Oxygen Affinities and Electrophoretic Patterns of Hemoglobins in Trout and Basses from Virginia*

JACK D. BURKE

Department of Anatomy
Medical College of Virginia, Richmond

Multiple hemoglobins in several different species of fishes were described by using electrophoresis in 1959 by Buhler and Shanks in the United States, Chandrasekhar in India, and Hashimoto and Matsuura in Japan. Consequently, using gene frequency data, variant hemoglobins have been studied in relation to such parameters as proportional changes of hemoglobins with growth in salmon (Hashimoto and Matsuura, 1960), and intraspecific variation in cod and whiting by Sick (1961). This paper is concerned with hemoglobin polymorphism as it is related to interspecific variation in *Micropterus dolomieui*, the smallmouth bass, and *Micropterus salmoides*, the largemouth bass, as well as *Salmo gairdneri*, the rainbow trout, and *Salmo trutta*, the brown trout. Since the brook trout, *Salvelinus fontinalis*, was available, it was also possible to compare the trout hemoglobins generically.

A striking example of distribution of fishes in freshwater is that of trout and catfishes. Trout are ordinarily found in cool, well aerated water having a high oxygen content, but catfishes can be found in shallow, warm water with a low oxygen concentration. A comparison of the results by Irving *et al.* (1941) on trout, with results reported by Haws and Goodnight (1962) for catfishes, shows that hemoglobin affinity for oxygen is greater in catfishes than in trout, when the measurements were made with similar temperatures and carbon dioxide tensions. With other conditions being favorable for reproduction and sustenance, it seems that the affinity of hemoglobin for oxygen is a limiting factor in the distribution of trout and catfishes. This may be a general ecophysiological relationship—even operating at the interspecific level—since it appears to be a factor in the distribution of fishes such as smallmouth and largemouth basses, as well as in trout. In this study is has been found that the affinity of hemoglobin for oxygen is different in basses and trout, and electropherograms on cellulose acetate membranes show hemoglobin polymorphism in the basses as well as the trout.

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Materials and Methods

The smallmouth bass were caught on artificial bait in or near the rapids at the Fall Line of the James River at Richmond, Virginia (mean oxygen content: greater than 7 mg per L). The largemouth bass were taken on both artificial bait and live minnows from different ponds and lakes in east-central counties of Virginia (mean oxygen content: less than 5 mg per L). All fishes were collected in the spring, summer, and fall months of the year. The fishes were weighed on a simple field balance described by Burke (1963), and the oxygen content of the water where the fishes were caught was determined by a modification of the Winkler method with a 10-ml syringe (Burke, 1962a). All of the trout were obtained from the State Trout Hatchery at Marion, Virginia.

Blood was removed from the fishes in the field, or in the laboratory if the transportation distances were short. The procedure of securing blood as described by Burke (1962b) was employed. Essentially, the technique used here is that the posterior part of the operculum was removed, a slit made through the posterior branchial chamber and the pericardium, the heart clipped, and the blood collected in a heparinized pipette as it "welled" into the pericardial cavity.

Hemoglobin solutions for oxygen affinity studies were prepared as follows. The blood was centrifuged, the plasma decanted with a vacuum pipette, and the cells washed three times in 0.11 M NaCl solution with intermittent centrifugation. The cells then were hemolyzed by suspending them in an equal volume of distilled water overnight in a refrigerator. After gentle agitation on a rotator, the suspension was filtered, centrifuged at high speed, and filtered again. The hemoglobin was made up in phosphate buffers with an ionic strength of 0.3 and a pH of either 7.4 or 6.8; all spectrophotometric readings at 640 mμ were made at 25 $\pm$ 1°C. Oxyhemoglobin affinity curves were determined by the spectrophotometric method of Burke and Powell (1962). After equilibration with air, percentages of oxyhemoglobin were determined for various oxygen tensions obtained manometrically using a tonometer, and the following equation: $y/100 = A_r -$

