperature sensitivity were found.

The emergent theme was that the polychaetes studied were not so much adapted to a range of environmental variables (since they often created their own environment) but rather to a way of life.

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1 PREFACE

There is a wealth of literature on the oxygen-equilibria of invertebrate respiratory pigments. A summary of the annelid data is given in Appendix 1. The first of these studies was published exactly 50 years ago by Barcroft & Barcroft. Recently however, Mangum (1970,1973) questioned the assumptions underlying the experimental approach of the older works and erected new criteria.

The problem was in extrapolating from *in vitro* experiments to *in vivo* function. With recent technical advances it is possible to measure pH, pCO_2 and pO_2 very accurately in microlitre quantities of anaerobically sampled blood. Thus it is worth re-examining older works and new information can be gained from much smaller animals than was hitherto possible. It is therfore hardly surprising that so many studies on the oxygen-equilibrium have been published since 1970.

In contrast with many of the more biochemically oriented contributions this thesis was an attempt to understand the function of the respiratory pigments through interpretation of the oxygen-equilibrium data. This was made possible with some knowledge of *in vivo* pH's and pO_2's and by using fresh whole blood for the experiments.

Two areas remain to be explored. The first is a precise understanding of the near environment and *in vivo* pCO_2 and pO_2 values. The lack of this information is partly due to the limitations in techniques of micro-analysis.

Secondly, it is not known whether the equilibrium properties are a fixed characteristic of invertebrate haemoglobins, or whether there are *in vivo* modifiers of $p50$ and N. These modifiers may result from changes in the proportions of monomer and dimer elements, each with different equilibrium properties, or from biochemical substances. It was only recently that the importance of organic phosphates in mammalian red

blood cells was realised and their effects on the equilibrium studied (Benesch & Benesch, 1969). There are no a priori reasons why the same vertebrate modifiers 2,3 DPG and IHP should be found in polychaete bloods but other substances may yet be found. A comparison of highly purified solutions of haemoglobin with whole blood may help to resolve this as well as the question of the significance of haemoglobin in polychaete coelomocytes. The answer must take into account the observation that many annelids have vascular haemoglobins which are, without exception, freely dissolved in the plasma in contrast with the coelomic cell haemoglobins.

These aspects are a chiaroscuro of hope for the future understanding of the functions of invertebrate respiratory pigments.

Table 2.1. The effects of propylene phenoxitol and MS222 on the pH of coelomic fluid and vascular fluid in Arenicola marina at 15°C.

Treatment	pHcf	aH	n	S	pHvf	aH	n	S
None	7.32	47.9	5	0.015	7.28	52.5	5	0.005
0.3% phenoxitol	7.27	54.3	4	0.015	7.28	52.5	4	0.005
1% MS 222	7.30	50.1	4	0.020	7.29	51.3	4	0.005

pHcf is the pH of coelomic fluid, pHvf the vascular fluid, aH is H^+ activity in nmol l^{-1}, n is the number of determinations, and S the standard deviation.

2 METHODS

2.1 Measurement of body fluid pH

For the purpose of this study, the electrochemical determination of pH may be defined as -log aH, where aH is the hydrogen ion activity. The National Bureau of Standards' (NBS) pH-scales and certified buffers were used although a small error may be introduced with polychaete fluids because they have a higher ionic strength (Hansson, 1973). A two-point calibration procedure with NBS-approved buffer standards ensured absolute accuracies of the order of ±0.005 pH unit. When it was required to use the precision buffer solution Types S1500 and S1510, attention was paid to the fact that the pH changed with temperature. Temperature corrections are given in Appendix 2.

Electrode drift sometimes occurs with poorly buffered body fluids (Wilson, 1970) and for most worms without a respiratory pigment it was necessary to test several samples in succession before a stable reading was obtained.

The influence of blood cells and dissolved proteins at the liquid junction boundary is another source of error resulting from bioelectric potential (Maas, 1970). Differences of ±0.01 pH unit between cell and cell-free suspensions were found, depending on haematocrit value. Packed cell volumes were therefore measured and expressed as a percentage (PCV).

Coelomic fluid and blood were sampled anaerobically after rapid dissection under light paraffin or directly with a microsyringe. However, it was suspected that in some cases, direct sampling resulted in higher pH readings when the syringe was operated against a high internal hydrostatic pressure. For these, and some of the more errant worms, a 0.3% propylene phenoxitol or 1% MS 222 (Sandoz) solution in sea water relaxed the animals sufficiently to aid sampling. Use of these narcotics did not appear to influence the pH of internal fluids, ($p > 0.5$). (See Table 2.1.)

Samples were analysed immediately on removal from the animal using a capillary microelectrode (G 298A) which forms part of the Radiometer BMS2 apparatus and the pH was read from the Radiometer PHM 71 analyser. Replicate measurements were made quickly in succession after collection of fluid into glass capillaries. Samples were drawn by suction into the microelectrode which required 25-µl samples for a reliable pH reading. The glass, reference electrodes and liquid junction were held at 15±0.02°C unless otherwise stated.

A seasonal survey of body fluid pH was made on a population of Arenicola marina at Whitstable in Kent. Worms were collected at approximately monthly intervals and returned to the laboratory in a vacuum flask and the blood and coelomic fluids were analysed for pH.

A variety of worms was collected during a period of 2 weeks in the spring from shores in South Devon and kept under laboratory sea water circulation for a few hours at 15°C before pH measurements were made. Packed cell volumes (PCV) were estimated for the coelomic fluids using a micro-haematocrit centrifuge (Hawksley).

Since the pH scale is logarithmic, arithmetic means were calculated from the antilogarithms of the raw data. Samples were compared using the non-parametric Wilcoxon two-sample test to assess levels of significance.

2.2 Detection and quantification of carbonic anhydrase

2.2.1 Histochemical methods

Of the several qualitative methods for demonstration of carbonic anhydrase activity, the histochemical technique of Hansson (1967) was chosen. However, during the course of investigation, histochemical methods were shown to be non-specific (Huther, 1972) and were therefore abandoned in favour of a kinetic approach.

2.2.2 Enzyme kinetics

The procedure for determining quantitative activity of carbonic

anhydrase in the hydration reaction of carbon dioxide was adapted from Maren (1960) utilizing the changing pH principle (Philpot & Philpot, 1936). A microtonometer forming part of the Radiometer BMS2 apparatus was used as a scaled down reaction vessel to give a reaction volume of 80 µl. During the latter part of the study, the volume was further reduced to 60 µl. Reactants and enzyme source were used in less than one-tenth the quantities described in Maren's method. Humidified CO_2 delivered at a rate of 100 ml min^{-1} maintained saturation of the reactants. The reaction temperature was 15.0±0.02°C.

Stock solutions of enzyme were prepared by dissolving bovine carbonic anhydrase (Koch-Light) or diluting lugworm blood in distilled water until approximately 5 µl halved the time necessary to complete the uncatalysed reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

and

$$CO_2 + OH^- \rightleftharpoons HCO_3^-.$$

2.2.3 Procedure

Once the CO_2 flow had been stabilized, 40 µl of phenol red indicator (12.5 mg l^{-1} 0.0026M $NaHCO_3$) were run into the vibrated microtonometer. Enzyme solution was added and followed by inhibitor when desired. The volume was made up immediately to 70 µl with distilled water. A brief pause of 2-3 min at this stage of the procedure allowed the enzyme and inhibitor to come into equilibrium with the resulting enzyme-inhibitor complex. Ten µl of carbonate-bicarbonate buffer (30 ml 1M Na_2CO_3 and 20.6 ml 1M $NaHCO_3$ to 100 ml with distilled water) were rapidly expelled from a microsyringe (Shandon) into the reaction vessel and timed from then. A run was completed when the indicator changed from pink to straw colour. Uncatalysed reaction times were determined with total inhibition of the enzyme by 10 µl 10^{-5}M acetazolamide (Koch-Light).

2.2.4 Inhibition

Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulphonamide) was chosen for carbonic anhydrase inhibition because it is stable, specific and potent in very low molar concentrations. It has no other known action either in vitro or in vivo (Maren, 1967). For the kinetic part of the study, acetazolamide was titrated against a fixed concentration of enzyme in the reaction vessel. Concentrations of enzyme and inhibitor are given in the results.

Complete inhibition of Arenicola carbonic anhydrase in whole animals was achieved when 50 µl acetazolamide (10^{-5}M in sea water) were injected into the coelom. The drug was also added to the sea water medium at a final concentration of 10^{-5}M and no enzyme activity could be posthumously detected. Controls were divided into two groups; one with no treatment, and the other with 50-µl intracoelomic injections of sterilized sea water.

2.3 Oxygen-combining capacity and oxygen equilibrium

Sampling small blood volumes for oxygen content analyses without sacrificing the accuracy and precision of the traditional methods is a major technical difficulty. Polychaetes often yield meagre quantities of fluid making it necessary to pool or dilute samples. The choice of method for studying oxygen relations in body fluids depends on the kind of information required.

From an ecological point of view, it is better to study oxygen relations in whole blood rather than in dilute solution. This approach has been taken for several invertebrates by Wolvekamp & Vreede (1941), Niesel & Thews (1961), Toulmond (1970 a) and Weber (1970, 1971a,b, 1973). The discrepancies arising from dilution were first discussed in detail by Hill & Wolvekamp (1936) and later by Wolvekamp (1961). These have not been fully explained although a number of causes is likely; subunit interaction and organic phosphate concentration are probably the most

important. Differences in oxygen equilibrium between haemoglobin in the cell and in solution have been noted by Forbes & Roughton (1931), Radford et al.(1967), and Forster (1972).

The manometric method (Van Slyke & Neill, 1924) remains the universal standard for accurate oxygen content determination. It gives direct and unambiguous information concerning the amount of oxygen in combination with a respiratory pigment. However, the need for relatively large volumes of blood (0.5 ml) renders it impractical for many species.

Spectrophotometric methods assume obeyance of the Bouger-Beer relationship. This assumption has been justified for very dilute solutions of respiratory proteins in the visible region of the electromagnetic spectrum. Light scattering from cell suspensions may once have been a source of error but modern instruments do not suffer this effect. The equilibrium data are usually presented as a saturation fraction rather than in volumes of combined oxygen as in the direct approach. High sensitivity, accuracy, and speed have popularised this technique over the past 25 years (Benesch et al., 1965)

Evacuation procedures used to prepare the deoxygenated form of the pigment may lead to rapid denaturation. Further problems with the spectrophotometric method may arise when the presence of haem compounds other than oxy- or deoxy- are anticipated. Several polychaetes are known to contain such compounds (Patel & Spencer, 1963 a; Dales, 1964, 1965). It is suspected that methaemoglobin does not form naturally as with vertebrates and a reductase system has yet to be demonstrated. However, methaemoglobin was found in freshly collected Capitella blood, and metchlorocruorin in Mercierella. Several polychaete haemoglobins may form methaemoglobins after storage and during purification.

An apparatus was recently described by Sick & Gersonde(1969) for continuous measurement of oxygen equilibrium curves. The measuring principle is based on the operation of a diffusion chamber through which

gas comes into rapid equilibrium with a thin layer of whole blood. Changes in light absorption are monitored as a function of pO_2.

Full saturation of a pigment is difficult to estimate because of the asymptotic nature of the equilibrium data. The p100 point has been accurately determined by varying the oxygen content of a red-cell suspension and measuring the resultant pO_2 decrease caused by a heart muscle preparation (Colman & Longmuir, 1963; Longmuir, 1964). A rapid electrode response and low critical pO_2 are requirements of the method and it is not suited for use with haemoglobin solutions.

The ultramicromethod described in this thesis was developed to overcome some of the limitations of the contemporary techniques.

2.3.1 Principle

Oxygen bound by haemoglobin may be released into physical solution if a blood sample is diluted with a large volume of ferricyanide solution. The increased oxygen tension resulting from the dissociation of oxygen from oxyhaemoglobin may be measured with an oxygen electrode. The oxygen content of the sample may then be calculated from the pO_2 increase and the solubility coefficient of oxygen in solution. The micromethod described in this thesis was based on the earlier method of analysis of 50-µl samples of mammalian blood described by Laver et al. (1965). I have analysed samples down to 0.8 µl of lower oxygen-combining capacity without sacrificing accuracy or precision. See Fig 2.3 p 20 Consequently, I was able to construct oxygen equilibrium curves from 10-50 µl samples of whole, undiluted blood.

2.3.2 Apparatus

The Radiometer Blood Micro System series 2 (BMS2) was designed for measurements on µl volumes of whole blood under anaerobic conditions to prevent the gain or loss of gas (Severinghaus, 1964) and was therefore suited for use with blood from small animals.

A direct reading pO_2 microelectrode (Radiometer E5046) was housed in a water jacket and equipped with an adaptor to fit a luer syringe. The

electrode required at least 70 µl for an oxygen measurement and because of the low cathode current (10^{-11} A mm Hg^{-1}) it was not necessary to stir the sample.

Blood samples of up to 50 µl were equilibrated at various pO_2's in one or more of the 4 microtonometers which form part of the Radiometer BMS2 apparatus.

Gases from cylinders of oxygen-free nitrogen and 1% oxygen in nitrogen were mixed in a Gallenkamp gas mixer. For higher pO_2's, mixtures of 20% or 100% oxygen with nitrogen were used. A continuous flow of humidified gas was passed through the tonometers and released to the atmosphere or checked for pO_2. Later in the study, the gas mixer was replaced with a 3-way system incorporating 3 flowmeters (Heterate, England) and allowed CO_2 to be blended in.

A 1 cm^3 glass tuberculin syringe (Chance or Summit, England) clamped in a rigid frame was calibrated to deliver a constant volume (Fig.2.1). A small glass bead of known volume inserted into the barrel ensured complete mixing of the contents. Blood was subsampled from the tonometer in 1-5 µl ± 0.5% graduated micropipettes (Yankee, U.S.A.). A diagram of the functional parts of the apparatus is given in Fig.2.2.

2.3.3 Reagent

A dilute ferricyanide solution (12 mmol l^{-1} $K_3Fe(CN)_6$) was freshly prepared and stored for not more than a few days at room temperature in a dark glass bottle.

2.3.4 Procedure

When the BMS2 apparatus had been adjusted to the required working temperature (±0.02°C), the pO_2 electrode was zeroed with oxygen-free nitrogen or Na_2SO_3 (1 mol l^{-1} 0.02 mol l^{-1} $Na_2B_4O_7.10\ H_2O$) and calibrated with air-equilibrated ferricyanide solution at the same temperature.

A freshly taken sample of blood was placed in the tonometer and agitated for at least 15 min to ensure equilibration. A sample of ferricyanide solution was taken into the glass syringe, capped, and

Figure 2.1. Picture of the glass syringe and clamp designed to deliver a constant volume. A sample of blood is shown being introduced from a graduated micropipette.

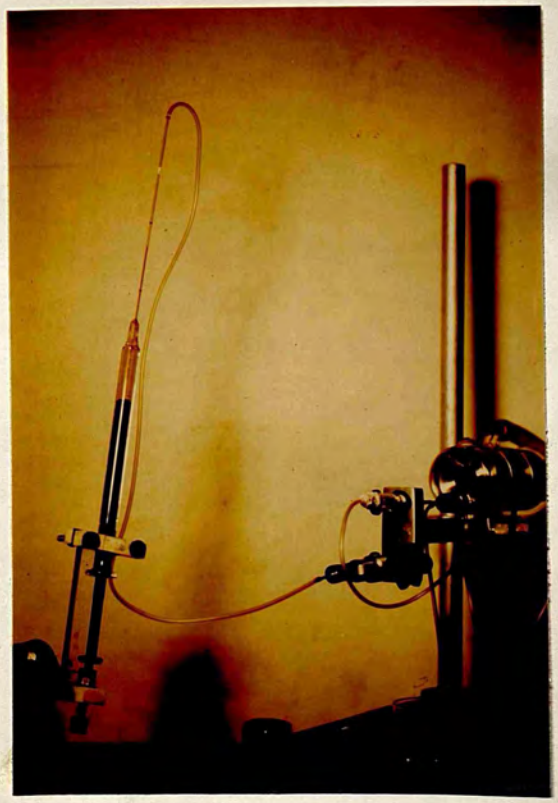
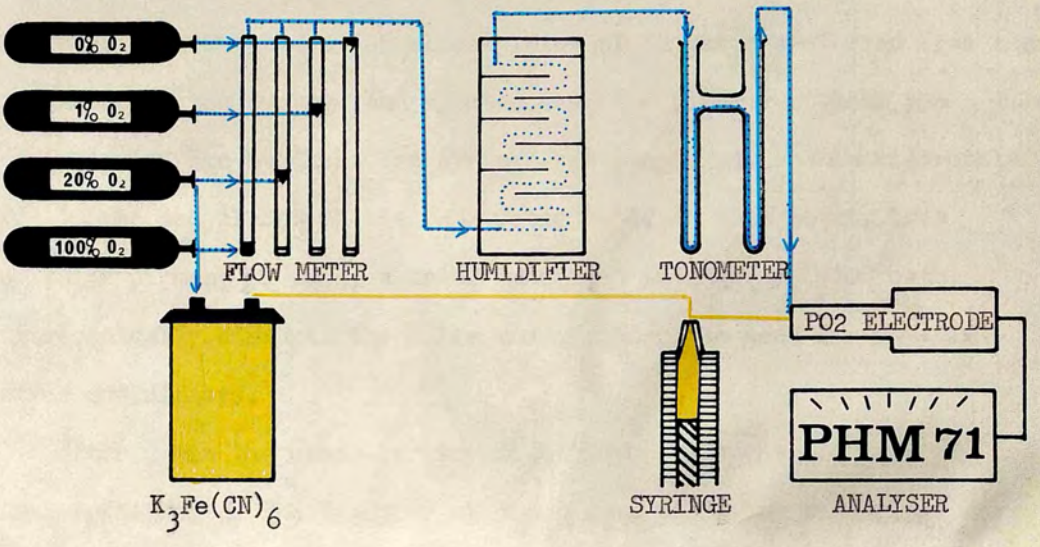


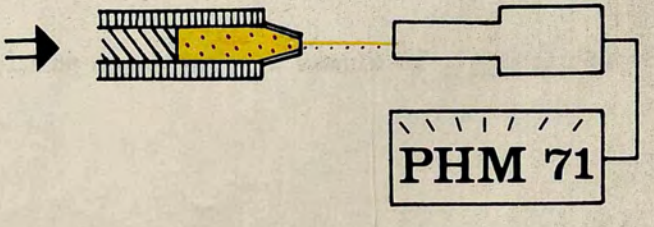
Figure 2.2. Diagram of the functional parts of the apparatus used in the determination of oxygen content analysis and equilibrium studies.

(i) The electrode is calibrated and the gas mixture is checked for $\underline{p}O_2$.



(ii) The blood is equilibrated with the gas and a sample is introduced into the syringe.

(iii) Oxygen is released from combination with the pigment and the contents of the syringe are analysed for $\underline{p}O_2$.



* Every point on an equilibrium curve represents a duplicate determination of oxygen-combining capacity at the applicable gas tension. The two measurements comprising each point were identical in all the points which have been plotted and therefore had no standard deviation (as in Fig.2.3). This zero standard deviation indicated the high precision of the method. The accuracy of the method was established with Arenicola blood (Section 5.3) for which many equilibrium curves have been published (See Appendix 1). Since the equilibrium data was calculated as % saturation (see Section 2.3.5) the accuracy and precision of the curves is a function of the electrode characteristics alone (see Section 2.3.6).

returned to one of the BMS2 ports. The syringe was clamped in an inverted position and a 1-5 µl sample of blood was transferred from the tonometer to the syringe and ejected onto the plunger without the admission of air bubbles. The syringe was capped and shaken vigorously for 10 sec and placed in the BMS2 water bath. At this point, if a cellular pigment is used, a brief immersion in a Kerry KS100 bath ultrasonically disrupts the cells and obviates the need for saponin or other emulsifiers.

After 2 min the blood-ferricyanide mixture was analysed for $\underline{p}O_2$ and the result noted. The transfer of blood from tonometer to syringe was completed as quickly as possible and, with practice could be done in 15-20 sec, one whole determination taking about 5 min.

* See opp.

2.3.5 Calculations

The oxygen content of a blood sample in vols% was calculated from the following formula derived from the Gas Laws:-

$$C_{O_2} = \frac{\alpha^{T}_{H_2O}}{760} \times \frac{V}{v} \times 100\left[\underline{p}O_{2_{fehb}} - 0.2093(P_{bar} - WVP)\right]$$

where

C_{O_2} = oxygen content in vols%

$\alpha^{T}_{H_2O}$ = Bunsen absorption coefficient of oxygen in water at T°C

V = volume in µl of ferricyanide in syringe

v = volume in µl of blood in syringe

$\underline{p}O_{2_{fehb}}$ = partial pressure of O_2 in mm Hg of blood-ferricyanide mixture

P_{bar} = barometric pressure in mm Hg

WVP = water vapour pressure in mm Hg at T°C.

At 15°C the oxygen-combining capacity of a sample of Arenicola blood was calculated as follows:-

$$C_{O_2} = \frac{0.0315}{760} \times \frac{275.4}{5} \times 100 \left[192 - 0.2093\ (766.6-12.8)\right]$$

$$= 8.5 \text{ vols\%}.$$

2.3.6 Possible sources of error

The ferricyanide method is based on the assumption that the Bunsen oxygen solubility coefficient of the dilute ferricyanide solution with or without the added blood sample, is equal to the oxygen solubility coefficient of water. Evidence to validate this assumption was presented by Laver et al. (1965) and the error is less than 0.05%. Bunsen coefficients of oxygen were taken from Weiss (1970) and are given in Appendix 3.

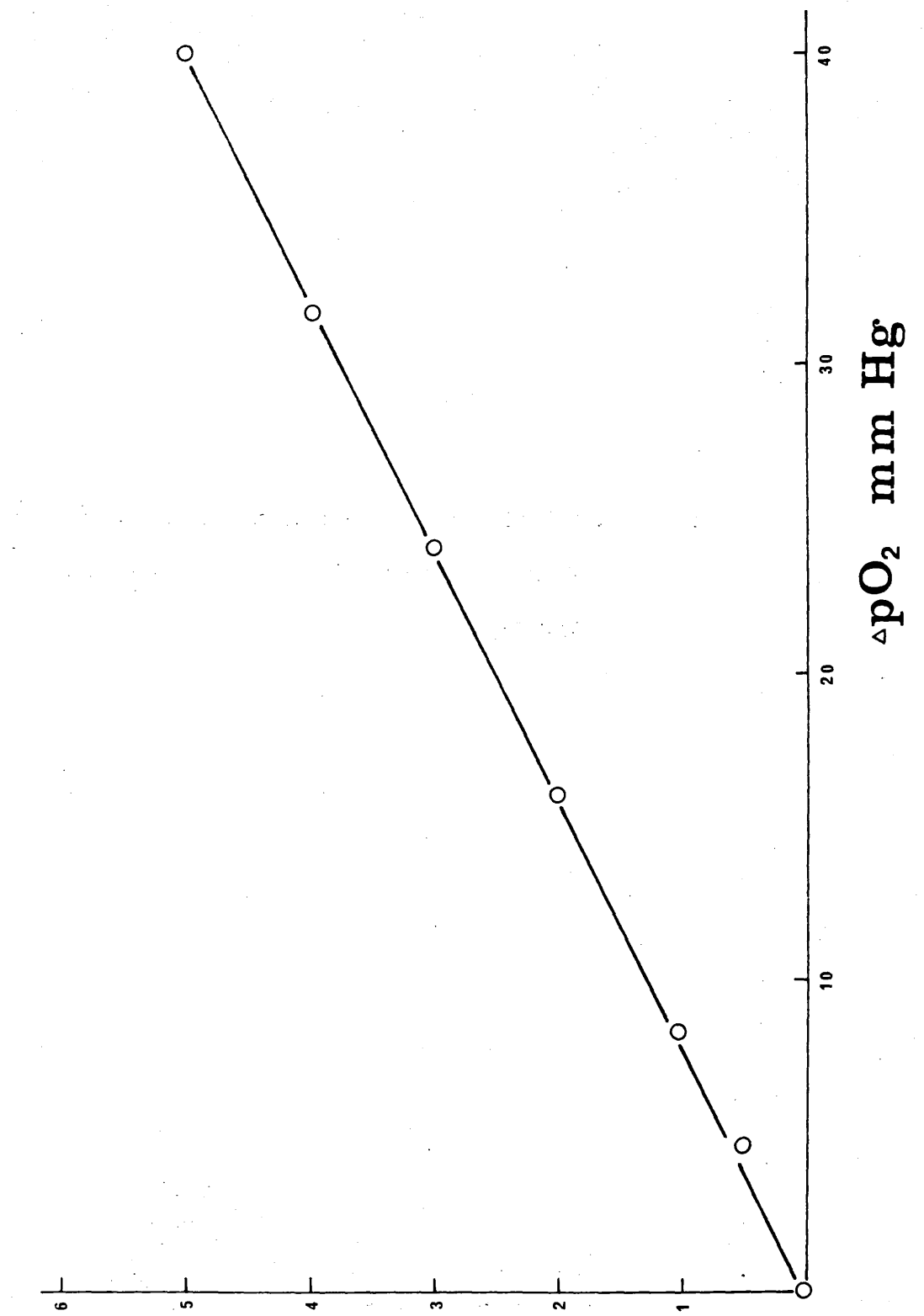
The precision of the method was determined with a sample of Arenicola blood showing the change in pO_2 proportional to the volume of blood added to the ferricyanide (Fig. 3.).

The characteristics of the E5046 electrode are such that drift is negligible if the electrode span is calibrated immediately before every measurement. Error in the measurement of pO_2 in the ferricyanide solution and the blood-ferricyanide mixture is essentially a constant percentage and thus cancels out in subtraction. If the accuracy of pO_2 measurement is ±1 mm Hg, a maximum standard deviation of 0.2 vols% results. In practice, the deviations were less.

2.4 Spectrophotometry

Respiratory pigments were characterized by measuring absorption maxima with Unicam SP500, Beckman DB-G, and Beckman DK-2 scanning spectrophotometers. It has been asserted that no spectrophotometric measurement should be considered reliable unless the instrument has been checked with suitable standards (Rand, 1969). An acid solution of potassium dichromate consisting of 50 mg $K_2Cr_2O_7$ l^{-1} 0.01N H_2SO_4 has an absorbance of 0.725 at 257 nm and 0.535±0.005 at 350 nm (Martinek et al., 1972). This solution and a similar one containing 100 mg $K_2Cr_2O_7$ l-1 were the standards used to calibrate the instruments.

Figure 2.3. Relation between the volume of blood injected into 305 µl ferricyanide solution and the change in the $\underline{p}O_2$ in the syringe. (See p201 for details).


blood volume (l x 10⁻⁶)
1
2
3
4
5
6
10
20
30
40
ΔpO₂ mm Hg

2.5 Estimation of blood volumes

2.5.1 Principle

The volume of a pigmented fluid may be found from the application of a spectrophotometric technique (Chapman, 1967). The absorbance of an uncontaminated sample of fresh blood of known volume is determined at a known dilution. An extract of the blood from the rest of the animal may be matched up by dilution to give a similar absorbance at a convenient wavelength and the total volume of blood can be calculated.

