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HEMOGLOBIN TRANSFORMATION DURING METAMORPHOSIS IN ANURANS

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A Critical Essay

Submitted to the Biology Department
in partial fulfilment of the requirements
for the degree Master of Arts
University of Oregon
May 1978

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INTRODUCTION

Hemoglobin (Hb), one of the most abundant vertebrate proteins, distributes oxygen among body tissues following oxygenation at the respiratory interface between organism and environment. During the course of natural selection, the Hb molecules of a particular species take on biochemical and biophysical parameters which facilitate adaptation to a unique environmental regime. Extensive research has shown that during the life history of a particular organism there may be a progressive expression of Hb types as characteristic alterations in oxygen availability and oxygen demand occur. Such is the case among anurans; during the discrete postembryonic transition period known as metamorphosis, new molecular forms of Hb appear which persist in the adult frog.

Since McCutcheon (1936) noted a difference in the Bohr effect between frog and tadpole Hbs, there has been mounting evidence suggesting extensive alterations to the Hb content of circulating red blood cells (RBCs) during metamorphosis. Electrophoretic studies have revealed a shift in Hb migration patterns (Herner and Frieden, 1961) which seems to correlate with the finding by Trader et al. (1963) that an increase in dibasic amino acids and a decrease in dicarboxylic amino acids occurs during the transition from tadpole to frog Hb. Further, unlike the human situation in which adult and fetal Hbs have an alpha chain in common, all the monomers comprising frog and tadpole Hbs seem to be different (Elzinga, 1964; Moss and Ingram, 1968a; Frieden, 1968; Aggarwal and Riggs, 1969; Wise, 1970; Maclean and Jurd, 1971; Watt, 1976). Though it has been shown that tadpole Hb does exhibit a Bohr effect when subjected to a large excess of ATP or inositol hexophosphate (Araki et al., 1973), the effect is absent in tadpole RBCs and is presumed to be absent under normal physiological conditions. Also lacking

among tadpole Hbs is the characteristic cooperativity displayed by frog Hbs (Watt, 1976). This phenomenon is due, most likely, to the virtual lack of reactive sulfhydryl groups in tadpole Hbs (Baglioni and Sparks, 1963; Hamada et al., 1964; Trader and Frieden, 1966; Aggarwal and Riggs, 1969). A chart listing the well-documented functional changes occurring during metamorphic Hb transformation is presented in Appendix B. These changes certainly represent significant alterations to the protein moiety of Hb molecules. As illustrated (see Table I), the differences between the Hbs of Rana grylio tadpoles and frogs in terms of amino acid composition are greater than the differences among adults of different cogeneric species, and are comparable to the differences between fetal and adult Hb in humans.

It is assumed that most of the observed Hb alterations are of direct or indirect adaptational value. Prior to metamorphosis the tadpole is an aquatic organism, but following metamorphosis the frog is essentially (with some exceptions) terrestrial. The higher oxygen affinity of tadpole Hb and the lack of correlation between oxygen binding capacity and pH are thought to be appropriate to aquatic life with its limited supply of dissolved oxygen, whereas the lower oxygen affinity and the decrease in binding capacity as pH increases (the Bohr effect) characteristic of frog Hb are more appropriate for a terrestrial life with increased oxygen demands and oxygen availability (Bennett and Frieden, 1962; Dodd and Dodd, 1976). The Bohr effect noted in frogs may also promote carbon dioxide discharge in the lungs; presumably the tadpole needs no such aid in voiding carbon dioxide since its gills are continually bathed in water. The appearance of the Bohr effect, it seems, is a striking example of molecular adaptation. Lenfant and Johansen (1967) as well as Woody and Justus (cited in Dodd and Dodd, 1976) provide evidence that urodels which remain aquatic after metamorphosis do not undergo the same extensive Hb alteration characteristic of anurans.

The heterogeneity of both tadpole and frog Hbs most likely

TABLE I

Number of different residues between anuran and human hemoglobins

Species	No. residues different per mole of Hb
R. grylio tadpole/Human adult	108
R. grylio frog/ Human adult	106
R. grylio tadpole/Human fetal	97
R. grylio frog/Human fetal	95
R. grylio tadpole/R. esculenta frog	86
Human fetal/Human adult	68
R. grylio tadpole/R. catesbeiana frog	63
R. grylio tadpole/R. pipiens frog	58
R. grylio tadpole/R. grylio frog	52
R. grylio frog / R. esculenta frog	38
R. grylio frog / R. pipiens frog	27
R. grylio frog / R. catesbeiana frog	8

The differences reported were determined by calculating the differences between the two Hbs for each amino acid, and summing these values. By using the standard deviations from the mean, the least possible difference was calculated in each case. The averages of the standard deviations were 1.3 ± 0.4 and 1.7 ± 1.5 for the R. grylio tadpole and frog Hbs respectively. The known compositions of the human Hbs were used in the calculations. The average values reported by Tentori et al. (1965) were used in the calculations for R. esculenta Hbs. (adapted from Trader and Frieden, 1966)

increase the versatility of the respiratory pigment by allowing efficient functioning under a wide range of physiological conditions (Watt, 1976). Many (Tata, 1972) feel that the Hb transformations occurring during metamorphosis can be distinguished from simple adaptational responses, however, in that the process is begun and often completed in anticipation of environmental change. These events, then, would represent initiation and promotion of a predetermined program of partially differentiated cells (Tata, 1972).

The Value of Studying Anuran Hemoglobin Transformations

The regulation of cell differentiation remains a major unresolved problem in developmental biology. By operationally defining the differentiated state as the capacity for synthesis of specialized proteins (Marks and Rifkind, 1972), the central issue in terms of genetic control becomes the elucidation of how specific proteins are favored when all cells are thought to contain the same genetic information (Hollyfield, 1966b). Because of their specificity and easily recognizable products (Hb), RBCs have been used widely to study the control of genome activation and expression (Saxén, 1976). Considerable insight into understanding such regulation can be gained by determining the cellular basis for the changing patterns of Hb synthesis noted during development and the degree of stability of Hb messenger RNA (mRNA) during erythroid cell differentiation. In all carefully studied species, Hb molecules have been noted to undergo transformation between the embryonic and adult stages of development (Ebert and Sussex, 1970). These changes possibly represent an orderly, inherent succession in gene expression (Hadorn, 1974).

Hb transformation in anurans represents one of the most promising systems for study of this differentiation process and its relation to hormonal action (Frieden and Just, 1970; Just and Atkinson, 1973) since some molecular changes involved

(such as the reduction of oxygen binding capacity) resemble changes occurring in the transition from fetal to adult Hb in humans (Hamada et al., 1966) and because most changes occurring in the anuran erythropoietic system are thought to be mediated in large part by thyroid hormones (Broyles and Frieden, 1973). From the time Karlson (1961) suggested that hormones affect mRNA synthesis, numerous investigations have been directed toward elucidating the mechanism by which hormones modulate protein synthesis; anuran metamorphosis, of course, is a classic example of the dramatic effects hormones can have on differentiation. It is thought (Tata, 1970) that studies of target tissues, such as the erythropoietic tissues, may yield much valuable information about hormone specificity and the intermediary mechanism(s) of regulation. Since metamorphosis occurs in free living forms and can be induced experimentally by administration of chemically defined hormones (Moss and Ingram, 1968b), anurans are naturally suitable for extensive research.

In terms of natural selection, study of cell lines will reveal some of the important molecular adaptations which have been selected to meet the requirements of different environments (Watt, 1976). Phenotypic differences between tadpoles and frogs are so great that one might suspect the two forms to be products of different genotypes. It may be that genes involved only in larval stages are selected independently of those involved only in the adult stages (Salthe and Mecham, 1974). Hb molecules are valuable to study in this regard because they are found in all vertebrates and perform a vital physiological function. With the exception of lamprey and hagfish Hbs, all known vertebrate Hbs consist of four polypeptide monomers of approximately 15,000 to 17,000 molecular weight with one heme group per monomer (Ingram, 1961). Goodman et al. (1975) recently analyzed the differences between 55 contemporary globins to deduce the geneology of the continually adapting Hb molecule. Because anurans are phylogenetically located between aquatic and terrestrial vertebrate forms, they seem to be of particular interest in the search for transitional molecules (Chauvet and Ocher, 1972).

An Overview of Anuran Metamorphosis

The literature dealing with anuran metamorphosis is extensive and has been reviewed frequently (Etkin, 1955,1964,1968; Weber, 1967a,1967b; Frieden 1967,1968; Frieden and Just, 1970; Tata, 1970,1971,1972; Dodd and Dodd, 1976). Soon after Gudernatsch (1912,1914) observed precocius metamorphosis following administration of thyroid hormones to tadpoles, Allen (1916,1927, 1932) demonstrated that thyroid hormones were in turn regulated to some extent by pituitary hormones. It has long since been established that anuran metamorphosis is mediated in some fashion by the thyroid under the influence of TSH secreted by the pars distalis portion of the pituitary. As the complexity of metamorphic interactions became more obvious, Taylor and Kollros (1946) established stages of larval development which still serve as an index to metamorphic events. For less rigorous considerations, larval development can be divided into three general periods (Bentley, 1976): a) a period of rapid growth designated as premetamorphosis, b) a period of reduced growth but increased differentiation designated as prometamorphosis, and c) metamorphic climax during which the tail is resorbed and the frog emerges to assume a terrestrial existence (see Appendix C for a brief summary of the morphological and biochemical systems changing rapidly during metamorphic climax). Premetamorphosis is found only in anurans and represents a true larval period during which considerable growth and development of larval structures occur (Dodd and Dodd, 1976). Transformation of the larval structures into a form suitable for adult use, and development de novo of structures and functions essential to the survival of the frog occur during the final two periods. Metamorphosis, it seems, allows the insertion of a postembryonic feeding stage into the anuran life cycle during which food reserves are accumulated in preparation for the change to a higher level of organization. This ability of the larva to exploit a different habitat than the adult allows an economizing of yolk material during reproduction (Dodd and Dodd, 1976).

