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A COMPARISON OF THE PRIMARY STRUCTURE OF HEMOGLOBINS FROM TWO MICROTINE TRIBES: MICROTINI AND LEMMINI

A

DISSERTATION

Presented to the Faculty of the
University of Alaska in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by

Lawrence K. Duffy, B.S., M.S.
Fairbanks, Alaska
May, 1977

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A COMPARISON OF THE PRIMARY STRUCTURE OF HEMOGLOBINS

FROM TWO MICROTINE TRIBES: MICROTINI AND LEMMINI

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THESIS ABSTRACT

The hemoglobins from each of three species of microtine rodent (Microtus xanthognathus, Dicrostonyx rubricatus, Dicrostonyx stevensoni) showed two α-chain subunits and a common β-chain subunit. The results of the determination of the covalent structure of the individual globin chains are presented. Amino terminal sequences (residues 1-20) were obtained for the α- and β-chains from the major component of M. xanthognathus. Sequences were also obtained for two cyanogen bromide-cleaved fragments from the major β-chain of M. xanthognathus (residues 56-109 and residues 110-146). Tryptic and carboxypeptidase digestions of the cyanogen bromide fragments and peptic digestion of the β-chain were also performed. The hemoglobin sequences obtained for the two species of lemmings (D. stevensoni and D. rubricatus) resulted from the alignment of the tryptic peptides from the isolated chains by homology with the known covalent structures of hemoglobin from the mouse and yellow-cheeked vole. The resulting hemoglobin sequences are compared with those of the white rat and house mouse. The amino acid substitutions observed for these hemoglobins confirm a high degree of variation in primary structure in rodent hemoglobins, even among closely related species, and suggest a more active evolution in Rodentia than in other mammalian orders. That the majority of amino acid replacements do not involve charged residues can account for the hemoglobin solubility and alkaline denaturation behavior. The independent occurrence of α-gene duplication between rodent taxa is inferred. Cladograms based on these hemoglobin sequences relate and unify different systematic descriptions of microtine tribes.

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CHAPTER I

INTRODUCTION

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CHAPTER I

INTRODUCTION

Hemoglobins perform the fundamental physiological role of transporting gases between the environment and the tissues over a very wide range of conditions. The process of evolution of the different hemoglobins from a common ancestor must, therefore, have been directed by the necessity of meeting the requirements dictated to any living system by its metabolic demands in relation to the particular environment (Brewer, 1972). This process has led to the development of complex rate controlling mechanisms, such as cooperativity of hemes, involving various structural interactions which allow for a more efficient transport of O_2.

Evolution is thus recorded in the genetically distinct forms of hemoglobin in the blood of vertebrates. The number and properties of the components vary from animal to animal, and descriptions of hemoglobin heterogenity in most species may be found in the literature (Prosser, 1973; Antonini and Brunori, 1972). According to the concept of proteins as evolutionary "clocks", change in amino acid sequence during evolution is primarily a time-dependent process (Zuckerkandl and Pauling, 1962). This process accounts for the correlation generally found between the amount of time that has elapsed since two species last shared a common ancestor and the degree to which the sequences of their proteins differ today. The validity of this correlation has been reviewed (Wu et al., 1974; Fitch, 1975; Johnson,

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1974; Nei and Chakraborty, 1976) and there is wide-spread agreement that such a correlation exists for many proteins (Van Valen, 1974). It should be noted, however, that this correlation with time depends on the species and the proteins compared (Holmquist et al., 1976). Recently, variation in the hemoglobins of species of the genus Microtus has been used to construct a phylogenetic progression (Genaux and Morrison, 1973a, b). An approximate constancy of the rate of nucleotide substitution seems to hold for a large group of organisms. A possible explanation for this approximate constancy may be that every phyletic line has undergone numerous episodes of rapid and slow evolution, and that the substitution rates are averages over vast periods of time and cycles of speciation, extinction and phyletic evolution (Lewontin, 1973; Simpson, 1944). Lewontin believes that the claimed "constancy" is simply a confusion between an average and a constant. Earlier, Hafleigh and Williams (1966) pointed out that, although a constant rate of modifications cannot be assumed, the longer the evolutionary time considered, the more regular is the rate function (see also, Dickerson, 1971; Langley and Fitch, 1974). It is this averaging process which allows a correlation with paleontological dates.

When a potentially useful protein appears, further mutations in the gene coding for the protein will often be advantageous and selected for, but after the protein has adopted a functional role, a much larger proportion of mutations would be detrimental and selected against (Goodman, 1975). Retention of mutations during this later

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period would result from genetic drift rather than natural selection. Morrison (1973) pointed out that evidence of taxonomic relationships depends on the nonfunctionality of the mutations (see also Zuckerkandl, 1976a, b). Thus, the use of "trees" in biological systematics involves a variety of assumptions. Primary among them is the assumption that the amount of divergence between two groups is proportional to the number of generations elapsed since their separation. In the evolution of proteins, if amino acid substitutions occur primarily by genetic drift, then the number of substituted residues does indeed increase in proportion to time. On the other hand, if selective processes have been responsible for amino acid substitutions, then the amount of divergence will be proportional to the rate of change of the selective environment and need not reflect the number of generations since separation. Both will give linear rates of evolution (Johnson, 1973).

Sometimes this pattern is interrupted by the process of gene duplication. The presence of a second locus, created by unequal crossing over, allows for the accumulation of formerly forbidden mutations (Ohno, 1970). The progeny of one duplicated gene will eventually become heterogenous in the population by both random mutations and recombinations, while natural selection will conserve the sequence (within limits) of the other gene to keep the organisms viable. Thus, after duplication, the two genes evolve independently within functional limits (Zuckerkandl, 1975). This process of gene duplication is evident in hemoglobin. Until rather recently it was accepted that

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there was only one structural locus for each of the polypeptide chains, e.g., in man: alpha, beta, gamma, delta and epsilon. However, the occurrence of multiple loci governing α-chain synthesis in many species has now been documented (Rucknagel and Winter, 1974; Weatherall and Clegg, 1972; Huisman, 1974; Hood, 1976), and genetic models have been constructed (Nute, 1974). The change in amino acid composition by means of gene duplication as a focal point could lead to refinement in the evaluation of an evolutionary progression and provide an alternative source of information about divergence times, even for taxonomic groups that have poor fossil records.

Phylogeny of Hemoglobin

While vertebrates ranging from the primitive hagfish to man possess hemoglobins, urochordates and cephalochordates lack hemoglobin. It is likely that so far as vertebrates are concerned the earliest gene for a hemoglobin polypeptide came into being at the onset of vertebrate evolution. On the other hand, the existence of hemoglobin in diverse invertebrate species suggests that a primordial gene capable of becoming a gene for hemoglobin has long been present in the animal kingdom (Ohno, 1970; Zuckerkandl, 1975; Brown, 1976).

The increasing number of hemoglobin sequences that is being established is now sufficient for drawing certain statistical conclusions concerning the trends of evolution and the divergence of species. This has been done by aligning the amino acid sequences and constructing phylogenetic "trees" by suitable computer algorithims

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(Goodman et al., 1974; Fitch, 1974; Holmquist et al., 1972; Dayhoff et al., 1965). The branching patterns and the numbers of nucleotide replacements on the links connecting ancestors and descendants on such "trees" point out 1) the approximate times in the evolutionary past for the gene duplications which produced the nonallelic loci coding for the different globin chain types (Goodman and Moore, 1973) and 2) the effects gene duplication had on rates of molecular evolution (Goodman et al., 1975).

One of the first attempts to describe the evolution of the hemoglobin molecule was that of Ingram (1961, 1963). He supposed that originally the hemoglobin molecule was a single peptide chain which could not show heme-heme interaction. During the evolution of the first hemoglobin chain (the α-chain in Ingram's scheme) there occurred a gene duplication followed or accompanied by translocation. The two duplicate α-chain genes could then evolve independently; one became the modern myoglobin gene. The ancestral α-chain gene evolved in such a way that its product had the property of dimerization in solution to form α_2-molecules and also heme-heme interaction. Later duplication of this α-chain led to the formation of a γ-gene which in turn led to the present day β-gene.

More recently, Goodman and colleagues traced the patterns of mutational change in the hemoglobin genes using 55 contemporary globin sequences. Their proposed scheme differed significantly from Ingram's although both are based on the process of gene duplication and on the early divergence of myoglobin.

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Goodman et al. (1975) assumed that homotetramers preceded heterotetramers and that tetrameric hemoglobins had evolved before the β-α gene duplication (see Ohno, 1970). Goodman and colleagues, unlike Ingram, further assume that the ancestral homotetramer had subunits more β-chain-like in polymer forming properties than α-chain-like. This second assumption is supported by the observation that homotetramers are readily formed by β-chains but not by α-chains (Benesch and Benesch, 1974).

Goodman et al. (1975) point out that lamprey hemoglobin[1] chains in the oxygenated state are monomers but in the deoxy state they form homodimers and transitory tetramers (Behlke and Schelee, 1970). The lamprey hemoglobin is presented by Goodman et al. (1975) as evidence that a hemoglobin with dimeric properties probably existed in the earliest vertebrates. They propose that this first vertebrate hemoglobin, like that of lamprey, released oxygen from its heme iron atoms more readily at reduced pH from an aggregated state. It would have been, therefore, a more useful protein than the homotetramers of β-chains which show no cooperativity. Natural selection could then lead to a hemoglobin more efficient at delivering oxygen to the tissues of larger-bodied, fast-moving animals - a heterotetrameric hemoglobin.

On the basis of the assumption that β-gene is closer

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[1]Zuckerkandl (1975b) cautions that rates of evolution and functions of hemoglobin dependent on the lamprey sequence may not be accepted at face value at this time. He would like to know "whether among several lamprey globin components that have not yet been sequenced there is one, perhaps a minor component, that resembles Gnathostome hemoglobin and myoglobin chains more than the sequenced lamprey chains do".

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evolutionarily to the β-α gene ancestor, Goodman and colleagues proposed a phylogeny for the hemoglobin to follow the periods of β-α-gene duplication.

1. In a common ancestor of teleosts and tetrapods, positive Darwinian selection acted on the nascent α-gene locus, and stabilizing selection acted at the β-gene locus[2]. The α-gene locus, as the more recent and relatively silent locus, had initially fewer constraints with respect to natural selection and thus could accumulate mutations more rapidly.

2. Once the older β_4 type homotetramer was replaced by the heterotetramer with a specialized α-chain, positive selection for a more differentiated β-chain intensified. The rate of beta evolution accelerated several fold between tetrapod and amniote ancestor, and functionally superior tetrameric hemoglobin emerged. This tetrameric hemoglobin possessed all the appropriate amino acid substitutions that are essential to the interchain cooperativity of present-day tetrameric hemoglobin. These are heme contacts, subunit contacts ($\alpha_1\beta_1$ and $\alpha_1\beta_2$) and salt bridges.

3. After the development of the heterotetramer with interchain cooperativity, both α- and β-loci have been subject to stabilizing selection. In the descent from the amniote ancestor to chicken and mammals, an abrupt slowing of both alpha and beta evolution occurred.

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[2]Stabilizing selection was stronger at the β-locus while positive Darwinian selection acted on the nascent α-locus. The existence of "silent proteins" has been demonstrated by Rigby et al. (1974) in a bacterial enzyme system (see also Zuckerkandl, 1975).

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In the early mammals, rates again accelerated but later became extremely slow in some lineages such as the human.

4. Goodman et al. (1975) propose that once the essential features of the hemoglobin tetramer were fixed in the preamniote ancestor, finer adaptations could be shaped through natural selection by substitutions at surface positions. Amino acid sites in mammal hemoglobins which have the highest number of amino acid substitutions are generally observed in the exterior or surface positions without defined functions.

Organismal Change and Protein Evolution

Salthe (1975) notes that natural selection is a process that operates from one generation to the next at the population level. Structures in the organism are seen to evolve because the organism as a whole is evolving. He believes that the bridge between different levels in respect to evolution is the total integrated phenotype. Since it is the net balance of many gene actions which guides organismal selection, the refined co-adaptations among proteins must await the overall adaptation of the organism. Thoday (1975) states that amino acid substitutions at seemingly nonfunctional positions in proteins may be viewed as the storage of potential variation without a lowering in the fitness of the organism. He points out that natural variation between organisms would be advantageous because a neutral allele could eventually cease to be neutral and could provide a potential source of genetic flexibility that does not involve loss of

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genetic stability. Protein polymorphisms which do not show any functional differences may provide a source of adapt response to future environments.

An alternative view has been proposed by Zuckerkandl (1976a, b). He views any amino acid substitution as having an effect on the functions of a protein and thus on the fitness of both the molecule and organism. For example, as substitutions are being selected on account of their advantage to one of the general functions of the protein molecule, very small negative selection factors will accumulate in relation to other general functions. In due course, the sum of these negative selection factors will become sufficiently distinct from zero to be significant. The sum of the fixations, that are each only slightly deleterious by itself to some general function, leads to a "selection vacuum". This vacuum waits to be filled by a substitution that is favorable to that general function. The mutation cycle would then repeat itself. In this theory Zuckerkandl proposes that the Red Queen Hypothesis[3] of Val Valen (1974) applies to protein evolution and provides for a limited component of randomness in amino acid substitutions, accompanied by selection. This theory can also be viewed as an extension of Fitch's covarion hypothesis[4].

Recently Wilson (1976) has pointed out that a correlation has often been between genetic similarity (estimated by protein comparisons) and organismal similarity (measured in terms of taxonomic distance). He believes that this correlation could result simply from

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[3,4]Glossary

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the fact that both structural genes and anatomy usually evolve at fairly steady rates. If genetic change and organismal change are each correlated with time, they will appear to be correlated with each other.

In order to find out whether organismal change is dependent on structural gene mutations, Wilson (1974, 1975) compared the rates of structural genetic change among taxonomic groups which have experienced different rates of organismal change. Because he believed that any type of genetic change that had occurred rapidly in mammals but slowly in frogs could be at the basis of organismal evolution, he compared placental mammals (rapid organismal evolution as exemplified by the 16 orders) with frogs (fairly uniform phenotypically with only a single order, Anura). He found that the anatomically similar frogs differed greatly at the protein sequence level, whereas mammals showed much less sequence variation in the proteins studied (albumin, hemoglobin and fibrinopeptides). Similar results were obtained in DNA annealing studies. Yet, placental mammals have experienced far more rapid karyotypic changes than have frogs (e.g. for Microtus oregoni 2n = 16 for M. xanthognathus 2n = 56). Frog species retain the ability to hydridize with one another and mammals usually do not (Moore, 1975). Studies on rates of evolution in frogs and in mammals (see the study of chimpanzees, King and Wilson, 1975) suggest that sequence change in structural genes occurs for the most part independently of organismal change. By contrast, regulatory change occurs in parallel with organismal change. Studies on pocket gopher (Nevo et

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al., 1974) suggest that there may be independence of rates of protein primary sequence from regulatory change in the evolution of the rodents.

Significance of this Work

It is well-accepted that the number of amino acid differences among homologous proteins correlates with the taxonomic separation of the proteins. A necessary prerequisite for computing a geneaology from protein structure is that the protein family be well-represented by amino acid sequence data. The present study increases the number of rodent hemoglobin structures known in the family Cricetidae.

The accumulation of cricetid hemoglobin sequences permits meaningful phylogenetic correlations among the various species. Nucleotide sequences for structural genes and the primary structures of the α- and β-chains for the hemoglobin molecule originate in molecular changes at the level of the gene and indicate rates of evolution. Comparison of amino acid sequences provides independent perspective on evolutionary divergence, the concepts of which have been until now largely based on morphological considerations alone.

Amino acid substitutions in the hemoglobins from several Microtus series have been reported (Genaux and Morrison, 1973a, b; Genaux et al., 1976). The hemoglobins from M. xanthognathus and Dicrostonyx have now been analyzed and have been compared to other microtine hemoglobins with respect to systematics.

The analysis of two populations of Dicrostonyx which differ in both chromosomal and morphological character allows a comparison of

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biochemical evolution with organismal evolution (Wilson, 1976).

The sequences derived from this study are useful for making comparisons among animals. They also are important in confirming and supplementing the previously available knowledge on vole hemoglobin structure and genetics.

Functional parameters of proteins derive from their amino acid sequences. A single amino acid substitution in hemoglobin may produce a change in a functional parameter by eliminating hydrogen bonds, salt bridges or hydrophobic interactions. Isolation and characterization of hemoglobins from rodent populations provide data with which to study single or multiple residue replacements. Differences in such parameters as O_2-binding, electrophoretic mobility, solubility, and denaturation of these hemoglobins can be adequately analyzed only by considering the covalent structure.

The structure of the minor electrophoretic component of *M. xanthoganthus* has also been studied in an attempt to discover functional effects due to the presence of this component. Since some North American species of mice and voles (i.e. *M. pennsylvanicus*) exhibit a polymorphism in relation to the number of hemoglobin components, the difference between the structure of the major and minor component might indicate their functional importance.

The basic questions I have tried to answer are: first, where are the structural differences in the major hemoglobins of *M. xanthoganthus* and *Dicrostonyx* and how do they fit into microtine systematics? Second, how do the minor hemoglobin components differ from the major

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in these species and could the existence of the minor component be advantageous?

Sequence Strategy

The rigorous solution of the house mouse hemoglobin sequence by Popp (1968, 1973a, b) and Gilman (1973a) has facilitated the elucidation of hemoglobin structures from other rodents. The strategy for the solution of the hemoglobin sequences of yellow-cheeked vole and collared lemming involved the isolation and characterization of the tryptic peptides of the aminoethylated globin chains. The peptides isolated could be aligned by homology with the house mouse sequence.

A complete set of peptides was not isolated from the tryptic digestions of the yellow-cheeked vole and cyanogen bromide cleavage was performed in time to use the automatic sequencer made available by Dr. F. R. N. Gurd. This work confirmed previous alignments of the *Microtus* tryptic peptides. Other digestions by chymotrypsin, pepsin and carboxypeptidase were used to shed light on "core" areas of the chains.

With the provision that the succeeding chapters depend on chapter II (Material and Methods), each chapter has been written so as to be essentially complete in itself.

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CHAPTER II

MATERIALS AND METHODS

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CHAPTER II

MATERIALS AND METHODS

ANIMALS

Animals used in this study were obtained from the animal colony of the Institute of Arctic Biology. The *Microtus xanthognathus* colony had been established from wild stock ($\sim$ 5 animals) in 1973 by Dr. R. Rausch with animals caught at Hess Creek, Alaska (approximate lat. 65° 40'N, long, 149° 07'W). The *Dicrostonyx rubricatus* were long-established in the colony. *D. stevensoni* from Umnak Island was described by Rausch and Rausch (1972).

The present colony of *M. pennsylvanicus tananaensis* was established by Dr. L. P. Stratton, Mr. W. Galster and myself with three animals in 1975. *M. pennsylvanicus pullatus* was established with animals from Hamilton, Montana. *L. sibiricus* used were from a colony established by Sarah Cambell. *M. oeconomus* were caught locally. The taxonomic designations for the several species listed in Table 2-1 are according to Hall and Kelson (1959); the geographic origins of the several species are included. With the exception of *M. xanthognathus* and *M. p. tananaensis*, the colonies were started from a number of animals and periodically additional wild caught animals were introduced to the breeding pool. On the other hand, in the colonies started from a few individuals it is not uncommon for the majority of animals of a specific colony to be related to a single founding individual, or even pair.

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TABLE 2-1

Species and Geographic Origin of
Rodents from which Hemoglobin was Obtained

SPECIES	GEOGRAPHIC AREA	
M. xanthognathus	Hess Creek (Yukon River)	77°N, 151°W
Dicrostonyx rubricatus	North Slope 1. Pt. Barrow	73°N, 155°W
	2. Beaufort Lagoon	70°N, 142°W
Dicrostonyx stevensoni	Umnak Island	53°N, 168°W
M. p. tananaensis	Fairbanks	65°N, 148°W
M. p. pullatus	Hamilton, Montana	
M. oeconomus	Fairbanks	65°N, 148°W
L. sibiricus	Pt. Barrow	73°N, 155°W
Acomys cahirinus	Egypt, Israel	

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REAGENTS

All chemicals were reagent grade.

TRIS is used for TRIS (hydroxymethyl) amino aminomethane

Urea (Fisher) solutions were passed through Rexyn 1-300 H-OH ion (mixed bed) exchange resin immediately before use to remove traces of cyanate.

Water was distilled then deionized by passage through an ion exchange column.

The chemicals for the Edman degradation were all "sequenal" grade (Pierce).

Enzyme preparations were used without preliminary preparation.

Ion exchange (DEAE-Sephadex and CM52) and gel filtration resins were prepared as directed by manufacturer's instructions.

High voltage paper electrophoresis of peptides was performed on Whatman Co. 3MM paper.

METHODS

Preparation of Dialysis Tubing

Union Carbide Corporation visking cellulose tubing was treated with approximately 2 gm of sodium bicarbonate and 0.1 gm of EDTA per liter of deionized water and brought to a boil. The prepared tubing was stored in deionized water at 5°C.

Concentration of Protein Solutions

Hemoglobin solutions were concentrated by ultrafiltration over

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UM 2 (M.W. < 2,000) or UM 10 (M.W. < 10,000) membranes on Amicon Diaflo cells of 50 ml capacity. Ultrafiltration was performed at 5°C under a nitrogen pressure of 40 lbs. per in^2.

Prior to the introduction of Amicon ultrafiltration equipment, the solution to be concentrated was placed in dialysis bags prepared as described above and left at 5°C overnight in air.

Polyacrylamide Gel Electrophoresis

The number of components of hemolysates was initially established by vertical polyacrylamide gel electrophoresis (E-C Apparatus). The operation of this apparatus is described by Shaughnessy (1974).

Vertical gels were prepared with TEB buffer (16.1 g TRIS, 1.56 g EDTA, 0.92 g Boric acid in 1 liter). Samples of fresh oxyhemoglobin or carboxyhemoglobin were applied to the gels, and electrophoresis was performed for 2 hours at 200 volts. The gel was cooled by circulating tap water. Vertical gels were stained with 1% amido swartz, and excess dye was removed by rinsing the gels with 5% acetic acid.

Cellulose Acetate Electrophoresis

Electrophoresis on cellulose acetate (Microzone, Beckman Instrument Co.) was carried out in the same buffer as that used for the acrylamide gel electrophoresis. Adequate separation of hemoglobin components required 45 minutes at 350 volts. Complete instructions for the use of the Beckman Microzone system are available in the Beckman Microzone Manual and their Methods Manual RM-TB-010A.

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Polypeptide chains were also characterized on cellulose acetate. Standard TEB buffers (TRIS-EDTA-Borate) were made 8 M in urea and 0.05 M in 2-mercaptoethanol. A small amount of hemolysate containing 2 to 10 percent hemoglobin was diluted by adding an equal volume of 2-mercaptoethanol (Schneider, 1974); the mixture was refrigerated for 2 - 8 hours. Electrophoresis was performed on a cellulose acetate strip which had been immersed in the urea buffer until thoroughly wet. The strip was then blotted lightly, and the samples were applied to it. Good separation was obtained by electrophoresis for 1 - 1 1/2 hours at 300 v, 2 ma.

