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THE COMPARATIVE AMINO ACID COMPOSITION OF FIVE HEMOGLOBINS FROM THE GENUS MICROTUS

A

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THE COMPARATIVE AMINO ACID COMPOSITION OF

FIVE HEMOGLOBINS FROM THE GENUS MICROTUS

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ABSTRACT

The amino acid composition was determined for the tryptic peptides from each of five hemoglobins from the genus Microtus and from human hemoglobin as a reference protein.

Twenty-five peptides identified from the alpha and beta chains of the single M. pennsylvanicus hemoglobin differed in 39 amino acids from the corresponding peptides from human hemoglobin; there were at least 23 amino acid differences from the corresponding peptides from white mouse strain C57BL hemoglobin. At least one amino acid difference was found in each of five peptides alpha-T2, alpha-T3, alpha-T4, beta-T5(6) and beta-T10 between the hemoglobins of M. pennsylvanicus and the major components of the multiple hemoglobins of M. abbreviatus, M. miurus and M. oeconomus.

The designation beta-T5(6) describes a unique peptide which has not been observed in any other hemoglobin. Two amino acids appear to have been deleted from the beta-T5 peptide of Microtus hemoglobin. Yet the net length of the tryptic peptide is unchanged because the nearby replacement of a lysine by a histidine adds the two amino acids of the otherwise dipeptide beta-T6 to tryptic peptide beta-T5.

In general, the observed amino acid substitutions involved neutral amino acids or replacements of charged amino acids which were not detectable in the peptide maps without amino acid analysis.

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Dio mi guidò.

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INTRODUCTION

"One gene - one enzyme" was the first clear statement of the fundamental relationship between proteins and the material of the genes which was supposed to govern the variations in the structure of those proteins, the enzymes (Beadle and Tatum, 1941; Horowitz and Leupold, 1951, reprinted in Peters, 1959). The statement has been amply verified and has, until recently, only been modified to read "one gene - one polypeptide" to accord with the subsequent understanding that some proteins are constituted of multiple, associated protein "subunits" which are polypeptide chains. The statement is presently in the process of being modified again with the discovery, which will be discussed presently, that the gene "message" may in some cases be subject to variable translation.

The gene is polynucleic acid material of which the smallest representative unit is the nucleotide. The genotype of a living organism is the total number of genes of that organism; in higher plants and animals the gene material is found in cell nuclei as the structures known as chromosomes. The genotype of a particular organism determines by way of protein intermediates its phenotype, the collection of chemical, physiological, and structural characteristics in which the organism resembles, or is distinguished from, other organisms. Over a period of many years taxonomists have developed systematic classifications for living forms in terms of classes, orders, species, etc. The

classifications generally have been made in terms of a large number of phenotypic characteristics. A chief result of comparative taxonomy has been improved understanding of probable evolutionary relationships among organisms and of adaptive relationships between organisms and their environments.

Particularly within the past fifteen years the possibility has developed to add to the phenotype the chemical reactions of organisms and their structures at the molecular level. In view of the demonstrated role of polynucleotides in governing the heredity of an organism, knowledge of the exact compositions and structures of polynucleotides from one organism to another should in time add to other taxonomic data. At the moment, techniques are still being devised to handle these enormously complicated polynucleotide molecules which are also relatively scarce in biological specimens.

Especial progress has been made recently in numerous laboratories, however, in the analysis of proteins which are primary products of the nucleic acid polymers. Proteins are centrally involved as catalysts, the enzymes, in all known biological processes including the biochemical syntheses of carbohydrates, lipids, proteins and nucleic acids themselves. Other proteins serve in various structural and transport capacities.

Pauling, et al. (1949) reported the discovery of the first abnormal human hemoglobin protein, the so-called sickle-cell hemoglobin or hemoglobin S, which is associated with the disease sickle-cell anemia. Electrophoretic studies showed that this protein differs from that of

normal adult human hemoglobin, Hb A, by two net positive charges per molecule. Quantitative analyses of the amino acid content of the two proteins performed at about the same time showed no significant differences. This suggested that the two proteins were, on the whole, very much alike. The discovery of hemoglobin S provided additional impetus to study the structure of the protein portion of hemoglobin.

When Ingram applied his new technique of peptide mapping to tryptic hydrolysates of hemoglobins A and S, he found only one difference among the 26 peptides which were produced from each protein. The difference was shown to be the result of the replacement of one glutamic acid by one valine in 287 amino acids of hemoglobin S (Ingram, 1958). A gross phenotypic abnormality had been traced precisely to its molecular origin.

In 1949, Sanger reported the results of the determination of the N-terminal amino acids of the two different polypeptide chains of bovine insulin by means of the reagent fluorodinitrobenzene which he had developed. This was a preliminary step in the remarkable achievement of Sanger and his colleagues which culminated in 1956, with the first elucidation of the complete covalent structure of a protein (Sanger, 1956). The particular importance of this work lies in its opening up the whole subject of chemical structural analysis of complex protein molecules, a task which seemed impossible a few years ago.

Spackman et al. (1958) introduced the first automatic quantitative amino acid analysis, a decisive contribution to the techniques which have been used in numerous complete structural analyses of proteins

after insulin. Many of these structural analyses have concerned proteins such as the human hemoglobins A and S mentioned above. Braunitzer et al. (1964) listed the substitutions of amino acids for fifteen different abnormal hemoglobins which like Hb S differ from Hb A by only one amino acid substitution in 287 positions. But the number of aberrant human hemoglobins now characterized (1968) exceeds 75.

