A two-state thermodynamic and kinetic analysis of the allosteric functioning of the haemoglobin of an extreme poikilotherm

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(Received 6 October 1983/Accepted 16 April 1984)

The blood of the extreme poikilotherm *Trematomus borchgrevinki* contains one major haemoglobin component, which may be separated from the minor species by ion-exchange chromatography. This haemoglobin shows co-operative CO-binding isotherms at pH 8.2. An analysis of the temperature-dependence of the binding curves has allowed the thermodynamic constants associated with the two-state allosteric parameters L , K_R and K_T to be measured. The binding of CO at lower pH (6.2) is characterized by the maintenance of the T-state to relatively high degrees of saturation. Kinetic investigations with the use of flash photolysis of the haemoglobin–CO complex under various conditions has allowed the determination of the thermodynamic parameters association with the T-state and R-state rate constants. An amalgamation of these data has allowed the mathematical simulation of predicted time courses for CO binding to this haemoglobin, by using the two-state model, which very closely represents those obtained experimentally. The overall findings show that this haemoglobin closely follows the two-state model of co-operative interaction.

The functioning of haemoglobin in terms of its ligand binding has been described by a range of models. For equilibrium ligand-binding studies it has been found that the use of a single equilibrium constant (related to p_{50}) to describe the overall binding process is inadequate for a number of species where the shape of the binding isotherm varies with the measurement conditions (Roughton *et al.*, 1955; Imai & Yonetani, 1975; Wyman *et al.*, 1977; Carey & Gibson, 1977). Analysis in terms of the Adair scheme (Adair, 1925; Imai & Yonetani, 1975) has been applied to this problem, but suffers from relatively large errors associated particularly with K_2 and K_3 no matter whether best-fitting (Imai & Tyuma, 1973) or analytical solutions are used (Gill *et al.*, 1978). The use of the Koshland *et al.* (1966) model, although suitable in principle, requires the determination of too many independent parameters to be of general use.

The two-state model, however, requires the definition of three parameters (L , C and K_R) for a tetrameric system, for which a direct measurement of fractional saturation of sites is available (Monod *et al.*, 1965). The two-state model assumes that a system exists in two quaternary states with different affinities for ligand (R state and T state), which, in the absence of ligand, equilibrate with

each other with an associated equilibrium constant L . Co-operativity is considered to arise from the fact that each of the quaternary states has a different intrinsic affinity for ligand, and furthermore during the course of ligand binding a concerted shift occurs from one state to the other. The ratio of the affinities of the two states is expressed by the constant C . In order to describe fully the binding curve of such a system, only one further parameter (K_R) is required.

Furthermore under appropriate conditions the two-state model may be applied to kinetic investigations (Hopfield *et al.*, 1971), and thus allows a complete description of ligand interactions with haemoglobin to be made by using both the equilibrium and kinetic measurements simultaneously (Saffran & Gibson, 1978, 1979; Brunori *et al.*, 1980). A number of studies of ligand binding to haemoglobin have been performed at a single temperature, particularly on fish haemoglobins, which are easily manipulated between states by changes in solution conditions. This property allows easy characterization of each state in terms of its associated parameters. However, few studies have been directed towards characterization of the thermodynamic constants associated with this model. In particular, measurements have been made on systems from fishes that are very active

swimmers and so show unusual thermodynamic behaviour (Giardina *et al.*, 1978; Barisas & Gill, 1979; Dickinson & Gibson, 1981; Morris & Gibson, 1982).

In the present paper we report an investigation on the major haemoglobin component of the blood of an extreme poikilotherm. *Trematomus borchgrevinki* is a member of a group of fishes found in Antarctica that have some unusual blood characteristics. This fish has a constant blood temperature of -1.9°C ($\pm 0.1^{\circ}\text{C}$), which is maintained because of the presence of glycoproteins in the blood, which prevent freezing (Qvist *et al.*, 1977; Osuga *et al.*, 1978; Jokumsen *et al.*, 1981). As such it is of interest to investigate the nature of the thermodynamic control of the haemoglobin of this lethargic extreme poikilotherm and compare these findings with previous data on haemoglobins of actively swimming fishes from more temperate climates. To this end we have made both equilibrium and kinetic studies over a range of conditions and used the combined data, in conjunction with the equations of Bateman (1910) and Hopfield *et al.* (1971), to obtain mathematical predictions of the time course of CO binding, which have been compared with the experimentally obtained progress curves.

