THE MOLECULAR BASIS OF A MATERNAL-FETAL OXYGEN SHIFT IN THE VIVIPAROUS SEAPERCH, *EMBIOTOCA LATERALIS*

by

ROLF LUDWIG INGERMANN

A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

June 1980

APPROVED: Robert C. Terwilliger

Robert C. Terwilliger

An Abstract of the Dissertation of

Rolf Ludwig Ingermann for the degree Doctor of Philosophy in the Department of Biology to be taken June 1980

Title: THE MOLECULAR BASIS OF A MATERNAL-FETAL OXYGEN SHIFT IN THE VIVIPAROUS SEAPERCH, EMBIOTOCA LATERALIS

Approved: _____
Robert C. Terwilliger

Teleost fish of the family Embiotocidae, the seaperches, are viviparous. Among the small number of viviparous families, the embiotocids are unique in the morphological specialization of the fetus for gaseous and nutrient exchange. Midway through fetal development, the fetus has very large, well vascularized fins terminating in vascularized fin spatulates. The enlarged fetal fins and spatulates lie in close contact with highly vascularized ovarian sheets, thereby forming a "pseudoplacenta". In viviparous animals, juxtaposition of fetal and maternal blood flows is a major factor involved in gaseous exchange. Also, viviparous animals generally utilize molecular mechanisms to increase the oxygen binding affinity of fetal blood thereby increasing the efficiency of oxygen exchange. These mechanisms usually involve either a difference in oxygen affinities of fetal and adult hemoglobins or a difference in red blood cell concentrations of allosteric modifiers of hemoglobin function. This study attempts to

elucidate such molecular mechanisms in the striped seaperch, Embiotoca lateralis.

Various electrophoretic, chromatographic, and spectrophotometric techniques have been used to characterize adult and fetal hemoglobins and intracellular environments of the red blood cells. This study shows that the purified fetal hemoglobin present midway through gestation is composed of two types of globin chains; one chain is unique to the fetus, the other is shared by the adult. Oxygen binding experiments show that this mid-gestation fetal hemoglobin has a higher oxygen affinity than adult hemoglobin. Fetal hemoglobin obtained near the end of gestation shows an electrophoretic pattern more similar to adult hemoglobin. This "late" fetal hemoglobin has an oxygen affinity intermediate between mid-gestation fetal and adult hemoglobins. This hemoglobin appears to be a structural and functional composite of fetal and adult hemoglobins. "Late" fetal hemoglobin appears to be a developmental transition state.

The allosteric modifier, adenosine triphosphate (ATP) reduces the oxygen affinity of these hemoglobins. ATP levels in adult red blood cells are significantly higher than levels found in the "late" fetal cells.

Different oxygen affinities of the adult and fetal hemoglobins facilitate the transfer of oxygen from the mother to the mid-term fetus. Near birth, however, the

fetal hemoglobin oxygen affinity approaches that of the adult pigment and the difference in ATP concentrations facilitates fetal oxygen uptake.

Extensive studies have been conducted on maternal-fetal oxygen shifts in mammals. Little work, however, has been done on this subject in the lower vertebrates. This is the first extensive study of the molecular basis for a maternal-fetal oxygen shift in a teleost fish.

VITA

NAME OF AUTHOR: Rolf Ludwig Ingermann

PLACE OF BIRTH: Flensburg, West Germany

DATE OF BIRTH: April 19, 1950

UNDERGRADUATE AND GRADUATE SCHOOLS ATTENDED:

University of California, Los Angeles
University of Oregon

DEGREES AWARDED:

Bachelor of Arts, 1972, University of California, Los Angeles
Master of Science, 1974, University of Oregon

AREAS OF SPECIAL INTEREST:

Respiratory, Reproductive, and Cell Physiology
Marine Biology
Toxicology of Environmental Contaminants

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow, Department of Biology, University of Oregon, 1974, 1979-1980

Graduate Teaching Fellow, Department of Biology, University of Oregon, 1975-1977

AWARDS AND HONORS:

Research Award, Sigma Xi, Scientific Research Society of North America, 1977

Research Award, Lerner Fund for Marine Research of the American Museum of Natural History, 1979

PUBLICATIONS:

Ingermann, R., Scheer, B.T.: Interaction of insulin, vasopressin, and sodium ion effects on toad bladder. American Zoologist 15, 590 (1975)

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation for the guidance and support of my advisor, Professor Robert C. Terwilliger.

I am also indebted to my parents and friends at the Oregon Institute of Marine Biology and in the Department of Biology, University of Oregon for their aid, encouragement, and suggestions. Dr. Lawrence Dangott, Mr. Nicholas Grinich, and Mr. Michael Roberts provided invaluable assistance in obtaining research animals. Mr. Craig Lilly and Dr. Stanton Cook provided help with atomic absorption analyses.

This work was partially supported by research grants from Sigma Xi, the Scientific Research Society of North America, and the Lerner Fund for Marine Research of the American Museum of Natural History.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The seaperches constitute a rather small group of teleost fishes, the family Embiotocidae. Twenty three species have been described. Two are found off Japan and Korea, one is a freshwater fish of Central California and the remainder are marine species found between Baja California and Alaska (Tarp, 1952). Embiotoca lateralis, the fish used in this study, is found from northern Baja California to southern Alaska (Hart, 1973). One of the more interesting characteristics of the embiotocids is that unlike the majority of fishes, which are oviparous, they are viviparous. Among the few viviparous fish families, the embiotocids are further outstanding in that 1. they have a minute amount of yolk available for fetal development, 2. the period of gestation is long, up to 6-7 months for E. lateralis, and 3. the young fish is at a very advanced state of development at parturition. Another unique feature of embiotocid viviparity is the morphological specialization of the fetus midway through gestation (Fig. 1a). At this developmental stage when the fetal fish is beginning to become covered with scales, it has very large, well vascularized fins terminating in

fin extensions or fin spatulates which emanate from between the fin rays. These enlarged fins and spatulates lie in juxtaposition with highly vascularized ovarian sheets forming a so-called pseudoplacenta. This anatomical arrangement is well suited to facilitate maternal and fetal gas exchange and perhaps nutrient transfer as was first suggested by Eigenmann in 1892. As development proceeds the body becomes covered with scales and grows larger relative to fin size. At parturition the animal looks like a miniature adult. The vascularized fin spatulates appear to be functionally important until birth when blood flow through them is terminated. The spatulates are eventually resorbed. With such remarkable morphological clues to a potential maternal-fetal oxygen transfer, it is equally surprising that a study of this process has not hitherto been undertaken in this animal.

The transfer of oxygen from adult to fetus in mammals has been studied extensively. Oxygen transfer in mammals is of course facilitated by the juxtaposition of fetal and adult circulatory systems. In mammals, several additional mechanisms have been found. These include structural adaptations of the fetal and maternal hemoglobins as well as modifications of the intracellular components of maternal and fetal erythrocytes. Although these mechanisms have been extensively studied in mammals,

few investigations have been carried out on lower vertebrates.

Fetal blood of almost all species examined has a higher oxygen binding affinity than does its corresponding maternal blood. Thus, fetal blood can be almost completely saturated with oxygen while the corresponding maternal blood is less than fully saturated. Two major mechanisms appear to contribute to the high oxygen affinity of fetal versus maternal blood. In the first case, the fetal hemoglobin can be a structurally and therefore functionally different hemoglobin than the maternal hemoglobin. In the second strategy, both fetus and adult can have identical or nearly identical hemoglobins. However, the intracellular environment of the fetal erythrocyte is different from that of the corresponding adult erythrocyte. Different concentrations of effective allosteric molecules can modify the respective hemoglobins' oxygen binding affinities unequally. Examples of the first molecular adaptation are found in goats and sheep. In these animals, the structure of the fetal hemoglobin is different from that of the adult hemoglobin (Huisman et al., 1969; Bard et al., 1978). Most vertebrate hemoglobins are tetrameric molecules composed of two different pairs of subunits or polypeptide chains, usually designated as an α chain and a β chain. Adult sheep hemoglobin is

described as an $\alpha_2 \beta_2$ tetramer. However, fetal sheep hemoglobin, also a tetrameric protein, is composed of two α chains which are identical to those of the adult hemoglobin and a pair of γ chains which are unique to the fetus. The γ chain of the fetus is different from its α chain as well as the β chain of the adult. If one strips fetal sheep hemoglobin, that is removes all possible allosteric modifiers, the oxygen affinity of the stripped fetal hemoglobin is greater than that of the stripped adult hemoglobin. The structural differences of the two hemoglobins, therefore, can be directly correlated with the different oxygen binding affinities of adult and fetal blood.

A variation on this theme is shown by human adult and fetal blood. Whole fetal blood, hemoglobin within erythrocytes, has a higher oxygen affinity than whole adult blood. Fetal and adult hemoglobins of humans, like those of sheep, are different structurally. However, stripped adult and fetal human hemoglobins have virtually identical oxygen binding properties in contrast to sheep hemoglobins. Most interesting is that the internal environments of the adult and fetal erythrocytes are also very similar. Especially noteable are the similar high concentrations of 2,3 diphosphoglycerate (2,3 DPG) present in both adult and fetal cells. 2,3 DPG has been shown

to be effective in lowering the affinity of hemoglobin for oxygen (Benesch and Benesch, 1967, 1969; Chanutin and Curnish, 1967). Further studies have shown that 2,3 DPG, a polyanion, binds to the adult hemoglobin at the central cavity of the tetramer, specifically to six positively charged amino acid residues contributed by the two β globin chains (Perutz, 1970). The central cavity, however, can only accommodate 2,3 DPG when the tetramer is in the deoxygenated state. As the tetramer become oxygenated, its conformation changes, the β chains are forced closer to one another, and the 2,3 DPG is sterically forced out of the cavity (Perutz, 1970). The 2,3 DPG binds in a 1 : 1 ratio with the hemoglobin tetramer and stabilizes the deoxygenated state. The result of 2,3 DPG binding is that the hemoglobin has a reduced oxygen binding affinity. The human hemoglobin α chains are associated with the tetramer's conformational changes upon oxygenation but are not directly involved in 2,3 DPG binding. Fetal human hemoglobin is composed of two α and two γ globin chains. The fetal and adult α chains are identical; the fetal γ chain differs by 39 of 146 amino acids from the adult β chains. One of these substitutions appears to be largely responsible for the different oxygen affinities of adult and fetal blood. Specifically, one of the histidines of the adult β chain is replaced by a

serine in the fetal γ chain. This results in the loss of two positively charged amino acid residues (one from each γ chain) from the central cavity (Lorkin, 1973). The loss of these two positively charged residues means that there are two less sites for the binding of the allosteric modifier, 2,3 DPG. This is therefore the major physiologically significant substitution. Although the intracellular 2,3 DPG titers are equally high in the two cell types, approximately 5 millimolar (one mole 2,3 DPG per mole hemoglobin tetramer), 2,3 DPG binds less strongly to fetal than adult hemoglobin. Thus, fetal hemoglobin is less stabilized in the deoxygenated state than adult hemoglobin. Fetal hemoglobin, therefore, shows a higher oxygen binding affinity than adult hemoglobin in the presence of equal amounts of allosteric modifier. Consequently the fetal blood has a higher oxygen binding affinity than adult blood. A similar mechanism for maternal-fetal oxygen transfer has been reported in the Japanese monkey (Takenaka and Morimoto, 1976).

The second major molecular mechanism for maternal oxygen transfer involves different fetal and adult erythrocyte concentrations of 2,3 DPG. Most mammals so far studied utilize this mechanism; these include rabbit, horse, pig, and mouse (Baumann et al., 1973; Bunn and Kitchen, 1973; Bauer et al., 1975; Jelkmann and Bauer,

1977; Petschow et al., 1978; Bard and Shapiro, 1979). Little difference if any can be noted between stripped adult and fetal hemoglobins with respect to electrophoretic or oxygen binding characteristics. 2,3 DPG apparently reduces the oxygen binding affinity of hemoglobin in the erythrocytes of these species in the same manner as described for human adult hemoglobin (Bunn and Kitchen, 1973). 2,3 DPG is found at intracellular concentrations of 1.6 $\pm$ 0.2 mole 2,3 DPG per mole hemoglobin tetramer for all the adult animals mentioned above. The corresponding fetal erythrocytes have intracellular organic phosphate titers which are 1.2 $\pm$ 0.4 mole 2,3 DPG / mole hemoglobin tetramer less than levels found in the adult red blood cells. It is this difference in 2,3 DPG concentrations which largely accounts for the higher oxygen binding affinity of fetal blood in these animals.

The major exception to the generalization that fetal blood has a higher oxygen affinity than adult blood has been reported in the domestic cat (Novy and Parer, 1969). Adult and fetal cat whole blood show identical oxygen binding characteristics. No difference is detected between adult and fetal stripped hemoglobins with respect to oxygen binding and electrophoretic mobility. Furthermore, cat hemoglobin, actually a mixture of two components, is only slightly affected by 2,3 DPG, which is present

intracellularly in very low concentrations (Taketa, 1973; Scott et al., 1978).

Another example in which fetal blood does not have a higher affinity than adult blood was found in a study of a hemoglobin abnormality. Women with the abnormal hemoglobin designated hemoglobin Rainier, have blood with a higher oxygen affinity than the blood of the fetuses that they carry. Nonetheless, fetal development is apparently normal (Adamson et al., 1969). Hemoglobin Rainier differs from normal hemoglobin by a single amino acid substitution, resulting in its having a much higher oxygen affinity than normal hemoglobin (Antonini and Brunori, 1971). Dawes (1967) reported that intrauterine transfusions of human adult blood to fetuses apparently results in normal fetal development. All infants which had nearly complete replacement of fetal red cells by adult cells were above the 50th percentile in birth weight. Therefore, it appears that there was no impairment of fetal growth after replacement of blood (Novy, 1972). A higher oxygen binding affinity of fetal versus adult blood does not seem to be mandatory for fetal survival in all cases. However, since the fetuses of most mammals have a blood with a higher oxygen binding affinity than the maternal blood, this affinity difference probably provides an adaptive advantage by facilitating the transfer of

oxygen across the placenta.

Little is known of the molecular basis for the maternal to fetus transfer of oxygen in the lower vertebrates, because very few lower vertebrates have been studied. A maternal-fetal shift in oxygen affinity of whole blood has been reported in the viviparous garter snake Thamnophis. According to Manwell (1960), oxygen binding properties of adult and fetal hemoglobin solutions are indistinguishable indicating structural similarity or identity. Pough (1978), however, presented evidence indicating that adult and fetal pigments are structurally and functionally different. There has been no resolution to this controversy. A recent study of the viviparous caecilian amphibian, Typhlonectes compressicauda, showed that fetal blood has a higher oxygen binding affinity than adult blood (Toews and MacIntyre, 1977). Furthermore, fetal and adult hemoglobins are functionally and structurally indistinguishable. The molecular basis of the maternal-fetal oxygen transfer is thought to be mediated entirely by different concentrations of adenosine triphosphate (ATP) in adult and fetal erythrocytes (Garlick et al., 1979). Many lower vertebrates have hemoglobin responsive to 2,3 DPG; however, 2,3 DPG is rarely found in high concentrations in the erythrocytes of these animals. The apparent functional role of 2,3 DPG in

mammals has been replaced by inositol pentaphosphate in birds (Vandecasserie et al., 1971; Bartlett and Borgese, 1976). ATP, and to a generally lesser extent, guanosine triphosphate (GTP) serve the same function in reptiles, amphibians, and fish (Coates, 1975; Bartlett, 1976a). These polyanions apparently reduce the oxygen affinity of hemoglobin in a manner similar to that of 2,3 DPG as previously described for human hemoglobin (Coates, 1975). The fetal blood of the ovoviviparous spiny dogfish shark, Squalus suckleyi, also has a higher oxygen binding affinity than does adult blood (Manwell, 1958). A difference in affinities is maintained by the hemoglobins. It is not clear, however, whether the protein was completely stripped of allosteric factors in these experiements. Enzymatic digestion of S. suckleyi hemoglobins followed by electrophoretic analysis has shown that a few peptide fragments from fetal hemoglobin are absent in adult hemoglobin digests yet all adult fragments can be found in the fetal pattern (Manwell, 1963). It appears that the fetal hemoglobin may be the adult hemoglobin with an extra piece of polypeptide chain. Alternatively, the fetal hemoglobin may be a multiple hemoglobin system containing one or more uniquely fetal chains. Nonetheless, the structures of adult and fetal hemoglobins are different, and it is proposed that this contributes to the oxygen

binding differences of whole blood. It appears unlikely that organic phosphate concentration differences are involved in the maternal-fetal oxygen shift in the shark. The intraerythrocytic ATP concentrations found in several species of sharks are much less than levels found in teleosts and the high intracellular concentration of urea may interfere with the hemoglobin-organic phosphate interaction (Coates, 1975). McCutcheon (1947) has reported, however, that diluted adult and embryonic red cell lysates from three species of viviparous ray show no difference in maternal-fetal oxygen binding characteristics. Perhaps rays utilize differences in organic phosphate concentrations to facilitate maternal-fetal oxygen exchange but that high dilution *in vitro* prevents the analysis of the effect.

The hemoglobins of only one viviparous teleost have been examined. Hjorth (1974) examined adult and fetal hemoglobins from an eelpout, *Zoarces viviparus*, and found them to be electrophoretically different. It seems likely that these different hemoglobins have different oxygen binding characteristics; however, neither this possibility nor the possibility of different intraerythrocytic environments has been studied further with respect to fetal oxygen uptake.

There have been no other published reports of which

I am aware pertaining to fetal oxygen uptake in the lower vertebrates.

In the mechanisms of mother to fetus oxygen transfer discussed above, the fetus generally utilizes either a unique hemoglobin or a much reduced intracellular level of organic phosphate as the major molecular mechanism to facilitate oxygen uptake at the placenta or pseudoplacenta. However, other aspects of the intracellular environment can also affect the oxygen affinity of hemoglobin either directly or indirectly. These other intracellular factors do not appear to be specific to any particular fetal developmental state but rather are consequences of the normal cell response to its immediate environment and metabolism. One of the most important factors is the hydrogen ion concentration. Most vertebrate hemoglobins show a decreased oxygen affinity as the hydrogen ion concentration is increased. This is referred to as the alkaline or normal Bohr effect. (Occasionally the opposite reaction is observed and this is referred to as the acid or reversed Bohr effect.) In human hemoglobin, the decrease in oxygen affinity with decreasing pH is due to the increased stabilization of the deoxygenated hemoglobin conformation by three pairs of amino acid salt bridges (Perutz, 1970; Baldwin and Chothia, 1979). In deoxyhemoglobin, the amino group of the terminal valine of the

α chains forms a salt bridge with the terminal carboxy groups of the arginine residue of the opposite α chains. This interaction is slightly sensitive to pH. Two other salt bridges which form in deoxyhemoglobin but not in oxyhemoglobin are very pH sensitive. A histidine on each β chain forms a salt bridge with an aspartic acid residue on the same chain, and another histidine on each α chain probably forms a bridge with an aspartic acid residue on the same chain (Perutz, 1970). The histidines involved have imidazole pK values of about 6.5; however, in the transition from oxy to deoxyhemoglobin, salt bridges form and these pK's are raised. The conformation of deoxyhemoglobin, but not oxyhemoglobin, brings the histidines close to negatively charged carboxylate groups. The proximity of the carboxylates stabilizes the protonated state of the imidazole rings thereby raising their effective pK into physiological pH's. Slight decreases in pH will lead to an increased fraction of protonated α-amino groups and histidine imidazoles. Formation of these bridges will generate a more stabilized deoxygenated hemoglobin conformation, and consequently, a decreased oxygen binding affinity.

Some fish hemoglobins show a so-called Root effect. This has been interpreted by some as an extreme Bohr effect (Giardina et al., 1975; Saffran and Gibson, 1978).

Hemoglobins showing this effect have a very low affinity for oxygen at low pH's and at low pH's do not become completely oxygenated at very high oxygen pressures (Root, 1931; Noble et al., 1970).

Carbon dioxide has a dual effect on hemoglobin (reviewed by Cameron, 1979). First, most carbon dioxide in the presence of red cell carbonic anhydrase forms carbonic acid. This product dissociates into protons and bicarbonate:

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

The effect of this reaction on hemoglobin is a decreased oxygen affinity due to a lower pH, that is, an increased stabilization of deoxyhemoglobin by the Bohr effect. In addition, carbon dioxide reacts with the unprotonated terminal α-amino groups:

$$R\text{-}NH_2 + CO_2 \rightleftharpoons R\text{-}NHCOO^- + H^+$$

(Kilmartin and Rossi-Bernard, 1969). The carbamate reduces oxygen affinity by stabilizing the deoxyhemoglobin conformation at the central cavity (Tomita and Riggs, 1971). Also, the liberated proton will reduce the oxygen affinity via the Bohr effect. Both polyanion binding and carbamation occur at the terminal amino groups of the β chains and are mutually exclusive events (Tomita and Riggs, 1971; Duhm, 1976). Conceivably, carbamation could occur independent of polyanion binding

at the terminal amino groups of the α chains. This apparently does not occur in most fish, however. No α chain from a teleost hemoglobin yet studied has a free α-amino group; most are probably acetylated (Riggs, 1979) and will not react with carbon dioxide. The direct effect of carbon dioxide on hemoglobin is therefore a function of pH, polyanion concentration, and carbon dioxide tension. The effect of polyanion concentration on human hemoglobin is apparently more important than the effect of carbon dioxide tensions at low pH (6.8) and the reverse appears true at high pH (7.6) (Arnone, 1974). Wood and Johansen (1973) and Haswell (1979) report the intracellular pH in teleost red cells to range from 7.1 to 7.5. Therefore, the *in vivo* effect of carbon dioxide may be more important than the effect of polyanion concentration in the fish red cells. Weber and Lykkeboe (1978) report, however, that the specific effect of carbon dioxide is completely suppressed by ATP and GTP in carp erythrocytes.

It is generally accepted that protons are passively distributed across the red cell membrane according to the Gibbs-Donnan equilibrium (Steen and Turitzin, 1968). This ion distribution, however, is a function of the intracellular concentrations of nonpermeable polyvalent molecules, most notably hemoglobin and organic phosphates. Duhm (1971, 1972, 1976) has reported that high human

intraerythrocytic concentrations of organic phosphates increase the ratio $[H^+]$ cell / $[H^+]$ plasma. Wood and Johansen (1973) reported that hypoxia-acclimated eels reduced red cell ATP concentrations and increased whole blood oxygen affinity. They concluded that the increased oxygen affinity was due primarily to an increased intracellular pH rather than less direct ATP-hemoglobin interaction. Apparently the reduction of intracellular ATP concentration caused the increase in intracellular pH via the Gibbs-Donnan equilibrium. High organic phosphate levels therefore decrease oxygen binding affinity by 1. binding directly to the hemoglobin and 2. by decreasing cell pH.

In general, the oxygen affinity of hemoglobin decreases with increasing salt concentration (Benesch et al., 1969); however the effect of salt depends on the type of ions involved (Vig and Aviram, 1978). Benesch et al. (1959) and Chiancone et al. (1972) have suggested that inorganic anions reduce oxygen binding in a manner analogous to that determined for the organic phosphates. They postulated that anions compete for a common binding site on the deoxyhemoglobin. More recently, additional mechanisms have been proposed (Nigen et al., 1976). However, the mechanisms by which monovalent anions or salts decrease oxygen affinity is essentially unknown

(Haire and Hedlund, 1977). The physiological importance of variations in salt concentrations remains obscure, and perhaps doubtful in cells containing high organic phosphate levels. Benesch et al. (1969) showed that a salt effect was not discernable for human hemoglobin in the presence of a physiological concentration of 2,3 DPG.

The intracellular divalent cation concentration has been shown to be significant in influencing oxygen affinity in some blood. Although these ions have little influence on hemoglobin directly (Rose, 1968; Weber and Lykkeboe, 1978), they will complex with nucleoside triphosphates thereby preventing the organic phosphate-hemoglobin interaction. This will effectively increase the oxygen affinity of the hemoglobin. The interaction of magnesium with 2,3 DPG is slight (Costello et al., 1977); the interaction of magnesium with ATP is very significant. The dissociation constant at 35°C and 0.1 ionic strength for the reaction:

$$(ATP)Mg^{2-} \rightleftharpoons (ATP)^{4-} + Mg^{2+}$$

is 1.9×10^{-5} M (Phillips et al., 1966). High magnesium concentrations essentially eliminate the interaction of ATP with hemoglobin but will not completely eliminate the interaction of GTP with hemoglobin (Weber, 1978). Calcium ions will cause similar effects. Weber and Lykkeboe (1978) have measured the nucleoside

triphosphate and magnesium concentrations in hypoxia and normoxia-acclimated carp. The magnesium and nucleoside triphosphate (primarily ATP) titers were 4.3 and 5.0 millimole / liter red cells respectively in hypoxia-acclimated animals; 4.7 and 9.0 mM/lt. cells respectively in normoxic animals. The magnesium concentration is probably sufficiently high to reduce the effectively free (hemoglobin-interactable) organic phosphate concentration. In contrast, calcium probably has a minor role as it generally is found in low intracellular concentrations (Diem and Lentner, 1970).