2.5.2 Procedure

Arenicola marina were anaesthetised with 0.3% propylene phenoxitol in sea water, blotted, and the total volume of the animal measured by water displacement. Blood was sampled directly from the dorsal vessel with a 20 µl glass micropipette (Drummond) after the coelomic fluid had been removed. The 20 µl were diluted with 4 ml phosphate buffer pH 7.4 (McIlvaine). The rest of the animal was ground with pestle and mortar to release blood from the tissues. An equal volume of buffer ensured no extreme changes in the pH which might have denatured the pigment. The preparation was spun at 30,000 g at 5°C for 20 min in an M.S.E. model 25 high-speed centrifuge.

The clear supernatant was matched up with the sample at 414 nm. The total volume of blood from the worm was calculated from the volume of buffer required for matching the standard.

2.5.3 Calculation

Volume of worm = 4.20 ml

volume of pure blood = 10 µl

matched volume = 212 ml

thus total blood volume = (212 x 0.01)/4 - 0.01

= 0.52 ml.

2.6 Haemoglobin determination from cyanmethaemoglobin

2.6.1 Principle

A known volume of blood was diluted with a cyanide-ferricyanide solution and haemoglobin concentration was estimated from the optical density of the solution at 540 nm by comparison with cyanmethaemoglobin standards.

2.62. Reagents

A modified Drabkin's solution was prepared from 0.05g KCN and 0.20g $K_3Fe(CN)_6$ dissolved in 1l distilled water and is stable at room temperature in a dark glass bottle for several days. Precautions were taken when handling potassium cyanide.

Diluted cyanmethaemoglobin standards were prepared from a 1 in 201 dilution of Diagen (Diagnostic Reagents) equivalent to 18g haemoglobin per 100 ml blood.

2.6.3 Calibration

Absorbancy scales vary up to 20% between different spectrophotometers (Rand, 1969) and it is thus necessary to construct a calibration graph to relate the absorbance reading to the amount of the substance which is to be measured. Absorbance was plotted against haemoglobin concentration from serial dilutions of the cyanmethaemoglobin standard (Fig.4).

2.6.4 Procedure

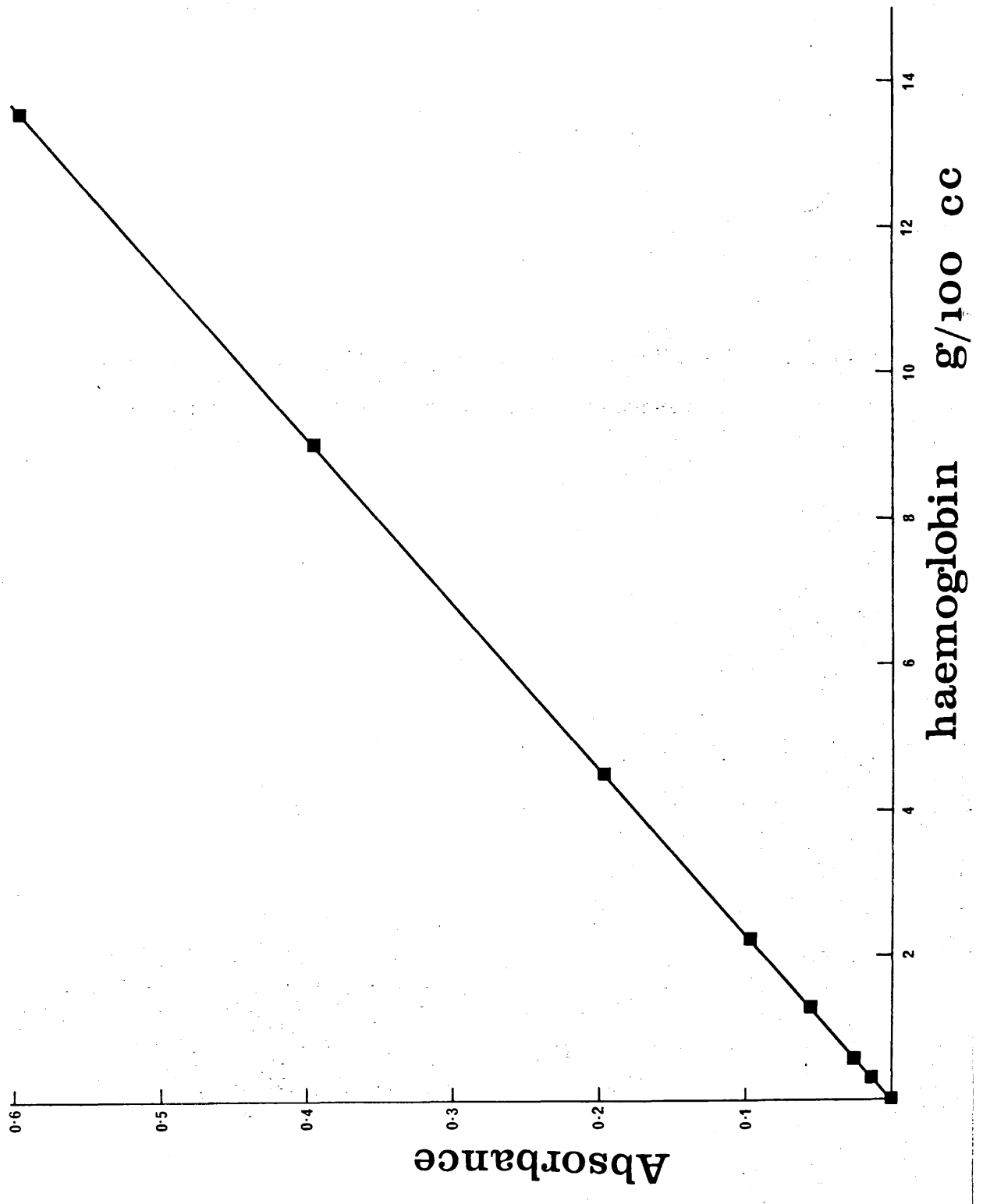
0.02 ml blood was added to 4 ml Drabkin's solution, stoppered, shaken, and left to stand at room temperature for 20 min. The absorbance was then measured with a Beckman DB-G spectrophotometer at 540 nm and the haemoglobin concentration estimated from the calibration graph or calculated from:-

$$c_1 = c_2 \times A_1/A_2$$

where c_1 and A_1 are concentration and absorbance of the unknown sample, and c_2 and A_2 the concentration and absorbance of the cyanmethaemoglobin standard.

Figure 2.4. Calibration graph for the estimation of haemoglobin concentration from cyanmethaemoglobin absorbance.

Every point represents one spectrophotometric measurement from one sample from each of the serially diluted Diagen standard solutions (see p25).


Absorbance
0·6
0·5
0·4
0·3
0·2
0·1
haemoglobin g/100 cc
2
4
6
8
10
12
14

2.7 Aerial respirometry

Measurements of oxygen consumption rates in moist air were made using constant pressure respirometers (Davies, 1966) placed in a water bath (±0.2°C) using worms which had been acclimated in shallow tanks of aerated sea water at 14°C for 10 days. The well was filled with 20% KOH and a cone of Whatman No.42 (starch-free) filter paper inserted to ensure complete and rapid removal of CO_2.

One experiment was performed in which a CO_2 buffer was substituted for KOH to maintain a constant proportion of CO_2 in the respirometer chambers. The buffer used (80 vols 3M $KHCO_3$: 20 vols 3M K_2CO_3) provided a 2.5% CO_2 atmosphere at 20°C.

CO_2 exchange was accelerated with 1mg bovine carbonic anhydrase (Koch-Light) ml^{-1} buffer, as recommended for use in manometric vessels by Burk (1961). Because of the long equilibration times required, the respirometers were assembled in a 2.5% CO_2 atmosphere in a polythene glove-bag. Readings were taken at 3 or 4 intervals every hour and continued for up to 10h. To simulate the natural condition, the respirometers were kept in darkness for the duration of the experiments. Wet weights were used.

3. HYDROGEN ION ACTIVITY IN POLYCHAETE BODY FLUIDS

3.1 Abstract.

1. Body fluids from a natural population of Arenicola marina fluctuated between pH 7.15 (70.8 nmol l^{-1}) and 7.51 (30.9 nmol l^{-1}) throughout the year and had no apparent correlation with temperature which ranged from 5 to 15°C.

2. Hydrogen ion activity ranged between pH 7.79 (16.2 nmol l^{-1}) and 7.41 (38.9 nmol l^{-1}) in the blood, and 7.66 (21.9 nmol l^{-1}) to 7.19 (64.6 nmol l^{-1}) in the coelomic fluid of Arenicola during 6 weeks of fasting.

3. Twenty-five polychaete species varied in body fluid pH from 6.84 (145 nmol l^{-1}) to 7.44 (36 nmol l^{-1}) in fresh, anaerobically handled samples at 15°C.

4. Coelomic fluid packed cell volumes ranged from less than 1 per cent to 78 per cent in different species.

3.2 Introduction.

The amplitude of variation in body fluid pH that might occur in invertebrates is not well known and even less is known about the causes of such variations or the potential regulating mechanisms which might damp the effects of fluctuating hydrogen ion activity. Mangum (1970) has drawn attention to the lack of information concerning pH values for blood and coelomic fluid in invertebrates. The problem of pH variation was partly resolved by a tripartite approach posing the following questions:

(i) What variations in body fluid pH occur at a given time? When an animal possesses a well-developed vascular system, differences in afferent and efferent blood pH must be presumed. In coelomic fluids

with or without a respiratory pigment there exists the possibility of pH gradients as suggested by Clark (1964). There is also the possibility of respiratory gradients in marine invertebrates based on the distribution of succinic dehydrogenase (Krishnaswamy et al., 1967).

(ii) What agencies promote or regulate hydrogen ion activity within a population and what potential is there for regulation? For example, seasonal changes accompanied by gamete production, temperature change or fasting should be considered along with the possible effects of anoxia on body fluid pH. The effects of external changes in pH on the internal state are not known. However, Amoureux (1961) noted a fall in the pH of interstitial water from pH 8.4 to 7.3 over the intertidal period in an estuary rich in polychaetes. The pH of the blackened layer of sediment at Whitstable was significantly lower (pH 6.6-6.9) than that at the surface (Perkins, 1957). These results are difficult to assess in relation to possible changes in body fluid pH without knowing the gas or ionic species which caused the observed fall. An accumulation of CO_2 in the near environment may have physiological significange at the sites of oxygen uptake where there is a functional Bohr shift.

Any gain or loss of hydrogen ions may be difficult to observe when their concentration is masked by the buffer actions of proteins and the carbonate-bicarbonate system.

(iii) Are there real interspecific differences in pH?

These questions and some of their implications have been examined in this section. The first two categories may be conveniently discussed with reference to Arenicola marina (L.), an invertebrate chosen as a model to demonstrate some of the possibilities outlined above. The last question was partly resolved by measurement of body fluid pH of different species under comparable conditions.

Reliable values for pH values of body fluids have been quoted by

Mangum & Shick (1972) for a number of marine invertebrates. Other values scattered throughout the literature do not meet Mangum & Shick's criteria for accurate measurement in respiratory fluids, namely: accurate temperature regulation to within 0.1°C, anaerobically handled samples, an open liquid junction between reference and glass electrodes, and the temperature of measurement the same as *in vivo*. The last criterion mitigates attempts to measure pH at one temperature and calculate its value at another because temperature coefficients of biological fluids vary considerably.

The limits of blood pH must be known in order to evaluate the importance of the Bohr shift in the functioning of respiratory pigments. The Bohr effect has been studied many times in *Arenicola* and failing reliable measurements of blood pH which may be assumed to prevail in a living animal, *Arenicola* is quoted as having no Bohr effect at pH 5.4-6.8 (Prosser & Brown, 1961), a slight effect at pH 7.6-7.9 (Allen & Wyman, 1952) and pCO_2 0-120 mm Hg (Wolvekamp & Vreede, 1941) and a more marked effect at pH 7.1-7.5 (Toulmond, 1970a).

Measurements of pH following some of the suggestions outlined above should therefore form a basis for better interpretation of the function of respiratory fluids.

3.3 Results.

A seasonal survey of *Arenicola* showed no seasonal trend which might be accounted for by temperature alone (Fig.3.1).

There was no significant difference in the pH of the coelomic fluid samples taken at successive points along the body of *Arenicola* ($P>0.5$), indicating that a pH gradient does not exist in the coelomic fluid of this animal.

The effect of starvation on the pH of the body fluids in a laboratory population of *Arenicola* is shown in Fig.3.2. An initial rise

Figure 3.1. The pH of the blood (■—■) and coelomic fluid (□—□), at monthly intervals, from a population of A.marina found at Whitstable. Mud temperatures at 20 cm depth at the time of collection are given. Every point is a mean pH from 10 worms measured individually. As the samples were not pooled, N = 10. Temperature of measurement = 15°C. Standard deviations are shown.

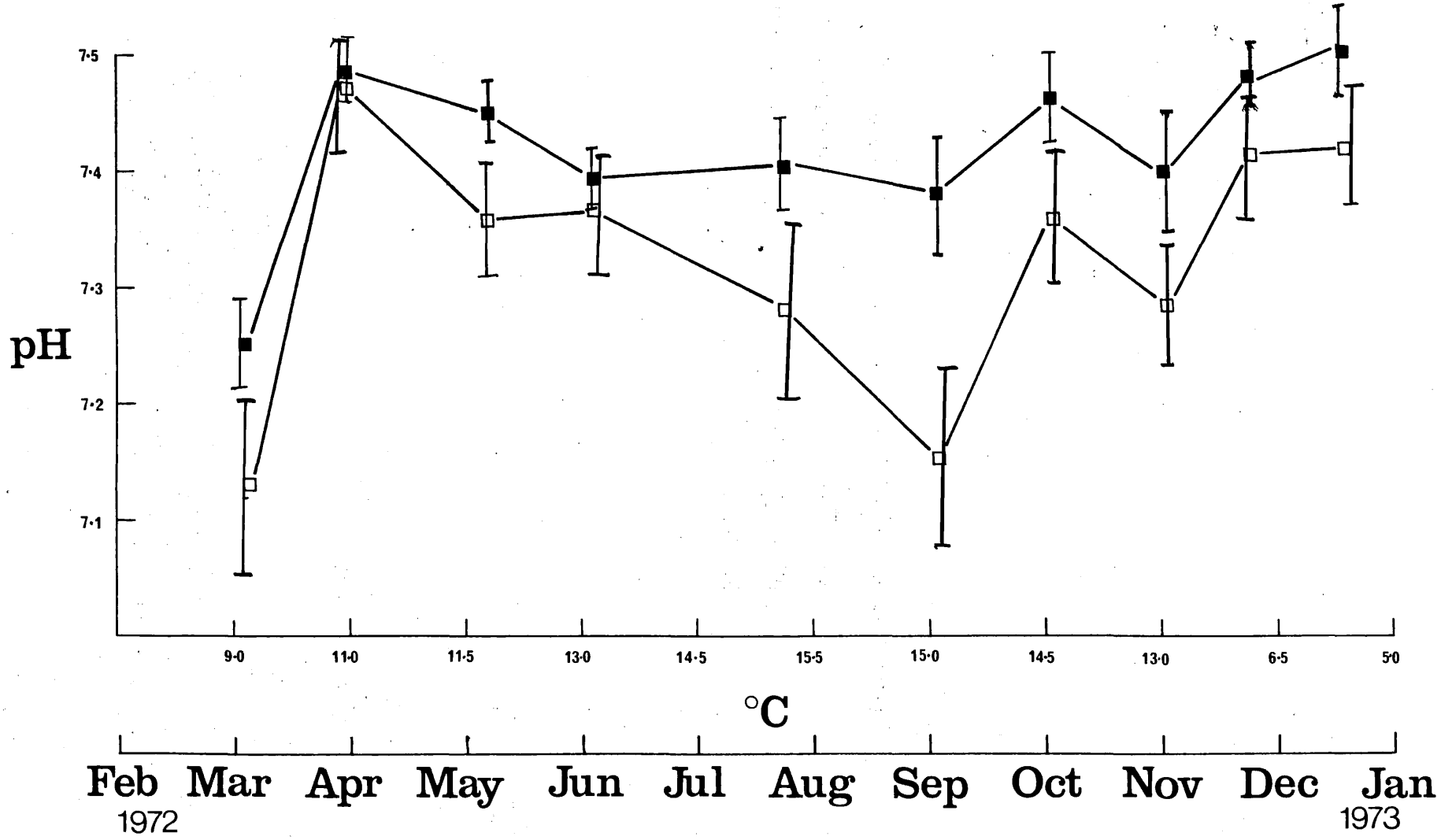
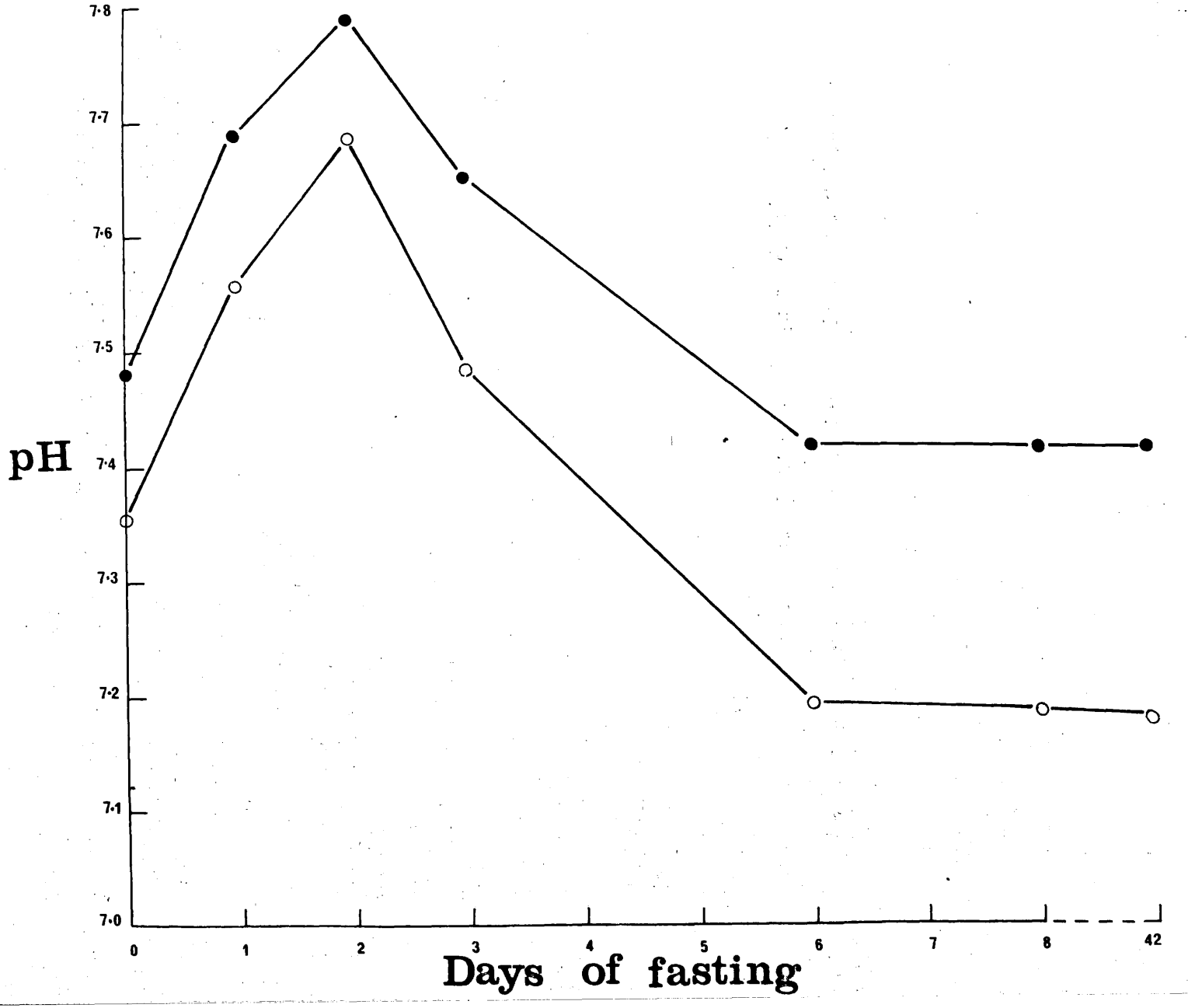

pH
7·5
7·4
7·3
7·2
7·1
9·0
11·0
11·5
13·0
14·5
15·5
15·0
14·5
13·0
6·5
5·0
°C
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
1972
1973

Figure 3.2. The effect of fasting on the pH of blood (●—●) and coelomic fluid (O—O) in A.marina. The worms were kept in running sea water at 15°C without food. Time zero is the time of collection and each point is a mean of 5 measurements from 5 individuals.


pH
7·8
7·7
7·6
7·5
7·4
7·3
7·2
7·1
7·0
0
1
2
3
4
5
6
7
8
42
Days of fasting

followed by a decline to a steady pH level may reflect depletion of metabolic reserves and changes in substrate.

Results of pH measurements from a variety of polychaete body fluids are summarized in Table 3.1 and indicate large interspecific differences.

3.4 Discussion.

We have become accustomed to thinking of tissues as exquisitely sensitive because the reaction of the blood is controlled to a fraction of a pH unit. But the range from pH 7.0-7.8 includes activities from 2.5 times the normal mammalian value (100 nmol l^{-1} at pH 7.0) to two-fifths of the normal (16 nmol l^{-1}) at pH 7.8, a range from 40 to 250 per cent of the value normally maintained. This is far greater than the range of potassium or sodium ions that could be tolerated.

One of the pitfalls of dealing with a logarithmic scale is that serious errors may result from averaging pH values instead of first converting them to antilogarithms. For example, the addition of two pH values and division of the sum by two is not a mean, but a square root! Likewise, the reporting of standard error will obscure any real differences in pH which might occur. The use of nmol l^{-1} to express hydrogen ion activity overcomes the problem of the pH scale which is the logarithmic scale of dilution. The mole concept in relation to chemical activity is discussed by Owen et al.(1970). Thus, there is now the opportunity to express hydrogen ion activity in terms of the basic SI unit, the mole. There is some degree of uncertainty in deriving concentration from reliable pH measurements but it is a reasonable assumption that for solutions as dilute as the body fluids, the activity coefficient is 1.00 (Mattock & Band, 1967). For these reasons, hydrogen ion activities and standard deviations in nmol l^{-1} were reported together with pH in the results.

Mangum (1970) and Mangum & Shick (1972) have emphasized the importance of temperature, not only as it affects the hydrogen ion activity (the pH of neutrality drops with a rise in temperature), but also in acclimation. Amphitrite cirrata acclimated at 10°C had a "mean" pH of 7.06 and those at 15°C, 6.90. Howell et al.(1973) observed that the pH of invertebrate body fluids decreased as their body temperature increased in precisely the same way that the pH of the neutral point of water decreased. This, they claimed was evidence that invertebrates regulated the pH of their extracellular fluids to maintain their relative alkalinity by manipulation of pCO_2 and HCO_3^-.

Another interesting feature shown by Mangum & Shick (1972) was the systematic drop with time from pH 7.4 to 6.5 in an isolated sample of Arenicola blood. The authors attributed this to deoxygenation and the value which might be achieved during respiratory stress at low tide. I can also confirm this observation with Arenicola blood and, indeed, with other polychaete body fluids. However, unlike an isolated sample, a whole animal has the means to remove CO_2 as suggested in Section 4.5 thus preventing respiratory acidosis. Furthermore, it is well known that a higher proportion of oxyhaemoglobin contains more free acid radicles than the deoxy-form, thus with deoxygenation, the pH rises, not falls. Toulmond (1970a) has shown that upon deoxygenation, the blood of A.marina rises by approximately 0.1 pH unit. On the other hand, Section 4.4 shows that for whole animals under anoxia, the pH of Arenicola falls to 6.98 after 5h. It is most probable that a marked fall in blood pH with time is due to cellular or micro-organism metabolism in the absence of anaerobic respiration. Toulmond (1973) has subsequently obtained similar data on the effects of anaerobiosis on the blood pH of Arenicola and proposes a major function of haemoglobin during low tide in the acid-base regulation of the blood, is to buffer against the acid

Table 3.1. Mean values of pH and hydrogen ion activity (a_H) at 15°C.

Species	Coelomic fluid					Vascular fluid			
	pH	*a*H	*N*	*S*	PCV (%)	pH	*a*H	*N*	*S*
Arenicolidae									
Arenicola marina (L.)	7·295	50·96	14	2·50	<1	7·290	51·40	13	3·50 Hb
Arenicolides ecaudata (Johnston)	7·170	67·30	5	12·23	<1	7·265	54·32	5	1·88 Hb
Ampharetidae									
Melinna palmata Grube	7·325	47·32	12	<1					
Aphroditidae									
Aphrodita aculeata L.	7·350	44·78	5	3·60	<1				
Hermonia hystrix (Savigny)	7·080	83·18	2		<1				
Arabellidae									
Arabella iricolor (Montagu)						7·023	94·41	2	Hb
Capitellidae									
Notomastus latericeus Sars	7·030	93·76	4	6·25	21 Hb				
Chaetopteridae									
Chaetopterus variopedatus (Renier)	6·840	145·63	3	2·14	<1				
Cirratulidae									
Cirriformia tentaculata (Montagu)	7·100	79·39	8	9·86	72	7·116	76·74	8	4·83 Hb
Flabelligeridae									
Pherusa plumosa (O. F. Muller)	7·440	36·01	2		<1 Cr				
Glyceridae									
Glycera sp.	7·330	46·54	2		<1 Hb				

Maldanidae									
Euclymene lombricoides (Quatrefages)	7·110	77·90	6	7·92	<1				
Nephytidae									
Nephtys caeca (Fabricius)	7·205	62·02	7	7·05	<1 Hb				
Nephtys hombergi Savigny	7·285	51·89	5	1·11	<1 Hb				
Nereidae									
Eunereis longissima (Johnston)	7·315	48·43	6	1·49	78				
Polynoidae									
Lepidonotus clava (Montagu)	6·980	104·3	3	0·69	58				
Sabellidae									
Megalomma vesiculosum (Montagu)	7·240	57·55	2		<1 Cr				
Sabella penicillus L.	6·920	120·75	8	20·6	60 Cr				
Terebellidae									
Amphitrite edwardsii (Quatrefages)	7·320	47·80	5	5·80	25 Hb	7·450	35·54	4	2·30 Hb
Amphitritides gracilis (Grube)	6·955	110·30	16		<1 Hb				
Lanice cochilega (Pallas)	7·330	46·64	4	7·17	27 Hb	7·388	40·74	2	Hb
Neoamphitrite figulus (Dalyell)	7·285	51·90	6	5·20	23 Hb	7·295	51·01	5	1·38 Hb
Terebella lapidaria L.	6·980	129·90	11	10·93	42 Hb				
Echiuroidea									
Thalassema neptuni Gaertner	7·435	36·57	5	2·81	53 Hb				
Sipuncula									
Golfingia elongata (Keferstein)	7·115	76·94	4	13·76	29 Hr				

N is the number of determinations and *S* is the standard deviation. Where *S* is not given, samples have been pooled. PCV's include all the cellular constituents of the fluid. Hb, haemoglobin; Cr, chlorocruorin; Hr, haemerythrin. All coelomic pigments are in corpuscles excepting Cr, and all vascular pigments are in solution excepting Hr.

metabolites which are produced (Dales,1950).

The large variation in hydrogen ion activity between individuals and species is endorsed by earlier fragmentary reports in the literature. Clark (1964) measured coelomic fluid pH of Nephtys hombergi with samples equilibrated in air. Control worms were found to fall in the range pH 6.4-6.6 and she suggested that these might be slightly higher than the in vivo values. These low values may have been a result of the length of time it took her to collect coelomic samples and they contrast with the higher values for this species in Table 3.1.

