Comp. Biochem. Physiol., 1969, Vol.**28**, pp. 1203 to 1223.

TEMPERATURE-INDUCED CHANGES IN THE OXYGEN EQUILIBRIUM CURVE OF THE BLOOD OF THE BROWN BULLHEAD, *ICTALURUS NEBULOSUS*

GORDON C. GRIGG

Abstract-1. The affinity of blood for oxygen is dependent on temperature, which would seem to present a disadvantage to those fishes which encounter large seasonal temperature changes. Considering the well-known acclimatory abilities of many fishes, it would seem reasonable to propose the occurrence of seasonal modification of blood oxygen equilibria to compensate for changes in temperature.

2. In *Ictalurus nebulosus,* blood from one group of fish acclimated at 24°C showed a consistently higher oxygen affinity compared with a group acclimated at 9°C, when measured at the same temperature. This shift, accompanying thermal metabolic acclimation, minimizes the effect of temperature on oxygen affinity.

3. The shift did not persist when dilute solutions of hemoglobin were studied.

4. No changes with acclimation were seen in the multiple hemoglobin pattern nor in blood pH.

5. The erythrocyte, rather than the plasma, appears to be the site of modification.

6. Some large changes in erythrocyte potassium accompanied thermal acclimation, but the relation of this to the shift in oxygen affinity is unknown.

INTRODUCTION

The loading and unloading properties of the blood of fishes have been shown to be well adapted to the environment and needs of the particular species (Prosser & Brown, 1961). However, these properties are temperature dependent, for an increase in temperature generally causes a large decrease in the oxygen affinity of the blood (Roughton, 1936; Antonini, 1965) and yet this has received little attention in respect to acclimation or acclimatization. In fishes living in an environment subject to strong seasonal changes in temperature, it would seem advantageous if some control were exerted on oxygen affinity so as to compensate for the effect of temperature. This seems even more likely when the ability of many fishes to undergo thermal metabolic acclimation (Bullock, 1955) is considered.

Physiologically significant ontogenetic changes in the oxygen equilibrium curve have been described in mammals birds, reptiles, amphibians and fishes (see review by Manwell, 1960, pp. 222-226). It seems reasonable to propose analogous seasonally reversible changes in oxygen affinity in those poikilotherms which are subjected to large seasonal changes in temperature.

There are several possible mechanisms which could modify oxygen affinity reversibly. Barcroft (1914) commented that pH, electrolytes and other substances within the erythrocytes have a marked effect on the oxygen equilibrium curve of whole blood. Seasonal alteration of one or several of these factors could bring about adjustment of the loading and unloading properties of the blood. In addition, multiple hemoglobin systems are common throughout the vertebrates (Gratzer & Allison, 1960) and are known to be at least partly responsible for ontogenetic changes in oxygen equilibrium curves (Manwell, 1960). Modification of the pattern of hemoglobin heterogeneity would seem to provide an ideal mechanism for seasonal adjustments of oxygen affinity.

Two poikilotherms have been examined previously for seasonal changes in the oxygen equilibrium curve, the European frog, *Rana esculenta,* and the brook trout, *Salvelinus fontinalis.* Kirberger (1953) and Straub (1957) worked with *R. esculenta* acclimated to different temperatures. They described changes in the oxygen equilibrium curve in addition to those which could be ascribed to the accompanying changes in oxygen capacity. Oxygen affinity was modified in some way so that at the same experimental temperature the oxygen affinity of the blood of the frogs having a warm history was higher than that of frogs having a cold history. This partially compensates for the effect of seasonal temperature changes on oxygen affinity. The control mechanism was not discovered. Black et al. (1966) compared oxygen equilibrium curves and the Bohr effect of blood from *S. fontinalis* in summer and winter. They found no differences between the two groups and concluded that the large Bohr effect tended to offset the disadvantageous effect of low temperatures on oxygen transport.

The fresh-water brown bullhead, *Ictalurus nebulosus* (Le Sueur), was examined in the present study. It is a eurythermal fish and shows a significant relationship between metabolic rate and thermal history (Tinker, unpublished data). It was already known to have a small Bohr effect and a high oxygen affinity

(Black, 1940; Haws & Goodnight, 1962), presumably as adaptations to waters high in carbon dioxide and low in oxygen. However, these conditions are most likely to be encountered in summer when the fish are most active and are in shallower waters to feed and breed (Pry, 1939). Hence, in the summer there is a greater demand for oxygen during the time that high temperatures lower the oxygen affinity of the blood. On the other hand, in cooler waters the increased oxygen affinity should inhibit the unloading of oxygen from the blood to the tissues. From an ecological viewpoint, therefore, any ability to reduce the effect of seasonal temperature changes on the oxygen equilibrium curve of *I. nebulosus* could be advantageous.

The aim of this study was to compare the respiratory properties of the blood of warm- and cold-acclimated *I. nebulosus,* particularly with respect to their affinity for oxygen at different temperatures, and to describe the mechanisms by which any adjustments are made.

MATERIAL AND METHODS

1. Material

The brown bullhead, *Ictalurus nebulosus,* has been introduced into Oregon from the eastern United States and large populations now live in many Oregon lakes and streams (Swan, personal communication). The fish used in this study came from either Dorena Reservoir 10 miles east of Cottage Grove, Oregon, or from a privately owned pond in Eugene, Oregon. The species was identified by reference to the keys of Bond (1961). The fish from Dorena were larger (150-700 g) than fish from the pond (35-110 g). Fish from Dorena were taken in a modified fyke trap loaned by the Oregon State Game Commission. Large numbers were caught in April and May during spawning, but at other times of the year, especially in winter, fish were obtained with some difficulty. Fish were taken from the pond by angling and this was successful except during the coldest months.

2. Acclimation protocol

In the laboratory, fish were acclimated to cold (9-10°C) and warm (24-25°C) conditions in insulated 55-gal polyethylene barrels, two barrels at each acclimation temperature. The warm barrels were each heated by a Briskeat heating tape, and the cold barrels cooled by circulation of refrigerant through a heat exchanger made by coiling plastic conduit tubing. The operation of each system was regulated by a Fenwal thermoswitch. The barrels were aerated and the oxygen level was consistently greater than 85 per cent saturated. A slow influx of fresh dechlorinated water provided gradual turnover of the water in each barrel. Throughout the study, fish were acclimated for a minimum of 3 weeks. Initially the fish were not fed, but in later experiments whole earthworms were provided.

3. Blood sampling and preparation of hemoglobin solutions

Blood was drawn from the heart into a syringe containing a few crystals of heparin to prevent clotting. Pooling of samples from different fish was avoided and no data from pooled samples are recorded, with the exception of four oxygen equilibrium curves of whole blood as marked in Fig. 2. Observations were always made on freshly drawn blood, the only exceptions being the analyses of ionic concentration in plasma and hemolysate samples which were frozen fresh and then stored for several weeks frozen in sealed tubes.

Fresh hemolysates were used for study of the oxygen equilibrium curve of hemoglobin in solution, for electrophoresis and ultracentrifugation. The erythrocytes were washed at least four times in physiological saline. Hemolysis was effected by shaking the red cells in distilled water (1.5 ml water : 1.0 ml cells) with a few drops each of toluene and chloroform. A clear concentrated solution of hemoglobin was then obtained by centrifugation for 30 min in a clinical centrifuge. For electrophoresis the hemolysate was used at this concentration, but for determination of oxygen equilibrium curves and for ultracentrifugation, the samples were diluted to 1-2% (according to determinations of iron content) with physiological saline buffered to pH 7.3 with $Na_2HPO_4^-$ NaH_2PO_4.