Materials and methods

Fish were captured during the Antarctic spring of 1982 at the southern tip of Ross Island. All fish were housed for at least 24 h in aquaria circulated with fresh seawater at -1.9°C to allow recovery from trauma before bleeding. Approx. 1 ml of blood was collected from each unanaesthetized fish by caudal venepuncture. Erythrocytes were washed three times in nototheriid saline (Dobbs & De Vries, 1975) before further manipulation. The erythrocytes were lysed by the addition of 3 vol. of distilled water. Cell debris was removed by centrifugation, and the haemolysate was immediately passed down a column ($40\text{ cm} \times 1.5\text{ cm}$) of Sephadex G-50 (Pharmacia) equilibrated with 50 mM-Tris/HCl buffer, pH 8.2. The haemolysate was then frozen and transported to New Zealand in the frozen state.

Isoelectric focusing was carried out on polyacrylamide tube gels according to the procedure of Riggs (1981) with 2% Ampholines (pH range 5–8) (Pharmacia). The sample was loaded in a dense sucrose solution, and separation was performed at 4°C by applying a current of 1 mA per tube. Gels were scanned with a Helena Quickscan densitometer (Helena Laboratories, Beaumont, TX, U.S.A.) provided with a 420 nm filter. Separation of the major haemoglobin component was achieved by ion-exchange chromatography on a

column ($25\text{ cm} \times 1\text{ cm}$) of DEAE-cellulose DE-32 (Whatman). Elution was achieved by using a linear gradient produced by mixing 100 ml of 10 mM-Tris/HCl buffer, pH 7.15, with 100 ml of the same buffer at a concentration of 0.1 M. The major band fractions were pooled and stored at -80°C in the carbonmonoxy form until required. Haemoglobin samples were stripped of bound CO by repeated sequential evacuation and oxygenation in the presence of a strong light (100 W lamp at approx. 10 cm).

For equilibrium binding studies deoxygenated haemoglobin was introduced anaerobically into a stoppered spectrophotometer cell containing a few grains of $\text{Na}_2\text{S}_2\text{O}_4$ to remove any adventitious O_2 . No gas space was present. CO solutions were prepared by appropriate dilution of a standard CO solution with deoxygenated buffer. Additions of dilute CO solutions were made with a Hamilton micro-syringe through a rubber septum. After mixing, the solution was allowed to equilibrate for at least 5 min before spectra were recorded. Spectra in the region 400–480 nm were recorded with an Aminco DW2a spectrophotometer (Aminco Corp., Silver Spring, MA, U.S.A.) after each addition. Titrations were performed at least twice, and each titration consisted of 30–40 additions. The end point for each titration was determined by equilibrating the contents of the spectrophotometer cell with 0.1 MPa (1 atm) pressure of pure CO.

Kinetic measurements were made with a KF-020 flash-photolysis apparatus (Applied Photophysics, London W.1, U.K.) capable of delivering a $5\text{ }\mu\text{s}$ pulse of light of 200 J energy. The output of this apparatus was passed into a DL901 transient recorder (Data Laboratories, Mitcham, Surrey, U.K.) and subsequently in digital form into a CompuCorp 625 minicomputer (CompuCorp, Los Angeles, CA, U.S.A.). Conversion into absorbance values and subsequent kinetic analysis were performed by using programs developed in this laboratory. Partial photolysis ($< 15\%$) was achieved by interposing neutral-density filters between the sample and photolysis lamps.

In both kinetic and equilibrium studies the sample was housed in a 1 cm-pathlength cell fitted with a micro-probe thermometer allowing direct measurement of the sample temperature. Temperature control in both cases was provided by a circulating-water bath (Grant Instruments, Cambridge, U.K.).

All experiments were performed with protein samples at a concentration of approx. $6\text{ }\mu\text{M}$. No corrections have been made for any dimer present. In the absence of any accurate measurements of the dissociation of this protein, we have assumed the data of Atha & Riggs (1976) and Ip & Ackers

(1977) to be applicable. In this case at pH 8.2 the protein could be expected to be present with a content of approx. 7% dimer in the liganded form with a negligible amount of dimer present in the unliganded form. These conditions were also chosen so as to allow direct comparison with previously reported data on other systems (Wyman *et al.*, 1977; Barisas & Gill, 1979; Dickinson & Gibson, 1981; Morris & Gibson, 1982).