Cole (1940) reported pH values for Echiurus pallassi (7.6), Amphitrite brunea (6.8) and Glycera dibranchiata (7.4). De Jorge et al. (1970) recorded pH 7.368± S.D. 0.086 for Sipunculus natans and 7.333± S.D. 0.030 for S.multisulcatus. The pH measurements were not made under strictly anaerobic conditions however.

Such wide variations are by no means confined to the invertebrates, for Eddy (1971) found variations of up to 0.5 unit in anaerobically sampled blood of rainbow trout kept under identical conditions. He further noted that variations became more pronounced at low CO_2 tensions and this situation is paralleled by marine invertebrates. These reports, and the results presented here, do not confirm the statement of Prosser & Brown (1961, p.62) that "All marine animals have a relatively constant hydrogen ion concentration." Whether relative to other ions, the sea or other animals, there is no evidence to support this claim.

There does not appear to be a correlation between the type of respiratory pigment or the design of the fluid compartments and the values of pH recorded in the results. It is worth noting that several figures lie outside the limits of pH which have been investigated for Bohr shift, e.g. chlorocruorin from Sabella pavonina (now penicillus) showed a Bohr effect at pH 7.4 to 8.0 (Fox, 1932) but the mean value found in this study was 6.92. This is particularly significant in view

of the enormous Bohr shifts of chlorocruorin (see Section 9). The terebellid Eupolymnia showed no Bohr effect between pH 7.2 and 7.7 (Manwell, 1959) but the two terebellid species in Table 3.1 showed average pH values of 6.95 and 6.89.

Variation in hydrogen ion activity in the body fluids of marine invertebrates may not have significance unless it can be shown that a Bohr shift operates.

4. CARBONIC ANHYDRASE ACTIVITY IN ARENICOLA MARINA (L.)

4.1 Abstract.

1. The catalytic effect of Arenicola blood on CO_2 hydration was compared with the activity of purified bovine carbonic anhydrase. The kinetic pattern of inhibition for both carbonic anhydrases by acetazolamide was found to be non-competitive.

2. Acetazolamide administration lowered the pH of coelomic fluid and blood within 1 hr during aerobic respiration. No significant change in pH was observed for treated worms kept under anaerobic conditions.

3. A primary role of the enzyme in the removal of metabolic CO_2 is suggested. A necessary consequence of this action is the limited control of body fluid pH by regulation of bicarbonate and hydrogen ions. This may be important in the blood where the haemoglobin equilibrium curve is known to have a positive Bohr effect.

4.2 Introduction.

Various invertebrate tissues are known to possess the enzyme carbonic anhydrase (carbonate hydrolyase E.C.4.2.1.1) from their effects on the rates of CO_2 hydration and carbonic acid dehydration according to the reactions:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$

and

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \qquad (2)$$

(Van Goor, 1948; Polya & Wirtz, 1965[a,b]). The course of (1) and (2) from left to right is referred to as the "hydration reaction" and the reverse, the "dehydration reaction".

The presence of carbonic anhydrase in the blood of Arenicola sp. was reported concomitantly with the separation of the catalytic system from haemoglobin in mammalian erythrocytes by Brinkman et al. (1932).

*I found no enzyme activity in homogenized gill tissue which had been washed in clean sea water to remove traces of blood.

A report by Prosser & Brown (1961, p232) that "carbonic anhydrase is found in coelomic fluid of Arenicola" is evidently a mistake based on a typographical error in Table 33 of Van Goor's reference to the original work by Brinkman et al.(1932). Although Brinkman et al.(1932) found high enzyme activity in Arenicola blood, its absence in coelomic fluid was not reported until later (Brinkman, 1933), and this was reaffirmed when Florkin (1935) and Clark (1948) found carbonic anhydrase in "plasma" (blood) but not in the "body liquid" (coelomic fluid) of Arenicola marina. Clark (1948) also examined various tissues of A.marina and showed the mean activity of lugworm and human blood to be of the same order.

I can find no original evidence for the statement in Prosser & Brown (1961, p232) that polychaete gills contain "a rich supply of the enzyme", and would suggest that this report is based on the view of Ferguson et al. (1937) who did find rich supplies in many crustacean gills. Clark (1948) found only a trace of activity in A.marina gills and I would agree that there is no more carbonic anhydrase in the gills than would be contained by the blood within these tissues.* see opp.

The presence of carbonic anhydrase in mammalian erythrocytes is an important factor in determining whether CO_2 can be released sufficiently rapidly during the passage of blood through the pulmonary capillaries. There are no haemoglobin-containing corpuscles in the blood of A.marina and both haemoglobin and carbonic anhydrase are in solution. No experiments concerning possible respiratory function of the enzyme are known for any invertebrate. There is an apparent association of the enzyme with calcium in oyster shell formation (Wilbur & Jodrey, 1955), but Nielsen & Frieden (1972b) point out that activity is much higher in the blood than in the mantle tissue of Crassostrea virginica and suggest that carbonic anhydrase is involved in the maintenance of the ionic strength of the blood. Addink (1971) demonstrated that the properties of carbonic anhydrase from the mantle muscle of the cephalopod Sepia officinalis were similar to the human and bovine isoenzymes, but

* had been adjusted to $28^{o}/_{oo}$ salinity (pH=8.2) and matched that of the medium from which the animals had been collected. Freshly made up synthetic sea water was used as a convenient source of sterile sea water which was required in large volumes. While some ionic activities may differ from those in natural sea water, and the worms' natural behaviour cannot be guaranteed, the use of synthetic sea water in these experiments may be justified from the two sets of control animals which were used.

the role of the enzyme in molluscan mantle has not been established.

A comparison of some of the kinetic properties of A.marina carbonic anhydrase (ACA) with a highly purified extract from bovine erythrocytes (BCA) therefore seemed a necessary preliminary for investigation of the role of ACA in whole animals, and the results of this study are presented here.

4.3 Materials and methods.

Lugworms were collected intertidally from a population at Whitstable, Kent, and kept at 15°C in a temperature-controlled aquarium under low illumination. No selection was made for body size or sex. Experimental and control animals were placed in plastic bowls containing aerated synthetic sea water (SeAquariums) which *(see opp) For experiments in anaerobic conditions, lugworms were kept separately in stoppered flasks of sea water under a continuous stream of nitrogen.

Coelomic fluid and blood were sampled anaerobically after rapid dissection under light paraffin. Replicate measurements were made quickly in succession after collection of fluid into capillaries. Samples were drawn by suction into a capillary microelectrode (Radiometer BMS2) and the pH determined with a Radiometer PHM 71 analyser. The electrode required 25-µl samples for a reliable pH reading. All pH measurements were made at 15.0±0.02°C (see Section 2.1). A two-point calibration procedure was performed before every run using NBS-approved buffer standards. Standard buffer values were corrected for temperature at 15°C (see Appendix 1) and were accurate to 0.005 pH units.

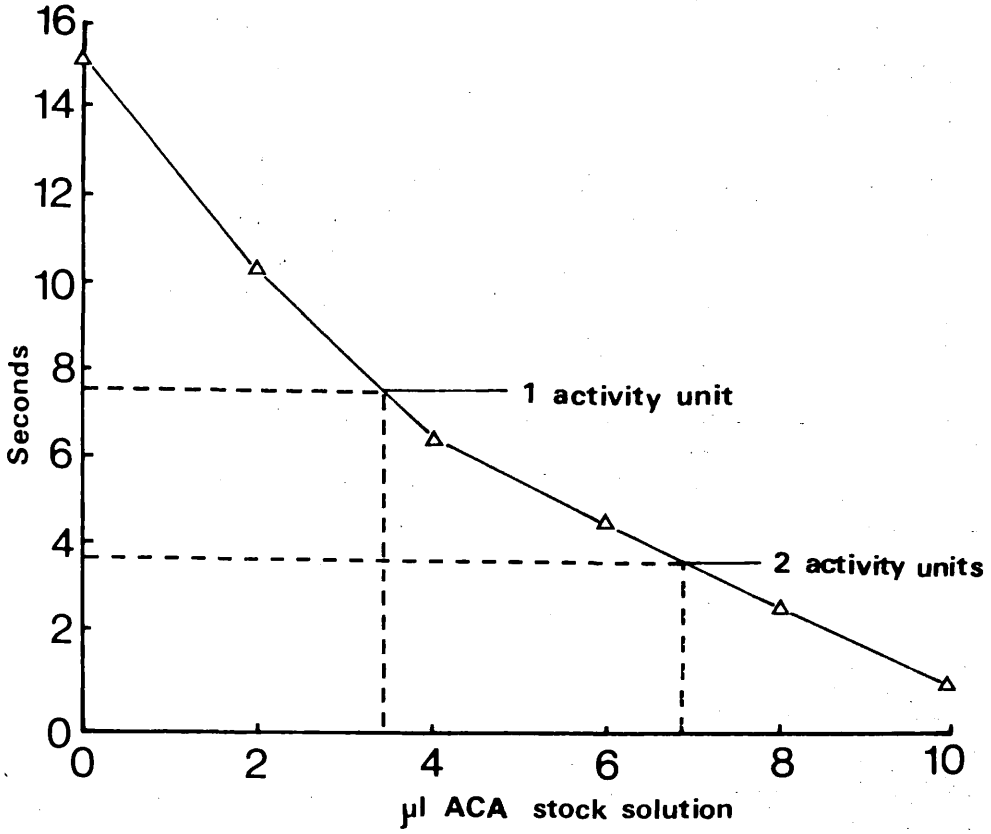
Since the pH scale is logarithmic, arithmetic means were calculated from the antilogarithms of the raw data. Samples were compared using the non-parametric Wilcoxon two-sample test to assess levels of significance.

4.4 Results.

The effect of varying ACA concentration on the time for the CO_2 hydration reaction to be completed is shown in Fig.4.1. One enzyme activity unit was defined as the amount of enzyme required to halve the time of the uncatalysed reaction. The enzyme unit is therefore an arbitrary unit defined in relation to the present system for measuring the CO_2 hydration rate.

The activity of ACA and BCA when two enzyme activity units were added to the system and titrated against acetazolamide is expressed in Fig.4.2. Activity decreased linearly with time over the range of enzyme concentration used. The amount of acetazolamide required to elicit one enzyme unit of activity when two units were added, corresponded to a molar concentration of 2.3×10^{-7}M for BCA and ACA. This value is known as the I50 for acetazolamide but is not comparable with other values of I50 using different assay systems or temperatures.

The results of experiments with whole animals are summarized in Table 4.1. Control group 1 had no treatment and group 2 was injected with 50 µl sea water. n is the number of animals and S.E. is the standard error of measurement. All experiments were held at 15.0±0.5°C and the pH was determined at 15.0±0.02°C. Controls 1 and 2 were not significantly different ($P > 0.2$). Reversibility of treated animals is shown after 24h for a group which had been given several changes of clean sea water. When coelomic fluid and blood from anaerobically maintained groups were equilibrated tonometrically with nitrogen for 40 min, no significant change in pH was observed ($P > 0.5$). However, in all groups maintained under aeration, equilibration of coelomic fluid with air raised the pH to 8.2±0.07, a value close to that of sea water. Blood from the same groups was raised to 7.72±0.09.


16
14
12
10
8
6
4
2
0
Seconds
1 activity unit
2 activity units
0
2
4
6
8
10
µl ACA stock solution

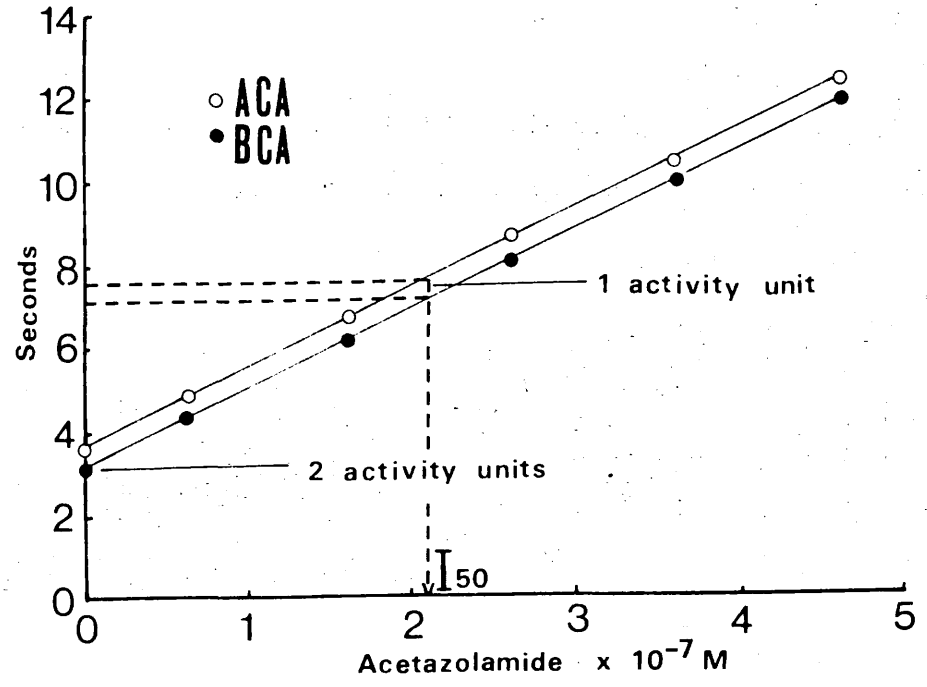
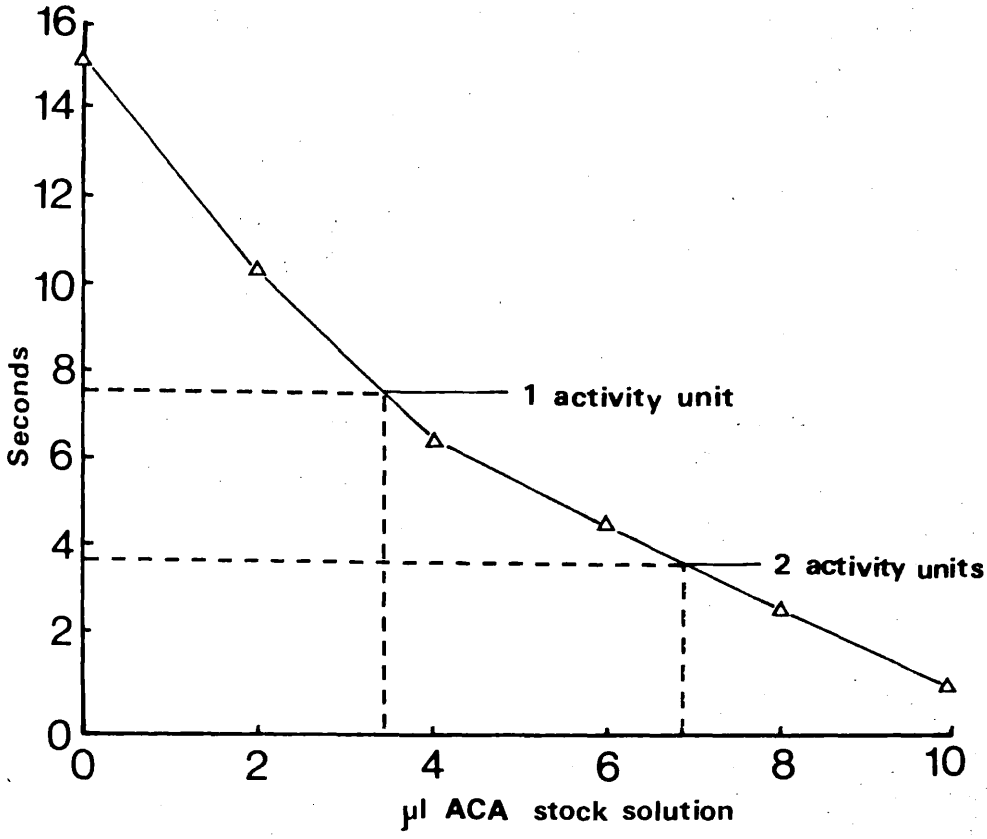

14
12
10
8
6
4
2
0
Seconds
○ ACA
● BCA
1 activity unit
2 activity units
I50
0
1
2
3
4
5
Acetazolamide x 10⁻⁷ M

Figure 4.1. Time for CO_2 hydration reaction to be completed as a function of ACA concentration.

Figure 4.2. Titration of two activity units of ACA and BCA against acetazolamide.


16
14
12
10
8
6
4
2
0
Seconds
1 activity unit
2 activity units
0
2
4
6
8
10
µl ACA stock solution

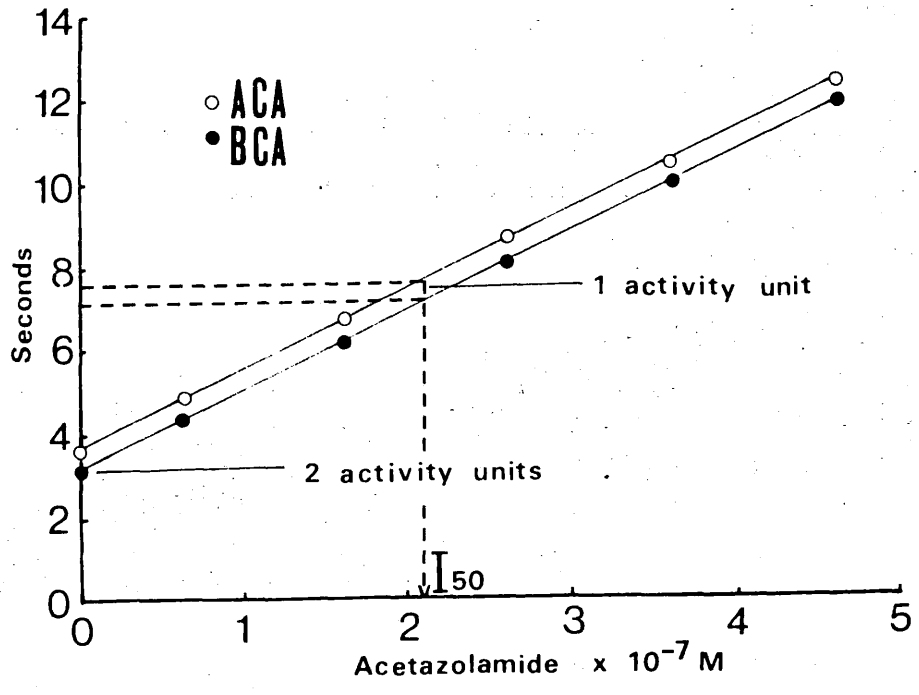

14
12
10
8
6
4
2
0
Seconds
○ ACA
● BCA
1 activity unit
2 activity units
I50
0
1
2
3
4
5
Acetazolamide x 10⁻⁷ M

Table 4.1. Effect of acetazolamide on the pH of body fluids from Arenicola kept at 15°C under aerobic and anaerobic conditions. N is the number of determinations. Measurements were made at 15°C.

	Coelomic fluid			Blood		
Treatment	pH	*N*	S.E.	pH	*N*	S.E.
Control 1 1 hr aerobic	7·545	10	0·009	7·670	8	0·011
Control 2 1 hr aerobic	7·542	5	0·012	7·668	5	0·014
Acetazolamide 1 hr aerobic	7·420	11	0·010	7·411	10	0·005
Control 1 5 hr aerobic	7·543	5	0·018	7·672	5	0·012
Acetazolamide 5 hr aerobic	7·415	6	0·014	7·403	5	0·027
Acetazolamide 24 hr aerobic	7·538	5	0·027	7·663	4	0·013
Control 1 5 hr anaerobic	6·951	8	0·028	6·991	6	0·031
Acetazolamide 5 hr anaerobic	6·993	10	0·022	7·030	9	0·026

4.5 Discussion.

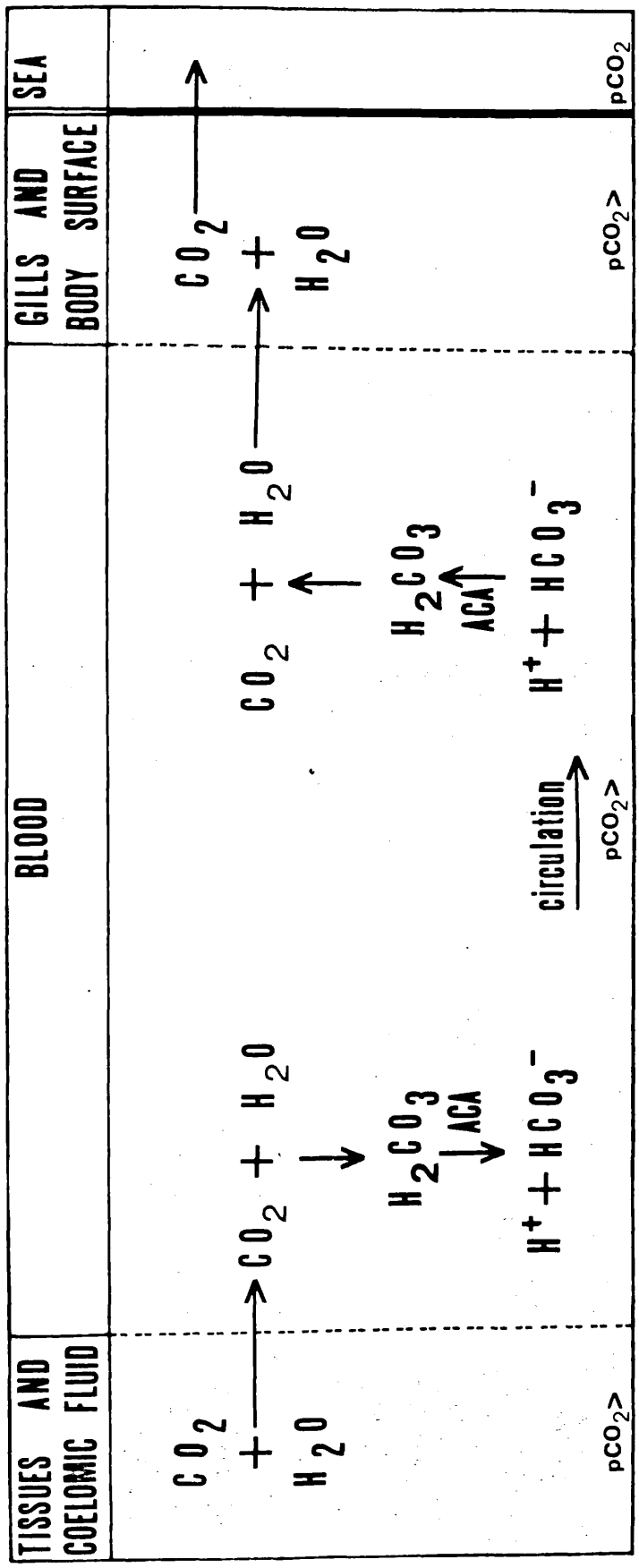
The results reveal a similar kinetic pattern of ACA and BCA activity in titration against the sulphonamide drug, acetazolamide. This pattern clearly demonstrates that acetazolamide does not compete with ACA for substrate, but inhibits the enzyme by the formation of an enzyme-inhibitor complex. This classical profile of non-competitive inhibition has already been shown for the carbonic anhydrases in mammals (Maren, 1967), the cephalopod mollusc, S.officinalis (Addink, 1971) and in oyster serum (Nielsen & Frieden, 1972 b).

A marked drop in the pH of coelomic fluid and blood resulted from acetazolamide-treated animals kept in oxygenated water. However, there was no significant difference in pH between the drug treated and control groups maintained under anaerobic conditions. This suggests that ACA participates in the removal of metabolic CO_2.

The blood is buffered by virtue of the haemoglobin in solution. Yet, the results show that this is not enough to buffer the blood against excess hydrogen ions arising from the staunched flow of CO_2 from sites of metabolic activity to the gills and body surface. A secondary role of ACA is therefore likely to be the control of body fluid pH by increased bicarbonate production in response to increased pCO_2. These possibilities have been considered by Nielsen & Frieden (1972a), but they suggest that the high enzyme activity in oyster serum is more likely to be involved in maintenance of ionic strength of the blood.

The experimental observations from A.marina could be accommodated if ACA were involved in respiratory transport of CO_2 by the scheme proposed in Fig.4.3.

The principle of diffusion demands that CO_2 passes along a pressure gradient from a region of high pCO_2 (the tissues and coelomic fluid) into a region of lower pCO_2 (the blood). A low blood pCO_2, maintained by


TISSUES AND COELOMIC FLUID
BLOOD
GILLS AND BODY SURFACE
SEA
$CO_2 + H_2O$
$CO_2 + H_2O \rightarrow H_2CO_3 \xrightarrow{ACA} H^+ + HCO_3^-$
$CO_2 + H_2O \leftarrow H_2CO_3 \xleftarrow{ACA} H^+ + HCO_3^-$
$CO_2 + H_2O$
circulation
pCO_2
$pCO_2>$
$pCO_2>$
$pCO_2>$
pCO_2

ACA in the hydration reaction to form bicarbonate, would assist in the removal of metabolic CO_2. The transport of bicarbonate is effected by the circulation of the blood to the gills and general body surface where the dehydration reaction occurs. Haemoglobin in A.marina is known to suffer a positive Bohr shift, the extent of which has been investigated recently by Toulmond (1970a). During periods of aerobic respiration it would therefore be an advantage to maintain a low blood pCO_2 (and hence higher pH) to avoid vitiating the oxygenational properties of the haemoglobin.

The mechanism of ACA operation in respiration appears to differ from the mammalian situation in two respects: (1) The pCO_2 in Arenicola blood is extremely low under normal conditions and presents difficulties in measurement. It would be useful to measure CO_2 directly, or indirectly from pH measurements via the Henderson-Hasselbalch equation. However, at low pCO_2's the equation is difficult to apply. Toulmond (1970b) demonstrated that for A.marina blood, the Henderson-Hasselbalch equation was inapplicable after establishing a non-linear relationship between blood pH and log pCO_2. The equation cannot be expected to hold at very low pCO_2's since as pCO_2 approaches zero, pH approaches infinity. (2) Because ACA is in solution and not confined to corpuscles, there can be no ion imbalance and hence no need for a chloride shift. Concerning the presence of carbonic anhydrase in mammalian erythrocytes, Krogh (1941)$_{p93}$ wrote "...it seems an unnecessary complication". Yet, in Arenicola the enzyme appears to have a similar role although in solution.

5 THE FUNCTION OF THE EXTRACELLULAR HAEMOGLOBINS OF ARENICOLA MARINA, NEANTHES (=NEREIS) VIRENS, AND CIRRIFORMIA TENTACULATA.

5.1 Introduction.

The polychaetes Arenicola marina (L.), Neanthes (=Nereis) virens (Sars) and Cirriformia tentaculata (Montagu) have well developed vascular systems containing a high molecular weight haemoglobin (ca. 3×10^6) free in solution. The idea that a vascular haemoglobin might serve as an oxygen store at low tide (Barcroft & Barcroft, (1924);Borden,(1931)) is now discredited for reasons given in Section 6.5. As these worms have no coelomic cell haemoglobin, their blood pigments might be expected to exemplify a simple transport function, although a "temporary storage" function is a necessary consequence of any transport system whenever activity is temporarily suspended.