Regulation of the transformation occurring during anuran metamorphosis seems to depend on a number of fundamental features characteristic of hormone action (Tata, 1970): a) most hormones exhibit multiple actions, b) the interactions between hormones and target organs indicate a high degree of inherent specificity, c) any one tissue may be responsive to more than one hormone, and different tissues may respond differently to the same hormone, and d) most hormones stimulate target organs while present in extremely low concentrations. The biochemical systems known to be extensively modified by such hormonal action during anuran metamorphosis are listed in Appendix E. Using thyroxine-cholesterol pellet implants, Kollros and Kaltenbach (1952) reproduced many tissue changes locally and independently of general systemic effects. Thyroxine, then, was shown to initiate a wide range of tissue responses. Cohen (1970) later demonstrated that the cross-sectional diameter and matrix density of mitochondria increase following thyroxine administration. Alterations in the biosynthetic behavior of target cells seem in many cases to be the direct consequence of hormone action. There are, however, a number of different mechanisms which could account for these altered patterns of protein synthesis (see Table II).

Several years ago Finamore and Frieden (1960) used incorporation of radioactive phosphate by R. grylio liver tissue to assay increases in RNA, DNA, and protein concentrations prior to morphological change. Later studies of RNA turnover in liver cell suspensions (Cohen, 1970) suggested that thyroxine treatment stimulates the synthesis of ribosomal RNA (rRNA), transfer RNA (tRNA), and mRNA before morphological changes can be observed. These findings agree well with Brown's (1964) thought that thyroxine is involved in some process which leads to an "unfolding" of genetic information (see Appendix F for an illustration of Brown's scheme of protein changes). It was also found by Cohen that the ratio of rRNA to liver weight increased during continued exposure to thyroxine, suggesting both translational and transcriptional events to be involved in the mechanism

TABLE II

Some Mechanisms Proposed For The Regulation Of New Or Preferential Enzyme Synthesis In Animal Cells

1. DNA synthesis
    a) New cell population
    b) Gene "amplification"
2. Gene activation
    a) New genes unmasked
    b) Increased rate of transcription
3. Messenger RNA utilization
    a) Selective transfer from nucleus to cytoplasm
    b) Cytoplasmic stability and activation
4. Regulation of translation
    a) Polypeptide chain assembly and release
    b) Polysome deployment and cell structure, attachment to membranes
5. Regulation of degradation of proteins

(from Tata, 1970)

of hormonal action in liver cells. Tata (1972) attempted but failed to demonstrate, by base analysis, sucrose density gradient fractionation, and DNA-RNA hybridization, the proportion of mRNA among the nuclear RNA synthesized *in vivo* at the onset of metamorphosis. Tata did show, however, that following the initial burst of induced RNA synthesis, a complex process leads to breakdown of 'old' RNA, alterations in polysome profiles, and redistribution of ribosomes attached to membranes of the endoplasmic reticulum (ER). Brucker and Cohen (1976) also observed differential alteration of enzyme activities and a proliferation of mitochondria and the ER network in liver cells of *R. catesbeiana* after *in vivo* thyroxine treatment. Dodd and Dodd (1976) report additional alterations to liver cell organelles (nuclei become irregular, the number of nucleoli increases, Golgi apparatus increases in size) as a result of thyroid hormone action. Though the initial site of thyroxine action remains unknown, these observations of biochemical alterations and changes in fine structure support the view that thyroid hormones probably mediate metamorphosis by interacting with genes rather than by catalyzing cytoplasmic events. Extensive modifications to the ER, of course, would not be expected in the case of RBCs (since Hb is not exported), but the alterations to RNA and protein profiles could very well be initiated in RBCs by the same mechanism operating in the liver cell. It has recently been found (Just and Robinson, 1977) that during prometamorphosis the plasma amino acid concentrations rise and then decrease about 20% during the final stages of metamorphosis. Such increased levels in the amino acid pools could facilitate increased protein synthesis

Finally, when dealing with any metamorphic event, it is well to consider how the physiological phenomena noted are related to external environmental conditions. The mechanisms by which living organisms respond physiologically to physical environmental pertebations appear to have been neglected in recent years. As concern for environmental quality increases, it seems worthwhile understanding how subtle changes can affect regulation of genetic expression. Anuran Hb transformation is not necessarily

a simple system for study, but it is a convenient one physiologically and economically.

The Sequence of Metamorphic Regulation

Without detailed reference to a multitude of specific alterations, it is instructional to consider Hb transformation in light of the overall regulatory sequence transpiring during metamorphosis. That Hb transformation occurs concomitantly with metamorphosis, of course, immediately suggests regulation via the prevailing hormonal state. Caution should be exercised, however, to avoid making assumptions based on mere phenomonology. It may well be that Hb transformation is a response, or can be a response, to some external or metabolic stimulus other than hormone action. The very nature of hormonal action alluded to previously suggests that tissues may respond similarly to various stimuli or may respond only to a particular concert of stimuli. The following hierarchy of regulatory actions directing metamorphosis is presented simply to establish the biochemical context whithin which Hb transformation naturally occurs.

First it must be assumed that metamorphosis occurs as a response either to favorable external conditions or to some appropriate internal metabolic state (or a combination of both!). Salthe and Mecham (1974) noted that there is extensive variability in metamorphic age and body size among temperate frogs, suggesting the interplay of numerous environmental factors. Before the turn of the century, Hertwig (1898) reported the influence of temperature on normal amphibian metamorphosis, and Etkin (1964) has since noted that within the range of 15 to 30°C, increased temperature preferentially accelerates metamorphosis over growth such that animals metamorphose at a smaller size when subjected to warmer conditions. He also proposes the presence of a cephalic temperature-sensitive mechanism which regulates the ratio of TSH and growth factor secretion. As would be expected, it has also been shown (Bounhiol *et al.*, 1964) that day length influences

metamorphosis in some way. Since the phenomenon was recorded for specimens having transected optic nerves, it is thought that light may be acting directly on hypothalamic structures of the diencephalon. This is, however, in contradiction to studies by Srebo (1962) which indicate that structures controlling metamorphosis in Xenopus laevis lie outside the diencephalon. As Etkin (1964) has suggested, consideration of endocrine-mediated responses to environmental changes has received inadequate attention in recent years. There are certainly additional environmental factors having subtle influences on metamorphic events, and the application of modern quantitative techniques would no doubt yield significant gains toward elucidating such factors. In the case of Hb transformation for example, it is not difficult to imagine how mechanisms capable of responding to decreased oxygen tension could contribute to the initiation of metamorphosis by facilitating TSH production.

With regard to the possible effect of internal metabolic states on metamorphic transformations, two observations will suffice. It was noted many years ago (D'Angelo et al., 1941) that starvation inhibits metamorphosis during the early prometamorphic period but starvation occurring during late prometamorphosis accelerates the metamorphic process. These results led Etkin (1964) to suggest the existence of a neurological mechanism sensitive to feeding behavior. Such a mechanism would promote emergence of frogs from the confines of a deteriorating or overcrowded pond. A different internal factor affecting time of metamorphosis was presented by Goin et al. (1968) who compared the relative amounts of nuclear DNA among several species and noticed that those possessing lesser amounts pass through the developmental stages most rapidly. This correlation between genetic material and developmental duration supports the current idea of metamorphic regulation operating at the genetic level.

The wealth of information concerning hypothalamic regulatory action during metamorphosis has been frequently reviewed in re-

cent years (Porter, 1972; Jørgensen, 1974; Dodd and Dodd, 1976). Hypothalamic control of the thyrotropic function mediated by the pars distalis is seemingly indispensable for normal metamorphosis. Specific neurons (Goos, 1969) release thyrotropin-releasing factor (TRF) at their terminals in the median eminence; TRF then flows by way of the portal circulation to the pars distalis where TSH secretion is stimulated. However, since the median eminence is not completely differentiated until after the beginning of metamorphic climax, progressively greater release of TRF is reflected in ever greater production of thyroxine in response to TSH. After the hypothalamus becomes sensitive to thyroxine during prometamorphosis, a positive feedback system develops to promote rapid acceleration of the initially slow paced metamorphic climax. Normal metamorphosis, it appears, is dependent upon normal development of the median eminence, and any factors regulating the onset or rate of metamorphosis would act by regulating, either directly or indirectly, development of the median eminence.

Though there is much difference of opinion (Oordt, 1974) as to which basophil type produces TSH in the pars distalis, TSH has long been known to be the link by which the pituitary regulates thyroid action (Smith, 1916,1920; Grant, 1931). Pituitary removal in X. laevis larvae causes immediate histological and ultrastructural changes in the thyroid follicular epithelium that lead to gross changes in cellular appearance (Regard and Mauchamp, 1973, as cited by Dodd and Dodd, 1976). Prolactin, which is also produced by the pars distalis but is inhibited by TRF, is known to stimulate growth in tadpoles. When TRF release is minimal, there is neither significant stimulation of TSH production nor prolactin inhibition. This would seem to be the basis for the phenomenon alluded to earlier while considering the effect of temperature on metamorphosis; growth primarily occurs during premetamorphosis until differentiation is stimulated at the expense of growth during prometamorphosis.

