Studies on Ligand Binding to Hemoglobins from Teleosts and Elasmobranchs

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SUMMARY

Hemoglobins from three sharks, the porbeagle (Lamna nasus), the dusky (Carcharhinus obscurus), and the mako (Isurus oxyrinchus), and four bony fishes, the big-eye tuna (Thunnus obesus), the swordfish (Xiphias gladius), the carp (Cyprinus carpio), and the smallmouth bass (Micropterus dolomieu), have been examined by a variety of techniques to determine both their molecular structure and their kinetic and equilibrium ligand-binding properties. For all species multiple hemoglobins were found in the hemolysates. These hemoglobins are tetrameric and exhibit cooperative ligand binding. The main components of the hemolysates from the oceanic species consist of either three or four electrophoretically separable globin chains. However, the hemolysates behave as if they contain only two distinct functional components which appear to be present within the same tetrameric molecule. These two types of ligand-binding sites exhibit different kinetic and spectral properties for the carbon monoxide-binding reaction and for the displacement of oxygen by carbon monoxide. At 20°C the differences between the rates of oxygen dissociation from these two components are largest for the hemoglobins of the mako shark (22.7 s⁻¹ and 1.86 s⁻¹) and those of the swordfish (28.2 s⁻¹ and 4.8 s⁻¹). Similar albeit smaller differences were observed for porbeagle, dusky, big-eye tuna, and carp hemolysates. The carbon monoxide-binding reactions of these hemolysates were also separable into a fast and slow component with all of the fish and shark hemoglobins appearing to interact with organic phosphates with a stoichiometry of 1 mole bound per mole of hemoglobin tetramer. At neutral pH inositol hexaphosphate lowers the rate of CO binding to swordfish hemoglobin by a factor of 10 to 20 but appears to affect only half of the heme sites. Although less dramatically, this organic phosphate also lowers the rate of CO binding to the shark hemoglobins.

The oxygen equilibrium curves of porbeagle and big-eye tuna hemoglobins change shape with temperature, but the concentration of oxygen at 50% saturation for the former is independent of temperature and that of the latter varies only slightly in going from 35°C to 5°C. The basis of this unusually small temperature dependence lies in a differential temperature response of the two types of functional components which appear to be present within the same tetramer. For big-eye tuna hemoglobin, the rate of oxygen dissociation for one of the components is the same at 3°C as it is at 20°C, approximately 50 s⁻¹. For porbeagle hemoglobin, the rate of ligand association to one of the components present in the deoxygenated hemolysate is 36 times smaller at 3°C than at 20°C. Both of these responses produce unusually low affinity species at 3°C.

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Over the past several years Carey and Teal (1) have described the elaborate, vascular countercurrent heat exchangers by which the porbeagle shark (Lamna nasus), the mako shark (Isurus oxyrinchus), and certain species of tuna fish are able to elevate the temperature of their muscle mass above that of the water in which they live. A recent review article about these warm bodied species has discussed the evolutionary advantage, in terms of the increased power available for swimming, which these species obtain from such heat-conserving mechanisms (2). In 1960 prior to this work, Rossi-Fanelli and Antonini studied the oxygen equilibrium curves of a crystallized preparation of hemoglobin from the blue-fin tuna (Thunnus thynnus) which is also warm bodied and found that the oxygen concentration required to saturate half the available heme sites, P₅₀, was virtually independent of temperature (3). As is shown in this paper and has been observed previously by Carey,¹ the P₅₀ values of

whole blood from these other warm bodied fishes and sharks are similarly little affected by temperature.

It was our intention to elucidate the mechanism by which the oxygen equilibrium curves are rendered temperature insensitive. To achieve this end it was necessary to examine various physico-chemical properties of hemoglobins both from warm bodied species and from cold bodied species whose hemoglobins are more normally affected by temperature (4). The molecular weights and the subunit composition of a number of fish and shark hemoglobins were therefore determined, and a variety of experiments was performed to examine the equilibrium and kinetic ligand-binding properties of these two groups of hemoglobins. On comparing the various results obtained with these proteins, we have been able to identify tentatively the kinetic basis of the temperature invariance of the oxygen equilibrium of porbeagle shark and of big-eye tuna fish (Thunnus obesus) hemoglobin.

MATERIALS

Hemoglobins—Specimens of the five oceanic species, the porbeagle (L. nausus), the mako (I. oxyrinchus), and the dusky shark (Carcharhinus obscurus), and the swordfish (Xiphias gladius) and the big-eye tuna (T. obesus) were obtained by research vessels operating out of Woods Hole, Mass. Blood was collected from these animals, the erythrocytes spun down, the supernatant and fluffy layer of white cells were decanted and discarded, and the red blood cells were frozen by adding them dropwise to liquid nitrogen. The frozen pellets were maintained under either liquid nitrogen or Dry Ice until used. For use, a portion of the frozen sample was thawed, and the red blood cells lysed by the addition of distilled water. The stroma was removed by centrifugation at 10,000 rpm for 15 min. In most cases the resultant hemolysate had a cloudy appearance, and both phosphate buffer (approximately 0.1 M phosphate, pH 7.0 buffered per 50 ml of hemolysate) and several hundred milligrams of Hefyo Super-Cel (Fischer Scientific Co.) were added to the solution, which, after standing for 30 min, was centrifuged at 15,000 rpm for 30 min. In all instances this procedure cleared the hemolysates. For those studies in which the phosphate content of the reactant solutions was not carefully controlled this hemolysate was used as the stock Hb022 preparation. For experimentation requiring hemoglobin free of organic phosphates and other small molecules, the hemolysates were passed over a column (6 x 55 cm) of G-25 Sephadex which was equilibrated with 0.05 M bis-tris (Aldrich) buffer, pH 7.0, containing 0.1 M NaCl.