$A_s/A_r - A_o$ where y is percentage oxyhemoglobin, A is absorbance, and r, s, and o represent "reduced" hemoglobin, partially oxygenated hemoglobin, and fully oxygenated hemoglobin, respectively. This procedure was modified from the methods described by Hall (1935), Riggs (1951), Redmond (1955), Rossi-Fanelli and Antonini (1958), and personal communication with Dr. Clyde Manwell of the University of Illinois, Urbana. At least three oxyhemoglobin affinity curves were determined, both at pH 7.4 and 6.8 on pooled blood samples, from two to four fish of each species that were sexually mature. Therefore, the points for each curve shown in figs. 3 to 7 represent mean values.

Before dilution, the hemoglobin solutions prepared for the determination of the oxyhemoglobin affinity curves were also used in spotting for electropherograms. Electrophoresis was carried out in a Gelman electrocab containing a barbital buffer with a pH of 8.6, and an ionic strength of 0.05 μ at 250 V for 1 hour at room temperature. At least three electrophoretic patterns were run on each hemoglobin sample. The patterns were developed on cellulose acetate membranes by staining with bromphenol blue or amido Black 10B, clearing in dilute acetic acid solution, and drying in air.

Results

Using paper electrophoresis, Buhler and Shanks (1959) reported three hemoglobin bands each for rainbow and brook trout, and two bands for largemouth bass. With similar experimental conditions, I was able to confirm their results, but when cellulose acetate membranes were used the hemoglobin patterns were found to resolve more clearly. The hemoglobins in rainbow trout resolved into six bands, four bands in brown trout and three bands in brook trout, as shown in fig. 1. Using free moving boundary electrophoresis, Buhler (1963) reports three distinct hemoglobins for rainbow trout. But Tsuyuki and Gadd (1963) report 16 hemoglobins in rainbow and 15 in brook trout with starch gel electrophoresis. In fig. 2 it is shown that three hemoglobins were found in the smallmouth bass, and four bands characterize the hemoglobins in the largemouth bass.

As shown in figs. 3, 4, and 5, re-

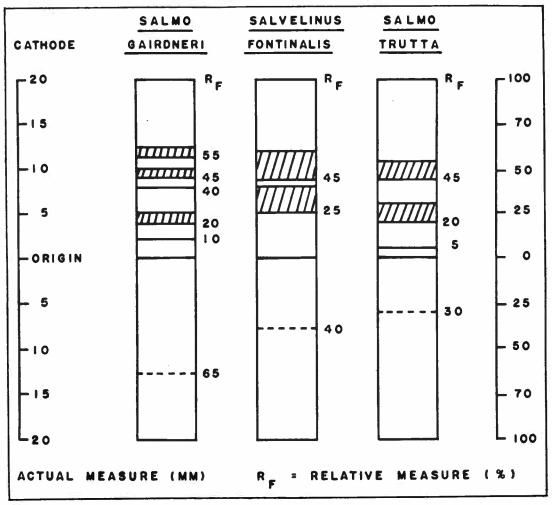

Fig. 1—Electrophoretic patterns of hemoglobins formed on cellulose acetate membranes are shown for trout: rainbow (*Salmo gairdneri*), brook (*Salvelinus fontinalis*), and brown (*Salmo trutta*). Distance (in millimeters) of migration is shown as well as percentage of relative measure (R_F). Except for the origin line, the bands are drawn in relative density to each other.