Hemoglobin Typing After Cystamine Treatment

Cystamine treatment (Wegman and Gilman, 1970) enables one easily to determine if hemoglobins differ in the number of free thiol groups. The positively charged S-ethylamine group of cystamine forms disulfide bonds with available thiol groups. A resulting difference in charge between two hemoglobins with different numbers of free thiols permits their electrophoretic resolution.

To 1 ml of hemoglobin solution in TEB buffer (pH 8.9 - 9.1) is added cystamine dihydrochloride (Sigma, M.W. 225.2) to a concentration of 0.25 M. The reaction proceeds rapidly at this pH, and after 10 minutes the hemoglobin can be electrophoresed.

Isoelectric Focusing

Synthetic ampholytes from LKB Instruments, Inc. with a pH range

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of 6 - 8 were used for the isolectric focusing of hemoglobin components. This was performed in either 110 or 440 ml glass column from LKB Instruments, Inc. as described by Behrisch and Johnson (1974). using a sucrose density gradient and at 4°C. The cathode solution was located at the bottom of the column. Fractions of 1 - 2 ml were collected, and absorbances were measured at either 540 or 254 nm. The pH of peak fractions was recorded.

Solubility Curves

Salting out of hemoglobins was performed following the method of Derrien as modified by Popp (1959) to determine solubility characteristics in K_2HPO_4 - KH_2PO_4 buffers at pH 6.7. Samples contained 0.1 ml of HbCO (2 to 5 percent) in a total volume of 5 ml of phosphate buffer (Table 2-2). After standing for 21 hours at 30°C samples were filtered through Whatman No. 1 filter paper, and the optical density of the filtrates was read at 540 nm on a Gilford Spectrophotometer.

Alkaline Denaturation Methods

A. Singer Test

To 0.1 ml of a solution of oxyhaemoglobin is added 1.6 ml of N/12 NaOH (pH 12.7) at 20°. After 1 min the hemoglobin solution is acidified (3.4 ml of a solution: 800 ml 2M $(NH_4)_2$ SO_2 + 1 ml 12 N HCl) and the mixture is filtered. The percentage of undenatured hemoglobin remaining in the filtrate is determined spectroscopically at 540 nm.

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B. Alternate Method

From 0.020 to .050 ml of a 5% solution of oxyhaemoglobin is added to a 1 ml cuvette containing .025N or .05N NaOH at 28°C. The solution is mixed and the denaturation with time is followed at 540 nm on a recording Gilford spectrometer.

Hemoglobin Preparation

Hemoglobin was isolated from the blood of the animals by either retro-orbital eye puncture, decapitation, heart puncture or a combination of the above. The red blood cells were packed by centrifugation at 2000 RPM for 10 minutes and then washed 3 times by re-suspension and centrifugation with 0.9% (w/v) NaCl. Following the final wash the cells were lysed by the addition of 1 - 1.5 volumes of water; the lysates were mixed and frozen. The erythrocyte membrane was removed by centrifugation for one hour at 10,000 x G. The hemoglobin solution was then decanted.

Separation of Components

Separation of the major and minor components of the hemolysate was carried out by chromatography on DEAE-Sephadex (A-50). The method of Huisman et al. (1966) employs elution by a 0.05 M TRIS-HCl gradient from pH 7.9 to 7.0. The initial pH of the column was 8.0, and the hemoglobin was dialyzed against a pH 8.0 TRIS-HCl buffer before loading. This procedure was subsequently modified to employ a single eluting buffer at pH 7.85 (Stratton, personal communication) instead of a gradient.

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Separation of α - β Chains

Globin was prepared from hemoglobin by precipitation in acid-acetone at -20° (Genaux, 1969). For the separation of globin into α- and β-chains the method of Clegg et al. (1966) was used, as modified by S. H. Boyer (personal communication to P. R. Morrison). A 1 x 30 cm column was filled up to 15 cm with CM 32 or CM 52 cellulose (Whatman) which had previously been suspended in starting buffer (.005 NaH_2PO_4). Dry globin was dissolved in 1 ml of starting buffer; the globin sample was layered on top of the equilibrated CM - cellulose column. Starting buffer was allowed to flow through the column for 1 - 2 hours in order to remove any unbound material. The flow rate was set at about .25 ml/minute after the end (0.45 M NaH_2PO_4) buffer was added and the gradient started. Freshly precipitated globin was necessary for a successful separation. Aminoethylated globin was found to also give a good separation, and unreacted chains appear as separate peaks.

Aminoethylation

Modification of cysteine residues in globin was carried out by a procedure of Jones (1964). Globin was dissolved (5 mg/ml) in 1 M TRIS, and subsequently urea was added (.5 g/ml) and stirred to dissolve. The solution was adjusted to pH 9.6 with conc. HCl and was flushed with nitrogen for 5 minutes. Mercaptoethanol (0.04 ml) was then added and the solution was left stoppered for 30 minutes to ensure reduction of the thiol groups. Finally ethyleneimine (0.1 ml)

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was added and the solution was left stoppered at room temperature for 2 hours. The aminoethylated globin (AE) was separated from salts and urea on a 3.5 x 100 cm column of Sephadex G-25 using 5% formic acid in water. The protein emerged in the void volume and was freeze dried (see also Lehman and Huntsman, 1974).

Cyanogen Bromide (CNBr) Cleavage

The reaction of cyanogen bromide with either the whole globin or with individual polypeptide chains was performed essentially according to the procedure of Tsao et al. (1974). Approximately 200 mg of protein were dissolved in 3 ml of ice-cold 70% formic acid. To this was added 2 ml of ice-cold CNBr solution (1 g of CNBr/10 ml of 70% formic acid) to provide a 1:1 (w/w) ratio of CNBr to protein. The reaction was allowed to proceed 36 hours in a nitrogen atmosphere at 4° with gentle stirring. The CNBr reaction mixture was diluted with H_2O and freeze dried.

Sequencing Techniques

All peptides sequenced in this study were subjected to automated Edman degradations as described by Dwulet et al (1975). A Beckman Model 890 C sequencer[1] was used with either the fast peptide - DMAA program or the fast - protein program (Dwulet et al., 1975). Cyanogen bromide peptide B2, which had several free ε-aminolysine residues, was first coupled with m-SPITC to decrease extraction

[1] See glossary for description of the Beckman sequencer

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losses. This was accomplished by first drying the peptide in the sequencer cup and then adding 1 mg of m-SPITC dissolved in 0.5 ml of DMAA buffer. The peptide was coupled for 1 hr and the buffer was removed by vacuum evaporation. When the peptide was dry, the desired program was started.

The amino acid phenylthiohydantoins were identified by gas chromatography on 2 mm x 4 ft. columns of 10% SP400 Chromosorb WHP in a Hewlett-Packard 5711A gas chromatograph as described by Pisano et al. (1972). Residues which were found to be acids or amides were also checked by thin-alyer chromatography (Inagami and Murakami, 1972). Residues which gave no peaks on the gas chromatograph were reconverted by 6 N HCl (Van Orden and Carpenter, 1964) to the free amino acids which were then determined on the amino acid analyzer (Spackman et al., 1958).

Cleavage of C-Terminal with Carboxypeptidase (CP)

Two alternative procedures were used depending on whether the C-terminal residue was a basic amino acid (Arg or Lys; procedure A) or not (procedure B).

A. To a solution of α chain (^{5mg}/5 ml H_2O) was added CPB, 0.020 ml, followed immediatel;y by addition of 5 ml of 0.2 N NH_4 $C_2H_3O_2$. After 15 minutes an additional 0.20 ml of CPA was added together with 0.010 ml of CPB. Samples of 1.8 ml were removed at designated intervals, and reaction was stopped by freezing.

B. To a solution of peptide which was suspended in 0.1 M NH_4

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$C_2H_3O_2$ was added CPA, 0.010 ml. The reaction was run at 37°C and stirred frequently. At various times aliquots were removed, and reaction was stopped by freezing. The lyophilized samples were dissolved in pH 2.7 buffer and run on a Technicon amino acid analyzer.

Tryptic Digestion

Tryptic digestion of 10 mg samples was performed under N_2 at 30° in fresh 1% NH_4HCO_3 solution with 0.1 ml of fresh trypsin solution (Worthington, 3X crystallized, 1 mg/ml 0.001 NHCl). After 4 hours the digest was diluted with water, and the pH was lowered to 3 with conc. HCl. The sample was then frozen and lyophilized. Dried digests were taken up in H_2O, centrifuged and relyophilized.

Chymotryptic digestion employed the same procedure with the replacement of trypsin by chymotrypsin (Worthington).

Peptic Digestion

Peptic digestion of 10 mg samples was performed under N_2 at 30°C in 0.05 MHCl with 0.1 ml of pepsin (Sigma - 2 mg/ml). After 6 hours the sample was frozen and lyophilized.

Fingerprinting

Two-dimensional displays of tryptic peptides were obtained on paper by high voltage electrophoresis in pH 6.4 pyridine-acetic acid buffer followed by descending chromatography in n-butanol-pyridine-acetic acid-water (30:20:6:24) solvent (Genaux and Morrison, 1973a).

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The resulting peptide maps were stained with ninydrin (0.2 g in 1 l. of acetone). Hydrolysates were prepared from the eluted peptides (6 M HCl for 24 hours at 105°C) and were analyzed using a Technicon amino acid analyzer.

Stoichiometry of The Peptide Data

Most of the amino acid data in the tables in this thesis approximate integral numbers of amino acids per peptide, with a latitude of ± 0.3 amino acids. There are three general explanations which can account for most of the observed departures from perfect stoichiometry:

1) loss of amino acid during analytical procedures
2) apparent gain of amino acids due to minor contaminants related to nearby peptides, and
3) sequence dependent staining and hydrolysis behavior.

It was found that after staining the peptide map with .020% ninhydrin, some of the peptide N-terminal residues were partially destroyed. Consequently, analysis of a peptide eluted from the stained peptide map sometimes gave a low value for the N-terminal amino acid.

The hydrolytic behavior of polypeptides includes some destruction of serine, threonine and proline, and some resistance towards acid hydrolysis of peptide bonds especially involving dipeptides of valine, leucine and isoleucine (Tristram, 1966). Savoy et al. (1975) amplified Tristram's study and showed that the liberation of arginine, leucine,

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isoleucine, valine, and phenylalanine is slow and not quite complete at 22 hrs of hydrolysis.

Assignment of Amide Residues

Asparagine and glutamine residues are hydrolysed to their acid forms during acid hydrolysis. Their presence in the sequence was determined by observing the electrophoretic mobility of the peptides at pH 6.4 which results because of the electric charge to the following amino acids: lysine (+1), arginine (+1), aminoethylcystein (+1), histidine (+1/2), aspartic acid (-1), glutamic acid (-1), asparagine (0) and glutamine (0) (Offord, 1966).

Special Amino Acid Staining

Tryptophan: either the excized peptide or the entire peptide map is dipped in fresh Ehrlich reagent (1g p-dimethylamino benzaldehyde, 10 ml conc. HCl and 90 ml acetone) and then dried. The ninhydrin-stained spots first turn a pale orange and fade completely. In a few minutes the typtophan peptides develop a purple color similar to the original ninhydrin color. If their concentration on the map is high, tryptophan peptides can be detected occasionally before ninhydrin staining by means of their florescence in UV light.

Proline: The paper is sprayed or dipped in a 2% isatin solution (Sigma). The Proline peptides turn blue after being heated in an oven for 2 - 5 minutes at 105°C.

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2, 3-Diphosphoglyceric Acid Determinations

Analysis of the 2, 3-diphosphoglyceric acid (DPG) concentration in whole blood was carried out by monitoring the change in absorbance of NADH which accompanied the oxidation of the DPG (Grisolia et al., 1969). The procedure was automated as described by Atkinson (1973) using an Autoanalyzer 1. After 0.010 ml of blood was added to 10 ml of a NaOH solution (5 mmol per liter), the samples were stored at 5°C till analyzed. The DPG concentrations of the samples were determined from standard curves prepared on the same day.

The isotopic enrichment of the carbamino adduct was performed at Indiana University by equilibrating the sample solutions with $NaH^{13}CO_3$ and $Na_2{}^{13}CO_3$ sealed under room pressures of CO_2. Special NMR tubes (Morrow, 1974) allowed the handling of deoxygenated hemoglobin samples. Samples were placed in either a Varian high resolution DP-60 or XL-100 NMR spectrometer modified for Fourier transform (FT) operation. After the NMR measurement each protein sample was analyzed for pH, total carbonate concentration and the degree of ^{13}C enrichment.

Mass Spectroscopy. Varian MAT CH7 or Associated Electrical Industries MS-9 magnetic sector mass spectrometers were used to monitor the degree of isotopic enrichment in the ^{13}C samples. Most of the analyses involved the measurement of the $^{13}C/^{12}C$ ratio in the atmosphere within the sealed NMR tubes. The nature of the specially constructed NMR tubes allowed anaerobic loading of the gas sample directly from the NMR tube (Morrow, 1974). Several mass scans were made of each sample, and the $^{13}CO_2/^{12}CO_2$ ratio was calculated from the

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integral areas of the parent peaks at 45 and 44 m/e units. Integration was done digitally by means of an on-line Texas Instruments 980 mini-computer.

Measurement of Total Carbonates. A Natelson Microgasometer model #600 with motorized shaker attachment #M-373-20, manufactured by Scientific Industries, Inc., was used to analyze the total carbonates present in small volumes of sample. The principle of operation of this instrument is identical to the classical Van Slyke manometric method in which the difference in gas pressure over a solution made first acidic and then basic is compared at constant volume and temperature. This difference is representative of the pCO_2 which with appropriate corrections is convertable to the total carbonate concentration. Complete operating instructions are available (Scientific Industries, Inc., Instruction manual #6).

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CHAPTER III

CHARACTERIZATION OF THE HEMOGLOBINS FROM ARCTIC MICROTINES

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CHAPTER III

CHARACTERIZATION OF THE HEMOGLOBINS FROM ARCTIC MICROTINES

INTRODUCTION

Hemoglobins of inbred strains of the laboratory mouse, *Mus musculus*, have been extensively studied with respect to physical properties and amino acid composition and sequence. Differences in electrophoretic properties can be correlated with β-chains (Hutton et al., 1962; Popp, 1962a) while differences in solubility are due to α-chain differences (Popp, 1962b; Russell and McFarland, 1974). These and other studies have resulted in a good understanding of the genetics of mouse hemoglobins.

Hemoglobins from species of wild rodents have been studied far less than those of laboratory mice. Hematological values and hematocrits for microtine hemoglobins have been reported (Dieterich, 1972, 1973; Sealander, 1966) and electrophoretic mobility has been applied as a taxonomic tool (Foreman, 1960, 1968; Johnson, 1968). Marchuwska - Koji (1966) found single hemoglobins with identical migrations in the three microtines, *Clethrionomys glareolus*, *Microtus arvalis*, and *M. agrestis*. Johnson (1974, Fig. 4, Page 38) showed that *M. p. drummondi*, *M. p. funebris* and *M. p. insperatus* also have only one hemoglobin component.

Recently Genaux and Morrison (1973a, b) reported detailed

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comparisons of tryptic peptides from a number of microtine hemoglobins. They noted that *M. p. pennsylvanicus* and *M. p. tananaensis* have one electrophoretically distinct hemoglobin whereas *M. miurus*, *M. oeconomus* and *M. abbreviatus* each have two. At pH 8.6 both of the single *M. pennsylvanicus* components have the same mobility as the major component of the other *Microtus* species.

Isoelectric Focusing

The technique of electrofocusing involves the separation of proteins of differing isoelectric points by electrophoresis in a pH gradient. It has been used extensively in the investigation of hemoglobins. Hemoglobin behaves as a dipolar ion which bears a net positive charge on the acid side of its isoelectric point and a net negative charge on the alkaline side. The isoelectric point of the protein molecule is the pH at which the effective number of positive and negative charges on the surface of the molecule cancel each other out and result in overall electrical neutrality. A protein molecule fails to move in an electric field at this pH (its pI). Table 3-1 compares the isoelectric points of hemoglobins which were examined during the course of this research. It can be seen that all the hemoglobins studied are very similar. The two hemoglobins from *M. xanthognathus* have pI 7.1 (major) pI 7.3 (minor). An overall conservation of surface charge in hemoglobins from different *Microtus* species can be seen from this data.

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TABLE 3-1

Comparison of Rodent Hemoglobin pI

Species	I	II	III	IV
Peromyscus m. bairdii[1]	7.32 (major)	7.20 (minor)		
Mus musculus	7.25			
Rat (Sprague - Dawley)[2]	7.40	7.20	7.10	6.90
Swiss mouse[2]	7.05	6.95	6.85	
Calomys callosus	7.35 (minor)	7.08 (major)		
M. oeconomus	7.30 (minor)	7.10 (major)		
M. xanthognathus[3]	7.30 (minor)	7.10 (major)		

[1]Data from P. Ramakrishnan, sucrose gradient, pH at 4°C.

[2]From Drysdale et al. (1970) pH @ room temperature in polyacrylamide gel.

[3]The major and minor hemoglobin components for M. xanthognathus, M. pennsylvanicus pullatus and Dicrostonyx torquatus were also separated by column chromatography on DEAE A 50 Sephadex. The minor component eluted first and was followed by the major component.

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Electrophoresis

Table 3-2 shows the relative distribution and mobility of several microtine hemoglobins. The ratio between major and minor forms is about 2:1 for M. oeconomus, M. xanthognathus and D. rubricatus and 3:1 for M. pennsylvanicus pullatus (Montana) and L. sibiricus.

Figure 3-1 shows the polypeptide chain patterns for several different microtine hemoglobins at pH 9.1. As would be expected from the similar migrations of their hemoglobins, all the Microtus species possess chains with similar mobilities. The minor α-chain has a greater mobility towards the cathode than the major α-chain. It is interesting that hemoglobins from all species having a major component with a relative mobility of approximately 1.0 (Table 3-1) are distinguished by β-chains which migrate with the same mobility (Fig. 3-1). D. rubricatus has a hemoglobin which moves faster toward the anode (relative mobility 1.1) as a consequence of the greater negative charge on the β-chain (like that of human β-chain, Fig. 3-1). It should be noted that Lemmus and Microtus hemolysates usually show a fourth trailing band which may be a non-heme protein. This band is not present in either major or minor hemoglobin chain patterns of M. pennsylvanicus pullatus or M. xanthognathus after separation on DEAE-Sephadex.

When the pH is lowered to about 7, the imidazols of histidine residues become 50 percent ionized (Table 3-3). Figure 3-2 shows that when the pH is 7.3, the minor α-chain of M. pennsylvanicus pullatus has a greater mobility in relation to the minor α-chain of M.

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TABLE 3-2

Distribution and Mobility of Microtine Hemoglobins

Species	Major Component				Minor Component			
	% of Total	N[1]	Relative Mobility[2]	N	% of Total	N	Relative Mobility	N
M. oeconomus	64 (±3)[3]	4	.99 (±.02)	7	36 (±3)	4	.75 (±.05)	7
M. pennsylvanicus pullatus	70 (±4)	11	.98 (±.03)	5	30 (±4)	11	.76 (±.05)	5
M. pennsylvanicus tananaensis	100		.97 (±.02)	5	0			
M. xanthognathus	64 (±2)	4	.97 (±.01)	6	36 (±2)	4	.75 (.05)	6
D. rubricatus	62	2	1.16 (±.06)	5	38	2	1.03 (±.04)	5
L. sibiricus	70	2	.98 (±.02)	4	30	2	.81 (.05)	4

1. Number of determinations.
2. Human hemoglobin was assigned a value of 1.0; migration direction was toward the anode (+).
3. ± Standard deviation of the mean.

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Fig. 3-1. Electrophoretic separation of hemoglobin chains on urea-cellulose acetate. 1, M. oeconomus; 2, M. pennsylvanicus tananaensis; 3, M. pennsylvanicus pullatus; 4, M. xanthognathus; 5, human; 6, D. t. rubricatus; 7, L. sibiricus; 8, M oeconomus hemolysate. Samples were treated with equal volumes of 2-mercaptoethanol at 5°C for 2-6 hr. and electrophoresis carried out in TRIS:EDTA:Borate buffer pH 8.6, 6 M urea, 0.05 M 2-mercaptoethanol. Amido Black stain, 350V, 2MA, 60 min. Migration direction was toward the cathode, in contrast to hemoglobins which migrate toward the anode. The chains were identified by peptide mapping after separation on a carboxymethyl cellulose column from which they eluted in the reverse order as observed in electrophoresis.

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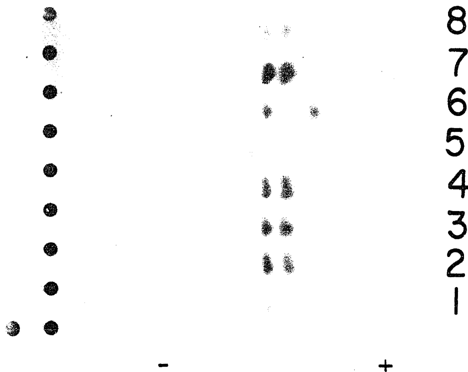

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Fig. 3-2. Electrophoretic separation of globin chains at pH 7.3 on urea-cellulose acetate. 1, 6, *M. xanthognathus* major component; 2, *M. xanthognathus* (whole hemolysate); 3, *M. oeconomus* (whole hemolysate); 4, *M. p. pullatus*, note slightly faster migration of leading band; 5, *D. rubricatus* (whole hemolysate).

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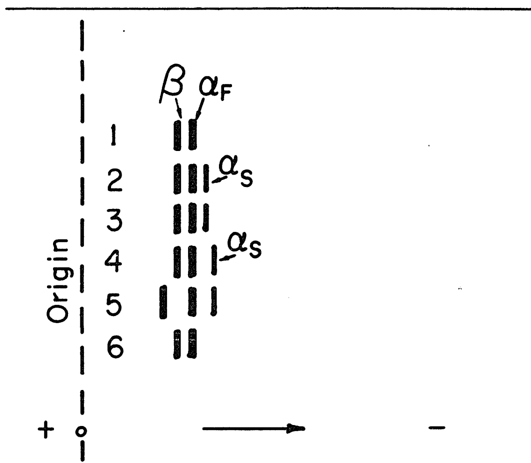

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TABLE 3-3

Degree of side group ionization in electrophoresis buffers[3]

("Charged") Amino Acids with ionizable side Groups	Side Group	pKa[1]	Percent Charged[2] Buffer pH 7.0	8.6	9.0
ASP	-COOH	4.6	99	100	100
GLU	-COOH	4.6	99	100	100
HIS	imidazole	7.0	50	2	1
TYR	phenolic	9.6	100	91	80
CYS	-SH	9.1	100	---	---
LYS	$-HN_2$	10.2	100	98	94
ARG	guanidyl	12	100	100	100

[1]pKa values are those of Tanford and Havenstern (1956).

[2]Percent charged values are from Johnson (1973).

[3]The surface charge of the protein molecule is the outcome of the charges on the above amino acids. As in all globular protein molecules, in hemoglobin the overall charge will depend not only on the total number of these side chains but also on their position. The electrical activity of an amino acid is influenced by neighboring amino acid side chains.

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xanthognathus and M. oeconomus. This suggests the presence of a histidine in M. p. pullatus minor α-chain which is not present in the other Microtus species. Electrophoresis of these hemoglobins after cystamine treatment showed a retention of the two band pattern. This indicates that the number of active cysteines are the same in each component. A change in mobility of both components indicated that the hemoglobin components were derivatized.