The affinity of hemoglobin for oxygen depends on the amount of oxygen already bound. The binding of oxygen to one heme of a tetramer facilitates oxygen binding by the other hemes in the same tetramer. A measure of this interaction is given by the Hill coefficient, h. Physiologically, this is an important property of hemoglobin. The greater the value of h, the generally greater will be the change of hemoglobin-oxygen saturation with changes in partial pressure of oxygen. This can significantly facilitate oxygen loading at the lungs, gills, and other respiratory structures and oxygen unloading to the tissues. The importance of differences in h values or cooperativity between fetal and adult hemoglobin in mammalian maternal-fetal oxygen shifts is unclear however.

There appears to be no difference in h values between fetal and adult sheep hemoglobin (Battaglia et al., 1970). In the Japanese monkey, h is higher for fetal than for adult hemoglobins, and h is not affected by 2,3 DPG. In humans, h is lower for fetal than for adult hemoglobins in the absence of 2,3 DPG yet very similar in the presence of 2,3 DPG (Takenaka and Morimoto, 1976).

The concentration of hemoglobin may also affect its oxygen affinity. Benesch et al. (1969) have reported that human hemoglobin concentration changes had no effect upon oxygen affinity. The concentrations they examined, however, were one to two orders of magnitude less than those found in erythrocytes. In contrast, Forster (1972), Laver et al. (1977), and Lykkeboe and Weber (1978) reported that human, human and sheep, and carp hemoglobins respectively showed a decrease in oxygen affinity with an increase in hemoglobin concentration. Hemoglobin concentrations used in their studies were close to those encountered physiologically. It appears that an increased hemoglobin concentration is associated with its lowered oxygen affinity but the explanation of the mechanism remains controversial. Gary-Bobo and Solomon (1968), Solomon (1971), and Hoffmann (1977) have reported that at physiological concentrations, hemoglobin molecules interact with one another resulting in a reduction in net

charge per molecule. This would be accomplished by either formation of salt bridges or by shielding charged groups from the solution by aggregation. How these changes would affect the function of the hemoglobin, however, remains unclear. Gros et al. (1978) repeated much of the earlier work. They came to the conclusions that hemoglobin charge and oxygen binding characteristics were independent of hemoglobin concentration. They postulated, however, that an increase in hemoglobin concentration might influence oxygen affinity indirectly by the law of mass action. Incomplete removal of organic phosphates would lead to an increased fraction of phosphate-bound hemoglobin with increased hemoglobin concentration and hence a decreased oxygen affinity. Despite the controversy over the molecular mechanism, it appears likely that hemoglobin concentration may directly or indirectly affect oxygen binding and therefore could be important in a maternal-fetal oxygen transfer system. This hemoglobin dilution hypothesis has no precedent in the maternal-fetal oxygen transfer literature. However, hemoglobin dilution by cell swelling has been reported (Lykkeboe and Weber, 1978) in hypoxia as compared to normoxia-acclimated fish. If an embiotocid fetus can be regarded physiologically as a hypoxic adult, this dilution phenomenon may be important.

In an attempt to integrate some of the factors known

to affect the oxygen affinity of hemoglobin as described above, it is advantageous to observe what might be happening in the circulation of a fetus. Respiration of fetal tissue results in a deposition of carbon dioxide into the blood. This carbon dioxide results in a decreased hemoglobin oxygen binding affinity through a decreased pH via the normal Bohr effect and possible carbamate formation. In addition, an increased chloride concentration, arising from the exchange of plasma chloride for erythrocytic bicarbonate (from the reaction: $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$), may further decrease oxygen affinity (Cameron, 1978). And finally, by the law of mass action, oxygen dissociates from the oxyhemoglobin and diffuses down its concentration gradient, from blood to the tissues. Upon returning to the maternal-fetal exchange surfaces (the placenta in mammals and most likely the fetal fins and ovarian sheets in the embiotocids,) the fetal blood undergoes similar but reversed changes and becomes oxygenated. Fetal blood entering the exchange surfaces has a lower pH than does adult blood. In humans, fetal blood from the umbilical artery has a pH of 7.3 and adult arterial blood has a pH of 7.4 (Bertles, 1974). A Bohr effects of fetal and adult hemoglobins facilitate fetal oxygen uptake as carbon dioxide, bicarbonate, and protons effectively leave the fetal cells, thereby increasing the oxygen

affinity of the fetal blood, and effectively enter the adult cells, thereby decreasing the oxygen affinity of the adult blood. Chloride may also affect oxygen affinity as described previously. Again, oxygen will diffuse down its concentration gradient from the oxygen-rich adult blood to the relatively oxygen-poor fetal blood. Fetal hemoglobin may also facilitate fetal oxygen uptake. Further facilitation of fetal oxygen uptake will occur if the fetal hemoglobin has a higher intrinsic oxygen binding affinity than the adult hemoglobin.

Molecular mechanisms of the maternal-fetal oxygen transfer which involve different fetal and adult intra-erythrocytic organic phosphate concentrations may utilize an additional property to facilitate fetal oxygen uptake. First, the lower organic phosphate titers of the fetal cells will lead to higher oxygen binding affinities relative to adult cells due to less stabilization of the deoxy-hemoglobin state by polyanions. In addition, lower levels of organic phosphates in fetal versus adult cells will lead to a smaller difference of intracellular pH between fetal and adult cells due to the Gibbs-Donnan effect. Acidification of the adult red cells at the exchange surfaces will still occur resulting in a decrease of adult blood oxygen affinity due to the Bohr effect. Therefore, in those animals utilizing different organic phosphate

levels between fetal and adult cells, fetal cells will have a higher internal pH relative to fetal cells, such as human fetal red cells, which maintain fairly high organic phosphate levels. This will lead to a high fetal red cell oxygen affinity and to an increased facilitation of fetal oxygen uptake at the respiratory exchange surfaces.

In many animals examined, fetal blood shows a higher oxygen carrying capacity, that is, a higher blood hemoglobin concentration, than adult blood. This is found in humans, sheep, and cats, and apparently favors quantitative oxygen uptake and delivery to the tissues. In contrast, rabbits, goats, and pigs show no difference between fetal and adult oxygen carrying capacities (Novy and Parer, 1969). A higher fetal oxygen carrying capacity may enhance quantitative fetal oxygen uptake; however, since some animals do not utilize this strategy, its contribution seems either small or associated with unfavorable side effects. These may include a decreased oxygen binding affinity associated with an increased cellular hemoglobin concentration or an unfavorably high blood viscosity if the oxygen carrying capacity rise is due to an increased number of erythrocytes (Driessen et al., 1979).

Finally, changes in the circulation of the adult and fetal animals may occur to enhance oxygen uptake by

the fetus, especially as it approaches parturition. An increased maternal blood flow rate through or an increased vascularization of the placenta (or pseudoplacenta) could increase the oxygen exchange efficiency and insure high oxygenation of fetal blood. This is involved in fetal sheep development (Rosenfeld et al., 1974).

EXPERIMENTAL OBJECTIVES

The seaperch, Embiotoca lateralis, presents a unique opportunity to study the molecular mechanisms of a maternal to fetal oxygen transfer system in a lower vertebrate. The remarkable morphological adaptations of the fetal fish strongly suggest that such a transfer occurs in this animal. The questions asked and experiments described in this thesis fall into four major areas. First, what are the structural and functional relationships between adult and fetal hemoglobins? Second, how do the adult and fetal cells compare qualitatively and quantitatively with respect to organic phosphates and divalent cations? Third, how do the major intracellular modifiers affect the oxygen binding affinities of adult and fetal hemoglobins? Finally, how do oxygen carrying capacities, hematocrits, and mean corpuscular hemoglobin concentrations of adult and fetal blood compare?

The overall aim of the studies described in this thesis is to elucidate the molecular mechanisms involved in facilitating the fetal uptake of oxygen from the maternal circulation in Embiotoca lateralis.

MATERIALS AND METHODS

Adult Embiotoca lateralis, standard length (tip of snout to end of hypural) greater than or equal to 22 cm, were obtained from the Cape Arago region of the Oregon coast. The animals were identified according to Hart (1973). Animals were either sacrificed immediately or kept in a 1200 liter tank with aeration and running sea water. Fetal fish obtained in April and early May (Fig. 1a) yielded hemoglobin termed mid-gestation or mid-fetal hemoglobin; more advanced fetuses obtained just prior to birth in June and July (Fig. 1b) yielded late-gestation or late fetal hemoglobin.

Adults were rendered unconscious by a blow to the head and were bled by severing the tail at the caudal peduncle. Fetal fish were rinsed in buffered saline several times and were bled by severing the tail and the ventral aorta.

Except for bleeding, all preparative procedures were conducted at 4-5°C. Blood was collected in an ice cold beaker containing 1% NaCl, 1 mM tris (hydroxymethyl) aminomethane (Tris), a small quantity of heparin and titrated to pH 8.0 with HCl. Erythrocytes were washed three times with 1% NaCl, 1 mM Tris, and HCl to pH 8.0

Fig. 1a Mid-gestation E. lateralis fetus obtained in April and early May. This developmental state is characterized by the enlarged, highly vascularized fins and fin spatulates.

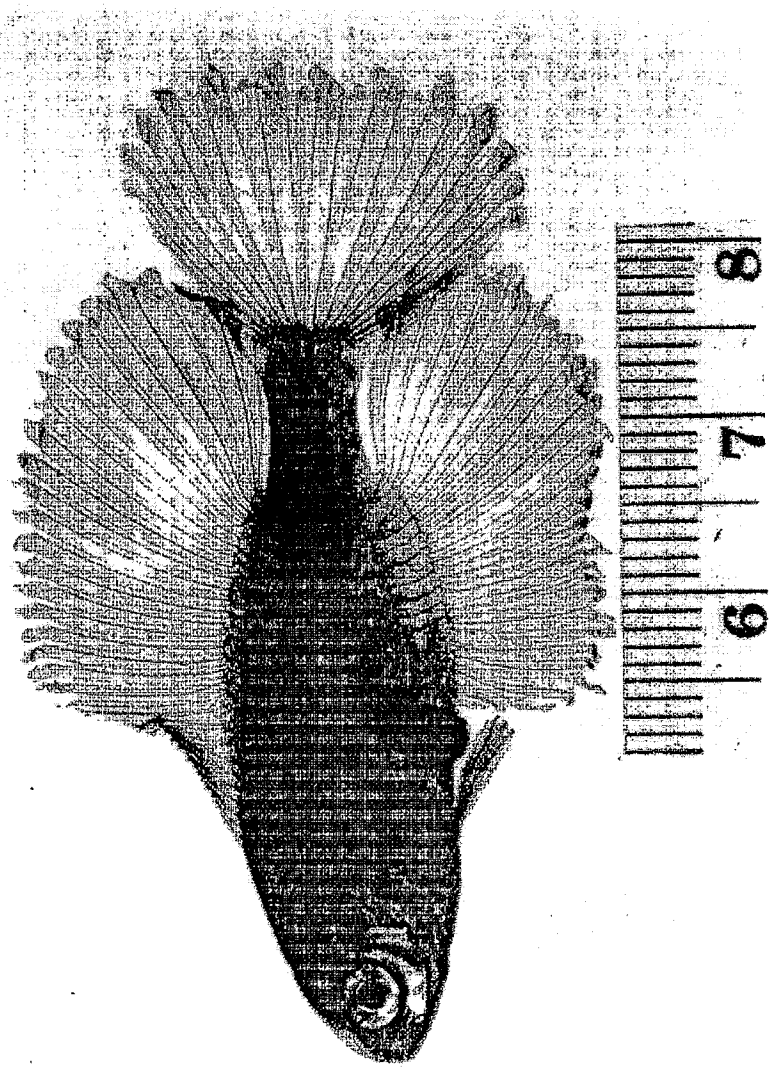
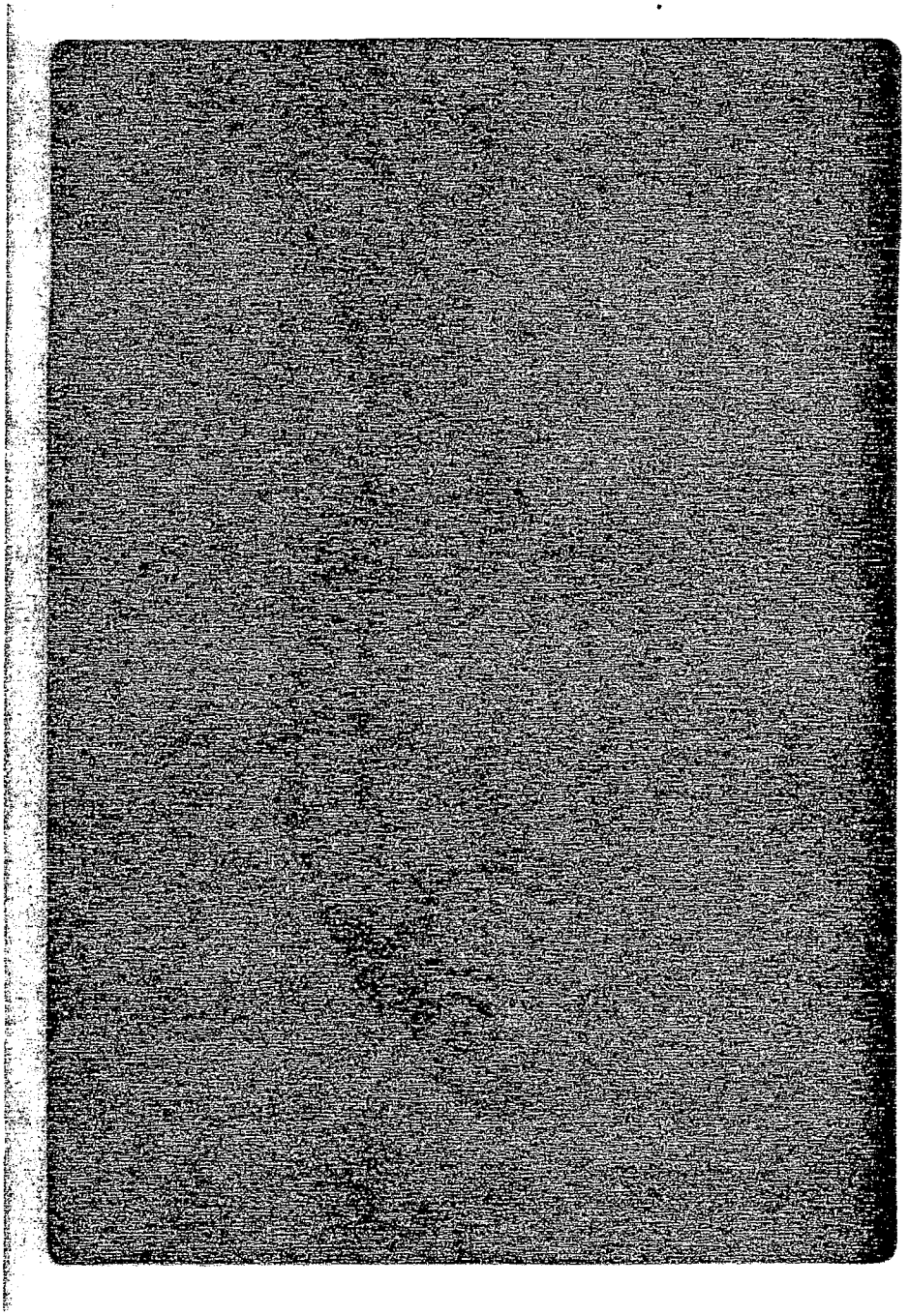

6
7
8

Fig. 1b Late gestation fetus obtained in June and early July. This developmental state shows adult-like body proportions.

Fig 1 b



with centrifugation at 120 g for 10 minutes. Packed cells were lysed with three times their volume of distilled water followed by centrifugation at 13,000 g for 15 minutes. The supernatant was then passed through a 62 x 2.2 cm column of Pharmacia Sephadex G-100 superfine gel filtration matrix equilibrated with 0.1 M NaCl, 0.01 M $MgCl_2$, 0.05 M HCl titrated to pH 8.0 with Tris. Hemoglobins were further stripped of organic phosphates by a modification of the methods of Hamasaki and Rose (1974) and Petschow et al. (1978). The hemoglobins were passed through a 37 x 1.9 cm column of Sigma Sephadex G-25-80 gel filtration matrix equilibrated with 0.1 M NaCl, 0.05 M HCl, and titrated to pH 7.3 with Tris.

HEMOGLOBIN STRUCTURE

Molecular weights of the intact hemoglobins were determined on a 100 x 1.9 cm column of Sephadex G-100 superfine equilibrated with 0.1 M NaCl, 0.05 M HCl, and titrated to pH 8.0 with Tris. Calibrants were blue dextran (molecular weight $\cong$ 2,000,000), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen A (25,700), and sperm whale myoglobin (17,200) (Sigma Chemical Co.).

Ion exchange chromatography of stripped hemoglobins

was conducted on 9.5 x 1.0 cm columns of diethylamino ethyl cellulose (DEAE) medium (Sigma Chemical Co.). Hemoglobins were converted to the carbon monoxide (CO) derivatives by addition of a small amount of sodium dithionite followed by bubbling with carbon monoxide. Prior to application to the column, approximately 10 ml of the CO-hemoglobin solution was dialyzed overnight against two 1 liter portions of 0.01 M ammonium bicarbonate (NH_4HCO_3). Elution was accomplished by a linear gradient of 0 to 0.5 M NaCl in 0.01 NH_4HCO_3; 500 ml of each solution.

Polyacrylamide disc gel electrophoresis of stripped, CO-hemoglobins was carried out according to the method of Ornstein (1964) and Davis (1964). Gels which were 7.5% in acrylamide at pH 8.9 were used with a 3% acrylamide stacking gel at pH 7.2. The resolving gel had an acrylamide to bisacrylamide ratio of 30 : 0.8 and electrode buffers were Tris / glycine pH 8.9 in the upper and Tris / HCl pH 8.1 in the lower reservoir. Phosphoric acid (or phosphate) was not used in any buffers; it was replaced with hydrochloric acid. Electrophoresis of the hemoglobins was also carried out in 7.5% gels at pH 8.0 and in 6% and 8.5% gels at pH 8.9.

Apohemoglobin was prepared by the method of Fioretti et al. (1976). Stripped, DEAE-purified hemoglobins were dialyzed against 0.01 M NH_4HCO_3 and heme groups were

removed from the hemoglobins with HCl and 2-butanone extraction at 0°C. Globin solutions were dialyzed against 0.01 M NH_4HCO_3, then frozen, and lyophilyzed. The dried globins were stored dessicated at -20°C.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of globins was carried out on 1.5 mm slab gels (Studier, 1973) with a discontinuous buffer system (Laemmli, 1970). Gel concentrations of 12.5, 13.5, and 15%, at a constant ratio of 30 : 0.8, were used. Upper and lower electrode buffers were 0.025 M Tris, 0.19 M glycine, 0.1% SDS, and 0.001 M EDTA (ethylenediaminetetraacetic acid) (pH 8.3). Globins were denatured by heating at 100°C for 2 minutes in 0.0625 M Tris, 2% in SDS, 5% in 2-mercaptoethanol, 1 mM PMSF (phenylmethylsulfonyl fluoride) (pH 6.8). Molecular weight calibrants were ovalbumin, α-chymotrypsinogen A, sperm whale myoglobin, lysozyme (molecular weight = 14,300) (Sigma Chemical Co.), and human apohemoglobin (α : 15,100; β : 15,900) (prepared as per Fioretti et al., 1976). Gels were stained in Coomassie Blue according to Fairbanks et al. (1971).

Urea gel electrophoresis of globins was performed at pH 2.2 in the presence of 6.25 M urea. The resolving gel was 5% acrylamide with an acrylamide to bisacrylamide ratio of 20 : 1.2. The technique was a modification

of those used by Panyim and Chalkley (1969) and Poole et al. (1974). Globins were incubated in a solution of 10 M urea (previously deionized with Amberlite MB-1), 5% acetic acid, and 1% 2-mercaptoethanol. Upper and lower reservoir electrolyte solutions were 5% acetic acid. Gels were preelectrophoresed and run at 2 ma per tube. After electrophoresis, the gels were stained with Coomassie Blue and destained in 10% acetic acid.

OXYGEN BINDING

Stripped hemoglobins were dialyzed overnight against two 500 ml buffer changes. Oxygen binding buffers were 0.1 M NaCl, 0.05 M HCl titrated to pH 7.0 and above with Tris, and below pH 7.0 with Bis-Tris (Sigma Chemical Co.). Oxygen equilibrium measurements were made at 20.0°C in glass tonometers (Benesch et al., 1965) using a Zeiss PMQ II spectrophotometer. The tetrameric hemoglobin concentration was approximately 12 μM. Prior to binding, hemoglobin solutions were Millipore filtered or centrifuged at 13,000 g for 15 minutes if any flocculent material was visible. The hemoglobin solution was pipetted into a tonometer and deoxygenated by evacuation and flushing with argon to prevent foaming. Absorbance scans from 600 to 500 nm were conducted periodically

with a Perkin-Elmer Coleman 124 spectrophotometer during the deoxygenation process. The pigment was judged to be deoxygenated when the scans showed a single, symmetrical absorbance peak at 560 nm. Prior to and during reoxygenation of the pigment, tonometers were equilibrated for at least 15 minutes in a 20.0°C water bath before taking absorbance measurements. Each experiment was terminated by recording the absorbance spectrum of the air-saturated pigment solution after equilibration at atmospheric pressure. Calculation of degree of oxygenation was based on changes in absorbance at 540, 560, 575, and 580 nm. pH values were determined immediately upon termination of the oxygen equilibrium measurements. At the end of binding experiments conducted at pH values below 7.0, some solid Tris was added to raise the final pH to above 7.0 in order to eliminate the Root effect and to determine the spectral properties of 100% oxygenated hemoglobin. Hemoglobin solutions below pH 6.5 were unstable with a tendency to form methemoglobin; in these binding experiments the methemoglobin-reductase system described by Hayashi et al. (1973) was utilized. The value for P_{50}, the oxygen tension for half saturation of the pigment expressed in millimeters of mercury and the Hill coefficient, h or cooperativity, were determined by plotting log $[y/(1-y)]$ versus log PO_2 (the Hill plot)

in which y is the fractional degree of oxygenation and PO_2 is the partial pressure of oxygen. Only data points between 25% and 75% oxygenation were used in the determinations. Oxygen binding experiments at various pH's were conducted in the presence and absence of 1 mM ATP (Sigma Chemical Co.). ATP was added from a concentrated solution prepared immediately before the experiments in a quantity necessary to achieve a final 1 mM ATP concentration in the hemoglobin solution.

Oxygen binding experiments on adult and late-gestation fetal hemoglobins were also conducted with subsaturating concentrations of ATP and magnesium. Stripped hemoglobin was concentrated over sucrose then dialyzed for six hours against six-500 ml buffer changes. Buffer was 1 mM HCl, containing calculated amounts of ATP and/or magnesium and titrated to pH 7.2 with Tris. The concentrated hemoglobin solution was diluted to the desired hemoglobin concentration with this buffer just prior to the binding experiments. Tonometers containing the hemoglobin solutions were weighed prior to initiation and following completion of the experiment. These data were used to determine the concentrations of ATP and/or magnesium present during the experiment. Final hemoglobin concentrations were determined by the cyanmet- derivative method of Drabkin and Austin (1935) using a 540 nm millimolar heme

extinction coefficient of 11.0 for the cyanmethemoglobin (van Assendelf and Zijlstra, 1975).

INTRACELLULAR ENVIRONMENT ANALYSIS

Adult and late-gestation fetal red cells were collected and washed as previously described. The pellet obtained from the last wash and centrifugation was resuspended in 1% NaCl, 1 mM HCl, and titrated to pH 8.0 with Tris. Two replicate aliquots of this suspension were diluted with a specific volume of distilled water to lyse the red cells for cyanmethemoglobin concentration determinations. The volume of the suspension remaining was recorded. The suspension was then recentrifuged and the pellet mixed with approximately five volumes of cold 0.6 M perchloric acid as described by Bartlett (1978a). The mixture was centrifuged at 1000 g; the supernatant saved, and the residue reextracted with two volumes of cold 0.3 M perchloric acid. This was then recentrifuged, pellet discarded, and the supernatant mixed with the previous supernatant. The combined supernatants were neutralized with concentrated KOH and the resulting potassium perchlorate precipitate was eliminated by centrifugation. The extracts were then diluted six-fold with distilled water and applied to a 22 x 1.9 cm column

of Dowex-1 X 8 ion exchange resin (200-400 dry mesh) (Sigma Chemical Co.). Prior to application, the ion exchange resin had been converted from the chloride to formate form by treatment with 5 M ammonium formate (Bartlett, 1959a). A standard phosphate mixture composed of inorganic phosphate, ATP, GTP, and 2,3 DPG was eluted with 1.5 lt. of a 0-5 M ammonium formate buffer made of four parts 5 M formic acid with one part 5 M ammonium formate. An extract of adult red cells was similarly eluted. An extract of late gestation fetal red cells was eluted with 1.0 lt. of the above buffer system as relatively little fetal blood could be collected. At the end of the fish runs, 200 ml of 1.0 M HCl was passed through the column to elute any inositol polyphosphates (Borgese and Nagel, 1978). Phosphates were eluted at about 0.5 ml per minute and 6-9 ml fractions were collected. The fractions were analyzed for nucleoside absorbance at 260 nm. Nucleoside concentrations were determined with molar extinction coefficients calculated from standard ATP and GTP solutions.