Cirriformia is a non-selective surface deposit feeder living in mucous lined burrows which it does not irrigate (Courtney, 1958). The burrows are not normally exposed by the tide but covered by shallow pools of water left by the receding tide. A strong odour of sulphide is characteristic of the reducing muds in which it lives. Although the worms lie in vertical burrows of anoxic mud up to 20 cm in depth, the distal parts of the branchiae project from the entrance of the burrow and thus the worm is not dependent on the oxygen content of the mud, but that of the overlying water (George 1964a,1964b). The oxygen transport properties of this haemoglobin are interesting in view of the very steep pO_2 gradient which must exist between the sites of uptake and release.

The polychaetes Arenicola and Neanthes are found in similar shore habitats, burrowing in fine sediments where the interstitial water is generally likely to be deficient in oxygen at low tide (Brafield, 1964). The two species are sometimes found together in the lower mid-littoral zone of estuaries. The oxygen deficiency is partly compensated for by the construction of burrows which are irrigated. The burrows of Neanthes,

about 10-15 cm below the surface, are consolidated by mucous linings and open to the surface by semi-permanent openings. If the course of a burrow is followed through the strong reducing conditions of the black sediments, a lighter zone of sand is seen surrounding the burrow indicating the presence of at least some oxygen. There is probably very little communication between interstitial water and the burrow (Walsby, 1970). The only apparent source of oxygenated water is that drawn in by irrigation movements. These movements in Neanthes, well described by Lindroth (1938), consist of a series of dorso-ventral waves passing posteriorly. The movements are intermittent and it has been suggested that they are initiated either by low burrow water pO_2 (Walsby, 1970) or by an endogenous pacemaker (Dales et al. 1970). The irrigation movements of Arenicola have been described in detail by Wells (1949) and consist of a tailward locomotion phase followed by headward, then tailward irrigation. Headward irrigation is the more conspicuous component whenever there is a plentiful supply of oxygen. Nevertheless, as the author pointed out, this is not evidence for the pO_2 initiation of activity per se since irrigation was not shortened or abolished by a decrease in pO_2.

The physiological properties of the blood of these three species can reveal the nature and extent of an adaptation to life in a low oxygen environment provided that the experimental approach relates to the conditions under which the animals might be expected to live.

5.2 Materials and methods.

Arenicola marina and Neanthes virens were collected from Southend-on-Sea, Essex, and Cirriformia tentaculta from Wembury, Devon. Worms were acclimated to the laboratory sea water circulation at 15°C for several days prior to experimentation.

Worms were narcotized with a 0.3 per cent solution of propylene phenoxitol in sea water and blotted to remove surface moisture. The blood was sampled directly from the dorsal vessel of Cirriformia and Neanthes

* Unlike the case of Arenicola, it was difficult to obtain a sample of whole blood from Nereis without traces of coelomic fluid. It must therefore be assumed that the highest measured capacity of 6.8 vols% from 5 individuals is the most realistic one.

with a fine hypodermic needle inserted at an acute angle into the vessel, which was visible beneath the skin. The body wall of Arenicola was cut slightly to one side of the mid-dorsal line, thus releasing most of the coelomic fluid. With the dorsal vessel exposed, blood was sampled with a syringe. Samples contaminated with coelomic fluid were discarded. All experiments were carried out using freshly taken blood.

5.3 Results.

Oxygen equilibrium data for whole blood from Arenicola marina are presented as the linear transformation from Hill's equation in Fig.5.1. The p50 at 15°C for Arenicola was 3.0 mm Hg (0.40 kN m^{-2}) and is the same as a figure quoted in Prosser & Brown (1961). A high oxygen-combining capacity of 8.3±0.6 vols % (duplicate measurements from 5 individuals) confirms the results of Borden (1931) and Barcroft & Barcroft (1924). The capacity of Neanthes blood at 15°C had a maximum value of 6.8 vols %. The Hill transformations in Fig.5.2 (see opp.) show the influence of temperature on the position but not the shape of the equilibrium curve in Neanthes. Affinity was increased from 4.0 mm Hg (0.53 kN m^{-2}) at 15°C to 2.0 mm Hg (0.27 kN m^{-2}) at 7.4°C and lowered to 7.5 mm Hg (1.00 kN m^{-2}) at 22°C. The increase in temperature which displaces the curve towards a lower affinity for oxygen presumably reflects the exothermic nature of the oxygen-binding process. The greater demand for oxygen at high temperatures generally shown by poikilotherms, is therefore partly offset by a decrease in affinity and thus easier unloading. However, extreme temperatures could possibly shift the curve out of the useful working range. The experimental temperatures were chosen to cover the range that the worms might experience over the year and were based on field measurements made by Kay (1972).

Fig.5.3 shows the apparent invariance of the equilibrium of Neanthes haemoglobin to hydrogen ion activity between pH 6.68 and 7.33. The value of Hill's coefficient, N, is shown to be somewhat pH dependent (N=1.1-1.2). The shift in p50 may be the result of dilution in the phosphate buffers

Figure 5.1. Hill plot of the equilibrium data of Arenicola marina haemoglobin at 15°C and pH 7.28.

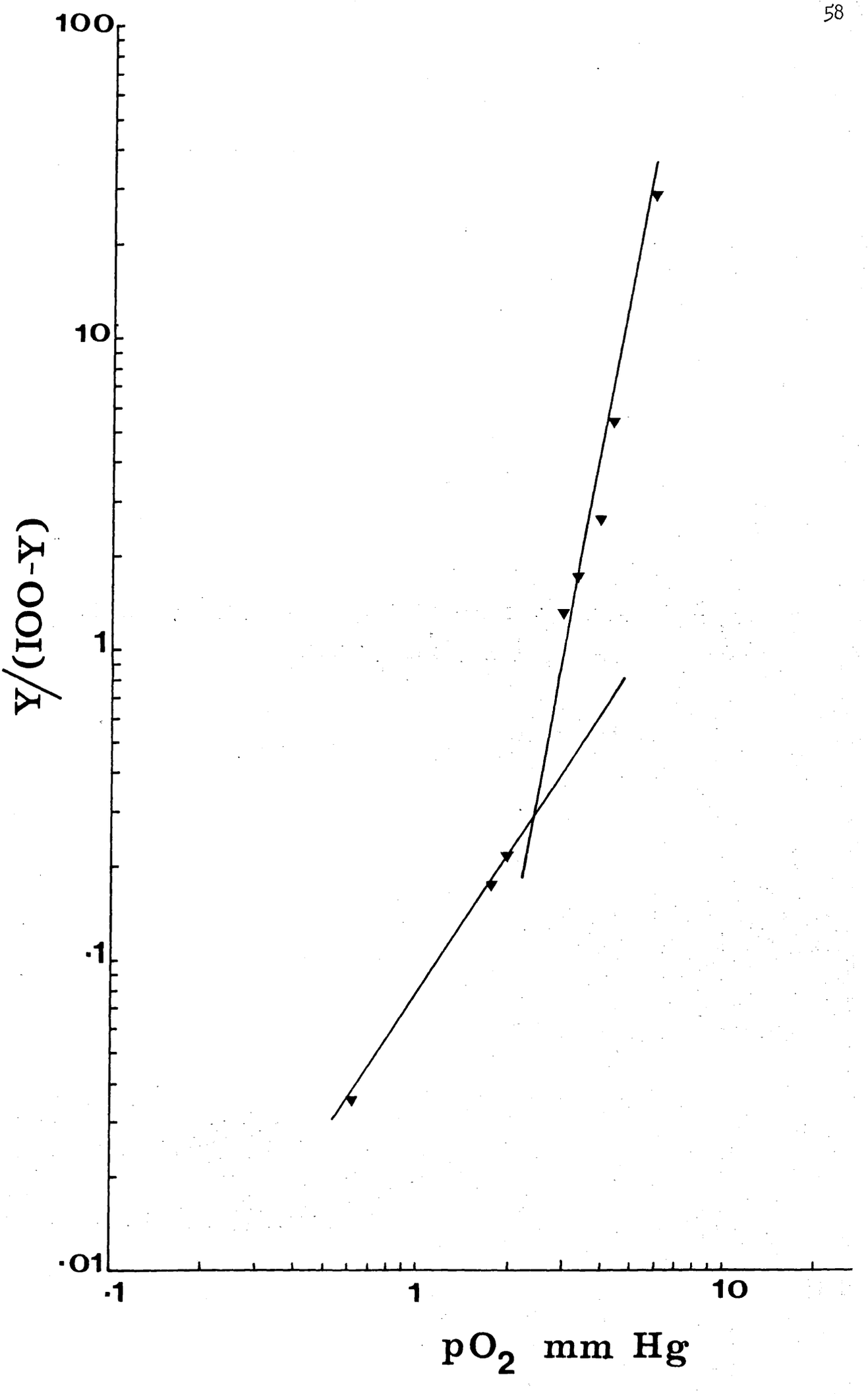

100
10
1
·1
·01
·1
1
10
Y/(100-Y)
pO2 mm Hg

Figure 5.2. Hill plots of the equilibrium data from Neanthes haemoglobin showing the effect of temperature on the position of the curve. pH = 7.28. Mr. A. P. Economides supplied the blood sample for this experiment.

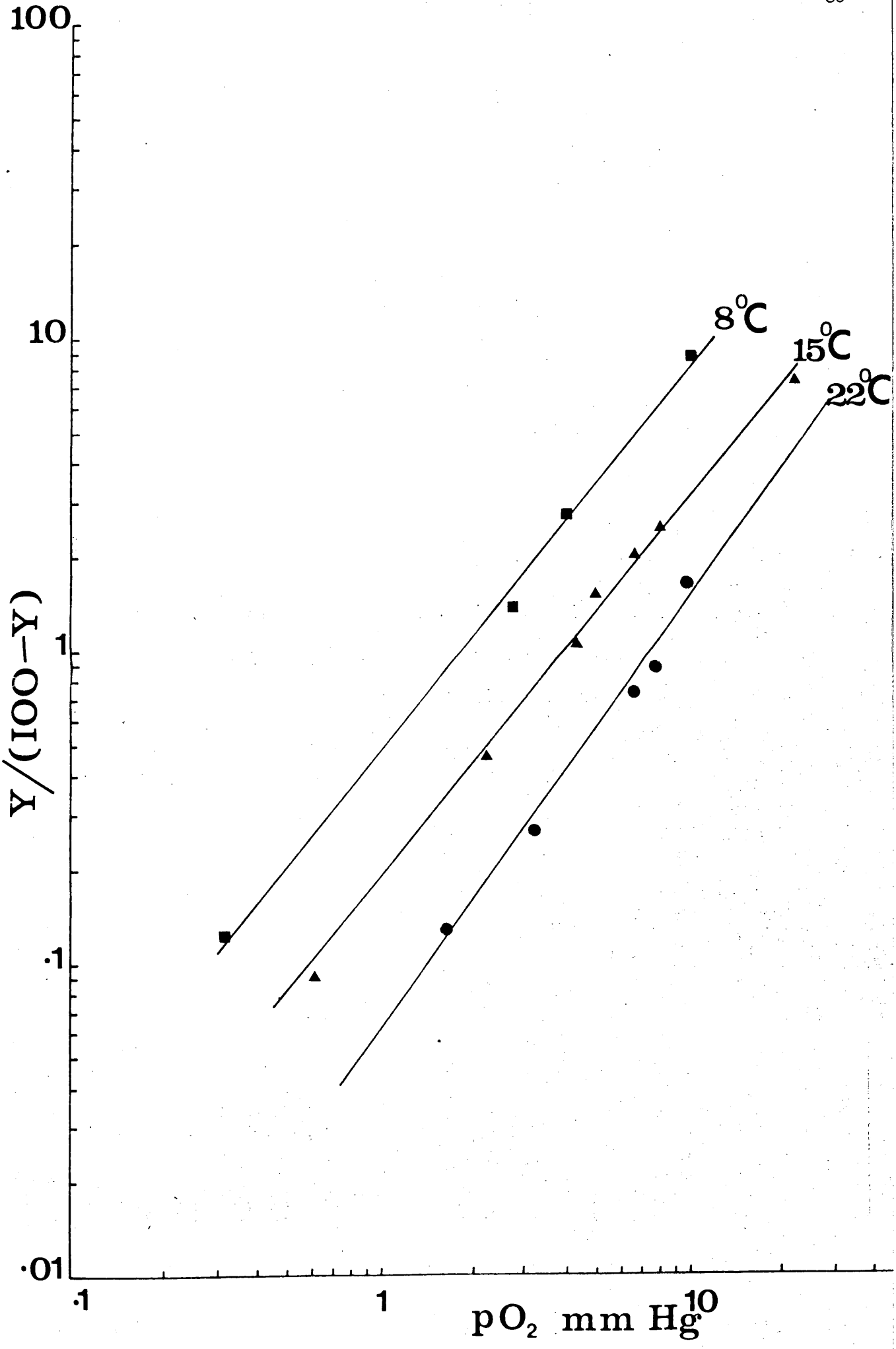

100
10
1
·1
·01
·1
1
10
Y/(100−Y)
pO2 mm Hg
8°C
15°C
22°C

Figure 5.3. Hill plot to show the lack of change in position of the equilibrium curve of Heanthes virens haemoglobin with hydrogen ion activity between pH 6.68 ■ and ▲7.33 at 15°C.

Mr.A.P.Economides supplied the blood sample for this experiment.

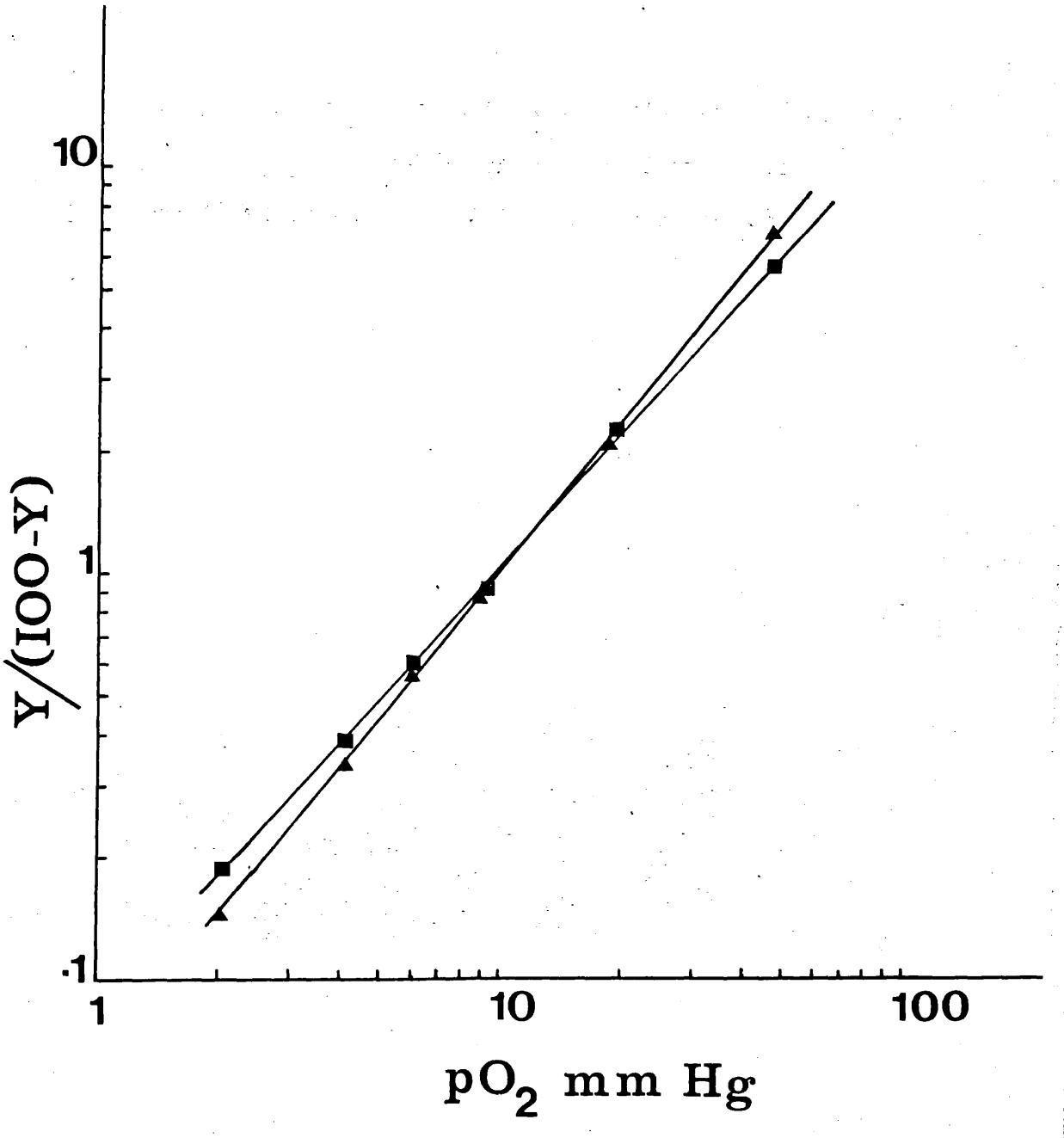
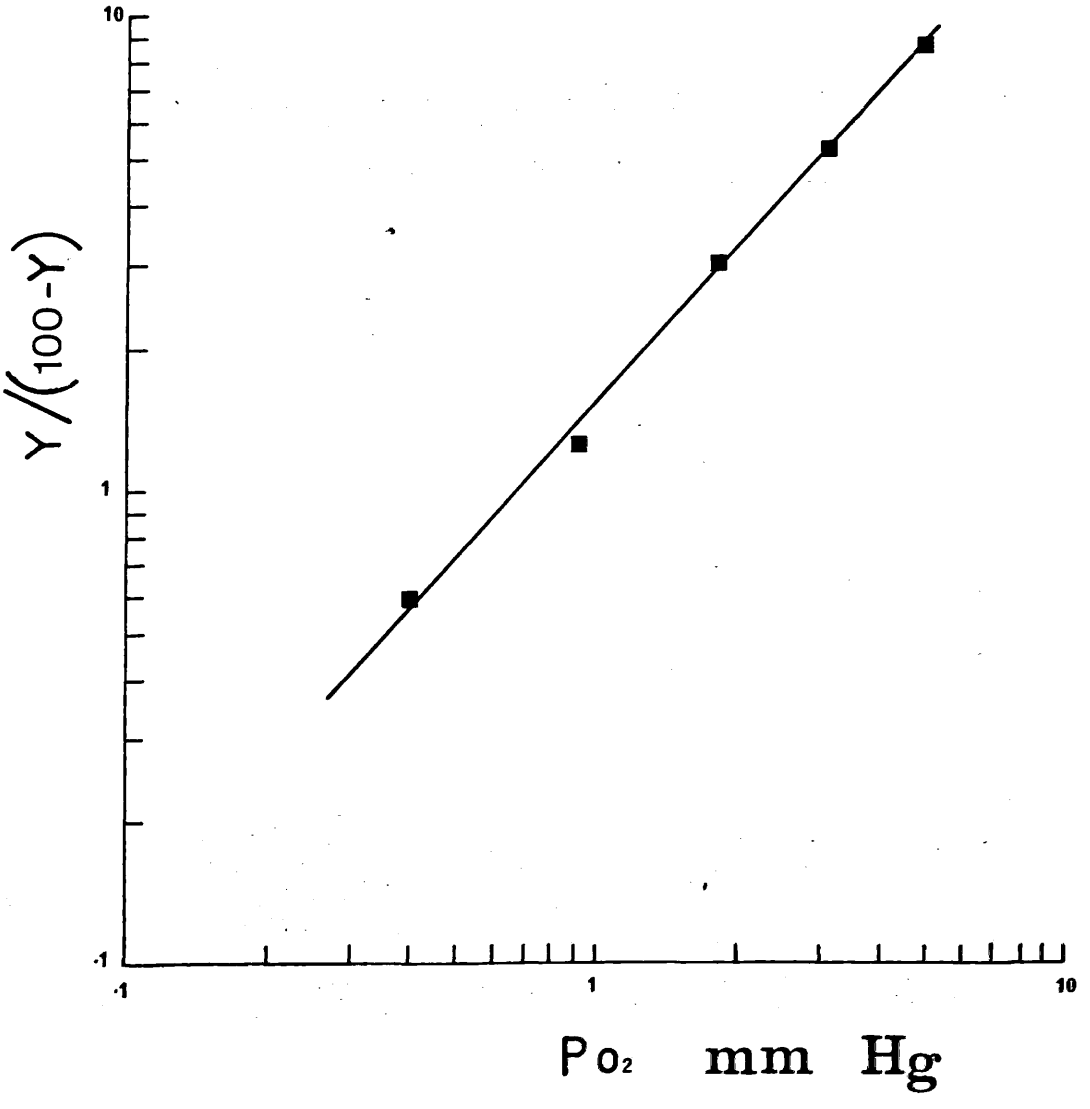

10
1
·1
Y/(100-Y)
1
10
100
pO2 mm Hg

Figure 5.4. Hill plot of the equilibrium data of *Cirriformia tentaculata* haemoglobin at 15°C, and pH 7.31.


10
1
·1
·1
1
10
Y/(100-Y)
Po2 mm Hg

* As was the case with Neanthes, it was difficult to be certain that the vascular blood of Cirriformia was not diluted by traces of coelomic fluid. The maximum value obtained from 5 worms of 7.0 vols% was probably the most realistic estimate of combining capacity.

which were used to test the Bohr shift.

The equilibrium for Cirriformia haemoglobin has the form of a rectangular hyperbola and fits the Hill approximation with a slope of 1.0-1.1 indicating no haem-haem interactions. This haemoglobin in interesting in that it has a very high affinity for oxygen ($p50$=0.8 mm Hg) and a high oxygen-combining capacity of 7.0 vols % at pH 7.31 and 15°C (Fig.5.4). * (see opp)

The Hill plots were fitted by regression analysis and N calculated from the slopes of the regression lines. Weak haem-haem interactions for Neanthes (N=1.3-1.4) contrasted with the strong facilitating interactions (N=5.4) in Arenicola.

5.4 Discussion.

The respiratory requirements of invertebrates vary enormously and have given rise to many interesting adaptations. The oxygen equilibrium data cannot, in themselves, furnish an adequate description of the function of haemoglobin. Additional information on behaviour, arterio-venous pO_2 and pH measurements, circulation rates, blood pressures, and rates of oxygen consumption in moist air and in water would be welcome.

Cirriformia is able to utilize oxygen down to the low ambient tension of 15 per cent saturation (Courtney, 1950). Its high affinity haemoglobin coupled with a high combining capacity is well adjusted to the conditions of steep pO_2 gradients in which the animal lives. A high affinity was also noted for Cirriformia sp. by Swaney & Klotz (1971) who also found a very large Bohr shift between pH 6.5 and 7.2. Since this range spans the in vivo pH value of pH 7.12 measured in C.tentaculata (Table 3.1), the existence of a functional Bohr effect is likely. Nevertheless, afferent and efferent blood pH's and conditions of the worm's near environment ought to be measured in order to interpret haemoglobin function with more precision.

Walsby (1970) demonstrated a fall in oxygen tension in Neanthes burrow water during the periods between bursts of irrigation. The high affinity of its haemoglobin may provide an adequate supply of oxygen from the burrow water during these rhythmical "emergencies", thus reducing or abolishing the anaerobic period which the animals might experience before irrigation is resumed. Walshe (1950) described a similar situation for the tubicolous larva of Chironomus.

Haemoglobin may be of additional value to Neanthes at low tide when the mud-flats are exposed. Under these conditions, the burrow entrances frequently become closed by the receding tide. Because of the lack of communication between burrow water and the interstitial medium, oxygen in the burrow is consumed without being replenished. In this situation it is reasonable to consider the blood together with the burrow water as an oxygen store. Experiments carried out by Economides (unpublished) using artificial beaches, showed that oxygen in Neanthes burrows dropped to 15% air-saturation at 15°C and 32°/oo salinity (3.12 kN m^{-2}) in both closed burrows and those with air above the openings over a period corresponding to a low tide. At this tension, the haemoglobin would be expected to function in oxygen transport.

When, as sometimes happens, the burrow entrances are exposed to air, irrigation must cease and the diffusion rate of oxygen across the air/water interface does not meet the demand for oxygen. Where surface pools remain, burrows may be reopened and normal irrigation cycles resumed. Neanthes was seen to reduce its muscle tone under nearly anaerobic conditions, this presumably being a behavioural adaptation to low oxygen.

Jones (1955) observed that the $\underline{p}O_2$ did not fall below 14 mm Hg (1.86 kN m^{-2}) in Arenicola burrows even after 5h exposure at low tide. At this tension, the blood can transport enough oxygen to meet the normal requirements of the animal, assuming a modest integumentary gradient of 10 mm Hg (1.33 kN m^{-2}) across the sites of uptake, and there is no need to

consider the blood as an oxygen store as did Borden (1931) and Barcroft & Barcroft (1924). Primary storage functions of the blood are usually ascribed only to the non-vascular piments of polychaetes (Jones, 1963). Neanthes and Arenicola do not possess coelomic pigments and their vascular fluids cannot be strictly regarded as storage systems.

On the basis of function, Neanthes and Arenicola fall into Jones's (1972) category of "low tension transport from low ambient pO_2". This grouping is characterized by modest integumentary gradients and allows for full venous saturation under "normal" conditions. Thus the pigment may assume an "emergency" role with every irrigation pause, even when the water above the burrow is well oxygenated. Cirriformia cannot be categorized so simply since it is the pO_2 gradient, rather than the ambient tension alone, for which the haemoglobin is adapted for functioning.

Because the sea temperatures are low, and CO_2 is highly soluble in water, marked arterio-venous pCO_2 gradients are unlikely and an afferent/efferent pH difference is a necessary requirement for an operational Bohr effect. It is therefore not surprising that Neanthes has no Bohr effect, but it is much more difficult to explain the moderate shift noted in Arenicola by Toulmond (1970a). A Bohr effect would be even less likely to be of significance in animals with low metabolic requirements and could even prove disadvantageous at low tide if the burrow water pO_2 accumulated.

Recent evidence suggests that the above account of the Bohr function of haemoglobin may be oversimplified. Toulmond (1973) found that the pH of the prebranchial blood in Arenicola dropped from 7.48 to 7.35 after 4h emersion and the internal pO_2 fell to 1.0 mm Hg. He deduced a pCO_2 increase from 0.8 to 2.4 mm Hg and a shift in the p50 from 2.3 to 3.4 mm Hg under the above conditions. But if, as Toulmond suggests, most of this pH drop may be accounted for by acid products of anaerobic respiration, then the deductions about pCO_2's and the Bohr effect may not be valid and the existence of a functional Bohr shift remains unconfirmed. It may be that,

during anaerobic periods, the haemoglobin assumes a prominent role in acid-base balance to protect the animal from acidosis.

Dales (1958) showed that although glycogen was metabolised during anaerobiosis in Arenicola, there was no accumulation of lactic or pyruvic acids. He concluded that other metabolic acids were involved and/or the products were excreted. On the other hand, in Neanthes, lactic acid was produced as glycogen was decreased during anaerobic periods (Economides, unpublished). Retention of these acids might be a disadvantage to Arenicola because of the Bohr shift, but of little consequence to Neanthes.

The sigmoid nature of the equilibrium curve is usually quantified by N, Hill's coefficient and is the slope of the double log plot of the data. Manwell (1963) considered sigmoid curves to be the result of natural selection for a very narrow range of internal pO_2's. He further suggested that haemoglobin in Arenicola served to maintain a low venous pO_2 and therefore protect the tissues from oxygen poisoning. The shape of the equilibrium curves of haemoglobin from Neanthes and Cirriformia approximates a rectangular hyperbola but in contrast with Arenicola, these bloods can give up the major part of their load only if the venous pO_2 is very low. Neanthes and Cirriformia were kept in aerated sea water for several months and did not develop the symptoms of oxygen poisoning observed in Arenicola by Manwell (1963), and it is therefore unlikely that these haemoglobins function to maintain a low internal oxygen tension. This theme has been further expanded in Section 7.4.