4. Solubility

The salting out of hemoglobin from each of several microtine hemolysates in phosphate buffers at pH 6.7 is shown in Figure 3-3. Hemoglobins from M. pennsylvanicus pullatus and from M. oeconomus are the most soluble in these phosphate solutions. Lemmus sibiricus and M. xanthognathus hemoglobins are the least soluble of the species studied. When the whole hemolysate from M. pennsylvanicus pullatus or from M. xanthognathus is salted out of solution, both the major and minor hemoglobins came out of solution together. Evidence for this was obtained when filtrates with different O.D. values were concentrated, dialyzed and subjected to electrophoresis. In all cases, both major and minor hemoglobins were found in approximately a 2:1 ratio in the soluble portion.

Major and minor hemoglobin components were separated by column chromatography on DEAE A 50 Sephadex and checked for purity and homogeneity by cellulose acetate electrophoresis before determination of solubility in concentrated potassium phosphate solutions. The

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Fig. 3-3. Salting out of microtine hemoglobins. Hemolysates were placed in varying concentrations of pH 6.7 phosphate buffer, equilibrated 21 hr. at 30°C, filtrate read to determine amount of hemoglobin remaining in solution. *M. pennsylvanicus* = *pullatus* (Montana).

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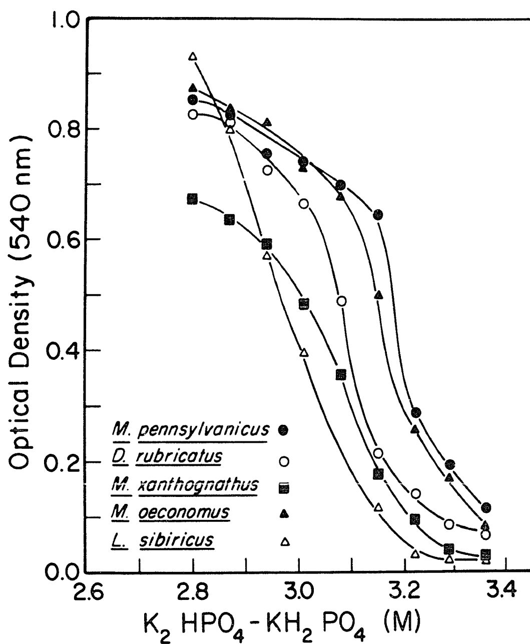

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isolated components of M. xanthognathus were found to have unique solubilities (Fig. 3-4). The same situation was found to exist for M. pennsylvanicus pullatus although the curves were shifted to the right compared to the corresponding curves for M. xanthognathus. This is shown in Figure 3-5 and compared with the single hemoglobin from M. p. tananaensis. The single hemoglobin of the Alaskan subspecies has a solubility similar to the whole hemoglobin of the Montana subspecies.

5. 2, 3-Diphosphoglycerate

The effect of organic phosphates on the oxygen binding properties of mammalian hemoglobins has been known for a number of years. It has been shown that binding of intracellular organic phosphates, notably 2, 3-Diphosphoglycerate (DPG), is stronger with deoxyhemoglobin than it is with oxy- or carboxy-hemoglobin (Benesch and Benesch, 1967). Thus the oxygen affinity of hemoglobin is decreased upon addition of DPG. A clear picture has emergedof the interactions between hemoglobin and organic phosphates (Benesch et al., 1975; Riggs, 1976; Bonaventura et al., 1975; Gray, 1973; Benesch and Benesch, 1974; Tomita and Riggs, 1970), and an evolutionary relationship between taxa has been proposed (Coates, 1975a, b).

At the same time, the importance of the concentration levels of DPG within the mammalian erythrocyte for an optimal regulation of the hemoglobin function has been studied (Riggs, 1976; Brewer and Eaton, 1968). Nobel and Brewer (1972) have pointed out that ATP and DPG levels vary quantitatively and are influenced by both heredity and

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Fig. 3-4. Salting out of *M. xanthognathus* hemoglobins. Conditions as for Fig. 3-8. Major and minor hemoglobins were separated by chromatography on DEAE A 50 Sephadex in 0.05 M TRIS buffer 7.8.

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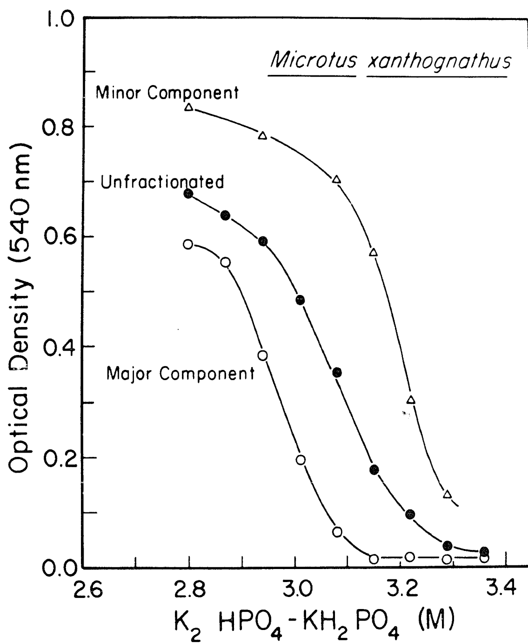

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Fig. 3-5. Salting out of *M. pennsylvanicus* subspecies. Major component of *M. p. pullatus* hemoglobin was separated on DEAE Sephadex in .05 M TRIS buffer pH 7.8.

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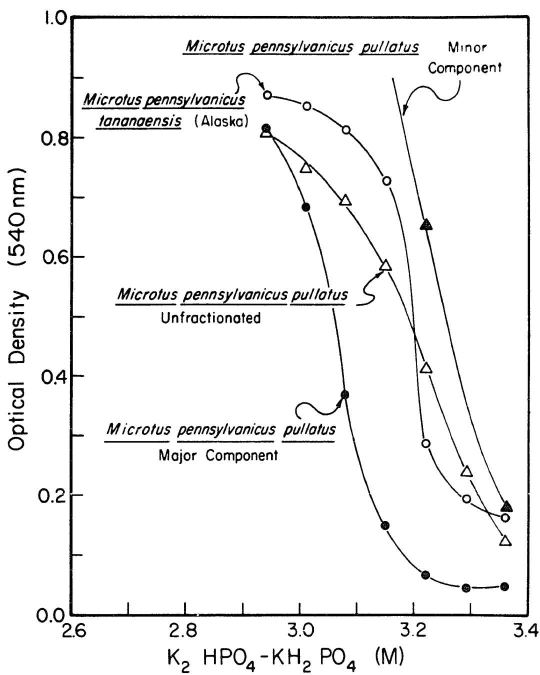

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environment. They were able to develop two strains of hooded rats which were true-breeding for either high (31 μmoles/gHb) or low (21 μmoles/gHb) levels of DPG from an initial population which had an average of 27 μmoles/gHb. They also showed a correlation of ATP level with 2-3 DPG levels and a difference in the oxygen dissociation curve between strains.

Little information has been available for DPG levels in northern microtines. Table 3-4 presents the results of a brief survey which was made of species in the IAB colony. The DPG levels were obtained by the method of Atkinson (1972). Although too few animals were examined to draw statistical conclusions, the results of this survey seem to indicate that microtine DPG levels are appropriate to body weight (Riggs, 1960, 1967) in relation to hemoglobin content. The muskrat data, showing a lower level of DPG, is consistent with its being the largest microtine. In the presence of DPG, mouse and human hemoglobins have been shown to have different Bohr effects, even though they had very similar Bohr effects in the absence of DPG (Riggs, 1976). The variability in DPG levels observed, either within or between subspecies of M. pennsylvanicus, indicates that different DPG genotypes may be present, similar to those observed in rats.

Discussion

Comparative solubility characteristics for several microtine hemoglobins have been obtained in concentrated potassium phosphate solutions. This method provides an alternative means to

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TABLE 3-4

2,3 DPG Levels in Microtine Rodents

Species	N	μMDPG/gHb	MDPG/MHb
Calomys callosus	1	20.5	1.3
Ondatra zibethicus	1	8.3	.5
Lemmus sibiricus	2	30.5 ± .06	1.9
Clethrionomys rutilus	1	22.6	1.4
M. oeconomus	2	23.0 ± .60	1.5
M. xanthognathus	4	24.5 ± 4.60	1.6
D. t. stevensoni	2	26.6 ± .90	1.7
D. t. rubricatus	2	24.2 ± 3.50	1.5
M. p. tananaensis	2	30.9 ± 1.25	1.9
M. p. pullatus	4	21.9 ± 5.00	1.4
(M. p. tan. x. M.p. pul.) F1	6	30.0 ± 1.60	1.9
Homo sapiens	4	11.5 ± 2.50	.7
Rat[a]		27 ± 5	1.7
Mouse[b]		24.8 ± 3	1.6

[a]Noble and Brewer (1972).

[b]Tomita and Riggs (1971).

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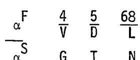
electrophoresis for differentiating between hemoglobins. Although the hemoglobins of *M. xanthognathus*, *M. oeconomus* and *M. pennsylvanicus pullatus* have identical electrophoretic mobility at pH 8.9, there are considerable differences in solubility as shown in Figures 3-3 to 3-5. When unfractionated hemolysates were salted out, both major and minor components came out together. This coprecipitation of two hemoglobins is in agreement with other experiments in which two hemoglobins mixed *in vitro* salted out together (Popp and Cosgrove, 1959). When major and minor hemoglobin components were compared for *M. p. pullatus* and *M. xanthognathus* it was found that the major hemoglobin was less soluble than the minor hemoglobin. The range of solubilities observed for these *Microtus* species overlaps the range reported for a variety of strains of the laboratory mouse (Popp and Cosgrove, 1959; Stratton and Popp, unpublished) and *Calomys* (Stratton and Duffy, unpublished) although *Microtus* species seem to possess the more soluble hemoglobin.

There is a minimum of three differences between the sequences of α^F and α^S chains of *M. xanthognathus* (Chapter IV).



α^F / α^S	4	5	68
α^F	V	D	L
α^S	G	T	N

The solubility is less for the major component because of the leucine at position 68. This conclusion is supported by data from strains of *Mus musculus* (Popp, 1962; Russel and McFarland, 1974). The C57BL strain has the highest solubility and has Asn at position α68. The valine at position α4 may also influence the solubility of the major component.

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Difference in solubility of the major hemoglobins of the species shown in Figure 3-3 may be explained by α-chain differences. Both L. sibiricus and M. xanthognathus hemoglobins have a valine residue at position 34 of the α-chain. This position is an $\alpha_1\beta_1$ subunit contact point (Lehmann and Huntsman, 1974). Calomys hemoglobin (Morrison et al., 1976) also has a valine residue at position α34 and it has a lower solubility in phosphate buffer. All other hemoglobins of species shown in Figure 3-3 have an alanine at position α34 and have a relatively high solubility in the phosphate buffer. This solubility behavior may be related to a difference in hydrophobicity between alanine and valine.

Differences in electrophoretic mobility between major and minor hemoglobins of Microtus species and subspecies can be ascribed to α-chain differences since their β-chains behave the same on electrophoresis at the pH where the hemoglobins move differently (Fig. 3-1). These Microtus species do not follow the pattern established for Mus musculus by Popp (1962a, b), Russell and McFarland (1974), and Hutton et al. (1962) in which electrophoretic differences in hemoglobin components are correlated with β-chain differences. The evidence presented in Figure 3-1 argues for the existence of multiple hemoglobin α-chain loci for several microtine species. With the exception of M. pennsylvanicus we have observed only a single phenotype in each of several species (Table 3-2). Several hypotheses are available with which to explain the presence of two hemoglobin components in the whole population sampled. The hypothesis of Nute (1974) is most

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consistent with these data. He proposes that the two structural loci per chromosome have come about through the process of duplication followed by mutation. The hemoglobin ratio, in turn, could mean that the genes in question are linked and that linked α-chain genes are not equally active in the production of their respective chains. Rather, one governs the synthesis of approximately twice as much chain as the other. A hemoglobin ratio of about 2:1 as found in M. oeconomus, M. xanthognathus and D. rubricatus (Table 3-1), would indicate a genotype having two duplicate alleles, each chromosome containing different linked α-chains genes produce hemoglobin in a 2:1 ratio (see Table B-1).

Differential control of duplicated structural genes could imply the functional diversification of duplicated regulatory genes. In man it would appear that the α-chain gene action, the ε-chain gene action and γ-chain gene action have different receptors for separate regulatory genes. The β- and δ-chain gene actions on the other hand, must still be under the control of the same regulatory gene, and therefore, they must have nearly identical receptors (Ohno, 1970). This may explain the differential synthesis of Microtus α-chains. Ohno (1970) believes that since the hybrid lepore chain, which has the amino terminal of δ-chain, is produced in as small amount as the δ-chain itself, the receptor base sequence at the head of the δ-chain gene is responsible for the poor productivity of this structural gene locus. The minor α-chain from M. xanthognathus may have a different receptor base sequence than the major α-chain.

An alternative explanation for the different amounts of α-chain

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synthesized is that there are two major α-chain genes (α^F) and one minor (α^S). If all genes are transcribed and translated equally, a 2:1 ratio would result. Such a situation was proposed for the γ-globin genes in man, but recent studies indicate that there are only two γ-globin genes. The two different γ-globin ratios must therefore be due to either differential gene transcription or m-RNA translation rather than to gene dosage.

Huisman (1974) believes that events leading to the duplication of the hemoglobin α loci in bison, goat and aoudad are associated with a considerable increase in the hemoglobin α-locus "efficiency". He points out that the Hb IIα (minor component) locus has become considerably more effective, resulting in an absolute increase in about 35% in the production of the IIα chains. However, as in the Equidae (Clegg, 1974), it appears that α-chain gene duplication must have occurred on a number of occasions during the evolution of ruminants. I think that any differences between species in amount of minor component would be a secondary effect of the gene duplication process. Feedback regulation to equalize β- and α-chains may be related to the observed "position effect".

Physiological Considerations

The existence of two distinct α-chains in microtines indicates that in the red cell there exists a hybrid hemoglobin tetramer possessing both α^F and α^S. However, hybrid hemoglobins formed from dissimilar dimers cannot be isolated by conventional electrophoresis

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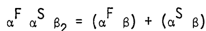
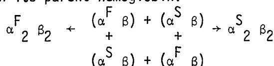
or chromatography because, during separation, the hybrid dissociates into unlike dimers as the fast component is removed from the equilibrium



$$\alpha^F \alpha^S \beta_2 = (\alpha^F \beta) + (\alpha^S \beta)$$

Each dimer can recombine to form a non-hybrid tetramer and then migrate with its parent hemoglobin.



$$\alpha^F{}_2 \beta_2 \leftarrow \begin{matrix} (\alpha^F \beta) + (\alpha^S \beta) \\ + \quad\quad + \\ (\alpha^S \beta) + (\alpha^F \beta) \end{matrix} \rightarrow \alpha^S{}_2 \beta_2$$

anode   cathode

Such hybrids have been demonstrated by isoelectric focusing of deoxygenated hemoglobins on polyacrylamide gels (Bunn and McDonough, 1974). The equilibrium constant for the dissociation of deoxyhemoglobin into dimers is several orders of magnitude lower than that for oxyhemoglobin. Deoxygenation prior to isoelectric focusing enhances the integrity of the tetramers and prevents the dissipation of asymmetrical hybrids during separation. This is probably due to intersubunit salt bonds which stabilize the deoxytetramer (Perutz, 1970).

The amount of hybrid formed is predictable from the binomial distribution ($\alpha^2 + 2\ \alpha\beta + \beta^2 = 1$). This strongly implies that the stability of the mixed-hybrid hemoglobin is equivalent to that of the parent hemoglobins.

In M. xanthognathus, the percentages of the different tetramers in the red cell as calculated from the binomial distribution are: fast - 43.5%, slow - 11.5% and hybrid-45%. Assuming a corpuscular hemoglobin concentration of 32 g/l, the intracellular fast hemoglobin would be 13.9 g/l, as compared to 14.4 g/l for the hybrid hemoglobin.

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Although there may be little selective difference, it seems that the hybrid is physiologically as important as the major component.

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CHAPTER IV

STUDIES ON THE PRIMARY STRUCTURE OF HEMOGLOBIN FROM *MICROTUS XANTHOGNATHUS*

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CHAPTER IV

STUDIES ON THE PRIMARY STRUCTURE OF HEMOGLOBIN FROM MICROTUS XANTHOGNATHUS

Introduction

Jones (1961) distinguished three types of heterogeneity in hemoglobin: maturation heterogeneity, genetic heterogeneity and minor component heterogeneity. With the exception of muskrat and some subspecies of meadow vole, microtines usually exhibit minor component heterogeneity in hemoglobin (see Chapter III). This is also the case for *Mus* (Gilman, 1974; Popp, 1968), *Rattus* (Garrich et al., 1975), and many other rodent species (Aste-Salazar and Morrison, 1962).

The microtine hemoglobins which display this heterogeneity have three electrophoretically distinct polypeptide chains, and it is of interest to identify which chain is duplicated and what is the specific chemical difference. Since there are 24 North American species in the genus *Microtus*, it would be valuable to extend the early observations on the hemoglobins of this genus made by Genaux and Morrison (1973a, b). According to favorable circumstances at the time, it was decided to study *Microtus xanthognathus*, the yellow-cheeked vole. This vole occurs only in boreal North America from west-central Alaska to the western shore of Hudson Bay.

The yellow-cheeked voles which were used in this study were obtained from a colony established by Dr. R. L. Rausch with several

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animals captured at Hess Creek, Alaska (see Rausch and Rausch, 1974, for a history of the animals). In the colony of yellow-cheeked voles the two-banded hemoglobin phenotype has been observed in each of approximately 60 animals sampled (over a three year period). At this time there is no data for the variability within this Hess Creek population or between it and other M. xanthognathus populations.

In the process of working with pooled samples and rounding off amino acid stoichiometry to integers, allelic heterogeneity may be missed in the colony population samples. For example, if 10% of the animal hemoglobin showed threonine at a certain position while 90% showed serine there, the chromatographic and electrophoretic properties of the peptide would probably not be affected, and the results would only indicate a serine residue at that position. However, it should be noted that previous studies on Microtus were carried out on material from individual animals (Genaux and Morrison, 1973a, b) and that the present data from pooled hemoglobins compare favorably with their data. Although genetic heterogeneity not involving charge changes could have been overlooked, the results of the present study are considered to indicate the lower limit for substitutions between species.

Characterization of Globin Chains

Figure 4-1 shows the polypeptide chain patterns obtained by electrophoresis on cellulose acetate in urea-TEB buffer (pH 8.9). The fast (MX^f) and slow (MX^s) hemoglobins themselves were isolated by

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Fig. 4-1. (6 M Urea) Cellulose acetate electrophoresis at pH 8.9 in 6 M urea demonstrates chain differences between fast and slow hemoglobin components of the yellow-cheeked vole. 1, unfractionated hemolysate; 2, CM-32 separated non-heme protein; 3, DEAE-separated fast component; 4, CM-32 separated α-chain from the fast component (α^f); 5, DEAE-separated slow component; 6, CM-32 separated α-chain from the slow component (α^s); 7, CM-32 separated β-chain; 8, human β- and α-chains. Anode (+) is at the left.

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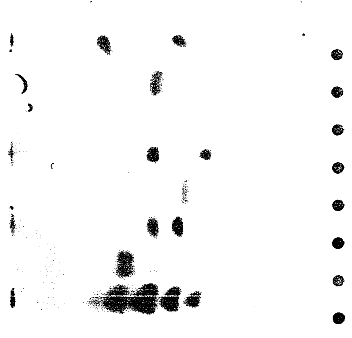

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DEAE-chromatography, and the globin chain components are shown at position 3 for MX^f and at position 5 for MX^s hemoglobin. These chain patterns can be compared with that for human hemoglobin at position 8. The α-chains differ in mobility, with α^f having more negative charge than α^s at pH 8.9.

Primary Structure of β-Chain

Figure 4-2 is a composite of several peptide maps for the tryptic β-chain peptides. The shaded spots on this map indicate the position of tryptic peptides observed only after the β-chain had been subjected to chemical modification by cyanogen bromide. Table 4-1 lists the composition for all of these peptides.

Cyanogen bromide (CNBr) cleaves at the carboxyl function in the peptide bonds of methionines in the β-chain. This results in peptides with a homoserine residue at their C-terminal. In the first application of this technique, cleavage of the methionines in the β-chain from hemoglobin of *Clethrionomys rutilis* was used to ascertain on which side of the methionine the histidine residue in peptide βT5 was located. Figure 4-3 shows the resulting map, and Table 4-2 compares the composition of peptide CNBr-βT5b to the similar peptide later found in β-chain from *M. xanthognathus* and *D. rubricatus*. The cyanogen bromide cleavage procedure was applied to both whole globin from *M. xanthognathus* and to its individual β-chain. The CNBr fragments were separated by gel filtration and were analyzed either by automatic sequencing or by tryptic digestion followed by peptide mapping.

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Fig. 4-2. Composite map of tryptic β-chain peptides of _M. xanthognathus_ hemoglobin. This map is an idealized representation of peptide locations. Suspected contamination by βT12 in the βT13 area of the map is not shown.

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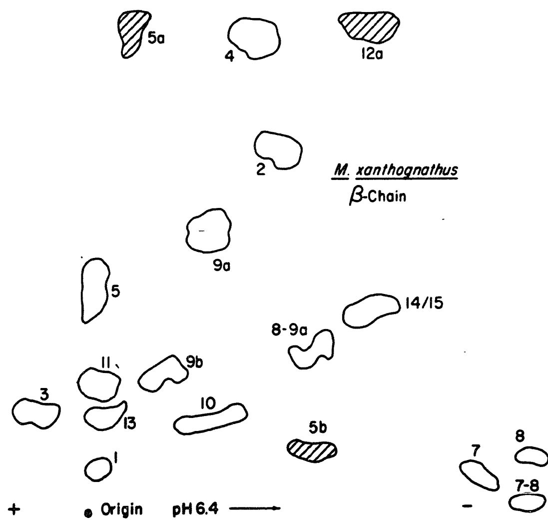

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Table 4-1: An integral number of residues is assumed when the data shown are within ± 0.3 residue of 1, 2, 3, etc. In other cases the integral number inferred is shown in brackets [ ] following the data. Tryptophan was detected in intack peptides by dipping the spot in a fresh solution of 1% p-dimethylaminobenzaldehyde in acetone: conc. HCl, 9:1 by volume. Values in ( ) excluded from relevant peptide.

Notes: 1, Values are low due to N-terminal sequences (Ala - Ala, Val - Ile, Val - Val, Leu - Leu, Phe - Phe); 2, after treatment with CNBr, Met was detected as homoserine (Beale and Kent, 1968). 3, These three peptides are not always well resolved with T12 sometimes appearing in both T13 and T11 regions on the map. Peptide T12 values were calculated from a T13 spot in which a high Met value was observed.