Many early studies have demonstrated thyroid hormones are responsible for initiating metamorphic regulation as dictated

by the hypothalamo-hypophyseal axis (Allen, 1916; Mayerouna, 1922; D'Angelo and Charipper, 1938; Etkin, 1955; Kaltenbach, 1959). Several years after Shellabarger and Brown (1959) identified the thyroid hormones as iodine-containing derivatives of tyrosine, it was established that tetraiodothyronine (T_4) and triiodothryonine (T_3) were the primary hormones produced by thyroid follicles (the term thyroxine is used in this discussion to denote the composite action of T_4 and T_3). The thyroid glands increase in size during larval development due to both proliferation of follicles and an increase in follicular volume such that at the beginning of climax the thyroid is large, epithelial height is maximal, and colloid vacuolization is extensive. It is the glandular epithelium which transports iodide from the blood and prepares it for bonding with tyrosine residues in the colloid protein (Gorbman, 1964). By the end of climax the gland appears inactive with flattened epithelium and distended follicles having dense acidophilic colloid (Saxén et al., 1957; Kaye, 1961; Etkin, 1964). From very early on (Schwind, 1933) thyroxine has been thought to be a non-specific stimulus carrying no differential "instructions" to its multitude of target organs. Rather, the multiple effects of thyroxine have been considered to be secondary manifestations of a fundamental biochemical event (Tata, 1964). It has even been suggested that vastly different events such as catabolic tail resorption and renewed anabolic activity in liver cells are initiated via a common mechanism involving directed protein synthesis (Frieden, 1967). Very recent work by Saleem and Atkinson (1978) indicates, however, a decreased rate of protein synthesis in the tadpole tail in response to in vivo administration of thyroxine. Transplant experiments have yielded dramatic evidence that the tissues themselves are in some way responsible for the indepenently unique morphological and biochemical changes exhibited under the influence of thyroxine (Porter, 1972). That thyroxine acts only to induce some pre-existing message has been demonstrated by Frieden (1967). If larvae are injected with T_3 while being maintained at a temperature of 5° C,

the characteristic response is rapid when the temperature is increased to 25° C after 60 days, even though studies indicate that only very low percentages of T_3 and T_4 remain metabolically active after such periods.

Two views have been expressed concerning the relationship between thyroxine concentration and the sequence of metamorphic events:

1) The Stoichiometric view (Etkin, 1955,1964,1968) assumes that each tissue requires a unique amount of hormone before metamorphic processes will be initiated. This amount can be accumulated either by high hormone concentrations acting over a short period of time or by low concentrations acting over a longer period of time. The normal metamorphic pattern of events, then, would be a function of the rate at which the total thyroxine requirements of each tissue are met.
2) The threshold view (Kollros, 1959,1961) assumes that each tissue has a certain minimum hormone level requirement that must be dynamically maintained before the typical response occurs. Accordingly, the normal metamorphic pattern of events would reflect individual tissue response to a rising molar concentration of thyroxine.

Although thresholds have been demonstrated during induced metamorphosis, the normal pattern of metamorphic events may well involve both stoichiometric and threshold relationships (Frieden and Just, 1970).

Regardless of how one chooses to envision the dynamics of thyroxine action, some as yet undefined biochemical mechanism mediates cellular response to thyroxine in terms of protein synthesis. The most likely site for hormonal influence on genetic expression seems to be the synthesis (transcription) or function (translation) of the cell's primary mobile informational macromolecule, mRNA (Frieden, 1967). Modification of the type or number of mRNA molecules would be reflected in protein synthetic patterns. This line of thought began when

Finamore and Frieden (1960) demonstrated an increased rate of phosphorus uptake and RNA turnover in liver cells during metamorphosis. Paik et al. (1961) also showed evidence suggesting a thyroxine-induced rearrangement of RNA molecules in liver cells. Tata (1965) later observed enhanced RNA synthesis in liver nuclei 24 hours before induced morphological changes began. The logic has been that certain tadpole genes are inactive because of the presence of repressors (methylated or acetylated histones?) on the operator genes, but are modified during metamorphosis (Frieden, 1967). More recently, however, Tata (1972) has suggested that regulation of protein synthesis is likely to be more complex than these models based on mechanisms described for prokaryotes. Mechanisms involving the selective transfer of RNA from the nucleus to the cytoplasm, or attachment of ribosomes to ER membranes in those cells synthesizing proteins for export, may prove to be the initial site(s) of interaction between thyroxine and its targets. Whatever the mechanism selecting RNA may be, hormonal stimulation gradually leads to an accumulation of polysomes that are precoded with hormone-specific messengers. The terminal stages leading to increased protein synthesis include an increase in cytoplasmic RNA, a shift toward heavier polysome aggregates (suggesting increased messenger content), and an elevation in the capacity of the ribosomes to incorporate amino acids (Tata, 1970). It may well be possible that some early hormonal events modifying RNA and protein synthesis may operate via activation of adenyl cyclase or some mechanism controlling membrane permeability. Until very recently (Saleem and Atkinson, 1978) there has been little evidence suggesting regulation at the translational level during metamorphosis.

When considering the specific induction of Hb transformation, both the environmental factors influencing the development of the median eminence and the mechanisms mediating thyroid regulation of protein synthesis must be analyzed. At the organismal level there may well be environmental factors capable of modifying hypothalamic development in response to respiratory

effectiveness. The nature of the Hb molecules present in the circulating RBCs determine in large part the efficiency of respiration and thus may contribute to the onset of metamorphosis. At the molecular level, the specialized protein synthesis occurring in differentiated RBCs provides a model system for experimentation directed toward elucidating the initial site(s) of interaction between thyroxine and protein synthesis and the biochemical pathway of induced metamorphic transitions.

Work Contributing To The Present Knowledge Of Hb Transformation

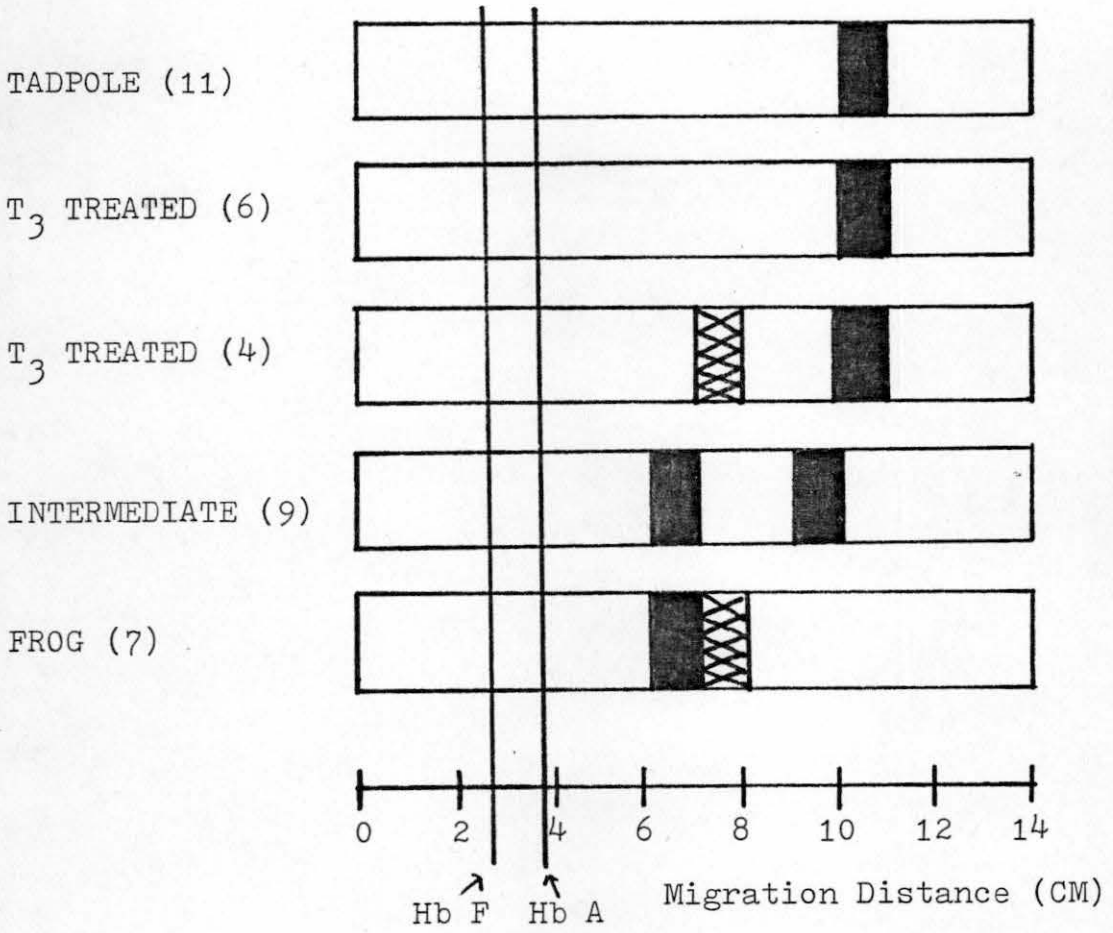
Evidence for Hb transformation during anuran metamorphosis was first found by McCutcheon (1936) who noticed that the oxygen equilibrium curves for R. catesbeiana tadpole and frog Hbs differ. Whereas isolated tadpole Hb is characterized by a hyperbolic binding curve with a high oxygen affinity, frog Hb displays a sigmoid binding curve with lower oxygen affinity which is typical of many air-breathing vertebrates. Riggs (1951) later confirmed McCutcheon's findings, noted the presence of a Bohr effect in frog Hb but not in tadpole Hb, and estimated tadpole Hb to have a molecular weight of 68,000. Riggs (1960) also noted a correlation of the Bohr effect in frog Hb with availability of reactive sulfhydryl groups. Hamada et al. (1964) reconfirmed earlier findings regarding oxygen binding and the Bohr effect, and after chromatographically separating the major components of frog and tadpole Hbs, found four reactive sulfhydryl groups per mole of frog Hb, but found no such groups in tadpole Hb fractions. This characteristic sulfhydryl reactivity of frog Hb has since been used to assay for frog Hb in the circulating blood of metamorphosing animals (Hamada et al., 1966). The existence of sulfhydryl groups in tadpole Hb seems yet unclear, however. Fingerprint studies by Baglioni and Sparks (1963) indicate the absence of methionine and cysteine or any form of disulfide bonding between polypeptides. Using an autoradiographic technique, Stratten's (cited in Frieden, 1967) preliminary data indicated, however, that all monomers of tadpole and frog Hb possess sulfhydryl groups.