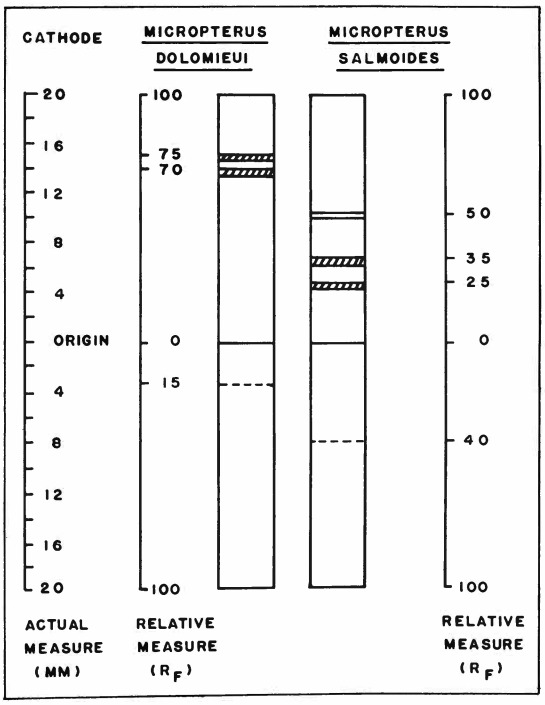

Fig. 2—Electrophoretic patterns of hemoglobins formed on cellulose acetate membranes are shown for the smallmouth bass (*Micropterus dolomieui*) and largemouth bass (*Micropterus salmoides*). Distance (in millimeters) of migration is shown as well as percentage of relative measure (R_F). Except for the origin line, the bands are drawn in relative density to each other.

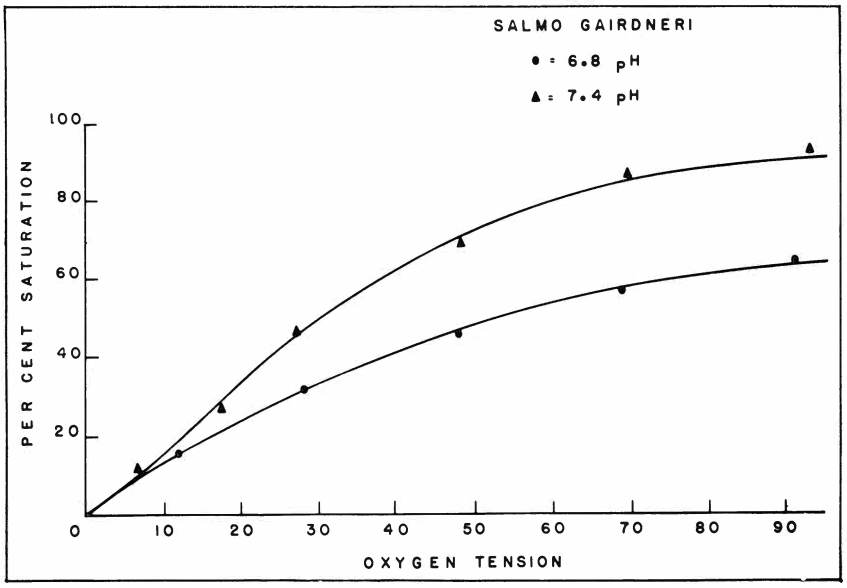

Fig. 3—Oxyhemoglobin affinity curves for rainbow trout (*Salmo gairdneri*) determined at 25°C.