Total phosphorus was determined for each fraction as per Bartlett (1959b). 1.0 ml from each fraction or phosphate standards and 0.5 ml of 5 M H_2SO_4 were pipetted into test tubes. Samples were heated at 150-160^{o}C for five hours, a small quantity of H_2O_2 was added, and

the solutions were heated for at least an additional two hours. To the residues were added 0.5 ml of 2% ammonium molybdate, 0.2 ml of Fiske-SubbaRow reagent (Sigma Chemical Co.), and 3.9 ml water. This mixture was then heated in a boiling water bath for seven minutes. Absorbance at 830 nm was recorded and unknowns determined from a phosphate standard curve.

The phosphates, eluted from the Dowex-1 X 8 column, were identified by elution positions of the standards, published elution positions (Bartlett, 1968, 1978a), and by analysis of total phosphorus.

Nucleoside triphosphate levels were determined with an enzymatic assay kit from Sigma Chemical Co. Aliquots of a suspension of washed adult and late fetal red cells were taken for determination of cyanmethemoglobin; others were used for the NTP assay. Equal volumes of cell suspension and 12% trichloroacetic acid (TCA) were mixed for the NTP assay. The mixture was centrifuged to remove proteins and membrane debris; the supernatant was split for NTP and magnesium analyses. The NTP determination utilized the following reactions:

ATP + 3-Phosphoglycerate $\rightleftharpoons$ ADP + 1,3-Diphosphoglycerate

catalyzed by phosphoglycerate phosphokinase

1,3-Diphosphoglycerate + NADH $\rightleftharpoons$ Glyceraldehyde-3-P + NAD + P_i

catalyzed by glyceraldehyde phosphate dehydrogenase The decrease in absorbance at 340 nm due to the oxidation of NADH was monitored. NTP was then calculated from the NADH extinction coefficient and the 1:1 stoichiometric relationship between ATP hydrolysis and NADH oxidation. NTP concentrations were then expressed as a mole NTP to mole hemoglobin tetramer ratio.

Magnesium levels in whole cell TCA extracts were determined by a modification of the Sky-Peck (1964) procedure. Equal volumes of unknowns or standards in 6% TCA, 0.0035% Clayton's thiazole yellow (MCB Manufacturing Chemists, Inc.) in 0.015% polyvinyl alcohol, and 2 M lithium hydroxide were combined. Absorbance was read immediately at 540 nm and unknown magnesium concentrations determined from a standards plot.

Magnesium and calcium levels in adult red cells were also determined by atomic absorption analysis with a Techtron AA-5 spectrophotometer. Washed red cells were lysed, centrifuged at 13,000 g for 20 minutes, and supernatants used for analysis. An air-acetylene flame was used. Magnesium was determined by absorbance at 285.21 nm; calcium by absorbance at 422.67 nm.

BLOOD PARAMETERS

Hematocrits and blood hemoglobin concentrations were determined for adult and late-fetal blood. Caudal peduncles of fish blotted dry were cut and blood collected in heparinized micro-hematocrit capillary tubes. These tubes were spun for two minutes in an International Micro-capillary Centrifuge, model MB. No further packing occurred with longer centrifuge times. Blood hemoglobin was determined by diluting a small, measured volume of blood into a large, known volume of distilled water. Cyanmethemoglobin concentration was then determined as previously described.

RESULTS

HEMOGLOBIN STRUCTURE

The apparent molecular weights of both adult and mid-gestation fetal hemoglobins are 54,000 as determined by Sephadex column chromatography (Fig. 2a, b). SDS slab gel electrophoresis of mid-gestation fetal, late gestation fetal, and adult hemoglobins each show a single band corresponding to a molecular weight of about 15,000 (Fig. 3). The banding pattern does not change when the hemoglobin is electrophoresed in either 12.5% or 15% acrylamide. If the mid-gestation fetal and adult hemoglobins are electrophoresed together on a single gel, only one band is observed. Human hemoglobin, electrophoresed under the same conditions, can be resolved into two bands.

Ion exchange chromatography of mid-gestation fetal and adult CO-hemoglobins on DEAE-cellulose shows essentially identical patterns. One major peak can be resolved in both samples. A trace of a minor component is present in the mid-gestation fetal hemoglobin sample that is not seen in the adult hemoglobin sample. (Fig. 4a, b).

Polyacrylamide disc gel electrophoresis of both

Fig. 2a Adult CO-hemoglobin chromatographed on Sephadex G-100. A, B, and C refer to the elution positions of bovine serum albumin (Mol. wt. = 68,000), ovalbumin (43,000), and α-chymotrypsinogen A (25,700), respectively. Not shown are the elution positions of blue dextran (~ 2 X 10^6) and sperm whale myoglobin (17,200). These had elution volumes of 0.106 and 0.214 lt., respectively. Absorbance at 280 nm: ○ ; absorbance at 540 nm: ● .

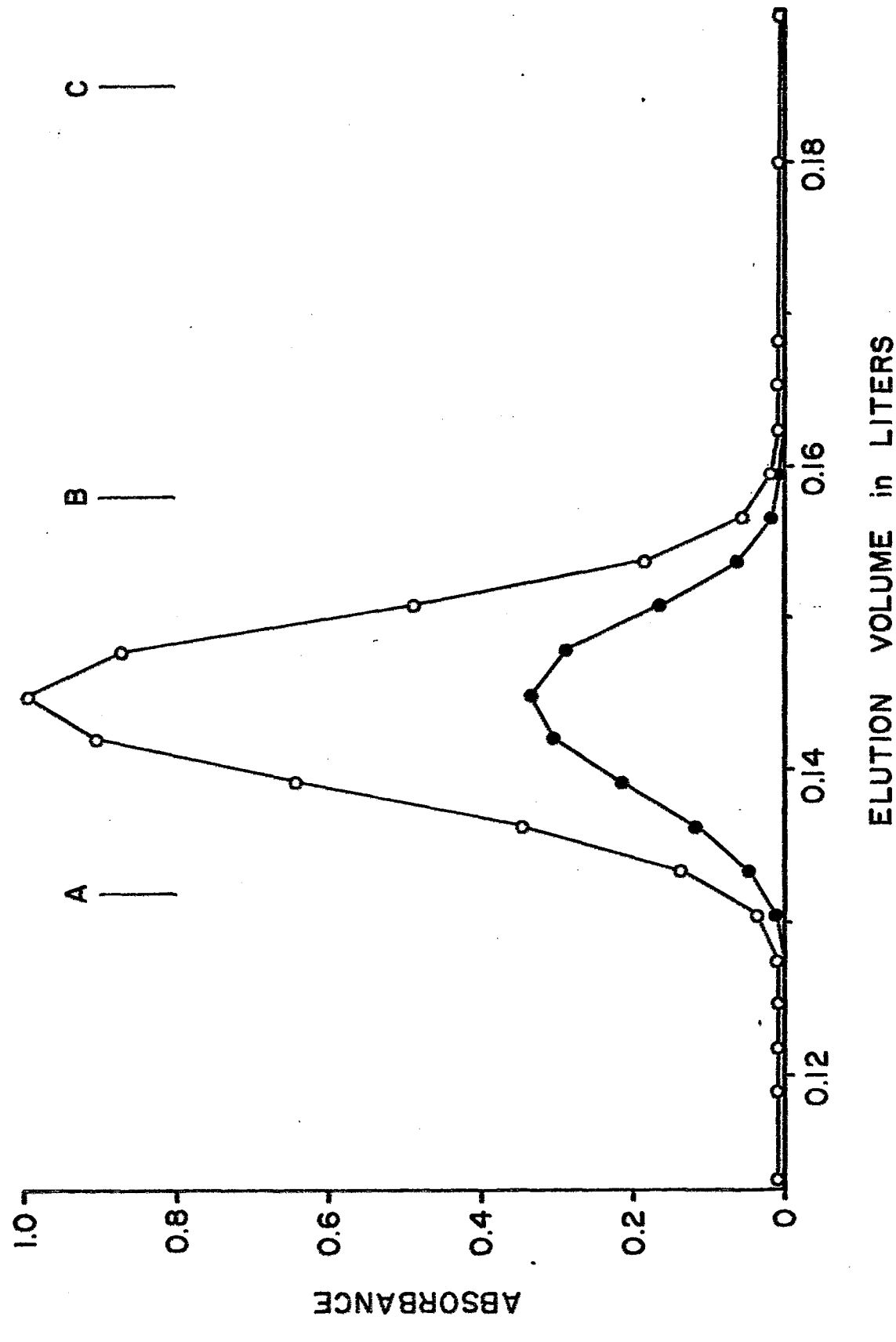

A
B
C
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0.12
0.14
0.16
0.18
ELUTION VOLUME in LITERS

Fig. 2b Mid-gestation fetal CO-hemoglobin chromatographed as described in Fig. 2a.

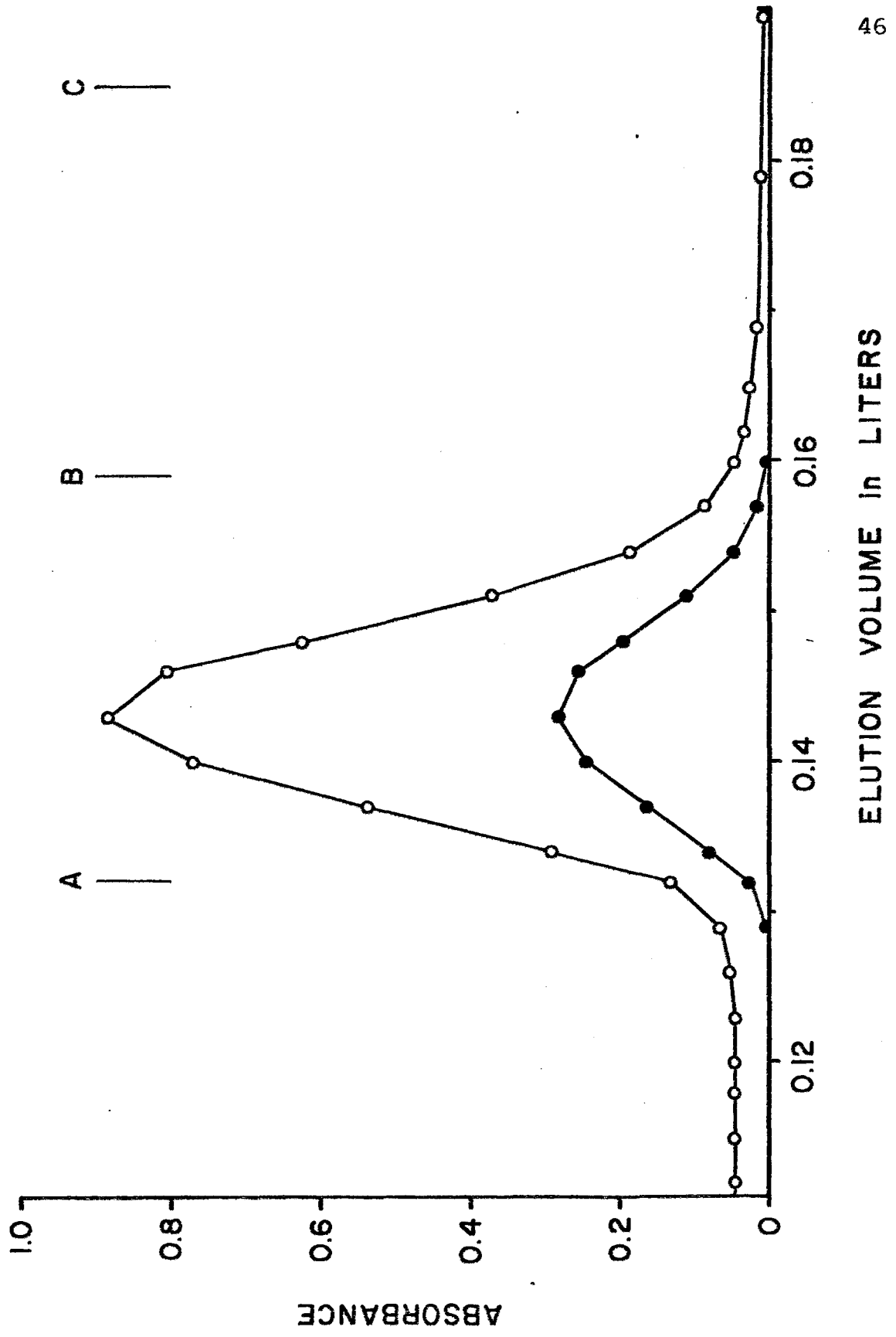

A
B
C
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0.12
0.14
0.16
0.18
ELUTION VOLUME in LITERS

Fig. 3 SDS slab gel electrophoresis. A is mid-gestation fetal, B is late gestation fetal, C is adult, D is mid-gestation fetal plus adult, and E is human apohemoglobin. Standard molecular weights are those of ovalbumin, α-chymotrypsinogen A., sperm whale myoglobin, and lysozyme (Mol. wt. = 14,300). The figure represents results obtained from a 13.5% gel.

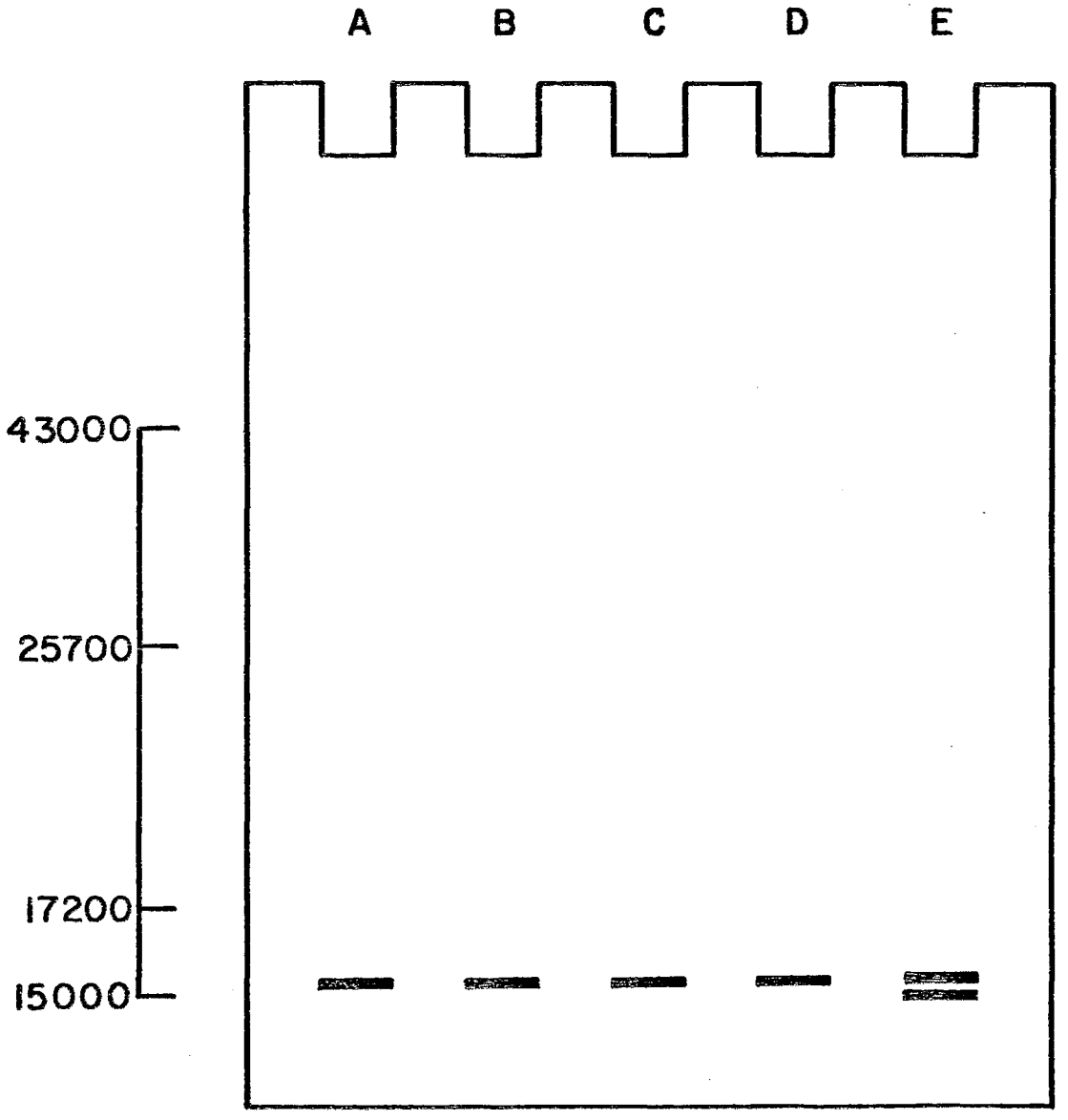

A
B
C
D
E
43000
25700
17200
15000

Fig. 4a Elution pattern of adult CO-hemoglobin from DEAE cellulose. Total elution volume is one liter and the salt gradient is 0 to 0.5 M NaCl in 0.01 M ammonium bicarbonate. The figure represents only a portion of the total elution, that portion containing the hemoglobin elution. Absorbance at 280 nm: -●- ; absorbance at 540 nm: -o- . The ratio of absorbance at 280 nm to absorbance at 540 nm is a relative measure of hemoglobin purity.

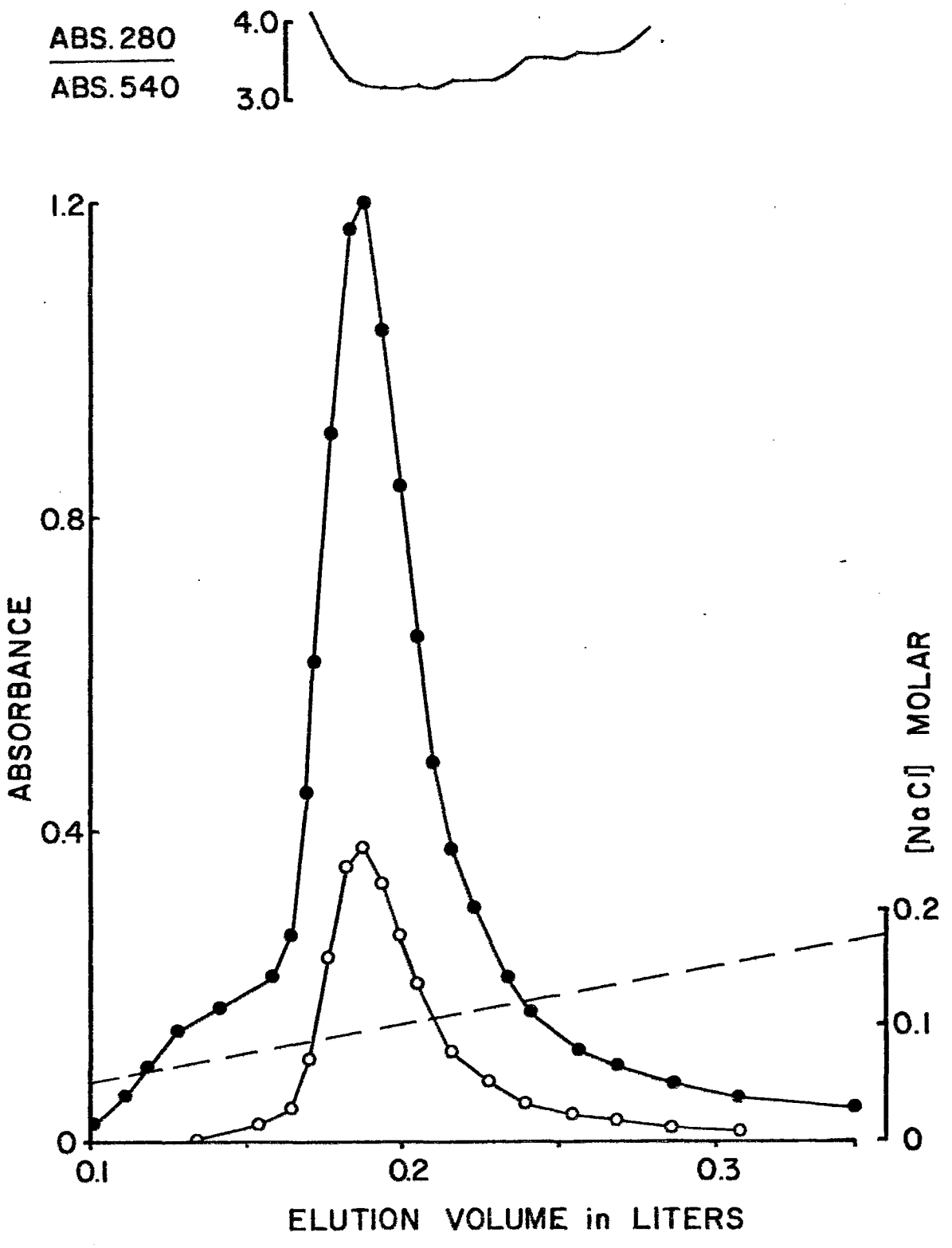
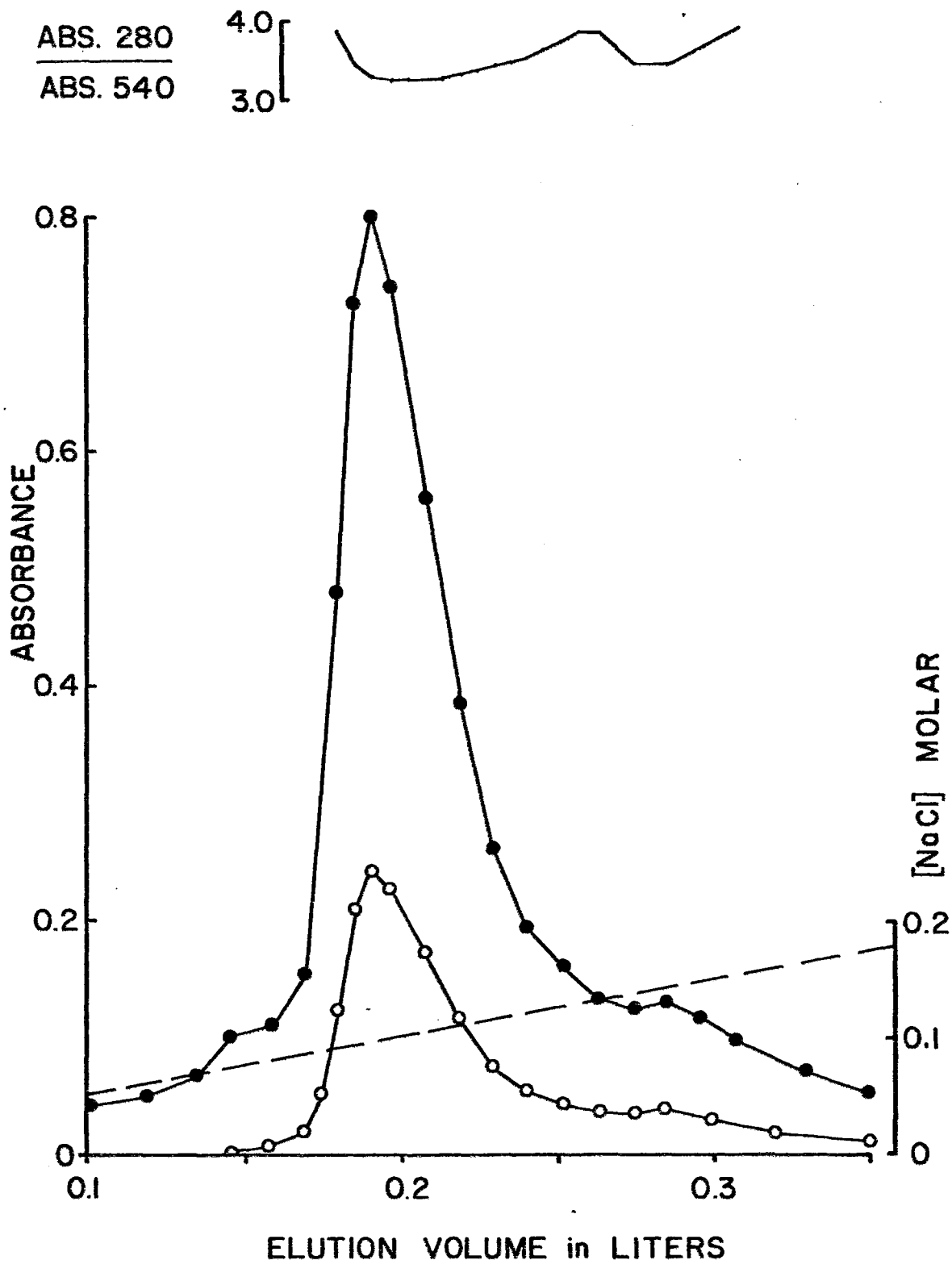

ABS. 280
ABS. 540
4.0
3.0
1.2
0.8
0.4
0
ABSORBANCE
0.2
0.1
0
[NaCl] MOLAR
0.1
0.2
0.3
ELUTION VOLUME in LITERS

Fig. 4b Elution pattern of mid-gestation fetal CO-hemoglobin. System is as described for Fig. 4a.