Chieffi *et al.* (1960), followed by Herner and Frieden (1961), studied the paper electrophoretic behavior of anuran Hbs in barbitol buffer of pH 8.6 and found tadpole Hb to migrate 1½ to 2 times as fast as frog Hb (see Figure I). A nonheme protein detected by Herner and Frieden was speculated to be the precursor for a new type of Hb; on the basis of an increased uptake of radioactive iron during spontaneous metamorphosis, they proposed synthesis of new Hb from this suspected porphyrinogen-globin via a pathway adapted from Eriksen (1957) (see figure II). Bennett and Frieden (1962) soon attributed the functional differences of the various Hb fractions to the changing character of the protein moiety (globin) since absorption spectra for various oxyhemoglobin solutions all exhibited the same shape with identical maxima at 541 and 578 millimicrons. These findings reinforce Herner and Frieden's assumption that the heme groups of the various Hbs are identical.

Amino acid analysis of frog and tadpole Hb fractions by Trader *et al.* (1963) revealed the expected increase of dibasic amino acids and decrease of dicarboxylic amino acids in frog Hb which would account for the lower electrophoretic mobility of frog Hb as compared to tadpole Hb noted earlier. Further, sedimentation studies indicated the unexpected apperarance of a 136,000 molecular weight octamer in *R. grylio* frog Hb. This new Hb fraction was found to increase in relative concentration gradually during metamorphosis to become the major component in frog hemolysates. Baglioni and Sparks (1963) also noted the presence of the heavy polymers, particularly in hemolysates of older frogs, and suggested that polymerization of Hbs may result from physiological changes associated with age. Hemolysates prepared without reagents other than toluene, however, showed no indication of the octamer. This result led Trader and Frieden (1966) to suggest that many of the dimerized Hb molecules reported may have formed only after hemolysis and may not exist *in vivo*. They attributed the variations among Ranidae in ability to dimerize Hbs as resulting from differences in the orientation of reactive sulfhydryl groups on the beta chains, and proposed a mechanism for dimer formation (see Figure III).

FIGURE I

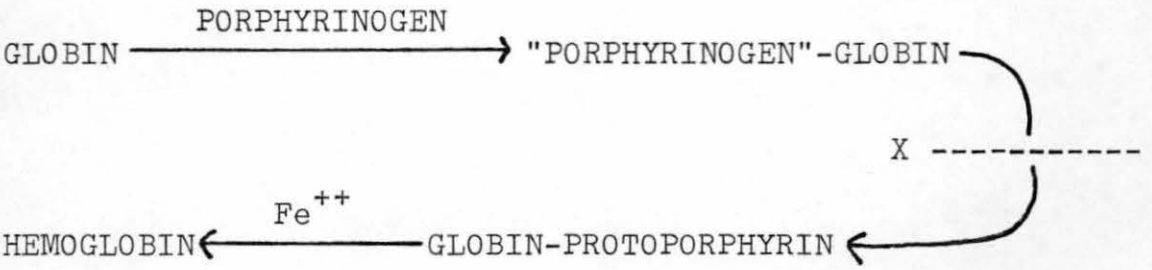
Changes in the Electrophoretic Character of
R. grylio Hemoglobin During Metamorphosis



T_3 refers to animals treated with triiodothyronine for 5 or 6 days. The numbers in parentheses are the numbers of animals in each group. The dark areas represent intense fractions; the crosshatched areas are lighter fractions. The migrations of the human hemoglobins F and A are included for comparison. Reduced mobilities are observed when thicker paper is used. (adapted from Herner and Frieden, 1961)

FIGURE II

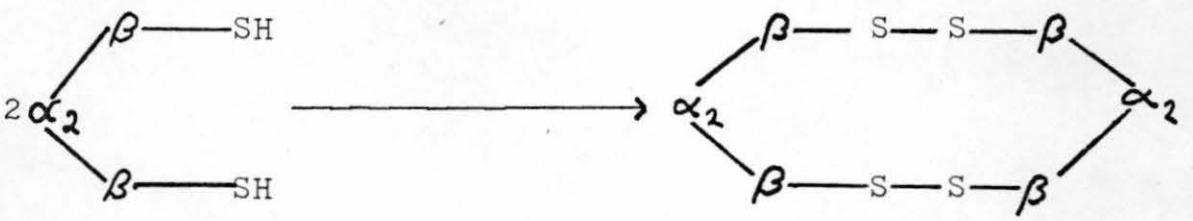
Possible Metabolic Pathway for the Synthesis of Hemoglobin



Regulation may occur at a point of protein modification indicated by X, as a result of iron availability, or as a result of globin precursor availability. (from Bennett and Frieden, 1962)

FIGURE III

A Model for Hemoglobin Dimerization



A necessary requirement for dimerization is that the monomers possess reactive sulfhydryl groups (-SH) so situated as to make dimer formation feasible and possible. Mere possession of reactive sulfhydryl groups is insufficient: adult R. pipiens hemoglobins have as many reactive sulfhydryl groups as R. grylio or R. catesbeiana hemoglobins, but never dimerize. Adult and fetal human hemoglobins have two reactive sulfhydryl groups, but they normally do not dimerize. (from Trader and Frieden, 1966)

Following these early studies demonstrating functional and structural changes during metamorphosis, many lines of research were initiated. Many investigators attempted to quantify and compare the various Hb types appearing during the larval and adult stages of several species, while others compared monomers at the molecular level with regard to amino acid composition, a task complicated greatly by the presence of multiple Hbs. As the differences became more highly resolved, cell lines and sites of erythropoiesis were analyzed in an attempt to determine the origin of the various Hb types and their subunits. An understanding of subunit relationships and the magnitude of changes occurring among subunits was required before a mechanism for induction of Hb transformation at the molecular level could be elucidated.

Soon after the work of Herner and Frieden, Baglioni and Sparks (1963) used starch gel electrophoresis to resolve three distinct tadpole Hbs and four frog Hbs for R. catesbeiana, with the most slowly migrating tadpole Hb having the same electrophoretic mobility as one of the major frog Hbs. Fingerprinting, however, indicated the two fractions to be quite different in compostion. Analysis of tadpoles undergoing induced metamorphosis revealed the existence of Hbs characteristic of both tadpoles and frogs. Using polyacrylamide gel electrophoresis, Moss and Ingram (1968a) confirmed the migration pattern noted by Baglioni and Sparks with somewhat greater resolution. Since the Hb had been converted to metHb-cyanide prior to electrophoresis and maintained in that state, it could be assumed that the multiple bands observed were not artifacts of variation in the heme oxidation state. Elzinga (1964) also used starch gel electrophoresis to separate the Hb components of R. catesbeiana tadpoles and frogs but found evidence of only three frog fractions and a variation of from two to five tadpole fractions. Differences in technique or specimen selection may well have contributed to the discrepancy but it must be noted that Moss and Ingram (1968a) also resolved Hb of R. catesbeina tadpoles into five characteristic fractions. Using acid starch gel

electrophoresis to separate subunits, Elzinga concluded tadpole and frog Hbs had no polypeptide chains in common, indicating a complete change in Hb synthesis at metamorphosis. Hamada and Shukuya (1966), also working with R. catesbeiana specimens, determined that the N-terminal and C-terminal amino acids of the alpha chains, but not the beta chains, were identical (glycine N-terminal and histidine C-terminal) for tadpole and frog Hbs. On the basis of these findings, coupled with tryptic and chymotryptic peptide maps, they suggested that, contrary to earlier findings already noted, a common alpha chain exists for the Hbs of frogs and tadpoles. Moss and Ingram (1968a), however, found the major tadpole Hb to have a valine N-terminal and on that basis concluded that the major tadpole Hb had no peptide chain in common with the major frog Hb. It is possible that conflicting end-group data has resulted from use of different tadpole subspecies since Wise (1970) has shown the amino acid composition of Hbs from different tadpole populations to show considerable variability.

Trader and Frieden (1966) studied the Hbs of R. grylio and found virtually the same electrophoretic patterns as Baglioni and Sparks had for R. catesbeiana, four frog Hb fractions and three more-rapidly migrating tadpole Hb fractions. Also reported in great detail were the amino acid compositions of the tadpole and frog hemolysates for R. grylio and comparative compositions for the adults of other species (see Appendix G). Very significant increases of methionyl and cystyl residues are found after metamorphosis as well as significant shifts in histidine, arginine, threonine, proline, glycine, valine, isoleucine, and tyrosine ratios. With the exception of tyrosine, all of these shifts would contribute or be compatible with the early observations regarding changes in electrophoretic mobility and the availability of reactive sulfhydryl groups. It seems likely that residues bearing sulfhydryl groups are present on all monomers, both tadpole and frog, but are not reactive or involved in disulfide bonds between monomers owing to configurational restraints prior to the appearance of frog Hb subunits. Considering the unique structure of proline,

a shift in the abundance of proline indicates a probable change of monomer conformation.