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Table 4-1. Composition of Tryptic Peptides from the β-Chain of *M. xanthognathus*

	T1	T2	T3	T4	T5	CNBr T5a	CNBr T5b	T7	T8-9a	T9a	T9b	T10	T11[3]	CNBr T12a	T12[3]	T13[3]	T14	T15
Lys	1.00	0.86			1.22		0.92	1.00	1.95	1.18	1.00	0.94			1.00	1.20	1.00	
His	0.87				1.05	0.90		0.98	1.05	0.82	0.91	0.89	0.79		2.09		1.00	1.00
Arg			1.00	1.00									0.86					
Asx	0.79		1.56[2]		2.03	1.10	1.20		0.72	1.07	1.80	1.01	2.20	1.00	1.00	1.02		
Thr	0.92			1.21								0.87				1.06		
Ser		1.38[1]			2.48[3]	3.16						2.02	(0.36)			0.92	1.00	
Glx	0.87		1.08	0.98	1.81	0.74	0.83					0.95	1.18			1.62[2]		
Pro				0.97									0.75			1.16		
Gly		1.76	2.02		1.90	1.06	0.71	1.07	1.30	1.06		0.93	(0.32)	1.05	1.65			1.39[1]
Ala	0.91	1.63[2][1]	4.70		3.01	2.23	1.14	0.81	2.10	2.23		1.37[1]	(0.57)		1.30	3.06	3.92	
Val	0.81		1.00	1.80	2.02	1.16	0.92		.75	0.59[1][1]			1.07		1.99	(0.44)	2.31[3][1]	
Cys-AE												0.51[1]						
Met					0.57[1]	+[1][2]								+[1][2]	0.15[1][1]			
Ile		1.14							0.64[1]	0.45[1][1]					0.23[2][1]			
Leu	1.21	0.86	1.10	2.06	1.50[1]	1.10			0.95	1.28	1.98	2.01	1.03	1.38[2][1]	3.03[4]	(0.65)	1.12	
Tyr																		0.89
Phe					2.30[3][1]	2.59[3][1]			0.83	1.25		0.96	1.01			1.62[2]		
Trp		+[1]		+[1]														
Residues	1-8	9-17	18-30	31-40	41-61	41-55	56-61	62-65	66-76	67-76	77-82	83-96	96-109	105-109	105-121	121-132	133-144	145-146

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Fig. 4-3. Composite map of tryptic β-chain peptides of the red-backed vole. Peptides 5a and 5b were observed after CNBr treatment of 2 mg of β-chain.

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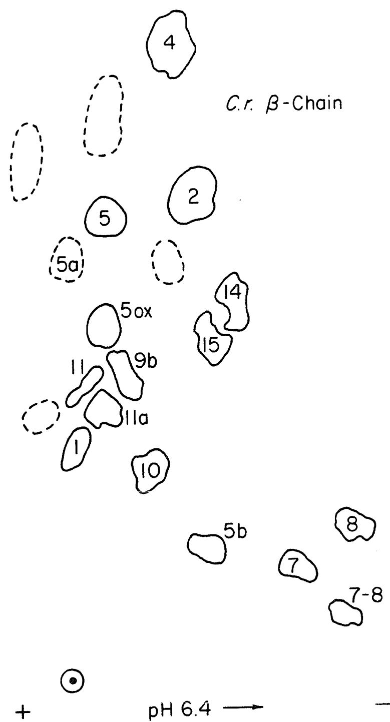

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TABLE 4-2

Composition of Peptide CNBr T5b From Three Microtines

	Gly	Asx	Ala	Glx	Val	Lys
M. xanthognathus	0.71	1.20	1.14	0.83	0.92	0.92
C. rutilis	0.75	0.95	1.04	0.95	1.06	1.00
D. rubricatus	0.88	0.60	1.33	0.86	1.00	1.00

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Tables 4-3, 4-4, and 4-5 give the results of automatic sequencer analysis. Figure 4-4 shows the map of the tryptic digest of the N-terminal (β1 - 55) obtained from cyanogen bromide cleavage and Table 4-6 shows the compositions of the peptides eluted from this map. After this fragment had been identified as the N-terminal CNBr peptide, other aliquots of the same fragment were treated with carboxypeptidase A for 15 minutes and then analyzed. This treatment yielded a C-terminal composition for which the following sequence is inferred (residues β52 - 55): Ser.Ala. Val.Homoserine.

In another study the β-chain was digested with pepsin, and the resultant peptides were separated by peptide mapping (Fig. 4-5). Table 4-7 gives the composition of the peptides that could be identified. The large number of peptides generated by pepsin limited the application of this technique.

Figure 4-6 gives the proposed sequence of the β-chain of M. xanthognathus hemoglobin. This sequence confirms and validates the identification and alignments for the tryptic β-chain peptides obtained previously from closely related microtines. Also shown in Figure 4-6 is the alignment for M. pennsylvanicus based on Genaux and Morrison (1973a and 1973b revised). With the exception of peptide T5, the β-chains from these two species appear to be identical. However, the results of the analyses for peptide T5 of M. xanthognathus and for its cyanogen bromide fragments do not support the postulate of two deletions at residue positions 52 and 54 of Microtus β-chain made earlier by Genaux and Morrison (1973b) on the basis of compositional

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TABLE 4-3

Automated Sequencer Analysis of β-Chain From *M. xanthognathus*

Residue Number	Gas Chromatography Silylation (-)	Gas Chromatography Silylation (+)	Hydrolysis	Sequence
1	V			Val
2			H	His
3	L/I	L		Leu
4	T			Thr
5		D		Asp
6	A			Ala
7		E		Glu
8			K	Lys
9	A			Ala
10	A			Ala
11	L/I	I		Ile
12	S			Ser
13	G			Gly
14	L/I	L		Leu
15	(W)[1]			---
16	(G)[1]			---
17			K	Lys
18	V			Val

Notes: 1. Low recovery
2. Lost during back hydrolysis
3. 3-sulfophenyl-isothiocyanate

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TABLE 4-4

Automated Sequencer Analysis of Cyanogen Bromide Fragment B2 from *M. xanthognathus* (m-SPITC[3] treated)

Residue Number	Gas Chromatography Silylation (-)	(+)	TLC	Hydrolysis	Sequence
56					---
57		N	N		Asn
58	A		A		Ala
59		Q	Q		Gln
60	V		V		Val
61				K	Lys
62	A				Ala
63				H	His
64	G		G		Gly
65				*[2]	---
66				K	Lys
67	V		V		Val
68	L/I	I	L/I		Ile
69				H	His
70	A		A		Ala
71	F				Phe
72	A		A		Ala
73		D	D		Asp
74	G		G		Gly
75	L/I	L	L/I		Leu
76				*[2]	---
77				*[2]	---
78	L/I	L			Leu
79			D		Asp
80			(N)[1]		---
81	L/I	L			Leu
82				K	Lys

Notes: see Table 4-3

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TABLE 4-5

Automated Sequencer Analysis of Cyanogen Bromide Fragment B3 From _M. xanthognathus_

Residue Number	Gas Chromatography Silylation (-)	(+)	TLC	Hydrolysis	Sequence
110	L/I	I	L/I		Ile
111	V		V		Val
112	L/I	I	L/I		Ile
113	V				Val
114	L/I	L	L/I		Leu
115	A		A		Ala
116				H	His
117				*2	---
118	L/I	L	L/I		Leu
119	G		G		Gly
120				*2	---
121		D	D		Asp
122	F		F		Phe
123	(T)[1]				---
124	(P)[1]				---

Notes: See Table 4-3

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Fig. 4-4. Separation of tryptic peptides from cyanogen bromide fragment B1. This map confirms peptide designations used in Figs. 4-2 and 4-3. B1 accounts for residues 1-55.

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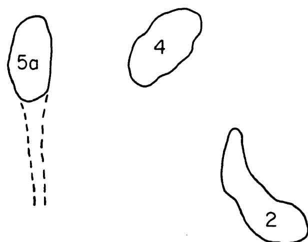
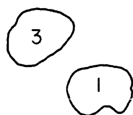


M. xanthognathus

CNBR Fragment B1



\+ ⊙ pH 6.4 ⟶ −

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TABLE 4-6

Composition of Tryptic Peptides From
CNBr Fragment B1 (1-55) of _M. xanthognathus_

Sample	T1	T2	T3	T4	T5a	
Lys	0.78	0.86				K
His	0.84				.90	H
Arg			[1][1]	1.00		R
Asp	1.26		2.15		1.10	B
Thr	1.17			1.27	(0.42)	T
Ser		1.38 [1]			3.16	S
Glu	0.73		1.07	0.94	.74	Z
Pro				1.10		P
Gly		1.76	1.74		1.06	G
Ala	1.00	1.63 [2]	4.02 [5]		2.23	A
Val	0.27 [1]		1.21 [2]	1.35 [2]	1.16	V
Cys						C
Met					+ [1][2]	M
Ile		1.14				I
Leu	0.88	0.86	1.00	1.78	1.10	L
Tyr				0.47 [1]		Y
Phe					2.59 [3]	F
Trp		[1]		[1]		W

1. Not analyzed due to system failure

2. Homoserine detected

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Fig. 4-5. Composite map of peptic β-chain peptides of the yellow-cheeked vole.

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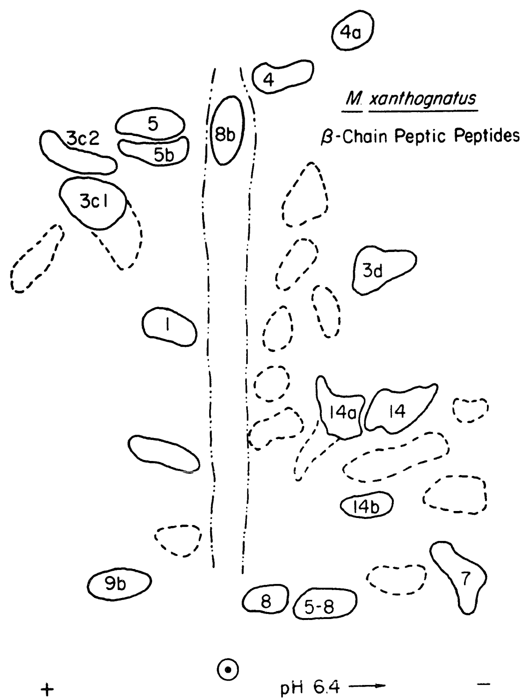

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TABLE 4-7

Composition of Peptic Peptides (soluble pH 6.4)
From the β-chain of M. xanthognathus

Sample	p1	p3c1	p3c2	p3d	p4	p4a	p5	p5b	p7	p8	p8b	p9b	p14	p14a
Lys					(0.40)				1.20	3.30	0.90		1.00	1.25
His	0.78				(0.40)		1.05	1.00	0.93	2.00	0.55 [1]		1.89	2.39 [2]
Arg				1.10	1.72									
Asp	1.18						0.73	1.31		2.36	1.35 [1]	1.02		
Thr	0.96				1.40	0.85				(1.10)	(0.32)			
Ser					(0.44)	(0.25)				1.85 [3]	(0.31)			
Glu		0.80	1.00		0.52		0.86	0.62		0.93	(0.35)			
Pro					1.24	0.85								
Gly	(0.23)		0.75	.43	0.96	(0.23)	1.11	0.90	0.84	2.85	1.21			
Ala	1.00	1.00	1.09		(0.44)	(0.19)			1.30	3.80	1.00		1.26	1.77
Val	0.65				1.64 [2]	2.11			1.00	2.65 [3]	(0.38)			
Cys														
Met														
Ile									(0.31)	.32	(0.37)			
Leu	0.94	1.47	1.15	1.10	2.64 [3]	1.21	1.30	1.20		1.00	(0.30)	1.00	0.83	1.00
Nle														
Tyr					0.92	0.70							0.74	0.93
Phe					1.72	(0.18)	1.43 [2]	1.30						
Trp					[1]									
Position	1-6	26-28	26-29	29-31	29-42	32-38	42-48	43-48	60-64	46-69	72-77	80-81	141-146	140-146

Fig. 4-6. Proposed sequence of the M. xanthognathus β-chain. Mus musculus (Popp and Bailiff, 1973a) and M. pennsylvanicus (Genaux and Morrison, 1973a, b; 1976) are shown for comparison. The single letter amino acid code is indicated as in Dayhoff (1972). Short arrows indicate residues sequenced. Residues underlined by long arrows resulted from carboxypeptidase digestion. The acidic and amide forms of aspartyl or glutamyl residues were obtained by sequencing or were deduced 1) by homology with Mus and 2) from the mobility of the respective peptides on the peptide map.

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```
SPECIES
           βT1                     βT2                        βT3                         βT4
MUS    'V H L T D A E K' A A V S G L W G K' V N A D E V G G E A L G R' L L V V Y P W T Q R'
                        (10)                  (20)                   (30)                 (40)
M.x.                          I.                       A. A.  A.
M.p.                          I.                       A. A.  A.

                        βT5                   βT6    βT7    βT8                 βT9
MUS    'Y F D S F G D L S S A S A I M G N A K' V K' A H G K' K' V I T A F S D G L N H L D N L K'
                       (50)                   (60)                   (70)               (80)
M.x.    F.   Z. H.           S.  V.           Q.                     H.    A.     K.
M.p.    V.   S. H.           =   =            Z.                     H.    A.     K.

                βT10                     βT11                      βT12
MUS    'G T F A S L S E L H C D K' L H V D P E N F R' L L G N M I V I V L G H H L G K'
                (90)                     (100)                   (110)        (120)
M.x.                                                                   A.
M.p.                                               [//////////////////////////////]

                βT13                     βT14
MUS    'D F T P A A Q A A F Q K' V V A G V A A A L A H K' Y H'
                       (130)             (140)
M.x.                   S.                S.       <------
M.p.                   S.                S.
```

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data from several other Microtus species. Since the T5 region appears to be the only place where differences exist between the β-chains from M. xanthognathus and M. pennsylvanicus, the stoichiometry for other Microtus T5 peptides has been re-examined. Earlier data for T5 showed only small differences in Phe, Ser and Glx. If losses are accepted for Phe due to incomplete hydrolysis of a Phe.Phe N-terminal (Garrick et al., 1974) and for Ser and Glx due to hydrolytic degradation in the 21-amino acid peptide (Tristram, 1966), then the T5 peptides can be brought into correspondence. The data for M. xanthognathus confirm the inclusion of residues 60 and 61 (Mus peptide T6) into the T5 peptide of Microtus species and show that the Lys residue at position 59 is replaced by a Gln residue. Further studies will determine if correction for hydrolytic losses should be applied to earlier Microtus peptide T5 data.

Primary Structure of α-Chains

The two electrophoretically differentiated α-chains from hemoglobin of M. xanthognathus were further characterized by peptide mapping (Fig. 4-7). There is a significant difference in electrophoretic mobility between peptides α^{f}T1, which has the greatest mobility toward the anode (spot 1F), and α^{s}T1, which appears in a region of electrically neutral peptides. Table 4-8 presents the amino acid composition for the tryptic peptides shown in Figure 4-7. It can be seen that the electrophoretic difference between α^{f}T1 and α^{s}T1 results from the number of negatively charged Asp residues in the two

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Fig. 4-7. Composite map of tryptic α-chain peptides from fast and slow components of M. *xanthognathus* hemoglobin. This representation is idealized since some αT12b and αT13 may be present in the αT9, αT8-9 region.

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Table 4-8. An integral number of residues is assumed when the data shown are within ± 0.3 residue of 1, 2, 3, etc. In other cases the integral number inferred is shown in brackets [ ] following the data. Cysteine was determined as S-(aminoethyl)-cysteine; tryptophan was detected in intack peptides by dipping the spot in a fresh solution of 1% p-dimethylamino-benzaldehyde in acetone - conc. HCl, 9:1 by volume. The symbols B and Z do not distinguish between the possible amide and free acid residues.

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TABLE 4-8: COMPARISON OF AMINO ACID COMPOSITION OF TRYPIC PEPTIDES FROM α FAST AND α SLOW OF *M. XANTHOGNATHUS* (MOLAR RATIOS)

		T1	T2	T3	T4	T5	T6	T7	T8	T8-9	T9	T10	T11	T12a	T14	
LYS	f	1.00	1.06	1.00		1.00	1.00	1.00	1.00	2.20			1.00			K
	s	1.00	1.00	1.00		1.26	1.00	1.03	1.00		1.06		1.00			
HIS	f				.98		1.87	1.02		3.20				1.06		H
	s				.76		2.02	1.00			2.88					
ARG	f				1.00							1.00			1.00	R
	s				.70							1.00			1.25	
ASX	f	2.11	1.27	.60[1]	.84		1.17			4.34[4]			1.74			B
	s	1.32	.90	1.00	.58[1]		1.39[1]				4.69[5]		2.37[2]			
THR	f		.74			1.86	.73			1.66[2]						T
	s	.91	.85			1.93	.92				1.78					
SER	f	.95					1.84			1.91				1.27		S
	s	.94					2.12				1.93					
GLX	f				1.60[2]		.98									Z
	s				1.47[2]		1.05									
PRO	f					1.00	.94			.90			.80			P
	s					1.13	.83				1.01		1.13			
GLY	f			.92	3.70		1.15	1.04		2.07						G
	s	1.15		.96	4.22		1.24	1.01			2.38[2]					
ALA	f			.92	3.10		1.28	.86		5.97						A
	s			.83	3.43[3]		.97	.93			5.71					
VAL	f	2.38[2]				1.62[2]	1.88			2.30			1.54[2]			V
	s	.90				2.11	1.73				1.88		2.23			
AE-CYS	f													.82		C
	s															
MET	f					.67[1]										M
	s					.30[1]										
ILE	f		.83		.94											I
	s		.99		.88											
LEU	f	1.04			1.08					6.40[6]		.82		1.86		L
	s	1.16			1.04						5.30	.83				
TYR	f				.74	.73	.82								.70	Y
	s				.75	1.02	.52[1]								.70	
PHE	f					.98	1.88						1.00			F
	s					1.12	1.88						1.08			
TRP	f			+[1]												W
	s			+[1]												
TOTAL	f	7	4	5	15	9	16	4	1	30		2	7	5	2	
	s	7	4	5	15	9	16	4	1		29	2	7		2	
Position		1-7	8-11	12-16	17-31	32-40	41-56	57-60	61	61-90	62-90	91-92	93-99	100-104	140-141	

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peptides. The peptides also differ in the number of valine residues. The presence of two valine residues in the α^fT1 peptide is unique to M. xanthognathus when compared to all other such αT1 peptides obtained from rodent hemoglobins. Sequential analysis of an amino-terminal cyanogen bromide fragment from α^f (α1 - 32) positively located the additional valine at position α4 and the aspartic acid at position α5 (Table 4-9). In a follow-up study, peptide maps were prepared from the tryptic digests of the CNBr fragments (α1 - 32) from both α^s and α^f. Comparison of the compositions obtained in each case for the four peptides (Tables 4-10 and 4-11) provides additional support for the identifications shown in Table 4-8.

Peptides αT12b and αT13, accounting for 35 amino acids, were not found among the soluble tryptic peptides. The compositions and sequences given for αT13 and αT14 were inferred from the results of timed, sequential digestion by carboxypeptidase of the α-chains (Table 4-12). Although these C-terminal compositions for α^f and α^s are very much alike, difference(s) cannot be ruled out.

Compositional differences were also observed between peptides αT9. An alternate peptide, αT8-9, appears if the Lys-Val bond between residues α61 and α62 is not completely cleaved. The additional lysine of αT8-9 causes the peptide to shift slightly toward the cathode (Fig. 4-7). This area of the map may be complicated further by the presence of αT1-2 and any αT12b which remains with the soluble tryptic peptides. The peptides with the best stoichiometry are presented in Table 4-8. It appears that α^sT9 has an Asx residue which replaces a

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TABLE 4-9

Automated Sequencer Analysis of α^f N-terminal CNBr Fragment From M. xanthognathus

Residue Number	Gas Chromatography Silylation (-)	(+)	TLC	Hydrolysis	Sequence
1	V				Val
2	L/I	L			Leu
3	(S)				---
4	V		V		Val
5		D	D		Asp
6		D	D		Asp
7				K	Lys
8	(T)				---
9		N	N		Asn
10	L/I	I	L/I		Ile
11				K	Lys
12		N	N		Asn
13	A		A		Ala
14					---
15	G				Gly
16				K	Lys
17	L/I	I			Ile
18	G				Gly
19	G				Gly
20				H	His

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TABLE 4-10

Composition of Tryptic Peptides
From CNBr Fragment A1 (Fast Component)
(α1 - 32)

	T1	T2	T3	T4	
Lys	1.00	1.00	1.00		K
His				1.25	H
Arg				1.00	R
Asp	1.86	1.00	1.13	0.87	B
Thr		1.01			T
Ser	0.97				S
Glu				1.50 [2]	Z
Pro					P
Gly			1.40 [1]		G
Ala			1.45 [1]		A
Val	1.56 [2]				U
Cys					C
Met					M
Ile		0.79		0.41 [1]	I
Leu	0.98			1.06	L
Tyr				0.78	Y
Phe					F
Trp					W

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TABLE 4-11

Composition of Tryptic Peptides T1 and T3
From CNBR Fragment A1 (Slow component)
(α1 - 32)

	T1	T3	
Lys	0.82	0.70	K
His			H
Arg			R
Asp	1.13	1.23	B
Thr	1.04		T
Ser	1.02		S
Glu			Z
Pro			P
Gly	1.24	1.21	G
Ala		1.29	A
Val	0.90		U
Cys			C
Met			M
Ile			I
Leu	0.82		L
Tyr			Y
Phe			F

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TABLE 4-12

Comparative Amino Acid Composition of M. xanthognathus α-Chain Carboxy Terminal (Molar Ratios)[1]

	CPB		CPB + CPA		
Time (min)	5 (α^s)	30 (α^s)	60 (α^s)	150 (α^s)	150 (α^f)
Arg	1.00	1.00	1.00	1.00	1.00
Tyr	0.97	1.38	0.98	0.92	1.25
Lys	0.85	1.29	0.95	1.43	1.45
Ser	---	1.72	0.71	1.70	2.08
Thr	---	1.30	0.68	.90	1.00
Leu	---	.67	0.68	1.60	2.15
Val	---	0.50	0.56	1.10	1.73
Ala	---	---	0.32	1.18	1.48
Phe	---	---	0.23	.70	1.15
Asp	---	---	---	.50	.87
His	---	---	---	.48	.35
Pro	---	---	---	---	---

Notes: 1. Amino acids are listed in order of their first appearance as one follows the sequence backwards from the C-terminal.

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Leu found in α^fT8-9. The amino acid is probably an Asn, a neutral residue that would not affect the mobility of the peptide.

Figure 4-8 shows the amino acid sequences for the hemoglobin α-chains from Mus and M. xanthognathus. The replacement at position α5 of one residue of aspartic acid in the α^s-chain explains the observed electrophoretic differences between the hemoglobin components α^s and α^f. Although the replacements in position α4 do not affect the electrophoretic mobility of the peptide, the chromatographic behavior is governed by the change in functional groups, from -H(Gly) to $-CH_3$(Ala) to $-CH(CH_3)_2$(Val) (Fig. 4-9). Differences in these residues may also be important in determining solubility differences between the hemoglobins MX^f and MX^s (see Chapter 3, Fig. 3-8).

Positions 4 and 5 in αT1 are highly variable among hemoglobin chains of rodents, and substitutions there are not expected to have any effect on oxygen binding. Recently Garrick et al. (1975) showed that the electrophoretic difference between rat α-chains also results from an Asp → Ala substitution at position α5 and that the P_{50} of the components were not greatly affected. Other neutral and acidic amino acids are commonly found at this position in many species (Dayhoff, 1972 and 1976 supplement).

It is also noteworthy that the major hemoglobin α-chain, α^f, of M. xanthognathus differs from the corresponding α-chain from M. pennsylvanicus. The three differences between these two α-chains are equal in number to the residue differences that occur between the two hemoglobin chains (α^f and α^s) of M. xanthognathus. This can be

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Fig. 4-8. Amino Acid Sequence Alignment for _M. xanthognathus_ Hemoglobin, α- Peptides. _Mus_ (_musculus_) is shown for comparison. The single letter amino acid code is indicated in Table 4-8. Arrows indicate residues sequenced from the N-terminal of MX^f. Bracketed area at C-terminal represents inferred alignment for αT13 of both MX^f and MX^s on the basis of carboxypeptidase digestions as indicated in Table 4-12.

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Amino Acid Sequence Alignment-Alpha peptides