In a number of the later experiments with these hemolysates the formation of methemoglobin at levels between 10 and 30% proved troublesome. This methemoglobin was reduced with dithionite (obtained from Hardman and Holden, Miles Platting, Scientific Co.) were added to the solution, which, after standing 30 min, was centrifuged at 15,000 rpm for 30 min. In most instances this procedure cleared the hemolysates. For those studies in which the phosphate content of the reactant solutions was not carefully controlled this hemolysate was used as the stock Hb022 preparation. For experimentation requiring hemoglobin free of organic phosphates and other small molecules, the hemolysates were passed over a column (6 x 55 cm) of G-25 Sephadex which was equilibrated with 0.05 M bis-tris (Aldrich) buffer, pH 7.0, containing 0.1 M NaCl.

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The carp (Cyprinus carpio) and the lamprey (Petromyzon marinus) were obtained from tributary streams of Lake Cayuga, Ithaca, N. Y. in June 1971. The former were bled from the caudal vein, and the latter by decapitation. The smallmouth bass (Micropterus dolomieu) were obtained from tributary streams of Lake Cayuga, N. Y., and used within 1 month of its preparation. Stripping away of small molecules was accomplished as previously described.

Solutions—Saturated solutions of CO and O2 were obtained by bubbling buffers or distilled water with the pure gas for 10 min. Dilutions were made into solutions which had been degassed by bubbling with N2. In experiments not requiring stripped hemoglobin, the buffers used were phosphate-citrate at pH 5.6, phosphate between pH 6.0 and 8.0, and borate at pH 9.0. In experiments with stripped hemoglobin, bis-tris buffers were used at pH 6.0 and 7.0, and Tris-HCl buffers at pH 8.0 and above. ATP, 2,3-diphosphoglycerate, and 1H+ were obtained from Sigma, and solutions were made up as previously described.

METHODS

Electrophoresis—Starch block electrophoresis of the hemolysates of the oceanic species at pH 9.1 was accomplished by the method of Poulik (6), with staining for heme by a mixture of 1% benzidine in 10% acetic acid containing several drops of 30% hydrogen peroxide. Polyacrylamide gel electrophoresis (7) was performed with a Buchler Polyacrylamid apparatus; aqueous gels at pH 9.1 were constructed with a 2.5% stacking gel and a 5% running gel. The hemoglobins were applied as cyanomethemoglobin which had been prepared by treating the hemolysates consecutively with potassium ferricyanide and potassium cyanide, both in slight molar excess to the heme concentration.

Six molar urea gels were prepared according to Reisfeld and Small (8); however, no stacking gel was employed. Globin was prepared with cold acid acetone (9), and 50 to 100 μg of these globin preparations were applied per tube. After electrophoresis, the gels were soaked in 5% trichloroacetic acid-5% sulfosalicylic acid to denature the proteins, which then were stained with Coomassie blue. Finally sodium dodecyl sulfate gels were run as described by Shapiro et al. (10). From this procedure an estimate of the molecular weight of the various globin components of these hemolysates was made by comparing the Rf of the protein under investigation with the Rf values of known standards as described by Weber and Osborn (11).

Ultracentrifugation—A Beckman model E analytical ultracentrifuge equipped with absorption scanning accessories was employed. Sedimentation equilibrium was obtained by centrifugation at 20,000 rpm overnight at 20°C, and the scanning attachment yielded a record of absorbance versus radial distance from the center of the rotor.

Rapid Mixing—The stopped flow apparatus has been described in detail by Gibson (12) and was equipped with on-line data acquisition capability as described by DeSa and Gibson (13).

Flash Photolysis—The flash apparatus used in these studies has been described by Parkhurst and Gibson (14) and Gray (19). In all cases the reactions were carried out at 22 ± 1°C. Titrations with organic phosphates were accomplished with the use of a 10-μl Hamilton syringe to make consecutive 2-μl additions of a concentrated stock solution into a cuvette whose total volume was 1.3 ml. At the end of these titrations the absence of oxygen was checked by adding several crumbs of dithionite and repeating the last photolysis experiment. In none of the experiments reported did the addition of dithionite alter the time course of the carbon monoxide recombination reaction.

Determination of O2 and CO Partition Coefficient—A solution equilibrated with 1 atmosphere of oxygen at pH 6.2 in 0.1 M
phosphate buffer containing 64 μM porbeagle hemoglobin and a solution of equal heme concentration containing 117 μM CO and 70 μM O₂ were prepared. The spectrum of each of these two solutions and of various mixtures of the two was then measured between 500 and 650 nm with a Cary 14 spectrophotometer. The percentages of HbOt and HbCO in each solution were calculated and these data were used to determine the partition coefficient, M (16).

Oxygen Equilibria—These curves were determined on whole blood samples with a modified version of the apparatus previously described by Duvelleroy et al. (17). The main difference was that instead of sliding a chamber containing deoxygenated blood under one containing O₂ to start the experiment, the deoxygenated blood was in a chamber which included a gas phase of pure N₂ and the experiment was initiated by flushing the vessel with O₂. In a comparison kindly arranged by C. Tung, the two versions of the instrument were used to analyze the same sample of human blood, and identical P₅₀ values were obtained. All oxygen equilibrium data are presented here in the form of Hill plots (18).