4. Hematocrit, red blood cell counts and oxygen capacity

Duplicate hematocrit determinations were made immediately upon withdrawal of the blood. After mixing in the heparinized syringe, blood was placed in a capillary tube and centrifuged for 3 min in a micro-hematocrit centrifuge. Centrifugation for longer periods of time did not result in tighter packing. Disagreement between duplicates was usually less than 1 per cent and never more than 2 per cent. Red blood cell counts were made by Mr. Roger White using the standard technique, but it was found that agreement between duplicates was improved by counting cells in all twenty-five squares on the hemocytometer. The average agreement between duplicates was 4.7 per cent (range 0.86-11.76 per cent). Measurement of the oxygen content of blood samples was made by the technique of Roughton & Scholander (1943), incorporating modifications for fish blood after Scholander & van Dam (1456) and

further modified according to the advice of Dr. Everett Douglas (personal communication, 1966). Determinations of oxygen capacity were made on blood equilibrated to air in a temperature-controlled tonometer (Finley et al., 1960). Values are expressed as volumes percentage of oxygen. Differences between duplicates averaged 2.5 per cent, representing an average precision of ±0.2 vol. per cent.

5. *Oxygen equilibrium curves of whole blood*

Oxygen equilibrium curves for whole blood were determined according to the technique described by Lenfant & Johansen (1965), after Haab et al. (1960). In tonometers (Finley et al., 1960) immersed in a water-bath at experimental temperature, one half of the well mixed blood sample was equilibrated to air and the other half to nitrogen. This provided samples of fully oxygenated and fully deoxygenated blood. Oxygen content in these samples was determined by the method described above. By mixing subsamples from each of these in different proportions, a series of mixtures was obtained with known oxygen contents. The oxygen tension (PO_2 mm Hg) and pH of each mixture were then determined using a Beckman Oxygen Macro-electrode and a Beckman Micro-blood pH Electrode, each connected to a Spinco Model 160 Gas Analyser. Both electrodes were housed in modular cuvettes through which water from the water-bath was circulated to stabilize the electrode at experimental temperature during both calibration and measurement. Determination of pH was precise to ±0.02. unit. In measurements of PO_2, duplicate or triplicate determinations an the same sample agreed within 2 mm in the upper range of the 0-160 scale and within 1 mm over the 0-60 scale. Because many of the values reported in this paper are in the range from 0-15 mm, accuracy of the electrode in this range was carefully checked.

6. *Oxygen equilibrium curves of hemoglobin solutions*

These were determined by a technique similar to that described by Riggs (1951), with the addition of an oxygen electrode in the tonometer as described by Johnston et al. (1967). The tonometer was made from a 100-ml three-necked distilling flask. The oxygen electrode, a gas entry port and a stopcock were mounted through rubber stoppers in the necks of the flask. A 10 x 2 mm optical cuvette was mounted on a side-arm protruding in the equatorial plane of the vessel. About 2 ml of the buffered 1-1.5 % hemoglobin solution were placed in the tonometer, which was then flushed with nitrogen gas. After an equilibration period, the tonometer was removed from the water-bath, tilted to fill the cuvette and the whole unit set in a Cary 14 recording spectrophotometer with the cuvette in the sample slot and covered with a dark cloth to prevent light leaks. A record was then made of the absorbance spectrum from 680-500 mμ. Initially, this step was repeated as a check on the complete deoxygenation of the sample. The tonometer was then returned to the water-bath and some air introduced. After 20 min for equilibration, the P_{O2} was read and another recording made of the absorbance spectrum. This step was repeated, and a series of spectra recorded at different measured oxygen tensions, up to full saturation at 1 atm. of air. Excellent coincidence of isobestic points was obtained in every case, so that one member of a family of spectra could be compared with another at 558 mμ in the determination of percentage of oxygen saturation of the pigment. The room, the water-bath and the sample chamber of the spectrophotometer were all maintained at 20°C.

7. *Electrophoresis*

Electrophoresis of hemolysates was carried out on both cellulose acetate strips and vertical acrylamide gels using a Tris-sodium-barbital buffer (pH 8.6) in each case. Electrophoresis on Gelman "Sepraphore III" cellulose acetate strips (2-5 x 17°0 cm) was performed in a Gelman Electrophoresis Chamber (51101), for 2 hr at 300 V. Positive identification o£ hemoglobin in the pherogram was made by staining with *o*-Dianisidine (Manwell, 1963). Ponceau S was used as a permanent stain. Strips were cleared and dried and the percentage composition of each hemoglobin component in each sample determined by scanning the strip with a Photovolt electronic spot photometer, Model 501 A. The output from this was recorded on a Varicord variable recorder 42 B and quantitated with an Integraph integrator, Model 49. Acrylamide gel electrophoresis was performed in a vertical gel chamber with a circulating buffer system. Details of the chamber and the technique are given by Blattler (1967). Electrophoresis for 3 hr at 250 V gave good separation. Gel concentrations of 5-10% were used and no spacer gel was found to be necessary. *o*-Dianisidine was used as a specific stain for hemoglobin and Amido Black as a general protein stain.

8. Ultracentrifugation

Determination of the sedimentation velocity of *I. nebulosus* hemoglobin was made on a 1 % solution of hemoglobin in distilled water. A Spinco Model E Analytical Centrifuge was used, with a titanium rotor, a single sector quartz-window cell and Schlieren optics. The runs were performed at 20°C and 60,000 rev/min (r_{av} = 6.62 cm). Once this speed was reached, five photographs were taken 8 min apart,

using a Wratten No. 29 Filter over the ,light source and Kodak Tri-X film cut to fit a film holder designed for the plate cassette. This film-filter combination gave a good picture of the Schlieren pattern even through the dense red of the hemoglobin. A bar angle of 65' was used. The value of sedimentation velocity, S, was determined and then converted to S_{20w}.

9. Ion analyses

Inorganic phosphate content of hemolysates was determined using the King modification of the Fiske & Subarrow technique as described by Lindberg & Ernster (1956). The degree of colour development was read at 660 mμ using a Beckman DB-G spectrophotometer. Results are expressed in mM PO_4^{2-}/100 ml cells.

Determinations of concentrations of sodium, potassium and calcium in both plasma and hemolysates were made using a Beckman DU flame photometer. Mixed standards were used, containing sodium (105 m-equiv./1), calcium (12.5 in-equiv./1) and potassium (25 m-equiv./1). Four or five intermediate dilutions of these were made to establish a curve. The samples were diluted twenty-five times, except for the measurements of potassium in the erythrocyte for which the hemolysates were diluted fifty times. In the hemolysates corrections were made for the amount of distilled water added in the initial hemolysis, so that the values expressed in m-equiv./1 refer to the concentration inside the erythrocyte.