```
Species
             T1          T2        T3                T4                      T5
MUS   'V L S G E D K'S N I K'A A W G K'I G G H A G E Y G A E A L E R'M F A S F P T T K'
                                             20                                      40
MXf          V D     T       N                     D                     V V Y

MXs            T     T       N                     D                     V V Y

                     T6                    T7 T8                           T9
MUS   'T Y F P H F D V S H G S A Q V K'G H G K'K'V A D A L A N A G A H L D D L P G A L S A L S D L H A H K'
                                             60                                      80
MXf                                      A                   T L         V           T

MXs                                      A                   T           V           T

       T10       T11         T12a                        T12b                            T13           T14
MUS   'L R'V D P V N F K'L L S H C'L L V T L A S H H P A D F T P A V H A S L D K'F L A S V S T V L T S K'Y R'
                                             110                                     130
MXf                                 ///////////////////////////////////////////// [                           ]

MXs                                 ///////////////////////////////////////////// [                           ]
```

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Fig. 4-9. Comparison of αT1 peptide locations of cricetid rodents.

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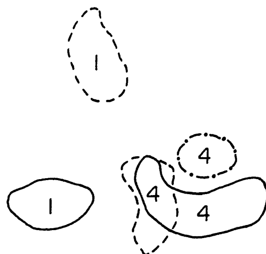

M.o. –·–·–
M.x. – – –
P.m. ——

Sequence AT1

M.o. Val-Leu-Ser-Gly-Asp-Asp-Lys
M.x. Val-Leu-Ser-Val-Asp-Asp-Lys
P.m. Val-Leu-Ser-Ala-Asp-Asp-Lys

\+ pH 6.4 ⟶ −

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interpreted to mean that selection pressure at hemoglobin loci has been similar within these microtine species since their divergence from a common ancestor.

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CHAPTER V

PRIMARY STRUCTURE OF

DICROSTONYX HEMOGLOBIN

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CHAPTER V

PRIMARY STRUCTURE OF DICROSTONYX HEMOGLOBIN

Molecular data have become an independent source of phylogenetic information by providing an objective way of choosing among alternative taxonomic affinities and sometimes pointing out unsuspected relationships.

One species of varying lemming has been recognized in Alaska: *Dicrostonyx groenlandicus* (Hall and Kelson, 1959). More recently, Rausch and Rausch (1972) proposed that *groenlandicus* was part of the holarctic torquatus group. Rausch believes that populations of this varying lemming group became fragmented or displaced southward during Würm time, with relic stocks persisting in unglaciated refugia and in periglacial tundra. Post-glacial dispersal of these populations resulted in a reoccupation of suitable habitat (Fig. 5-1). Rausch states that "while manifesting a high degree of developmental homeostasis, some of these geographic isolates have undergone chromosomal reorganization with genotypic change to the extent that the taxa studied (*exsul*, *nelsoni*, *richardsoni*, *rubricatus*, and *stevensoni*) evidently are reproductively isolated as well. By these criteria, the "torquatus-group" in North America appears to be a superspecies."

For the present, I will consider both *rubricatus* and *stevensoni* populations to be separate *Dicrostonyx* species. This is based on the

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Fig. 5-1. Species' ranges of *Dicrostonyx* (from Rausch and Rausch, 1972).

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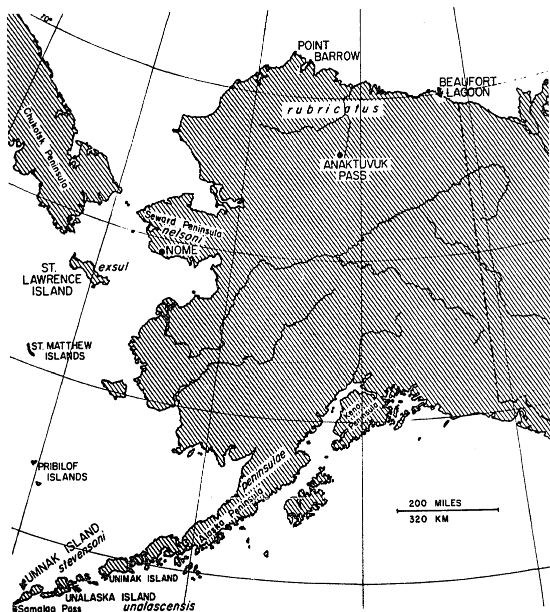

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following two observations of Rausch and Rausch:

1. Breeding studies showed that the F_1 males of a cross between D. stevensoni and D. rubricatus were sterile.

2. D. stevensoni and D. rubricatus have different diploid chromosomal complements (although the fundamental number is the same).

The Dicrostonyx genus shows distinctive morphological differences from other microtines so that it has been proposed that it be listed as an independent tribe of the subfamily Microtinae. There even seems to be a wider taxonomic gap between it and Microtus than between Lemmus and Microtus (Hooper and Hart, 1962). Kretzar (1950) states that it "will yet be classed with the more ancient independent branches of microtine-like forms". A comparison of hemoglobins will provide an additional source of phylogenetic information on both Dicrostonyx and Lemmus and allow a more direct way of relating Dicrostonyx to other microtines.

Dieterich (1972) noted the absence of major hematologic differences for D. rubricatus as compared to other microtine genera. Johnson (1974) reported that D. stevensoni had two hemoglobin components. Stratton et al. (1976) also found two hemoglobin components for D. rubricatus and proposed the existence of a second nonallelic minor α-chain locus in these two Dicrostonyx species based on the electrophoretic separation of globin chains. They also described the solubility of D. rubricatus hemoglobin in phosphate buffer.

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Characterization of the Globin Chains

Figure 5-2 compares the electrophoretic mobility of hemoglobins from Dicrostonyx and Lemmus. As in Microtus, there are two different hemoglobin components for each species because of the presence of two different α-chains, a minor (α^s) and a major (α^f). It can also be seen in Figure 5-2b that the origin of the electrophoretic difference between Dicrostonyx hemoglobin components and Lemmus hemoglobin components resides in the β-chains of the hemoglobins from these two genera.

Structure of β-Chain

Figure 5-3 compares the β-chain peptide locations of Dicrostonyx with those from Microtus and Ondatra (muskrat). The only significant difference in electrophoretic and chromatographic mobility between Microtus and Dicrostonyx is in peptide T13. Peptide T13 of Dicrostonyx and Ondatra move farther toward the anode than does T13 of Microtus (and Lemmus, not shown). The addition of a negative charge in T13 of Dicrostonyx β-chain accounts for the greater mobility of its hemoglobin components, as compared to Microtus and Lemmus hemoglobin mobility. Although peptide T13 from muskrat β-chain has an extra negative charge, muskrat hemoglobin has the same mobility as Microtus hemoglobin because of the presence of an additional lysine residue in peptide T6 (Genaux et al., 1976). Table 5-1 compares the compositions of this peptide in several microtine species. The replacement of an alanine residue by an aspartic acid residue has been placed by

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Fig. 5-2. Electrophoretic patterns of *Dicrostonyx* and *Lemmus* hemoglobins.

(a) Hemoglobins at pH 8.9; position 1, *Lemmus sibiricus*; position 2, *Dicrostonyx rubricatus*.

(b) Chains in urea-cellulose acetate at pH 8.9: position 3, *D. r.* α^f chain; position 4, *D. r.* α^s chain; position 5, *Lemmus* chains; position 6, *Dicrostonyx* chains.

Note: *Lemmus lemmus* had an identical pattern to *Lemmus sibiricus* and *D. stevensoni* had a pattern identical to *D. rubricatus*.

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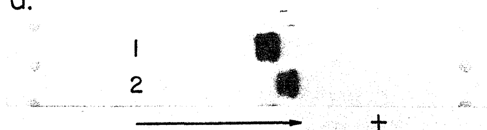
a.



b.

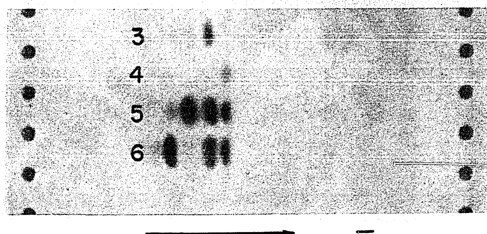


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Fig. 5-3. Composite peptide map of three microtine genera β-chains. Position of βT13 should be noted. Muskrat, (≡); *Dicrostonyx*, (////); *Microtus* (spots in common, clear). On later maps, a peptide 10a was detected slightly above and to the right of peptide 10.

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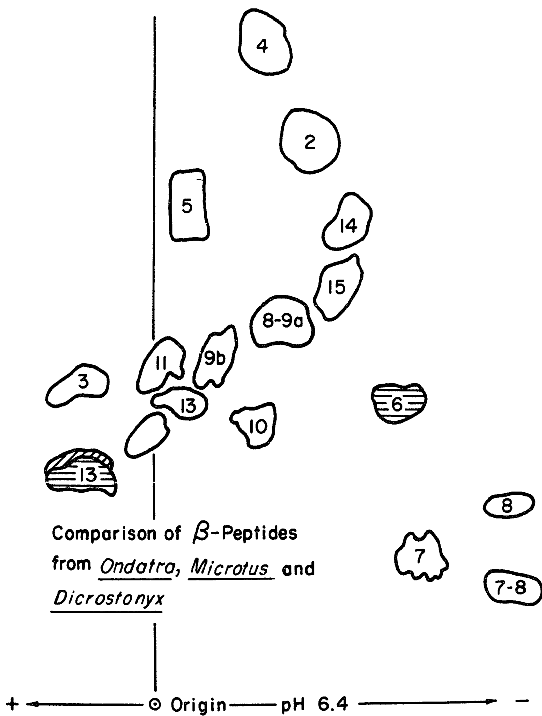

Comparison of β-Peptides from *Ondatra*, *Microtus* and *Dicrostonyx*

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TABLE 5-1

COMPARISON OF MICROTINE T13 PEPTIDES

	D.r.	O.z.	M.x.	L.s.
Lys	1.00	1.02	1.20	1.00
His				(0.20)
Asp	1.79	1.52	1.02	1.00
Thr	0.83	1.19	1.06	1.00
Ser	1.04	0.97	0.92	1.06
Gln	1.86	2.25	1.62	2.04
Pro	1.43	1.32	1.16	1.22
Gly				(0.38)
Ala	2.23	2.21	3.06	3.11
Val			(0.44)	
Leu			(0.65)	(0.63)
Phe	2.06	1.92	1.62	1.78

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homology with other mammals at position β125 (H3). Carboxypeptidase digestion gave the following C-terminal composition for this peptide from D. stevensoni hemoglobin: Lys (1.00), Gln (0.49), Phe (0.71), Ser (0.33) and Ala (0.51).

It should be noted that this position has a functional role in $\alpha_1\beta_1$ subunit contacts (Lehman and Huntsman, 1974). It contacts residue 34 (B15) in the α-chain. Ondatra and Dicrostonyx α-chains differ at position α34 (Val vs. Ala).

Using the compositions presented in Table 5-2, the tryptic peptides of Dicrostonyx can be aligned with the homologous peptides of M. xanthognathus and Mus and then compared to Lemmus (Fig. 5-4). There are no differences observed between Lemmus and Dicrostonyx peptides T1, T2, T3, T7, T11 and T15.

Peptide T2: The values for alanine and glycine varied in this Dicrostonyx peptide. Alanine sometimes was lower than expected and glycine higher. In the alignment (Fig. 5-4) a conservative interpretation is made and no difference is reported. An alternative alignment could be:

A/G A I S G L W G K

Peptide T3. The composition of these Dicrostonyx and Lemmus peptides differ from that of Microtus. This peptide is very variable in rodent species as can be seen in Table 5-3. The glycine for alanine substitution did not alter the position of the peptide on the map.

Peptide T4. Unlike the homologous Lemmus peptide, this peptide

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Table 5-2. An integral number of residues is assumed when the figures shown are within ± .03 residues of 1, 2, 3 etc. In other cases the integral number inferred is shown in brackets [ ]. Data in parentheses ( ) are attributed to sources outside the given peptide and are excluded from the stoichiometry.

a. Data from C. T. Genaux (personal communication).

b. *Dicrostonyx* peptide is interpreted in relation to CNBrT5a and CNBrT5b (see text).

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TABLE 5-2

COMPARISON OF THE AMINO ACID COMPOSITION
OF THE TRYPTIC PEPTIDES FROM DICROSTONYX AND LEMMUS[a]
β CHAINS

		T1	T2	T3	T4	T5[b]	T7	T9a	T8-9a	T9b	T10	T11	T13	T14	T15
Lys	Dr	1.00	1.10			1.16	1.00	1.00	1.80	1.00	1.00	1.00	1.00	1.00	
	Ds	1.30	1.00				1.00	1.00	1.84	1.00	1.00	1.10	1.10	1.00	
	Ls	0.86	1.00			0.62	0.93	.61 [1]		0.80	1.00	0.90	1.00	1.25	
His		0.73				0.70	0.95	.63	1.00	0.85	0.93	0.82		1.30	1.00
		0.60					0.96	.54	.96	0.40 [1]	1.08	0.94		.96	1.00
		0.80				1.32	1.00			0.25 [1]	1.16	0.94		1.00	1.17
Arg				1.00	1.00										
				0.84	1.00										
				0.85	0.84										
Asx		1.16		1.98		2.23		.93	0.91	1.74	1.15	1.74	1.79		
		1.12		2.23				.91	1.04	2.10	0.97	1.85	1.55 [2]		
		1.17		2.28		1.94		.92		2.34 [2]	1.16	1.70	1.00		
Thr		0.73			1.16	(0.67)						(.16)	0.83	(0.60)	
		1.11			1.30							(.33)	1.14		
		1.15			0.86	0.64 [1]		.87			1.10		1.00	0.97	
Ser			1.10			2.65 [3]					3.12		1.04	1.41 [1]	
			.94								3.07	(.33)	1.14	.62 [1]	
			0.76			1.85					2.08		1.06		
Glx		1.00		1.08	1.50 [1]	1.56 [2]					0.93	1.05	1.86		
		0.88		1.02	1.07						0.88	0.91	1.85		
		1.19		1.11	1.05	1.27					1.24	1.20	2.04		
Pro					0.86							0.95	1.43 [1]		
					0.83							1.27	1.19		
					1.00							1.03	1.22		
Gly			2.33	1.87	0.93	2.26	1.04	2.07	2.34		0.93	(0.16)		2.06	
			2.56 [2]	1.70	0.73		0.93	2.24	2.14		1.16	(0.38)		2.29	
			1.85	2.18		2.05	1.33	2.38			0.65	(0.47)		1.92	

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TABLE 5-2 (continued)

		T1	T2	T3	T4	T5	T7	T9a	T8-9a	T9b	T10	T11	T13	T14	T15
Ala	Dr	1.33	1.47 [2]	3.65 [4]		1.76 [3]	0.71	1.24	1.08		0.90		2.23	2.70	
	Ds	1.35	1.42 [2]	3.62 [4]			0.73	1.02	1.18		1.51	(0.75)	2.13	2.96	
	Ls	0.91	1.64 [2]	3.99		4.16	1.00	1.22			1.48	(0.77)	3.11	3.04	
Val		0.76		1.40 [2]	0.70	1.40 [2]		0.33 [1]	0.68 [1]			1.05		2.10 [3]	
		0.50 [1]		1.54 [2]	1.01			0.40 [1]	0.61 [1]			1.20		2.25 [3]	
		0.67 [1]		2.10	2.35 [2]	1.74		0.48 [1]				1.06		2.98	
Met						0.33 [1]									
						0.35 [1]									
Ile			1.29					0.28 [1]	0.51 [1]						
			.72					0.43 [1]	0.71 [1]						
			1.00					0.61 [1]							
Leu		1.20	1.04	0.95	1.63 [2]	2.13 [1]		1.06	1.20	2.27	1.81	0.84		1.11	
		1.05	0.86	1.03	1.84			0.97	1.13	1.70	2.26	1.20		1.09	
		1.06	1.00	0.94	1.65 [2]	2.63 [2]		1.04		2.12	2.32	0.76	(0.63)	1.25	
Phe						1.76 [3]		1.03	0.85		0.70	0.91	2.06		
								0.78	1.07		1.13	0.90	1.79		
						1.12 [2]		0.82			1.12	0.88	1.78		
Tyr					0.18										0.52
					0.27										0.51
					0.41										0.51
Trp			+ [1]		+ [1]										
			+ [1]		N.A.										
			+ [1]		+ [1]										
AE-CYS											0.31				
											0.25				
											0.41				
		8	9	13	10	21[b]	4	10	11	6	13	9	12	12	2

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Fig. 5-4. Amino acid sequence alignment for peptides from β-chains. Inferred amino acid substitutions are indicated relative to the determined sequence for the white mouse (Mus, Popp, 1973) and rat (L. Garrick, personal communication). Blank positions indicate identity with Mus. The single letter amino acid symbols are given in Dayhoff (1972).

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```
SPECIES
          βT1              βT2                     βT3                   βT4
MUS     'V H L T D A E K'A A V S G L W G K'V N A D E V G G E A L G R'L L V V Y P W T Q R'
                         (10)               (20)                (30)                (40)
D.R.                       I.                       A.     A.           [G. ]
L.S.                       I.                       A.     A.
RAT                          N.               P.    D.

                         βT5                 βT6    βT7  βT8              βT9
MUS     'Y F D S F G D L S S A S A I M G N A K'V K'A H G K'K'V I T A F S D G L N H L D N L K'
                         (50)               (60)                (70)                (80)
D.R.    [F.   Z.H.       S./G.      V.]     Q.                  H.     G.      K.
L.S.    [F.   A.H.L.     T.         V.      Q.   ]                     G.      K.
RAT                                                             N.     N.

                     βT10                  βT11                 βT12
MUS         'G T F A S L S E L H C D K'L H V D P E N F R'L L G N M I V I V L G H H L G K'
                         (90)               (100)               (110)               (120)
D.R.          S.                                       K.[                              ]
L.S.                                                   K.[                              ]
RAT                 N.   T.

                   βT13              βT14
MUS     'D F T P A A Q A A F Q K'V V A G V A A A L A H K'Y H'
                        (130)               (140)
D.R.            D.       S.         G.S.
L.S.                     S.         G.T.
RAT           (?)                     S.
```

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TABLE 5-3

Comparison of Some Rodent βT3 Peptides

	18	19	20	21	22	23	24	25	26	27	28	29	30
Human	V	N	V	D	E	V	G	G	E	A	L	G	R
Mus (C57BL)			A										
Mus (C34H/HEJ)			S										
Mus (AKR)			S										
Mus (Cervicolor)			S		A			A					
Mus (Car.)			P										
Microtus (Xant.)			A		A	A		A					
Ondatra (Zib.)					A								
Clethrionomys (Rut.)					A								
Lemmus (Sibiricus)			A		A			A					
Dicrostonyx (Rub.)			A		A			A					
(Stev.)			A		A			A					
Calomys (Callosus)					E/D			A					
Peromyscus		K	P	Z	A	I							
Rat			P		D								

showed a glycine for valine substitution in Dicrostonyx. This peptide contacts the α-chain, and both valines have been reported to be invariant in mammals. Recently R. T. Jones (personal communication) has reported a Val → Ile substitution in some carnivores, and Morrison et al. (1976) have reported a similar substitution in the β-chains of Calomys callosus. It should be noted that the glycine could also be substituted at position α34. The Lemmus peptide shows the expected two valines.

Peptide T5. This peptide from D. rubricatus hemoglobin seems to contain from 19 to 21 amino acids. In D. stevensoni, the data for this peptide (not shown in Table 5-2) indicate only 19 amino acids; it is short one serine and one phenylalanine residue. The peptides for D. stevensoni and D. rubricatus are both found above peptide βT11 as shown in Figure 5-3. The Lemmus peptide also shows approximately 19 - 21 amino acids. In none of the three species of lemmings was peptide βT6 (Val - Lys) observed.

Cyanogen bromide cleavage of the β-chain from D. rubricatus yielded a gray tryptic peptide in the map location expected for the six C-terminal residues (βT5b). As in Microtus and Clethrionomys, this confirmed the presence of methionine in βT5 of Dicrostonyx and supported the Lys → Glx substitution at position β59. A peptide (β5a) was found but was short of the expected 15 amino acids: Phe (1.83, Asx (1.07), Glx (0.97), Ala (1.66), His (1.12), Gly (1.48), Ser (2.40), Leu (1.05), homoserine (0.31) and Val (0.80). Although further work with carboxypeptidase and amino peptidase M will clarify

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the question of hydrolytic loss, the present data (approximately 20 amino acids) indicates the lack of deletions at positions 52 and 54. Figure 5-4 shows the proposed substitutions between Dicrostonyx and Lemmus T5 peptides assuming a composition of 21 amino acids.

Peptide T9a. The data from this Lemmus and Dicrostonyx peptide support a lysine substitution at position 76 of the globin chain. This is similar to the situation found in the minor β-chain of Mus (Gilman, 1974; Popp, 1973). The C-terminal six residues (T9b) are clearly represented in the chain-separated preparations. The two lemming genera differ by a two base change in the β69 codon (His → Thr). The glycine at position β72 has also been reported in Clethrionomys (Genaux et al., 1976). The map positions of tryptic peptides from the badger β-chain (Han et al., 1976) support identification of peptides 9a, 8 - 9a and 9b, as do the sequence studies on M. xanthognathus.

Peptide T10. The composition of this Dicrostonyx peptide differs by one amino acid from that of Lemmus (Thr vs. Ser). A minimum solution would have an amino acid replacement in only one position (β84) as shown in Figure 5-4. On the other hand, it is possible for the serine to be in position β86 as is the case reported for Clethrionomys. In this alternative alignment, there are two changes involved:

Ls   G T F A S L S E L H C D K

Dr   G A F S S L S E L H C D K

The composition in Table 5-2 is supported by the composition of a

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trace peptide 10a which sometimes appears above peptide 10.

Peptide T14. This peptide differs between the two genera of lemmings. In Dicrostonyx peptide T14 there is a serine residue while in the Lemmus peptide there is a threonine residue. Both peptides from the two genera show a glycine residue not found in Microtus. In Figure 5-4, this is placed at position 138 by homology with muskrat. In marsupial hemoglobins there is a glycine residue sequenced at position 142 (Whittaker and Thompson, 1974).