Results

Isoelectric focusing indicates the presence of three haemoglobin components in the erythrocytes of *T. borchgrevinki* with isoelectric points of 7.2, 6.4 and 6.15 (Fig. 1a). By using ion-exchange chromatography it has been possible to separate the major component haemoglobin (Fig. 1b), and this represents the material used in all work outlined below.

Equilibrium studies

CO binds to the deoxygenated form of the haemoglobin, shifting the Soret maximum from 430 nm to 419 nm. This process is characterized by

clear isosbestic points at 424.5 nm and 453 nm. At pH 8.2 both the position and the shape of the binding curves are temperature-dependent. In order to obtain parameters relating to the two-state model, the following procedure was carried out. The absorbance changes associated with the addition of a certain amount of added CO were transformed to yield Hill plots (Fig. 2). By obtaining results at very low and very high degrees of saturation, limiting unitary slopes may be obtained, which when extrapolated on to the abscissa of the Hill plot allow evaluation of K_T and K_R , i.e. the equilibrium constants associated with binding of ligand to the tense and relaxed states respectively (Levitzki, 1978). These parameters, together with $[X]$ (the concentration of free ligand present at half saturation), were used in eqn. (1) (Levitzki 1978) to obtain values of the allosteric constant L at various temperatures:

$$L = \frac{(1 + [X]/K_R)^3 (1 - [X]/K_R)}{(1 + [X]/K_T)^3 ([X]/K_T - 1)} \quad (1)$$

Table 1 gives a summary of the measured parameters as a function of temperature.

Fig. 1. Separation of *T. borchgrevinki* haemoglobin (a) Isoelectric focusing of *T. borchgrevinki* haemoglobin in polyacrylamide gels in the region pH 5–8. —, A_{420} ; O, pH. (b) Chromatographic separation of *T. borchgrevinki* haemoglobins by using an ionic-strength gradient of Tris/HCl buffer at pH 7.15. ●, A_{420} ; O, concn. of buffer. For experimental details see the text.

Fig. 2. CO-binding curves for *T. borchgrevinki* haemoglobin. The binding isotherms are shown for *T. borchgrevinki* haemoglobin in 10 mM-Tris/HCl buffer, pH 8.2. The values were obtained at 7°C (●) and 23°C (▲). The continuous lines were calculated by using eqn. (2) of the text. Other curves have been omitted for clarity.

Table 1. Two-state parameters for reaction of *T. borchgrevinki* haemoglobin with CO. For explanation see the text.

	7°C	11°C	17.5°C	23°C
K_T	9.8×10^5	7.7×10^5	4.8×10^5	3.2×10^5
K_R	1.75×10^7	1.3×10^7	7.3×10^6	5.0×10^6
C	0.0558	0.0585	0.0675	0.0631
L	5.00×10^3	2.88×10^3	4.56×10^2	3.47×10^2

By using the measured temperature-dependence of each of the three main allosteric constants (L , K_R and K_T) it is then possible, with the use of Van't Hoff plots, to obtain the free-energy, enthalpy and entropy changes associated with each of these parameters (Table 2). Fig. 3 highlights the effect of temperature on the allosteric constant L , which expresses the relative stability of each of the two states in the absence of ligand. This parameter is particularly important, as it is this constant that is combined with kinetic data to allow prediction of kinetic progress curves.

At pH6.2 the binding curves for CO show a more extreme behaviour. Under these conditions the T-state affinity appears to have the same value as at pH8.2, but as this state is stable to higher saturations the lower unitary slope is maintained to higher saturation. However, because of this effect, which is increased slightly by the presence of inositol hexaphosphate, at pH6.2 it is not possible to determine the position of the upper unitary slope portion of the binding curve and so obtain an accurate value of K_R (Fig. 4).

Table 2. Two-state thermodynamic parameters for reaction of *T. borchgrevinki* haemoglobin with CO
Data given are for 25°C. For explanation see the text.