Evidence of a different nature was submitted by Maniatis et al. (1969) to demonstrate the existence of multiple Hbs. After inducing R. catesbeiana tadpoles to produce antibodies against their own frog Hb, the same specimens produced an uncharacteristic Hb following metamorphosis, suggesting an immunological difference between tadpole and frog Hb.

With the exception of Hamada and Shukuya (1966) there seems to be general agreement that there are multiple frog and tadpole Hbs which have no polypeptide chains in common (Baglioni and Sparks, 1963; Elzinga, 1964; Trader and Frieden, 1966; Frieden, 1968; Moss and Ingram, 1968; Aggarwal and Riggs, 1969; Wise, 1970). Though there are differences of opinion as to the number of tadpole and frog Hb types existing and the presence of a common alpha chain, it is worthwhile attempting a unified model to consolidate the data. If it is assumed that each polypeptide monomer is coded from a separate gene, there are a minimum of eight structural genes (1 common + 4 tadpole + 3 frog) specifying a total of eight or nine fractions in larval and adult Ranidae (Watt, 1976). Recently Watt and Riggs (1975) have analyzed in great detail the structure and function of the four major isolated components of R. catesbeiana tadpole Hb. Their analyses indicate that the beta chain of the four Hb fractions are unique and that at least three of the alpha chains are unique. Though the C-terminal residues of the tadpole alpha and beta chains are identical to those of frog Hb and human Hb, the beta chain N-termini include a six-residue segment missing from the beta chains of frog Hb. Since phosphate binding occurs in the region between the two beta chain N-termini in mammalion Hbs, this N-terminal difference between tadpoles and frog Hbs may be the basis for the increased allosteric effect of organic phosphate on tadpole Hbs. After this type of analysis is carried out for frog Hb and the Hbs of other anurans, an accurate assessment can be made of the number of genes involved in Hb transformation during metamorphosis. It is noteworthy that the transition toward slower electrophoretic mobility during metamorphosis mimics the overall tendency toward slower migrating Hbs in the transition from the lower amphibia to the higher reptiles noted by Dessauer et al. (1957).

Erythroid Cell Lines

The evidence for a significant transition in Hb types occurring during metamorphosis has been interpreted to indicate thyroxine inhibition of the proliferation of tadpole cell lines containing tadpole Hb and thyroxine induction of frog cell lines (Moss and Ingram, 1965; Hollyfield, 1966a; Frieden, 1967; Watt and Riggs, 1975). Appearance of small crenulated RBCs in circulation during metamorphosis (Hollyfield, 1966a) would seem to support this idea; the crenulated cells increase in number as metamorphosis proceeds and gradually lose their wrinkled appearance. It seems as though thyroxine may induce the premature appearance of these new cells in young tadpoles. McCutcheon (1936) noted that R. catesbeiana RBCs become more elliptical after differentiation and Holtfreter (1947) observed that crenulation occurred readily in young cells exposed to only slight changes in medium salt concentration. The appearance of immature RBCs has more recently been shown (Osaki et al., 1974) by a dramatic increase of iron uptake from frog transferrin by RBCs during metamorphosis. Total liver iron peaked at the beginning of metamorphic climax followed by a tripled increase in ferretin (an enzyme which mobilizes liver iron) reducing activity. RBC and heme distruction reached a maximum just prior to this as shown by biliverdin concentrations in the gall bladder. The two areas of disagreement seem to be the time required for completion of the indicated transition, and the occurrence of tadpole and frog Hbs within the same RBC.

Many regard the Hb transition to be complete by the end of tail resorption (Theil, 1967; Moss and Ingram, 1968a, b) while others (Trader et al., 1963; Benbassat, 1970, 74a) have demonstrated that it generally takes longer. Since most metamorphic changes seem to be complete by the end of tail resorption, this may be a point of some importance. Results from carboxymethyl cellulose columns (Hamada et al., 1966) and disc gel electrophoresis (Moss and Ingram 1968b) seem to have demon-

strated a complete Hb transformation at metamorphosis. It has been suggested, however (Benbassat, 1970), that the relatively high initial thyroxine levels used in these studies to induce metamorphosis may have accelerated the transition such that the normal sequence was not observed. Benbassat (1970) studied Hb transition during spontaneous metamorphosis in R. catesbeiana specimens from local ponds and in R. pipiens fertilized and grown under laboratory conditions. On the basis of changes in morphology, red cell autoradiography, and polyacrylamide gel electrophoresis, he found the transition to begin in R. catesbeiana during front leg emergence and reach completion four to ten weeks later. He also noted the temporary appearance during metamorphic climax of red cell proteins exhibiting electrophoretic patterns different from those of either frog or tadpole Hb. If such an intermediate state exists, it may well be the basis for the discrepancies noted earlier in regard to the number of Hb types present. Benbassat (1974a) later observed the changes in Hb synthesis in vitro and again found that during metamorphic climax a small amount of tadpole Hb continued to be synthesized after frog Hb had been induced. It seems, then, that the transition in naturally metamorphosing cells is a gradual process involving the replacement of the cell population(s) containing tadpole Hb with another cell population(s) containing frog Hb with the possible transitory appearance of an intermediate cell population. Yet to be determined is whether these cell populations are the products of new cell lines or the result of renewed proliferation of altered tadpole cells which have become capable of synthesizing frog Hb.

Though it has been reported that RBCs of metamorphosing R. catesbeiana tadpoles contain either tadpole or frog Hb, but not both (DeWitt, 1968; Rosenberg, 1970; Maniatis and Ingram, 1971b), there has been increasing evidence that such is not necessarily the case. Shukuya (1966) first offered evidence that both tadpole and frog Hbs were present within single RBCs, and it would seem that this idea is consistent with the earlier observations of non-heme proteins within tadpole RBCs which are

somehow involved in metamorphosis (Herner and Frieden, 1961; Theil, 1967). More recently, Maclean and Jurd (1971) artificially induced anemia in X. laevis frogs by both blood extraction and phenylhydrazine injection, and observed the increased synthesis of both tadpole Hb and a frog Hb normally present in very small concentrations. This resynthesis of tadpole Hb is not confined to a small population of cells, and though Xenopus is a continuously aquatic species, it does produce different Hbs during adult life. Not only have Jurd and Maclean (1970) demonstrated via immunofluorescent techniques that RBCs contain both tadpole and frog Hbs during metamorphosis in Xenopus, but they have also demonstrated a considerable flexibility inherent in the Hb synthetic pathway. Benbassat (1974b) also used immunofluorescent techniques to study R. catesbeiana Hb production during metamorphosis. Using antisera produced by rabbits immunized with tadpole and frog Hbs, staining at metamorphic climax revealed that 16% of the RBCs reacted to frog antisera while 98% reacted to tadpole antisera, suggesting the coexistence of both types of Hb in the same RBCs. These results are contrary to those of Maniatis and Ingram (1971b) and it is suggested that either the antisera specificities differed or the staining sensitivities differed. Using an immunofluorescent double-labeling technique, Jurd and Maclean (1974) again showed some cells of metamorphosing Xenopus tadpoles to stain differentially, indicating a mixture of tadpole and frog Hbs. Combining new techniques, Meints and Forehand (1977) recently demonstrated erythropoietically stressed frogs (R. catesbeiana) to synthesize an uncharacteristic pattern of Hbs which includes tadpole fractions. Anemic (induced by blood extraction) tadpole, metamorphosing, and frog specimens were subjected to isotopic labeling and their Hbs analyzed by polyacrylamide disc gel electrophoresis. The resynthesis of a tadpole fraction is evident and seems to support a model suggested by Benbassat (1974a). He suggested that single cells are able to produce both tadpole and frog Hb fractions but normally do not do so concurrently. This model seems compatible with our understand-

ing of the genetic complement within each cell and the demonstrated ability of stressed animals to produce uncharacteristic Hb patterns. Application of a new electrophoretic technique described by Anyaibe and Headings (1977) which is capable of separating different Hbs within individual RBCs could prove very valuable in elucidating the RBC's apparent ability to alter protein synthesis under appropriate stimulus. Though it has been assumed again recently that Hb transition during metamorphosis results from the appearance of a new cell line (Watt, 1976), it must not be presumed that circulating RBCs necessarily lack the ability to respond to stress (or thyroxine?) by altering protein synthetic patterns.