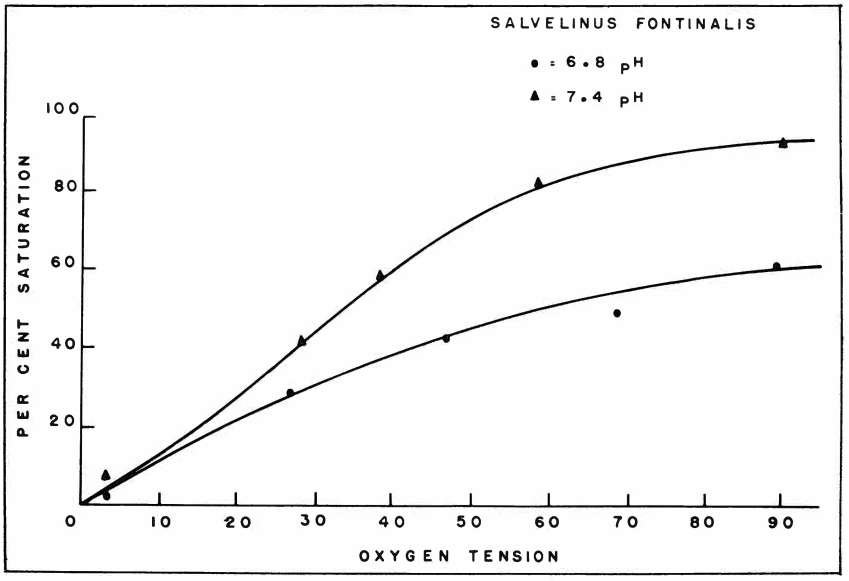

Fig. 4—Oxyhemoglobin affinity curves for brook trout (*Salvelinus fontinalis*) determined at 25°C.

spectively, the oxyhemoglobin affinity curves in rainbow, brook, and brown trout are different. At pH values of 7.4 and 6.8, rainbow trout hemoglobin was 50% saturated at 29 and 53 mm of Hg, whereas in brook trout hemoglobin the $T_{1/2\ sat}$ = 34 and 56, and it was 31 and 45 mm of Hg in brown trout hemoglobin. At the same pH values where Hb = HbO_2, the oxygen tension was 14 and 93 mm of Hg in the smallmouth bass, but 9 and 30 in the largemouth bass as indicated in figs. 6 and 7.

Discussion

Studies by Krogh and Leitch (1919) showed that blood of different fishes was peculiarly sensitive in combining with oxygen in the presence of different concentrations of carbon dioxide. Earlier, Bohr *et al.* (1904) found that the affinity of hemoglobin for oxygen decreases with an increase in carbon dioxide tension, and Christiansen *et al.* (1914) reported the reciprocal effect. The term "Bohr effect" is now used to include both of these conditions. In the

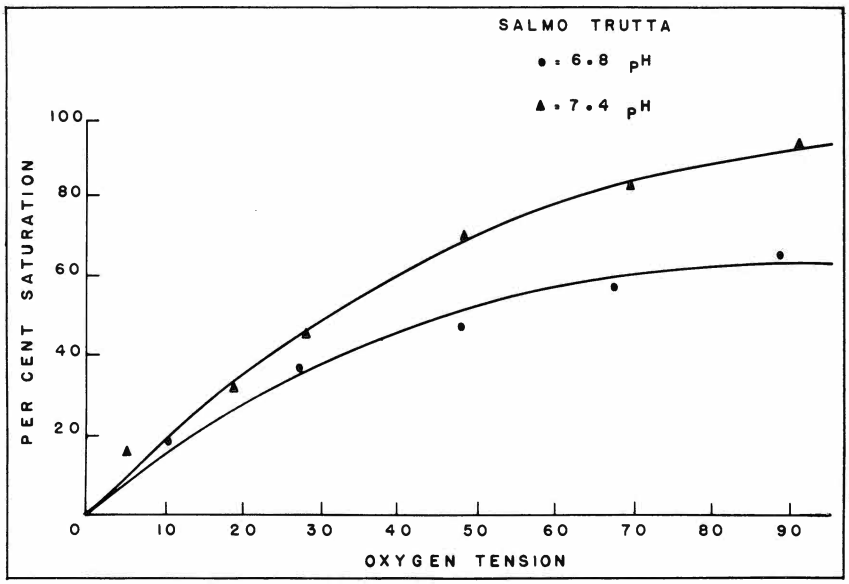

Fig. 5—Oxyhemoglobin affinity curves for brown trout (*Salmo trutta*) determined at 25°C.