ABS. 280
ABS. 540
4.0
3.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0.2
0.1
0
[NaCl] MOLAR
0.1
0.2
0.3
ELUTION VOLUME in LITERS

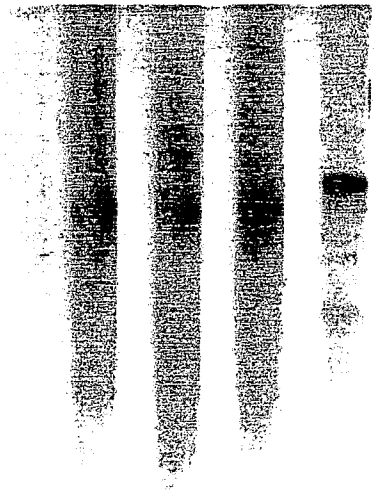
mid-gestation fetal and adult CO-hemoglobins shows consistently a single major band (Fig. 5). When mid-gestation fetal and adult hemoglobins are electrophoresed together, only a single band is seen. This band may be slightly wider than the bands obtained when either of the hemoglobins are electrophoresed separately. Occasionally, a slightly faster migrating minor component is detected in addition to the major band. Electrophoresis of cyan-methemoglobin or CO-hemoglobin at different gel concentrations or pH's does not change the resolution nor the consistency of the pattern.

Disc gel electrophoresis of mid-gestation fetal, late gestation fetal, and adult apohemoglobins at low pH (2.2) in the presence of 6.25 M urea are shown in Fig. 6a,b,c. The mid-gestation fetal hemoglobin shows two major bands. The late gestation fetal hemoglobin shows a pattern that includes the two globin chains seen in the mid-gestation fetal pattern; however, there are two additional chains with faster mobilities. When the mid-gestation fetal and late gestation fetal samples are electrophoresed together as seen in Fig. 6d, the top two globins co-electrophrese. The adult hemoglobin shows a third pattern (Fig. 6c). The top band appears to be identical to the most slowly migrating chain present in both mid-gestation fetal and late gestation fetal patterns;

Fig. 5 Polyacrylamide disc gel electrophoresis of mid-gestation fetal (MF), adult (A), mid-gestation fetal plus adult (M&A), and human CO-hemoglobin (H). Cathode is at the top of the photograph.


⊖
⊕
M F A M&A H

Fig. 6 Low pH, urea disc gel electrophoresis of mid-gestation fetal (A), late gestation fetal (B), adult (C), mid- plus late gestation fetal (D), mid-gestation fetal plus adult (E), and human apohemoglobin (H). Anode is at the top of the photograph.



MF A M&A H

this is evident when mid-gestation fetal and adult apohemoglobins are co-electrophoresed (Fig. 6e). However, the second major band of the mid-gestation fetal pattern, which is also present in the late gestation fetal sample, is absent in the adult globin pattern. The most cathodal migrating band of the late gestation fetal hemoglobin is present in the adult hemoglobin sample as a strong component. The most anodally migrating bands of the mid-gestation fetal, late gestation fetal, and adult apohemoglobins co-electrophorese and are likely the same subunit chain. Under the same electrophoretic conditions, human apohemoglobin separates into its component α and β chains as shown in Fig. 6h.

Several experiments indicate, therefore, that the adult and fetal hemoglobins are structurally very similar or identical. Gel permeation chromatography shows the adult and fetal hemoglobins to be indistinguishable with respect to intact molecular weight. SDS electrophoresis shows no obvious difference in molecular size among the component globins of the hemoglobins from the different developmental states examined. Ion exchange chromatography of adult and fetal hemoglobins on DEAE cellulose indicates that the net surface charges or surface charge distributions of these molecules are very similar or identical. Finally, a polyacrylamide disc gel electrophoresis,

carried out under a variety of conditions, indicates that adult and fetal hemoglobins are structurally very similar or identical, as based on the electrophoretic mobility (that is, mobility as a function of net exposed molecular charge and molecular size) of intact molecules at alkaline pH.

In contrast, electrophoresis of apohemoglobins in the presence of a denaturing agent (6.25 M urea) at low pH, after incubation in a reducing agent and 10 M urea, suggests that a structural difference exists between adult and fetal hemoglobin subunits. Under these electrophoretic conditions, it appears that half of the mid-gestation fetal, late gestation fetal, and adult hemoglobin tetramer is indistinguishable from one tetramer to the next, the other half is apparently different. Late gestation fetal hemoglobin appears to be a mixture of mid-gestation fetal and adult hemoglobins. The differences seen in the urea gel experiments of protein from different stages of development are interpreted as differences in protein structure for the following reasons. First, prior to the electrophoresis, hemoglobin from red blood cells of the different developmental stages was purified in the same way. The purified apohemoglobins were all treated identically, including incubation in urea from the same stock solutions before

electrophoresis and electrophoresed at the same time using the same electrophoretic conditions. In order to confirm differences, samples were co-electrophoresed in the same gel. Second, when the experiments were repeated on samples which had been purified from different specimens of fish, the same results were observed. Third, SDS electrophoresis indicates the subunit molecular weights of these fish hemoglobins to be very similar or identical. Different electrophoretic mobilities in low pH, urea electrophoresis are therefore likely to be due to charge differences and hence, chemical differences between these proteins (Poole et al., 1974). It may be that the electrophoretic bands seen are artifactual, that they do not reflect the true subunit structure of the fish hemoglobins. One of the most prevalent problems with urea denaturation is that cyanate can be formed, even at low pH, and react with the protein (Hirs, 1967). Spurious bands could be produced. Although this has not happened with human apohemoglobin under these experimental conditions, one cannot discount its happening with the fish hemoglobins; the fish apohemoglobins may be more receptive to this process. Such possible artifact formation may still reflect a difference in protein structure, albeit slight, and perhaps function. Another problem could be that in preparation of the pigments, some artifacts, such

as deamination or glycosylation, could be more likely in the fetal cells than in the adult. Again, this may still indicate a difference in protein structure. Further studies are necessary to conclude with certainty that these adult and fetal hemoglobins are indeed different proteins. This is especially necessary in that the proteins seem to be very similar to one another as is shown by the properties of their native configurations.

OXYGEN BINDING

Oxygen binding experiments were conducted on hemoglobins stripped of organic phosphates. The effectiveness of the stripping procedure was monitored by assaying the putative stripped hemoglobin sample for total phosphorus. The results are expressed in moles NTP per mole hemoglobin tetramer and are the average of two determinations using adult red cell lysates. Red cells lysed with distilled water and centrifuged at 13,000 g for 15 minutes to remove cell debris have a mole NTP to mole hemoglobin ration of 3:2. When the lysate supernatant is passed through the Sephadex G-100 column equilibrated with a magnesium-containing buffer, a ten-fold reduction in phosphorus content is obtained. Subsequent chromatography of the hemoglobin solution through Sephadex G-25 results in

a further reduction of phosphorus by one half, or 0.16 mole NTP per mole hemoglobin. Finally, a NTP to hemoglobin molar ratio of less than 0.01 can be obtained after a single six hour dialysis of this material against 500 ml of a pH 7.5 buffer. Stripped hemoglobin is defined in this thesis as hemoglobin which has been passed through Sephadex G-100 with a magnesium buffer, Sephadex G-25, and dialyzed overnight against at least two 500 ml buffer changes.

Oxygen binding data of mid-gestation fetal, late gestation fetal, and adult stripped hemoglobins are shown in Fig. 7 and Table 1. In Fig. 7, Log P_{50} values are plotted versus pH at 20° C. It appears that throughout the pH range examined, stripped mid-gestation fetal hemoglobin has the highest oxygen binding affinity and stripped adult hemoglobin has the lowest. Stripped late gestation fetal hemoglobin appears to show an oxygen affinity intermediate between the affinities of the mid-gestation fetal and adult hemoglobins.

Below pH 7.5, the ϕ values (Δ Log P_{50}/ Δ pH) are about -0.8, -0.8, and -0.9 for mid-gestation fetal, late gestation fetal, and adult hemoglobins respectively. All three stripped hemoglobin preparations show a Bohr effect which is similar in magnitude. Above pH 8.0, the adult hemoglobin shows a slight normal Bohr effect, the

Fig. 7 Oxygen binding studies of stripped mid-gestation (●)fetal hemoglobin and late gestation (○) fetal hemoglobin and adult (△) hemoglobin. Hemoglobin concentration is approximately 12 micromolar.

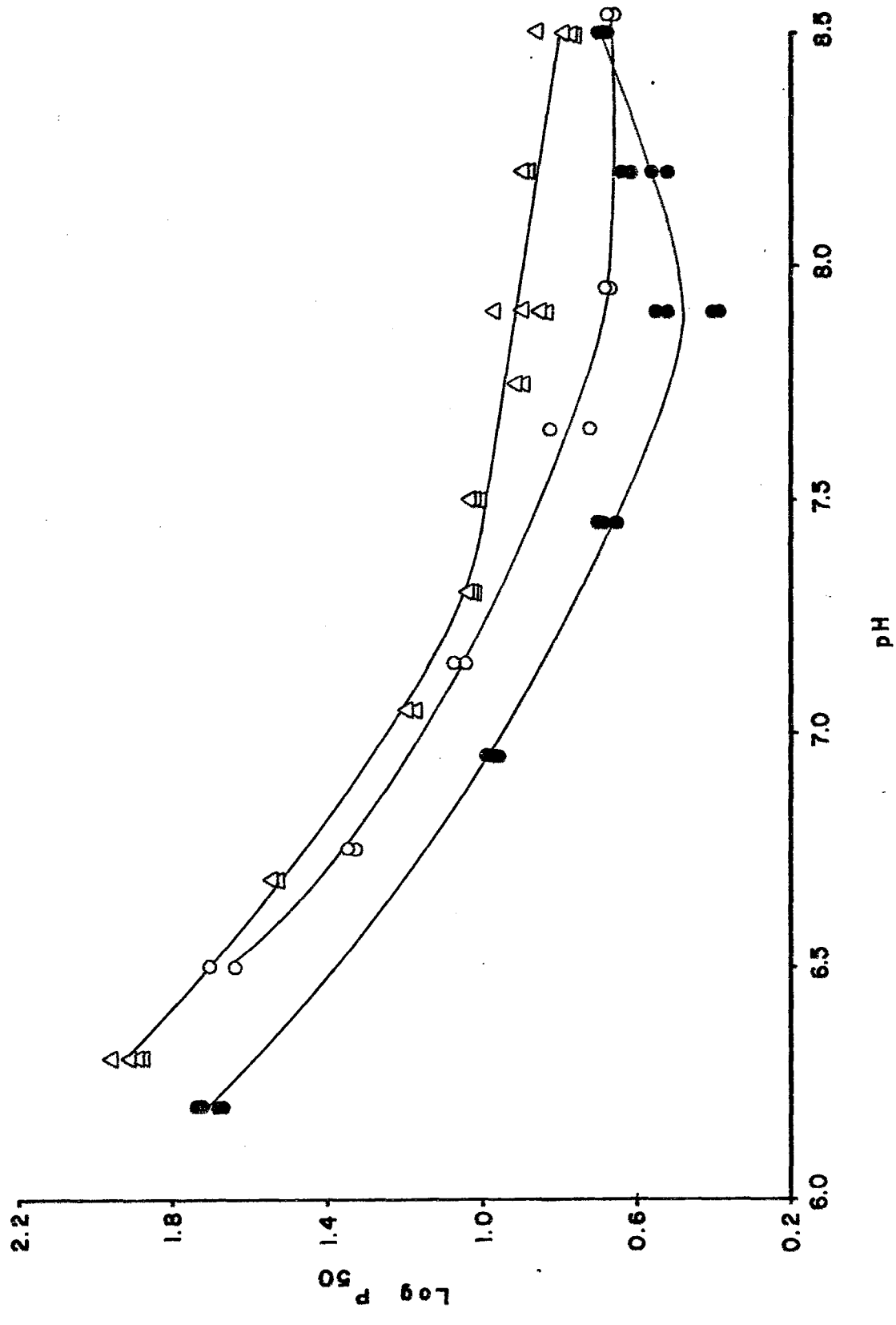

Log P_{50}
2.2
1.8
1.4
1.0
0.6
0.2
6.0
6.5
7.0
7.5
8.0
8.5
pH

TABLE 1: P_{50}, expressed in torr ($\pm$ 1 S.D.), as a function of several pH's for stripped hemoglobins of the adult, mid-gestation fetus, and late gestation fetus.

pH	Adult	Mid-gestation fetus	Late gestation fetus[b]
7.9	7.8 $\pm$ 1.2 n=4	3.0 $\pm$ 0.6 n=4	4.7 - 5.0
7.45	9.4 $\pm$ 0.2[a] n=4	4.8 $\pm$ 0.3 n=3	6.9 - 8.2
7.0	16.9[a] (avg. of 17.1, 16.7)	8.4 $\pm$ 0.1[a] n=4	13.8 - 14.6

a: P_{50}'s shifted to designated pH, pH change being 0.05 units in each case, using a Bohr factor (Δ Log P_{50}/ Δ pH) of ϕ = -0.8.

b: P_{50} ranges estimated by extrapolation of data shown in Fig. 7.

mid-gestation fetal hemoglobin appears to show a slight reversed Bohr effect and the late gestation fetal hemoglobin seems to show an intermediate response to pH.

Addition of 1 mM ATP to the various stripped hemoglobin samples reduces the oxygen binding affinity of each sample at pH values below approximately 7.8 (Fig. 8). P_{50}'s estimated at pH 7.1 from oxygen binding curves are 8, 12, and 14 torr without ATP and 13, 21, and 27 torr with ATP for mid-gestation fetal, late gestation fetal, and adult stripped hemoglobins respectively. As the pH is lowered below about 7.8, the effect of 1 mM ATP increases; approximately to the same extent for each hemoglobin sample. The ϕ values between pH 6.5 and 7.5 for mid-gestation fetal, late gestation fetal, and adult hemoglobin in the presence of 1 mM ATP are approximately -1.0, -1.3, and -1.0 respectively. The Bohr effect may be slightly stronger in the presence of 1 mM ATP than in its absence.

Fig. 9 shows plots of the Hill coefficient, h or cooperativity, versus pH. It appears that the lowest h values are obtained at low pH, that 1 mM ATP has little, if any, effect on h, and that cooperativity may be higher for adult than mid-gestation fetal hemoglobin.

Results of oxygen binding experiments therefore indicate that the mid-gestation fetal, late gestation

Fig. 8 The effect of 1 mM ATP on hemoglobin oxygen affinity. With (●) and without (○) ATP for adult (A), late gestation (B), and mid-gestation (C) fetal stripped hemoglobins.

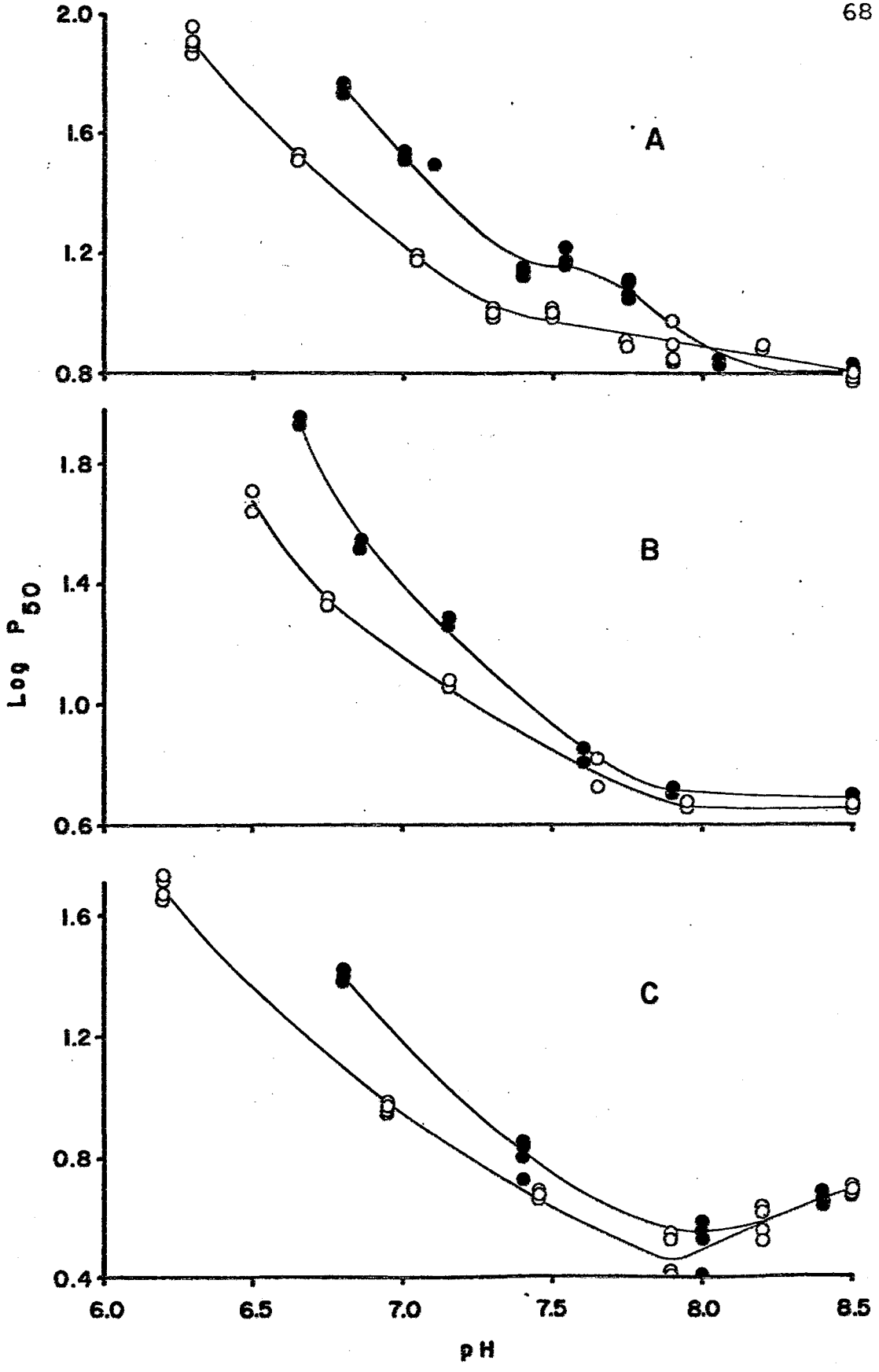
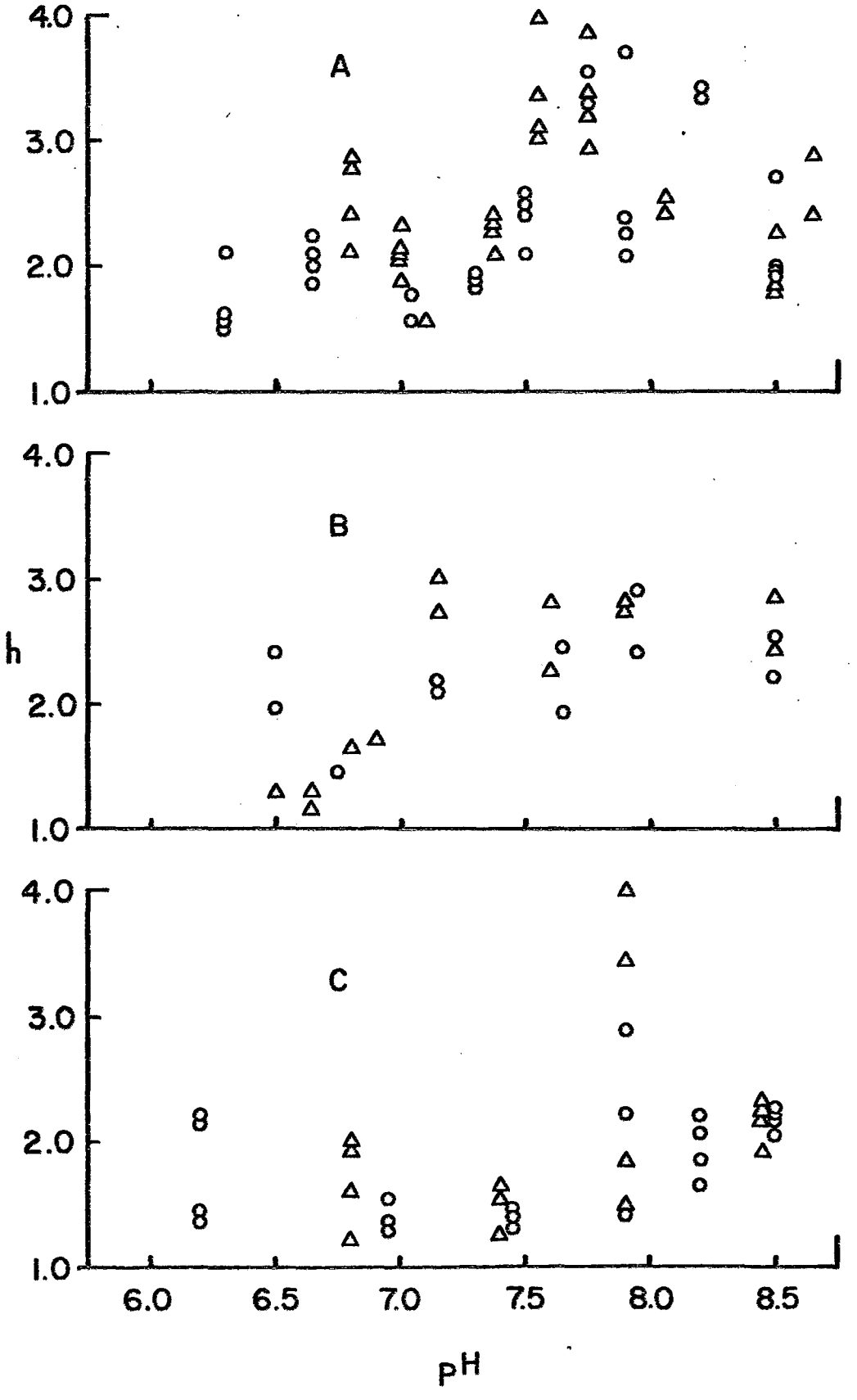

2.0
1.6
1.2
0.8
A
1.8
1.4
1.0
0.6
B
Log P50
1.6
1.2
0.8
0.4
C
6.0
6.5
7.0
7.5
8.0
8.5
pH

Fig. 9 The effect of pH changes and presence of ATP on the cooperativity, h, for adult (A), and late (B) and mid-gestation fetal (C) stripped hemoglobins.

⊖ : no ATP, △ : 1 mM ATP.