Sites Of Erythropoiesis

In addition to problems presented by multiple Hbs of unspecified origin having unique physical and functional properties, there has been disagreement concerning the sites of erythropoiesis. Very early it was assumed that there was a shift in the erythropoietic sites of R. catesbeiana and R. pipiens during metamorphosis, from the kidney in the tadpole to the spleen in the frog (Jordan and Speidel, 1923; Jordan, 1933), and that these sites produced different types of Hb (McCutcheon and Hall, 1937). Foxon (1964) later presumed the marrow of the long bones to be the final RBC maturation site in adult frogs, and designated the spleen as an intermediate erythropoietic site in R. catesbeiana and R. pipiens (the liver was designated as the intermediate site in R. temporaria). Soon correlations were being made between new populations of cells appearing during metamorphosis, as indicated by Hb alterations, and the shift in erythropoietic sites (Moss and Ingram, 1965; Hollyfield, 1966a). This seems a reasonable assumption since it is generally held that in the case of mammals and birds (Wilt, 1967) that a differentiated RBC stops Hb synthesis, and the development of a new stem cell is required for biosynthesis of different Hb types. Maniatis and Ingram (1971a) investigated

maturation sites for tadpoles, metamorphosing animals, and frogs of R. catesbeiana by comparing the percentage of immature, hemoglobin-containing erythroid cells in circulating blood, liver tissue, spleen tissue, kidney tissue, and bone marrow. The liver was found to be the primary maturation site during the tadpole stages while the marrow served that function in frogs; portions of the spleen indicated an erythropoietic function in both tadpoles and frogs. It must be noted, however, that since only cells containing Hb were counted, these results do not identify the stem cells, which may originate elsewhere and be transported to the erythropoietic sites during the maturation process. After dissecting out suspected erythropoietic sites in bled R. catesbeiana tadpoles, polyacrylamide disc gel electrophoresis and in vitro labeling experiments were performed by Broyles and Frieden (1973) in an attempt to demonstrate that the three major tadpole Hbs represent the products of different erythropoietic sites. The data suggest that tadpole kidneys promote the synthesis of a Hb fraction not produced by the other tissues. Deutsch and Broyles (1975) later used phenylhydrazine injections to show a preferential loss of one Hb fraction from R. catesbeiana tadpole circulation in an attempt to support the growing view that the Hbs found in different cell populations originate in different erythropoietic tissues. The thorough study noted earlier by Watt and Riggs (1975) included an attempt to associate the changing oxygen affinities of Hbs with cell populations and erythropoietic sites. They proposed that the development of the tadpole is associated with the successive proliferation of three distinct populations of RBCs - first from the kidney, then from the liver, and finally from the bone marrow following metamorphosis - and that these changes are associated with a progressive decrease in oxygen affinity of the Hbs. This apparent expression of a differential lineage must, as Marks and Rifkind (1972) have suggested in the case of the mouse, reflect the interaction of a programmed cell line and changing environmental factors.

Hemoglobin Synthesis

The idea of switching Hb structural genes in RBCs during metamorphosis by some regulatory mechanism has has been considered since Baglioni and Sparks (1963) suggested that each different polypeptide monomer is coded for by a different gene. As Price (1964) indicated, it is difficult to account for the appearance of new proteins unless the protein synthesizing systems are affected. After Moss and Ingram (1965) reported an initial decline of *in vitro* Hb synthesis in tadpole RBCs followed by synthesis of frog-like Hb during thyroxine administration, DeWitt (1968) suggested the RBCs were synthesizing frog Hb. McMahen, Jr. and DeWitt (1968) then attempted to examine RNA production by RBCs during progressive stages of thyroxine-induced metamorphosis. Measuring labeled uridine incorporation, RNA synthesis appeared to decline rapidly during the initial eleven days and then show a marked increase in synthesis. From this correlation of RNA synthesis with the change in Hb synthesis noted by Moss and Ingram (1965), they postulated that thyroxine exerts both a repressive and an inductive regulatory action on Hb synthesis by halting proliferation of one cell line and inducing another. While Frieden (1967) considered the ability of thyroxine to directly or indirectly repress one set of genes while stimulating the expression of others, Moss and Ingram (1968b) suggested a clonal selection theory requiring no direct action by thyroxine on structural genes but rather on the proliferation or maturation of a different cell line. Studies by Maclean *et al*. (1973) on RNA synthesis in *Xenopus* RBCs indicate that, indeed, most of the RNA seemed to be ribosomal and transfer RNA, suggesting that the RBC nucleus is nearly incapable of mRNA production after leaving the erythropoietic site.

These initial findings suggest several possible ways for thyroxine or some other factor to cause induction and repression of specific Hb proteins (adapted from Frieden and Just, 1970): a) regulation of stem cell maturation such

that characteristic Hbs are produced in response to the hormonal state or biochemical environment, b) regulation of the maturation sites such that characteristic Hbs are produced in response to a specific tissue environment, c) regulation of Hb synthesis in circulating RBCs, and d) regulation via a combination of these mechanisms. Using radioactively-labeled amino acids and thymidine, Just and Atkinson (1972) obtained evidence in support of the proliferation of a new cell tissue which would seem to be incompatible with option c above. Following thyroxine injection, there is a rise in thymidine incorporation followed later by a rise in amino acid incorporation (see Figure IV); electrophoretic analysis shows a concomitant rise in the production of frog Hb. This is the first demonstration in this system of an induced DNA synthesis followed by protein synthesis and seems to be good evidence for a new cell line proliferating and beginning to produce its characteristic pattern of Hbs.

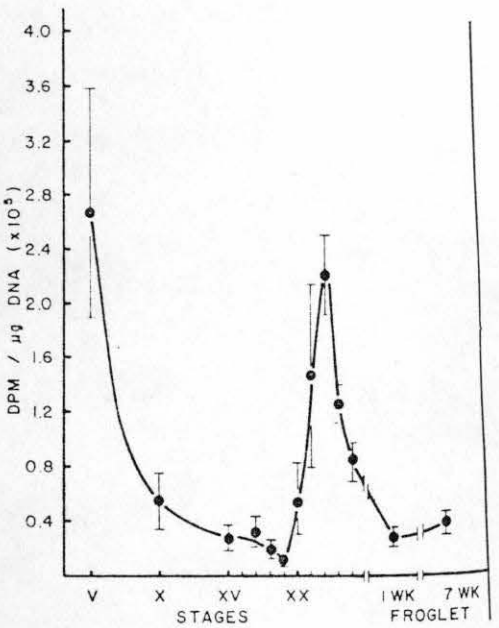
Proposed Model Of The Inductive Sequence

Though regulation of gene expression and protein synthesis at the molecular level is yet to be elucidated in regards to anuran Hb transformation, the following model is offered in an attempt to unify the presented data and provide a framework for future investigation. To facilitate continuity in logic and argument, references to works already cited have been omitted.

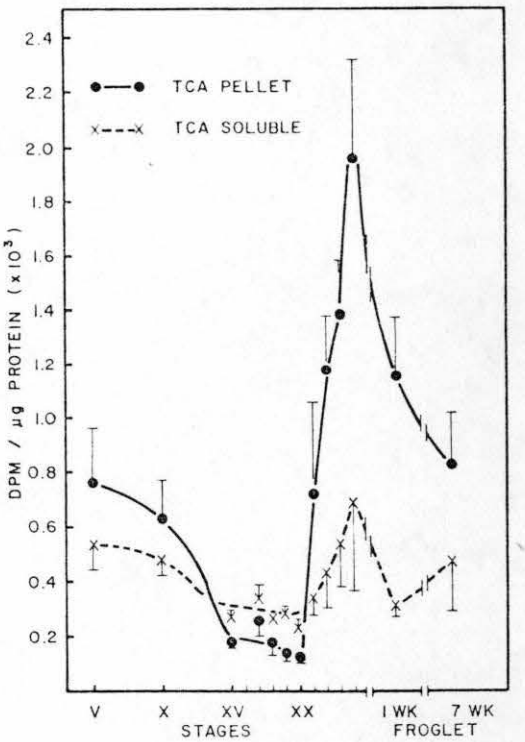
During the normal RBC growth cycle, stem cells give rise to RBCs which reside during some undefined maturation time in an erythropoietic tissue. Though nucleated, the RBCs probably show little mRNA synthetic ability after entering the circulation where they remain until removed by some specialized tissue, probably the spleen. During times of erythropoietic stress, cells may leave the maturation site earlier than normal and exhibit a greater or lesser degree of mRNA synthetic versatility. Experiments utilizing thyroxine administration indicate that there is a decreased synthesis of tadpole Hb

FIGURE IV

DNA and Protein Synthesis Following Thyroxine Administration



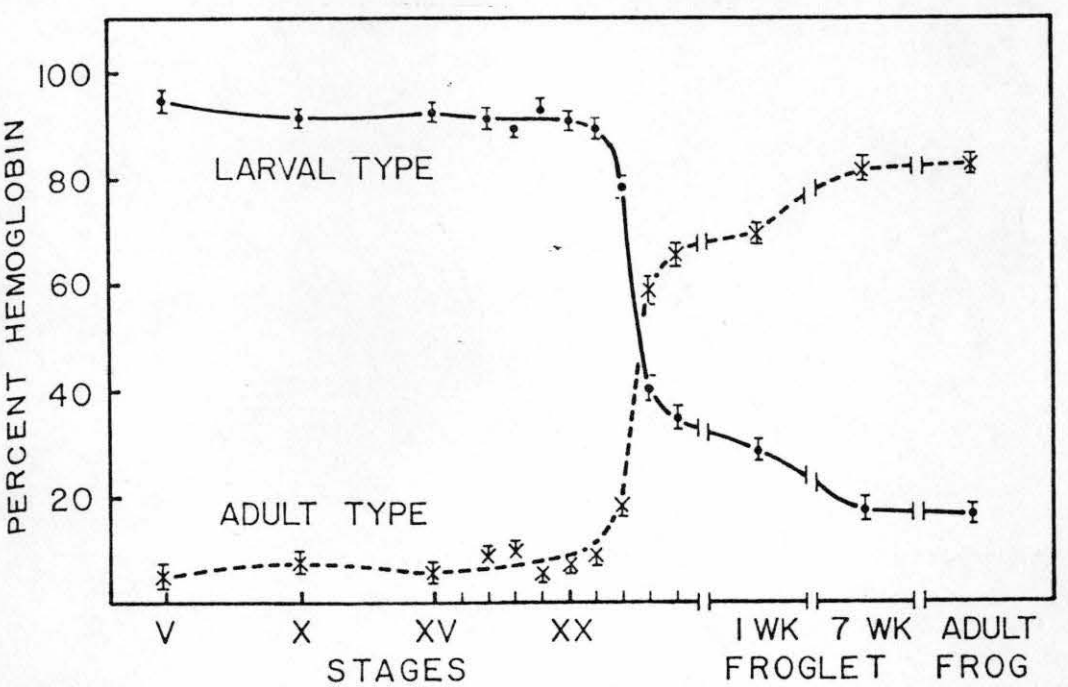
A. Incorporation of ^{3}H-thymidine *in vitro* into tadpole RBCs at different stages of metamorphosis



B. The *in vivo* incorporation and acid soluble pool of ^{14}C-amino acid mixture in RBCs of tadpoles at different stages of metamorphosis.