Data Analysis—Analysis of the kinetic data in terms of two independent components was obtained by fitting the observed time courses to the sum of two exponentials

$$y = A_{f} e^{-rt} + A_{s} e^{-st}$$

under conditions where the concentration of ligand was at least four times the total concentration of heme (pseudo-first order conditions). Fits were made with an iterative least squares program in a PDP 8/S computer. A notation for the reaction of hemoglobin with various ligands has already been described (19). We are using k and l for the oxygen and carbon monoxide reactions, respectively. These symbols are primed for association reactions (e.g. k' and l') and unprimed for dissociation reactions. The fitting programs for the replacement reactions (see Table III) have been thoroughly discussed (20). The subscripts f and s refer respectively to the fast and slow process.

RESULTS

Molecular Weight Determinations

Electrophoresis—Unlike many mammalian hemolysates (20), all those of fishes and sharks so far examined contain several electrophoretically separable hemoglobins (21). This heterogeneity has already been established for the carp (22), the lamna-py (23), and the big-eye tuna (24), and as shown in Fig. 1 none of the hemolysats from the other oceanic species proved an exception when examined by starch block electrophoresis at pH 9.0. Patterns essentially similar to these were also observed with a polyacrylamide aqueous gel system at pH 9.1. On both supports the big-eye tuna, the swordfish, and the mako shark hemolysates were observed to have two major components, whereas the dusky and the porbeagle hemolysates have three.

The molecular weights of the subunits of these hemoglobins were investigated with the use of disc gel electrophoresis techniques with runs being made at pH 9.0, with the gel and the upper bath containing 1% sodium dodecyl sulfate. The procedure allows, as described under “Methods,” the estimation of molecular weight. A standard curve with 10 pure proteins had been prepared at Cornell and gave a linear relationship for log MW versus RF, for a molecular weight (MW) range from 10,000 to 70,000. In Table I the RF values observed for globins prepared from the hemolysates of the fishes and sharks are given along with the MW calculated from the standard curve. In all cases the subunit molecular weights are approximately equal to those of other globins (25). Only the swordfish showed more than one band when stained for protein with Coomassie blue, and in this case the band of protein corresponding to a MW of 18,800 appeared to stain more darkly.

A further experiment was then performed in which electrophoresis of the cyanomet form of the hemolysates (cyanomethe-
moglobin) was first carried out in an aqueous gel system, the major band excised from the gel, protein eluted from the band with water, and then globin, prepared from this eluent by cold acid acetone treatment (9) was run on a 6 M urea gel. In these experiments the major hemoglobin component of the porbeagle hemolysate produced four weak bands, while those of the mako, the dusky, and the swordfish gave three bands, one of which stained more darkly than the other two. Human hemoglobin gave two strongly staining bands under these conditions, and for some undiscovered reason, the major hemoglobin component of the big-eye tuna hemolysate demonstrated no distinct banding but only a severely smeared pattern. (Repetition of this entire experiment yielded equivalent results.)

Ultracentrifugation— Hemolysates from the five oceanic species were examined at pH 7.0 in 0.1 M phosphate buffer equilibrated with 1 atmosphere of CO, at 2 and 20 μM heme by sedimentation equilibrium techniques. The data for the dusky shark hemolysate are shown in Fig. 2 as plots of the log of absorbance versus the square of the distance from the center of the rotor. The straight lines obtained at both heme concentrations indicate that the solutions were homogeneous with respect to the molecular weights of those species which absorbed light at the observing wavelengths. The slopes of the lines gave a molecular weight of 59,480 at 20 μM heme and 55,060 at 2 μM. These molecular weights are consistent with those of a tetramer whose subunit molecular weight is that given by the data in Table I, which was obtained by disc gel electrophoresis in sodium dodecyl sulfate. Table II gives the values of the molecular weights for porbeagle, dusky, mako, swordfish, and big-eye tuna hemoglobin, and in all cases, the linearity of the plots of the log of the absorbance versus the radius squared and the values for the slopes indicate that these hemolysates contain homogeneous solutions of hemoglobin tetramers. Comparing these results with the data obtained by running globin preparations of the major electrophoretic components on urea gels, it would appear that these tetramers have three to four distinct subunits, a structure even more complex than the αβ2 structure of mammalian hemoglobins. Similar subunit heterogeneity has recently been reported for the hemoglobins of the salmon, Oncorhynchus keta (20). In this species as appears to be the case with the major component of the porbeagle hemolysate some of the intact tetramers consist of four different globin chains.

Oxygen Dissociation

Olson et al. (20) have shown that detailed analysis of the time course for the replacement of oxygen from saturated mammalian hemoglobins by carbon monoxide requires the recognition of two distinct ligand-binding sites, the α and β chains. Evidence for at least two distinct functional units was also observed in the CO replacement reactions for the oxygenated fish and shark hemoglobins. The curve marked A in Fig. 3 shows the kinetic profile of the replacement reaction obtained by mixing porbeagle oxyhemoglobin with buffer equilibrated with 1 atmosphere of CO. The time course is biphasic and can be represented accurately by the sum of two exponentials (Equation 1) with 53% rapidly reacting material. This replacement reaction was carried out at several oxygen concentrations, and the two rate constants at each O2 concentration were determined by fitting the observed curves to Equation 1. Plots were then constructed of the reciprocal of the apparent first order rate constants versus O2 concentration. As was shown by Gibson and Roughton (27), such plots yield straight lines which have a y intercept equal to the reciprocal of the rate constant for the dissociation of the first O2 molecule from fully saturated oxyhemoglobin (k). The slope of these plots is equal to k'/k12 (CO), where k' and P are the association constants for the reaction of the last molecule of O2 and CO, respectively, with tetrameric hemoglobin. By means of this procedure, the values of k and k1 for porbeagle hemoglobin at pH 6.0 were determined to be 13 and 1.5 s−1, respectively. These kinetic parameters for the seven species were obtained according to the methods of Olson et al. (20), and the results are shown in Table III.