RESULTS

1. Erythrocytes and oxygen capacity

Two properties of blood are of great importance in its role in transporting oxygen, the oxygen capacity of the blood per unit volume and the conditions at which this oxygen can be loaded and unloaded. Bullheads acclimated at 9°C had a significantly larger percentage cell volume than those acclimated at 24°G, although the total ranges in each group overlap considerably (Table 1). Determinations made on sixteen of these samples showed that oxygen capacity is linearly related to hematocrit (Fig. 1). It can be seen that *I. nebulosus* have lower oxygen capacities after warm history than after cold history, corresponding to their respective hematocrits. Haws & Goodnight (1962) reported a decrease in hematocrit in *I. nebulosus* during the summer months. 'The present data on oxygen capacity and hematocrit are in agreement with those of Black (1940).

TABLE 1-COMPARISON OF HEMATOCRITS IN WARM (24°C) AND COLD (9°C) ACCLIMATED *I. nebulosus*

Treatment	Hematocrit	*N*	S.D. of sample	t_{84}	*P*
24°C history	26.0 (18-38)	48	± 5.40	5.33	0.001
9°C history	32.5 (23-45)	38	± 5-73		

Mean values are shown, followed by the range (in parentheses).

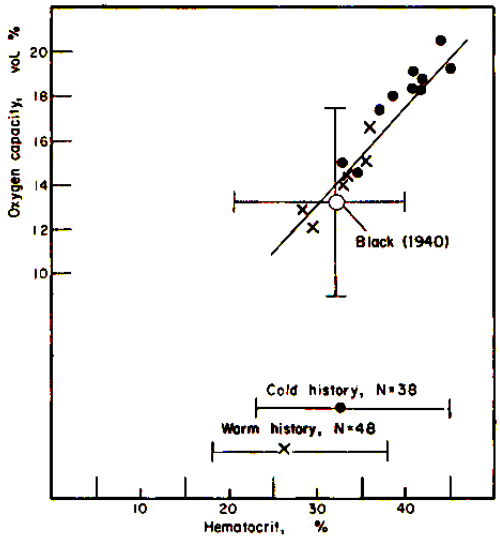

FIG. 1. The relationship between hematocrit and oxygen capacity in warm acclimated (x 's) and cold-acclimated (closed circles) *I. nebulosus*. The ranges of data reported by Black (1940) are indicated, and also the range of hematocrits of warm- and cold-history bullheads in the present study.

At one stage during this study it seemed that the higher hematocrits in cold history fish could be due partly to an increase in individual cell volume. To test this, thirty red cell counts were made with accompanying hematocrit readings. If an empirical relationship between these two parameters is expressed as

$$Y = a + bX,$$

where Y = hematocrit and X = number of red blood cells (millions), then a should equal zero and *b* would be an expression of the individual erythrocyte volume in relation to the way in which the cells pack together. A larger individual cell volume is represented by a larger value of *b* as long as the packing remains the same. Within one species therefore, comparison of b values after different experimental treatments allows an assessment of the effect of this treatment on the volume of individual erythrocytes. In *I. nebulous* this comparison was made between warm- and cold-acclimated groups and equations derived as follows:

$$Y=7.4970+14.2920X \text{ (warm history)},$$
$$Y=4.1491+15.4201X \text{ (cold history)},$$

Statistical comparison of the *b* values by Student's t-test yielded $t_{28} = 0.3251$ which does not indicate a significant difference between the two groups, so it was concluded that no change in red cell volume accompanies thermal acclimation. Neither of the a values is significantly different from zero.

2. Oxygen equilibrium curves of whole blood

Oxygen equilibrium curves were determined at both 9 and 24°C for each acclimation group. The oxygen tension at half-saturation, P_{50}, was taken as representative of a particular curve, at the pH measured on the same half-saturated sample of blood. Lines were fitted to these points by the method of least squares (Fig. 2), with the general equation:

$$Y = a + bX,$$

where Y = log P_{50} and X =pH.

The value of *a* is physiologically meaningless by itself, but is related to oxygen affinity and so affords a means for statistical comparison of the oxygen affinities of the blood in each of the four experimental categories. The value of *b* in this equation is an expression of the Bohr effect. Comparison of the slopes and intercepts of these equations (Table 2) shows that pH does have a significant effect on the oxygen affinity, but the magnitude of this Bohr effect is not modified by either thermal history or experimental temperature because no significant differences exist between the slopes. There are, however, significant differences between the oxygen affinities of blood from warm and cold-history fish measured at either 9 or 24°C, cold-history fish having blood with a significantly lower oxygen affinity than warm-history fish at the same temperature. This is consistent with the findings of Kirberger (1953) and Straub (1957) for *R. esculenta* and with the hypothesis outlined in the introduction. The pH values measured at P_{50} of each oxygen equilibrium curve were compared by t-tests between experimental categories. No significant differences were found, indicating that the observed alteration in oxygen affinity is not the result of a change in pH accompanying thermal acclimation. It is interesting that there was such a wide variation in pH (almost 0.8 unit) of *I. nebulosus* blood equilibrated to atmospheric carbon dioxide tensions and half saturated with oxygen. It has already been shown that oxygen affinity of tile blood of *I. nebulosus is* significantly related to pH. The Bohr effect can be defined quantitatively by

$$\Phi=\Delta \log P_{50} / \Delta \text{ pH}$$

so that the average value for Φ in *I. nebulosus is* -0.3079, which is not high compared with the values tabulated by Prosser & Brown (1961) but does represent a considerable percentage change in P_{50} with pH, because of the low P_{50}

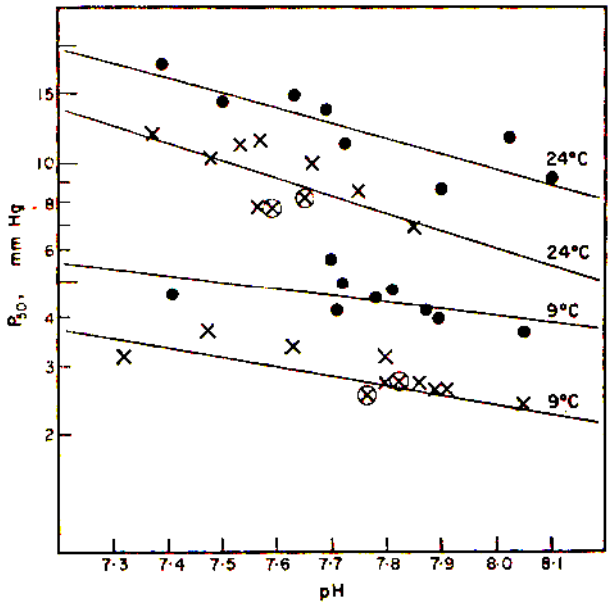

FIG. 2- The relationship between P_{50} and pH (at P_{50}) of cold-history (9°C) (closed circles) and warm-history (24°C) (x 's) I. *nebulosus,* measured at 9°C and 24°C: as marked on each line. Each point represents a measurement on a different fish, except those points with a circle about them which are derived from blood pooled from two or three fish. Lines were fitted by the method of least squares, and statistical comparisons of these lines are shown in Table 2.