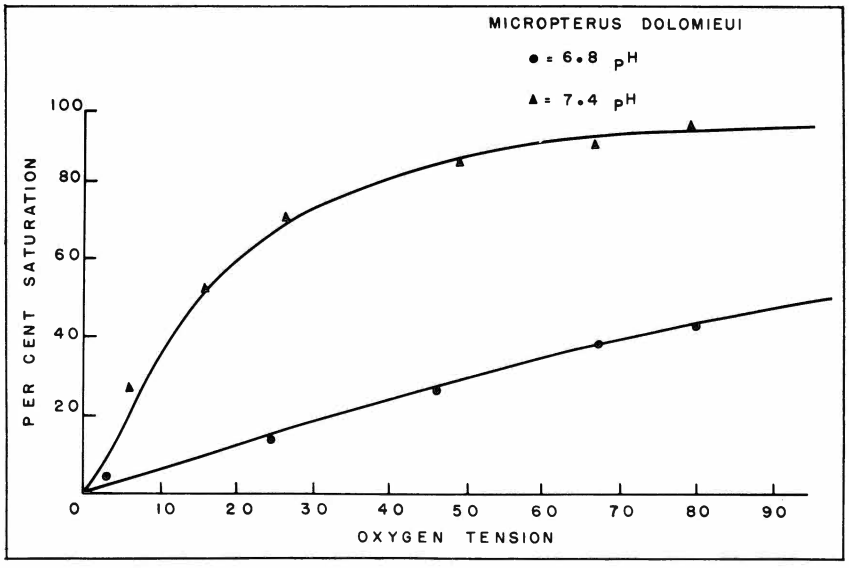

Fig. 6—Oxyhemoglobin affinity curves for smallmouth bass (*Micropterus dolomieui*) determined at 25°C.

work by Irving *et al.* (1941), the Bohr effect was established for various fishes. A comparison of the Bohr effect for the trout results shown in figs. 3, 4, and 5 may be shown by the ratio where $R = \Delta \log p^{50}/\Delta$ pH. For rainbow, brook, and brown trout, "*R*" was calculated to be (−0.44), (−0.36), and (−0.27), respectively. Not only does the rainbow trout have a greater Bohr effect, but its hemoglobin also has a greater affinity for oxygen as indicated previously. These two characteristics may allow the rainbow trout to sustain a greater activity than the brown (or brook) trout, because more oxygen can be unloaded to the tissues as blood pH decreases, and allow it to tolerate warmer water containing less oxygen (Fry, 1957). Interspecific hemoglobin differences between rainbow and brown trout are shown in fig. 1 when the electrophoretic patterns are compared; the generic differences between *Salmo* and *Salvelinus* are also shown.

As shown in fig. 2, interspecific dif-

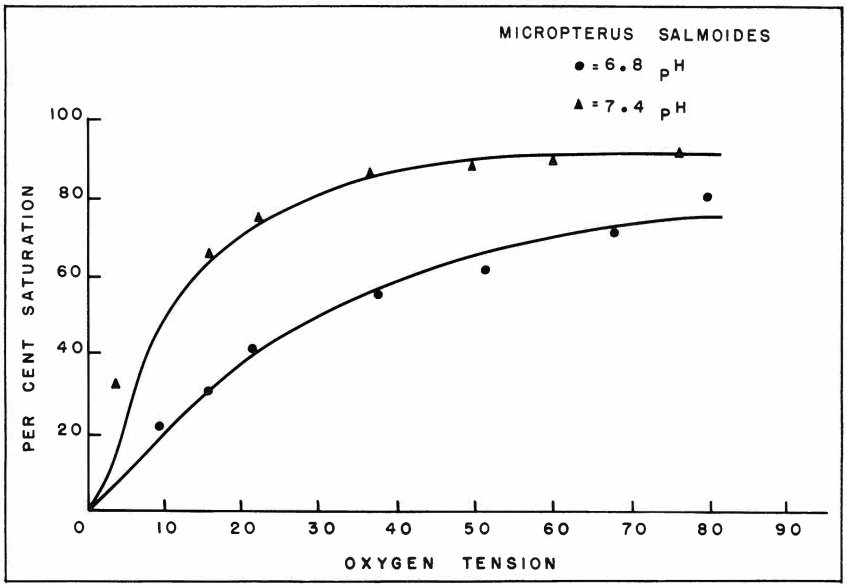

Fig. 7—Oxyhemoglobin affinity curves for largemouth bass (*Micropterus salmoides*) determined at 25°C.