Of all of the hemolysates examined only that of the small-mouth bass reacted homogeneously. The 12-fold difference in oxygen dissociation rates for the fast and slow components in
from CO-O₂ replacement experiments apply only to the last step in ligand binding (\(\text{Hb}_xX + X \rightleftharpoons \text{Hb}_xX_2\)) since they are determined under conditions where the hemoglobin molecules contain a minimum of three bound ligands (20, 27). The reaction of oxyhemoglobin with dithionite is more complicated (28) and exhibits a time course which reflects not only the rate of release of the first oxygen molecule but also the rate of release of subsequent molecules from the partially saturated intermediates that are generated during the reaction. In the limiting case where the rates of dissociation from all partially saturated intermediates are much larger than the rate of dissociation from saturated tetrameric oxyhemoglobin, the observed rate for the dithionite reaction will be 4 times the rate of dissociation from \(\text{Hb}_4(\text{O}_2)_4\) and, in the absence of intramolecular heterogeneity, the time course will be exponential. If intramolecular subunit heterogeneity does occur, the initial rate for this limiting case will be \(2(k_f + k_s)\) and the amplitudes of the fast and slow phases will be equal to \(k_f/(k_f + k_s)\) and \(k_s/(k_f + k_s)\), respectively. For the case of porbeagle hemoglobin, the time course would still be expected to be closely exponential since the amplitude of the slow phase would comprise only 6% of the total reaction.

If the two oxygen dissociation rates seen for porbeagle hemoglobin in curve A of Fig. 3 were the result of functionally distinct tetramers (intramolecular heterogeneity), the reaction of the oxygenated hemolysate with dithionite would also exhibit a markedly biphasic character, under all conditions, with the amplitudes of the two phases being approximately equal to those observed in the replacement reactions. As shown by curve B in Fig. 3, the dithionite reaction of porbeagle hemoglobin is not biphasic but, rather, exhibits a virtually homogeneous time course not unlike those observed for mammalian oxyhemoglobins. The lag observed at the beginning of the reaction represents the time required for the dithionite to consume all of the free oxygen present. Thus, the two rates observed in the replacement reaction, \(k_f\) and \(k_s\), must arise from heterogeneity within the same tetrameric molecule. Further, the rate of the first order portion of the dithionite reaction is approximately equal to \(2(k_f + k_s)\) indicating a substantial amount of cooperativity for the dissociation of oxygen from porbeagle shark hemoglobin.

By means of lower heme concentrations the carbon monoxide replacement reaction for porbeagle oxyhemoglobin was followed at a variety of wave lengths in the Soret absorption region, and the difference spectra of the fast and slow components were determined. As shown in Fig. 4, the position and relative intensities of these spectra are nearly identical with those obtained for human hemoglobin (20), except that the spectrum of the component which has the more rapid \(O_2\) dissociation velocity here resembles the spectrum, in human hemoglobin, of the \(\alpha\) chain which has in all mammalian hemoglobins the lower \(O_2\) dissociation velocity. This correspondence of the \(\alpha\) chain type difference spectrum with the rapid \(O_2\) dissociation rate was observed with all three shark hemolysates. In contrast, the component which had the slower \(O_2\) dissociation velocity in telost hemoglobins always had the \(\alpha\) chain type difference spectrum.

Another difference between the fishes and the sharks is the dependence of the \(O_2\) dissociation rates on pH. From Table III it is apparent that there is no significant difference between the replacement reaction at pH 6.0 and at pH 7.0 for the porbeagle hemolysate. A similar pH independence was observed with the dusky and the mako hemolysates, for which the time course of the CO replacement reaction with an air-saturated solution of oxyhemoglobin at pH 6.0 was superimposable with the time

### Table III

<table>
<thead>
<tr>
<th>Species</th>
<th>pH</th>
<th>Fast k₇</th>
<th>(k_f/k_s)</th>
<th>(l_f/k_f)</th>
<th>(l_s/k_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Sharks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porbeagle</td>
<td>6.0</td>
<td>52</td>
<td>13.0</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>54</td>
<td>13.5</td>
<td>0.15</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>49</td>
<td>8.5</td>
<td>1.29</td>
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</tr>
<tr>
<td>Mako</td>
<td>7.0</td>
<td>54</td>
<td>22.7</td>
<td>0.14</td>
<td>1.86</td>
</tr>
<tr>
<td>Dusky</td>
<td>7.0</td>
<td>50</td>
<td>17.7</td>
<td>0.16</td>
<td>5.33</td>
</tr>
<tr>
<td><strong>B. Fishes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big-eye tuna</td>
<td>7.0</td>
<td>50</td>
<td>42.8</td>
<td>0.19</td>
<td>15.3</td>
</tr>
<tr>
<td>Swordfish</td>
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<td>58</td>
<td>28.2</td>
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<td>4.88</td>
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<td>Carp</td>
<td>7.0</td>
<td>44</td>
<td>5.9</td>
<td>0.14</td>
<td>2.64</td>
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<tr>
<td>Smallmouth bass</td>
<td>7.0</td>
<td>100</td>
<td>15.4</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

the mako hemolysate is the largest so far reported for any hemoglobin sample (20). It is noteworthy that, even though these hemolysates have multiple hemoglobin components, the replacement reaction can be fitted successfully by assuming that, kinetically, there are only two types of functional heme sites, possessing different rates of oxygen dissociation. Further, these two ligand-binding groups are present in nearly equal amounts. In those experiments then, the gross electrophoretic heterogeneity notwithstanding, the replacement reactions of the fish and shark hemolysates resemble those of mammalian hemoglobins in which two kinetic components are also observed and have been identified with the \(\alpha\) and \(\beta\) subunits (20).