TABLE 2-STATISTICAL COMPARISON OF P_{50} AS A FUNCTION OF pH IN COLD-HISTORY (9°C) AND WARM-HISTORY (24°C) *I. nebulosus,* MEASURED AT BOTH 9°C AND 24°C (SEE FIG. 2)

History	Experiment	Equation	N	Comparison of *a*	Significance of *b*
9°C	24°C	Y= 4.0729-0.3809X	8	t_{16} = 16.5 P<0-001	P<0·01
24°C	24°C	Y = 4.4571-0.4538X	10		P < 0.02
9°C	9°C	Y= 2.0772-0.1818X	9	t_{18} = 3.79 P<0-01	P>0.2
24°C	9°C	Y= 2.1423-0.2152X	11		P<0-01

When the complete oxygen equilibrium curves are compared between each of the four experimental categories (Fig. 3), it can be seen that the direction of shift of the curves during thermal acclimation is toward a conservative position. When the effect of temperature on oxygen affinity is examined (Fig. 4), almost exact similarity is seen between acute measurements on blood from fish with different thermal histories, except that oxygen affinities of blood from cold-history fish are lower at any temperature than those from warm-history fish. Over a period of time, therefore, thermal acclimation minimizes the effect of temperature on the oxygen affinity of the blood.

Erythrocytes from warm-history fish were suspended in plasma from cold history fish, and vice versa. This was done to determine whether or not the observed adjustment was influenced or modified by something in the plasma. Oxygen equilibrium curves were determined (Table 3) and when these data are compared with Fig. 4 it is clear that oxygen affinity is a function of the cells and not the plasma. The factor or factors responsible for modification of oxygen affinity must be localized in the erythrocyte itself.

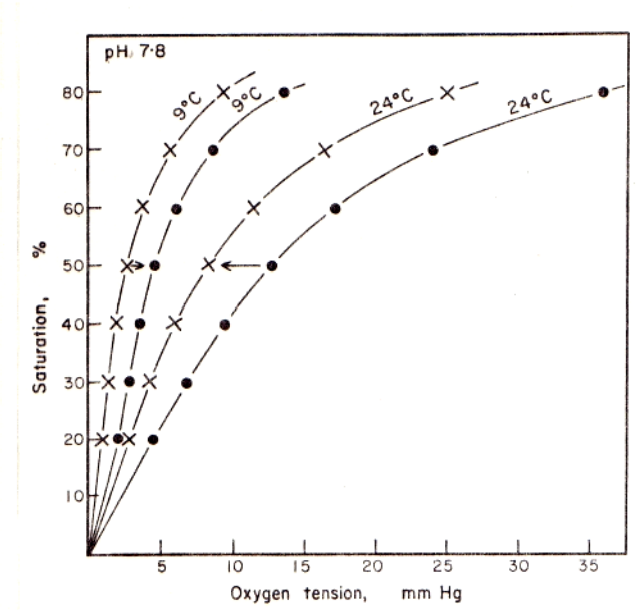

FIG. 3. Oxygen equilibrium curves of warm-acclimated (x 's) and cold-acclimated (closed circles) I. *nebulosus* measured at the temperatures marked on the curves and converted to pH 7.8. Arrows indicate the direction of shift in oxygen affinity which accompanies thermal acclimation. Each point is the average of at least eight values and the statistically significant differences between the P_{50} values are shown in Table 2.

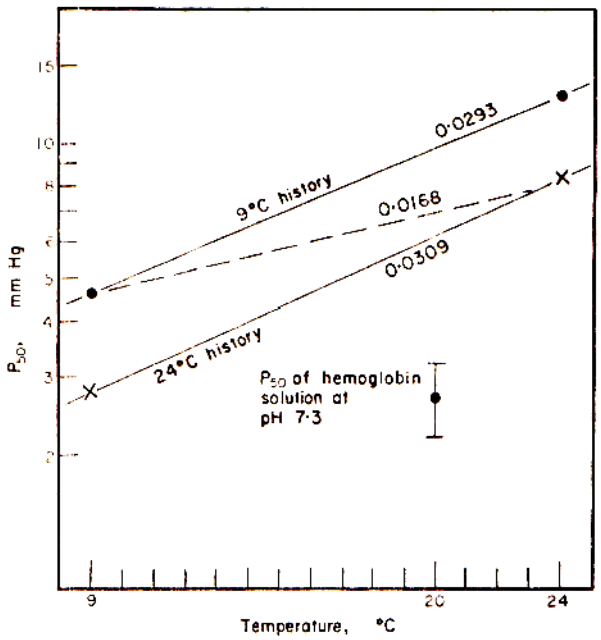

FIG. 4. The effect of temperature on the oxygen affinity of the blood of cold-history (closed circles) and warm-history (x's) *I. nebulosus* Each point represents the average of at least eight determinations and slopes are marked on each line. Statistically significant differences have been demonstrated (Table 2). The range of P_{50}'s for bullhead hemoglobin solutions at 20°C is shown far comparison.

TABLE 3-DETERMINATIONS OF P_{50} (ADJUSTED TO pH 7.8) IN SUSPENSIONS OF CELLS FROM ONE FISH IN THE PLASMA FROM ANOTHER WITH A DIFFERENT THERMAL HISTORY

Thermal history		Experimental temperature	P_{50} (pH 7.8)
Of cells	Of plasma		
24°C	9˚C	24°C	6.8
9˚C	24˚C	24°C	13.5
24°C	9˚C	9˚C	3.3
9˚C	24˚C	9˚C	5·1

When the oxygen equilibrium curves are plotted with oxygen content as a function of P_{O2} and the described changes in oxygen capacity due to thermal acclimation are taken into account, the result is Fig. 5. At first glance it would seem that there is no sign of acclimation. However, if there were no change in oxygen affinity, the oxygen equilibrium curves of blood from warm-history fish measured at 9 and 24°C would be consistently to the right of those for cold-history fish, in response to the change in oxygen capacity, but this is not the case. Quite clearly, the curves from warm-history fish cross from the right to the left of the curves from the cold-history fish measured at the same temperature. It would seem advantageous for the hematocrit to increase in winter as a partial offset to the high oxygen affinity of the blood. On the other hand, it would then seem disadvantageous for the hematocrit to decrease in summer when the fish are most active. The reasons for these changes are unknown.

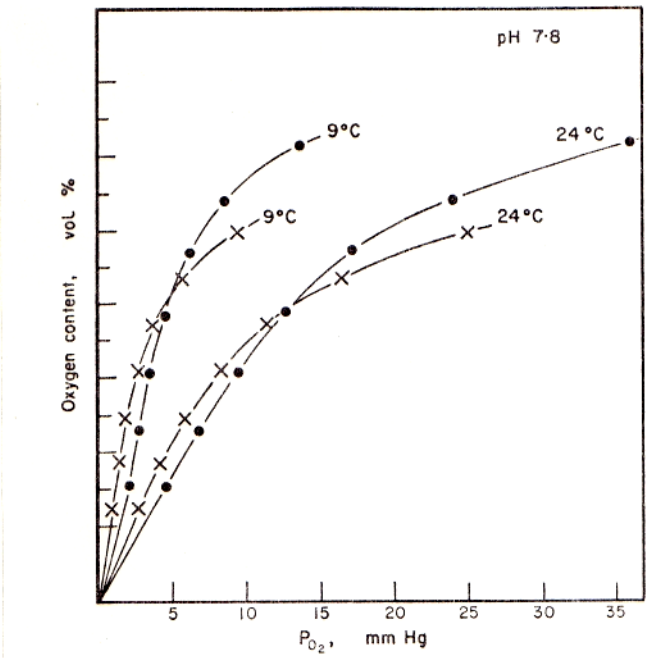

FIG. 5. Oxygen equilibrium curves of blood from cold-acclimated (closed circles) and warm- acclimated (x 's) I. nebulosus at both 9 and 24°C, with oxygen content plotted as a function of oxygen tension