Haem-haem interactions may, according to Manwell (1964), function in invertebrates by facilitating the transfer of oxygen to myoglobin in the muscles of animals possessing this pigment. Neanthes and Cirriformia do not appear to have myoglobin, but it would be interesting to have information for other worms on the distribution of myoglobin and its possible correlation with haem cooperativity of vascular haemoglobins. Haem interactions may be eliminated by extensive purification as noted by

Manwell (1963[a]), and fresh whole blood or cell suspensions may be a more reliable guide to the interpretation of *in vivo* function . To realise the adaptive significance of these parameters, it is therefore preferable to examine carefully, the conditions under which the respiratory pigment is believed to function.

6 HAEMOGLOBIN FUNCTION IN TEREBELLA LAPIDARIA L., AN INTERTIDAL TEREBELLID POLYCHAETE.

6.1 Abstract.

Equilibrium data for the cell haemoglobin of Terebella lapidaria have been determined for three temperatures within the range likely to be experienced by the animals. The p50 of the cell haemoglobin is 2.0 mm Hg at 15°C, and is sensitive to temperature. H^{o} was calculated to be -13 kcal mol^{-1} (54 kJ mol^{-1}). The p50 of the vascular haemoglobin is 3.0 mm Hg at 15°C, suggesting a transfer system. Hill's coefficient, N, is 1.33-1.48 for the cell haemoglobin indicating some haem-haem interaction; the vascular haemoglobin shows strong interaction, N=2.7, but N tends to 1.0 at low pO_2's. The cell haemoglobin shows no significant Bohr shift within normal physiological pH's (pH 6.50-7.28). Oxygen-combining capacity, haemoglobin concentration and total blood volume have been estimated. The respiratory rate of normal and CO-treated worms in moist air and in an air-CO_2 mixture has been determined. The respiratory rate of normal and CO-treated coelomic cells was measured. The significance of these data is discussed in relation to activity and survival of the worms during the intertidal period. We conclude that O_2-transport is possible in moist air but that activity is reduced at low pO_2's. The cell haemoglobin would provide a store for only part of the 3-4h period for which the worms are commonly exposed.

6.2 Introduction.

Terebella lapidaria L. is a small terebellid common in crevices in the mid-littoral zone of rocky shores in the Plymouth area. The coelom contains abundant coelomocytes charged with haemoglobin in sufficient quantity to give the worm a brick red colour. T.lapidaria also has a discrete vascular system with haemoglobin dissolved in the plasma. When

covered by the tide the worm irrigates its burrow intermittently. When revealed by the tide it cannot do so, and is left in a thin film of water or in moist air for 3-4 hours. We thought it was of some interest therefore to determine the oxygen equilibrium characteristics of these pigments.

Many terebellids have coelomocytes containing haemoglobin. Terebella cells are notable for the high concentration of the pigment. Coelomocyte haemoglobins have been described from the terebellids Pista pacifica by Terwilliger and Koppenheffer (1973), Thelepus crispus by Garlick & Terwilliger (1974) and Amphitrite ornata by Mangum (personal communication). Other polychaetes with cell haemoglobins which have been investigated are the opheliid Travisia pupa (Manwell, 1960a), Glycera gigantea (Weber, 1973) and G.dibranchiata (Hoffmann & Mangum 1970, Vinogradov et al. 1970, Mizukami & Vinogradov 1972, Seamonds et al. 1971 a,b, and Mangum & Carhart 1972). Most studies have been made with purified extracts in buffer. Weber (1973) however, has determined the oxygen equilibria for whole G.gigantea cells using the diffusion chamber method of Sick & Gersonde (1969) and Mangum & Carhart (1972) have also determined the oxygen equilibria of G.dibranchiata cells in suspension. We have used whole blood or whole coelomic fluid using the micromethod described in Section 2.3.

6.3 Materials and methods.

Specimens were collected from rocky crevices of the mid-littoral zone at Wembury in Devon.

Coelomic fluid volumes were estimated by photometric determination of extracted haemoglobin. Erythrocytes were disrupted ultrasonically and centrifuged at 5,000 g to remove debris and the haemoglobin extract was passed through a 300 x 10 mm alumina column to remove fats. Absorbance was measured at 540 nm in a Beckman DB-G spectrophotometer.

*Aerial respiration rates were determined using the method described in Section 2.7. The worms used in these experiments were selected to eliminate differences in the respiratory rate due to size. The mean fresh weight and standard deviation were 0.34 ± 0.5 g. One to 3 worms were used for each determination as where the oxygen consumption rate was low (e.g. at 0.5°C) it was convenient to use more than one animal in the respiration chamber. The numbers of animals and determinations are given in the results in Table 6.1 together with the experimental temperatures and other treatment.

Erythrocytes and coelomic fluid were tested for the presence of carbonic anhydrase by estimating their effect on the rate of CO_2 hydration (Section 2.2.).

Oxygen-combining capacities and equilibrium data were obtained from whole blood at 9°, 15° and 25°C at pH 6.46 and 7.28 using the micro-method described in Section 2.3 based on the principle that bound oxygen is released by excess ferricyanide solution, the increase in oxygen tension being measured with a Radiometer E5046 electrode in conjunction with the BMS2 apparatus.

Animals were bled soon after return to the laboratory and fresh whole blood was used for all equilibrium studies. Coelomocytes were collected by making a small incision in the body wall. Blood was collected from the dorsal vessel in glass capillaries after opening the body cavity, rinsing with sea water, and drying with filter paper.

The pH of whole coelomic fluid was measured anaerobically with a Radiometer G 298A capillary microelectrode and PHM 71 analyser (Section 2.1). The effect of pH on the equilibrium state was examined after resuspension of cells in a similar volume of phosphate buffer adjusted to 0.54M with NaCl.

The presence of large quantities of fat in the coelomocytes made it impossible to estimate haemoglobin concentration directly by the cyanmethaemoglobin method. Haemoglobin concentration was therefore estimated by measurement of oxygen-combining capacity based on the assumption that 1 vol.% is equivalent to 0.746 g haemoglobin per 100 ml blood.

* See opp.

6.4 Results.

6.4.1 Aerial respiration

Rates of oxygen consumption at several temperatures are summarised in the form of regression equations in Table 6.1. Fresh weights have been used throughout. Also presented are rates for worms treated with

carbon monoxide and those kept in respirometers in which CO_2 was maintained at 2.5%. Oxygen consumption with time was linear in all experiments after initial adjustment to thermal shock. This is typically illustrated in Fig 6.0. The initial rate change explains the small projected intercepts in the regression equations. Monoxide treatment, observing the precautions mentioned by Jones (1963) slightly depressed the consumption rate at 20°C. However, 2.5% CO_2 greatly reduced the oxygen consumption.

A more detailed analysis of the temperature effect on quiescent VO_2 is given in the Arrhenius plot of Fig.6.1 and indicated a complex relationship not simply described by invariance or dependence of rate with temperature. The range of temperatures covered the extremes that the animals might encounter in nature.

6.4.2 Oxygen equilibrium

Equilibrium data for the cellular haemoglobin at 3 temperatures are plotted as Hill transformations in Fig.6.2. The vascular blood data for 15°C are similarly presented in Fig.6.3 and are biphasic. The lower p50 (2.0 mm Hg) of the cell haemoglobin contrasts with a vascular p50 of 3.0 mm Hg at 15°C, suggesting a transfer system. The vascular pigment circulating through the gills could transfer oxygen to the higher affinity coelomic haemoglobin.

Hill's coefficient, N, is a measure of the interaction between haems. For cell haemoglobin this was 1.33-1.48 indicating some interaction. The vascular haemoglobin is biphasic indicating strong interactions (N = 2.7) but N tends towards 1.00 at very low pO_2's.

The lack of change in cell haemoglobin p50 between pH 6.50 and 7.28 is shown in Fig.6.4. These pH values span the measured in vivo pH of 6.90. The procedure apparently altered the position, but not the shape of the curve. The slight change in N (1.2 to 1.4) is without physiological significance.

Table 6.1. Regression solutions for O_2 consumption rates of Terebella lapidaria in moist air.

Temperature ^{o}C	0.5	7.3	12.2	20.0	28.2	20.2 + CO	20.2 + CO_2
Regression equation	y = 0.31x + 95	y = 1.13x + 38	y = 1.69x + 26	y = 2.11x + 71	y = 1.74x - 16	y = 1.83x - 90	y = 0.61x + 13
VO_2 µl O_2 g^{-1} h^{-1}	19	68	101	127	104	110	37
N (number of worms)	14	10	10	10	22	10	10
n (number of determinations)	8	10	10	10	8	10	6

See Sections 2.7 and 6.3 for details. The constancy of the respiratory rates may be assumed from Fig.6.0.

Figure 6.0. Graphs of oxygen consumption per gram fresh weight versus time showing the constancy of the rate after a brief initial adjustment. Details of the experimental procedure are given in Sections 2.7 and 6.3. A linear relationship was observed for the duration of all the experiments which was up to 10 h in some instances. Each rate was chosen from one experiment to illustrate clearly the linearity which is not self-evident from the analysed data summarizing all the experiments which have been presented in Table 6.1.

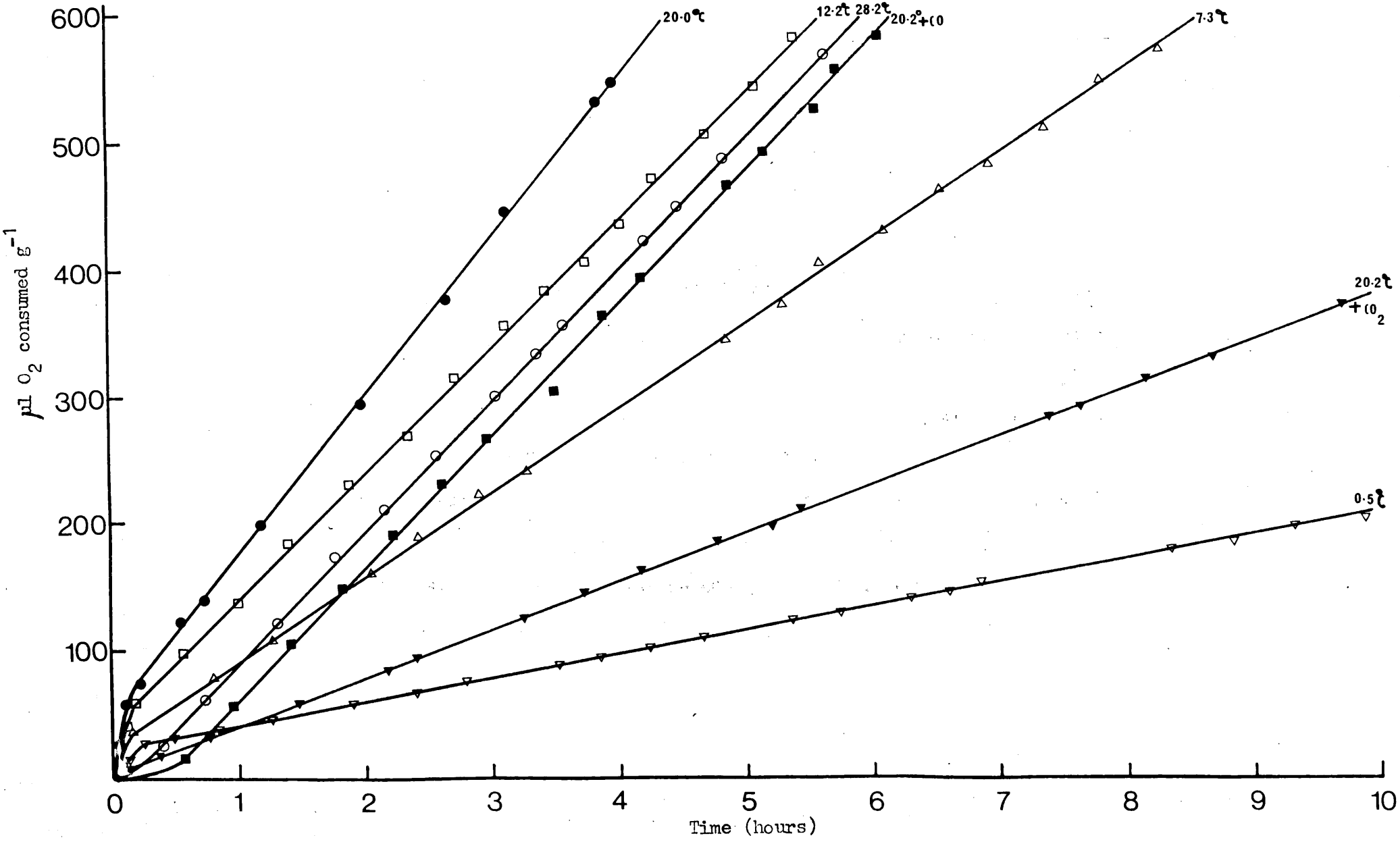

600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
µl O2 consumed g-1
Time (hours)
20·0°C
12·2°C
28·2°C
20·2°C+(O
7·3°C
20·2°C
+(O2
0·5°C

Figure 6.1. Arrhenius plot of oxygen consumption rate versus temperature for Terebella in moist air.

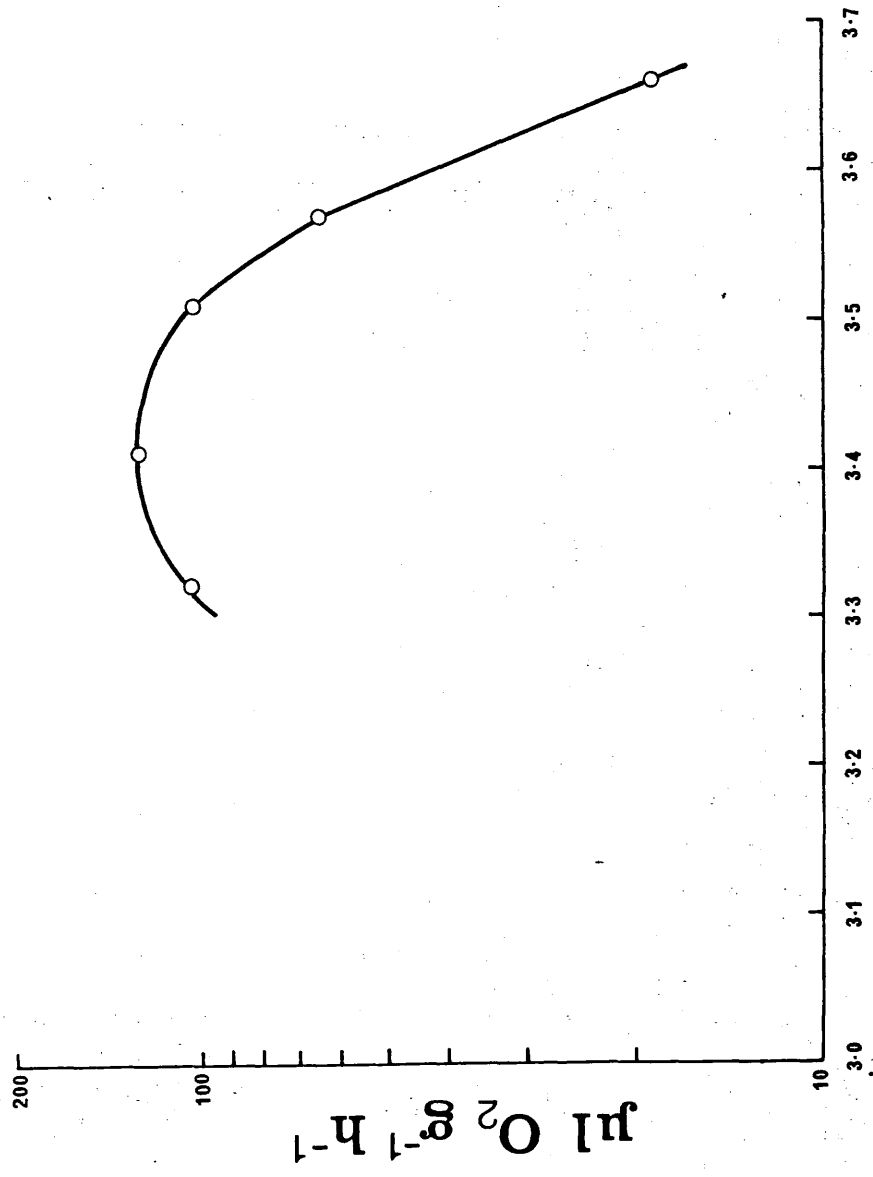

µl O2 gg-1 h-1
200
100
10
3·0
3·1
3·2
3·3
3·4
3·5
3·6
3·7
1/T(10³ x °K⁻¹)

Figure 6.2. Oxygen equilibrium data for the coelomic cell haemoglobin of Terebella at 9°, 15°, and 25°, pH 6.90 plotted according to the Hill equation.

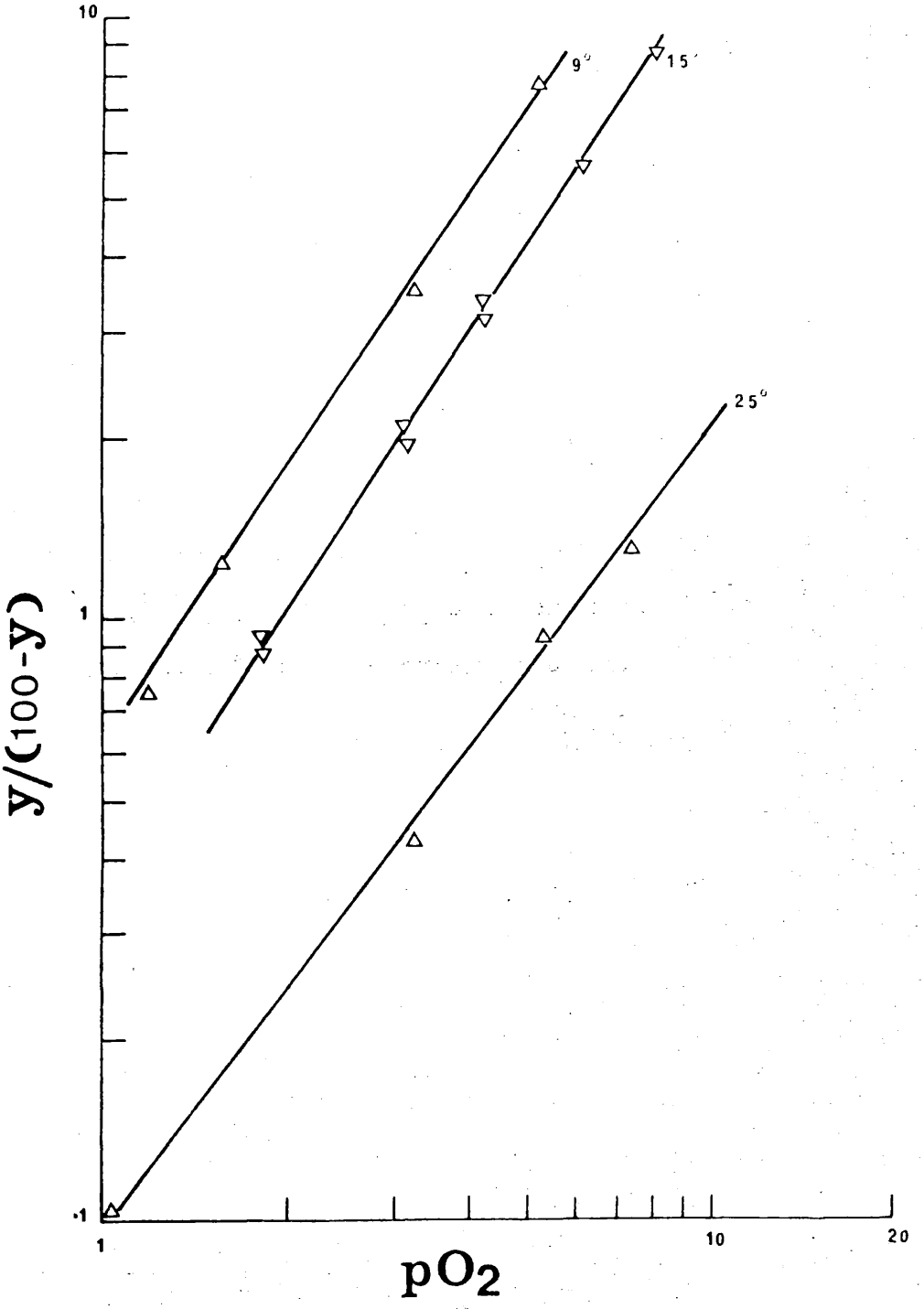

10
1
·1
1
10
20
9°
15°
25°
y/(100-y)
pO2

Figure 6.3. Oxygen equilibrium data for the vascular haemoglobin of Terebella at 15°C.

In Terebella, the volume of vascular blood was too small to make a pH measurement.

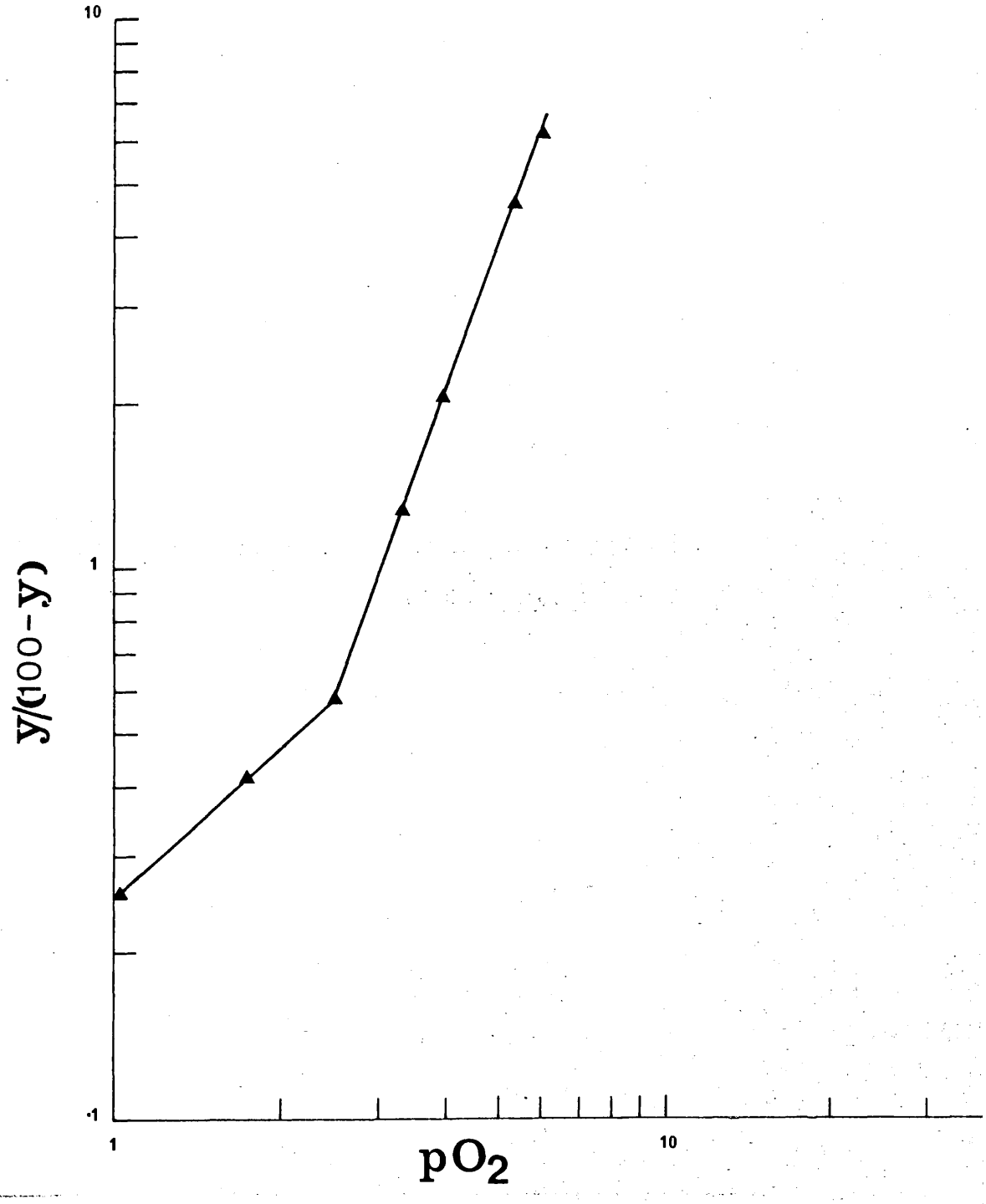
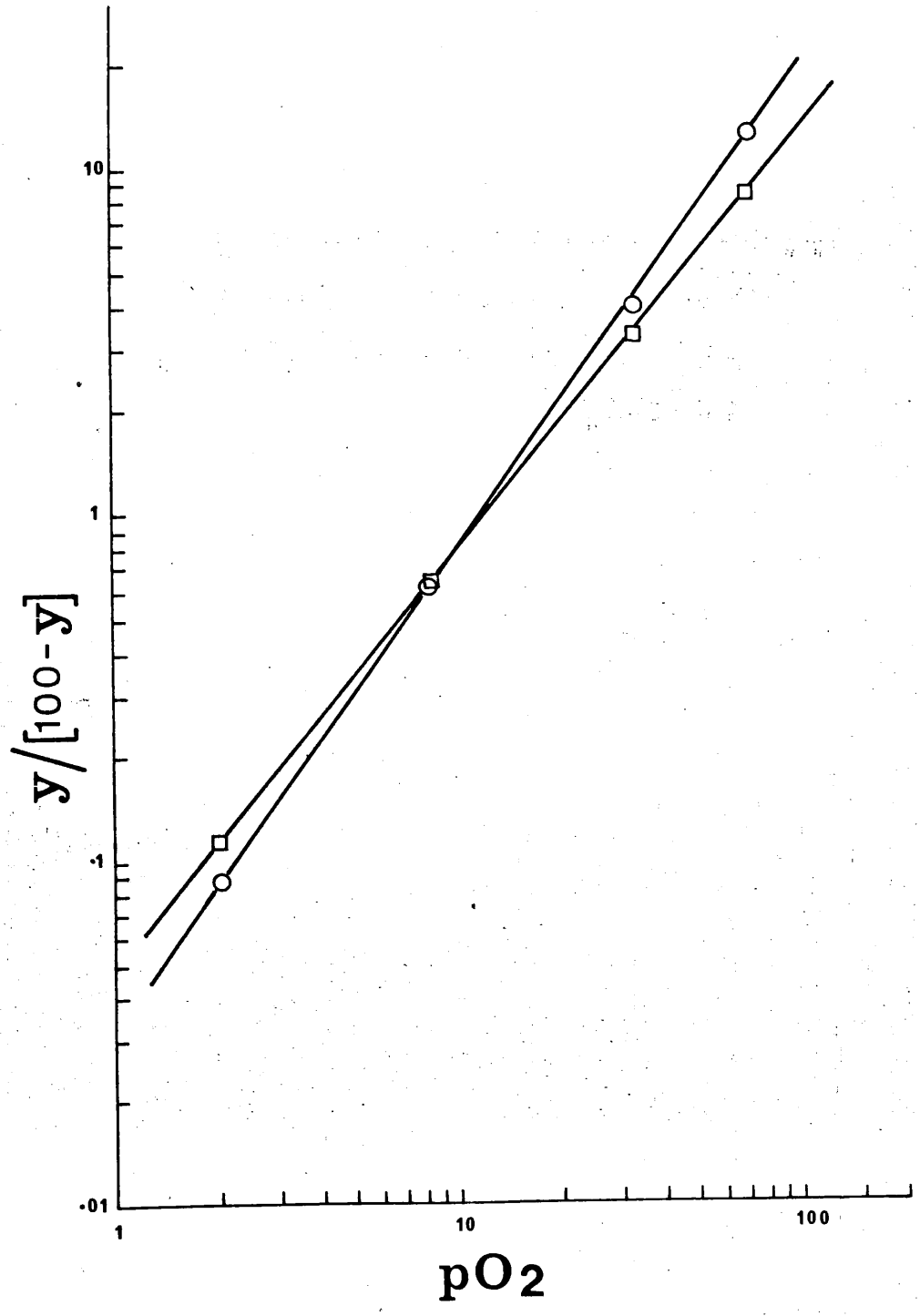

10
1
-1
y/(100-y)
1
10
pO2

Figure 6.4. Hill plots to show the effect of hydrogen ion activity on the oxygen equilibrium of the coelomic cell haemoglobin at 25°C. O= pH 6.50 and □ = pH 7.28.