The various constants \((k_s, k_f, l_f/k_f,\) and \(l_s/k_s)\) obtained...
course of the same reaction carried out at pH 7.0. However, it is well known that the $O_2$ affinities of many teleost hemolysates decrease markedly as the pH is lowered through this region (29-31). Noble et al. (22), for example, have recently shown with carp hemolysates that the $O_2$ dissociation rate at pH 5.6 is substantially greater than it is at pH 7.0.

Because of the large differences in $k_f$ and $k_s$ for porbeagle hemoglobin, it was expected that the partition of the fast and slow components between $O_2$ and CO might also differ and, therefore, that the spectra of solutions containing varying proportions of HbCO and HbO2 would lack an unique isosbestic point. If this were true, a plot of $(O_2)/(CO)$ versus $(HbO2)/(HbCO)$ would yield a curve instead of a straight line. Indeed, when such spectra were prepared, an initial isosbestic point at 574 nm was observed at high percentages of HbCO, and this intersection point moved progressively to shorter wave lengths (finally to 571.5 nm) as the percentage of HbCO decreased. These data plotted as $(HbO2)/(HbCO)$ versus $(O_2)/(CO)$ are shown in Fig. 5. The best approximation to the data points is given by the smooth curve which was calculated assuming that there are two independent species, distributed in a 50:50 fashion, one with a partition coefficient, $M$, equal to 18.5 and another with an $M$ value of 145. The ratio of these partition coefficients, 7.8, is nearly the same as the ratio of $k_f:k_s$ at pH 6.0, which is 8.7.

**Carbon Monoxide Binding**

Gray and Gibson (32) have observed differential rates of CO binding to the $\alpha$ and $\beta$ chains within mammalian hemoglobins. These differences were only apparent in the presence of phosphate compounds at pH values less than 8.0. The shark and fish hemolysates investigated here also exhibit spectral and kinetic heterogeneity in the CO binding reaction. However, the factors which produce this behavior are not as easily classified. As shown in Fig. 6, the time course of the reaction of CO with stripped dusky hemoglobin in 0.05 M bis-tris, pH 7.0, was observed at three different wave lengths, 432 nm where the entire reaction is obtained (assuming the components contribute equal spectral weights at this maximum in the difference spectrum) and at wave lengths on either side of the 425 nm isosbestic point. On the shorter wave length side of the isosbestic point the reaction is rapid, but on the longer wave length side of isosbestic point and at 432 nm the reaction is slower and slightly accelerating. The addition of inositol hexaphosphate affects the rate of the reaction on either side of the isosbestic point but exerts its greatest effect on the slow component (Fig. 6B). Furthermore, IHP increases the apparent acceleration in the composite time course at 432 nm. In contrast to dusky hemoglobin, spectrally homogeneous behavior is observed for the mako hemolysate in the absence of phosphates, and although the composite reaction at 432 nm for mako hemoglobin in the presence of IHP is closely exponential, at the wave lengths near the isosbestic points the reaction is clearly separated into a rapid and a slow phase indicating the presence of intramolecular subunit heterogeneity.

The kinetic characteristics of carbon monoxide binding to the shark hemoglobins are summarized in Table IV. For each species, spectral heterogeneity could be demonstrated, but with the exception of the stripped porbeagle hemolysate, the overall time courses measured at 432 nm were either accelerating or exponential. Without the use of extensive fitting procedures it is impossible to estimate the rates of CO binding to these spectral components, which are presumed to represent functionally...
distinct chains within the same tetramer, and thus, only initial rates for the composite curves are given in Table IV. In the case of stripped porbeagle hemoglobin, the time courses were distinctly biphasic and could be represented by the sum of two exponentials (Equation 1).

Similar phenomena were also observed with teleost hemolysates, except IHP and low pH generally only affected half of the heme sites. For instance, with stripped swordfish hemoglobin at pH 7.0 where the over-all reaction with CO was accelerating, it was possible to separate the reaction into a rapid and a slow phase on, respectively, the short and long wave length side of the 425 nm isosbestic point. At lower pH or in the presence of IHP the time course was dramatically altered; however, only 50% of the heme sites were affected. Again, all the characteristics of these reactions of teleost hemolysates with CO are described in Table V. Of these teleost hemolysates only the carp hemoglobin reacted homogeneously under all conditions, in agreement with previous studies by Noble et al. (22).

**Flash Photolysis**

In order to estimate the stoichiometry of IHP binding carbon monoxide recombination reactions were carried out by flash photolysis techniques which allowed multiple, consecutive additions of IHP to the same hemoglobin sample. After each addition of IHP, the carbon monoxide hemoglobin was photolyzed and the recombination reaction monitored. As more and more IHP was added, the reaction developed an increasing amount of slowly reacting hemoglobin. The maximum amount of slowly reacting material was obtained with 10 μM IHP for a 40 μM solution of porbeagle carbon monoxide hemoglobin, that is a stoichiometry of 1:4, in agreement with other studies on tetrameric hemoglobinins (32). A similar experiment with swordfish hemo-