Reaction	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS (J · deg · mol ⁻¹)
T $\rightleftharpoons$ R	-12.6	-91.4	-26.4
T-state ligation	-31.1	-50.4	-64.7
R-state ligation	-37.85	-58.4	-68.9

Fig. 3. Temperature-dependence of the allosteric constant for *T. borchgrevinki* haemoglobin

The temperature-dependence of L , derived from curves such as those in Fig. 2, is indicated (●), together with the extrapolated value at the physiologically significant temperature (▲). For explanation see the text.

Kinetic studies

By manipulation of solution conditions and the strength of the photolytic flash employed it is possible to obtain kinetic constants relating to each state in isolation from the other. To obtain the rate constant for ligand binding to the R state partial photolysis (< 12%) was performed on the haemoglobin-CO complex at pH8.2. By using the data from Table 1 it is possible to obtain the average value for the number of ligands bound to the tetramer at which the concerted switch from the T state to the R state is predicted (Hopfield *et al.*, 1971). Calculations show that this value ranges from 2 to 2.7 over the temperature range used in the kinetic studies.

Under conditions of < 15% photolysis in the presence of excess CO, Gibson (1956) has shown that recombination occurs predominantly to Hb(CO)₃. Although it cannot be directly proven that this species will reside in the R state, the above calculations are suggestive of this being the case. Furthermore, recombination kinetics after partial photolysis do show a simple single exponential time course, which would be expected for binding to an R-state system. Also, under these conditions full photolysis leads to recombinations that show a marked co-operative, accelerating time course (Fig. 5), presumably reflecting initially slow T-state binding accompanied by conversion and fast binding to R-state haemoglobin as the reaction progresses. Both of these observations lead us to believe that at high pH partial photolysis does indeed lead to recombination kinetics characteristic of R-state binding, as has been previously

Fig. 4. Low-pH binding curves for *T. borchgrevinki* haemoglobin

The binding isotherm for CO and *T. borchgrevinki* haemoglobin in 10 mM-Tris/HCl buffer, pH6.2, at 24°C is shown both in the presence (▲) and in the absence (●) of added 1 mM-inositol hexaphosphate.

Fig. 5. *T. borchgrevinki* haemoglobin-CO reaction curve at high pH

The accelerating reaction curve seen for the reaction of 100 μM -CO with 6 μM -haemoglobin monitored at 420nm is shown. The reaction was performed at 23°C in 50mM-Tris/HCl buffer, pH8.2, after full photolysis of bound CO.

reported for the case of shark haemoglobin (Dickinson & Gibson, 1981).

T-state rate constants are normally very much more difficult to obtain than are R-state parameters, as binding to the initial T state is associated with progressive conversion of the haemoglobin tetramer into the R state as the reaction progresses. In the present study use has been made of changes in solution pH and the addition of inositol hexaphosphate to stabilize the T state preferentially, even up to relatively high degrees of saturation. Equilibrium data (Fig. 4) indicate that at low pH and in the presence of inositol hexakisphosphate the T state is stabilized over and above that at neutral pH. This is evident from the extended initial unitary slope part of the binding curve, which is maintained up to relatively high saturation (Dickinson & Gibson, 1981). Under these solution conditions it could therefore be expected that recombination with CO after full photolysis would occur predominantly to T-state molecules, which would be converted into the R state only in the last stages of the reaction and so would reflect essentially only T-state binding, as the conversion into the R state occurring so late as to have negligible effect on the progress curve. This

Fig. 6. Temperature-dependence of reaction rates of T-state and R-state *T. borchgrevinki* haemoglobin with CO. The temperature-dependence of the T state (●) and of the R state (▲) *T. borchgrevinki* haemoglobin-CO combination rates are shown. T-state rates were obtained by full photolysis at pH 6.2 in the presence of 1 mM-inositol hexaphosphate, and R-state rates were determined by partial photolysis at pH8.2 as outlined in the text.

suggestion is borne out by the experimental findings that at pH 6.2 in the presence of inositol hexakisphosphate full photolysis yields a single exponential time course showing linear semi-logarithmic plots for > 90% of the excursion.

From the temperature-dependence of the R-state and T-state rate constants it was possible to obtain values of the associated activation energy for each binding process (Fig. 6). The values obtained were 42 kJ/mol for the T state and 67.2 kJ/mol for the R state.