3. Oxygen equilibrium curves of hemoglobin in solution

The oxygen equilibrium curve exhibited by whole blood is a function of the hemoglobin itself and the cellular environment within which it functions. Comparisons of oxygen equilibrium curves of 1-1-5% solutions of hemoglobin from warm- and cold-history *I. nebulosus* were made at 20°C; (Fig. 6). The difference in oxygen affinity seen in whole blood from warm- and cold-history fish does not persist in dilute hemoglobin solutions, and a single curve was fitted to the data. The oxygen affinity of the hemoglobin solutions is much higher than that of the whole blood and this is consistent with data from other fishes (Manwell, 1.957). The P_{50} values for the whole blood of *I. nebulosus* reported by Black (1940) and Haws & Goodnight (1962) at 0 mmHg of CO_2 are much lower than those reported in this paper. Their values are lower even than the P_{50} values for dilute solutions of hemoglobin from *I. nebulosus* (above). Over a range of carbon dioxide tensions from 0-36 mm Hg (no pH data given), Black reported P_{50} to range from1.4-14.0 mm Hg at 15°C. This range is not inconsistent with present data over the pH range considered.

4. Oxygen equilibrium curves plotted according to Hill's equation

This method of representation allows further comparisons of oxygen equilibrium curves of whole blood and hemoglobin solutions. By plotting the logarithm of Y/100- Y (where Y =percentage saturation) against log P_{O2} a straight line is obtained which approximates the oxygen equilibrium curve. The slope of this line at P_{50} (where Y/100- Y = 1.0) is equal to the "sigmoid coefficient" known as *n*. This is considered to be a measure of the interaction between the different oxygen binding sites of the hemoglobin tetramer. When n = 1.0, interaction is zero and the oxygen equilibrium curve is part of a rectangular hyperbola. When values of *n* are greater than about 1.6, the heme-heme interactions are sufficient to produce a noticeably sigmoid oxygen equilibrium curve. The oxygen equilibrium curves obtained from whole blood and solutions of hemoglobin from *I. nebulosus* were plotted according to this method (Fig. 7). The values of *n* for each equilibrium curve are shown in Table 4. As expected from the sigmoid curve exhibited by the hemoglobin solution (Fig. 6), compared with. the hyperbolic equilibria seen in whole blood (Fig. 3), the value of it for the solution is higher

than those from the whole blood. This difference in shape between oxygen equilibria of whole blood and hemolysates has been discussed by Prosser & Brown (1961). Although the differences are small, the curves for cold-history fish measured at both 9 and 24°C have a greater sigmoidal tendency than similar curves for warm-history fish. This is most clearly shown by the curve at 9°C from cold-history fish, in which $n = 1.5$ In both warm- and cold-acclimated fish, oxygen equilibrium curves measured at 9°C have a tendency in the straight-line representation for the slope to increase at lower oxygen tensions. Again, this is particularly noticeable in the cold-history fish and could imply a physiological advantage facilitating unloading at low temperatures.

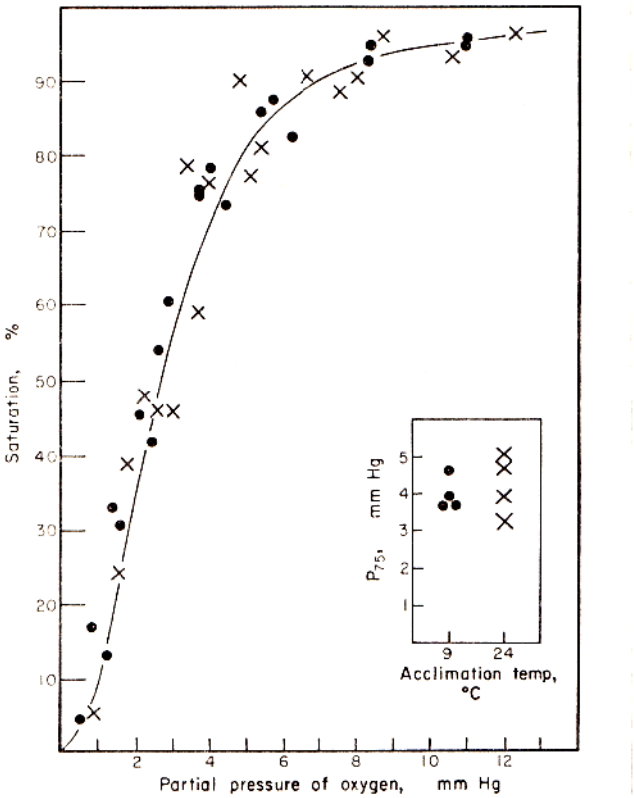

FIG. 6. Oxygen equilibrium curves of 1-1.5% solutions of *I. nebulosus* hemoglobin in saline buffered at pH 7.3 Cold-history (closed circles) and warm-history (x's) fish are compared. Temperature, 20°C. Individual curves from four fish were determined in each group, and each point represents an individual determination. Inset: Comparison of P_{75} values obtained from warm- and cold-acclimated fish.

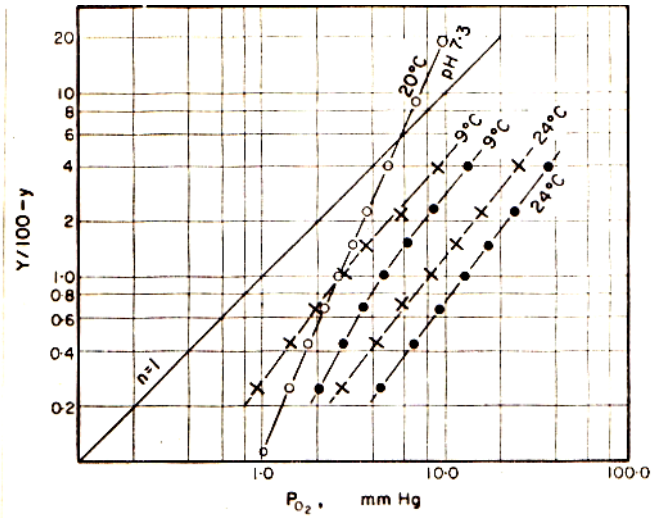

FIG. 7. Oxygen equilibrium curves of whole blood at pH 7-8 (symbols as before) and hemoglobin solutions (open circles) at pH 7.3, plotted according to Hill's equation. The temperature of measurement is marked on each line. Each point is the average of at least eight determinations.

TABLE 4-THE VALUES OF *n* IN HILL'S EQUATION, FOR WHOLE BLOOD AND HEMOGLOBIN SOLUTIONS FROM *I. nebulosus*

Thermal history	Experimental temperature	n	
24°C	24°C	1.23	Whole blood
24°C	9°C	1.25	Whole blood
9°C	9°C	1.50	Whole blood
9°C	24°C	1.32	Whole blood
9°C and 24°C combined	20°C	2.22	Hemoglobin solution

5. Do brown bullheads in natural conditions seasonally adjust the oxygen affinity of their blood?

At various times during the year as fish were available, oxygen equilibrium curves were determined on blood from fish immediately after removal from the lake. A temperature profile was determined at the trap-site whenever fish were collected. There was never any evidence of a thermocline although no midsummer observations were made. Temperature range along the profile varied from 0-2°C from top to bottom, and the determination of the oxygen equilibrium curve was made at the average temperature at the time of collection. In this way, seasonal changes in P_{50} with temperature were compared with the acutely measured effect of temperature on oxygen affinity (Fig. 8). In fish from Dorena Reservoir, oxygen affinities of their blood varied much less than would be expected over the observed temperature range if no seasonal adjustment had occurred. It seems clear that adjustment of oxygen affinity to compensate for temperature changes occurs under natural conditions as well as in the laboratory.