**Table IV**

**Rate of carbon monoxide binding to shark hemoglobins**

CO-binding experiments with the unstripped hemolysates, stripped hemolysates, and stripped hemolysates plus 300 μM inositol hexaphosphate were carried out in 0.05 M bis-tris or 0.05 M borate (pH 9.0) buffers. Time courses which were neither homogeneous nor accelerating were fitted to Equation 1 yielding values of $\tau_f$ and $\tau_s$. Reaction solutions contained 3 μM deoxy-hemoglobin and 40 μM CO after mixing, and observations were made at 432 nm through a 2-cm path length cell in the stopped flow apparatus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Condition</th>
<th>$\tau_f$</th>
<th>$\tau_s$</th>
<th>Fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porbeagle</td>
<td>pH 7.0, stripped</td>
<td>2.48</td>
<td>0.55</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>pH 7.0, plus IHP</td>
<td>1.13</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, stripped</td>
<td>1.88</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Mako</td>
<td>pH 7.0, stripped</td>
<td>2.62</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Dusky</td>
<td>pH 7.0, stripped</td>
<td>2.62</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

**Table V**

**Rates of carbon monoxide binding to fish hemoglobins**

CO-binding experiments with stripped hemolysates and stripped hemolysates plus 300 μM IHP were performed under conditions identical with those described for the shark hemolysates in Table IV.

<table>
<thead>
<tr>
<th>Species</th>
<th>Conditions</th>
<th>$\tau_f$</th>
<th>$\tau_s$</th>
<th>Fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swordfish</td>
<td>pH 6.0, stripped</td>
<td>2.18</td>
<td>0.46</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>pH 6.0, plus IHP</td>
<td>2.72</td>
<td>0.17</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>pH 7.0, stripped</td>
<td>3.48</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, plus IHP</td>
<td>2.28</td>
<td>0.29</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>pH 9.0, stripped</td>
<td>3.90</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>plus or minus IHP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big-eye tuna</td>
<td>pH 6.0, stripped</td>
<td>2.07</td>
<td>0.14</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>pH 6.0, plus IHP</td>
<td>2.48</td>
<td>0.11</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>pH 7.0, stripped</td>
<td>3.16</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, plus IHP</td>
<td>3.03</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Smallmouth bass</td>
<td>pH 6.0, stripped</td>
<td>2.33</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, plus IHP</td>
<td>1.88</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, stripped</td>
<td>2.18</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Carp</td>
<td>pH 6.0, stripped</td>
<td>1.87</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 6.0, plus IHP</td>
<td>1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, stripped</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.0, plus IHP</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
globin again gave a stoichiometry of 1:4, even though, in this case, only 43% of the sample became slowly reacting. That IHP does indeed interact with all of the swordfish tetramers is suggested by the data in Table V. With swordfish hemoglobin the presence of IHP at pH 6.0 not only decreased the slow rate of ligand binding but it actually increased the rapid rate, thus exerting an effect on all the heme sites.

In other flash photolysis experiments, 2,3-diphosphoglycerate and ATP were also found to lower the rate of CO binding to both fish and shark hemolysates, and as with IHP, the effects are much greater on the teleost hemolysates.

**Oxygen Equilibrium and Temperature Dependence**

Hill plots for the oxygen equilibrium of swordfish, mako, porbeagle, and big-eye tuna hemolysates are given in Fig. 7. These data demonstrate the various possible responses of oxygen binding to changes in temperature. Swordfish hemoglobin has a $Q_{10}$, the change in the free concentration of oxygen at 50% saturation with a 10° change in temperature, of very nearly 2.0 which is similar to the value reported for mammalian hemoglobins. The mako and the big-eye tuna hemoglobin have $Q_{10}$ values of about 1.25, and the porbeagle, a $Q_{10}$ of almost 1.0. The dusky hemolysate exhibits equilibrium binding curves that are qualitatively similar to those of the swordfish hemolysate. The equilibrium curves for the swordfish and mako samples are very nearly parallel and linear while those for the porbeagle and the big-eye tuna hemolysates are neither parallel nor linear but exhibit, instead, decidedly different shapes at the two extremes of temperature. For both species the plots are more curved at 5° than at 35°, which suggests that the mechanism of ligand binding to porbeagle and big-eye tuna hemoglobins is changing with temperature.

Carbon monoxide binding and oxygen dissociation experiments were performed at 3° in an attempt to determine the kinetic expression of these peculiar temperature dependencies. In Table VI the rates of dissociation at 3° for the porbeagle hemoglobin, both the rate and the shape of behavior is contrary to that of all the other species examined, for which both $k_f$ and $k_s$ are substantially decreased at 3° (Table VI), and to that of mammalian hemoglobins which exhibit values of about 3 for the $Q_{10}$ of oxygen dissociation. Experiments similar to those shown in Fig. 3 provided evidence that the two rates of oxygen dissociation occur for the tuna hemolysate at 3° also represent two types of subunits within the same tetramer, and not different tetramers.

The rates and characteristics of the CO binding reactions at 3° are also given in Table VI. For the dusky, big-eye tuna, and swordfish hemolysates, the rate of CO binding decreases about 3-fold in going from 20° to 3°. Similar temperature effects have been observed for CO binding to mammalian hemoglobins. For example, the rate of CO binding to human hemoglobin decreases about a-fold in going from 20° to 3°. Similar temperature effects have been observed for CO binding to mammalian hemoglobins. For example, the rate of CO binding to human hemoglobin decreases from $1.5 \times 10^8$ M$^{-1}$ s$^{-1}$ to about $4 \times 10^6$ M$^{-1}$ s$^{-1}$ over the same temperature range. However, in the case of stripped porbeagle hemoglobin, both the rate and the shape of