4.0
3.0
2.0
1.0
A
B
C
h
6.0
6.5
7.0
7.5
8.0
8.5
pH

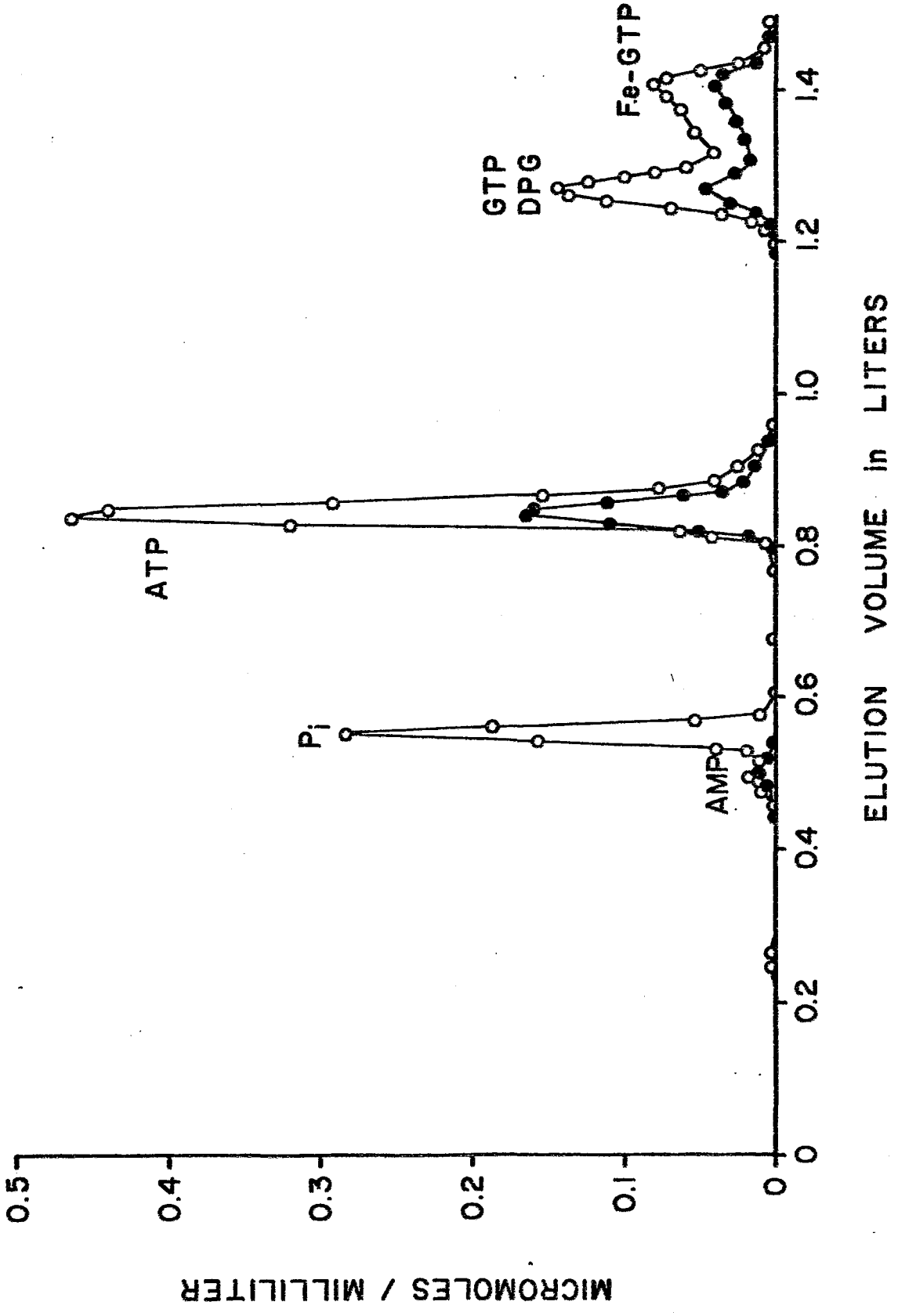
fetal, and adult stripped hemoglobins have different affinities for oxygen. As shown in Table 1 and Fig. 7, stripped mid-gestation fetal hemoglobin appears to have the highest, adult hemoglobin the lowest, and late gestation fetal hemoglobin an intermediate oxygen affinity. As indicated in Table 1, the P_{50}'s are close to one another yet at the pH's examined (which include the physiological, intracellular range) there is no overlap of the standard deviations. Furthermore, the late gestation fetal hemoglobin P_{50}'s estimated from Fig. 7 lie between those of adult and mid-gestation fetal hemoglobins without overlapping their standard deviations. The oxygen binding affinities of the stripped adult and mid-gestation fetal hemoglobins appear, therefore, to be significantly different. A possible challenge might be that some residual phosphate is present in the adult hemoglobin sample whereas the fetal hemoglobin samples have less phosphate. It has been shown, however, that very little phosphate remains with the adult hemoglobin after it has gone through the stripping procedure. Oxygen binding experiments with 1 mM ATP show that this allosteric modifier reduces the oxygen affinity of each pigment at pH values below approximately 7.8. Below this pH and in the presence of 1 mM ATP, mid-gestation fetal hemoglobin appears to retain a higher oxygen affinity than

adult hemoglobin; late gestation fetal hemoglobin again appears to have an intermediate oxygen affinity. If the oxygen binding affinity differences noted for stripped hemoglobins were due to residual phosphate, binding experiments at 1 mM ATP (an ATP to hemoglobin tetramer molar ratio of about 80) should eliminate the residual phosphate effect; a difference of affinities between adult and fetal hemoglobins in maintained, however. It appears, therefore, that the hemoglobins from animals at different developmental stages are functionally different. These results are consistent with the interpretation for structural data that these animals have different hemoglobins.

INTRACELLULAR ENVIRONMENT ANALYSIS

Separation of a standard mixture of inorganic and organic phosphates by ion exchange chromatography on Dowex-1-formate is shown in Fig. 10. Inorganic phosphate, ATP, and GTP recovery was 102%, 92%, and 94% respectively as determined by the total phosphate assay and absorbance of the nucleosides at 260 nm. These recovery values and the absence of any minor peaks on the elution profile indicate that little breakdown of the nucleotides occurred prior to and during the analysis. The elution position of

Fig. 10 Ion exchange analysis of a known mixture of inorganic and organic phosphates. The mixture contains 6.0, 7.1, 6.4, and 1.5 micromoles of inorganic phosphate, ATP, GTP, and 2,3 DPG, respectively. Elution was accomplished with linear gradient of 0-5 M ammonium formate buffer; total elution volume was equal to 1.5 lt. ● concentration of nucleoside based on 260 nm absorbance; -o- concentration of total phosphorus.


MICROMOLES / MILLILITER
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ELUTION VOLUME in LITERS
ATP
P_i
AMP
GTP
DPG
Fe-GTP

2,3 DPG can be inferred from the phosphorus assay curve and from previous reports of elution position under similar experimental conditions (Bartlett, 1976a). As reported by Bartlett (1976b) the GTP elution curve shows a bimodal pattern: the first peak corresponds to free GTP; the second peak corresponds to an iron complex as can be seen in Fig. 10. Figs. 11a and b show the elution profiles of adult and late gestation fetal red cell extracts. The major phosphate constituent of both fetal and adult red cells is ATP. ATP accounts for approximately 82% of the nucleoside triphosphate pool; the remainder is GTP. These proportions are very similar in both adult and late gestation fetal red cells. Fetal red cells appear to have a higher amount of inorganic phosphate than do adult cells. Fetal red cells contain 0.9 and adult cells, 0.3 mole inorganic phosphate per mole hemoglobin tetramer. Neither cell type contains any measurable amounts of inositol polyphosphates. A small peak in the fetal elution profile representing about 1% to 2% of the total soluble phosphorus may represent 2,3 DPG. This peak is not present in the adult elution pattern.

The enzymatic nucleoside triphosphate analysis of a standard ATP solution showed a recovery of 95%. Analyses of adult and late gestation fetal red cells, summarized in Table 2, yield mole NTP per mole hemoglobin tetramer

Fig. 11a Ion exchange analysis of E. lateralis adult red cell acid extract. System is as described for Fig. 10 with the exception that after elution with the ammonium formate buffer, 0.2 lt. of 1 M HCl was run through the column to free any inositol polyphosphates.

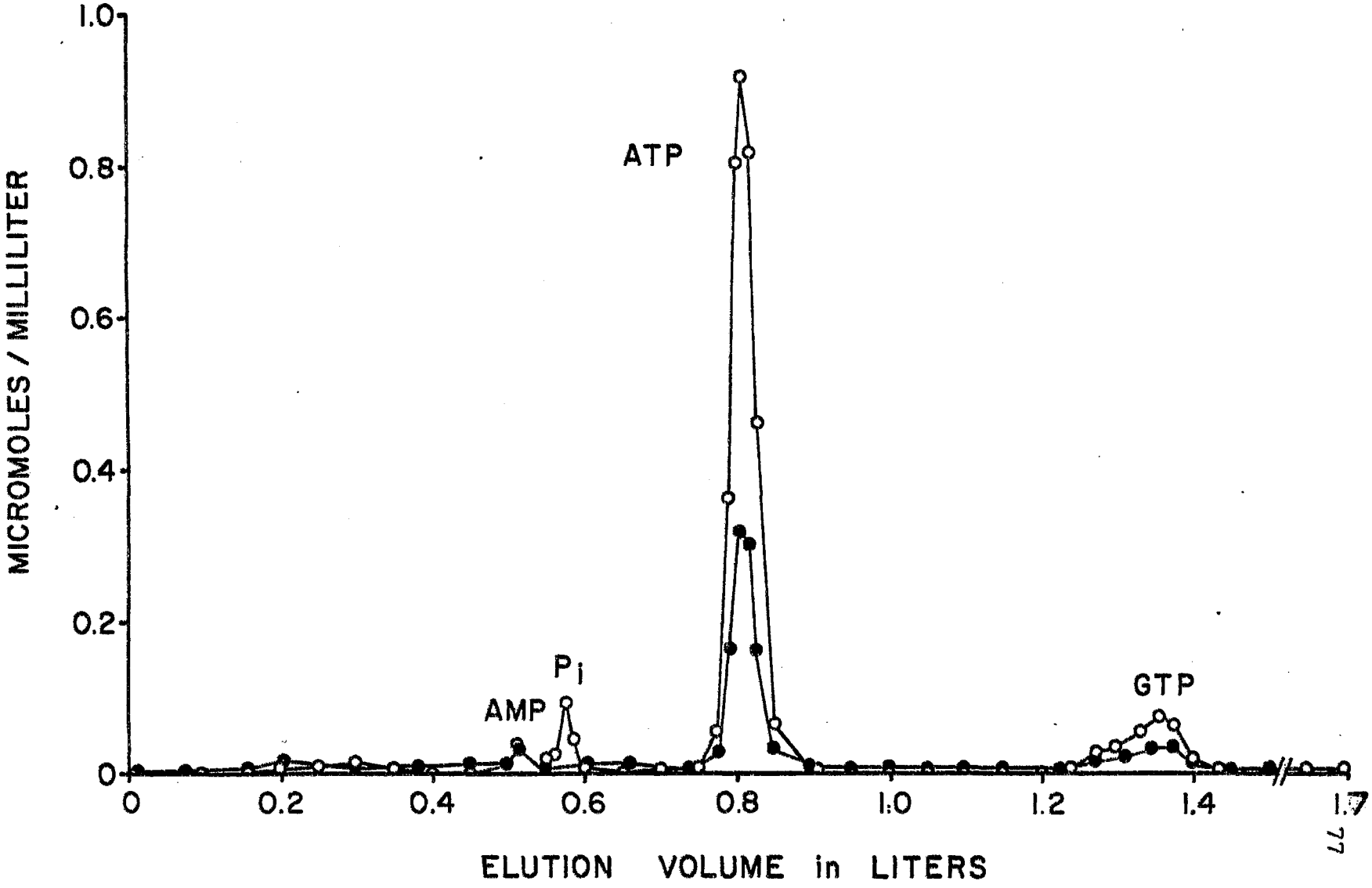

MICROMOLES / MILLILITER
1.0
0.8
0.6
0.4
0.2
0
ATP
Pi
AMP
GTP
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.7
ELUTION VOLUME in LITERS

Fig. 11b Ion exchange analysis of late gestation fetal red cell acid extract. System is as described for Fig. 11a with the exception that total elution volume of the ammonium formate buffer was 1.0 lt.

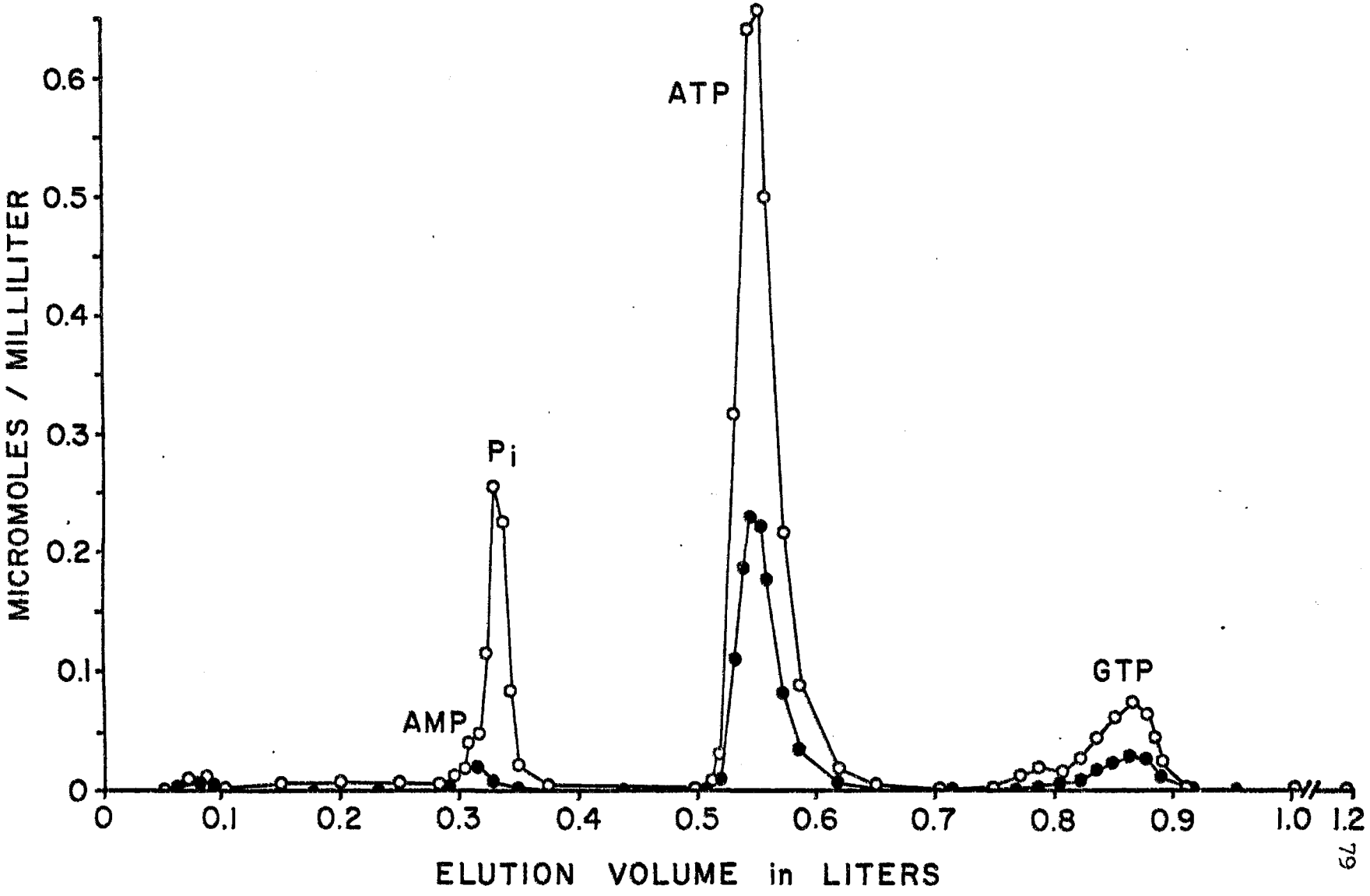

MICROMOLES / MILLILITER
0.6
0.5
0.4
0.3
0.2
0.1
0
ATP
Pi
AMP
GTP
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.2
ELUTION VOLUME in LITERS

TABLE 2: Results of enzymatic assays for nucleoside triphosphate expressed as moles NTP per mole hemoglobin tetramer.

Shown is the set of total data and the subset of results for fetuses and their mothers.

Total Data

	Adult	Late Fetus	Young
	2.90 2.96	1.43 1.54	2.68
	2.95 3.12	1.41 1.20	2.61
	2.38 2.96	2.28	2.60
	2.47 2.20	2.46	
	2.49	2.26	
Avg.	2.71 ± 0.33	1.80 ± 0.52	2.63 ± 0.04

Data for Fetuses and Their Mothers

Fetus	Adult	Difference
2.46	2.96	0.50
2.26	3.12	0.86
1.20	2.20	1.00
1.54	2.69	1.15
	Avg.	0.88 ± 0.28

ratios of 2.7 ± 0.3 (n=9) and 1.8 ± 0.5 (n=7) respectively. The red blood cells of young fish analyzed two days after birth had a molar NTP / hemoglobin ratio of 2.6 ± 0.1 (n=3). Analyses of NTP levels in blood cells of adults and their respective fetuses showed differences in molar NTP / hemoglobin ratios of 0.9 ± 0.3 (n=4). It appears, therefore, that adult cells contain more ATP and GTP per hemoglobin tetramer than do late gestation fetal red cells and that the amount of ATP in fetal cells approaches the ATP concentration of the adult quickly after birth.

The washing of red cells elevates cell NTP levels. The molar NTP to hemoglobin tetramer ratio in adult unwashed cells is 2.3 ± 0.2 (n=6). This is 87% of the value obtained from washed red cells. Storage of adult and late gestation fetal red cells on ice for four hours results in a decrease in cell NTP. Duplicate analyses of NTP in cells immediately after washing and in stored, washed cells shows that s rage results in an 11% NTP drop in both adult and late gestation fetal red cells.

The concentration of magnesium ions in whole cells is shown in Table 3. The measurements were carried out on TCA extracts of whole cells. The magnesium concentration reported as mole magnesium per mole hemoglobin tetramer for adult and late gestation fetal cells is

TABLE 3: Magnesium assays, results expressed as moles magnesium per mole hemoglobin tetramer.

A) TCA Extract of Whole Cells

	Adult	Late Fetus	Young
	2.18 3.38	2.32 2.51 2.86	2.61
	2.98 2.49	2.37 2.57 3.09	2.48
	2.58 2.79	2.48 2.59 3.18	2.71
	3.30 2.15	2.46 2.53 2.85	
Avg.	2.73 ± 0.47	2.65 ± 0.28	2.60 ± 0.12

B) TCA Extract of Red Cell Lysate Supernatant

Adult samples

Values obtained: 1.61, 1.70, 1.71, 1.60, 1.55

Avg. 1.63 ± 0.07

C) Atomic Absorption Spectroscopy of Red Cell Lysate Supernatant

Adult samples

Values obtained: 1.25, 2.13, 1.47

Avg. 1.62 ± 0.46

Calcium was too low to detect

2.7 ± 0.5 (n=9) and 2.6 ± 0.3 (n=12) respectively. The red cells of young fish two days after birth, have a molar magnesium to hemoglobin ratio of 2.6 ± 0.1 (n=3). Magnesium determinations of adult cells, lysed with distilled water and centrifuged to remove cell debris prior to TCA extraction, show a molar magnesium to hemoglobin ratio of 1.6 ± 0.1 (n=5). These data were obtained using a colorimetric test for magnesium and agree well with data obtained by atomic absorption spectroscopy (Mg/Hb = 1.6 ± 0.5, n=3). Calcium was also assayed by atomic absorption spectroscopy and could not be detected. This indicates that the molar ratio of calcium to hemoglobin is less than 0.1.

Oxygen binding experiments were carried out on stripped adult and late gestation fetal hemoglobins at low ATP concentrations. The ATP levels used ranged from 0 to 65 μM with hemoglobin and chloride concentrations of 12 μM and 1 mM respectively. The pH was 7.1 ± 0.1. The results are shown in Figs. 12a and b. There appears to be a linear relationship between the organic phosphate concentration and its effect on oxygen affinity over the concentration range examined. Adult and late gestation fetal hemoglobins appear to respond very similarly to low, apparently subsaturating, concentrations of ATP. The data indicate that the interactions and binding

Fig. 12a Sensitivity of adult stripped hemoglobin to low ATP. Hemoglobin concentration is 12 M and the mean P_{50} (no ATP) is 15.5 torr. The final pH determinations indicated that bindings occurred at pH 7.1 $\pm$ 0.1. A linear regression of P_{50} change versus increasing ATP concentration yields a slope of 0.1 torr / μM ATP.

○ : adult hemoglobin plus ATP

◇ : hemoglobin plus 46 μM Mg

◆ : hemoglobin plus 46 μM Mg and 46 μM ATP

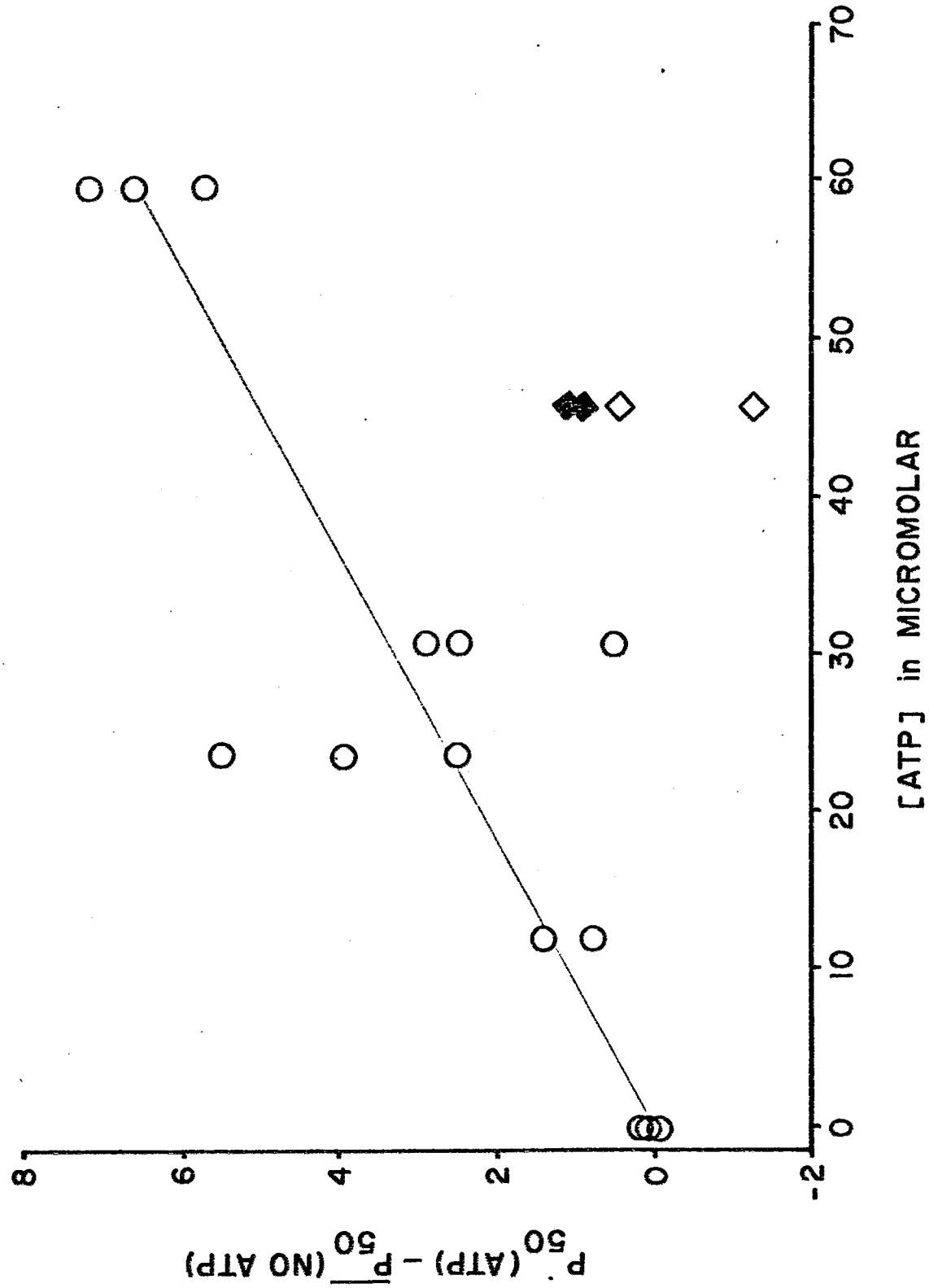
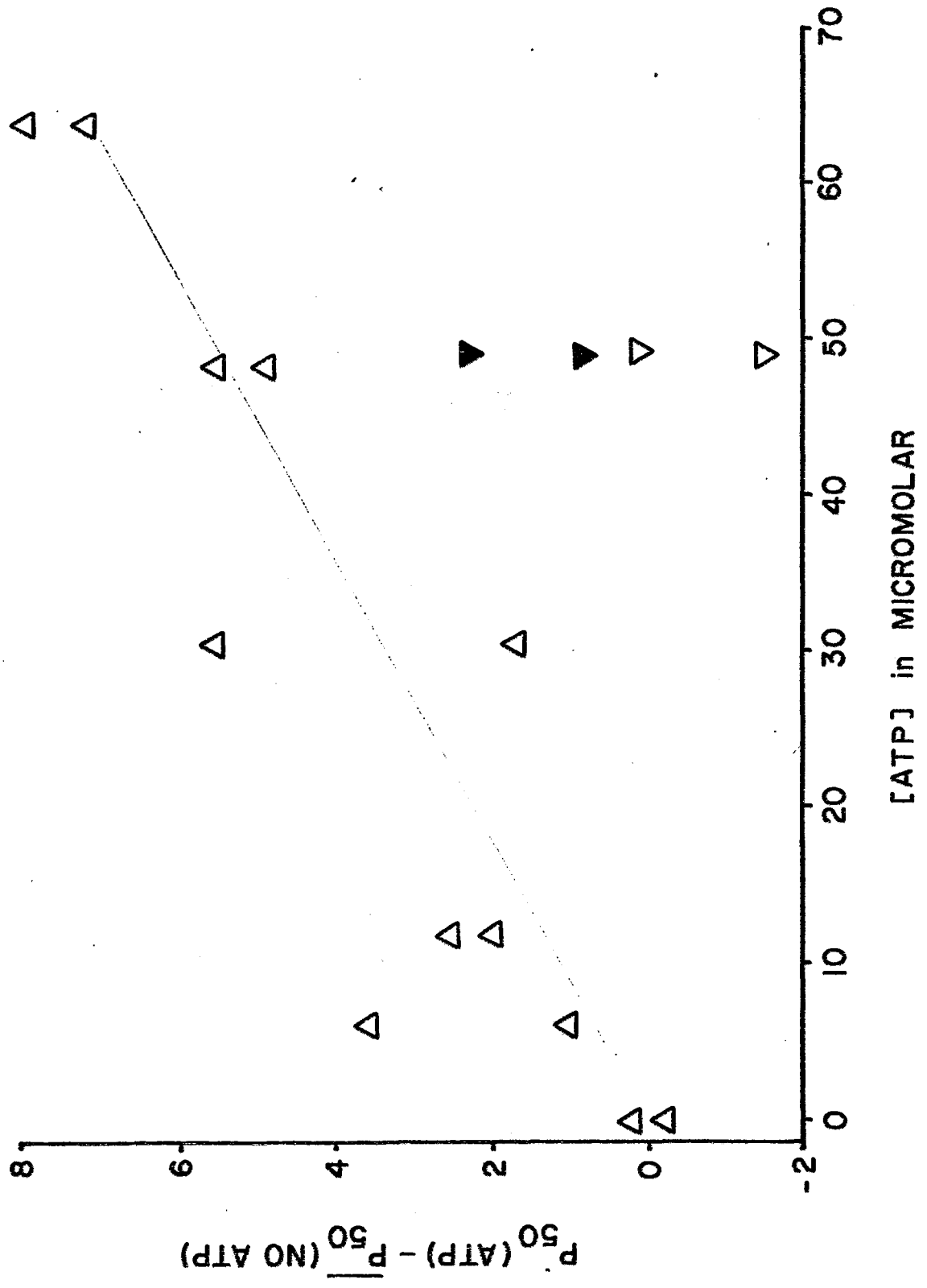

P'_{50} (ATP) – $\overline{P_{50}}$ (NO ATP)
8
6
4
2
0
-2
0
10
20
30
40
50
60
70
[ATP] in MICROMOLAR

Fig. 12b Sensitivity of late gestation fetal hemoglobin to low ATP. The mean P_{50} (no ATP) is 9.8 torr. The slope of the ATP-only bindings is also 0.1 torr / μ M ATP.