10
1
-1
-01
y/[100-y]
1
10
100
pO2

6.4.3 Oxygen-combining capacities.

A mean value of 3.6±0.2 vols % was obtained from the coelomic fluid of 12 worms at 15°C. The vascular blood from a pooled sample of 20 worms was estimated to have an oxygen-combining capacity of 3.1 vols %.* This value is similar to that obtained by Mangum & Carhart (1972) for Glycera dibranchiata cells.

6.4.4 Haemoglobin concentration.

Measurement of the haemoglobin content of coelomocytes using the cyanmethaemoglobin method was made difficult because of the fats in the cells. Some animals, however, yielded haemoglobin which did convert to a transparent cyanmethaemoglobin solution suitable for spectrophotometric measurement and we have no reason to believe that these had abnormal haemoglobin contents. An estimate of concentration using this method was 4.2 g haemoglobin per 100 ml coelomic fluid. The coelomocytes take up 42% by volume of the coelomic fluid (Table 3.1). A different approach to the determination of haemoglobin concentration which is not influenced by haemoglobin derivatives such as methaemoglobin, is based on the assumption that 1 vol.% is equivalent to 0.746 g haemoglobin per 100 ml blood.

Both the cyanmethaemoglobin method and oxygen capacity depend on haem units. From the data given above, the mean concentration of cell haemoglobin was 2.7 g% and the vascular haemoglobin 2.3 g%. In any case we found no evidence of methaemoglobin formation in either fresh blood or in solutions stored for several days after extraction from cells following passage through a 300 x 10 mm G25 Sephadex column. There was no rise in absorption near 630 nm which would have indicated methaemoglobin.

* Only two determinations could be carried out on the 10µl of vascular blood from the pooled sample, both of which yielded a capacity of 3.1 vols %.

*Nevertheless, an approximate estimate of the volume of vascular blood was obtained by collecting the fluid from punctured vessels after the coelom had been drained, rinsed and blotted dry. This volume varied between 0.8 and 1.0µl in worms with a mean fresh weight of 0.34 ± 0.05 g. It is clear therefore, that the vascular blood is less than 1% of the total body volume.

and coelomic fluid

6.4.5 Blood volume.

A mean value of 37% of the total body volume represents the coelomic fluid. Estimates ranged from 28% to 46% in 16 worms with a mean fresh weight of 0.34±0.05 g. It was not possible to calculate the small volume of vascular blood using the same technique.* Nevertheless, knowing the amounts of blood which could be collected in micropipettes for the oxygen equilibrium studies, it may be assumed that the vascular blood is less than 1% of the total body volume.

* See opp.

6.4.6 Carbonic anhydrase.

This enzyme is known to occur in several polychaetes where it probably expedites the removal of CO_2 (Section 4.3). I could not detect it either in the coelomocytes or in the coelomic fluid.

6.4.7 Respiration of cells.

The coelomocytes, while containing haemoglobin, are of course actively respiring. I measured the decline in oxygen tension within the syringe used to obtain anaerobically a sample of whole coelomic fluid, using Te Radiometer E5046 electrode. There were no gametes. The results of these determinations are shown graphically in Fig.6.6. The decline in oxygen tension from 23-11 mm Hg represents a consumption rate of 6.2 μl O_2 ml^{-1} h^{-1} whole coelomic fluid. This rate decreased below 11 mm Hg. After treatment with CO., the VO_2 was maintained down to the critical $\underline{p}O_2$ of 0.5 mm Hg. The difference gives a measure of the contribution made by the oxygen held in the cell haemoglobin at $\underline{p}O_2$ values less than 11 mm Hg. The critical $\underline{p}O_2$ was reached in 27 min without the haemoglobin (CO-treated) but the cells were able to continue respiring for about 1h when normal.

Figure 6.5. Plot of 1/p50 versus $1/T^{\circ}K$ in the temperature range 9 to $25^{\circ}C$ for the exothermic reaction of oxygen with the coelomic cell haemoglobin. See p89 for details.

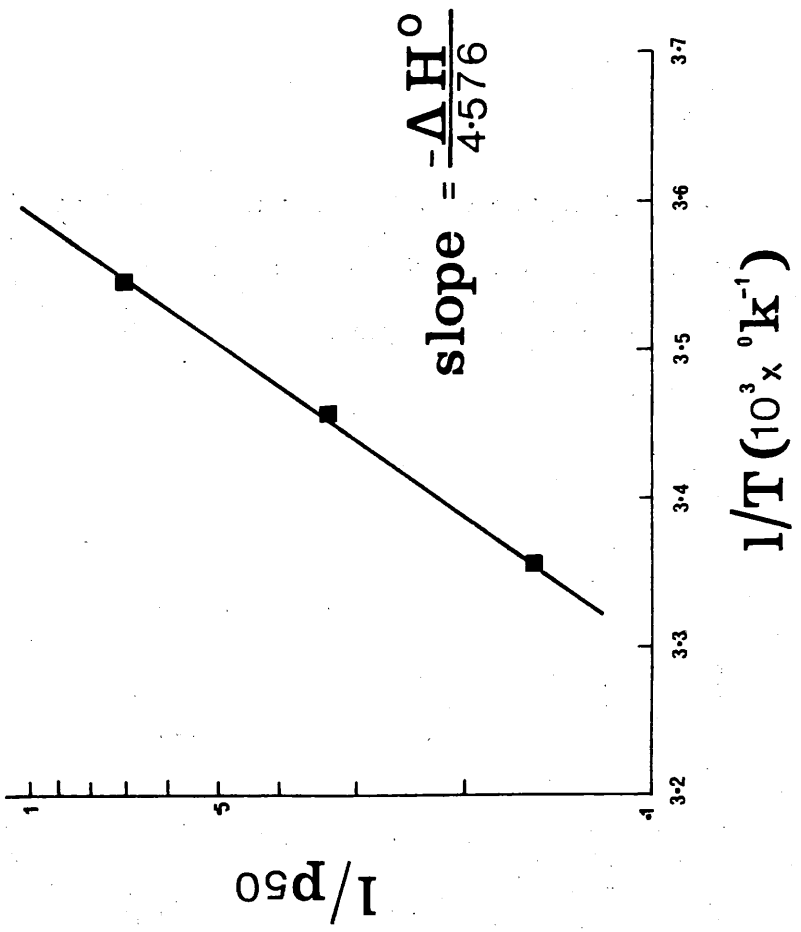
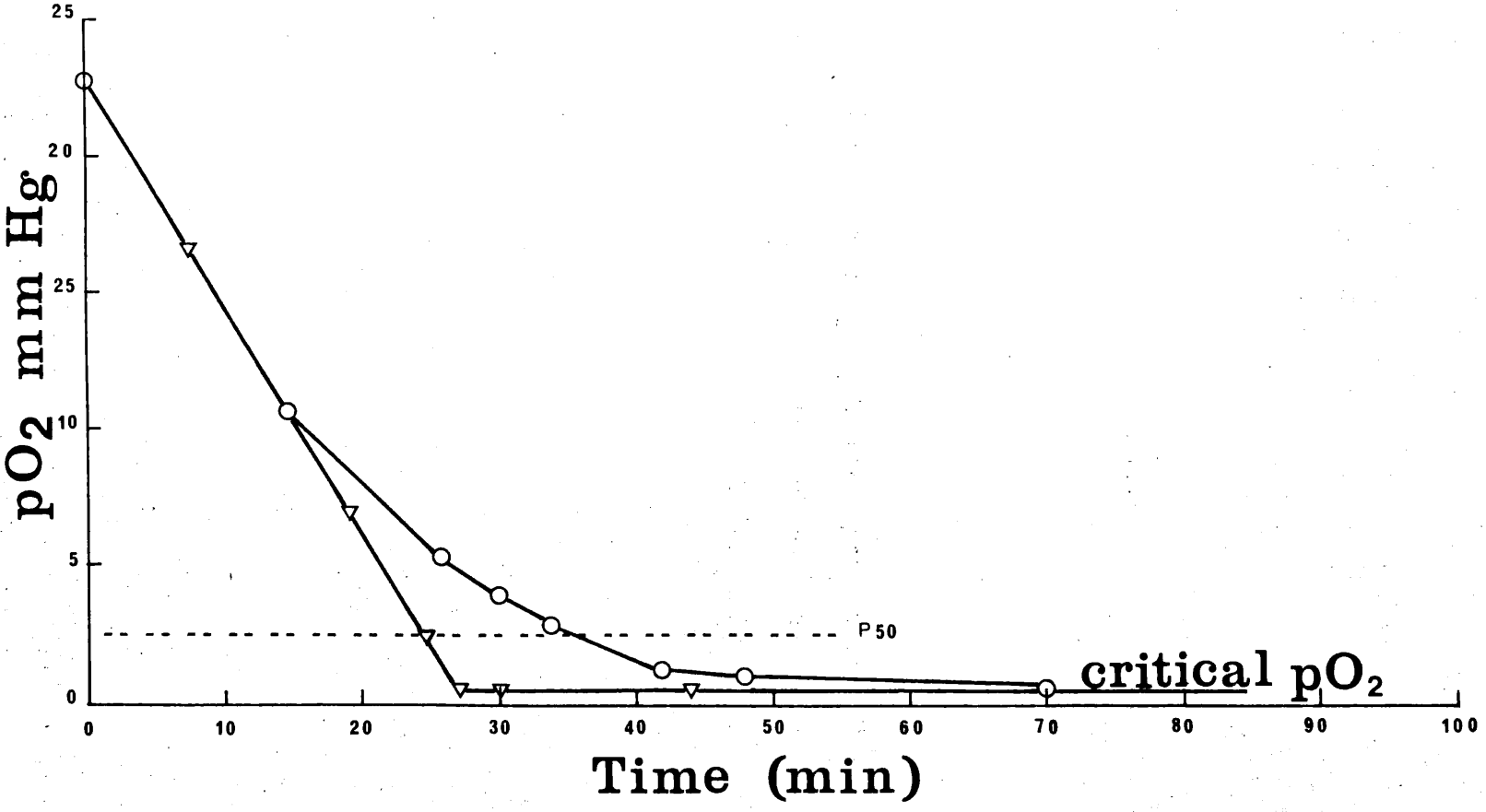

1/p50
1
5
·1
3·2
3·3
3·4
3·5
3·6
3·7
1/T (10³ x °k⁻¹)
slope = -ΔH°/4·576

Figure 6.6. The respiratory rate of Terebella coelomocytes at 20°C with (o) and without (▽) haemoglobin. The volume of fluid is 256 µl.


25
20
25
10
5
0
pO2 mm Hg
P50
critical pO2
0
10
20
30
40
50
60
70
80
90
100
Time (min)

6.5 Discussion.

The sensitivity of the moderately low p50 to temperature of the cell haemoglobin is demonstrated in Fig.6.2, the p50 rising with increasing temperature. This reduction of oxygen affinity is related to the exothermic nature of the oxygenation reaction. The quantitative relationship between the values of the equilibrium constant (1/p50) and the absolute temperature of the reaction are given in Fig.6.5. From the integrated form of the van't Hoff equation,

$$\Delta H^{o} = 4.576 \ \frac{T1.T2}{T2-T1} \ \log\left[\frac{p50'}{p50''}\right] \text{ cal mol}^{-1},$$

where

ΔH^{o} = heat of oxygenation

T1,T2 = lower and higher temperatures in ^{o}K

p50',p50" = half-saturation pO_2's at T1 and T2.

ΔH^{o} for the Terebella coelomic haemoglobin was calculated to be -13 kcal mol^{-1} (54 kJ mol^{-1}) which falls within the range -10 to -15 kcal mol^{-1} (42-63 kJ mol^{-1}) observed for most haemoglobins (Eley, 1943; Antonini & Brunori, 1971). This is interpreted as indicating a definite configurational change in the protein moiety upon oxygenation, oxyhaemoglobin being the more "ordered" form of the protein.

Recently Weber (1972) postulated that the low ΔH^{o} of -2.2 kcal mol^{-1} (9.2 kJ mol^{-1}) for Arenicola marina haemoglobin may represent an eco-physiological adaptation by safeguarding the high oxygen affinity under conditions of high temperature. Rossi-Fanelli & Antonini (1960) suggested that the very low ΔH^{o} of -1.8 kcal mol^{-1} (7.5 kJ mol^{-1}) of tuna haemoglobin was also related to the rapid temperature changes the fish experiences. Terebella lapidaria might be expected to experience some temperature variation especially on return of the tide yet the p50 is

sensitive over the range the worm is likely to experience. Garlick & Terwilliger (1974) found a ΔH^{o} of -8.4 kcal mol^{-1} (35 kJ mol^{-1}) for the coelomic cell haemoglobin of the polychaete Thelepus crispus.

The suggestion that an invertebrate blood might serve as an oxygen store to supply the animal when the tide was out was originally advanced by Barcroft & Barcroft (1924) for Arenicola marina. Borden (1931) confirmed the Barcrofts' figures which seemed to indicate that the haemoglobin could store enough oxygen for a substantial period. It is unfortunate that these arguments were ever made for they were based on inaccurate and absurdly high blood volumes (see p24), the most recent determinations by Toulmond (1971) and Alyakrinskaya (1972) confirming the conclusions of Eliassen (1955) that the length of time such a store could last was limited to a few minutes. While it seems unlikely therefore that vascular haemoglobin could act as a long term store owing to the relatively small blood volumes, this does not preclude the possibility that cellular haemoglobins could do so.

Terebella irrigates its tube by pulsations of the body wall when covered by the tide, and recordings of activity ~~using methods described before~~ (Dales, 1961) demonstrated the usual pattern of activity in such worms which is one of irrigation alternating with short periods of rest. It is possible that the store of oxygen in the coelomocytes is of use during pauses in irrigation when the pO_2 may fall in the water immediately surrounding the worm. When the tide is out, the crevices drain and the worms are essentially in moist air or retain only a film of water around their bodies. Under these conditions the gills collapse and the contribution of the vascular system may be reduced, but because the p50's of both vascular and cell haemoglobins are 3-4 mm Hg at 15°C the haemoglobin could function down to low pO_2's by virtue of the thin

dorsal body wall and circulation of the coelomocytes within. Applying a diffusion coefficient for oxygen across muscle (Krogh 1941) a $\dot{V}O_2$ of 127 µl O_2 g^{-1} h^{-1} could be maintained. The coelom is continuous in the main part of the body, the dorsal wall being only 0.1-0.2 mm in thickness and presents an area of about 120 mm^2 in a worm of 0.32 g fresh weight. Thus aerial respiration could be continued and the store would be drawn upon if the $\underline{p}O_2$ fell.

Use of KOH in respirometers to remove CO_2 presents an unnatural situation to the animal. Replacement of KOH with a CO_2-buffer containing carbonic anhydrase overcomes this difficulty and while it may be objected that the $\underline{p}CO_2$ maintained was abnormally high, the depression of $\dot{V}O_2$ found (Table 6.1) may be significant if crevices become stagnant.

In Terebella, the volume of vascular fluid is too small to be of use for any extended period. I estimated the total blood volume to be less than 1% of the total body volume (Section 6.4.5), the coelomic fluid however, comprises 37% of the body volume. A worm with a fresh weight of 0.34 g will have 125 µl of coelomic fluid containing coelomocytes. Since the oxygen capacity was estimated to have a mean value of 3.6 vols %, this would provide 4.5 µl O_2 (the amount in physical solution will not be more than 0.3 vols %). Conditions of low $\underline{p}O_2$ could occur either when the tide is in and the worm ceases to irrigate, or when the tide is out, the air stagnates within the crevice. We have no knowledge of the $\underline{p}O_2$ in crevices during a tidal cycle but measurements of $\dot{V}O_2$ in moist air indicate that aerial respiration can be carried on. Under conditions of poor ventilation the $\underline{p}O_2$ might fall. We found a $\dot{V}O_2$ of 127 µl O_2 g^{-1} h^{-1} at 20°C in moist air. If the $\underline{p}O_2$ fell to 3-4 mm Hg and the worm drew upon the oxygen held by the coelomocyte haemoglobin then all would be used by a worm of 0.34 g fresh weight continuing to respire at a $\dot{V}O_2$ of 127 µl O_2 g^{-1} h^{-1} in about 6 min. Nevertheless, the argument of Hoffmann

* The plastic bags provided a "crevice" in which the worms lay in two planes of compression and to some extent simulated the natural situation while enabling the coelomic fluid to be quickly sampled. Coelomic fluid samples were taken directly from the coelom with a syringe and analysed for combined oxygen using the method described in Section 2.3.

and Mangum (1970) that stores are of use under conditions of continuously declining $\underline{p}O_2$'s when VO_2 is likely also to decline in oxyconformers, cannot be ignored. Their application of the equation

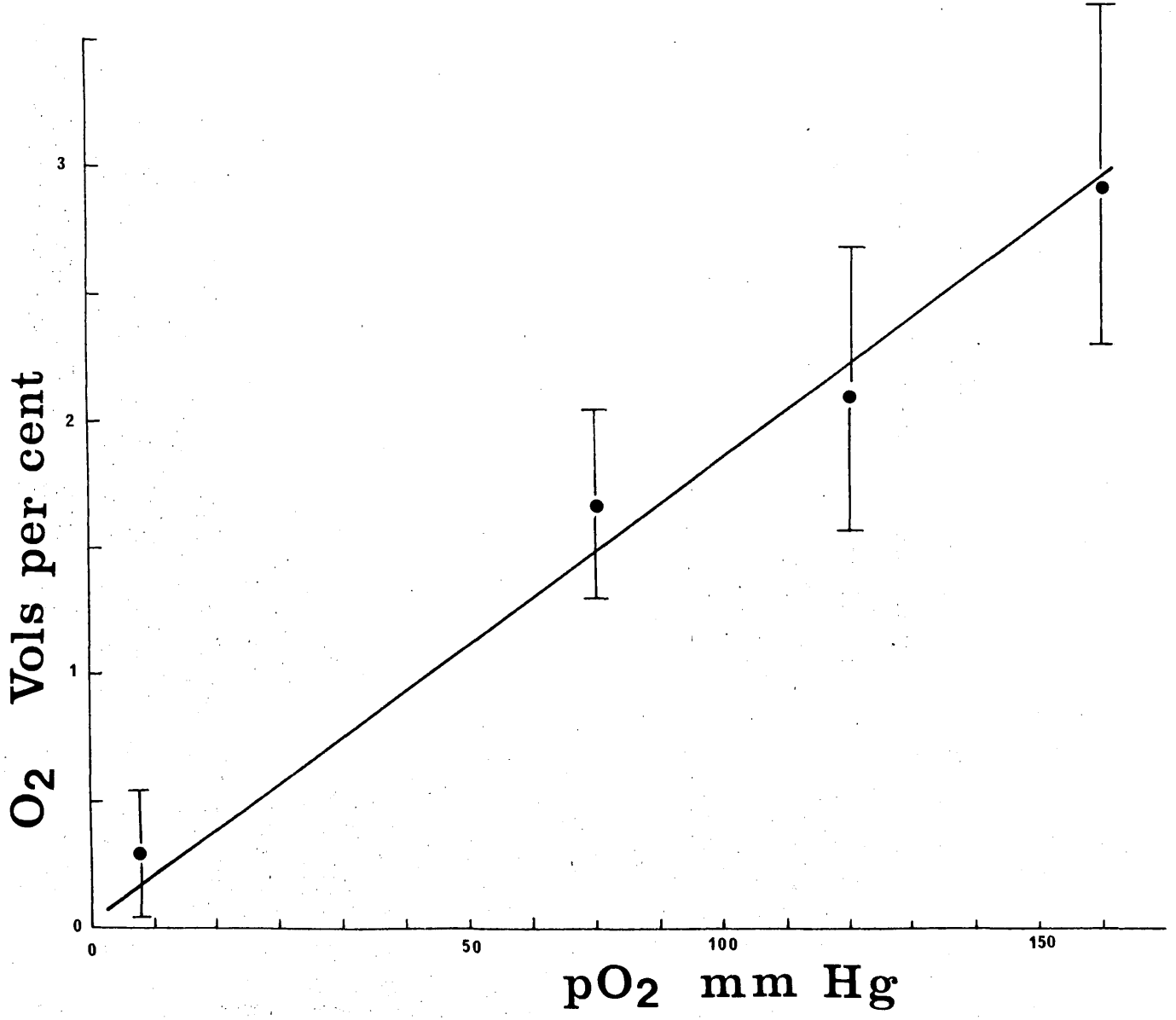
$$\frac{dY}{dT} = -kY$$

(where "Y" is the total amount of oxygen available and "k" is an empirical constant obtained from the decrease of Y with time, T) to such a situation in Glycera dibranchiata, showed that the length of time before aerobic shutdown was reached was considerably extended. The length of time an oxygen store would last on the assumption that VO_2 was maintained is in fact, roughly trebled. It may also be argued that temperatures lower than 20°C would give much less VO_2 when quiescent, so that the store would last longer. Even so, in Terebella, this would not reach the length of time for which the worms are regularly exposed, but it might be sufficient if the worm was able to respire aerobically at first and needed its store for part only of the intertidal period. It is also possible that metabolism is partly or wholly anaerobic at low $\underline{p}O_2$'s. I have found T.lapidaria most abundant around mid-tidal level where exposure may be as long as 3-4h.

To test some of these ideas I kept worms in plastic bags through which humidified air/nitrogen mixtures were maintained for 4h.* Samples of whole coelomic fluid were taken from within the bags and the oxygen contents determined. Worms were left under $\underline{p}O_2$'s approximating 160, 120, 80, and 8 mm Hg. The results are shown in Fig.6.7. After 4h the worms in 160 mm and 120 mm Hg were fairly active; those at 80 mm Hg showed some movement; those at 8 mm Hg were quiescent. It can be seen from Fig.6.7 that the oxygen content of the coelomic fluid is directly related to the external $\underline{p}O_2$ under these conditions. Further, that the O_2-content of the coelomic fluid is very low well above the $\underline{p}50$ of the haemoglobin. If

*(see opp.)

Figure 6.7. The relationship between the amount of oxygen carried by the coelomic haemoglobin and the external $\underline{p}O_2$ for whole worms equilibrated for 4h in moist air, and other gas mixtures. $T = 20°C$. Each point is the mean of 10 determinations and the standard deviations are shown.


O_2 Vols per cent
3
2
1
0
0
50
100
150
pO_2 mm Hg

the cellular haemoglobin was acting merely as a store and the worms were unable to respire aerobically, then we would expect the O_2-content to decrease with time irrespective of external $\underline{p}O_2$ so that after 4h the content at higher $\underline{p}O_2$'s would be much the same as those at ~~higher~~ the lower values. That this is not so indicates some transport at high $\underline{p}O_2$'s. The inactivity and low O_2-content after 4h at a $\underline{p}O_2$ of 8 mm Hg suggests that not only is the store of oxygen exhausted but that the gradient across the surface is insufficient to maintain a VO_2 required for normal activity.

These speculations are not at variance with the results presented in Fig.6.6. The area enclosed between the two curves represents the contribution by the haemoglobin contained within the cells to the maintenance of normal VO_2 at $\underline{p}O_2$'s lower than 10 mm Hg. The plane of bisection of this area on the vertical axis will, by definition, indicate the $\underline{p}50$ of the haemoglobin and this precisely confirms that shown in Fig.6.2. Fig.6.6 also demonstrates that the critical $\underline{p}O_2$ below which VO_2 fails to be maintained is the region of 0.5 mm Hg, for at that point cellular haemoglobin is virtually deoxygenated.

Two aspects now demand attention in order to better judge the significance of these results: the microclimatic conditions within the crevices in which Terebella lives and secondly, its respiration under natural conditions in water.

The term 'opportunistic' as used here has been used by a number of ecologists (e.g. Mozley (1960) in "Consequences of Disturbance" H.K.Lewis & Co., London) to describe the ability of a species to

1) occupy new territory readily

2.) reproduce at a relatively fast rate so that:-

3) tend to take over the exclusive occupancy of a disturbed area

4) and are transient features in a landscape.

7 THE FUNCTION OF THE CELLULAR HAEMOGLOBINS IN CAPITELLA CAPITATA (FABRICIUS) AND NOTOMASTUS LATERICEUS SARS (CAPITELLIDAE:POLYCHAETA)

7.1 Abstract.

1. Coelomic cell haemoglobins in Capitella capitata and Notomastus latericeus are rapidly oxidised in air and form a brown precipitate believed to be a haematin.

2. Both species have high-affinity haemoglobins ($p50$=3.0 mm Hg) and oxygen-combining capacities of 3-4 vols %.

3. The absence of a Bohr effect was demonstrated by the insensitivity of the oxygen equilibrium to pH 6.76 and 7.44.

4. It is suggested that capitellid haemoglobins function under low ambient pO_2's and may also protect the worms from the toxic action of sulphide.

7.2 Introduction.

Capitella capitata (Fabricius) is an opportunistic* species living in highly organic, reducing sediments which other species have failed to colonise. For this reason it has been used as an indicator of organic pollution (Wass, 1967). This species inhabits temporary burrows which may, or may not be vertical. The substrate is usually fine or muddy sand (Wolff, 1973). Eisig (1887) described irrigation movements similar to those found in the oligochaete Tubifex, whereby the hind end of the worm is wafted to and fro in the water overlying the burrow. This has been observed by the authors in the laboratory but not in the field. In addition to this, water is drawn into the gut via the anus. This enables the worm to present a larger surface for gaseous exchange.

Notomastus latericeus Sars inhabits permanent mucous lined burrows in a wide range of substrates although it is most common in sandy mud. In this species the abdominal neuropodia are slightly expanded to form small gills.

*(see opp)

The probe was pushed into the soft sediment so that the membrane-covered cathode was situated 8-10 cm below the surface. A reading was taken after a period of about 10 min to allow the sediment time to recover from distortion. Three measurements were made in different places where Capitella was found and 4 where Notomastus occured. These readings were taken near the water line where the animals were most abundant and during the time of dead low tide. These results were not intended for rigorous statistical analysis but rather to show that (a) in the regions where these animals are found, the oxygen levels were low enough to preclude the possibility of the peculiar denaturing effect observed in the laboratory under higher oxygen levels occurring in nature and (b) to gain an indication of the pO_2 gradient across the worm's body wall which might be useful in interpreting haemoglobin function in these capitellids.