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature</th>
<th>$k_f$</th>
<th>$k_s$</th>
<th>$Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Sharks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porbeagle</td>
<td>20°</td>
<td>2.48</td>
<td>0.55</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>3°</td>
<td>0.07</td>
<td>4.50</td>
<td>0.46</td>
</tr>
<tr>
<td>Dusky</td>
<td>20°</td>
<td>2.02</td>
<td>17.7</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>3°</td>
<td>0.80</td>
<td>1.5</td>
<td>0.58</td>
</tr>
<tr>
<td>B. Fishes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big-eye tuna</td>
<td>20°</td>
<td>3.16</td>
<td>0.79</td>
<td>42.8</td>
</tr>
<tr>
<td></td>
<td>3°</td>
<td>1.00</td>
<td>0.13</td>
<td>65.0</td>
</tr>
<tr>
<td>Swordfish</td>
<td>20°</td>
<td>3.48</td>
<td>28.2</td>
<td>4.88</td>
</tr>
<tr>
<td></td>
<td>3°</td>
<td>1.1</td>
<td>0.14</td>
<td>11.9</td>
</tr>
</tbody>
</table>

**Fig. 7.** The effect of temperature on oxygen binding to shark and fish blood. Oxygen equilibrium curves were obtained for whole blood as described under "Methods." These data were plotted according to Hill (18) as the logarithm of $Y/(1 - Y)$, where $Y$ is the proportion of oxy-heme sites, versus the logarithm of the free oxygen concentration. $A$, swordfish blood; $B$, mako shark blood; $C$, porbeagle shark blood, and $D$, big-eye tuna blood.
the time course of CO binding are dramatically altered by decreasing the temperature. At 3° the time course becomes exponential with a rate which is 8 times smaller than that of the slow component and 36 times smaller than that of the fast component observed at 20°. Also, there is no effect of IHP on CO binding to porbeagle hemoglobin at 3°. This dramatic temperature effect on ligand binding to porbeagle hemoglobin was also checked with oxygen. At pH 6.0, 20°, oxygen binding to unstripped porbeagle hemoglobin proceeded with an accelerating time course, exhibiting a maximum rate of 210 s⁻¹ when mixed with air-equilibrated buffer. At 3° and the same oxygen concentration, the reaction was biphasic (60% slow) with a slow rate of 30 s⁻¹. This change in the time course of the oxygen reaction with a change in temperature is a further indication that the mechanism of ligand binding to porbeagle hemoglobin varies with temperature.

**DISCUSSION**

Several recent investigations of the ligand-binding behavior of mammalian hemoglobins, especially human hemoglobin A, have shown that the α and β chains cannot be regarded as functionally equivalent. The degree of these subunit differences appears to be a function of the size and type of ligand studied, with the reactions of n-butyl isocyanide exhibiting the largest amount of heterogeneity (5), but even with the smaller ligands, O₂ and CO, α and β differences can be readily demonstrated (20, 32). The expression of these chain differences is modified by changes in pH and ionic strength and is strongly affected by the presence of organic phosphates. Similar effects are not at all unexpected for fish and shark hemoglobins, and because of the gross electrophoretic heterogeneity, an even more complicated situation is possible. However, under many conditions, both teleost and elasmobranch hemoglobins behave as if they contain equal amounts of only two functionally distinct binding sites, which are present within the same tetramer.

First, with the elasmobranchs, although spectral heterogeneity can be demonstrated in every case, an abundance of data indicates that the various tetramers present in the individual shark hemolysates are similar functionally. For example, with unstripped porbeagle hemoglobin the dithionite oxygen dissociation reaction at pH 6.0, the carbon monoxide-binding reaction at pH 5.6, 7.0, and 9.0 and the oxygen-binding reaction at pH 9.0 and 5.6, all proceed with either an exponential or an accelerating time course, as do the CO binding reactions of all forms of dusky and mako hemoglobin. It appears then that these hemolysates can be regarded as consisting of a single type of functional hemoglobin tetramer which exhibits chain differences. This type of intramolecular heterogeneity, as opposed to different tetrameric species, would then account for the lack of true isosbestic points observed in most of the kinetic experiments.

The inherent complexities in studying hemolysates of teleost bloods were appreciated as early as the 1930's (29-31). At sufficiently high pH the oxygen equilibrium curves of these bloods are homogeneous, but at low pH these curves become undulatory (30). It is interesting to note that the pH at which these curves become distorted appears to be considerably higher in whole blood than in the hemolysate. Besides those species investigated by Root et al. (29-31), more recent studies have demonstrated the occurrence of similar effects with hemolysates from cel, trout, salmon, and blue-fin tuna blood (3, 34-36). For the first three species, the hemolysates have been fractionated and the resultant purified hemoglobin components have been shown to be affected to different extents by salts and pH. All of these results predict that the kinetics of ligand binding to fish hemolysates should be highly complex, and indeed, this is the case.

For swordfish hemoglobin the relative spectral positions of the fast and slow components observed in carbon monoxide binding at neutral pH where the over-all reaction is accelerating is the reverse of that reported by Gray and Gibson (32) for phosphate-treated mammalian hemoglobins. As with shark hemoglobins, this particular spectral heterogeneity is hard to reconcile by assuming the presence of two distinct tetramers in view of the time course of the over-all reaction. However, an explanation assuming the existence of two unlike tetramers is possible in the case of the kinetic heterogeneity observed in the carbon monoxide-binding reactions of the fish hemoglobins at low pH or in the presence of phosphates.