△ : late fetal hemoglobin plus ATP

▽ : hemoglobin plus 49 μ M Mg

▼ : hemoglobin plus 49 μ M mg and 49 μ M ATP


P_{50} (ATP) - $\overline{P_{50}}$ (NO ATP)
8
6
4
2
0
-2
0
10
20
30
40
50
60
70
[ATP] in MICROMOLAR

affinities of the adult and late gestation fetal hemoglobins with ATP are indistinguishable. Since magnesium was found to be present in fairly high intracellular concentrations, oxygen binding experiments were carried out on stripped adult and late gestation fetal hemoglobins with magnesium, with and without ATP. The magnesium concentration employed was about 50 μM. The oxygen binding results indicate that magnesium alone has little or no effect on oxygen affinity. Equal molar concentrations of magnesium and ATP, however, apparently result in a significant drop in the magnitude of the ATP effect. Consistent with published reports (Rose, 1968; Weber and Lykkeboe, 1978), magnesium appears to eliminate much of the ATP-hemoglobin interaction without directly affecting the hemoglobin molecule.

Intraerythrocytic analysis by ion exchange chromatography indicates, therefore, that ATP is very likely the major organic phosphate in both adult and late gestation fetal red cells. The absence of an ADP peak from the elution profiles, however, is unexpected in light of the apparently high nucleoside triphosphate concentrations. The apparent lack of nucleoside diphosphates causes some concern over the validity of the experimental method. The validity of the results and method is supported by the following findings. First, very high molar

ratios of nucleoside triphosphates to diphosphates have been reported using this ion exchange method for a variety of lower vertebrate red cells (Bartlett, 1976a; Borgese and Nagel, 1978). Furthermore, Bartlett (1979) has shown that a very high ratio of nucleoside triphosphates to diphosphates is not likely a consequence of the methodology. Using this ion exchange method, he obtained molar nucleoside triphosphate to diphosphate ratios of 0.03, 0.53, and 0.12 for eel red cell, liver, and skeletal muscle. Second, molar nucleoside triphosphates to hemoglobin tetramer ratios obtained by two different methods, ion exchange chromatography and enzymatic assay, agree well. Recovery of nucleoside triphosphate standards from the ion exchange column was 93%; recovery from the ion exchange column of nucleoside triphosphates from red cell extracts was 90% according to the enzymatic assays. The two methods, therefore, appear to yield similar results. The two methods utilize, however, acid extraction to obtain soluble phosphates from the cell matrix. Although seemingly unlikely, the acid extraction may act as, or stimulate, kinase activity thereby converting nucleoside di- to triphosphates. It is possible that E. lateralis red cells contain a high concentration of nucleoside triphosphates relative to diphosphates and that the ion exchange method employed was not sufficiently

sensitive to indicate the presence of a small amount of ADP. Further studies are necessary to conclude with certainty the exact nature of the red cell organic phosphates. Nonetheless, both ion exchange analysis and enzymatic assays indicate that adult red cells contain higher concentrations of total nucleoside triphosphates than do late gestation fetal cells. Since the oxygen binding experiments with low chloride and ATP concentrations indicate that stripped adult and late gestation fetal hemoglobins have oxygen affinities sensitive to low ATP titers, the difference between adult and fetal red cell oxygen affinities will probably be accentuated by the intraerythrocytic organic phosphate concentration differences. Intracellular magnesium concentrations will probably not eliminate an organic phosphate effect on red cell oxygen affinity since much magnesium appears to be associated with cell membranes rather than soluble polyanions such as ATP.

BLOOD PARAMETERS

The hematocrit, blood hemoglobin concentrations, and cell hemoglobin concentrations were measured for the late gestation fetus, young two days after birth, and adult. The results are shown in Table 4. Hematocrit

TABLE 4: Blood parameters: hematocrit, blood hemoglobin concentration, and cell hemoglobin or mean corpuscular hemoglobin as a function of the animal's developmental state. Young were animals two days after birth.

	Hematocrit	$[Hb]_{Blood}$	$[Hb]_{Cell}$
Late Fetus	36.6 ± 2.7 % n=16	$(8.8 \pm 0.8) \times 10^{-4}$ M n=8	2.4×10^{-3} M
Young	26.1 ± 6.3 % n=7	$(8.5 \pm 0.8) \times 10^{-4}$ M n=3	3.3×10^{-3} M
Adult	20.6 ± 4.2 % n=4	$(8.7 \pm 0.3) \times 10^{-4}$ M n=9	4.2×10^{-3} M

determinations showed a significant difference between adult and late gestation fetal blood. Adult blood has a hematocrit of 20.6 $\pm$ 4.2% (n=4) and the late gestation fetal blood 36.6 $\pm$ 2.7% (n=7). Young two days after birth appear to have a value, 26.1 $\pm$ 6.3%, which in intermediate between those of the adult and late gestation fetus. A possible criticism of these results in that animals were not completely blotted free of surface moisture or that tissue fluids diluted the blood. In either case, the resulting hematocrits would be underestimates, rather than overestimates, of actual levels; that is, if anything the hematocrits would be slightly larger than determined values. Since the fetal and young animals were of similar size and treated identically, comparisons of their blood parameters are likely to be accurate. Adult animals contain relatively large volumes of blood which quickly pour from the caudal artery after the peduncle is severed. Blood was taken as close to the several caudal artery as posible and it is unlikely that the blood was diluted by surface moisture or tissue fluids. Furthermore, hematocrit tubes never indicated the presence of tissue chunks which would lead to overestimates of hematocrits. As one can see from the standard deviations, the values were very reproducible for adult and late gestation fetal blood. Concentrations of hemoglobin in the blood for

late gestation fetus, newborn, and adult are indistinguishable, 0.87 mM hemoglobin tetramer. Since samples for hematocrit and blood hemoglobin determinations were taken in identical manners, the criticisms and justifications of method and results are those previously expressed for the validity of hematocrit comparisons. The calculated values for intracellular hemoglobin concentration indicate a cellular hemoglobin dilution in fetal relative to adult cells. An alternative, though unlikely, possibility is that the intracellular hemoglobin concentrations are the same, but that the difference in hematocrits is due to enlarged organelles (such as the nucleus) in the younger animals, or that the blood of younger animals is mixed with a large proportion of non-hemoglobin containing cells. This latter possibility seems unlikely, as the packed blood cells resulting from the hematocrit determinations did not show any major fraction of colorless cells. The mean corpuscular hemoglobin concentrations were determined from the hematocrits and blood hemoglobin concentrations and appear to be 2.4, 3.3, and 4.2 mM hemoglobin tetramer for late gestation, young, and adult red cells respectively.

DISCUSSION

It is concluded that E. lateralis utilizes the strategy of having different fetal and maternal hemoglobins as well as different concentrations of organic phosphates in maternal versus fetal blood to facilitate a diffusion of oxygen from adult to fetal fish. There is a possibility that hemoglobin dilution, within the red cell of the fetus, also contributes to this effect. These characteristics of the hemoglobins and the red cells are part of a general physiological adaptation for viviparity in this fish which include the striking morphological specializations of the fetus and mother.

The highly vascularized fins and fin spatulates of the E. lateralis fetus lie in close contact with the vascularized ovarian sheets of the mother. This juxtaposition of the respiratory exchange surfaces permits oxygen to diffuse down its concentration gradient from maternal to fetal blood. A higher oxygen affinity of the fetal versus adult blood should enhance the concentration gradient and increase the efficiency of fetal oxygen uptake. This difference in affinities probably exists in most viviparous animals. It is due, in part, to the Bohr effect which affects both fetal and

adult hemoglobins. At the placenta or pseudoplacenta, carbon dioxide, bicarbonate, protons, and chloride effectively leave the fetal red cell (probably as CO_2 and HCO_3^-, Forster, 1973; Cameron, 1978, 1979). This increases the oxygen affinity of the fetal red cells. These molecules and ions effectively enter the adult red cell (also as CO_2 and HCO_3^-), thereby decreasing maternal red cell oxygen affinity. In addition, E. lateralis utilizes different hemoglobins, different organic phosphate concentrations, and possibly hemoglobin dilution to accentuate the difference in oxygen affinities of adult and fetal blood. The results of this study are discussed with respect to hemoglobin structure and erythorocytic environment (allosteric modifiers).

HEMOGLOBIN STRUCTURE

The hemoglobins of both fetal and adult E. lateralis are tetrameric with apparent molecular weights of 54,000 on Sephadex G-100 and subunits with molecular weights of 15,000 by SDS gel electrophoresis. The molecular weight value of 54,000 is alightly lower than might be predicted or is found for other vertebrate hemoglobins (Riggs, 1970). One explanation for the low molecular weight may be that a reversible tetramer-dimer dissociation occurs. The

molecular weight determined may be the average molecular weight of tetramers and dimers; it may therefore be an indication of a tendency to dissociate. Fynn and Sullivan (1975) reported that some shark hemoglobins readily dissociate to dimers. In contrast, Edelstein et al. (1976) reported that fish hemoglobins, including one shark species, have extremely low tetramer-dimer dissociation constants. In general, those tetrameric fish hemoglobins which have been studied in detail dissociate appreciably less than do mammalian hemoglobins. Thus, it appears unlikely that the relatively low molecular weight determined for E. lateralis hemoglobin by Sephadex chromatography is due to a tetramer-dimer dissociation. A more likely possibility is that some hemoglobins adsorb to Sephadex as reported by Wilkens (1970) and Iuchi (1973). This would yield a lower molecular weight value than expected. E. lateralis hemoglobins, from all developmental states studied, electrophorese in SDS as a single 15,000 molecular weight polypeptide chain. The chains from the developmental states examined co-electrophorese. It appears, therefore, that the size of the polypeptide chains does not change appreciably during development. SDS gel electrophoresis of human hemoglobin under the same conditions results in two bands. These

two bands correspond to the two human hemoglobin chains, the α and β chains, which are composed of 141 and 146 amino acids respectively (Zuckerkandl, 1965). The subunits of E. lateralis hemoglobins, therefore, probably differ less in molecular weight from one another than do human α and β chains.

Ion exchange chromatography of either mid-gestation fetal or adult CO-hemoglobin results in the elution of a single major hemoglobin component. They both eluted from the column at virtually identical NaCl concentrations. The strength of binding to the DEAE cellulose matrix and salt concentration required to displace the protein are determined primarily by the surface charge distribution of the protein. It appears that the surface charge distributions of adult and mid-gestation fetal hemoglobins are very similar if not identical.

Polyacrylamide disc gel electrophoresis separates molecules on the basis of net electrical charge. Electrophoresis of mid-gestation fetal and adult CO-hemoglobins consistently results in a single major band. In this respect, their behavior is similar to that seen with DEAE ion exchange chromatography. The major bands appear to co-electrophorese although the resulting band may be somewhat wider than the individual bands. This may indicate some difference between mid-gestation fetal and

adult hemoglobins Minor bands are seen in addition to the major component. However, their pattern is not consistent from one experiment to the next. Human CO-hemoglobin electrophoresed under the same conditions shows a single major with several minor bands. Dissociation of the CO-hemoglobin to deoxyhemoglobin or oxidation to methemoglobin may have given rise to the minor bands. Despite the minor peaks and their inconsistent behavior on electrophoresis, one can conclude that the major components of the adult and fetal hemoglobins appear to be very similar with respect to net charge at the pH values examined.

One of the main reasons for concluding that adult and fetal hemoglobins are structurally different is based on the urea gel electrophoresis data. In the presence of concentrated urea at low pH with reducing agent, apohemoglobin dissociates into individual globin chains and at pH 2.2, the chains are almost maximally protonated. Under these conditions, mid-gestation fetal apohemoglobin separates into two major components, two globin chains. Late gestation fetal apohemoglobin separates into one major and three lighter staining components; the major and anode-most of the lighter staining components correspond to the two mid-gestation fetal globins. The other two late gestation fetal lighter staining globins correspond

to the two lighter staining components of the adult pattern. The adult pattern also contains a single major component which corresponds to the major late gestation fetal component and to the anodal mid-gestation fetal component. One single globin chain, therefore, appears to be common to all developmental states. The mid-gestation fetal and adult hemoglobins appear to be composed of additional distinct globin chains. The late gestation fetal hemoglobin represents a transition state between the mid-gestation fetal and adult hemoglobins. The developmental states might be described by utilizing the human fetal and adult tetramer composition symbolism (Bank et al., 1980) where $\alpha_2\gamma_2$, $\alpha_2\gamma_2 + \alpha_2\beta_2 + \alpha_2\delta_2$ and $\alpha_2\beta_2 + \alpha_2\delta_2$ represent the mid-fetal, late fetal, and adult tetrameric states respectively. This interpretation may explain the inability to resolve the proteins better by gel electrophoresis as the only difference between adult and fetal hemoglobins are those of the half-molecules. α_4 and β_4 molecules might be better resolved. The tetramer composition model assumes that the tetramers are a symmetrical A_2B_2 type. Although this is generally true for vertebrate hemoglobins (Coates, 1975), Tsuyuki and Ronald (1971) have shown that some salmon hemoglobins have asymmetric compositions; that is, have A_2BC or ABCD subunit structures. Homotetramers,

A_4 or B_4, are also possible. Nonetheless, the patterns obtained by urea electrophoresis indicate that a single globin component comprises approximately half of the total protein and that this component is common to all the developmental states studied. In animals possessing distinct fetal hemoglobins including sheep, human, and baboon (Kitchen and Brett, 1974; De Simone and Mueller, 1979), adult and fetal hemoglobins share a common globin chain; the other chain is distinctly fetal or adult. It appears that E. lateralis could also fit into this pattern.

The difference between adult and fetal electrophoretic patterns is not likely to be an artifact of technique for the following reasons. Human hemoglobin, run under the same conditions, yields the expected results for an A_2B_2type molecule. The electrophoretic patterns for E. lateralis hemoglobins are reproducible from one preparation to another. The adult and fetal hemoglobins were prepared, treated, and run under identical conditions. Although urea solutions develop reactive cyanate ion on standing (Hirs, 1967), cyanate production does not appear to be a responsible explanation for the different mid-gestation fetal, late gestation fetal, and adult apohemoglobin electrophoretic patterns.

The electrophoretic differences seen among

mid-gestation fetal, late gestation fetal, and adult apohemoglobins in the presence of urea at low pH are slight and probably represent subtle structural differences. This may explain why no structural differences between mid-gestation fetal and adult hemoglobins could be detected by the chromatographic and electrophoretic techniques mentioned previously, especially if the hemoglobins are $\alpha_2\beta_2$ and $\alpha_2\gamma_2$. On the other hand, minor changes in hemoglobin structure can have significant physiological consequences. For example, replacement of one amino acid (glutamic acid) by another (valine) in human β chains results in sickle hemoglobin (Bunn et al., 1977). Sickle hemoglobin polymerizes upon deoxygenation resulting in a distortion of the red cell, an increase in blood viscosity, and a decrease in oxygen affinity (Bertles, 1974).

OXYGEN BINDING

The second major reason for concluding that adult and fetal hemoglobins are structurally different is that they have different affinities for oxygen. Oxygen equilibrium studies show that at the pH's examined, stripped mid-gestation fetal hemoglobin has the highest oxygen binding affinity and stripped adult hemoglobin has the lowest.

Consistent with the electrophoretic data which suggest that late gestation fetal hemoglobin is structurally intermediate between mid-gestation fetal and adult hemoglobins, late gestation fetal hemoglobin shows an intermediate oxygen binding affinity. The quantitative differences in P_{50} values of mid-gestation fetal and adult hemoglobins are comparable to the differences noted by Manwell (1958) for the adult and fetal hemoglobins of the shark, S. sucklevi.

Mid-gestation fetal, late gestation fetal, and adult stripped hemoglobins appear to respond very similarly to pH changes at pH values below 7.5. This may reflect a structural similarity between fetal and adult hemoglobins. Above pH 8.0, however, the Bohr effects of the pigments appear different. The adult hemoglobin seems to show a smaller normal Bohr effect and the mid-gestation fetal hemoglobin seems to show a slight reversed Bohr effect. The late gestation fetal hemoglobin shows an intermediate response to pH changes. Although the P_{50} values determined above 8.0 have little if anything to do with the physiology of the animals, oxygen equilibria data obtained at these pH's appear to reemphasize the inherent structural differences between mid-gestation fetal, late gestation fetal, and adult hemoglobins.

ATP is the major organic phosphate of E. lateralis

adult and fetal red blood cells. Addition of 1 mM ATP to the hemoglobin solution (an ATP to hemoglobin tetramer molar ratio of about 80) significantly reduces the oxygen binding affinity of the hemoglobin *in vitro*. ATP appears to lower the oxygen affinity of the mid-gestation fetal, late gestation fetal, and adult hemoglobins similarly from a pH of about 6.7 to 8.5. In other words, through out this pH range, in the presence or absence of ATP, mid-gestation fetal appears to have the highest and adult hemoglobin has the lowest oxygen affinity, with late gestation fetal hemoglobin an affinity intermediate between the other two. This observation argues against the possibility that the affinity differences noted for "stripped" hemoglobins are the result of incomplete stripping of the hemoglobins. This is further evidence that the adult and fetal hemoglobins are structurally and functionally different. The effect of ATP on the oxygen affinities of these hemoglobins is inversely related to the pH. Above pH 8.0, ATP has no affect on any of the pigments; this may be due to deprotonization of the functional groups at the organic phosphate binding site at high pH (Benesch *et al.*, 1969); Gillen and Riggs, 1977).

The mid-gestation fetal, late gestation fetal, and adult stripped hemoglobins appear to have a Bohr effect

of ϕ equal to about -0.8 to -0.9. This is a large Bohr effect relative to that reported for most animal hemoglobins (Prosser, 1973). A hemoglobin with a high pH sensitivity may be functionally important in facilitating oxygen unloading at the relatively acidic tissues as well as oxygen loading at the respiratory exchange surfaces of both fetal and adult fish. 1 mM ATP appears to increase the value of ϕ to about -1.0. Thus, the presence of ATP may further accentuate the oxygen loading and unloading responses of hemoglobin to the tissue pH's.

The Hill coefficient, "h", is an indication of the extent of heme-heme interaction, which is the facilitation of hemoglobin oxygenation after the first oxygen molecules have bound to the protein. The plot of h versus pH is very scattered (Fig. 9). Some of this scatter may be explained by the proposed hemoglobin heterogeneity. A combination of hemoglobins with different oxygen binding characteristics could yield an oxygenation curve (that is, a plot of percent hemoglobin oxygenation versus oxygen concentration) which deviates in shape from the sigmoid plot of a single tetrameric, vertebrate hemoglobin. A Hill plot of such data from a heterogeneous hemoglobin system may yield a non-linear graph. Depending on which data points are considered, a variety of slopes, or h values, could be obtained from a straight line drawn

through available data points. The plot of h versus pH is too scattered to allow conclusional interpretations; nevertheless, several inferences can be made. It appears that h may decrease with decreasing pH. Furthermore, it appears that high levels of ATP have relatively little effect on h. Finally, it appears that h is higher for adult than mid-gestation fetal hemoglobin over most of the pH range examined. A similar difference of h values exists for adult and embryonic rabbit hemoglobins (Jelkmann and Bauer, 1978), the opposite occurs in the Japanese monkey (Takenaka and Morimoto, 1976), and there appears to be no difference in h values between adult and fetal human hemoglobins (Takenaka and Morimoto, 1976; Tyuma and Schimizu, 1969). A decreased h values for E. lateralis fetal hemoglobin indicates that oxygenation and deoxygenation can take place over a larger oxygen concentration range. This appears to be a disadvantage to fetal oxygen loading and unloading. On the other hand, it may be an advantage if the oxygen tension of the maternal blood ever drops below the P_{50} of the fetal blood. In such a situation hemoglobin with a low h could pick up more oxygen than one with a high h if the p_{50}'s are equivalent. A possible difference in cooperativity of fetal versus adult hemoglobin is consistent with the contention that these hemoglobins are structurally different.

Gel electrophoresis in urea indicates that adult and mid-fetal hemoglobins are structurally different and that late fetal hemoglobin is a composite of those hemoglobins. Quantitative and qualitative data from oxygen binding experiements also support this hypothesis. The higher oxygen affinity of fetal hemoglobin strongly suggests that it has a major role in facilitating fetal oxygen uptake. Furthermore, the molecular mechanism proposed for E. lateralis is similar to that seen in the ruminants and possibly in the shark, S. suckleyi.

INTRAERYTHROCYTIC ENVIRONMENT

2,3 DPG is the major and ATP a minor regulator of hemoglobin function in most mammals (Rapoport and Guest, 1941; Scott et al., 1977). ATP is the major hemoglobin regulator in most fishes (Coates, 1975); however, other potential organic phosphate modulators are found in fish red blood cells. Parks et al. (1973) reported high GTP levels in erythrocytes of eels and lemon sharks. GTP apparently is the major intraerythrocytic modifier in at least six other species of fish (Bartlett, 1978a; Borgese et al., 1979; Leray, 1979). In addition, other organic phosphates such as uridine triphosphate, inositol diphosphate, and inositol pentaphosphate are present in

moderate concentrations in some fish red cells (Bartlett, 1978b and c; Borgese and Nagel, 1978). It was, therefore, essential to find out what organic phosphates are present in E. lateralis erythrocytes as different organic phosphates have effects of different magnitudes on the hemoglobin (Benesch and Benesch, 1969; Hamasaki and Rose, 1974). Qualitative analysis of adult and late gestation fetal red cells clearly shows ATP to be the major organic phosphate in both types of erythrocytes. ATP accounts for approximately 82% of the nucleoside triphosphate pool in the cells of both; the remainder is GTP. On a molar basis, the ratio of ATP and GTP to hemoglobin tetramer was found to be 2.1 and 0.5 respectively for adult erythrocytes and 1.8 and 0.4 respectively for fetal erythrocytes.

Fish respond to experimental hypoxia by reducing their nucleoside triphosphate levels and thereby increasing their blood oxygen affinities (Wood and Johansen, 1973; Wood et al., 1975; Greany and Powers, 1977; Weber and Lykkeboe, 1978). Weber and Lykkeboe (1978) reported that hypoxia in carp leads to a greater reduction in erythrocyte GTP than ATP; this is true even though ATP is the major cell organic phosphate. They speculated that GTP plays a greater role than ATP in the allosteric regulation of blood oxygen affinity. A lower organic

phosphate content of the E. lateralis late fetal versus adult red cells probably results in a higher oxygen affinity of the fetal versus adult blood. The late gestation fetus may be considered, at least to some extent, as a hypoxic adult (to be discussed in more detail later). If this is the case, GTP does not appear to play a greater role than ATP in dealing with hypoxia in E. lateralis. GTP is less than 20% of the total NTP and the GTP to ATP ratio is very similar in fetal and adult erythrocytes.

There appear to be no other organic phosphates in late gestation fetal and adult red cells of E. lateralis in sufficient concentration to affect oxygen binding. Inorganic phosphate is present however. Watt (1976) reported that phosphates reduce oxygen affinity in the following order of decreasing effectiveness: 2,3 DPG > ATP > ADP > AMP > P_i; all apparently binding to the same site of the tetramer. Hamasaki and Rose (1974) have shown that the binding of inorganic phosphate to hemoglobin is at least 300 times weaker than is the binding of ATP. Thus, although the molar P_i to hemoglobin ratio is 0.9 and 0.3 for late gestation fetal and adult erythrocytes respectively, it is unlikely that P_i at these concentrations will exert any measurable effect on the hemoglobins.