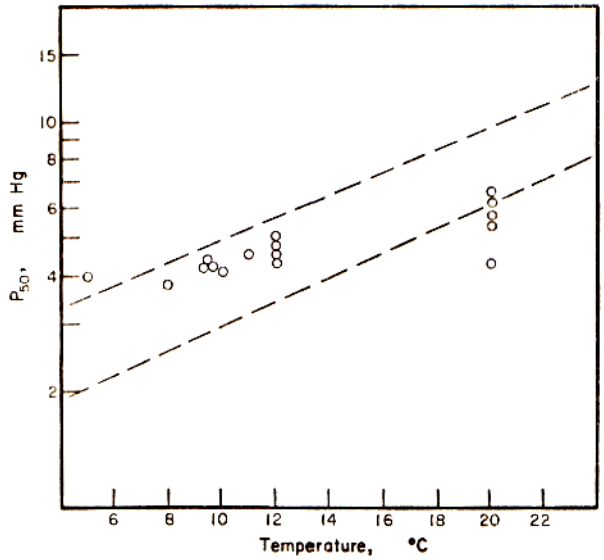

FIG. 8. Field data. P_{50} (at pH 7.8) is plotted against temperature for fish from Dorena Reservoir. P_{50} was measured at the temperature of the water from which the fish were caught.

6. Hemoglobin heterogeneity

The hemoglobins of many teleost species are electrophoretically heterogeneous (Buhler & Shanks, 1959), and this seems even to be the general rule in this group (Wilkins & Iles, 1966). It would seem reasonable that seasonal changes in hemoglobin heterogeneity could effect physiological adjustment. Electrophoresis of hemolysates from *I. nebulosus* on both cellulose acetate and acrylamide gel resulted in the separation of four major components and three (or very occasionally four) minor components. The possibility was considered that this diversity was an artifact caused by fewer hemoglobin tetramers, each dissociating into a mixture of monomers and dimers. By ultracentrifugation of the hemolysate the sedimentation velocity, S_{20w}, was found to be 4.4, a value consistent with those found for tetrameric hemoglobin in other animals. If much dissociation of the hemoglobin into its constituents had occurred, a lower sedimentation velocity would be expected. Furthermore, the shape of the Schlieren peak was

symmetrical, which would be unexpected if the hemolysate contained large proportions of dissociated subunits. In addition, advantage was taken of the molecular sieving abilities of the acrylamide gel, but the relative migration distances of each band in 5% and 10% gels were the same, indicating that the bands represent similar molecular sizes. It was concluded that the multiple bands are not artifacts, and do represent real diversity in the hemoglobin complement of *I. nebulosus.*

TABLE 5- PERCENTAGE COMPOSITION OF EACH ON THE SEVEN HEMOGLOBIN COMPONENTS IN *I. nebulosus*

Component	24°C history (% composition)		9°C history (% composition)		Comparing two histories t_{20}	P
1	2.86	(0.6-8.9)	2.82	(1.2-2.4)	0.054	N. S.
2	8.97	(5.5-19.4)	9.79	(5.6-15.0)	0.541	N. S.
3	13.87	(7.0-20.8)	11.84	(9.2-16.7)	1.243	N.
4	23.68	(18.6-28.6)	26.67	(24.0-33.3)	2.306	0·05 *
5	22.76	(12.9-28.0)	20.11	(15.4-25.3)	1.568	N. S.
6	16.37	(9.7-23.4)	17.79	(11.6-24.2)	0.776	N.S.
7	11.53	(2.3-20.3)	10.99	(5.6-16.3)	0.291	N.S.

Groups of fish with different thermal histories are compared. Mean values are shown, followed by the total range (in parentheses).

* Further analysis of this component by comparing it to components 3 and 5, showed no differences between the groups which are significant.

Analyses were made of the percentage composition of each of the seven consistently present hemoglobin components in twelve warm-history and ten cold history fish (Table 5). Although hemoglobin heterogeneity would seem to provide an ideal mechanism for physiological adjustments, no consistent differences can be seen between warm- and cold-history fish. Similar analyses were also made of the migration distances of each component but again no differences between acclimation groups are apparent.

7. Determinations of ion concentration

It seems clear that the properties of hemoglobin in *I. nebulosus* are not altered by thermal acclimation, so the observed changes in oxygen affinity must be induced by a change in some property of the cellular environment. The dependence of oxygen affinity of hemoglobin solutions on the concentrations of various salts is well known (Antonini, 1965; Riggs, 1965). With this in mind, analyses were made of concentrations of selected ions in hemolysates from cold- and warm-acclimated fish, and in the plasma for comparison (Table 6). Huisman & van Veen (1964) found that inorganic phosphate concentration in the erythrocyte is probably of importance in relation to the ontogenetic changes in oxygen affinity in the chick. However, no differences were found in similar analyses comparing brown bullheads with warm and cold history, so the concentration of $P0_4^{2-}$ ions is probably not of importance in the observed shift in oxygen affinity. In general, oxygen affinity of hemoglobin solutions can be decreased by increasing the concentration of NaCI or KCl in the solution (Antonini, 1965). In whole blood from I. *nebulosus* with a cold history, the oxygen affinity has been shown to be lower than that in warm-history fish. The cold-history fish exhibit considerably lower concentrations of both sodium and potassium in their erythrocytes (Table 6). Hence, this change in ion concentration is in the wrong direction to account for the observed modification of oxygen affinity, according to the studies on hemoglobin in solution. However, the work of Huisman *et al.* (1964) has pointed out that little is known or understood about the modifying effects of salts on oxygen affinity of hemoglobin inside the erythrocyte. At this stage it is unknown what relation, if any, the observed changes in ion concentration bear to the adjustment of oxygen affinity with thermal history.

TABLE 6-THE CONCENTRATIONS OF SODIUM, POTASSIUM. AND CALCIUM IN PLASMA AND ERYTHROCYTE AND INORGANIC PHOSPHATE IN' THE ERYTHROCYTE

	Cold history	Warm history	t	P	Ratio of erythrocyte to plasma	
					Cold	Warm
Sodium (m-equiv. /1)						
Plasma	90.21(14)	117.10(14)	3.79	0.001		
Erythrocyte	26.97(11)	48.42(12)	2.68	0.02	0.30	0.41
Potassium (m-equiv./1)						
Plasma	2.71 (14)	2-78(14)	0,85	N.S.		
Erythrocyte	52.05 (11)	77.12(12)	4.38	0.001	19.21	27.74
Calcium (m-equiv./1)						
Plasma	2.71 (14)	2.23(14)		N.S.		
Erythrocyte	0·1 (11)	0·1 (11)		N.S.		
Inorganic $PO_4$2- (mM/100 ml cells)						
Erythrocyte	0.532(12)	0.491 (13)	1.096			

Comparison is made between warm (24°C) and cold (9°C) acclimated *I. nebulosus.* Mean values are given, with the number of determinations (in parentheses).