By way of comparison, several differences between these elasmobranch and teleost hemolysates should be pointed out. For instance, these shark hemoglobins possess a respectable Bohr effect. In the case of porbeagle hemoglobin, the rate of CO binding at pH 6.0 is half that observed at pH 9.0 (Table IV), and the rate of the dithionite-oxygen dissociation at pH 6.0 is three times larger than at pH 9.0. These hemoglobins also bind IHP which further lowers the rate of CO binding at neutral or low pH (Table IV), yet this decrease in rate is relatively modest. In contrast, and with the possible exception of big-eye tuna hemoglobin's interactions with IHP, both changes in pH and the addition of IHP produced much more marked effects on the ligand-binding properties of these fish hemolysates. The magnitude of these effects as measured by the rate of carbon monoxide binding is quite striking. When the rate of CO binding at pH 7.0 is compared to the rate of the slow component observed at pH 6.0 (or in the presence of IHP), ratios of 20 and 15 are obtained for swordfish and bass hemoglobins, respectively. Furthermore, ATP and 2,3-diphosphoglycerate which are more likely to be present and play a significant regulatory role in vivo, have a more marked effect on most of these fish hemolyats than they do on the shark hemolysates. Big-eye tuna hemoglobin, however, does not appear to be influenced by the presence or absence of these organic phosphates at either pH 6 or pH 7 (Table V). Further, the relationship of the rapid and the slow rates of CO replacement of O₂ to the observed difference spectra are reversed for shark and teleost hemoglobin (Fig. 4), with the latter resembling all mammalian hemoglobins studied to date.

All these hemolysates investigated herein differ substantially from human hemoglobin in regards to certain physicochemical properties. Thus, whereas the liganded form of human hemoglobin has a tetramer dimer dissociation constant of approximately 2 μM (37), the ultracentrifugation data presented here indicate that the corresponding subunit dissociation constants for the shark and fish hemoglobins must be at least an order of magnitude lower. Furthermore, all these hemoglobin samples were found to lack a rapidly reacting form of hemoglobin on partial photoysis (38). They do exhibit a slightly increased rate of carbon monoxide binding after partial photolytic breakdown, but this enhanced rate was only 3- to 5-fold greater than the rate of binding to the fully deoxygenated hemoglobin samples, and not 30-fold as is seen for human hemoglobin. Thus, for both fish and shark hemoglobins the relationship of the various ligand binding constants must be quite unlike those observed for human hemoglobin (39).
One intriguing observation concerned the rates of ligand binding to the unstripped porbeagle hemolysate. The time course for CO binding to this hemoglobin preparation at both pH 9.0, 20°C, and pH 7.0, 3°C, was accelerating, yet biphasic time courses were observed for the same reactions with samples which were stripped of small molecules by passage through a Sephadex column. Curiously, the addition of inositol hexaphosphate did not restore the accelerating time courses for CO binding. The observation at pH 9.0 is the more interesting since phosphates would not be expected to play a regulatory role at high pH. Thus, it would appear that some other type of compound is present in the unstripped porbeagle hemolysate and is able to regulate the reactivity of the hemoglobin molecules.

Phosphate binding to some of these fish hemoglobins could partially explain the observation by Root and Irving (31) that, with certain teleost hemoglobins, the pH required to produce an equivalent loss of oxygen-binding capacity was one pH unit lower for the hemolysates than for whole blood. Kisch (40) determined that the hematocrit and the total heme of many of these fish bloods are quite low, and thus it could be expected that upon hemolysis the concentration of both effector molecules (possibly organic phosphates) and the hemoglobin would decrease to between one-fifth to one-tenth of their concentration within the red cell. This dilution would tend to dissociate the small molecules from the tetramer and therefore increase the oxygen affinity.

Ligand Binding at Low Temperature—Some conclusions can now be offered regarding the unusually small temperature dependence of the oxygen equilibria of hemolysates from the two warm bodied species that have been studied. For both porbeagle shark and big-eye tuna hemolysates, an unusual change in a kinetic property of one type of component, presumably one type of subunit within the tetramer, occurs in response to a decrease in temperature. For big-eye tuna hemoglobin, the rate of oxygen dissociation from the fast component at 20°C is essentially unchanged at 3°C, thus producing an unusually low affinity species at low temperature (Table VI). For porbeagle hemoglobin, the carbon monoxide association rates of both components seen at 20°C decrease dramatically with decreasing temperature and this also leads to an abnormally low affinity species at 3°C. In contrast, for mammalian hemoglobins which have a Q_{10} for the half-saturation point in O₂ binding of approximately 2, the association rates are very little affected by decreasing temperature, but the rates of ligand dissociation are substantially lowered (19).

In this case of parallel molecular evolution then these two warm bodied species have arrived at the same effect, little change in P_{50} with temperature, by virtue of alterations in different kinetic constants. Both changes, however, cause the mechanism of ligand binding, as represented by the shape of the O₂ equilibrium curve, to vary in response to temperature; this variation is seen in Hill plots (Fig. 7) as varying amounts of curvature at different temperatures. Especially in the case of porbeagle hemoglobin, this curvature in Hill plots allows the curves to nearly overlap at 50% saturation (log Y/(1 - Y) = 0), but still be separated at higher and lower degrees of saturation.

The mako shark which also has a P_{50} with an unusually small temperature dependence has Hill curves which are parallel at different temperatures. Presumably such behavior would be expected if either the rate of dissociation or the rate of association of both chains were effected unusually, but equally, with temperature. Rossi-Fanelli and Antonini reported that even though the P_{50} of blue-fin tuna hemoglobin was unaffected by temperature, the O₂-binding curves at various temperatures were overlapping (9). Here again, all chains within the tetramer must have unusual temperature dependencies of either the binding or the dissociation constants or both for this behavior to occur.

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Melvin E. Andersen, John S. Olson, Quentin H. Gibson and Francis G. Carey


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