Structure of α-Chains

As was found to be the case for hemoglobins from M. xanthognathus and L. sibiricus, the two electrophoretically different α-chains of D. rubricatus could also be differentiated by peptide mapping. The similarity between major and minor α-chains and also between α-chains of the two Dicrostonyx species was apparent upon peptide mapping. Several differences however were noted (Fig. 5-5):

1. In the map of the slow-component α-chain (α^s) from Dicrostonyx peptide T1 appeared to be missing. This is analogous to the situation in M. xanthoganthus. A spot in the map of D. rubricatus α^s-chain was located below peptide T11, and this spot had the same composition (Table 5-4) as the peptide αT1 from the minor α-chain of rat.

2. Both component α-chains of D. rubricatus showed a spot (designated T4') below tryptic peptide T4. This spot was not observed in the α^f-chain map from the fast (major) component of D. stevensoni,

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Fig. 5-5. Composite map of α-chain tryptic peptides from *Dicrostonyx* species.

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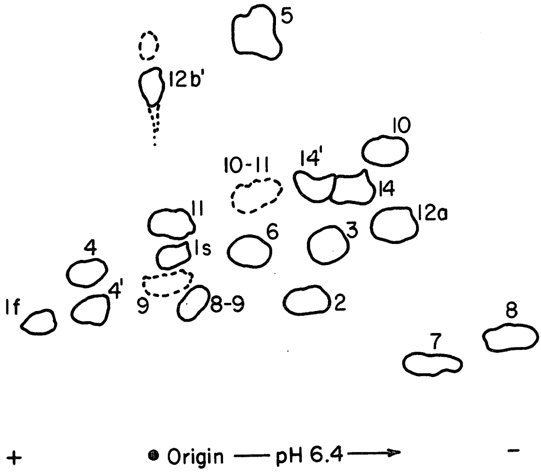

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TABLE 5-4

Composition of Tryptic Peptides from α-Chains of <u>Lemmus</u> and <u>Dicrostonyx</u>

		T1	T2	T3	T5	T6	T7	T10	T11	T12a	T14
Lys	$Dr\alpha^F$	0.84	1.00		1.00	1.00	1.00		1.00		
	$Dr\alpha^S$	1.00	1.11		0.73	0.75	1.00		0.70		
	$Ds\alpha^F$	1.06	1.00	1.15	1.00	1.00	1.00		0.91		
	$Ls\alpha^F$	1.00	1.02	0.94	0.96	1.07	0.97		1.05		
His						1.99	0.89			1.00	
						1.70	1.08			0.79	
						2.05	1.06			1.00	
						2.24	1.07			0.96	
Arg								1.00			1.00
								1.00			1.00
								1.00			1.00
								1.06			1.00
Asx		2.26	1.17			1.23			1.85		
		1.30	1.20			1.40 [1]			2.30		
		1.73	1.21	1.02		0.98			2.49 [2]		
		1.82	1.00	0.74		1.14			2.26		
Thr			0.88		1.75	0.75					
			0.80		2.18	1.29					
			0.89		2.87	0.99					
			0.76		1.70	0.85					
Ser		1.04				2.22				1.05	
		0.40 [1]				2.43 [2]				1.25	
		0.93				2.34 [2]				1.16	
		1.05				1.55 [2]				0.73	
Glx						1.08					
						1.00					
						0.81					
						1.12					
Pro					0.98	1.10			0.60		
					0.79	1.25			0.71		
					0.95	0.72			0.80		
					0.94	1.00			0.76		
Gly		1.12				1.28	1.05				
						1.04	1.13				
		1.04		0.72		0.99	1.24				
		1.17		1.18		1.08	1.70				
Ala					1.22	1.40 [1]	0.95				
		1.60 [2]			0.89	1.08	0.89				
				0.72	1.02	1.02	0.97				
				1.26		1.06					
Val		0.78			1.17	1.79			2.11		
		0.30 [1]			1.01	1.87			1.67 [2]		
		0.77			1.01	1.70			1.93		
		0.88			2.17	2.06			1.10 [2]		
Met					0.20 [1]						
					0.21 [1]						
					0.41 [1]						
					0.26 [1]						

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TABLE 5-4 (continued)

		T1	T2	T3	T5	T6	T7	T10	T11	T12a	T14
Ile	Dra^F		0.94								
	Dra^S		0.87								
	Dsa^F		0.81								
	LSa^F		1.00								
Leu		1.04						0.70		1.77	
		0.50 [1]						0.60 [1]		1.33 [2]	
		0.79						0.69 [1]		1.72	
		0.84						0.80		1.80	
Phe					0.87	2.12			1.00		
					0.84	1.83			0.73		
					0.90	1.89			0.92		
					0.93	1.99			0.81		
Tyr					0.76	0.78					0.42
					0.47 [1]	0.46 [1]					0.45
					0.37 [1]	0.40 [1]					0.65
					0.59 [1]	0.67 [1]					0.56
Trp				+[1]							
				+[1]							
AE-CYS										0.78	
										0.50 [1]	
										0.60 [1]	
										0.62 [1]	

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but it was observed in the map of D. stevensoni minor α-chain (α^s).

3. A spot not seen in maps from M. xanthognathus appeared to the left (anodic side) of αT14 in Dicrostonyx species. This was designated αT14'.

4. A peptide in the area expected for T3 was isolated from the minor α-chains, but its composition was radically different from the T3 of D. stevensoni major α-chains.

Table 5-4 presents the compositions for the tryptic peptides isolated from the α-chains of Dicrostonyx and from the major α^f-chain of Lemmus. Data from the two species are compared and are consistent with the similar mobilities obtained in the peptide maps. The only compositional differences observed in the Dicrostonyx genera were between α^f and α^s-chains in peptide T1, T3 and T4. When peptides of the major α-chains were compared with those of Lemmus α-chain (Fig. 5-6), no differences were observed in peptides T1, T2, T6, T10, T11, T12a and T14.

Peptide T1: This peptide showed a difference in composition as obtained from the major and minor α-chains from D. rubricatus. The α^f peptide showed two aspartyl residues and a single glycine while the α^s peptide showed two alanine residues and a single aspartyl residue. This agrees with the electrophoretic mobility of the peptides. Positions 4 and 5 seem to be more variable than the other positions in this peptide, and this is shown in the following summary:

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Mus	V L S G E D K*
Rat I	V L S A D D K*
Rat II	V L S A A D K*
Mx^f	V L S V D D K*
Mx^s	V L S G T D K
Dr^f	V L S G D D K
Ds^f	V L S G D D K
Dr^s	V L S A A D K
Ls^f	V L S G D D K
Oz	V L S G E D K*
Mo^f	V L S G D D K*
Apo	V L S G E D K*

* sequenced

Peptide T3. D. stevensoni α^f-chain shows the expected microtine peptide. A tryptophan positive spot appeared in the expected area of the D. rubricatus α^s map, but the isolated peptide could only tentatively be considered αT3. The Lemmus α^f and α^s chains T3 peptides were identical to that obtained from D. stevensoni.

Peptide T4. Table 5-5 presents the results from analyses of several peptides 4 and 4'. Those peptides obtained from the major α-chains in D. rubricatus and D. stevensoni are similar, and both have an isoleucine residue. The results indicate a difference between the α^f- and α^s-chains from D. rubricatus; D. rubricatus α-chain does not contain isoleucine. The presence of methionine has been reported in this peptide in one other species. The Ile → Met mutation involves a

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TABLE 5-5

Composition of αT4 Peptides From *Dicrostonyx*

	D.r[b] α^S4	D.r[b] α^S4	D.r[b] α^S4'	D.r[b] α^S4'		D.s. α^F4		D.r[a] α^F4	D.r[a] α^F4'	
Lys	(0.09)	N.A.	(0.05)	(0.12)		(0.28)		(0.06)	(0.10)	
His	0.93	N.A.	0.84	1.08	1.0	0.91	1.0	0.83	0.90	1.0
Arg	0.85	N.A.	1.07	1.00	1.0	0.84	1.0	1.00	1.00	1.0
Asp	1.05	1.81	1.81	2.13	1.5	0.83	.5	0.40	1.15	.5
Thr	0.21	0.42				(0.21)		(0.12)	(.11)	
Ser			(0.20)	(0.28)		(0.34)			(.10)	
Glu	2.87	1.67	2.61	2.50	2.5	2.60	2.5	2.03	3.35	2.5
Pro										
Gly	3.15	2.60	3.15	2.64	3.0	2.70	3.0	[2.54]	2.40	3.0
Ala	3.31	2.84	3.31	2.84	3.0	3.60	4.0	4.21	4.10	4.0
Val						(0.31)			(0.25)	
Cys										
Met	+	0.23	0.38	0.04	1.0			(0.09)	+	
Ile						0.64	1.0	0.62		1.0
Leu	0.97	1.00	0.88	1.13	1.0	1.15	1.0	1.12	0.90	1.0
Tyr	0.55	0.46	0.46	0.84	1.0	0.40	1.0	0.69	0.55	1.0
Phe						(0.05)		(0.18)	(.14)	
Σ					15.0		15.0			15.0

a, b = Different Preparations

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single base change between the corresponding codons. As indicated in Table 5-5, peptides 4 and 4' have approximately the same composition, but there is great variability in the data for the acidic residues. The appearance of two such spots is consistent with the presence of methionine for two reasons.

1. Methionine can be in either the oxidized or reduced form. This will give two different spots when chromatographed in our system (Lehman and Huntsman, 1974, page 364).

2. Isoleucine and methionine are expected to have different chromatographic mobilities.

The presence of αT4' in the α^f and α^s-chain maps of D. rubricatus and the α^s-chain map of D. stevensoni may indicate that these chains represent a mixed group of similarly charged α-chains and the methionine substitution is a polymorphism in the population. A similar situation is believed to exist in the rat β-chains (L. M. Garrick, personal communication).

Taking the best data for Dicrostonyx α-chains the following alignments are proposed:

Ls^f	I G G H A G D Y G A E A L E R
Dr^f	I G A H A G $\frac{D}{E}$ Y G A E A L E R
Dr^s	M G N H A G $\frac{D}{E}$ Y G A E A L E R
Ds^f	I G A H A G $\frac{D}{E}$ Y G A E A L E R

It should be again noted that these peptides were prepared from pooled hemoglobins.

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Peptide T5. This Dicrostonyx peptide had a composition similar to that of several Microtus species but was different from that of M. xanthognathus and L. sibiricus.

Mx	M F V V Y P T T K
Dr	M F A V Y P T T K
Ls	M F V V Y P T T K

Peptide T9. The data for this peptide (Table 5-6) lack precision because of the effects of hydrolysis on such a large peptide. Also, the presence of peptide T14' may indicate that peptide T9 is cleaved by trypsin into two peptides (9a and 9b) as in the α-chains of rat (Garrick et al., 1975). Until further information is obtained, an alignment cannot be proposed.

Peptide 12b. This peptide has been identified in Ondatra and Clethrionomys (Genaux et al., 1976). The Dicrostonyx rubricatus and Lemmus peptides (Table 5-6) are found in the same area of the map as those of Ondatra and Clethrionomys) and are similar in that they contain isoleucine. Several chymotryptic peptides were isolated after digestion of the tryptic core from α^S-chain of D. rubricatus (Table 5-7). These fit areas of αT12b and αT13.

Figure 5-6 shows the proposed alignments for the major α-chains of hemoglobins from Dicrostonyx and Lemmus and compares them to Mus and Rattus.

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TABLE 5-6

Comparison of Tryptic Peptides From α Chains of *Lemmus* and *Dicrostonyx* (Whose identity are Tentative)

		T9	T8-9	T12b	T14'	UNID.
Lys	$Dr\alpha^F$	1.00		1.00		
	$Dr\alpha^S$		1.45		1.00	1.00
	$Ds\alpha^F$		1.50	1.36	1.00	1.00
	$Ls\alpha^F$			.77		
His		3.25		1.70		
			2.20			
			2.40	1.64		
				1.41		
Arg			(0.32)			
Asx		5.41		2.41		
			5.05		1.67	1.90
			3.66	1.68	2.09	1.00
				2.93		
Thr		2.44		1.61		
			2.08			
			2.13	1.20		
				1.92		
Ser		3.69		2.03		
			3.50			
			2.67	1.45		
				1.34		
Glx				0.78		
				(0.59)		
				(0.27)		
Pro		+[1]		1.16		
			1.30			
			1.35	1.76		
				2.18		

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TABLE 5-6 (continued)

		T9	T8-9	T12b	T14'	UNID.
Gly	$Dr\alpha^F$	N.A.		1.37		
	$Dr\alpha^S$		2.07			1.33
	$Ds\alpha^F$		2.85	1.08		0.83
	$Ls\alpha^F$			0.91		
Ala		N.A.		3.97		
			5.00		1.22	
			6.45	3.36	0.75	
				4.22		
Val		1.75		1.86		
			1.65		0.91	1.00
			2.91	2.01	1.05	1.09
				1.86		
Met						
Ile				0.86		
			(0.37)	0.78		
				0.91		
Leu		5.00		3.16		
			4.52		1.14	0.61
			5.25	3.72		
				3.20		
Phe		(0.60)		1.02		
			(0.67)	1.23		
				1.00		
Tyr						
Trp						
AE-Cys						

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TABLE 5-7

Composition of Chymotryptic Peptides From α^s Chain Core of D. rubricatus

Amino Acid	Cl3b	X	Cl3a
Asp		1.68	1.23
Thr	.77	0.30	t
Ser	1.37	1.56	1.08
Glu		t	
Pro			
Gly		0.63	(0.25)
Ala		1.30	0.89
Val		t	0.30
Cys			
Met			
Ile			
Leu		1.00	0.92
Nle			
Tyr			
Phe + NH_3		+	0.95
Lys	1.00	1.00	0.72
His			
Arg			
Trp			

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Fig. 5-6. Amino acid sequence for α-chain from major component. Notes, as in Figure 5-4. Mus from Dayhoff (1972) and rat from Garrick et al. (1975).

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```
SPECIES ____________________________________________________________
                   aT1       aT2      aT3                aT4
MUS      'V L S G E D K'S N I K'A A W G K'I G G H(A G)E Y G A E A L E R'
                              (10)                  (20)                  (30)
D.T.R.             D.     T.      [N.T.       ]          E.
                                                          D.
D.T.S.             D.     T.       N.          A.
L.S.               D.     T.       N.          A.

RAT              A.D.     T.       N.C.              G.       E.      Q.
____________________________________________________________________
                     aT5                         aT6          aT7 aT8
MUS       'M F A S F P T T K'T Y F P H F D V S H G S A Q V K'G H G K'K'
                              (40)                  (50)                  (60)
D.T.R.             V.Y.                                   A.
D.T.               V.Y.                                   A.
L.S.             V.V.Y.

RAT                  A.             N/S  I.      P.        A.
____________________________________________________________________
                                   aT9                   aT10     aT11
MUS       'V A D A L A N A G A H L D D L P G A L S A L S D L H A H K'L R'V D P V N F K'
                              (70)                  (80)                  (90)
D.T.R.   [//////////////////////////////////////////////////////]
D.T.S.   [//////////////////////////////////////////////////////]
L.S.     [//////////////////////////////////////////////////////]

RAT                       K.             Z. V.       T.
____________________________________________________________________
            aT12a                      aT12b                     aT13        aT14
MUS       'L L S H C'L L V T L A S H H P A D F T P A V H A S L D K'F L A S V S T V L T S K'Y R'
         (100)                (110)                 (120)                 (130)                 (140)
D.T.R.          [         G.      I.Q.                   ][///////////////]
D.T.S.          [         G.      I                      ][///////////////]
L.S.            [         G.  B.  I.                     ][///////////////]