Neither species possesses a blood-vascular system, but the coelom contains large numbers of cells charged with haemoglobin which gives the worms their characteristic dark red appearance.

Hoffmann & Mangum (1970) suggested that the coelomic cell haemoglobin in the polychaete Glycera dibranchiata might serve as a temporary oxygen store for the periods between irrigation bursts in addition to a transport function at higher ambient oxygen levels. This dual function was supported by data on the oxygen contents of coelomic fluid from whole worms equilibrated at various pO_2's (Mangum & Carhart (1972).). However, a "temporary store" is a necessary consequence of any transport system when ambient tensions are temporarily lowered.

The respiratory physiology of capitellids is interesting because they live in very low oxygen environments. In consequence, one would expect the oxygen equilibrium characteristics of their haemoglobins to be adapted for life with little oxygen.

7.3 Materials and methods.

Capitella capitata and Notomastus latericeus were collected on a low spring tide from opposite banks of the Yealm Estuary in Devon, where the interstitial water was tested for oxygen using a Y.S.I. model 54 portable meter and oxygen probe. (contd. opp).

It was noted that capitellid haemoglobin rapidly denatured on contact with air. This phenomenon was studied with Notomastus blood using a Beckman DK-2 spectrophotometer. Worms were bled by coelomic puncture soon after collection and the blood was kept anaerobically on ice.

Fresh, whole blood was analysed for combined oxygen using 3-5 µl samples (Section 2.3). Oxygen-equilibrium data were obtained from the oxygen contents of samples equilibrated with gas mixtures containing progressively more O_2 to avoid haemoglobin denaturation. The results were analysed by logarithmic transformation according to

the Hill equation.

In vivo pH values were estimated from measurements made on whole blood collected under light paraffin using a Radiometer G 298A micro-electrode and PHM 71 analyser.

7.4 Results and discussion.

On contact with air capitellid haemoglobin forms a derivative reminiscent of the "brown form" reported in Arenicola marina by Patel & Spencer (1963 a). The reaction was almost instantaneous in pure oxygen. Capitellids do not come into contact with high pO_2's in nature and we have not seen the denatured haemoglobin in the field. However, it can be induced in the laboratory by keeping the worms in aerated sea water for several days. The reaction will occur in the dark. This contrasts with the findings of Mangum & Winkle (1973) who observed that C.capitata showed no ill effects from exposure to high oxygen levels.

Fox & Taylor (1955) suggested that invertebrates which live normally in low oxygen environments could be poisoned by high oxygen levels. Torres & Mangum (1974) could find no evidence for oxygen poisoning in mud-dwelling polychaetes after prolonged exposure to hyperoxia. In considering animals which live in poorly oxygenated environments, Manwell (1960 a)[c] proposed that haemoglobin might function by maintaining low internal pO_2's thus protecting the tissues from oxygen poisoning. It is difficult to see how the unstable capitellid haemoglobins could function in this way.

The spectral properties of fresh haemoglobin showed α and β maxima at 575 and 540 nm and a Soret band at 414 nm, typical of many other haemoglobins in the oxy- state (Prosser, 1973). With time and air saturation, the α and β maxima decreased in intensity and shifted

away from the red region of the spectrum until absorption bands could no longer be detected. The result was a brown precipitate which was soluble in 1N NaOH. I believe this derivative to be an alkali haematin. We could not therefore synthesise a methaemoglobin by oxidation.

For comparison, I attempted to produce the "brown form" from Arenicola marina blood which Patel & Spencer found in crude oxyhaemoglobin solutions after a few days in storage. I could not detect any change in the position of the absorption maxima or their intensity, and the ability to combine reversibly with oxygen was not diminished in blood which was kept in our laboratory for several weeks.

An unstable coelomic haemoglobin apparently occurs in the polychaete Travisia pupa (Manwell, 1960 a) and attempts to reduce it with dithionite resulted in precipitation of the protein. Capitellid haemoglobin behaved similarly after the addition of dithionite.

Notomastus haemoglobin did not form a sulphaemoglobin with H_2S as is characteristic of mammalian haemoglobins. As in Arenicola, a secondary function of the pigment may be to protect the worm from the toxicity of sulphide by its catalytic action of sulphide oxidation Patel & Spencer, 1963 b). Capitellids are often found in sediments with a distinct odour of sulphide and a similar catalytic function may be ascribed to their haemoglobins. Work on sulphide tolerance and measurements of concentration in nature is in progress.

The oxygen equilibrium curves are hyperbolic and described by Hill's equation:

$$\underline{y} = \frac{100\ \underline{p}/\underline{p}50}{1 + \underline{p}/\underline{p}50}$$

where $\underline{y}$ is the per cent of oxyhaemoglobin, $\underline{p}$ is the partial pressure of oxygen (mm Hg) and $\underline{p}50$ is the value of $\underline{p}$ at which the proportions of oxyhaemoglobin and deoxygenated haemoglobin are equal. A linear

transformation from the data is given in Fig.7.1 and the slope of 1.0-1.1 indicates the lack of haem-haem interactions.

The oxygen-combining capacities of blood from Capitella and Notomastus were 3-4 vols % and showed a high affinity for oxygen, the p50 being 3 mm Hg (0.4 kN m^{-2}) for both species.

The equilibrium of Notomastus haemoglobin was insensitive to hydrogen ion activity at pH 6.76 and 7.44 and these pH values span the range of pH measured in vivo (Table 3.1). This result confirms the emerging generalization that polychaete cells containing haemoglobin do not show a Bohr shift; namely, Eupolymnia crescentis (Manwell, 1959); Glycera dibranchiata (Mangum & Carhart, 1972); G.gigantea (Weber, 1973); Thelepus crispus (Garlick & Terwilliger, 1974); Terebella lapidaria (see Fig.6.4). This contrasts with the Bohr effect found in mammalian red cells. However, Bohr shifts have been found in the extracellular haemoglobins of Arenicola marina (Toulmond, 1970[a]) and Nephtys hombergi (Weber, 1971[b]) although the functional significance of these shifts is in doubt.

The pO_2 of the interstitial water from the richly organic sediments inhabited by Capitella was 8-12 mm Hg (1.1-1.6 kN m^{-2}) and that from the cleaner sediments with Notomastus, 18-23 mm Hg (2.4-3.1 kN m^{-2}) at 11-13°C and 28°/oo salinity. (see p.97). Thus the loading tension of the haemoglobins is well-matched with the availability of oxygen in the near environment. Allowing for a modest diffusion barrier across the thin body wall, the equilibrium p50 appears to be closely adjusted to the unloading pO_2.

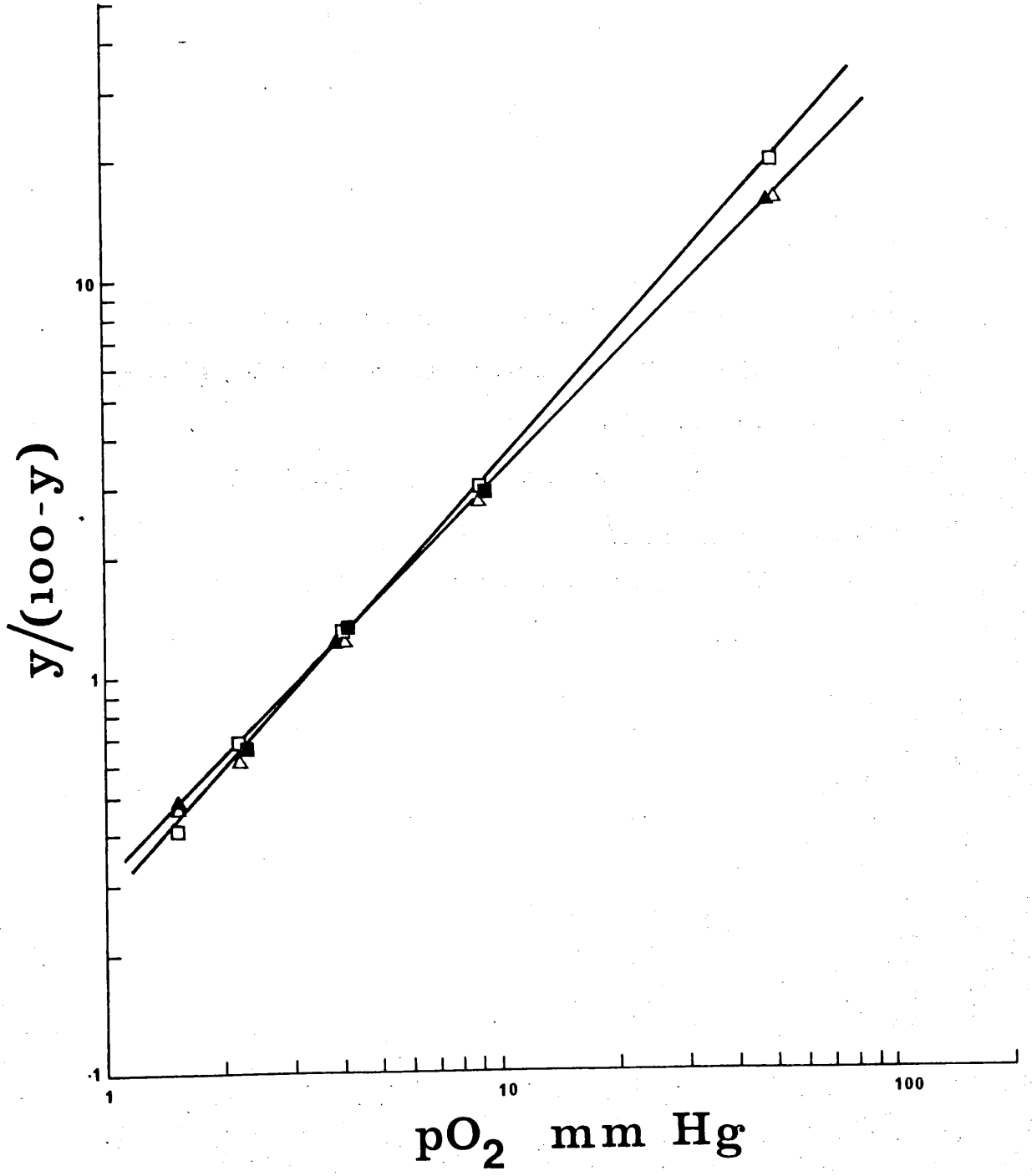
Some circulation of the respiratory pigment is afforded by peristaltic waves passing along the body wall by muscular contraction. These observations are consistent with the postulated function of capitellid haemoglobin in transport at low ambient pO_2.

Figure 7.1. Oxygen equilibria of coelomic cell haemoglobins at 15°C, using the linear transformation log $y/(100-y)$ as a function of log pO_2.

□ *Capitella* haemoglobin, pH 7.42; ■ pH 6.76, n=1.1.

△ *Notomastus* haemoglobin, pH 7.44; ▲ pH 6.80, n=1.0.

The low pH's were obtained with CO_2 in the equilibration gas.


y/(100-y)
10
1
.1
1
10
100
pO2 mm Hg

In the presence of haemoglobin, oxygen diffuses at a rate faster than would be predicted from the concentration difference. The effectiveness of haemoglobin in this process of "facilitated diffusion" is elegantly presented in the quantitative treatment given by Kreuzer (1970). The physiological implication of this phenomenon is that *ad hoc* functions of "storage" and "transport" are oversimplifications. We have used the term "transport" in a loose sense and since capitellids lack a specialised respiratory surface, the term "diffusive transport" used by Manwell (1960 a) to describe the situation in *Travisia* may be more precise.

One important aspect which demands attention in order to extrapolate with more confidence from oxygen equilibrium data to *in vivo* haemoglobin function, is the measurement of internal pO_2's of tissues and body fluids. Without this knowledge one cannot be certain of the actual loading and unloading conditions under which the haemoglobins are assumed to function.

8 OXYGENATIONAL PROPERTIES OF HAEMERYTHRIN IN THE BLOOD OF MAGELONA PAPILLICORNIS MÜLLER (POLYCHAETA:MAGELONIDAE)

8.1 Abstract.

1. The oxygen equilibrium characteristics of whole blood of Magelona papillicornis Müller have been determined and compared with the whole coelomic fluid of the sipunculid Golfingia elongata (Keferstein) using the same methods.

2. Magelona papillicornis haemerythrin is shown to have a low affinity for oxygen ($p50$ = 13 mm Hg (1.733 kN m^{-2}) at 15°C) but a high oxygen-combining capacity (6.2 vols % at 15°C) indicating an adaptation to function at high ambient oxygen tensions.

3. G.elongata fluid was shown to have a higher affinity ($p50$=4 mm Hg (0.533 kN m^{-2}) at 15°C) but a lower oxygen capacity (3.5 vols % at 15°C) indicating adaptation to low ambient pO_2's.

4. Hill's coefficient, N, = 1.0 in M.papillicornis indicating no interaction between combining centres. As recorded for several other sipunculid haemerythrins, that of G.elongata shows slight interaction (N = 1.2).

8.2 Introduction.

Haemerythrin is a non-haem iron protein which combines reversibly with molecular oxygen. The distribution of this pigment is restricted to a few species of marine invertebrates where it is always found in erythrocytes and never free in solution. It apparently occurs in all sipunculids, in two priapulids (Priapulus and Halicryptus), in two brachiopods (Lingula and Glottidia), and the single polychaete genus Magelona. Phylogenetic relations among these groups are uncertain. On the basis of haemerythrin "fingerprinting", Manwell (1963[b]) has suggested that brachiopods and sipunculids may be closer allies than hitherto suspected.

However, there are large enough differences in "fingerprint" maps to indicate polyphyletic origins. Joshi & Sullivan (1973) came to a similar conclusion after characterizing Lingula haemerythrin.

Haemerythrin was reported in Magelona papillicornis by Benham (1897) and Dehorne (1932). The annelid pigment has not been characterized, although the blood is coloured pink when oxygenated and is colourless under a stream of nitrogen. Dr.C.P.Mangum tells us that a sea water suspension of blood from Magelona sp. showed absorption maxima at 330 and 500 nm (Beckman DK-1A) and was indistinguishable from sipunculid blood. These observations suggest that there can be little doubt that the pigment in question is haemerythrin.

M.papillicornis is a small slender worm living in clean or shelly sand commonly below extreme low water of spring tides and is essentially a sub-littoral worm. It lives in vertical burrows which are dug by eversion of the proboscis and by its spade-like head. The long tentacles are protruded from the burrow for feeding, and presumably also for respiration. The vascular system, described in detail by McIntosh (1878), provides afferent and efferent vessels to those tentacles. As far as we are aware the burrow itself is not irrigated. Worms as slender as Magelona would hardly be expected to need a respiratory pigment if the whole body surface was bathed in well-oxygenated water.

Very little is known about the functional properties of haemerythrin. Oxygen equilibrium data are given for the haemerythrins in the sipunculids Phascolosoma (now Golfingia) sp. (Harrian, 1927; Kubo, 1953), G.agassizii (Manwell, 1958), Dendrostomum zostericolum and Siphonosoma ingens (Manwell, 1960 b), Sipunculus nudus (Florkin, 1933; Bates et al. 1968) and the brachiopod Lingula unguis (Manwell, 1960 d). Nothing is known about the functional properties of the priapulid or annelid pigments.

Recent interest in haemerythrin has centered on its behaviour as a model oligomeric protein. The protein isolated from Golfingia gouldii is an octomer composed of identical subunits of molecular weight 13,500. Every subunit contains two iron atoms and binds one molecule of oxygen (Klotz & Keresztes-Nagy, 1963).

In contrast with haemoglobin, haemerythrin contains iron atoms directly co-ordinated to protein side chains. Evidence from Rill & Klotz (1971) and York & Fan (1971) based on the extinction of the pigment's absorption maxima by tetranitromethane, suggests that tyrosine is an important Fe-co-ordinating ligand in contrast with the histidine ligands of the haemoglobins. As with haemoglobin, haemerythrin can be converted into the met- form accompanied by a valence change (FeII-III) but the pigment has no affinity for carbon monoxide.

There are several reasons which deem it preferable to study blood-oxygen relations in whole rather than diluted blood (Wolvekamp, 1961). The microtechnique used in this study was based on the principle described by Laver et al.(1965) for measuring combined oxygen in 50-µl samples of whole blood. We have scaled down the volumes with little sacrifice in accuracy and precision (see p 20). We were able to analyse 1-µl blood samples and construct an oxygen equilibrium curve from 9 µl of Magelona blood by incorporating a gas mixing apparatus into the system. The same procedure was adopted with Golfingia blood and served as a comparison.

The annelids present a strikingly diverse pattern of structural and functional modifications to satisfy the demands for oxygen under widely different ecological situations (Dales, 1967). The occurrence of haemoglobin is widespread in this phylum and a similar plasma protein, chlorocruorin, is a peculiarity exclusive to four families. The occurrence of haem-erythrin in a single polychaete genus is therefore puzzling.

Haemerythrin in the minor coelomate phyla is contained within

corpuscles in the coelom or in the vascular system. In Magelona the corpuscles within the blood vessels are derived from the vessel walls (Dehorne, 1949). The numerous corpuscles are enucleate spherical cell fragments 0.25-1.00 µm in diameter. On contact with sea water the corpuscles loose their spherical shape and a fibrous clot is formed. An ultrastructural study of this remarkable situation is in progress and it is intended to publish these findings separately.

8.3 Materials and methods.

Golfingia elongata was collected at low tide from the Yealm Estuary, Devon. Magelona papillicornis was dredged in Cawsand Bay, Cornwall, from approximately 6 m. About 150 worms were sorted from the dredgings of 5 runs. A few Magelona alleni were collected at low spring tide by R.P. Dales from Salcombe, Devon.

The Magelona were very small (20 x 0.5 mm) and blood was collected from individuals by puncturing the thorax, whereupon tiny droplets of blood were released and taken into glass micropipettes. The body was blotted dry and the operation performed on a dry glass slide under a microscope. A pooled sample of approximately 9 µl was all that could be obtained from 150 worms. Blood was sampled directly from the large coelomic cavity of Golfingia with a syringe. Blood was used immediately after collection.

8.4 Results.

The experimental data for the oxygen equilibria obtained are shown in Fig.8.1 in terms of a Hill plot, showing the dependence of log $y/(100-y)$ upon log pO_2. Hill's coefficient, N, derived graphically from the slope of the lines, is 1.0 for Magelona and 1.2 for Golfingia indicating only a slight co-operative interaction between sites in Golfingia but no interactions in Magelona haemerythrin. Very slight, or no interactions are characteristic of all the sipunculid haemerythrins so far examined.

Figure 8.1. Oxygen equilibrium data for Magelona (■—■) and Golfingia (□—□) haemerythrins plotted according to Hill's approximation, at 15°C. The pH of Golfingia coelomic fluid was 7.12. The volume of blood taken from Magelona was too small for a pH measurement.

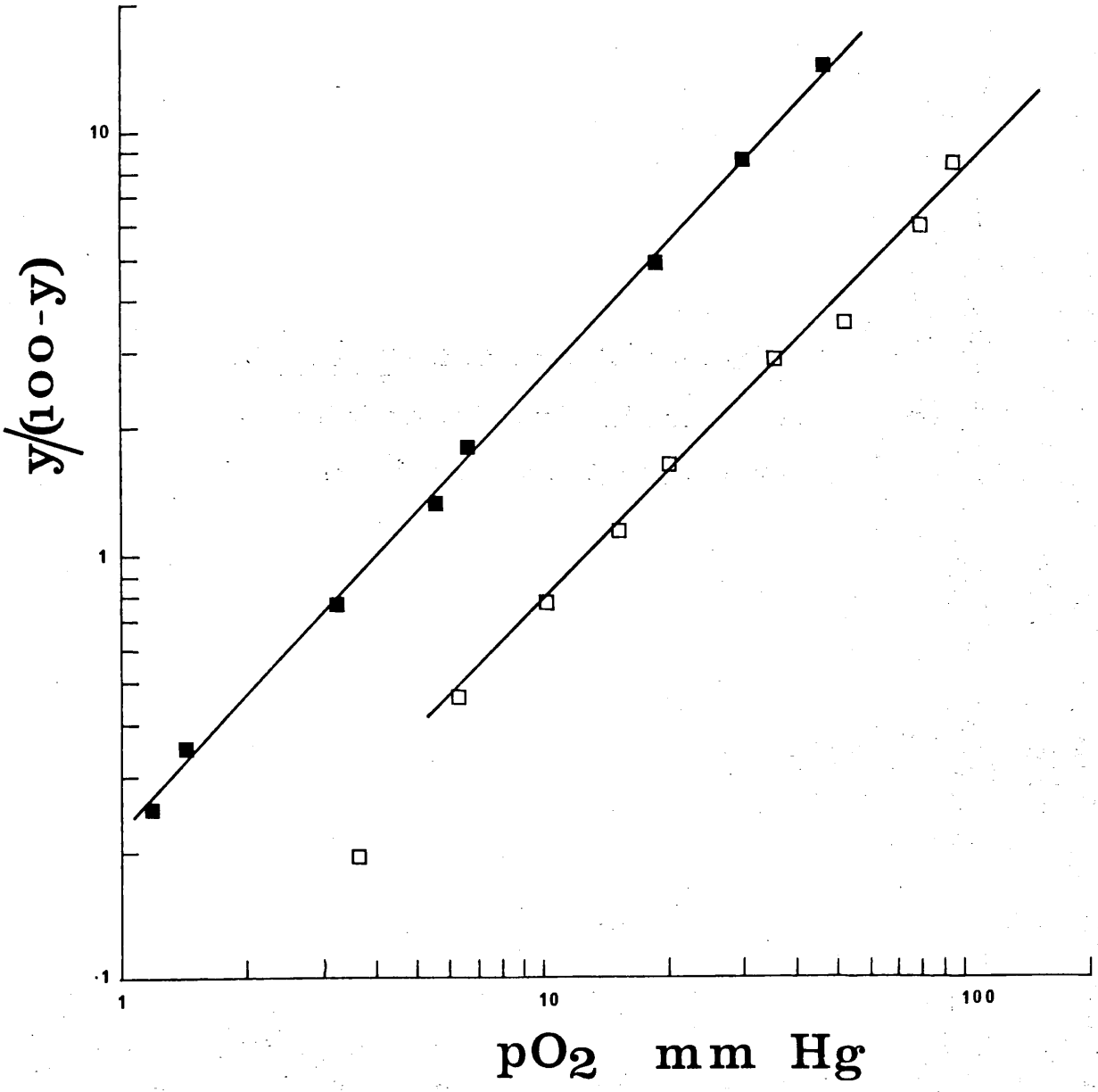

y/(100-y)
10
1
·1
1
10
100
pO2 mm Hg

Table 8.1. Summary of oxygen equilibrium data of haemerythrins. N is Hill's coefficient and p50 is the partial pressure at half saturation.

Species	p50 mm Hg ($kN\ m^{-2}$)	O2 capacity (vol.%)	N	Conditions of p50	Reference
Siphonosoma ingens	1.0 (0.13)	-	1.0	18.5°C, vascular cells	Manwell (1960b)
	1.8 (0.24)	-	1.0	18.5°C, coelomic cells	Manwell (1960b)
Golfingia elongata	4.0 (0.53)	4.2	1.2	15°C, coelomic cells	This study
Brachiopoda					
Lingula unguis	8.0 (1.07)	-	1.7-1.8	22-24°C, pH 6.7-7.6	Manwell (1960d)
Polychaeta					
Magelona papillicornis	13.0 (1.73)	6.2	1.0	15°C	This study
Magelona alleni	-	3.6	-	15°C	This study

The value for n for other haemerythrins is given in Table 8.1. The only haemerythrin known to have significant site to site interactions is Lingula (Manwell, 1960d).

Magelona haemerythrin is shown to have a low oxygen affinity ($p50$ = 13 mm Hg (1.73 kN m^{-2}) at 15°C) but a high oxygen-combining capacity (6.2 vols % at 15°C). Golfingia is shown to have a higher affinity ($p50$ = 4 mm Hg (0.53 kN m^{-2}) at 15°C but a lower oxygen capacity (3.5 vols% at 15°C).

8.5 Discussion.

Magelona is essentially a sub-littoral species and therefore does not have the problem of being cut off from its regular supply of oxygen at low tide. A $p50$ of 13 mm Hg (1.73 kN m^{-2}) suggests that Magelona haemerythrin functions at high ambient oxygen tensions. The blood is circulated through a pair of stout tentacles in a discrete vascular system and it is therefore likely that the pigment functions as a transport substance rather than as a store of oxygen which the animal would possibly never use.

Golfingia, on the other hand, possesses an extensive coelom containing haemerythrin with a $p50$ of 4 mm Hg (0.53 kN m^{-2}). In this case, the haemerythrin must represent an adaptation to low ambient tensions and the possibility of a storage function of the pigment at low tide cannot be ruled out. In the sipunculids examined by Manwell (1958, 1960b) there is a separate but smaller vascular system possessing haemerythrin with different physiological characteristics from the coelomic pigments. He has postulated an oxygen transfer system from one cavity to the other that is compatible with the ecology and respiratory physiology of sipunculids. Solutions of haemerythrin are also known to facilitate diffusion in vitro (Wittenberg, 1963). In Sipunculus nudus, Florkin (1933) demonstrated a coelomic pO_2 of 20 mm Hg for well-aerated specimens.

A high oxygen capacity and large volume of fluid suggest a storage function at low tide. It is found up to 30 cm in mud and does not make irrigatory movements. As the body wall is unspecialized, oxygen stores may be loaded during surface excursions and the haemerythrin fully charged. The high oxygen affinity and hyperbolic equilibrium curve endorse this hypothesis. The properties of Golfingia haemerythrin are similar and a similar function seems likely. The tentacle arrangements in Golfingia described by Gibbs (1973) may also be related to respiratory needs.

The results presented here lend weight to Manwell's (1963a) claim that respiratory pigments have been subject to selection and represent an adaptation to environmental oxygen. The polychaete blood pigments show a great pattern of functional versatility, and "fingerprinting" (Manwell, 1963a) has shown that the respiratory pigments are phylogenetically labile.

9 THE RESPIRATORY SIGNIFICANCE OF CHLOROCRUORIN

9.1 Abstract.

There has been much recent work on the physiological and structural characteristics of annelid haemoglobins but no studies have been made on chlorocruorins. The most recent work is that of Manwell (1964) who described the oxygen-equilibrium of Pherusa chlorocruorin, and Antonini et al. (1962b) who confirmed and extended the earlier work of H. Munro Fox (1932) on Sabella. I have examined the oxygenational properties of chlorocruorin in Pherusa and in Myxicola using whole blood.Both Pherusa and Myxicola pigments have large Bohr shifts, the chlorocruorin of Myxicola alone being fully oxygenated at normal pH in air. The oxygen capacity of whole blood is very large contrasting with that of animals having haemocyanin, and the significance of this is discussed. No Bohr shifts have been described for annelid cell haemoglobins and only slight Bohr shifts have been described for vascular haemoglobins, these being of doubtful significance. The significance of the Bohr shift of chlorocruorin is discussed in relation to the habits and environment of these animals and is compared with the large shifts found in cephalopods.