Simulations

Mathematical simulations of co-operative time courses have made use of the equations derived by Hopfield *et al.* (1971) and Bateman (1910), under conditions in which it is assumed (a) that equilibrium between allosteric states is faster than ligand binding, (b) that ligand is in large excess and (c) that ligand-dissociation rate is negligible relative to the binding rate (Brunori *et al.*, 1980). In this case the ligand-binding reaction reduces to a series of sequential pseudo-first-order steps, for which Bateman (1910) has derived an analytical solution:

$$[\text{Hb}_0] = [\text{Hb}] e^{-\lambda_1 t}$$

$$[\text{Hb}_i] = [\text{Hb}] \left(\prod_{n=1}^i \lambda_n \right) \sum_{n=1}^{i+1} \frac{e^{-\lambda_i t}}{\prod_{\substack{j=1 \\ j \neq n}}^{i+1} (\lambda_j - \lambda_n)}$$

$$[\text{Hb}_4] = [\text{Hb}] - ([\text{Hb}_{0-3}])$$

Fig. 7. Simulation of reaction profiles for *T. borchgrevinki* haemoglobin and CO

A comparison of simulated (—) reaction profiles, obtained by using kinetic and equilibrium parameters, is shown together with experimentally obtained values: curves are shown for reaction at 33°C (■), 26°C (●) and 18°C (▲), carried out in 50mM-Tris/HCl buffer, pH 8.2.

in which $[Hb_i]$ is the concentration of haemoglobin in the i th liganded state, $[Hb]$ the total haemoglobin concentration and $\lambda_i = k_i [CO]$. Hopfield *et al.* (1971) have shown that in this case k_i can be related to the binding rates of the R and T states of haemoglobin in accordance with the two-state model as follows:

$$k_i = (4 - i + 1) [f_{i-1} k_T + (1 - f_{i-1}) k_R]$$

where

$$f_i = \frac{L(K_R/K_T)^i}{1 + L(K_R/K_T)^i}$$

Thus, by using these equations and the measured temperature-dependences of L , K_R , K_T , k_R and k_T , we have simulated two-state-model time courses and compared them with those obtained experimentally (Fig. 7).

Discussion

It has been shown that *in vivo* the erythrocytes of *T. borchgrevinki* exist at -1.9°C with an internal pH of 8.2 (Qvist *et al.*, 1977). A few experiments have been reported on the whole haemolysate that indicate that at the physiological pH O_2 is bound co-operatively with a p_{50} of 1.6 kPa (12 mmHg) (Wells & Jokumsen, 1982). Although it has

previously been reported that the erythrocytes of the Antarctic Nototheniidae contain only one form of haemoglobin (Wells *et al.*, 1980), by use of the better sensitivity and resolving power of the techniques applied in the present study it is apparent that the erythrocytes of *T. borchgrevinki* contain one major and two minor species of haemoglobin. The major haemoglobin is present at approx. 80% of the total and is easily separated from the minor components.

The isolated major fraction shows co-operative ligand binding at pH 8.2, with an associated Hill coefficient only slightly lower than that reported for the whole haemolysate (Wells & Jokumsen, 1982).

The applicability of the two-state model to the reactivity of this haemoglobin may be judged in a number of ways. The CO-binding isotherms match very closely the saturation characteristics predicted by the two-state model (Levitzki, 1978) according to the equation:

$$Y = \frac{L[X]/K_T (1 + [X]/K_T)^3 + [X]/K_R (1 + [X]/K_R)^3}{L(1 + [X]/K_T)^4 + (1 + [X]/K_R)^4} \quad (2)$$

as shown in Fig. 2. Furthermore, on examination of the binding of CO to the haemoglobin at pH 6.2, both in the presence and in the absence of inositol hexaphosphate, it is clear that the value of K_T at this pH is very close to that from measurements at pH 8.2. Both pH and inositol hexaphosphate increase the saturation up to which the unitary slope is maintained (the T state is dominant), but do not significantly alter the T-state affinity. This is exactly what is predicted from the two-state model where allosteric effectors alter only L and not K_R or K_T . It should be noted, however, that this simple behaviour is not always the case for fish haemoglobins (Saffran & Gibson, 1979; Dickinson & Gibson, 1981). When considering the temperature-dependence of the CO-binding isotherms of *T. borchgrevinki* haemoglobin it is informative to compare these data with other two-state temperature-dependence analyses. In actively swimming fish both extremes of temperature-dependence are found. Trout haemoglobin I shows essentially a temperature-independent shape of its binding curves, which it has been proposed allow efficient delivery of O_2 to tissues independent of external temperature (Brunori, 1975). At the other extreme tuna, which may have a muscle temperature as much as 15°C higher than ambient during active swimming, shows such a large dependence on temperature that the shift in the binding curves is actually in the opposite sense to that seen in mammalian systems (Carey & Gibson, 1977). The