DISCUSSION

1. Oxygen equilibrium curves of different species in relation to the environment

The influence of temperature on the oxygen equilibrium curve was studied by Barcroft & Hill (1909), and Krogh & Leitch (1919) were the first to consider this in relation to fishes. Being unaware of the diversity of properties exhibited by hemoglobin in different animals, they considered that hemoglobin would exhibit a very high oxygen affinity at the low temperatures to which many fishes are subjected, and that unloading of the pigment would be inhibited. However, whole blood from a series of fishes had a much lower oxygen affinity at physiological temperatures than they had predicted on the basis of observations on mammalian blood. In fact, they concluded that the loading and unloading properties of blood from carp, eel, pike, cod and plaice are well adapted for function at the natural temperatures and oxygen and carbon dioxide contents at their respective environments. To explain the apparent discrepancy, they suggested that the containment of hemoglobin within the erythrocyte "makes possible the adaptation of the hemoglobin to extremely different respiratory conditions without interfering with the general composition of the blood.

The special adaptation of the blood oxygen affinity to the particular respiratory needs of the species is now generally recognized (Redfield, 1933; Wolverkamp, 1961) and attributed largely to interspecific differences in hemoglobin. The extent of adaptation of the oxygen equilibrium curve of fishes has been explored by many authors (e.g. Willmer, 1934; Black, 1940; Fish, 1956; Grigg, 1967). The oxygen affinity of blood from many species is consistently within a realistic range when measured at a temperature natural for that species although these temperatures range from -1.86 to +30°C. It is now well established that species with different respiratory needs or in different environments have evolved oxygen transport systems whose properties match these demands.

2. Seasonal adjustment of the oxygen equilibrium curve

Considering the fitness of the respiratory properties of the blood for the special needs of a given fish species, it seems reasonable to suggest that a fish living in an environment subject to strong seasonal changes would be at an advantage if it could make adjustments to suit these variable conditions.

In this paper, the effects of temperature have been considered. However, it could be just as important to adjust the respiratory properties of the blood to accommodate changes in other environmental parameters, oxygen saturation for example. Changes in temperature and oxygen saturation could be expected to be most pronounced in fresh waters in the temperate zones. In these waters, low oxygen tensions are most likely to be encountered in the summer, at the same time that higher temperatures tend to decrease the loading ability of the blood. Conversely, in winter, lower temperatures tend to inhibit the unloading of oxygen to the tissues. It would seem that any ability to modify the respiratory properties of the blood in response to seasonally changing environmental demands would confer survival value.

There has been very little work done in this area. Prosser *et al. (1957)* acclimated goldfish to low concentrations of oxygen and found that the critical oxygen tension was lowered accordingly. This result was attributed to increased hemoglobin concentration in the blood of fish acclimated to low saturations of oxygen, even though the differences were not statistically significant and the data are therefore insufficient to sustain this conclusion. The possibility of modification of the oxygen equilibrium curve was mentioned but dismissed. Straub *(1957)* established that *Rana esculenta is* able to modify the oxygen affinity of its blood and so compensate for the effects of temperature. However, Black *et al. (1966)* found no seasonal changes in the oxygen equilibrium curves of *Salvelinus fontinalis.* The present work describes adjustments in the oxygen affinity of the blood in response to thermal acclimation of *I. nebulosus* and is an addition to this topic, but it is clear that more animals should be examined in this respect.

Two properties of blood are of great importance in its role in transporting oxygen, the oxygen capacity of the blood per unit volume and the conditions at which this oxygen can be loaded and unloaded.

Oxygen capacity is related to the concentration of hemoglobin within the red cells and the concentration of the red cells in the blood. In general, oxygen capacity and hematocrit are related linearly in any one species, so that hematocrit can be used as a measure of oxygen capacity once this relationship is established. This would be untrue if, as a result of different experimental treatments, the amount of hemoglobin within red cells or the volume of individual red cells were altered in response to these treatments. Hematocrit of blood from *I. nebulosus* was found to be significantly lowered by a warm (24°C), compared with cold (9°C) thermal history. As has been outlined earlier, it was decided that this lowered hematocrit in warm-history fish is the result of a decrease in red blood cell counts rather than in individual red cell volume. This is of importance in the present context because changes in red cell volume could result in modification in the oxygen affinity of whole blood.

The conditions at which oxygen is loaded or unloaded by the blood are determined by the shape of its oxygen equilibrium curve, its affinity for oxygen and the sensitivity of this to modifying factors such as pH and temperature. In the Introduction and earlier in this section, it was suggested that fish in certain environmental situations would benefit from an ability to modify the oxygen affinity of their blood in compensation for the effects of temperature. As described fully in the Results, it was found that *I. nebulosus* has this ability. This conclusion is based on data gathered in vitro. Some minor changes in the shape of the oxygen equilibrium curve (measured by *n*) were observed during thermal acclimation, but their significance is difficult to evaluate. The sensitivity of oxygen affinity to p or temperature changes was the same regardless of thermal history. However, over the temperature range examined, the oxygen affinity of the blood from warm-acclimated bullheads was consistently lower than that from cold-acclimated bullheads. The problem now is to explain what causes this change in oxygen affinity.

3. The control of oxygen affinity of the blood

When the results in Table 3 are compared with Fig. 4, it seems probable, that the change in oxygen affinity is effected by a factor in the erythrocyte rather than in the plasma. There are two possibilities. :Either the hemoglobin itself could be altered during thermal acclimation, or a change could occur in some other factor in the cytoplasm so that the properties of the hemoglobin are modified. On the basis of results in Table 5 and Fig. 6, it seems clear that the change in oxygen affinity is not the result of a change in the nature of the hemoglobin itself. It must therefore result from a change in the level. of interaction between the hemoglobin and some other factor in the cytoplasm. Higher concentrations of both sodium and potassium were observed in erythrocytes from *I. nebulosus* after warm history compared with cold history (Table 6). This change in concentration is apparently in the wrong direction to account for the observed changes in oxygen affinity, assuming that it is valid to extrapolate from observations on hemoglobin in solution to hemoglobin *in situ.* This assumption may be incorrect, and it is unknown

whether or not the observed changes in ionic concentration bear any relationship to the adjustment of oxygen affinity during thermal acclimation of I. nebulosus.

In the erythrocyte, hemoglobin is in a situation very different from the dilute solution in which it is usually studied. It is at a higher concentration, which is thought to account for some of the differences between oxygen equilibria of whole blood and hemoglobin solutions. It is exposed to concentrations and combinations of salts and other substances which are different from those in study solutions. When several hemoglobin components are present, it is probable that the observed properties are the result of interactions between the components. In fact Huisman *et al.* (1964), studying the oxygen equilibria of hemolysates from adult chickens, found evidence of interactions between the two hemoglobin components. In *I. nebulosus it* may well be that, although the multiple hemoglobin system does not appear to alter during thermal acclimation, by interaction between its members it may contribute to the overall oxygen equilibrium properties of the blood and the potential for adjustment of these properties.