In order to better quantify actual amounts of organic phosphates in adult and late gestation fetal erythrocytes,

an enzymatic assay for total nucleoside triphosphate was used. Storage of red cells prior to the assays (analogous to the time required to collect sufficient quantities of fetal blood) resulted in a decline of NTP concentrations; washing the red cells resulted in an increase. Since adult and fetal cells were collected in a similar manner involving storage and washing, the determined intracellular NTP titers are probably very close to in vivo levels and the comparison of the values in adult and fetal cells is valid. Molar ratios of NTP to hemoglobin tetramer were 2.71 $\pm$ 0.33 for adult and 1.80 $\pm$ 0.52 for late gestation fetal cells. The difference is highly significant based on the "Student's t" distribution. Furthermore, when NTP to hemoglobin tetramer levels were examined for fetuses and their mothers, adult levels were always higher. The magnitude of the difference between E. lateralis adult and late gestation fetal red cell NTP per hemoglobin was found to be 0.9. This value is very similar to the organic phosphate concentration difference between adult and fetal red cells for rabbit, horse, pig, and mouse. The difference in these animals is 1.2 moles 2,3 DPG per mole hemoglobin (Baumann et al., 1973; Bunn and Kitchen, 1973; Bauer et al., 1975; Jelkmann and Bauer, 1977; Petschow et al., 1978; Bard and Shapiro, 1979). These animals have no unique fetal hemoglobin

and the difference in 2,3 DPG levels accounts for the higher oxygen affinity of fetal versus adult blood. The functional importance of the difference in NTP concentrations between E. lateralis adult and late gestation fetal red cells is further supported by the following findings. First, adult and fetal hemoglobins are sensitive to ATP and undoubtedly to GTP and these organic phosphates are found in higher concentrations in adult versus fetal cells. Second, eel, flatfish, minnow, and carp acclimated to hypoxia show reduced levels of NTP / hemoglobin and increased blood oxygen binding affinities relative to normoxic controls (Wood and Johansen, 1973; Wood et al., 1975; Greany and Powers, 1977; Weber and Lykkeboe, 1978). The decrease from normoxic to hypoxic NTP / hemoglobin levels in these fish is smaller than the difference in red cell NTP / hemoglobin levels found between adult and late fetal E. lateralis. The results of this study are therefore in accord with the proposal that a difference in red cell organic phosphate concentrations facilitates oxygen uptake by the E. lateralis late fetus.

Oxygen binding experiments were conducted on stripped adult and late gestation fetal hemoglobins in the presence of different concentrations of ATP. The data show that these pigments respond similarly, if not identically, to

low amounts of ATP. Human fetal blood has a higher oxygen affinity than adult blood due to the lower affinity of fetal versus adult hemoglobin for 2,3 DPG. This molecular basis for a maternal-fetal oxygen transfer does not appear to be utilized by E. lateralis even though the fetal hemoglobin is structurally different from that of the adult.

Several oxygen binding experiments were conducted with a low concentration of magnesium, with and without an equal concentration of ATP. The results indicate that magnesium alone has little, if any, effect on the oxygen affinities of late gestation fetal and adult E. lateralis hemoglobins. The results of the binding experiments with magnesium and ATP indicate that magnesium prevents most of the ATP from binding with the hemoglobin. Magnesium, and supposedly calcium, binds to ATP forming a fairly stable complex. ATP binds to hemoglobin more strongly than does the ATP-magnesium complex. Magnesium, therefore, tends to increase oxygen affinity of hemoglobin by blocking the ATP-hemoglobin interaction (Rose, 1968; Weber, 1978; Weber and Lykkeboe, 1978; Burton, 1980).

Magnesium levels were determined for whole adult and late gestation fetal red blood cells. There were no statistically significant differences in total magnesium concentration between the fetal and adult cells. The

levels found were approximately 2.7 moles of magnesium per mole hemoglobin tetramer. If complete complexing of this amount of magnesium with cell NTP were to occur, there would be no free NTP to interact with hemoglobin in either fetal or adult red cells. Magnesium could eliminate an organic phosphate effect from the maternal-fetal oxygen transfer. When lysed adult red cells were centrifuged to remove cell membrane debris, the supernatant (that fraction containing the hemoglobin) contained substantially less magnesium. Magnesium was detected in concentrations of about 1.6 moles of magnesium per mole hemoglobin tetramer rather than 2.7. Much magnesium, therefore, appears to be associated with cell membranes. This association has been confirmed for other types of cells in other animal systems (Rose, 1968; Sanui, 1970; Rubin, 1975; Sanui and Rubin, 1979). It is likely that the non-membrane-bound levels of magnesium are equivalent in adult and late gestation fetal red cells. If complete complexing occurs between non-membrane-bound magnesium (1.6 Mg/hemoglobin) and NTP in adult (2.7 NTP/hemoglobin) and fetal red cells (1.6 NTP/hemoglobin), the adult cells would still have substantially higher free NTP concentrations capable of interacting with hemoglobin. It seems unlikely, therefore, that red cell magnesium concentrations will eliminate the difference in hemoglobin-

interactable organic phosphate titers between adult and late gestation fetal red cells. This difference is probably appreciable and is very likely involved in the maternal-fetal oxygen transfer. Non-membrane-bound calcium was not detectable in adult red cells and therefore will probably not affect the maternal-fetal shift.

It appears that the blood of the late fetus will have a higher oxygen affinity than adult blood due to two major molecular mechanisms. First, the fetus has a structurally distinct hemoglobin with a higher oxygen affinity than adult hemoglobin. Second, the fetus has a significantly lower red cell nucleoside triphosphate level relative to the adult. This is the first report of a dual mechanism of this kind being involved in the maternal-fetal oxygen transfer.

BLOOD PARAMETERS

In humans, sheep, and cats, fetal blood has a higher oxygen carrying capacity, that is, a higher blood hemoglobin concentration, than does adult blood (Novy and Parer, 1969). There was no difference in oxygen carrying capacities between adult and late gestation fetal E. lateralis blood. The hematocrits (proportion of whole blood volume occupied by the erythrocytes), however, were

significantly different between adult and late gestation fetal blood. Fetal cells occupied about 1.8 times more blood volume than the adult cells. The late gestation fetal mean corpuscular hemoglobin concentration (by definition the blood hemoglobin divided by the hematocrit) is consequently about 60% of the adult value. Therefore, although blood hemoglobin levels are equivalent in adult and late gestation fetal cells, the hemoglobin appears to be more dilute in the fetal cells. It has been shown by several researchers that hemoglobin dilution in the range of hemoglobin concentrations encountered in fish red blood cells is associated with an increased oxygen binding affinity (Bellingham et al., 1971; Forster, 1972; Laver et al., 1977; Lykkeboe and Weber, 1978). Hemoglobin dilution in fetal cells therefore probably contributes to a slightly higher oxygen affinity of the late gestation fetal versus adult blood in E. lateralis.

SUMMARY OF FINDINGS

It is probable that at mid-gestation, fetal blood of E. lateralis has a higher oxygen affinity than adult blood. At this developmental state, fetal hemoglobin is structurally unique and has a higher oxygen affinity than adult hemoglobin. At an advanced developmental

state, fetal blood appears to contain a mixture of fetal and adult hemoglobins. This mixture of stripped hemoglobins retains a higher oxygen affinity than stripped adult hemoglobin but lower than mid-gestation fetal hemoglobin. Additionally, late gestation fetal red cells contain lower levels of organic phosphates than do adult red cells. Low concentrations of organic phosphates will probably increase oxygen affinity of the fetal blood through a lack of a direct effect on hemoglobin and through maintainence of high pH via the Gibbs-Donnan effect. Finally, hemoglobin dilution in fetal cells will probably increase cell oxygen binding affinity as previously outlined.

SPECULATIONS ON THE MATERNAL-FETAL SHIFT

EMBRYONIC HEMOGLOBINS AND ONTOGENETIC ORGANIC PHOSPHATE CHANGES

Mammals appear to have one or more embryonic hemoglobins. These mammalian hemoglobins are synthesized from the yolk sac, as opposed to liver or myeloid, erythropoiesis (Kitchen and Brett, 1974). Hemoglobins, therefore, occur in the embryo prior to complete formation of the placenta where they may facilitate oxygen uptake from the

maternal interstitial fluid (Lorkin, 1973; Kitchen and Brett, 1974). These hemoglobins are replaced by fetal or adult hemoglobins early in gestation. Several mechanisms appear to be involved in embryonic hemoglobin- and red cell-oxygen interactions. Huehns and Farooqui (1975) found no difference in oxygen binding properties of human embryonic and fetal red cells. Both cell types, however, have a higher oxygen affinity than have adult cells. Intraerythrocytic concentrations of 2,3 DPG and pH do not appear to be a function of gestational age (Bard and Teasdale, 1979). This appears to be true in sheep as well (Bard et al., 1978). Two interesting situations occur in the mouse and rabbit. Neither animal possesses a fetal hemoglobin. Bauer et al. (1975) and Wells (1979) reported that stripped embryonic mouse hemoglobin has a higher oxygen affinity than adult mouse hemoglobin. Furthermore, adult red cells contain approximately 1.3 more moles of 2,3 DPG per hemoglobin than do embryonic cells. Wells (1979) found that fetal red cell 2,3 DPG concentrations decrease during gestation, presumably to partially counter the increasing proportion of adult to embryonic hemoglobin. In contrast, Petschow et al. (1978) found that 2,3 DPG concentrations are relatively constant but ATP levels drop during mouse development. They reported that the oxygen affinity of the blood was

highest just prior to birth. It appears, therefore, that organic phosphate levels decrease during development and that this occurs as embryonic hemoglobin is being gradually replaced by adult hemoglobin. Rabbits have an embryonic hemoglobin characterized by a high oxygen affinity, yet embryonic blood has only a slightly higher oxygen affinity than adult blood. The oxygen affinity of the blood increases during intrauterine development, occurring even with a switch to adult hemoglobin early in gestation. The increase in oxygen affinity is largely mediated directly and indirectly (via an influence on pH) by decreasing concentrations of both 2,3 DPG and ATP (Jelkmann and Bauer, 1977; 1978).

A decrease in red cell organic phosphate concentrations during development occurs in at least some amphibians. The red cells of Rana catesbeiana tadpoles contain over twice as much ATP and 2,3 DPG as do adult cells yet the oxygen affinity of adult blood is much less than that of larval blood (Araki et al., 1971; Johansen and Lenfant, 1972). The difference in affinities is apparently due to different larval and adult hemoglobins (Johansen and Lenfant, 1972; Watt, 1976). Similarly, metamorphosis in the Mexican axolotl is associated with a change in hemoglobins, a net decrease in total red cell organic phosphates, and a decrease in blood oxygen

affinity. In this animal, metamorphosis involves a decrease in 2,3 DPG but an increase in ATP (Gahlenbeck and Bartles, 1970).

In light of the preceeding considerations, it becomes tempting to speculate on fetal respiration in E. lateralis early in gestation. I have noticed that very small fetuses (standard length of about 1 cm) have blood prior to any elaboration of the fins. This developmental state is probably analogous in some respects to the mammalian embryonic state. It appears very possible that E. lateralis utilize an embryonic hemoglobin early in development. As the embiotocid fetus develops and approaches parturition, its body becomes covered with scales and the relative size of the highly vascularized fins decreases, approaching adult proportions at birth (Wiebe, 1968; Webb and Brett, 1972a). In addition, I have observed that the advanced fetus is tightly packed within the ovarian sac. This packing may hinder free opercular movement and gill respiration; thus, the area of the functional respiratory exchange surfaces probably decreases as the fetus approaches birth. Although the hemoglobin's oxygen affinity decreases as development proceeds (due to increased proportion of adult hemoglobin), oxygen affinity of the blood may increase due to decreasing levels of organic phosphate. An increase

in the blood oxygen affinity may offset the effects of a decrease in respiratory exchange surface area during development. This study has shown NTP / hemoglobin to be low (relative to the adult) in the late gestation fetus; this ratio may be much higher earlier in development.

HEMOGLOBIN SWITCH IN LATE GESTATION

E. lateralis hemoglobin from the late gestation fetus appears to be a composite of distinctly fetal and adult hemoglobins. It appears to represent a transition from mid-gestation fetal to adult hemoglobin. Partial replacement of fetal hemoglobin by adult hemoglobin prior to birth has been shown to occur in other maternal-fetal oxygen shifts as well. At birth, lamb red cells contain less than or equal to about 20% adult hemoglobin (Bard et al., 1976). Stump-tail macaques contain approximately 10% adult hemoglobin at birth (Kitchen and Brett, 1974). In newborn baboons and humans, adult hemoglobin comprises about 40% of the total hemoglobin (Zuckerkandl, 1965; Kitchen and Brett, 1974; De Simone and Mueller, 1979). Although the structure of E. lateralis hemoglobin was not examined in the newborn, the late gestation fetus appears to contain hemoglobin which is probably greater than 60%

adult hemoglobin; at birth this may be even higher. With respect to its hemoglobin complement, late gestation fetal red cells are very similar to adult red cells. The major physiological differences between late gestation fetal and adult red cells lie in the intracellular environments. These differences may be more a function of adult and fetal organismal environments than genetic programming.

E. LATERALIS FETUS AS A HYPOXIC ADULT

In several respects, the late gestation fetal red cells are probably very similar to red cells of adult animals acclimated to hypoxia. The late gestation fetus may be considered a hypoxic adult with respect to respiratory physiology at the red cell level. Numerous studies have shown that red cells of fish acclimated to hypoxia contain less NTP than do controls (Wood and Johansen, 1973; Wood, 1978; Weber and Lykkeboe, 1978). Greaney and Powers (1977) have postulated that the effect of hypoxia on organic phosphate levels is mediated by the red cell mitochondria in fish. A lowering of the red cell oxygen content reduces the rate of oxidative phosphorylation and hence ATP, and presumably GTP, synthesis. The decrease in NTP increases blood oxygen

affinity in three ways. First, less NTP results in less NTP-stabilization of the deoxyhemoglobin molecular state. Second, less NTP results in higher intracellular pH's due to the Gibbs-Donnan equilibrium. The effect is a larger oxygen affinity mediated by the Bohr effect. Third, less ATP results in cell swelling and hemoglobin dilution. Cell volume is controlled by the active extrusion of sodium by the sodium and potassium-dependent ATPase. A reduction in the ATP supply to the sodium pump results in an accumulation of fluid within the cells or cell swelling (Hoffmann, 1977; MacKnight and Leaf, 1977). Hemoglobin dilution results in an increased oxygen affinity due either to an alteration of the hemoglobin-hemoglobin interaction and/or a decreased hemoglobin-organic phosphate interaction. An increase in oxygen available should reverse the above effects and decrease oxygen affinity of hemoglobin in the red cell.

The late gestation fetal E. lateralis red cells contain significantly less NTP than do adult cells by approximately 60%. If the preceeding postulates concerning the control of oxygen affinity by oxygen availability are correct, one would expect the newborn to have approximately adult values of NTP and cell volume soon after birth. The present study has shown this to be true. At two days after birth, neonatal red cell NTP concentrations

were essentially 100% of adult levels. At that time, hematocrit and red cell hemoglobin concentration were midway between late gestation fetal and adult levels. The results indicate a fairly quick transition. Therein may lie the adaptive significance of the late gestation fetal-maternal shift mechanism in E. lateralis. A rapid decrease in oxygen affinity after birth would facilitate oxygen release at the tissues and probably allow increased animal activity. An increased ease of oxygen unloading may be a benefit in feeding and/or predator avoidance.

A major question remains unanswered. Why does E. lateralis not maintain a higher fetal versus adult red cell oxygen affinity by keeping organic phosphate levels low and thereby eliminate the need for a fetal hemoglobin? One possible answer is that a high oxygen affinity fetal hemoglobin may allow mid-gestation fetal red cells to contain high organic phosphate titers and still maintain a blood oxygen affinity above that of adult blood. High organic phosphate concentrations in fetal cells could be involved in various synthesis or transport processes. Perhaps synthesis and transport activities become less as development proceeds as indicated by low NTP concentrations in fetal cells prior to birth. Another possibility is that at mid-gestation a high oxygen affinity, relative to that of late gestation fetal blood,

is required for some unknown reason and two mechanisms, a fetal hemoglobin and a low fetal red cell organic phosphate level, are employed. Perhaps the physiological emphasis of the blood shifts from an oxygen loading to an unloading strategy (see explanation below) as development proceeds.

PHYSIOLOGICAL IMPLICATIONS OF A HIGH FETAL BLOOD OXYGEN AFFINITY

A shift of the hemoglobin saturation curve to the left, an increase in affinity, allows the hemoglobin to become saturated at a lower oxygen tension. This shift facilitates oxygen loading when environmental oxygen is apparently of limited availability. Oxygen loading is of primary importance under such limiting circumstances; this is supported by the observations that animals of essentially aqueous environments have blood with high oxygen affinities. Excluding pathological conditions, all fetuses studied (with the exception of the domestic cat) have blood oxygen affinities higher than the respective maternal blood. This includes the mammals and the lower vertebrates. Adult fish respond to hypoxia by increasing their blood oxygen affinity. McCutcheon (1936) and Riggs (1951) reported that larval hemoglobin of bullfrog tadpoles

has a higher oxygen affinity than has adult hemoglobin. Also, prior to hatching, embryonic bird blood has a higher oxygen affinity than has adult blood (Lomholt, 1975; Wells, 1979).

A shift of the hemoglobin saturation curve to the left implies that fetal tissue oxygen levels must be low in order to allow the required amount of oxygen to leave the blood. Too low a tissue oxygen level may result in a decrease in oxygen consumption with a decrease in oxygen tension of the blood (Meschia, 1978). A high oxygen affinity could result in metabolic restrictions on fetal growth and development. The fetus therefore may exist near or on the verge of inadequate tissue oxygenation in order to insure the ability to obtain oxygen from the maternal circulation.

The risk of inadequate tissue oxygenation may be the rationale for the cat having adult and fetal bloods of equal oxygen affinity. It also appears to be the basis for the finding that most adult mammals respond to hypoxia by increasing, instead of decreasing, their organic phosphate (2,3 DPG) levels. Such increases of organic phosphate facilitates the delivery of oxygen to the tissues (Lenfant et al., 1968; Duhm and Gerlach, 1971).

With respect to fetal respiration, the problem of

getting oxygen into the fetal circulation is likely of higher physiological priority than is the oxygen unloading at the tissues. This is supported by the finding that essentially all fetal bloods have higher oxygen affinities than their respective maternal bloods. The relative importance of oxygen loading, however, may vary. Assuming identical Hill coefficients for adult and fetal hemoglobins (basically true, Tyuma and Schimizu, 1969; Takenaka and Morimoto, 1976; Jelkmann and Bauer, 1978), a reasonable estimate of loading importance can probably be made by considering the ratio of adult blood P_{50} versus fetal blood P_{50}. A high ratio represents a large difference of oxygen affinities and a stress on oxygen loading. A low ratio indicates less stress on loading; more on unloading. The ratio of adult P_{50} to fetal P_{50} is 2.0, 1.7, 1.5, 1.2, and 1.0 for sheep, rhesus monkey, pig, man, and cat bloods respectively (Novy and Parer, 1969). The ratio is 2.4 for the viviparous amphibian T. compressicauda (Toews and MacIntyre, 1977). The high oxygen affinity of fetal versus adult E. lateralis hemoglobin and the difference in cell organic phosphate titers indicate that the P_{50} adult to P_{50} fetal blood ratio for this fish is also likely to be high. Other factors such as oxygen carrying capacity, cardiac output, and extent of fetal-maternal blood contact are also

undoubtedly involved to ensure adequate oxygen delivery to the fetal tissues.

The fetal blood of Embiotoca lateralis undoubtedly has a higher oxygen affinity than that of the adult blood in vivo. This appears probable due to a unique fetal hemoglobin with high oxygen affinity relative to that of adult hemoglobin, low fetal red cell organic phosphate concentrations, and a low mean corpuscular hemoglobin concentration. Differences in oxygen carrying capacities does not appear to be involved, at least in late fetal development. It appears that fetal oxygen loading as opposed to unloading may have a very high physiological priority.

APPENDIX

ADDITIONAL DATA

Since the completion of the initial writing of this thesis, information has been obtained on the mid-gestation fetal red cell organic phosphate concentration, hematocrit, and blood hemoglobin level. These data, along with data previously shown in Tables 2 and 4, are shown in Tables 5,6, and 7.

Table 5 shows the total nucleoside triphosphate concentrations of fetuses and their mothers of mid-gestation and late gestation fetal red cells. In every case examined, adult NTP concentrations are always higher than fetal concentrations. There is no statistically significant difference in red cell NTP concentrations between these fetal developmental stages. Further, the difference in NTP concentrations between the fetuses and their mothers cannot be statistically distinguished between these two fetaldevelopmental stages. Table 6 shows the mole NTP per mole hemoglobin tetramer ratios and intracellular NTP concentrations which were calculated from the intracellular hemoglobin concentrations shown in Table 7. It appears that throughout at least much of

TABLE 5: Total nucleoside triphosphate concentrations of mid-gestation and late gestation fetuses and their mothers (in mole NTP/mole hemoglobin tetramer)

Mid-Gestation

	Fetus	Adult	Difference
	2.03	2.57	0.54
	1.92	2.59	0.67
	1.84	2.86	1.02
	2.00	2.56	0.56
	2.06	2.51	0.45
Avg.	1.97 ± 0.09	2.62 ± 0.14	0.65 ± 0.22

Late Gestation

	Fetus	Adult	Difference
	2.46	2.96	0.50
	2.26	3.12	0.86
	1.20	2.20	1.00
	1.54	2.69	1.15
Avg.	1.86 ± 0.59	2.74 ± 0.40	0.88 ± 0.28

TABLE 6: Total nucleoside triphosphate concentrations as mole NTP per mole hemoglobin tetramer and intracellular millimolar concentration for all developmental stages examined.

	M NTP / M Hb_t	$[NTP]_{Cell}$ [a]
Mid-Fetus	1.97 ± 0.09 n=5	4.3 mM
Late Fetus	1.80 ± 0.52 n=7	4.3
Young	2.53 ± 0.04 n=3	8.7
Adult	2.68 ± 0.27 n=14	11.3

a: calculated from cell hemoglobin concentration (Table 7) and NTP to hemoglobin ratios.

TABLE 7: Hematocrit and blood and cell hemoglobin concentrations as a function of *E. lateralis* developmental stage.

	Hematocrit	$[Hb]_{Blood}$	$[Hb]_{Cell}$
Mid-Fetus	48.9 ± 3.9 % n=10	(11.0 ± 0.8) X 10^{-4} M n=7	2.2 X 10^{-3} M
Late Fetus	36.6 ± 2.7 % n=16	(8.8 ± 0.8) X 10^{-4} M n=8	2.4 X 10^{-3} M
Young	26.1 ± 6.3 % n=7	(8.5 ± 0.8) X 10^{-4} M n=3	3.3 X 10^{-3} M
Adult	20.6 ± 4.2 % n=4	(8.7 ± 0.3) X 10^{-4} M n=9	4.2 X 10^{-3} M

fetal development, fetal red cells maintain a constant, low intracellular NTP concentration, rising quickly after birth to adult levels. Table 7 shows that the hematocrit is highest at the earliest developmental stage examined and decreases as fetal development proceeds. The blood hemoglobin concentration, an indication of blood oxygen carrying capacity, is about 25% higher in the mid-gestation fetus than in the late gestation fetus, young, and adult. This is consistent with a higher fetal oxygen carrying capacity in humans, sheep, and cats as reported by Novy and Parer (1969). The late gestation fetus has an apparent oxygen carrying capacity very similar, or identical, to that of the adult. This is consistent with a lack of a higher fetal oxygen carrying capacity in pigs, goats, and rabbits (Novy and Parer, 1969). The intracellular hemoglobin concentration, calculated from hematocrit and blood hemoglobin concentration, appears to be constant throughout at least the last half of gestation; adult concentrations are reached apparently soon after birth. The difference in fetal hematocrits with a constant, low intracellular hemoglobin concentration may be due to a greater number of cells per unit volume blood in the mid-gestation than late gestation fetus.

A constant, low NTP concentration in fetal cells during development relative to adult cells indicates that

throughout at least the latter half of gestation, NTP concentrations will have a constant effect on red cell oxygen affinity. Changes in NTP titers or hemoglobin concentration will apparently not counter the effect of replacing high oxygen affinity fetal hemoglobin with lower affinity adult hemoglobin as term approaches. The net effect as the fetuses become larger and more developed appears to be a decreasing oxygen affinity and hence, a possibly greater physiological emphasis on oxygen unloading of the fetal blood at the tissues than on oxygen loading at the fins and gills. Further, the low concentration of NTP and hemoglobin in mid-gestation and late gestation fetal cells is consistent with the hypothesis that the E. lateralis red cell NTP and hemoglobin titers (and hence to some extent, oxygen affinity) are a function of oxygen availability. Fetal red cells, which are likely to be exposed to less oxygen than are adult cells, may synthesize less NTP by oxidative phosphorylation than do adult cells. NTP appears to lower the hemoglobin oxygen binding affinity by directly binding with hemoglobin and indirectly by decreasing intracellular pH (by the Gibbs-Donnan equilibrium) and increasing intracellular hemoglobin concentration (by active transport of salts and consequently water out of the cell). Lower fetal than adult red cell NTP concentrations, therefore,

probably lead to higher fetal than adult red cell oxygen binding affinities and the magnitude of the NTP effects may be controlled by oxygen availability.