C. The distribution of hemoglobin types during the life cycle of bullfrogs.

(from Just and Atkinson, 1972)



followed by an increased synthesis of frog Hb, and there is a preliminary increase of DNA synthesis prior to Hb synthesis. During this time there is a switching of erythropoietic sites and a proliferation of a new cell line. Interposed there is a period of erythropoietic stress which causes the release of immature RBCs producing an intermediate pattern of Hbs. Before and after the switching of erythropoietic sites, the characteristic Hb pattern being produced is determined by the biochemical microclimate of the erythropoietic tissues which are, in turn, regulated by the hormonal state of the animal. The availability of the appropriate amino acid ratios and/or nucleotide precursors may play a role in determining the characteristic Hb pattern to be produced. Intracellularly, the thyroxine level may regulate the availability of the necessary genes for transcription, the transfer of specific mRNA into the cytoplasm, the aggregation of polyribosomes, or the synthesis of appropriate receptor proteins sensitive to other external factors. The latter idea is attractive in that different erythropoietic tissues seem to produce different Hb patterns, possibly because of receptors sensitive to metabolites unique to a specific tissue. Thyroxine may operate, then, by determining the activity of an erythropoietic site and/or by determining translational or transcriptional regulation within maturing cells.

This model of induction following the rise of thyroxine levels as well as the previous discussion concerning control of metamorphosis by the median eminence suggest possible areas of future investigation. Rather than consider the induction of Hb transformation only in terms of what happens following a rise in thyroxine levels, it may well be worthwhile to consider the role respiratory efficiency may have in contributing to the development of the median eminence. Perhaps there are hypothalamic receptors sensitive to oxygen tension and/or decreasing respiratory efficiency, and the progressive shift to Hbs of lower oxygen affinity may reflect specific alterations initiated by a hypothalamic attempt to maintain maximum metabolic effeciency during the energy-expensive processes of growth

and differentiation. By maintaining the normal variables such as day length and temperature at a fixed value, one could alter oxygen tension under laboratory conditions and measure the thyroid response. It should be noted, however, that Maclean and Jurd (1971) found the exclusively aquatic axolotl to also exhibit a Hb transition, suggesting that the shift may be an inherent amphibian response to the metamorphic hormonal state. Also it has been noted by Frieden (1967) that there seems to be no increase in oxygen consumption during spontaneous metamorphosis. Considering the frogs variety of respiratory surfaces, however, and the probable increased circulation time between the respiratory surface and the body tissues in the frog, this phenomenon may be a difficult one to establish. Despite a lack of recent work at this level, hopefully the time will come when investigators deem it important to learn in more detail how environmental factors mediate the internal response.

In regard to elucidating the origin of particular cell types, it seems a system discussed by Perlman et al. (1977) may be of considerable use. Utilizing complementary DNA transcribed from the mRNA of young RBCs, hybridization experiments could be performed using suspected stem cells. Assays to determine the degree of similarity to mRNA found in the tissues should indicate the origin of the true stem cells, and a cell-free translational system could be used to assay the polypeptides coded for in an attempt to correlate their relationship to known Hb subunits. Perhaps suspected non-heme precursors in cells lacking Hb could also be assayed in an attempt to determine whether or not erythropoietic sites are actually the sites of stem cell origin. Battaglia and Melli (1977) used a wheat germ translation system to synthesize four globin subunits characteristic of X. laevis and were able to estimate the number of globin gene copies in the genome. Also, in regard to erythropoietic sites, Gusella and Housman (1976) found that erythroid differentiation in murine erythroleukemia cells can be induced by the presence of purines, purine analogues, and other polar solvents. The various suspected erythropoietic

sites could be analyzed for concentrations of nucleotide bases and polar solvents in an attempt to quantify any differences in biochemical microclimate. Erythroid cells from the tissues of maturation could also be tested for synthetic products in vitro as the concentrations of nucleotide bases and polar solvents are varied systematically.

The possible contribution of respiratory efficiency to the development of the median eminence and the specific sites of stem cell origin and RBC maturation seem to be the prime areas for immediate investigation. Once these matters are elucidated, a more meaningful search can be made for the intracellular mechanisms by which thyroxine or other factors directly regulate Hb synthesis. Because the usual endocrine response is a modulation of previous synthetic behavior rather than a complete shift in synthetic patterns, direct evidence for the mechanism of Hb transformation in response to thyroxine has been difficult to obtain (Frieden and Just, 1970).

Summary

The Hbs of anuran tadpoles and frogs have long been known to exhibit differences in functional characteristics. Because of the nature, distribution, and physiological function of Hb, as well as the phylogenetic position of anurans, this transformation of Hb during metamorphosis has been thought to be an ideal system in which to study the regulation of genetic expression and protein synthesis. The occurrence of this transformation during metamorphosis has led most researchers to suspect that the regulation was a direct or indirect response to rising levels of thyroid hormones. The ultimate dependence of thyroid action on hypothalamic control has led to the suggestion that factors contributing to the development of the median eminence ultimately dictate the hormonal state, and hence, the metamorphic events such as Hb transformation.

The Hbs occurring at all stages of anuran development have been isolated and characterized by a variety of chromatographic,

and electrophoretic techniques. It has been generally agreed that the subunits appearing in frog Hbs are different from those of tadpole Hbs with the possible exception of a common alpha chain for the major frog and tadpole Hbs. The correlation of these altered Hb patterns with a change in cell populations and erythropoietic sites has led to the view that a transition occurs such that the cell lines produced by tadpole erythropoietic sites are repressed in favor of frog cell lines maturing in different sites and displaying Hb patterns resulting from the expression of different genes. It is suggested that the hormonal state and/or factors specific to particular erythropoietic sites regulate mRNA synthesis to such an extent during erythroid cell maturation that circulating RBCs exhibit a characteristic Hb composition. Futher investigations must be carried out to elucidate both the effect of respiratory efficiency on development of the median eminence, and the biochemical environment of stem cell sites and erythroid cell maturation sites before the intracellular induction of particular Hb subunits can be evaluated.

APPENDIX A

Abbreviations Used:

ATP - adenosine 5'- triphosphate

C-terminal - the amino acid bearing the terminal carboxyl group of a polypeptide

DNA - deoxyribonucleic acid

ER - endoplasmic reticulum

Hb - hemoglobin

mRNA - messenger RNA

MW - molecular weight

N-terminal - the amino acid bearing the terminal amino group of a polypeptide

RBC - red blood cell, reticulocyte

RNA - ribonucleic acid

rRNA - ribosomal RNA

T_3 - triiodothryonine
T_4 - tetraiodothryonine } thyroxin, thyroxine

TRF - thyrotropin-releasing factor

tRNA - transfer RNA

TSH - thyroid stimulating hormone, thyrotropin

Anuran Species Discussed:

Rana catesbeiana - Northern Bullfrog

Rana esculenta - European Edible Frog

Rana grylio - Southern Bullfrog, Pig Frog

Rana heckscheri - River Swamp Bullfrog, River Frog

Rana pipiens - Leopard Frog, Northern Leopard Frog

Rana temporaria - Common Frog

Xenopus laevis - South African Clawed Toad, Clawed Frog

APPENDIX B

Comparison of the Properties of Tadpole and Adult Bullfrog Hemoglobin

Property	Tadpole	Frog	Reference
pO_2 for 50% saturation	4 mm	14 mm	1
Bohr effect	None	Typical	1
Heme spectrum	Same	Same	2
Methemoglobin formation	Resistant	Less resistant	3
Alkali denaturation	Resistant	Less resistant	3
Electrophoretic mobility (on paper, starch block, gel, etc.)	Fast	Slower	3,4,5
Number of protein components	4	5	5
Average molecular weight	68,000	68,000	4,6
Dimerizable	0	80%	4
Protein chains	$\alpha_2^T\beta_2^T$	$\alpha_2^F\beta_2^F$	5
N-termini	Val	Gly	5
	N-Acetylgly	N-Acetylgly	5
C-termini	His, ala	His, glu	6
Amino acid analysis			
Residues of acidic A.A. in 65,000 g	104	98	4
Residues of basic A.A. in 65,000 g	52	62	4
½ Cystine	0	8	4

Key to references: 1. Riggs (1951); 2. Trader et al. (1963); 3. Herner and Frieden (1961); 4. Trader and Frieden (1966); 5. Moss and Ingram (1968a); 6. Hamada and Shukuya (1966).