9.2 Introduction.

Chlorocruorin was discovered in 1868 by Lankester, yet it remains the least known respiratory pigment presumably because its distribution is limited to a few tubicolous polychaetes. It is a dichroic iron-containing protein which appears green in tentacles and thin blood vessels but dark red when viewed in the large gut sinus. It resembles haemoglobin in having a haem structure, the difference being in the substitution of a formyl for a vinyl radicle on one pyrrole ring. Chlorocruorin has a high molecular weight (2.8×10^6) with 80 haems per molecule in Sabella spallanzanii but may dissociate into smaller subunits at high pH's (Antonini et al. 1962a). As in haemoglobin, oxygen combines with iron in

the ratio of 2:1.

Chlorocruorin always occurs in solution, never in cells; always in vessels and never in the coelom. In this respect, it is reminiscent of the high molecular weight annelid haemoglobins. The two pigments may even occur together in some serpulids (Fox, 1949).

Biosynthesis of chlorocrurohaem in the heart-body of Flabelligera affinis (Kennedy & Dales, 1958), extra-vasal tissue of Sabella penicillus, (Dales & Pell, 1970) and Spirorbis spp. (Potswald, 1969) closely resembles the process occuring in the haemoglobin-containing polychaetes (Mangum & Dales, 1965).

Differences in the anatomy of the blood systems of Flabelliderma commensalis and Pherusa plumosa may reflect habitat differences, the latter burrowing in soft muds containing less oxygen than the rocky substrates colonized by F.commensalis (Spies, 1973). Myxicola infundibulum lives in gelatinous tubes in muddy shores, but unlike other sabellids, it does not irrigate its tube (Wells, 1952).

In view of the fact that so little is known about chlorocruorin and none has been described with a high oxygen affinity or a negligible Bohr effect, I examined the bloods of Pherusa and Myxicola.

9.3 Material and method.

Pherusa plumosa were collected from Cullercoats, Northumberland and Myxicola infundibulum from South Devon on low spring tides.

Worms were anaesthetized with 1 per cent MS222 (Sandoz) and, after dissection, the blood could be collected in glass capillaries from the large gut sinus free from coelomic fluid.

Absorption spectra were recorded from a Beckman DK-2 spectrophotometer and the positions of the maxima were ascertained.

Oxygen equilibrium and combining capacities were determined as before.

9.4 Results.

A dilute solution of blood from Pherusa gave α and β absorption maxima at 606 and 557 nm and a Soret band at 433 nm in the oxy- state. Deoxygenation with nitrogen displaced the Soret peak towards the red end of the spectrum at 442 nm. A similar spectrum was obtained from Myxicola. These spectra are in agreement with the data given by Fox (1926), Crescitelli (1945), Antonini et al. (1962a) and Spies (1973) for other chlorocruorins.

Because of the difficulty experienced in getting whole blood from Pherusa free from traces of coelomic fluid, the highest estimations of oxygen-combining capacity were probably the most accurate. From 4 pooled samples from 15 worms, the highest capacity was 7.2 vols %. Blood from Myxicola was easier to sample and gave consistent capacities ranging from 7.8 to 8.1 vols %. The only figure given in the literature is that of Sabella penicillus which has a capacity of 10.2 vols % (Fox, 1949).

Like other chlorocruorins, that of Pherusa did not saturate in air. The fact that the blood was only 65% saturated in air accounts for the very low oxygen affinity (Fig.9.1). An interactionless, monophasic plot from Hill's approximation is shown in Fig.9.2 with a slope, N=1.0. A similar equilibrium curve was obtained for another flabelligerid (Pherusa inflata) by Manwell (1964).

Myxicola is unique in reaching full saturation at less than air tensions (Fig.9.1). It also has a higher oxygen affinity (see Table 9.1) than Pherusa although it is very much lower than that shown by most haemoglobins. A similar p50 and biphasic Hill plot was shown by other sabellid chlorocruorins with N approaching 5 in the mid-region of the curve and less than 1 at low pO_2's (Fox, 1932; Antonini et al., 1962b; Manwell, 1964) as illustrated in Fig.9.3.

A large "normal" Bohr effect was observed for both species (Figs 9.2-3)

Figure 9.1. Oxygen equilibria of chlorocruorin showing the sigmoid curve of Myxicola ▼ (pH 7.18 and 20°C) reaching full saturation, and the failure of the hyperbolic curve of Pherusa ■ (pH 6.96 and 15°C) to do so at atmospheric oxygen tensions.

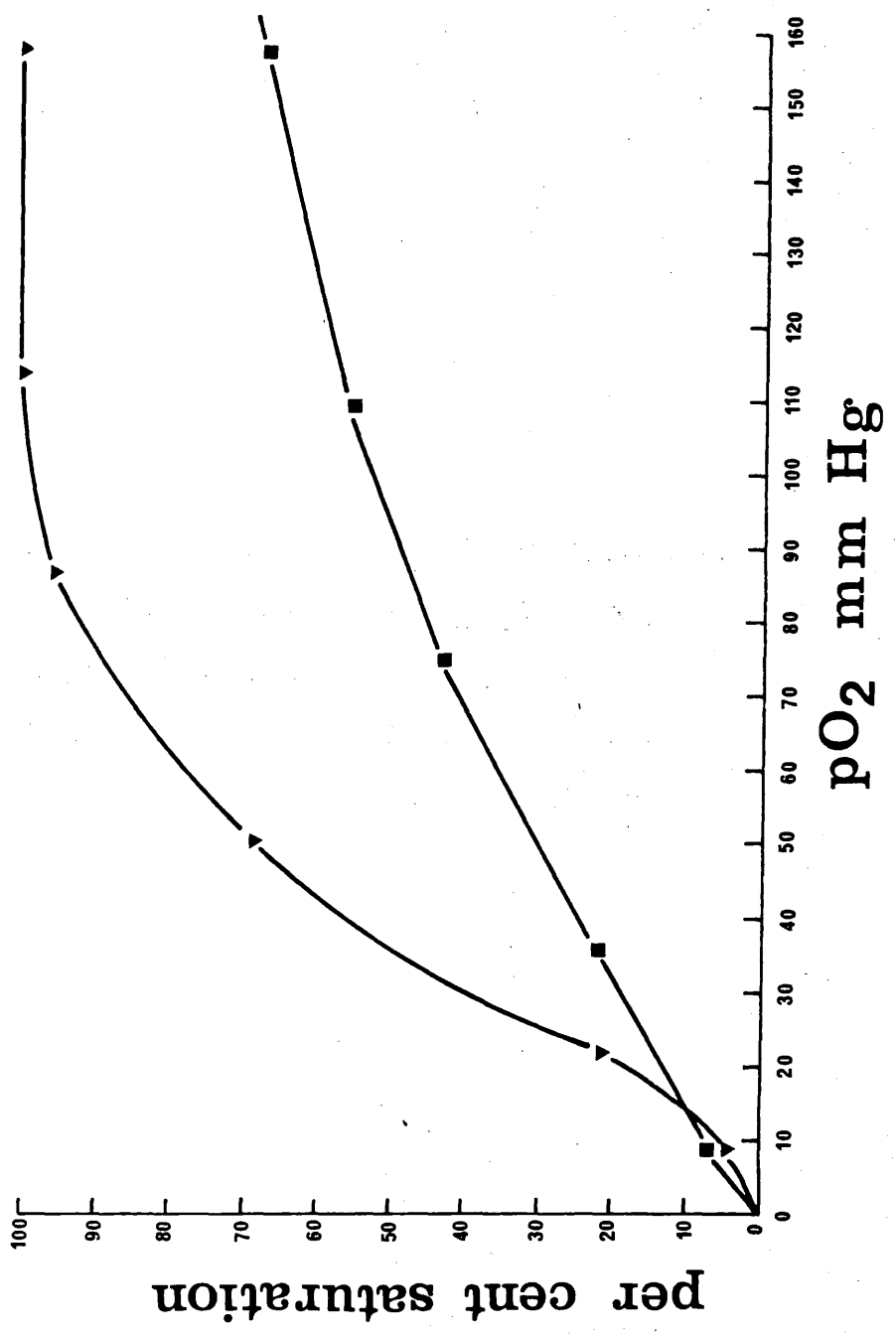

per cent saturation
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
pO2 mm Hg

Figure 9.2. The effect of hydrogen ion activity on the oxygen equilibria of chlorocruorin from Pherusa plumosa using the linear transformation "$\log[y/(100-y)]$" as a function of "log p" at 15°C. Blood was equilibrated without CO_2 ▲ (pH=6.95) and with CO_2 in the equilibration mixtures ■ (pH=6.53). The lines are drawn with a slope of N=1.0.

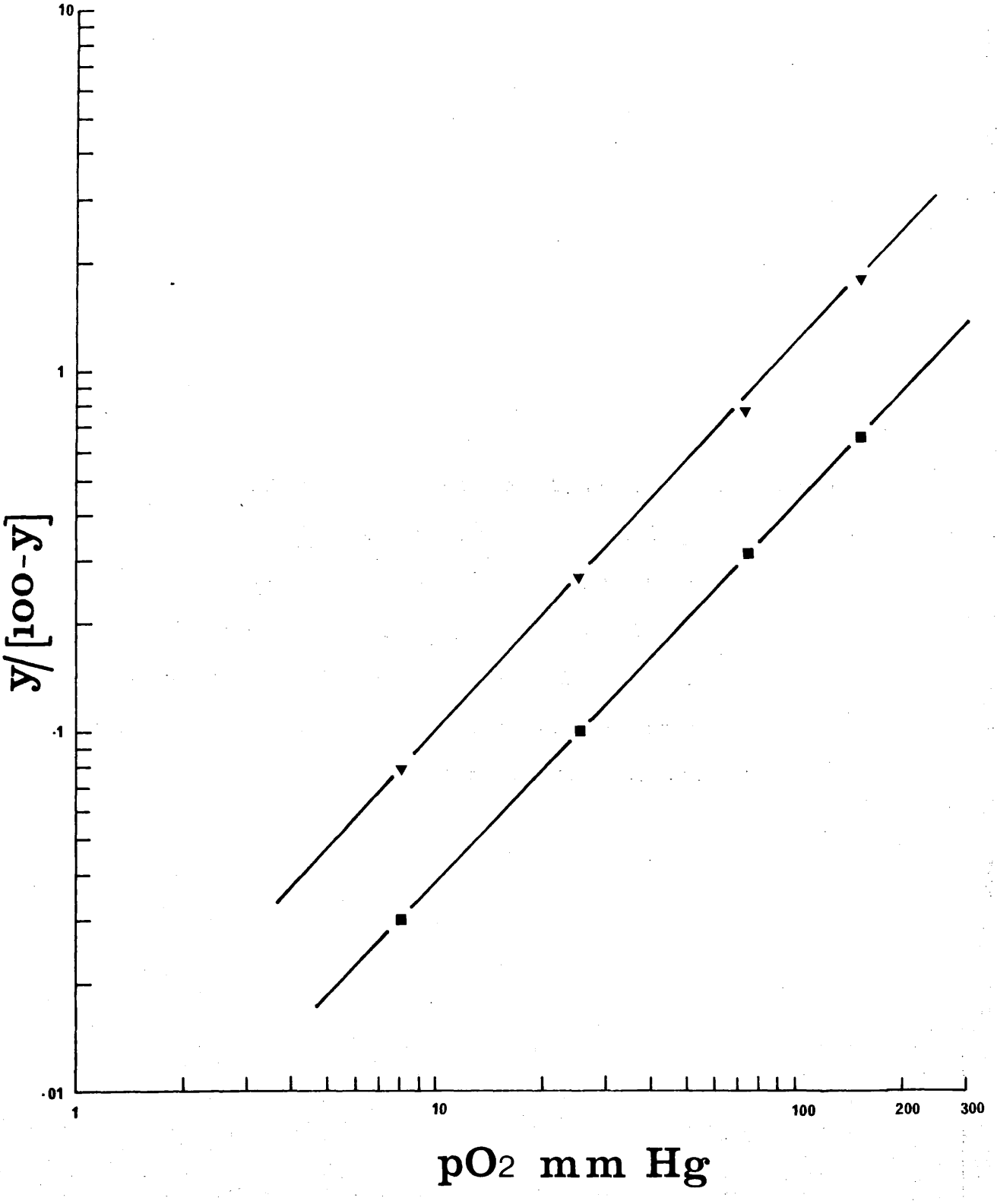
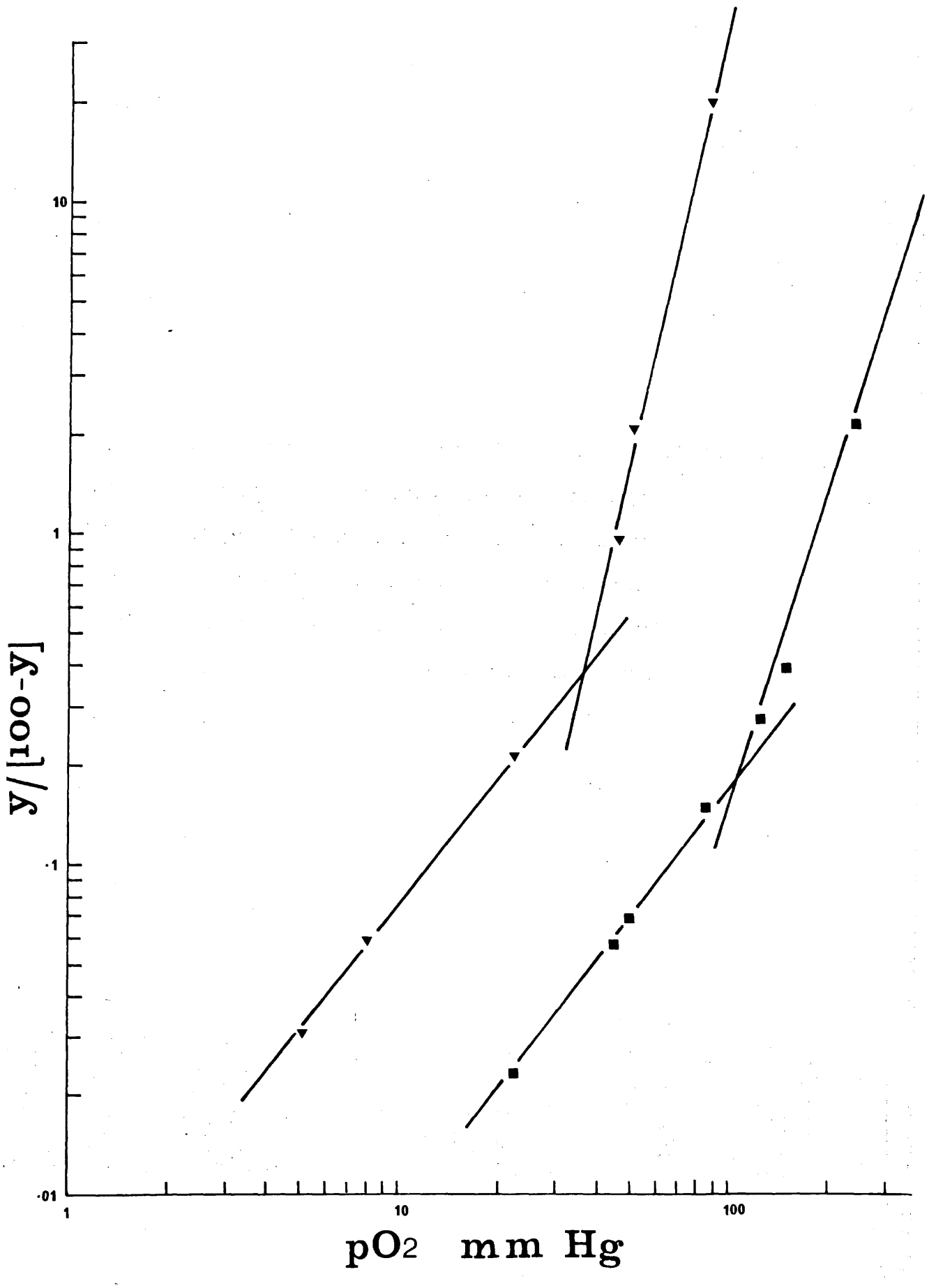

10
1
·1
·01
y/[100-y]
1
10
100
200
300
pO2 mm Hg

Figure 9.3. The effect of hydrogen ion activity on the oxygen equilibrium of blood from Myxicola infundibulum, using the linear transformation given for Fig.9.2 at 20°C. Chlorocruorin was equilibrated without CO_2 ▲ (pH=7.20) and with CO_2 ■ (pH=6.81). The graph shows the biphasic nature of the equilibrium with a slope of $\underline{N}$=4-5 in the mid-range and less than 1 at low $\underline{pO_2}$'s.


10
1
·1
·01
y/[100-y]
1
10
100
pO2 mm Hg

and compares with that observed for other chlorocruorins (Fox, 1926; Antonini et al. 1962b).

9.5 Discussion.

The similarities between haemoglobin and chlorocruorin are structural rather than functional. Yet, the functional similarities with the cephalopod haemocyanins are striking. Most apparent is the marked sensitivity of the oxygen equilibrium to hydrogen ion activity. The large Bohr shift in haemocyanin is correlated with a good circulation of the blood and a well-developed system of afferent and efferent vessels. A well-developed afferent/efferent system appears also to be a characteristic of chlorocruorin-containing polychaetes (Schlieper, 1927; Spies, 1973). The Bohr shift can be functional only when the ambient pCO_2 is very low and with pH differences between afferent and efferent blood. These differences need not be very great for the Bohr shift to operate, for in the cephalopod Loligo, it is only 0.13 pH unit (Jones, 1972).

At first, it is difficult to see what positive advantage such a large Bohr shift might confer upon the polychaetes. However, when it is remembered that the pH changes are likely to be extremely small, then the shift could operate and assist oxygen unloading at the tissue level. This represents a possibility not realised in the adaptive diversity of function in haemoglobin evolution where, at best, slight Bohr shifts of unproven significance have been noted in Arenicola (Toulmond, 1970b) and Nephtys (Weber, 1971b).

The oxygen-equilibrium of chlorocruorin in Sabella spallanzanii is relatively insensitive to changes in temperature as indicated by a ΔH^{o}=-4.5 kcal mol^{-1} (-18.8 kJ mol^{-1}) (Antonini et al. (1962b). Low values of ΔH^{o} have been interpreted as an adaptation to rapid temperature change in the environment for the tuna fish (Rossi-Fanelli et al.,1964) and the polychaete Arenicola marina (Weber, 1972). It is difficult to

interpret the sabellid heat of oxygenation in the same way as it is a predominantly sublittoral species with a low metabolic rate and unlikely to meet marked temperature fluctuations.

Sabellids exhibit a giant fibre response analogous to that shown in cephalopod molluscs, and this enables a rapid escape into their tubes. They do not stay withdrawn for very long, presumably because the $\underline{p}CO_2$ would accumulate and hinder oxygen loading at the sites of uptake. Polychaetes with chlorocruorin are essentially sublittoral species and their respiratory surfaces are bathed in well-oxygenated water of low $\underline{p}CO_2$ even though the tubes may extend into anoxic mud.

In contrast with the diverse properties of oxygen equilibrium in the annelid haemoglobins, all chlorocruorins appear to have low oxygen affinities, as do the haemocyanins, and are therefore assumed to transport oxygen under conditions of high ambient $\underline{p}O_2$ (Jones, 1972). However, in contrast with the high oxygen-combining capacity of chlorocruorin, the haemocyanins have low capacities of about 3-4 vols %. Presumably, the higher capacity of chlorocruorin can increase the total turnover of oxygen and allow for effective transport under lower $\underline{p}O_2$'s than the haemocyanins with their low capacities.

If the convention that the $\underline{p}50$ represents the unloading tension and $\underline{p}95$ the loading tension is applied, then it is difficult to see how these pigments might assist in oxygen transport. Yet, the work of Ewer & Fox (1940) with respiratory rates of normal and CO-treated Sabella surely suggests that chlorocruorin transports oxygen over an external $\underline{p}O_2$ range down to 30 mm Hg. With an external $\underline{p}O_2$ of 30 mm Hg, and allowing for a modest diffusion barrier across the gill surfaces, the $\underline{p}O_2$ at the points of oxygen delivery must be much lower. Although I have not measured *in vivo* pH and $\underline{p}O_2$ in these worms, it cannot be true that the $\underline{p}50$ corresponds with the unloading tension. This tacit assumption in

oxygen equilibrium interpretation seems even more unreasonable in view of the evidence given in Table 9.1. An unloading tension of 260 mm Hg cannot be conceived if the conditions of pH apply. In order to interpret the chlorocruorin equilibrium data from a physiological standpoint, assumptions must be made about *in vivo* and near environment pO_2's and pH's. Some progress has been made in this direction with *in vivo* pH measurements (see Table 3.1)[p39] and it is interesting that measurements of pH are below the range used to study the sabellid Bohr shift by other workers.

While the sabellids show sigmoid equilibrium curves and the flabelligerids show hyperbolic curves, there are no data from the other polychaete families (serpulids and ampharetids) which possess chlorocruorin.

APPENDIX 1

Summary of oxygen equilibrium data from annelid haemoglobins

Species	p50	N	Vols %	Bohr	pH	pCO_2	T°C	Fluid	Reference
POLYCHAETA									
Arenicolidae									
Arenicola sp.	1.8	3.5	5.7-8.7	+	7.3		20	vascular	Barcroft & Barcroft, 1924
Arenicola marina	2-4	1.9-2.5	9.7	+	7.7-7		15	vascular	Toulmond, 1970b
Arenicola marina	2.0-2.5	6			7.4		18	vascular	Prosser & Brown, 1961
Arenicola marina	3-4			-			20	vascular	Prosser & Brown, 1961
Arenicola marina	1.6-2.0		2.0-8	+			20	vascular	Wolvekamp & Vreede, 1941
Arenicola marina	2.0	4.8		+	7.45		20	vascular	Weber, 1970,1972
Arenicolides branchialis	3.6	4.6		+	7.0		20	vascular	Weber, 1972
Abarenicola claparedii	4.5	4.7		+	7.4		20	vascular	Weber, 1972
Abarenicola pacifica		3.5		-				vascular	Manwell, 1963a
Abarenicola vagabunda				-				vascular	Manwell, 1963a
Arenicola cristata	5.0-6.7			-	7.6-7.9		20	vascular	Allen & Wyman, 1952
Arenicola cristata	3.6-4.1	5.7		-	7.6		22	vascular	Waxman, 1971
Arenicola sp.	5			-	5.4-6.8		20	vascular	Prosser & Brown, 1961

APPENDIX 1

Summary of oxygen equilibrium data from annelid haemoglobins

Species	p50	N	Vols %	Bohr	pH	pCO_2	T°C	Fluid	Reference
Nephtyidae									
Nephtys hombergi	7.6	1.0-1.2	1.8	+	7.0-7.4		15	coelomic	Jones, 1955
Nephtys hombergi	6.0	1.0-1.2	4.8	+	7.0-7.4		15	vascular	Jones, 1955
Nephtys hombergi	12.4	1.3-1.7		-	7.3		20	coelomic	Weber, 1971b
Nephtys hombergi	15.5	1.2-1.8		-	7.3		20	vascular	Weber, 1971b
Glyceridae									
Glycera dibranchiata	7.0 & 4.4		7.7	+	7.4-6.8			coelomic	Hoffmann & Mangum, 1970
Glycera dibranchiata	5.6	1.0		-	7.4-7.9		22	coelomic	Mangum & Carhart, 1972
Glycera dibranchiata	5.0	1.1		-	6.1-8.4			coelomic	Seamonds and Forster, 1972
Glycera dibranchiata	10	1.2-1.4		+	6.3-9.3		20	coelomic	Mizukami & Vinogradov, 1972
Glycera gigantea	7.3	1.4-1.8		-	7.0-8.0		22	coelomic	Weber, 1973
Terebellidae									
Eupolymnia crescentis	36	1.1		-	7.2		10	coelomic	Manwell, 1959
Neoamphitrite robusta	30-50	1.0		-			20	coelomic	Manwell, 1959
Thelepus crispus	3.1	1.54		-	5.5-9.2		20	coelomic	Garlick & Terwilliger, 1974

APPENDIX 1

Summary of oxygen equilibrium data from annelid haemoglobins

Species	p_{50}	N	Vols %	Bohr	pH	pCO_2	T°C	Fluid	Reference
POLYCHAETA									
Opheliidae									
Travisia pupa	0.08	1.0		-	7.0-7.5		22	myoglobin	Manwell, 1960a
Travisia pupa	0.36	1.0		-	7.0-7.5		22	coelomic	Manwell, 1960a
Travisia pupa	0.5-1.1	1.0		-	7.0-7.5		22	vascular	Manwell, 1960a
Onuphidae									
Diopatra neapolitana	5.4	1.5-2.7						vascular	Manwell, 1960c
Lagis koreni	4.2	1.9-2.9						vascular	Manwell, 1960c
Cirratulidae									
Cirriformia sp.	0.04-4	1.0-1.4		+	6.5-7.2		20	vascular	Swaney & Klotz, 1971
Lumbrinereidae									
Lumbrinereis tetraura	4.5	2-2.5		+	7.4		20	vascular	Weber, 1971a
Nereidae									
Perinereis cultifera	1.5-2.0		5.7					vascular	Weber, 1971a

APPENDIX 1

Summary of oxygen equilibrium data from annelid haemoglobins

Species	p50	N	Vols %	Bohr	pH	pCO_2	T°C	Fluid	Reference
POLYCHAETA									
Aphroditidae									
Aphrodita aculeata	1.1	1.0		-	7.43		20	neural	Wittenberg *et al.*, 1965
Aphrodita sp.	2.0	1.0		-	7.40		20	neural	Manwell, 1960c
Halosydna brevisetosa	2.0	1.0		-	7.38		24	neural	Manwell, 1960c
OLIGOCHAETA									
Lumbricus terrestris	3.5-4.8	1.8		-			10	vascular	Manwell, 1959
Lumbricus terrestris	8			-	7.3		20	vascular	Haughton *et al.*, 1958
Allolobophora terrestris	6			-	7.3		20	vascular	Haughton *et al.*, 1958
Glossoscolex gigantea	7			-	7.5-7.8			vascular	Johansen & Martin, 1966
Alma emini	2			-		1		vascular	Beadle, 1957
Tubifex tubifex	10 - 140		6	-		0-38	15	vascular	Palmer & Chapman, 1970
Tubifex tubifex	0.6						17	vascular	Fox, 1945
Tubifex tubifex	0.67-2.2	1.9-2.5		+	6.0-8.6		20	vascular	Scheler, 1960

APPENDIX 2.

The precision buffer solutions consisted of:-

Type S1500	Type S1510
3.402g KH_2PO_4	1.816g KH_2PO_4
4.450g $Na_2HPO_4 \cdot 2H_2O$	9.501g $Na_2HPO_4 \cdot 2H_2O$
1.000kg water	1.000kg water

Conversion table for pH at various temperatures

T^oC	pH value for S1500	pH value for S1510
0	6.984	7.531
5	6.951	7.497
10	6.923	7.469
15	6.900	7.445
20	6.881	7.426
25	6.865	7.410
30	6.853	7.397
35	6.844	7.386

APPENDIX 3.

Bunsen coefficients x100 for oxygen in water.

Temperature (°C)	Bunsen coefficient
0	4.910
1	4.777
2	4.650
3	4.529
4	4.413
5	4.303
6	4.196
8	3.998
10	3.816
12	3.649
14	3.495
16	3.354
18	3.224
20	3.105
22	2.994
24	2.892
26	2.798
28	2.711
30	2.630
32	2.556
34	2.487
36	2.423
38	2.364

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