LITERATURE CITED

Adamson, J.W., Parer, J.T., Stamatoyannopoulos, G.: Erythrocytosis associated with hemoglobin Rainier: oxygen equilibria and marrow regulation. J. Clin. Invest. 48, 1376-1386 (1969)

Antonini, E., Brunori, M.: Hemoglobin and myoglobin in their reactions with ligands. Amsterdam: North-Holland Publishing Co., 1971

Araki, T., Kajita, A., Shukuya, R.: The effect of organic phosphates on the allosteric properties of Rana catesbeiana hemoglobins. Biochem. Biophys. Res. Comm. 43, 1179-1185 (1971)

Arnone, A.: Mechanism of action of hemoglobin. Ann. Rev. Medicine 25, 123-130 (1974)

Baldwin, J., Chothia, C.: Haemoglobin: the structural changes related to ligand binding and its allosteric mechanism. J. Mol. Biol. 129, 175-220 (1979)

Bank, A., Mears, J.G., Ramirez, F.: Disorders of human hemoglobin. Science 207, 486-493 (1980)

Bard, H., Fouron, J.C., Grothe, A.M., Soukini, M.A., Cornet, A.: The adaptation of the fetal red cells of newborn lambs to extrauterine life: the role of 2,3 diphosphoglycerate and adult hemoglobin. Pediat. Res. 10, 823-825 (1976)

Bard, H., Fouron, J.C., Robillard, J.E., Cornet, A., Soukini, M.A.: Red cell oxygen affinity in fetal sheep: role of 2,3-DPG and adult hemoglobin. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 45, 7-10 (1978)

Bard, H., Shapiro, M.: Perinatal changes of 2,3-diphosphoglycerate and oxygen affinity in mammals not having fetal type hemoglobins. Pediat. Res. 13, 167-169 (1979)

Bard, H., Teasdale, F.: Red cell oxygen affinity, hemoglobin type, 2,3 diphosphoglycerate, and pH as a function of fetal development. Pediatrics 64, 483-487 (1979)

Bartlett, G.R.: Methods for the isolation of glycolytic intermediates by column chromatography with ion exchange resins. J. Biol. Chem. 234, 459-465 (1959a)

_____: Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466-468 (1959b)

_____: Phosphorus compounds in the human erythrocyte. Biochim. Biophys. Acta 156, 221-230 (1968)

_____: Phosphate compounds in red cells of reptiles, amphibians, and fish. Comp. Biochem. Physiol. 55A, 211-214 (1976a)

_____: Iron nucleotides in human and rat red cells. Biochem. Biophys. Res. Comm. 70, 1063-1070 (1976b)

_____: Water-soluble phosphates of fish red cells. Can. J. Zool. 56, 870-877 (1978a)

_____: Phosphates in red cells of two South American osteoglossids: Arapaima gigas and Osteoglossum bicirrhosum. Can. J. Zool. 56, 878-881 (1978b)

_____: Phosphates in red cells of two lungfish: the South American, Lepidosired paradoxa, and the African, Protopterus aethiopicus. Can. J. Zool. 56, 882-886 (1978c)

_____: Phosphate compounds in red cells, liver and skeletal muscle of the American eel, Anguilla rostrata. Copeia 2, 360-363, 1979.

Bartlett, G.R., Borgese, T.A.: Phosphate compounds in red cells of the chicken and duck embryo and hatchling. Comp. Biochem. Physiol. 55A, 207-210 (1976)

Battaglia, F.C., McGaughey, H., Makowski, E.L., Meschia, G.: Postnatal changes in oxygen affinity of sheep red cells: a dual role of diphosphoglyceric acid. Am. J. Physiol. 219, 217-221 (1970)

Bauer, C., Tamm, R., Petschow, D., Bartels, R., Bartels, H.: Oxygen affinity and allosteric effects of embryonic mouse haemoglobins. Nature 257, 333-334 (1975)

Baumann, R., Teischel, F., Zoch, R., Bartels, H.: Changes in red cell 2,3-diphosphoglycerate concentration as cause of the postnatal decrease of pig blood oxygen affinity. Resp. Physiol. 19, 153-161 (1973)

Bellingham, A.J., Detter, J.C., Lenfant, C.: Regulatory mechanisms of hemoglobin oxygen affinity in acidosis and alkalosis. J. Clin. Invest. 50, 700-706 (1971)

Benesch, R., Benesch, R.E.: The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem. Biophys. Res. Comm. 26, 162-167 (1967)

_____: Intracellular organic phosphates as regulators of oxygen release by haemoglobin. Nature 221, 618-622 (1969)

Benesch, R.E., Benesch, R., Yu, C.I.: The oxygenation of hemoglobin in the presence of 2,3-diphosphoglycerate. Effect of temperature, pH, ionic strength, and hemoglobin concentration. Biochemistry 8, 2567-2571 (1969)

Benesch, R., MacDuff, G., Benesch, R.E.: Determination of oxygen equilibrium with a versatile new tonometer. Analyt. Biochem. 11, 81-87 (1965)

Bertles, J.F.: Human fetal hemoglobin: significance in disease. Ann. N.Y. Acad. Sci. 241, 638-652 (1974)

Borgese, T.A., Nagel, R.L.: Inositol pentaphosphate in fish red blood cells. J. Exp. Zool. 205, 133-140 (1978)

Borgese, T.A., Nagel, R.L., Roth, E., Murphy, D., Harrington, J.: Guanosine triphosphate (GTP): the major organic phosphate in the erythrocytes of the elasmobranch Mustelus canis (smooth dogfish). Comp. Biochem. Physiol. 60B, 317-321 (1978)

Bunn, H.F., Forget, B.G., Ranney, H.M.: Human hemoglobins. Philadelphia: W.B. Saunders Co., 1977

Bunn, H.F., Kitchen, H.: Hemoglobin function in the horse: the role of 2,3-diphosphoglycerate in modifying the oxygen affinity of maternal and fetal blood. Blood 42, 471-479 (1973)

Burton, R.F.: Adenosine triphosphate as a determinant of magnesium levels in cytoplasm. Comp. Biochem. Physiol. 65A, 1-4 (1980)

Cameron, J.N.: Chloride shift in fish blood. J. Exp. Zool. 206, 289-295 (1978)

_____: Excretion of CO_2 in water-breathing animals--a short review. Marine Biol. Let. 1, 3-13 (1979)

Chanutin, A., Curnish, R.R.: Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. Arch. Biochem. Biophys. 121, 96-102 (1967)

Chiancone, E., Norne, J.E., Forsen, S., Antonini, E., Wyman, J.: Nuclear magnetic resonance quadrupole relaxation studies of chloride binding to human oxy- and deoxyhaemoglobin. J. Mol. Biol. 70, 675-688 (1972)

Coates, M.L.: Hemoglobin function in the vertebrates: an evolutionary model. J. Mol. Evol. 6, 285-307 (1975)

Costello, A.J.R., Marshall, W.E., Omachi, A., Henderson, T.O.: ATP binding to human hemoglobin in the presence and basence of magnesium ions investigated with ^{31}P NMR spectroscopy and ultrafiltration. Biochim. Biophys. Acta 491, 469-472 (1977)

Davis, B.: Disc gel electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, 404-427 (1964)

Dawes, G.S.: New views on O_2 transfer across the placenta. In: The scientific basis of medicine, annual reviews. London: Athlone Press, 74-89, 1967

De Simone, J., Mueller, A.L.: Fetal hemoglobin (HbF) synthesis in baboons, Papio cynocephalus. Analysis of fetal and adult hemoglobin synthesis during fetal development. Blood 53, 19-27 (1979)

Diem, K., Lentner, C. (Editors): Giegy scientific tables. Basle, Switzerland: Ciba-Geigy, (7th ed.) 1970

Drabkin, D.L., Austin, J.H.: Spectrophotometric studies. II. Preparation from washed blood cells; nitric oxide and sulfhemoglobin. J. Mol. Biol. 50, 471-479 (1935)

Driessen, G.K., Heidtmann, H., Schmid-Schönbein, H.: Effect of hemodilution and hemoconcentration on red cell flow velocity in the capillaries of the rat mesentery. Pflügers Arch. 380, 1-6 (1979)

Duhm, J.: Effects of 2,3-diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes. Pflügers Arch. 326, 341-356 (1971)

_____: The effect of 2,3-DPG and other organic phosphates on the Donnan equilibrium and the oxygen affinity of the blood. In: Oxygen affinity of hemoglobin and red cell acid base status. Alfred Benzon Symposium IV-Copenhagen, May, 1971, Academic Press, 583-598, 1972

_____: Dual effect of 2,3-diphosphoglycerate on the Bohr effects of human blood. Pflügers Arch. 363, 55-60 (1976)

Duhm, J., Gerlach, E.: On the mechanism of the hypoxia-induced increase of 2,3 diphosphoglycerate in erythrocytes. Pflügers Arch. 326, 254-269 (1971)

Edelstein, S.J., McEwen, B., Gibson, Q.H.: Subunit dissociation in fish hemoglobins. J. Biol. Chem. 251, 7632-7637 (1976)

Eigenmann, C.H.: On the viviparous fishes of the Pacific coast of North America. Bull. U. S. Fish Comm. 12, 381-478 (1892)

Fairbanks, G., Steck, T.L., Wallach, D.F.H.: Electrophoretic analysis of the human erythrocyte membrane. Biochemistry 10, 2606-2616 (1971)

Fioretti, E., Ascoli, F., Crunori, M.: Preparation of apohemoglobin trout IV and reconstitution with different hemes. Biochem. Biophys. Res. Comm. 68, 1169-1173 (1976)

Forster, R.E.: The effects of dilution in saline on the oxygen affinity of human hemoglobin. In: Oxygen affinity of hemoglobin and red cell acid base status. Alfred Benzon Symposium IV-Copenhagen, May, 1971, Academic Press, 162-165, 1972

_____: Some principles governing maternal-foetal transfer in the placenta. In: Foetal and neonatal physiology, Sir Joseph Barcroft Centenary Symposium, London, Cambridge University Press, 223-237, 1973

Fyhn, U., Sullivan, B.: Elasmobranch hemoglobin: dimerization and polymerization in various species. Comp. Biochem. Physiol. 50B, 119-129 (1975)

Gahlenbeck, H., Bartels, H.: Blood gas transport properties in gill and lung forms of the axolotl (Ambystoma mexicanum). Resp. Physiol. 9, 175-182 (1970)

Garlick, R.L., Davis, B.J., Farmer, M., Fyhn, H.J., Fyhn, U.E.H., Noble, R.W., Powers, D.A., Riggs, A., Weber, R.E.: A fetal-maternal shift in the oxygen equilibrium of hemoglobin from the viviparous caecilian, Typhlonectes compressicauda. Comp. Biochem. Physiol. 62A, 239-244 (1979)

Gary-Bobo, C.M., Solomon, A.K.: Properties of hemoglobin solutions in red cells, J. Gen. Physiol. 52, 825-853 (1968)

Giardina, B., Ascoli, F., Brunori, M.: Spectral changes and allosteric transition in trout haemoglobin. Nature 256, 761-762 (1975)

Greaney, G.S., Powers, D.A.: Cellular regulation of an allosteric modifier of fish hemoglobin. Nature 270, 73-74 (1977)

Gros, G., Rollema, H.S., Jelkmann, W., Gros, H., Bauer, C., Moll, W.: Net charge and oxygen affinity of human hemoglobin are independent of hemoglobin concentration. J. Gen. Physiol. 72 765-775 (1978)

Haire, R.N., Hedlund, B.E.: Thermodynamic aspects of the linkage between binding of chloride and oxygen to human hemoglobin. Proc. Natl. Acad. Sci. 74 4135-4138 (1977)

Hamasaki, N., Rose, Z.B.: The binding of phosphorylated red cell metabolites to human hemoglobin A. J. Biol. Chem. 249, 7896-7901 (1974)

Hart, J.L.: Pacific fishes of Canada. Ottawa: Fisheries Research Board of Canada, Bulletin 180 (1973)

Haswell, M.S.: Red blood cell pH in the channel catfish. Am. Zool. 19, 901 (1979)

Hayashi, A., Suzuki, T., Shin, M.: An enzymatic reduction system for metmyoglobin and methemoglobin and its application to functional studies of oxygen carriers. Biochim. Biophys. Acta 310, 209-216 (1973)

Hirs, C.H.W.: Reduction and S-carboxymethylation of proteins. In: Methods in enzymology. Vol. 11, C.H.W. Hirs, ed., Academic Press: New York, 199-203, 1967

Hjorth, J.P.: Genetics of Zoarces populations. VII Fetal and adult hemoglobins and a polymorphism common to both. Hereditas 78, 69-72 (1974)

Hoffmann, E.K.: Control of cell volume. In: Transport of ions and water in animals. London: Academic Press, 285-332, 1977

Huehns, E.R., Farooqui, A.M.: Oxygen dissociation properties of human embryonic red cells, Nature 254, 335-337 (1975)

Huisman, R.H.J., Lewis, J.P., Blunt, M.H., Adams, H.R., Miller, A., Dozy, A.M., Boyd, E.M.: Hemoglobin C in newborn sheep and goats: a possible explanation for its function and biosynthesis. Pediat. Res. 3, 189-193 (1969)

Imaizumi, K., Imai, K., Tyuma, I.: On the validity of the spectrophotometric determination of oxygen saturation of hemoglobin; the wavelength dependence of observed oxygen equilibrium parameter values. J. Biochem. 83, 1707-1713 (1978)

Iuchi, I.: Chemical and physiological properties of the larval and the adult hemoglobins in rainbow trout, Salmo gairdnerii irideus. Comp. Biochem. Physiol. 44B, 1087-1101 (1973)

Jelkmann, W., Bauer, C.: Oxygen affinity and phosphate compounds of red blood cells during intrauterine development of rabbits. Pflügers Arch. 372, 149-156 (1977)

_____: Embryonic hemoglobins: dependency of functional characteristics on tetramer composition. Pflügers Arch. 377, 75-80 (1978)

Johansen, K., Lenfant, C.: A comparative approach to the adaptability of O_2-Hb affinity. In: Oxygen affinity of hemoglobin and red cell acid base status. Alfred Benzon Symposium IV-Copenhagen, May, 1971, Academic Press, 750-783, 1972

Kilmartin, J.V., Rossi-Bernardi, L.: Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups. Nature 222, 1243-1246 (1969)

Kitchen, H., Brett, I.: Embryonic and fetal hemoglobin in animals. Ann. N. Y. Acad. Sci. 241, 653-671 (1974)

Laemmli, H.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature 227, 680-685 (1970)

Laver, M.B., Jackson, E., Scherperel, M., Tung, C., Tung, W., Radford, E.P.: Hemoglobin-O_2 affinity regulation: DPG, monovalent anions, and hemoglobin concentration. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 43, 632-642 (1977)

Lenfant, C., Torrance, J., English, E., Finch, C.A., Peynafarje, C., Ramos, J., Faura, J.: Effect of altitude on oxygen binding hemoglobin and on organic phosphate levels. J. Clin. Invest. 47, 2652-2656 (1968)

Leray, C.: Patterns of purine nucleotides in fish erythrocytes. Comp. Biochem. Physiol. 4B, 77-82 (1979)

Lomholt, J.P.: Oxygen affinity of bird embryo blood. J. Comp. Physiol. 99, 339-343 (1975)

Lorkin, P.A.: Fetal and embryonic hemoglobins. J. Med. Genetics 10, 50-64 (1973)

Lykkeboe, G., Weber, R.E.: Changes in the respiratory properties of the blood in the carp, Cyprinus carpio, induced by diurnal variation in ambient oxygen tension. J. Comp. Physiol. 128, 117-125 (1978)

MacKnight, A.D.C., Leaf, A.: Regulation of cellular volume. Physiol. Rev. 57, 510-573 (1977)

Manwell, C.: A "fetal-maternal shift" in the ovoviviparous spiny dogfish, Squalus suckleyi (Girard). Physiol. Zool. 31, 93-100 (1958)

_____: Comparative physiology: blood pigments. Ann. Rev. Physiol. 22, 191-244 (1960)

_____: Fetal and adult hemoglobins of the spiny dogfish, Squalus suckleyi. Arch. Biochem. Biophys. 101, 504-511 (1963)

McCutcheon, F.H.: Hemoglobin function during the life history of the bullfrog. J. Cell. Comp. Physiol. 8, 63-79 (1936)

_____: Specific oxygen affinity of hemoglobin in elasmobranchs and turtles. J. Cell. Comp. Physiol. 29, 333-344 (1947)

Meschia, G.: Evolution of thinking on fetal respiratory physiology. Am. J. Obstet. Gynecol. 132, 806-813 (1978)

Nigen, A.M., Bass, B.D., Manning, J.M.: Reactivity of cyanate with valine-1 (α) of hemoglobin. J. Biol. Chem. 251, 7638-7643 (1976)

Noble, R.W., Parkhurst, L.J., Gibson, Q.H.: The effect of pH on the reactions of oxygen and carbon monoxide with hemoglobin of the carp, Cyprinus carpio. J. Biol. Chem. 245, 6628-6633 (1970)

Novy, M.J.: Alteration in blood oxygen affinity during fetal and neonatal life. In: Oxygen affinity of hemoglobin and red cell acid base status. Alfred Benzon Symposium IV-Copenhagen, May, 1971, Academic Press, 696-714, 1972

Novy, M.J., Parer, J.T.: Absence of high blood oxygen affinity in the fetal cat. Resp. Physiol. 6, 144-150 (1969)

Ornstein, L.: Disc electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci. 121, 321-349 (1964)

Panyim, S., Chalkley, R.: High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. 130, 337-346 (1969)

Parks, R.E., Jr., Brown, P.R., Cheng, Y.C., Agarwal, K.C., Kong, C.M., Agarwal, R.P., Parks, C.C.: Purine metabolism in primitive erythrocytes. Comp. Biochem. Physiol. 45B, 355-364 (1973)

Perutz, M.F.: Stereochemistry of cooperative effects in haemoglobin. Nature 228, 726-739 (1970)

Petschow, R., Petschow, D., Bartels, R., Baumann, R., Bartel, H.: Regulation of oxygen affinity in blood of fetal, newborn, and adult mouse. Resp. Physiol. 35, 271-282 (1978)

Phillips, R.C., George, P., Rutman, R.J.: Thermodynamic studies of the formation and ionization of magnesium (II) complexes of ADP and ATP over the pH range 5 to 9. J. Am. Chem. Soc. 88, 2631-2640 (1966)

Poole, T., Strayer Leach, B., Fish, W.W.: Analysis of polypeptide molecular weights by electrophoresis in urea. Analyt. Biochem. 60, 596-607 (1974)

Pough, F.H.: Ontogenetic change in molecular and functional properties of blood of garter snakes, Thamnophis sirtalis. J. Exp. Zool. 201, 47-56 (1978)

Prosser, C.: Comparative animal physiology. Philadelphia: W.B. Saunders Co., 3rd ed., 1973

Rapoport, S., Guest, G.M.: Distribution of acid-soluble phosphorus in the blood cells of various vertebrates. J. Biol. Chem. 138, 269-282 (1941)

Riggs, A.: The metamorphosis of hemoglobin in the bullfrog. J. Gen. Physiol. 35, 23-40 (1951)

_____: Properties of fish hemoglobins. In: Fish physiology. Vol. 4, W.S. Hoar and D.J. Randall, eds., Academic Press: New York, 209-252, 1970

_____: Studies of the hemoglobins of Amazonian fishes: an overview. Comp. Biochem. Physiol. 62A, 257-271 (1979)

Root, R.W.: The respiratory function of the blood of marine fishes. Biol. Bull. 61, 427-456 (1931)

Rose, I.A.: The state of magnesium in cells as estimated from the adenylate kinase equilibrium. Proc. Natl. Acad. Sci. 61, 1079-1086 (1968)

Rosenfeld, C.R., Morriss, F.H., Makowski, E.L., Meschia, G., Battaglia, F.C.: Circulatory changes in the reproductive tissues of ewes during pregnancy. Gynecol. Invest. 5, 252-268 (1974)

Rubin, H.: Central role for magnesium in coordinate control of metabolism and growth in animal cells. Proc. Natl. Acad. Sci. 72, 3551-3555 (1975)

Saffran, W.A., Gibson, Q.H.: The effect of pH on carbon monoxide binding to menhaden hemoglobin. Allosteric transitions in a Root effect hemoglobin. J. Biol. Chem. 253, 3171-3179 (1978)

Sanui, H.: pH dependence of the effect of adenosine triphosphate and ethylenediaminetetraacetate on sodium and magnesium binding by cellular membrane fragments. J. Cell Physiol. 75, 361, 368 (1970)

Sanui, H., Rubin, H.: Measurement of total, intracellular, and surface bound cations in animal cells grown in culture. J. Cell Physiol. 100, 215-226 (1979)

Scott, A.F., Bunn, H.F., Brush, A.H.: The phylogenetic distribution of red cell 2,3 diphosphoglycerate and its interaction with mammalian hemoglobins. J. Exp. Zool. 201, 269-288 (1977)

Sky-Peck, H.H.: A method for determination of magnesium in serum and urine. Clin. Chem. 10, 391-398 (1964)

Solomon, A.K.: The state of water in red cells. Sci. Am. 224, 89-96 (1971)

Steen, J.B., Turitzin, S.N.: The nature and biological significance of the pH difference across red cell membranes. Resp. Physiol. 5, 234-242 (1968)

Studier, F.W.: Analysis of bacteriophage T-7 early RNA's and proteins on slab gels. J. Mol. Biol. 79, 237-248 (1973)

Takenaka, C., Morimoto, H.: Oxygen equilibrium characteristics of adult and fetal hemoglobin of Japanese monkey (Macaca fuscata). Biochim. Biophys. Acta 446, 457-462 (1976)

Taketa, F.: Structure of the Felidae hemoglobins and response to 2,3-diphosphoglycerate. Comp. Biochem. Physiol. 45B, 813-823 (1973)

Tarp, F.H.: A revision of the family Embiotocidae (the surfperches). California Department of Fish and Game, Bulletin 88 (1952)

Toews, D., MacIntyre, D.: Blood respiratory properties of a viviparous amphibian. Nature 266, 464-465 (1977)

Tomita, S., Riggs, A.: Studies of the interaction of 2,3-diphosphoglycerate and carbon dioxide with hemoglobins from mouse, man, and elephant. J. Biol. Chem. 246, 547-554 (1971)

Tsuyuki, H., Ronald, A.P.: Molecular basis for multiplicity of Pacific salmon hemoglobins: evidence for in vivo existence of molecular species with up to four different polypeptides. Comp. Biochem. Physiol. 39B, 503-522 (1971)

Tyuma, I., Shimizu, K.: Different response to organic phosphates of human fetal and adult hemoglobins. Arch. Biochem. Biophys. 129, 404-405 (1969)

van Assendelft, O.W., Zijlstra, W.G.: Extinction coefficients for use in equations for the spectrophotometric analysis of hemoglobin mixtures. Analyt. Biochem. 69, 43-48 (1975)

Vandecasserie, C., Schenk, A.G., Leonis, J.: Oxygen-affinity studies of avian hemoglobins. Chicken and pigeon. Eur. J. Biochem. 24, 284-287 (1971)

Vig, I., Aviram, I.: The effects of salts on the oxygen affinity of hemoglobin. FEBS Lett. 90, 247-249 (1978)

Watt, K.W.: Functional and structural studies on bullfrog tadpole hemoglobin. Ph.D. dissertation, University of Texas at Austin, 1976

Webb, P.W., Brett, J.R.: Respiratory adaptations of prenatal young in the ovary of two species of viviparous seaperch, Rhacochilus vacca and Embiotoca lateralis. J. Fish. Res. Bd. Canada 29, 1525-1542 (1972a)

_____: Oxygen consumption of embryos and parents, and oxygen transfer characteristics within the ovary of two species of viviparous seaperch, Rhacochilus vacca and Embiotoca lateralis. J. Fish. Res. Bd. Canada 29, 1543-1553 (1972b)

Weber, R.E.: Functional interaction between fish hemoglobin, erythrocytic nucleoside triphosphates, and magnesium. Acta Physiol. Scand. 102, 20A-21A (1978)

Weber, R.E., Lykkeboe, G.: Respiratory adaptations in carp blood, influences of hypoxia, red cell organic phosphates, divalent cations, and CO_2 on hemoglobin-oxygen affinity. J. Comp. Physiol. 128, 127-137 (1978)

Wells, R.M.G.: Haemoglobin-oxygen affinity in developing embryonic erythroid cells of the mouse. J. Comp. Physiol. 129, 333-338 (1979)

Weibe, J.P.: The reproductive cycle of the viviparous seaperch, Cymatogaster aggregata, Gibbons. Can. J. Zool. 46, 1221-1234 (1968)

Wilkins, N.P.: The subunit composition of the hemoglobins of the Atlantic salmon (Salmo salar L.). Biochim. Biophys. Acta 214, 52-63 (1970)

Wood, S.C., Johansen, K.: Organic phosphate metabolism in nucleated red cells: influence of hypoxia on eel Hb-O_2 affinity. Neth. J. Sea Res. 7, 328-338 (1973)

Wood, S.C., Johansen, K., Weber, R.E.: Effects of ambient P_{O_2} on hemoglobin-oxygen affinity and red cell ATP concentrations in a benthic fish, Peuronectes platessa. Resp. Physiol. 25, 259-267 (1975)

Zuckerkandl, E.: The evolution of hemoglobin. Sci. Am. 212, 110-118 (1965)

Typed by: Lilah L. Chambers