ferences also occur in the hemoglobin patterns of smallmouth and largemouth basses. These differences may be related to the two ecophysiological factors differentiating the trout. First, the hemoglobin of the largemouth bass has a greater affinity for oxygen than does that of the smallmouth bass, as shown above. Assuming this difference to be genetic, it would explain the ability of the largemouth bass to inhabit waters with a lower oxygen content, lower turnover rate, and higher temperature. Conversely, since the hemoglobin of the smallmouth bass has a lower affinity for oxygen, this species would tend to be restricted to those waters where the temperature is lower and the oxygen content higher. Such conditions are associated with mountain lakes and streams, or fast flowing streams with rapids.

The second factor is the Bohr effect; this is, as the acidity increases, the affinity of hemoglobin for oxygen decreases. The Bohr effect difference between the two species is seen when the curves in fig. 6 ($R = -1.37$) are compared with those in fig. 7 ($R = -0.73$). The decreasing oxyhemoglobin affinity in the smallmouth bass is noticeably associated with an increasing Bohr effect. This relation is advantageous when metabolic demands require a high oxygen unloading tension in tissues (Foreman, 1954). It may also explain why it is that, as the acid metabolites form in cellular respiration (Prosser and Brown, 1961), the hemoglobin is buffered at a lower pH and more oxygen is unloaded to the cells. Thus, the smallmouth bass takes on a characteristic fighting habit when hooked, where an increasing supply of oxygen would be necessary to sustain greater activity. This unusual activity is indicated in a statement by Henshall (Harlan and Speaker, 1951) as follows: "He has the arrowy rush and vigor of a trout, the untiring strength and bold leap of a salmon . . . the gamest fish that swims."

Therefore, interspecific hemoglobin differences—as shown by certain parameters such as electrophoretic patterns, oxyhemoglobin affinities, and Bohr effects—are important in establishing the limits, sometimes overlapping, of the habitats which may be occupied by smallmouth and largemouth basses, as well as trout.

Summary

1. Hemoglobin solutions were prepared from pooled samples of blood taken from each of the following species; *Salmo gairdneri*, the rainbow trout; *Salvelinus fontinalis*, the brook trout; *Salmo trutta*, the brown trout; *Micropterus dolomieui*, the smallmouth bass; *Micropterus salmoides*, the largemouth bass.

2. Hemoglobin electrophoretic patterns for each species were developed on cellulose acetate membranes.

3. Oxyhemoglobin affinity curves were determined spectrophotometrically on different hemoglobin solutions from each species.

4. Interspecific differences concerned with hemoglobin electrophoretic patterns, oxyhemoglobin affinities, and the Bohr effect were shown for both trout and basses.

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"Some of my friends have even asserted that a Ph.D. thesis should be the greatest scientific work a man has ever done and perhaps ever will do, and should wait until he is thoroughly able to state his life work. I do not go along with this. I mean merely that if the thesis is not in fact such an overwhelming task, it should at least be in intention the gateway to vigorous creative work. Lord only knows that there are enough problems yet to be solved, books to be written, and music to be composed! Yet for all but a very few, the path to these lies through the performance of perfunctory tasks which in nine cases out of ten have no compelling reason to be performed. Heaven save us from the first novels which are written because a young man desires the prestige of being a novelist rather than because he has something to say! Heaven save us likewise from the mathematical papers which are correct and elegant but without body or spirit. Heaven save us above all from the snobbery which not only admits the possibility of this thin and perfunctory work, but which cries out in a spirit of shrinking arrogance against the competition of vigor and ideas, wherever these may be found!"

Norbert Weiner, *The Human Use of Human Beings: Cybernetics and Society.* Garden City, New York: Doubleday and Company, Inc., 1954, p. 133.