(adapted from Frieden and Just, 1970)

APPENDIX C

Morphological and Biochemical Systems Changing Rapidly at the Metamorphic Climax

System	Type of Change	Stage(s)
Blood		
Protein-bound iodine	80% Decrease	XIX-XXIII
Erythrocyte	Change in cell population	XX-XXIII
Hemoglobin	Switch from larval to adult hemoglobin	XXI-XXV
SH groups of red cells	Appearance of 4 or 8 SH groups	XXIII-XXV
Skin		
5-Hydroxytryptophan decarboxylase	10 X Increase	XX-XXIII
Serotonin	30 X Increase	XXI-XXV
Na pump	Initiated	XXI
Foregut	Appearance of peptic activity	XXII
Liver		
NH_3 Excretion	75% Decrease	XIX-XXI
Urea excretion	15 X Increase	XIX-XXI
Urea cycle enzymes	2 - 5 X Increase	XX-XXIV
Tail		
Morphological	90% Reduction	XX-XXIII
Many enzymes	2 - 35 X Increase	XXI-XXIII
Bones		
Cranial, visceral	Initiation of ossification	XX

The stages referred to are those devised by Taylor and Kollros, 1946.

For references regarding the data presented in this table, see Frieden and Just, 1970.

(adapted from Frieden and Just, 1970)

APPENDIX D

Adaptive and Nonadaptive Features of Amphibian Metamorphosis

In an earlier review (Bennett and Frieden, 1962) the adaptive significance of the many anatomical and chemical changes that occur in the tadpole during metamorphosis was emphasized. The adaptive nature of these changes supercedes the many useful descriptions of metamorphosis as an extended embryological process paralleling fetal to adult changes. Listed here are several of the most impressive changes accompanying anuran metamorphosis, which especially contribute to adaptation to land:

1. Tail regression and limb development leading to more powerful locomotion on land.
2. The shift from ammonotelism to ureotelism reflecting the change in environmental water availability.
3. Change in the hemoglobins reflecting the greater availability of oxygen to the frog.
4. Increase in serum proteins, particularly serum albumin, reflecting homeostasis and maintenance of the circulatory volume.
5. Changes in digestive enzymes and in intestinal design reflecting the necessary adjustment to a significant alteration of the diet.

These transitions are perhaps only the most obvious examples of metamorphic events that have an apparent direct adaptive value or serve as a basis for morphological or other changes that lead to improved adaptation.

In considering the many biochemical and morphological events of metamorphosis, Dr. Alan Kent has pointed out that many of these changes may be related to effects of thyroid hormones on developing vertebrates, e.g., the maturation of the nervous system in mammals; epiphysis closure in birds and mammals; effects on feathers, a skin derivative in birds; and even certain enzyme inductions in fetal and neonatal mammals. On the other hand, there are a group of events unique to metamorphosis and apparently controlled by the thyroid. Many of these changes appear to have adaptive value. In amphibian evolution, useful, adaptive changes came under the influence of the thyroid hormone, and

these are superimposed on other more general responses to the thyroid hormone. Thus the metamorphic transition in amphibians stands out as one of the major contributions of a hormone to biological evolution.

(from Frieden and Just, 1970)

APPENDIX E

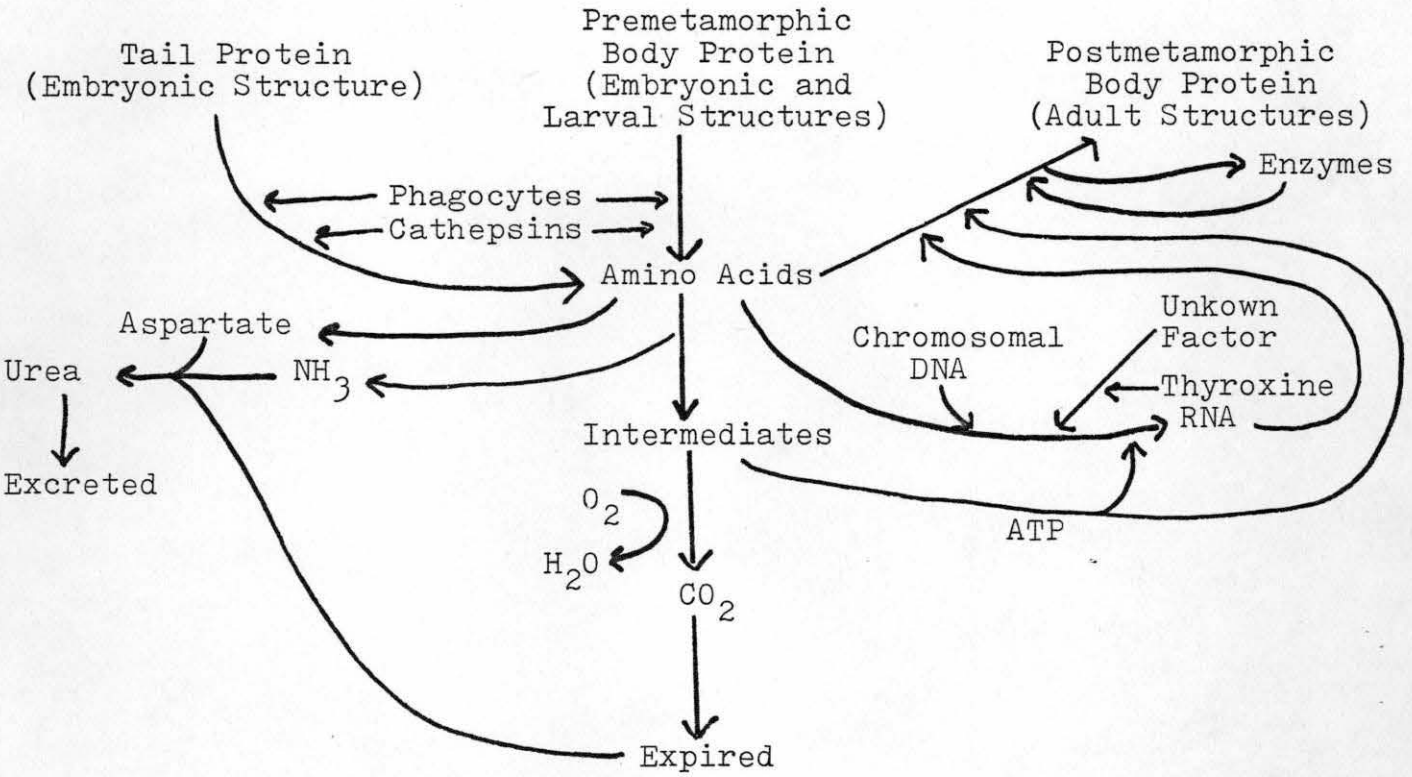
Biochemical Systems Known to be Extensively Modified During Anuran Metamorphosis

(adapted from Frieden, 1967, and Frieden and Just, 1970)

Tissue, organ	Biochemical System	Effect	Role in metamorphosis
Whole animal	Respiration	No increase; decrease in some species	Calorigenic response not associated with metamorphosis
Liver	RNA and protein biosynthesis	Increased and different	Thyroxine-mediated genetic expression via DNA
	Urea production	Increase in urea cycle enzymes	Transition from ammonotelism to ureotelism
Tail	Hydrolytic enzymes of lysosomal type	Stimulation of the biosynthesis of cathepsin, phosphatase, RNAase, DNAase, beta-glucuronidase, etc.	Accounts for tail resorption, adaptation to rapid movement
Blood	Erythrocytes: hemoglobin (Hb)	Repression of tadpole Hb, induction of frog Hb synthesis (increase of adenyl cyclase)	Adaptive oxygen capture
	Serum protein biosynthesis (in liver)	Increase in serum albumin, ceruloplasmin	Improved homeostasis
Pancreas			Greater enzyme and hormone secretion
Acinar cells	DNA, RNA, proteins	Regression, then regrowth; increase in acid phosphatase, a new alpha-amylase	
Islet cells	Insulin	Stimulation of insulin secretion	
Intestine	Digestive enzymes	Shift from carbohydrases to proteases	Change from herbivorous to carnivorous
Skin	Collagen biosynthesis	Collagenolysis in tail; deposition in back, head	Skin strengthening
Eye	Light-sensitive pigments	Shift to rhodopsin	Repression of porphyropsin synthesis
Limb buds	Proteins, nucleic acid	Development and growth of tissues (skin, etc.)	Locomotion on land

APPENDIX F

Protein Changes Occurring During Amphibian Metamorphosis



(Adapted from Brown, 1964)

APPENDIX G

Amino Acid Compositions of Anuran and Human Hemoglobins

Amino Acid	*R. grylio* tadpole	*R. grylio* frog	*R. catesbeiana* frog	*R. pipiens* frog	*R. esculenta* frog	Human adult
Aspartic acid	58	52	56	61	58	50
Glutamic acid	46	46	46	45	44	32
Lysine	34	36	38	41	41	44
Histidine	31	43	43	44	52	38
Arginine	18	26	25	18	17	12
Half-cystine	0.1	9	9	9	6	6
Threonine	27	21	19	22	21	32
Serine	38	37	35	36	29	32
Proline	26	20	20	20	21	28
Glycine	42	35	37	36	38	40
Alanine	58	63	63	63	74	72
Valine	43	38	41	44	38	62
Methionine	2	5	4	3	4	6
Isoleucine	28	16	19	16	17	0
Leucine	65	69	66	63	69	72
Tyrosine	14	20	20	18	20	12
Phenylalanine	31	30	31	30	30	30
Ammonia	59	61	58	59	49	28

The above figures indicate the average number of residues found per 65,000MW hemoglobin; for more detail regarding the error factor in these estimates see Trader and Frieden, 1966. For more information on the values given for *R. esculenta*, see Tentori et a., 1965. (Adapted from Trader and Frieden, 1966)

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