extreme temperature-dependence of the tuna haemoglobin has been identified as arising from the very large enthalpy change associated with the allosteric constant L (336 kJ/mol), which may have a role to play in maintaining the high temperature of the muscle mass during active swimming. *T. borchgrevinki* haemoglobin binding curves show a moderate dependence in shape on temperature, but maintain a relatively constant ratio of K_R to K_T owing to their similar temperature-dependence. This behaviour is similar to that seen for human haemoglobin, for which the binding isotherm changes very little in shape with changes in temperature (Roughton *et al.*, 1955; Imai & Tyuma, 1973) and may represent an adaptation to a situation where constant body temperature is maintained. *T. borchgrevinki* haemoglobin does, however, show sufficient enthalpy associated with the allosteric constant L (−91.4 kJ/mol) to produce significant shifts in the equilibrium between the R state and the T state over the experimentally amenable temperature range, with a change in L by a factor of approx. 20-fold between 7°C and 23°C, in contrast with human haemoglobin, which exhibits a change in L of only approx. 2-fold over the same temperature range (Imai & Tyuma, 1973).

A common finding in other studies of this type has been heterogeneity within either state. Both tuna and shark haemoglobins show binding heterogeneity in the R state (Morris & Gibson, 1982), whereas menhaden haemoglobin shows heterogeneity in the T state (Saffran & Gibson, 1979). A number of studies involving use of the two-state analysis have been made by Gibson and co-workers, but in these cases, owing either to the heterogeneity outlined above or to high affinity, not all the allosteric constants have been measured directly, but one or more have been derived by best-fitting procedures. Only in the case of trout haemoglobin I (Wyman *et al.*, 1977; Barisas & Gill, 1979; Brunori *et al.*, 1980) has a study comparable with the one reported in the present paper been carried out on a homogeneous system with full two-state analysis of both equilibrium and kinetic properties. In the case of trout haemoglobin I it may be noted that the value of the enthalpy change associated with the allosteric constant L is very similar to that obtained in the present study. Thus, although with *T. borchgrevinki* haemoglobin it is clear that at high temperature it should be possible to produce a haemoglobin system present preferentially in the R state even in the unliganded form, owing to instability of this protein at temperatures above 40°C it has not been possible to demonstrate this directly in the same way as has been done for trout haemoglobin I (Brunori *et al.*, 1980).

From the reported properties, if we assume the validity of extrapolation of thermodynamic data down to −1.9°C, we can predict that at this temperature *in vivo* the value of the allosteric constant for this haemoglobin is approx. 5×10^4 , and so at the physiological temperature the T state is dominant. The close similarity of the enthalpy associated with T-state and R-state ligand binding suggests that, unlike the case in many other haemoglobins, the relative affinities of both states would remain essentially constant, and could not be expected to contribute to any enhancing of cooperativity at low temperature as in the case for trout I (Barisas & Gill, 1979).

In summary, it would appear that *T. borchgrevinki* haemoglobin obeys closely the simple two-state model of allosteric function without any indication of heterogeneity within states. The thermodynamic data show the presence of a temperature-dependent system that *in vivo* would have no direct role to play. It may well be that the very low environmental temperature constantly encountered by these fish and the raised solubility of O₂ in seawater, together with its slow swimming habits, have negated the evolutionary necessity of an O₂-transporting system responsive to changes in temperature. As such, the thermal effects reported in the present paper may represent an 'intrinsic' rather than functional molecular property of haemoglobin.

The University Grants Committee (N.Z.) and the Antarctic Division of the D.S.I.R. (N.Z.) provided financial support for mounting the expedition to Antarctica. Professor A. De Vries kindly provided laboratory facilities at McMurdo Base, and Dr. Wells and Dr. Tetens assisted in the catching and bleeding of fish. Equipment was purchased from grants from the University Grants Committee (N.Z.) and the Golden Kiwi Scientific Distribution Fund.

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