4. Temperature, poikilothermy and homeostasis

Throughout physiological studies the implication is ever present that some degree of homeostasis is advantageous. Because of the effect of temperature. on many rate -functions in biological systems, it is not surprising that the peak of homeostasis is achieved in birds and mammals, in which body temperature precisely regulated. Because of the high specific heat of water and the opportunity for heat exchange at the respiratory surface, aquatic poikilotherms function at near ambient temperature. If this temperature is subject to large fluctuations the many reaction equilibria are being constantly altered and the degree of homeostasis is minimal. In situations where the temperature fluctuations are long term (seasonal), compensations are often made for the effects of temperature so that some way the rate function is modified to lessen the effects of temperature. The acclimation is best known in metabolic compensations for temperature (Bullock 1955). However, in *R. esculenta* (Straub, 1957) and in *I. nebulosus,* adjustments oxygen affinity in response to temperature are analogous to metabolic acclimation in that they represent homeostatic mechanisms with respect to temperature change

Acknowledgements-The author wishes to thank Professor Robert W. Morris and Professor Frederick W. Munz for their advice, assistance and encouragement, and Mr. Ralph Swan of the Oregon State Game Commission for his advice about catching the fish.

REFERENCES

ANTONINI E. (1965) Interrelationship between structure and function in hemoglobin an myoglobin. Physiol. Rev. 45, 123-170.

BARCROFT J. (1914) The Respiratory Function of the Blood. Cambridge University Press London.

BARCROFT J. & HILL A. V. (1909) The nature of oxyhaemoglobin, with a note on its molecular weight. J. Physiol. 39, 411-428.

BLACK E. C. (1.940) The transport of oxygen by the blood of freshwater fish. *Biol. Bull.* Woods Hole 79, 215-229.

BLACK E. C., KIRKPATRICK D. & TUCKER I1. H. (1966) Oxygen dissociation curves of the blood of brook trout (Salvelinus fontinalis) acclimated to summer and winter temperatures. J. Fish. Res. Bd Can. 23, 1-13.

BLATTLER D. P. (1967) A study of the association-dissociation of urease by quantitative electrophoresis. Ph.D. Thesis, University of Oregon.

BOND C. E. (1961) Keys to Oregon Freshwater Fishes. Technical Bulletin 58, Oregon Agricultural Experiment Station, Corvallis.

BUHLER D. R. & SHANKS W. E. (1959) Multiple haemoglobins in fishes. Science 129, 899.

BULLOCK T. H. (1955) Compensation for temperature in the metabolism and activity of poikilotherms. Biol. Rev. 30, 311-342.

FINLEY T. N., LENFANT C., HAAB P., PIIPER J. & RAHN H. (1960) Venous admixture in the pulmonary circulation of anaesthetised dogs. J. appl. Physiol. 15, 418-424.

FISH G. R. (1956) Some aspects of the respiration of six species of fish from Uganda. J. exp. Biol. 33, 186--195.

FRY F. I?. J. (1939) The position of fish and other higher animals in the economy of lakes. The Am. Ass. Adv. Sci. 10, 132-142.

GRATZER W. B. & ALLISON A. C. (1960) Multiple hemoglobins. Biol. Rev. 35, 459-506.

GRIGG G. C. (1967) Some respiratory properties of the blood of four species of Antarctic fishes. Comp. Biochem. Physiol. 23, 139-148.

HAAB P. E., PIIPER J. & RAHN H. (1960) Simple method for rapid determination of an oxygen dissociation curve of the blood. J. appl. Physiol. 15, 1148-1149.

HAWS T. G. & GOODNIGHT C. J. (1962) Some aspects of the hematology of two species of catfish in relation to their habitats. Physiol. Zool. 35, 8-17.
HUISMAN T. H. J. and VAN VEEN J. M. S. (1964) Studies on animal hemoglobins-111. The possible role of intercellular inorganic phosphate on the oxygen equilibrium of the hemoglobin in the developing chicken. *Biochim. biophys. Acta 88,* 367-374.
HUISMAN T. H. J., VAN VEEN J. M. S., DOZY A. M. & NECHTMAN C. M. (1964) Studies on animal hemoglobins--II. The influence of inorganic phosphate on the physico-chemical properties of the hemoglobin of the adult chicken. *Biochim. biophys. Acta* 88, 352-366.
JOHNSTON W., JAMES T. W. & BARBER A. A. (1967) Oxygen binding characteristics of lobster hemocyanin and its subunits. *Comp. Biochem. Physiol. 22,* 261-271.
KIRBERGER C. (1953) Temperaturadaptation der Sauerstoffbindung des Blutes von *Rana esculenta* L. *Z. vergl. Physiol.* 35, 153-158.
KROGH A. & LEITCH I. (1919) The respiratory function of the blood in fishes. *J. Physiol.* 52,288.300.
LENFANT C. & JOHANSEN K. (1965) Gas transport by hemocyanin-containing blood of the cephalopod *Octopus dofleini. Am. J.* Physiol. 209, 991-998.
LINDBERG O. & E RNSTER L. (1956) Determination of organic phosphorus compounds by phosphate analysis. *Methods Biochem. Anal. 3,* 1-22.
MANWELL C. (1957) The respiratory pigments. Ph.D. Thesis, Stanford University. MANWELL. C. (1960) Comparative physiology: blood pigments. A. Rev. Physiol. 22, 191-244.
MANWELL C. (1963) The blood proteins of cyclostomes. A study in phylogenetic and ontogenetic biochemistry. In *The Biology of Myxine* (Edited by BRODAL A. & FANGE R.). Universitetsforlaget, Norway.
PROSSER C. L., BARR L. M., PINC R. D. & LAUER C. Y. (1957) Acclimation of goldfish to low concentrations of oxygen. Physiol. Zool. *30,* 137-141.
PROSSER C. L. & BROWN F. A. (1961) *Comparative Animal Physiology.* W. B. Saunders, Philadelphia.
REDFIELD A. C. (1933) The evolution of the respiratory function of the blood. *Quart. Rev.* Biol. 8, 31-57.
RIGGS A. F. (1951) The metamorphosis of hemoglobin in the bullfrog. J. *gen. Physiol. 35,* 23-40.
RIGGS A. F. (1965) Functional properties of hemoglobins. Physiol. *Rev.* 45, 619--673.
ROUGHTON F. J. W. (1936) The thermo-chemistry of the oxygen-haemoglobin reactionII. Comparison of the heat as measured directly on purified hemoglobin with that calculated directly by the Vant Hoff isochore. *Biochem. J. 30,* 2117-2133.
ROUGHTON F. J. W. & SCHOLANDER P. F. (1943) Micro-gasometric estimation of the blood gases--1. Oxygen. J. *biol. Chem.* 148, 541-550.
SCHOLANDER P. F. & VAN DAM L. (1956) Micro-gasometric determination of oxygen in fish blood. J. *cell. comp. Physiol.* 48, 529--532.
STRAUB M. (1957) Weitere Untersuchungen sur Temperaturadaptation der Sauerstoffbindung des Blutes von *Rana esculenta* L. *Z. vergl. Physiol.* 39, 507-523.
WILKINS N. P. & ILES T. D. (1966) Haemoglobin polymorphism and its ontogeny in herring *(Clupea harengus)* and sprat *(Sprattus sprattus). Comp. Biochem. Physiol. 17,* 1141-1158.
WILLMER E. N. (1934) Some observations on the respiration of certain tropical fresh-water fishes. *J. exp. Biol. 11,* 283-306.
WOLVERKAMP H. P. (1961) The evolution of oxygen transport. In *Functions of the Blood* (Edited by ROBB-SMITH A. H. T.). Academic Press, New York.

Key Word Index-Temperature; oxygen equilibrium curve; teleost; *Ictalurus nebulosus;* temperature adaptation; haemoglobin; erythrocyte; plasma.