RAT          F.                  C.       G.          M.
```


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TABLE 5-8

Species Comparison

EVOLUTIONARY PARAMETER

	Physiological		Protein[d]		Chromosomal[e]		Behavior[F]
	Winter coat color	M. max[c]	αHb	βHb	FN	2N	
D. stevensoni	No change[a]	5.9 - 7.7	0(50%)	0(80%)	54	34	docile
D. rubricatus	white[b]	7.0 - 8.6	0(50%)	0(95%)	54	33	aggressive

a) Field observation (Nelson, 1929), Lab observation (Rausch and Rausch, 1972)
b) D. Feist (personal communication), Rausch and Rausch (1972)
c) P. Morrison and A. Porchét (personal communication)
d) This study - *D. stevensoni* used as base of comparison
e) Rausch and Rausch (1972)
f) A. Porchét (personal communication)

Conclusion

With respect to Wilson's theory (1976) of an organismal evolution rate which differs from the evolutionary rate of proteins, this data on Dicrostonyx (Table 5-8) implies that both rates are different in Dicrostonyx. On the basis of Wilson's theory, I had expected to find no amino acid differences between the populations since the average rate for an amino acid mutation fixation in the α-chains of hemoglobin is approximately one substitution per 1 to 5 million years (Popp, 1973; Fitch and Langley, 1976), and the assumed separation of subspecies occured during the late Pleistocene. This work study did not find any differences, although the αT3 data needs repeating. The hemoglobin data from Dicrostonyx α^S supports suggestions that like morphological characters, proteins from rapidly evolving populations show more variability than do those from slowly evolving groups.

It can be seen from this and other studies (Genaux et al., 1976; Garrick et al., 1975) that rodents, which are evolving rapidly (Van Valen, 1974), show a greater degree of sequence variation than do primates, which are believed to be evolving slowly (Fitch and Langley, 1976).

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CHAPTER VI

DISCUSSION

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CHAPTER VI

DISCUSSION

The aim of modern taxonomy is not only to describe, identify and arrange organisms in convenient categories, but also to understand evolutionary histories and mechanisms. For this purpose it is necessary to take stock of all characteristics of the organisms that show modifications. The characters which have conventionally been employed for taxonomic studies of microtines are mainly morphological (Hooper and Hart, 1962). More recently Rausch and colleagues (Rausch and Rausch, 1972, 1974 and Nadler et al., 1976) have used cytological, ecological and geographical characters. However, there are a number of instances in which none of these kinds of characters are helpful in drawing conclusions regarding taxonomic relationships of animals. Even when these characters appear to be adequate for taxonomic studies, biochemical characters such as the primary structure of proteins will be helpful in confirming and clarifying the conclusions. As Van Valen (1973) states "it is important to know where there have in fact been unusual rates of evolution. . . many microtine (arvicoline) and probably many murid rodents. . . exemplify extremely rapid rates of evolution." This work has attempted to supply useful data on hemoglobin evolution in these rodents.

The evolution and systematics of hemoglobins from _Microtus_ and _Dicrostonyx_ can be visualized in several different ways. One way is simply to compare the percentage difference between the hemoglobins of

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these two species with the hemoglobins of other microtines. The results of such a comparison are shown in Table 6-1 for the α-chains and in Table 6-2 for the β-chains. These comparisons show as expected that microtines are similar to microtines and approximately equidistant from Mus.

Another way, probably a better way to describe the data, is to construct a cladogram or phylogenetic progression. In this construction a lineage which gives a minimum number of mutational events is assumed. Because whole organisms evolve, I have used "trees" consisting of branches which radiate from identical nodal positions in order to represent a common ancestral organism which carried both α- and β-genes. The cladograms (Figure 6-1 for α-chains and Figure 6-2 for β chains) interrelate the two microtine tribes from this study as well as other known rodent taxa and are based on the amino acid sequences summarized in the appendix (Fig. 3-1, 2).

1. Microtine Stem

The sequences presented for M. xanthognathus and the two species of Dicrostonyx confirm the microtine stem proposed by Genaux et al. (1976). There are a minimum of four β-chain stem positions: 11, 22, 44, 72, (βT12 not included). The α-chain shows six stem positions that differ from the Hesperomyini: 4, 12, 19, 35, 36, and 73 (αT12b not included).

From the microtine stem node (Microtinae) the average species has acquired five substitutions (range: 9 - 3) in the α chain. In the β

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TABLE 6-1

Percentage Difference Between the Amino Acid Sequence of Several Rodent Hemoglobin α-Chains[d]

	M.x.	L.s.	D.t.	C.r.	O.z.	P.m.	C.c.	Mus
Rodentia Microtine								
Microtus xanthognathus	0	3	3	5	7	11	9	12
Lemmus sibiricus		0	2	4%	4%	12	11	9
Dicrostonyx torquatus			0	2%	5%	11	11	9
Clethrionomys rutilus[a]				0	7	8	10	9
Ondatra zibethicus[a]					0	10	11	12
Cricetine[b]								
Peromyscus maniculatus						0	8	9
Calomys callosus							0	7
Murid[c]								
Mus								0

a. Genaux et al. (1976)
b. Morrison et al. (1976)
c. Dayhoff (1972)
d. Minimum differences with αT12b not considered.

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TABLE 6-2

Percentage Difference Between the Amino Acid Sequences of Several Rodent Hemoglobin β-Chains[d]

	M.x.	L.s.	D.t.	C.r.	O.z.	P.m.	C.c.	Mus
Rodentia Microtine								
Microtus xanthognathus	0	6	6	5	8	12	12	11
Lemmus sibiricus		0	6	7	8	14	12	12
Dicrostonyx torquatus			0	7	7	14	10	15
Clethrionomys rutilus[a]				0	6	13	8	9
Ondatra zibethicus[a]					0	13	7	11
Cricetine[b]								
Peromyscus maniculatus						0	13	13
Calomys callosus							0	8
Murid[c]								
Mus								0

a. Genaux et al. (1976)
b. Morrison et al. (1976)
c. Popp (1973)
d. Minimum differences with βT12 not considered, and only positive T5 differences counted.

Figure 6-1. Cladogram relating α-chains in rodents.

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MICROTINE HEMOGLOBIN: ALPHA-CHAINS

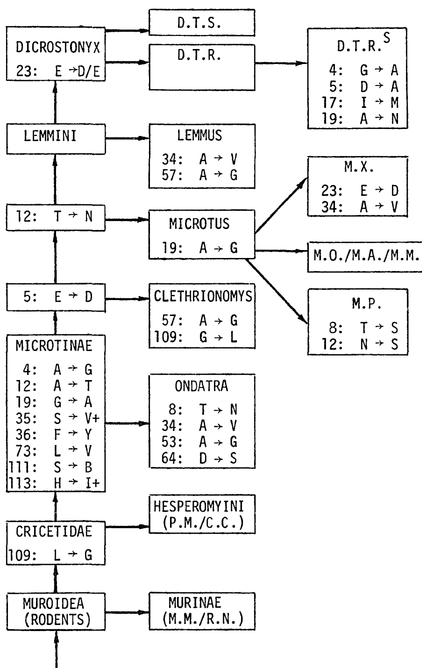

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Figure 6-2. Cladogram relating β-chains in rodents.

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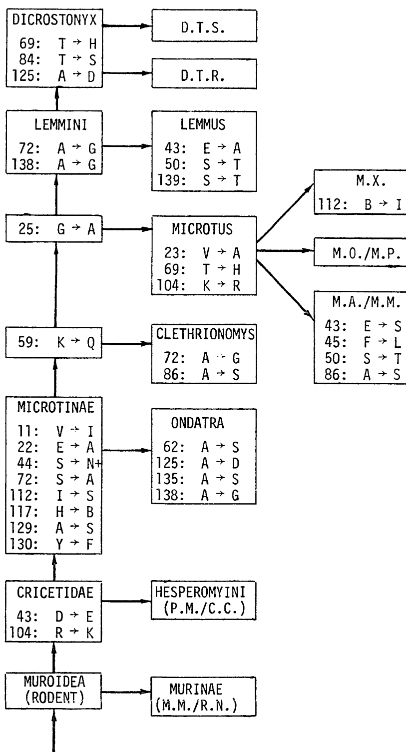
MICROTINE HEMOGLOBIN: BETA-CHAINS



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chain the average number of substitutions from the stem node is six (range: 9 - 3). Although the sample size is marginal (eight species), the data tend to obey a fundamental statistical law of evolutionary biology: for a given time period, if a lineage accumulates an amount (X) of genomic differences per locus from an ancestral population, then any other lineage descending from this ancestral population will also accumulate an average of X genomic differences from that ancestral population during the same time period (Salthe, 1975).

Table 6-3 compares the estimates of the dates of microtine and murid divergence based on either the number of amino acid differences between hemoglobin β-chains or mutational steps in the phylogenetic progression shown in Figure 6-2. The rate of mutational fixation is based on Dayhoff's estimate (1972, p 50) of one substitution per hemoglobin every 2.3 million years. This rate is in close agreement with those derived by Nei (1972) and Dickerson (1971). The estimates in Table 6-3 differ by a factor of three. This difference is due to a underestimation of mutational events by counting only differences. The variable positions in hemoglobin are usually restricted to two or three possible amino acids. These amino acids usually have similar side chains and in the past substitutions among these amino acid have been termed conservative, or more recently, neutral (Kimura and Ohta, 1971). Because of such conservative substitutions, the probability of observing back or parallel mutations is increased. These types of mutations are ignored by the difference counting method. Morrison et al. (1976) indicated that these types of mutations may be as high as

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TABLE 6-3

Comparison of Difference Counting Method (I) and Phylogenetic Progression Method (II) in Estimating Time Since Divergence From MUS

	I		II	
	Diff.	M.y.	Prog. Steps	M.y.
L.s.	12	13.8	18	39.5
D.r.	15	17.2	19	43.7
C.r.	9	10.3	13	29.9
O.z.	11	12.6	15	34.5
M.x.	11	12.6	16	36.8
Ave		13.3		36.8

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5% in the crecitidae. A crude estimate of the time of divergence of microtines from murids is not possible at present on the basis of rat and white mouse hemoglobins which differ greatly from each other. More murid sequences are necessary to arrive at an accurate average number of mutations. The natural grouping of microtines into the subfamily Microtinae is supported by the many nodal positions still present in the microtine node after the lemming data have been added to the cladograms. The microtine node has 14 (α+β) common positions compared to two for the node of tribe Hesperomyini. The microtine species are farther from the muroid stem than are the Hesperomyini species, although Peromyscus is near the range of several microtine species. Morrison et al. (1976) have pointed out that Calomys and Peromyscus "might better represent separate subfamilies within the Crecitidae". A similar situation is observed for the two murid genera, Mus and Rattus (Genaux et al., 1976; Garrick et al., 1975).

2. Lemming Stem

The study on M. xanthognathus supports previous proposals for the Microtus stem (Microtus) for hemoglobin (Genaux and Morrison, 1973a, b; Genaux et al., 1976). A possible reinterpretation of positions involving peptide βT5 is presented in cladogram Figure 6-2. Initially, the sequences for Dicrostonyx when combined with Lemmus data did not give a clear lemming stem node which differentiated α- and β-chains from Clethrionomys and Microtus. By assuming parallel mutations at positions α57 and β72 in Clethrionomys hemoglobin, an

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identical progression of nodal points can be obtained for both hemoglobin chains.

Position β69: Species of Microtus and Dicrostonyx have a histidine residue at this β-chain position while other microtines show a threonine. The Thr → His mutation involves two base changes in the codon (AG- → GAC/U). Two single base changes through either asparagine or proline could account for this substitution. Proline is unlikely since position β69 is in the middle of the E helix, and proline would disrupt the tertiary structure of the hemoglobin.

If one makes the customary assumption that Microtus and Dicrostonyx evolved from the stem body of microtines, either of two mutational mechanisms can be evoked to explain the two nucleotide changes. The first mechanism is a simultaneous change in two adjacent nucleotides of the β69 codon. This is a distinct departure from the prevailing view that a spontaneous mutation results entirely from unitary excitation, tautomerization or structural rearrangement of a single base during replication. The coincidence of a two-base change has a small probability. This view may be open to question as indicated by a recent study on the Celebes Black Ape.

Murata and Thompson (1976) described a hemoglobin polymorphism in this ape involving variant β-chains. Finger-printing and amino acid analysis of the tryptic peptides from the two chain types showed that they differ by a single amino acid substitution (Lys - Asp), which requires a two-nucleotide change in the corresponding codon. In their discussion they point out that the "whole spectrum of changes found in

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higher organism may in fact closely parallel the array of mutations induced by Yanofsky et al. (1968) in the structure of trytophan synthetase from E. coli using ultraviolet irradiation". Among a number of mutations altering the α-subunit of that enzyme, one substitution (Glu → Met) has a two-nucleotide change.

An alternative mechanism to simultaneous replacement of two nucleotides in the β69 codon would be two independent single-nucleotide replacements involving an ancestral Dicrostonyx and ancestral Microtus in an unlikely kind of parallel mutations. This would require

1. The mutation to asparagine or lysine in both the Microtus and Dicrostonyx ancestors but not the Lemmus and Clethrionomys ancestors.

2. A second step of mutation in the β69 codon from the asparagine or lysine to histidine residue.

3. Eventual fixation of the histidine in the populations.

The only possible test for either of these mechanisms is to find a Microtus or Dicrostonyx population with asparagine. An asparagine residue has been found in rat at position β69 (L. Garrick, personal communication; Weiser et al., 1976). It should also be noted that a similar two-nucelotide change occurs in the β69 codon in Peromyscus (Thr → Gln, Morrison et al., 1976). Patterns in the rate of acceptance of point mutation (Dayhoff, 1972, p 97) indicate that both Thr → Gln and Thr → His, although not common, do occur in many protein families.

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Positions α8 and α12: These positions are believed to be mutations in the M. pennsylvanicus lineage. They are subject to a high incidence of mutations in mammalian hemoglobins as is illustrated by mammalian hemoglobins (Dayhoff, 1972 and 1976).

Position α34: The effect of this position on the solubility of the hemoglobin molecule may be related to its involvement as an $\alpha_1 \beta_1$ contact. It contacts amino acid residues 124, 125 and 128 in the β-chain. Position α35 and α36 are also $\alpha_1\beta_1$ contacts. The major hemoglobins from M. xanthognathus, L. sibiricus, O. zibethicus and C. callosus all have valine at this position α34. These species are all on different branches of Figure 6-1, and this mutation from the stem alanine must represent a series of parallel mutations. In both of the minor hemoglobins, from Lemmus and from M. xanthognathus, valine is also found at position α34 indicating a recent origin for the duplicated gene.

The progession shown in Figure 6-1 indicates that the valine substitution was one of the last mutations fixed in each lineage. In the α-chains from M. xanthognathus and L. sibiricus, the valine appears following speciation and after the accumulation of 3 - 5 changes in the α-chain. Other closely related species must be studied to test the validity of this conclusion. The parallel mutation concept involved here is supported by data from the α chain of Hb-IV from trout which also has Val-Val at positions 34 and 35.

Position α57: In the α-chains of hemoglobin from Clethrionomys and Lemmus position α57 is occupied by a glycine residue, whereas in

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the Dicrostonyx α-chain position $\alpha57$ is occupied by an alanine. This would suggest a Lemmus-Clethrionomys branch point. The β-chain data suggest a Lemmus-Dicrostonyx branch (i.e. positions $\beta25$, and $\beta138$). This problem arises when only a few close species are evaluated. This problem exists in other globin studies but may not be observed when distant species are compared. To be consistant, I have the α-chain nodes parallel the β-chain node. In this progression Clethrionomys is assumed to have two parallel mutations.

The conclusions concerning the lemming lineages are regarded as a suggestive working hypothesis, and the following data are also listed for comparison (Hooper and Hart, 1962):

1. In Clethrionomys, Dicrostonyx and Lemmus, the posterior part of the hard palate are similar.
2. Lemmus and Dicrostonyx have spongy walls of the bulbae while in Clethrionomys the walls have no spongy tissue.
3. Lemmus and Clethrionomys have glans which are similar in structure while Dicrostonyx is unique.

The three genera resemble each other to some extent, but a definite pattern does not seem to emerge from the morphological data. The cladograms indicate that the three lineages diverged at approximately the same period of time (± 2.3 million years - one change per node) and that the Microtus and lemming lineages may be more closely related to each other than to Clethrionomys.

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3. Gene Duplication

Multiple forms of hemoglobins, which result from either allelism or duplications in the structural loci of polypeptide chains, are common in rodents (Weiser et al., 1976). In several species of the subfamily Microtinae (family Cricetidae), urea cellulose-acetate electrophoresis of individual hemoglobins has indicated that there are two α-chains. These two α-chains were present in all members of the populations sampled. Thus, animals of the general Microtus and Dicrostonyx normally contain two hemoglobins in the ratio of 2:1. Structural studies (see chapters IV and V) have shown that there is very little difference between the two α-chains. It therefore appears that recent duplication followed by point mutations has given rise to the genes responsible for the two adult α-chains in these genera. In this respect my results are similar to those of Hilse and Popp (1968) who found multiple α-chains in Mus musculus. They found that C3H mice had two α-chains whose synthesis was controlled by two α-chain genes. They also point out that the two genes must be closely linked, on the basis of their recombination frequency which is less than 0.01.

Using M. xanthognathus as an example, it can be seen that the α-chain gene duplication in the Microtinae probably occurred independently of the α-chain duplication in the Muridae. If the duplication occurred before the murids diverged from the microtines, the number of differences between the α-chains of M. x. f and M. x. s should be the same as the number of differences between the α-chains of M. x. f and C3H mice, or of M. x. f and rat α-chains. This is not the case

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since there are at least 13 differences (with an incomplete sequence) between the α-chains of M. x. and C3H mice while there are only two differences between the α-chains of C3H mice and three differences between those of M. x. Similarly for the rat, there are three differences between its α-chains and a minimum of 14 differences from MX α-chains.

The argument for independent duplication of α-chain loci can even be extended to apply within the subfamily Microtinae based on 1) hemoglobin component ratio 2) differences in subspecies of M. pennsylvanicus and 3) sequences in Ondatra, M. oeconomus and Lemmus and Dicrostonyx.

Nute (1974) has proposed that species with hemoglobin component ratios of approximately 66/34 demonstrate a "position effect" of linked genes which determine that amount of hemoglobin chains produced. This has been shown to be the case with human γ-genes. He believes that two α-chain genes, when linked, govern the synthesis of the same amount of α-chain as does a single allele on the homologous chromosome. If this model is true, then microtines have linked α-chains. It can be assumed that in the descent of the α-chain genes in the line leading to the yellow-cheeked vole a gene duplication occurred and a mutation in the DNA in one of the two α-chain genes caused the α-chains to differ in charge. This process would give rise to the two hemoglobin components observed. The comparisons of these minor chains indicate that they have probably arisen from the major α-chain separately within each species. When the minor α-chain of M.

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oeconomus is compared to that of M. xanthognathus a species specific residue can be seen in position α34. Also, the differences between αT1 peptides of Dicrostonyx and those of M. xanthognathus would imply a separate evolution, as would the differences in αT3 between the hemoglobins from D. stevensoni and D. rubricatus. Lemmus sibiricus has hemoglobin α-chains which are similar at positions α34 and α57, indicating independent duplication in relation to other microtines. When the major α-chains are compared, a common ancestry is very evident. The same α^{f}T1 is common to most microtine species, indicating that it is probably the ancestral form. A similar situation occurs in the Muridae in that the minor α-chains of rat and white mouse are as far apart as are the major α-chains in terms of members of substitutions.

Independence of gene duplication between rodent taxa is also supported by studies on the hemoglobin of muskrat (Genaux et al., 1976), which shows only one hemoglobin on electrophoresis indicating either a single unduplicated gene or duplicated genes whose products are not distinguishable by a difference in charge.

Data from muskrat hemoglobin have not revealed any pairs of homologous peptides which differ from one another in uncharged residues, thus supporting the existence of a single hemoglobin gene.

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Multiple Hemoglobins

Gene duplication at the α-chain locus has lead to multiple hemoglobins in *Microtus xanthognathus*, *Dicrostonyx rubricatus* and several other microtines. I propose that the two hemoglobins in individuals of these species represent a homozygous condition with both α-chain loci present on a single chromosome. The homologous chromosome containing these α-chain genes would be identical with respect to these genes. In the past, two hemoglobins were usually assumed to represent the heterozygous conditions of two alleles in the population. A recent study by Mazur and colleagues (Weiser et al., 1976) has demonstrated a similar situation in both rats and guinea pigs - two separate suborders of rodents. Alleles are probably also present but they do not effect the chemical isolation of the hemoglobin components or change the phenotypic definition of the species' hemoglobins. Mazur also proposes that variation in hemoglobin component ratios in adult rodents are due to nonuniform biosynthesis of the individual chains during maturation of the erythroid cell. He suggests that this phenomenon may be a general one among mammalian multiple hemoglobins. This proposal eliminates the need for a position effect and supports the concept of gene regulation and specific receptor sites as proposed by Britton and Davidson (1971).

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Conclusion

I have discussed two aspects of molecular evolution: hemoglobin divergence and gene duplication. The results reported in this thesis show that the evolution of hemoglobins in the tribes lemmini and Microtini has been faster than that reported for primates. The cladograms show that the primary structure of hemoglobins from microtines support the designation of their being a subfamily and possibly, in the future, even being given family rank when other rodent data is acquired. The cladograms also agree with two different systematic descriptions of Microtine tribes, that of Simpson and that of Ognev.

The difference in α^S chains between Microtine species suggests that gene duplication has occurred independently in each genera. The lack of differences between the two Dicrostonyx species could indicate that the rate of evolution of the species is different from the rate of evolution of the hemoglobin molecule.

This study has also confirmed the value as proposed by Genaux and Morrison (1973b) of comparing hemoglobin structure as a tool for studying taxonomic relationships among rodents. However, another lemming genus, such as Synaptomys, should be studied to confirm the Dicrostonyx distance from its branch points. Also, as Morrison (in Morrison et al., 1977) has pointed out, other rodent families have not been investigated and the study of their hemoglobin can provide additional information on early rodent evolution. Studies of D. t. exul, D. t. richardsoni and D. hudsoni would be valuable in understanding the formation of sibling species and the process of gene duplication in microtine hemoglobins.

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APPENDICES

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APPENDIX A

SEPARATIONS

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APPENDIX A

SEPARATIONS

This section groups together various physical comparisons of the hemoglobins and also records the results of the purification and separation of the α- and β-chains. This section provides a basis for comparison when further studies of microtines are performed.

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Figure I-1. Electrophoretic separation of some microtine hemoglobins, pH 9.1 in 0.13 M Tris:EDTA:Borate buffer on cellulose acetate. 1,8 *M. oeonomus*; 2, *M. pennsylvanicus tananaensis* (Alaska); 3, *M. pennsylvanicus pullatus* (Montana); 4, *M. xanthognathus*; 5, human; 6, *D. t. rubricatus*; 7, *L. sibiricus*. Amido Black stain, 350V, 3MA, 45 min. running time with anode to the right.

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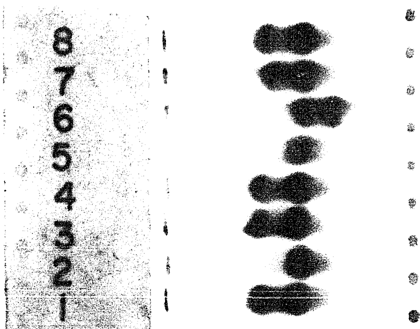

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Figure I-2. Electrophoretic comparisons of microtine hemoglobins before and after cystamine treatment. 1, *M. xanthognathus* (+ cystamine); 2, *M. xanthognathus*; 3, *D. t. rubricatus*; *D. t. rubricatus* (+ cystamine) 5, *L. sibiricus* (+ cystamine) 6, *L. sibiricus*.

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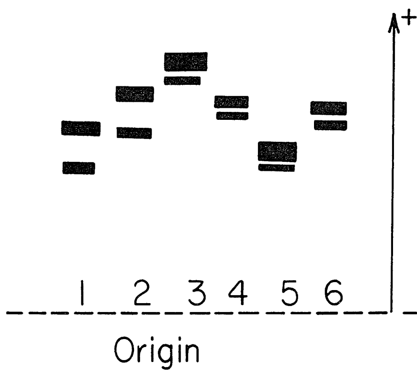

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Figure I-3. Electrophoresis patterns of Acomys. A densitometer tracing of chain pattern on urea-cellulose acetate (whole lipate). B. densitometer tracing of chain pattern on urea-cellulose acetate (major component only - after purification on a DEAE column). C. hemoglobin patterns of M. xanthognathus (position 1) compared to Acomys (position 2). D. pattern from which densitometer tracing was made.

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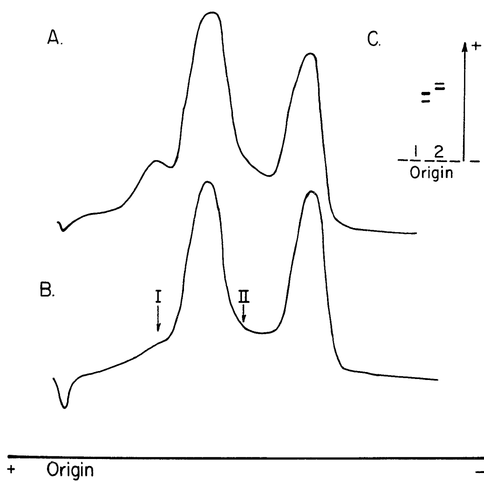

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D.

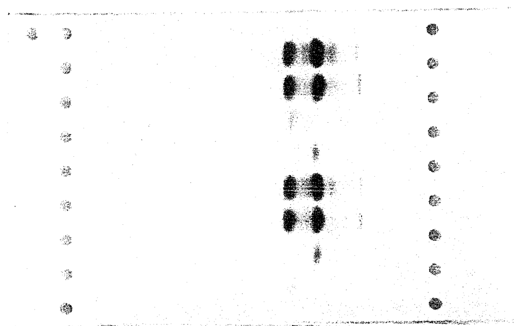

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Figure I-4. Alkali denaturation behavior of _M. xanthognathus_.

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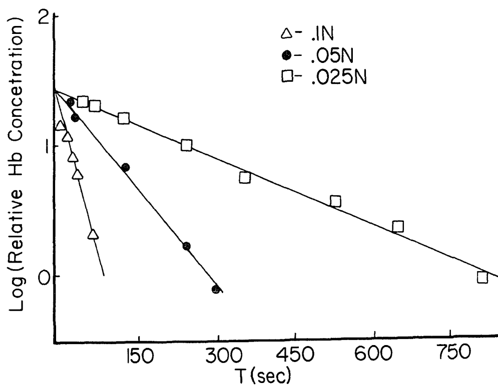

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Figure I-5. Results of electrofocusing experiment on carboxy-hemolysate of M. xanthognathus. The experiment was run for 40 h at 400V and 5°C over a gradient of pH 5.8. This column was drained at approximately .5ml/min about 1.01 ml fractions were collected. The pH values were plotted on the chart obtained from the recording of the UV absorption during the emptying process. Open circles, pH; solid line, absorption at 254 nm (Gilson Biochemical Monitor). The peak at fraction 10 was a non-heme substance.

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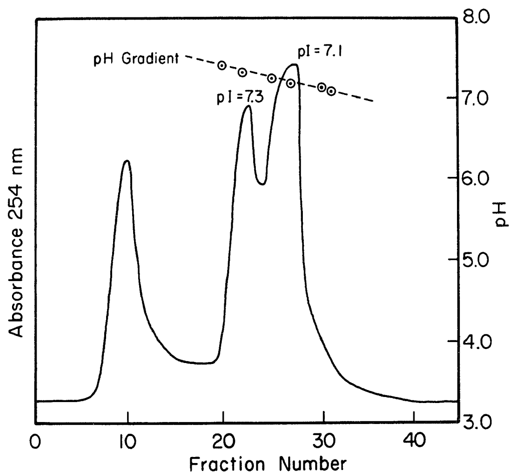

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Figure I-6. Separation of major and minor hemoglobin components of *M. xanthognathus* on DEAE. Pool I, minor component; Pool III, major component.

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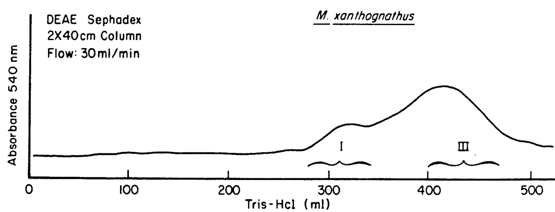

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Figure I-7. Electrophoretic characterization of minor and major hemoglobins of *M. xanthognathus* after separation by column chromatography on DEAE A 50 Sephadex. 1, 5, 8, whole hemolysate; 2, 3, 7, minor component; 4, met Hb (oxidized) form of minor component; 6, major component.

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8
7
6
5
4
3
2
1

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Figure I-8. Chain separation of M. xanthognathus whole globin on CM-32 column (1 cm x 15 cm): Flow rate was approximately .21 ml/min, and 3 ml fractions were collected.

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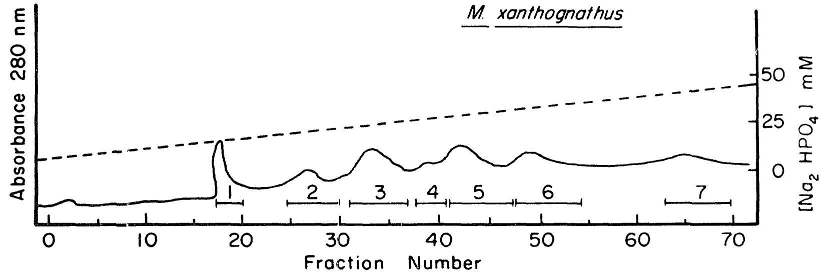

Figure I-9. Chain separation of *M. xanthognathus* minor component after separation from major component by DEAE Sephadex chromatography.

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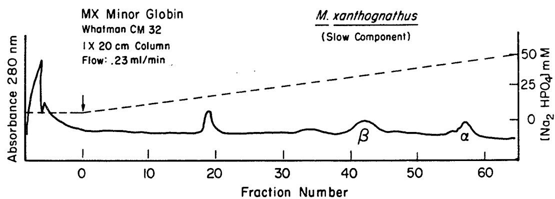

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Figure I-10. Gel filtration pattern for the initial purification of the peptides obtained from the cyanogen bromide cleavage of *M. xanthognathus* whole globin. The peptide mixture was applied to a 2.6 x 195 cm column of Bio-Gel P-10 (200 - 400 mesh) and eluted with 10% acetic acid.

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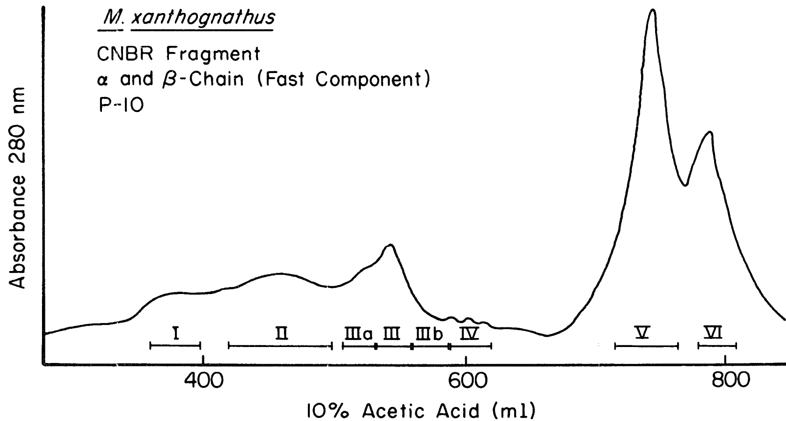

Figure I-11. Gel filtration pattern for the initial purification of β-chain cyanogen bromide peptides from *M. xanthognathus*. The peptide mixture was applied to a 2.6 x 18- cm column of Sephadex G-50 (superfine) and eluted with 10% acetic acid.

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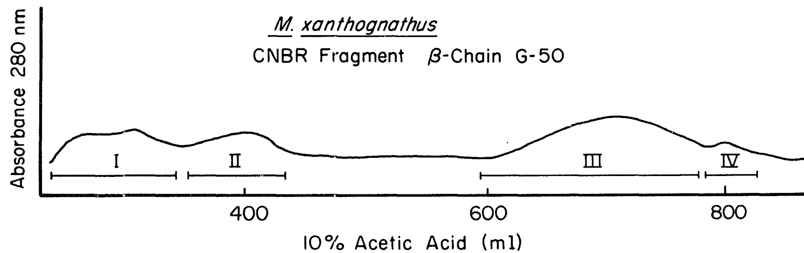

Figure I-12. Map of tryptic peptides from N-terminal cyanogen bromide fragment of the α^f (fast) globin chain from M. xanthognathus.

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CNBR Fragment A1 (Fast Component)

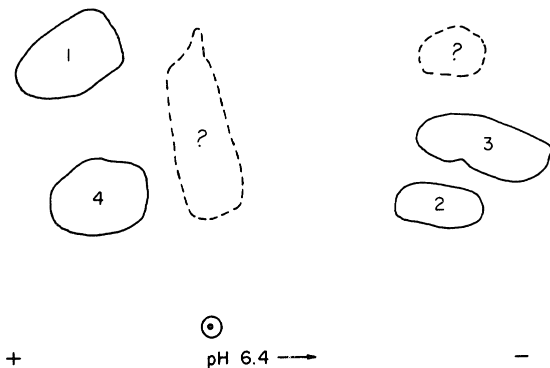

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Figure I-13. Gel filtration pattern for the initial purification of the α-chain from the minor (slow) hemoglobin component from _M. xanthognathus_.

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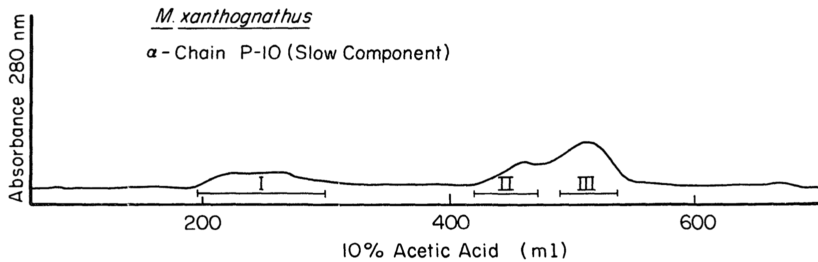

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Figure I-14. Map of tryptic peptides from N-terminal cyanogen bromide fragment of the α^S (slow) globin chains from *M. xanthognathus*.

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CNBR Fragment(Slow Component)

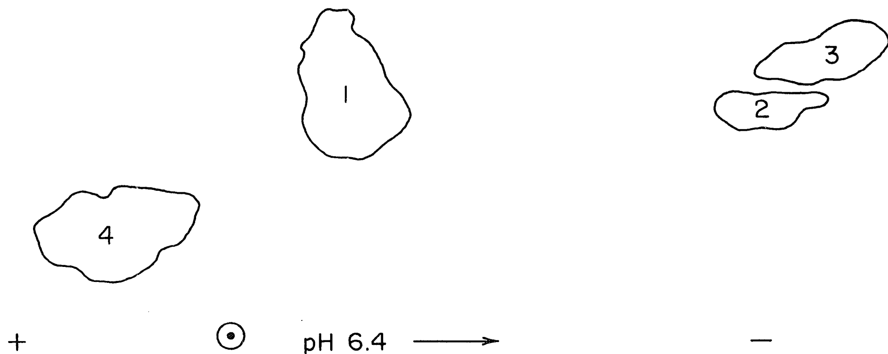

Figure I-15. Chromatogram illustrating the separation of globin chains from *D. stevensoni* on CM52.

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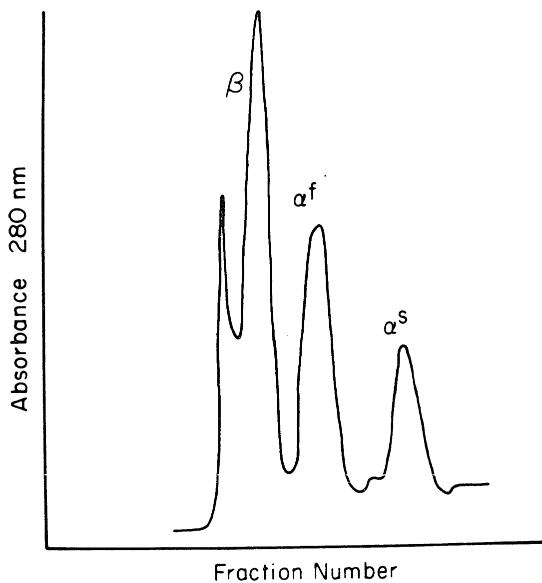

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Figure I-16. Chromatogram illustrating the separation of globin chains from the *D. rubricatus* on CM52 after aminoethylation.

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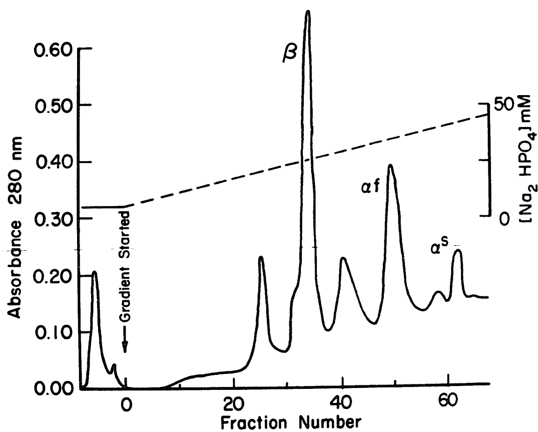

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Figure I-17. Chromatogram illustrating the separation of globin chains from the slow component of *D. rubricatus* on CM32.

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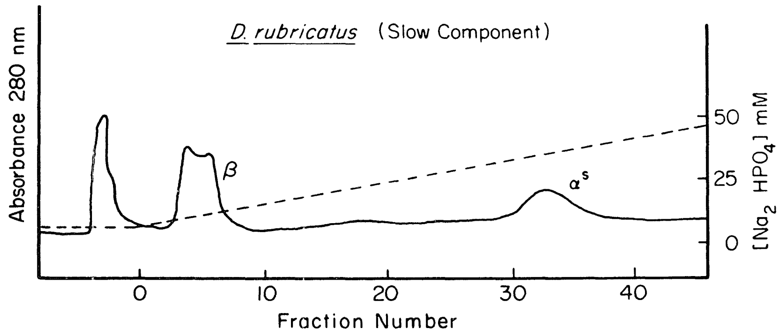

Figure I-18. ^{13}C NMR spectra of M. xanthognathus enriched oxy-hemoglobin equilibrated with 40 mM total carbonates. Top: major component, Bottom: minor component.

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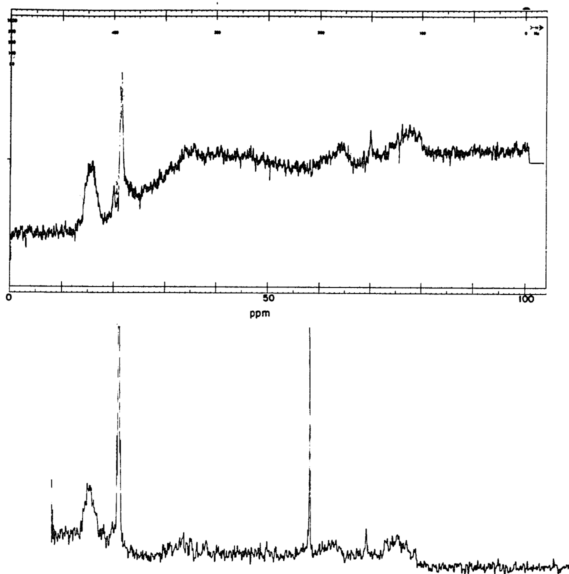

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Figure I-19. ^{13}CNMR spectra of M. xanthognathus enriched deoxy-hemoglobin. Insert: minor component, bottom: major component.

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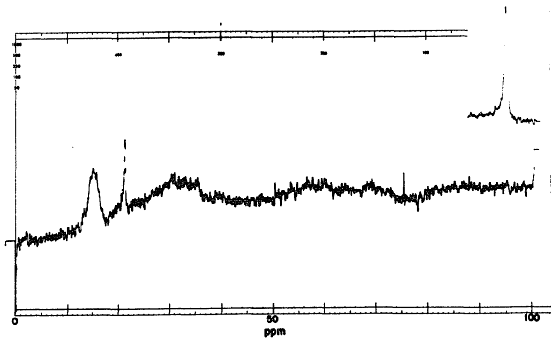

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APPENDIX B

BREEDING STUDY

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APPENDIX B

BREEDING STUDY

The hemoglobin of M. pennsylvanicus has been shown to vary between subspecies (Stratton and Duffy, 1976). M. p. tananaensis showed only one hemoglobin component during electrophoresis while M. p. pullatus showed two, a major (fast) and minor (slow). Figure II-1 compares the quantity of α^S chains from both parent species and the F1 offspring of their cross. Table B-1 summarizes the data obtained from 17 F1 offspring. Backcrossing the F1 offspring with M. p. tananaensis did not produce any offspring with the M. p. tananaensis phenotype (N = 9). However, in the F_2 generation (F_1 x F_1) 5 out of 12 offspring had the M. p. pullatus phenotype.

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Figure II-1. Densitometer tracing of amido black stained hemoglobins of <u>Microtus pennsylvanicus</u> after separation by urea-cellulose acetate electrophoresis at pH 8.9.

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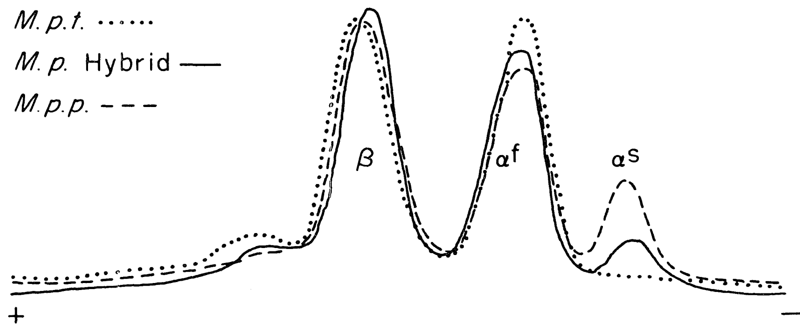

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TABLE B-1

Comparison of M. pennsylvanicus Hemoglobin Types

Subspecies	Genotype (assumed)	Pattern	Phenotype F/S (%)	$\beta: \alpha^f: \alpha^s$ (%)	$\frac{\alpha^s}{\alpha^f}$
M. p. pullatus (Hamilton, Mt.)	$\alpha^f \alpha^s$ $\alpha^f \alpha^s$	double	70/30	50:35:15	.4
M. p. tananaensis (Fairbanks, Ak.)	α^f α^f	single	100/00	50:50:00	0
F_1 (M.p.t. x. M.p.p.)	$\alpha^f \alpha^s$ α^f	double	85/15	50:42.5:7.5	.2

APPENDIX C

RODENT SEQUENCES

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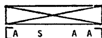
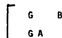
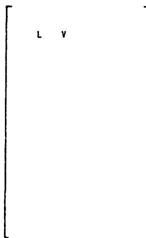
αT1 ' ' ' αT4 ' αT5 ' αT6 ' '' αT9 ' ' αT11 ' ' αT12b ' αT13 '

10 20 30 40 50 60 70 80 90 100 110 120 130 140

RAT D	NC G E Q A S I P AK Z V C A M
MUSG	S G A A
MURIDAE	A
MUROE	T IKAA G A E A E ASF P F V H A TN G L L G T L S GD V
Family Cricetidae	
CRIC.	G
Subfamily Cricetinae, tribe Hesperomyini	
HESP.	A
C.c.	A VR D V G B
P.m.D	A [A S A A] G A
Subfamily Microtinae, [tribe Microtini]	
MICG	T A VY V B I
O.zG	N T A VVY G S V
C.rGD	T A VY G V L
tribe Microtini	
Subgenus Microtus	
MICGD	N G
M.xVD	N D VVY L V
M.oGD	N VY
M.pGD	S T VY
Subgenus Stenocranius	
M.aGD	N VY
M.mGD	N VY
tribe Lemmini	
LEMMD	N A VY
L.sGD	N A VVY G
D.rGD	N A VY
D.sGD	N A VY

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      •   BT2   •    BT3     •     BT4  •        BT5     •   •   ••       BT9    •      BT10   •   BT11 •     BT12      •   BT13    •    BT14   • •
         10        20        30        40         50       60        70         80        90      100         110      120       130       140

RATTUS     N         D                  Y             I                 N              N                           G     E        F
MUS                                     Y             I                    N                                       G              F        A
MURIDAE                                 Y             I                                                            G              F
MURO DA AAV G    NADEV A        V       F DSF     SAS V  AK  A       T S   K       T  AS                R   MI I   H L   D        Y        S
   Family Cricetidae
CRIC.                                      Z                                                            K
   Subfamily Cricetinae, tribe Hesperomyini
HESP.                                      Z                                                            K
C.c.        S                   A          Z                                                            K
P.m.     L        KPZ I A       I          Z I     A         G      Z                                   K                        S         T
   Subfamily Microtinae, [tribe Microtini]
MIC       I          A                     ZH                          A                                K      S     B           SF
O.z.      I          A                     ZH                S         A                                K      S     B       D   SF   S   G
C.r.      I          A                     ZH             Q            G              S                 K      S     B           SF
   tribe Lemmini
LEMM.     I          A A                   ZH             Q            G                                K                        SF       G
L.s.      I          A A                   AHL    T       Q            G                                K                        SF       GT
D.r.      I          A A        G          ZH             Q          H G            S                   K                    D   SF       G
D.s.      I          A A        G          [                ]        H G            S                   K                    D   SF       G
   tribe Microtini
     Subgenus Microtus
MIC       I          AA A                  ZH             Q                                                                      SF
M.x.      I          AA A                  ZH             Q          H A                                                         SF
M.o.      I          AA A                  [                ]                                                                    SF
M.p.      I          AA A                  ZH             Q                                                                      SF
     Subgenus Stenocranius
M.a.      I          AA A                  ZHL    T       Q                           S                                          SF
M.m.      I          AA A                  ZHL    T       Q                                                                      SF
         10        20        30        40         50       60        70         80        90       100        110      120       130        140
```

TABLE C-1

Percentage of Hemoglobin Chain Positions Which Show Amino Acid Replacements

	β-Chains	α-Chains	N
Rodent	24	23	14
Murid	10	14	2
Cricetine	20	17	12
Hesperomine	10	8	2
Microtine	12	10	10

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GLOSSARY

Adaptation - any structure or process that makes an organism more fit to survive and to reproduce in comparison with other members of the same species.

Allele - each gene controls a specific set of developmental processes, and each occupies a particular locus, or place on a chromosome; when there are several forms of a single gene in a population, each segregating form is called an allele.

$Alpha_1Beta_1$ Subunit Contact - a "dimer" contact; the $Beta_1$ subunit rotates by about 4° relative to the $Alpha_1$ subunit.

$Alpha_1Beta_2$ Subunit Contact - a "heme-heme" contact; the $Beta_2$ subunit rotates by 13.5 relative to the $Alpha_1$ subunit.

Amniote - said of organism or group of organisms that possess a membrane which encloses the embryo. Used in the sense of "higher" vertebrate (reptiles, birds, and mammals).

Back Mutation - the mutation of a codon back to an ancestral form. The rate of back mutations is believed to be low because it is assumed to be the product of the rate of any sort of mutation at that codon and the inverse of the number of different possible ways the given codon can mutate.

Balancing Selection - natural selection for a heterozygous condition at a particular locus, that is, it preserves a balanced polymorphism over many generations. Sometimes used as synonymous with heterosis.

Cephalochordate - a subphylum of Chordata that contains the single class Amphioxi, i.e. lancelets.

Clade - a species or set of species representing a distinct branch in a phylogenetic tree.

Cladogram (tree) - a phylogenetic progression that depicts only the splitting of species and groups of species through evolutionary time.

Covarions - concomitantly variable codons: the range of permissible fixations at one site must change as a function of fixations at other sites, and that the consequence of one mutation often depends on the occurrence of others.

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Domain - a limited stretch of amino acid sequence in a polypeptide chain.

Diploid - A single, complete set of chromosomes is a genome. When there is only one genome per cell, the organism is haploid. The number of chromosomes of a genome, the haploid number, is generally designated by n. When there are two genomes, the organism is diploid (2n).

Evolutionary Clock - substitutions of amino acids in protein chains occur at uniform rates in time. Knowing the mutational distances between the proteins of representatives of two groups of organisms, and having an estimate of the substitution rate, one can arrive at an approximate date for the separation of the ancestral lines of these organisms.

Evolutionary Noise - fixations of amino acid mutations succeed each other in a circle around the different partial molecular functions involved. The progression along this circle is considered noise. The noise can be significant because it increases the potential for a functionally significant mutation.

Evolutionary Potential (plasticity) - the ability to become modified as a response to ecological catastrophes.

Fixation - the complete prevalence of one gene form (allele), resulting in the complete exclusion of another.

Founder Effect - the genetic differentiation of an isolated population due to the fact that by chance alone its founders contained a set of genes statistically different from those of other populations.

Fundamental Number - the number of chromosome arms.

Gel Filtration - method for separating mixtures of macromolecules by size. Separation depends upon the ability of the smaller components of the mixture to penetrate into the interior of the particles, while the larger molecules are restricted to the inter-particle space and, therefore, flow through the column more rapidly.

Genetic Drift - change in gene frequencies by change processes alone. It is the result of a sampling error that causes the random changes from one generation to the next.

Genetic Sufficiency - the species survives; it is neither perfectly nor fairly adapted. All surviving species are equally adapted.

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Genotype - the genetic constitution of an individual organism; usually with the reference to a single trait or set of traits.

Heme-heme Interaction - binding at one heme facilitates the binding of oxygen at another heme on the same tetramer.

Heterozygous - having different alleles of a given gene on the pair of homologous chromosomes carrying that gene.

Homologous - having a general definite relation to others in a series; in zoology, having a correspondence in position or development.

Ingram Effect - restrictions placed on the evolutionary plasticity of individual gene products because of the number of other products with which they have to interact such that molecules having to interact with many different gene products evolve more slowly than do those interacting with fewer different gene products.

Lineage Group - a group of species allied by common descent. It is an evolving gene pool or group of closely related gene pools

Linkage Group - group of genes that are members of the same chromosome.

Locus - the location of a gene on the chromosome.

Natural Selection - the differential contribution of offspring to the next generation by individuals with different genetic types but belonging to the same population. In this thesis, it is used in the sense of being divisable into positive selection and stabilizing selection.

Neutral Mutations - mutations whose fixation in evolution occurred as a result of fortuitous or random processes and not as a result of natural selection. A neutral gene is one whose phenotypic expression is not accessible to natural selection.

Neutralist - one who argues that the great majority, though not quite all the amino acid differences that one finds in a particular protein in different species are the results of amino acid substitutions that have little or no effect on the function of the protein.

Non-Darwinian - evolution by random walk; given many genetic variants that are selectively neutral, their frequencies in populations will drift at random until some of them are lost and others reach fixation.

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Nonfunctionality - when under the given environmental conditions, the phenotypic expressions are identical from the point of view of natural selection.

Peptide Mapping (fingerprinting of proteins) - two-dimensional separation of peptides from enzymatic digestions of proteins. The method is a combination of electrophoresis on paper (or thin layer), followed by chromatography at right angles.

Pleiotropic genes - have more than one function, that is, the gene product may have widespread influences on many structures.

Phenotype - the observable properties of an organism; the expression of the hereditary material in some particular environment.

Phylogeny - the evolutionary history of a particular group of organisms; usually describes a trait in relation to ancestor-descendant groups. Should have not more than two branches separating taxa at any point of divergence.

Point Mutation - a change along a very narrow portion of the nucleic acid sequence.

Polymorphism - a population in which two or more distinct inherited varieties coexist at frequencies too great to be attributed to mutation is said to exhibit a genetic polymorphism. A population is considered to be polymorphic if the rarest variety has a frequency greater than one percent.

Position Effect - the position of the gene on the chromosome relative to other genes has an effect on the degree of transcription.

Positive Selection (directional selection) - selection that operates against one end of the range of variation and hence tends to shift the trait toward the opposite end.

Proportionality Assumption - over long intervals, the directional selection on any protein is proportional (or positively related to that of the phenotype as a whole.

Red Queen Hypothesis - a selectionist's theory derived from Lewis Carroll's "Alice In Wonderland" based on the statement of the Red Queen to Alice, "Now, here you see, it takes all the running you can do to keep in the same place". It implies that an increase in momentary fitness by one species causes an equal total decline in momentary fitness among ecologically interacting species.

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Salt Links - pairs of residues held together by noncovalent, electrostatic interactions between oppositely charged groups.

Selectionist - one who ascribes all amino acid substitutions to natural selection.

Silent Gene - the locus is present but synthetically ineffective.

Stabilizing Selection - natural selection in which most point mutations are seen to be deleterious. This selection operates against the extremes of variation in a population and hence tends to stabilize the population around the mean. Can be divided into normalizing selection and canalizing selection.

Stochastic - referring to the properties of mathematical probability. A stochastic model takes into account variation in outcome that are due to change alone.

Structural Genes - any gene coding for a protein.

Subspecies - usually defined narrowly as a geographical race; a population or series of populations occupying a discrete range and differing genetically from other geographical races of the same species.

Teleological - concepts are fully teleological if they involve the notion that functional considerations are necessary and sufficient to explain a structure of phenomenon.

Tetrapod - a term coined to describe those classes of gnathostamatous chordates which have limbs as distinct from fins. The group contains the amphibia, reptiles, birds, and mammals.

Teleost - a suborder of rayed fin gnathostamatous chordates containing the true bony fishes.

Unequal Crossover - a crossover event involving different loci of hemologous chromosomes or different segments or homologous genes.

Urochordates - subphylum of chordates containing the sea-squirts and their allies.

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