Although a complete amino acid analysis of hemoglobin A was reported in 1950, it was not until 1961-1963 that the complete primary structure was reported (Braunitzer et al., 1961; Hill and Konigsberg, 1962; Schroeder et al., 1963). Hemoglobin A consists of four polypeptide chains, each of which is associated with a heme prosthetic group. Two of the chains, which are identical with 141 amino acids each, have been designated alpha (α) chains; the other two chains of 146 amino acids are also identical with each other and have been designated beta (β) chains. The hemoglobin molecule can be written chemically as $\alpha_2\beta_2$.

SURVEY OF HEMOGLOBIN COMPOSITION AND STRUCTURE.

Among the vertebrates, tetrameric hemoglobins of the formula $\alpha_2\beta_2$ have generally been found with the following exceptions:

1) hemoglobins of lamprey are monomeric and have molecular weights of approximately 17,500.

2) hemoglobins of mammalian muscle, the myoglobins, are monomeric and have molecular weights of approximately 17,500.

3) certain abnormal human hemoglobins are homotetrameric with formulas β_4 and γ_4.

4) many hemoglobins of amphibians and reptiles may be described as tetrameric, but they possess the property of polymerizing to octamers and higher polymers. This property has been observed in one case in man and in certain strains of mice with multiple hemoglobins (Bonaventura and Riggs, 1967).

5) one order of the fishes apparently has no respiratory pigment.

There is a monumental literature concerned with the qualitative characterization of hemoglobins of fishes, amphibians, reptiles, birds and mammals, by electrophoresis, molecular weight determination, hybridization, rates of denaturation, oxygen equilibrium studies, and by other means. Added significance may accrue to these properties as knowledge of the structures for the various hemoglobins becomes available.

Examples of structural analyses for fish and cyclostome hemoglobins come from the work of Braunitzer and co-workers. Exact amino acid analyses have been obtained for peptides of lamprey globin with 156 amino acids (Rudloff et al., 1966), and the peptide analyses and exact amino acid sequence have also been determined for carp hemoglobin, the α chain of 142 amino acids (Hilse and Braunitzer, 1968).

The knowledge of the structure of normal, adult human hemoglobin represents a substantial basis for studies of the primary structure of other hemoglobins. All examinations to date of hemoglobins from mammalian species have shown the same basic arrangement, $\alpha_2\beta_2$. That extensive segments of the alpha and beta polypeptide chains in different species are exactly the same as their homologous counterparts suggests

a common origin for all alpha chains and for all beta chains. For example, mouse and human have different amino acids in only 17 of 141 positions in the alpha chain. A common origin for beta chains and for the gamma chains of fetal human hemoglobin is suggested by the presence of only 39 points of difference in the sequence of 146 amino acids. A much older evolutionary kinship of alpha and beta chains is reflected in the retention in the human chains of 60 identical homologous pairs of amino acids. Four of the homologous pairs are evident in the first eleven residues of the two chains:

α val-LEU-ser-PRO-ala-asp-LYS-thr-asn-VAL-lys-

β val-his-LEU-thr-PRO-glu-glu-LYS-ser-ala-VAL-

Primate Hemoglobins.

There is extensive, accurate knowledge of the structures of human hemoglobin variants. These structures are generally closely related to the structure of normal adult human hemoglobin which was elucidated in the fundamental studies cited above. These will not be cataloged in detail, and the reader is referred to the compilation prepared by Dayhoff and Eck (1968).

The hemoglobins from approximately ten other primates which have been investigated to date have furnished the most extensive data for comparison of hemoglobin chains from a single group of species according to Nolan and Margoliash (1968). They note that chimpanzee hemoglobin is apparently identical in composition with human hemoglobin. Hemoglobin from the gorilla differs from human hemoglobin by one amino acid in each of the alpha and beta chains. Buettner-Janusch and Hill (1965) have

reported the amino acid compositions for large segments of the alpha and beta chains from hemoglobins of gibbon, potto, bush baby, sifaka, brown lemur, ruffed lemur, ring-tailed lemur and baboon. A range of 0-40 differences has been observed among the amino acids from the alpha chains and among the amino acids from the beta chains of the primates.

Hemoglobins of Rodents.

Extensive recent studies of hemoglobin from strains of white mice and from rabbit will be discussed below in some detail. There are very few studies of other rodent hemoglobins reported which have been carried beyond the electrophoresis stage, e. g. electrophoretic studies of fetal hemoglobins of mice have recently been reported.

Foreman (1964) reported qualitative evaluations of peptide maps for six species of Peromyscus in terms of the positions and intensities of 55 ninhydrin-staining spots. He also presented a composite drawing of maps of Clethrionomys gapperi and Microtus pennsylvanicus which indicated differences involving at least 13 peptides.

The N-terminal amino acids of the alpha and beta chains of adult rat hemoglobin were reported to be the same as those in human (Maeda, 1966).

The number of spots was reported in peptide maps of chymotryptic digests of trypsin-resistant "cores" from rat hemoglobins Hb_3, Hb_4 and Hb_5 (Marinkovic and Kanazir, 1966).

Hemoglobins of Other Mammalian Orders.

Among other mammalian orders there has been reported recently a survey and "structural evaluations" of hemoglobins from buffalo, bovine,

sheep, and goat on the basis of peptide map patterns (Ramakrishnan and Barnabas, 1967; Baloni et al., 1968).

Another comparative "structural" study which has been perpetrated recently by means of peptide mapping augmented by specific staining reactions is that (Kitchen et al., 1968) in which two of seven polymorphic hemoglobins from white-tailed deer have been contrasted with three polymorphic hemoglobins from sheep on the basis of peptide patterns alone (supported ? by data on peptide sequences of sheep hemoglobins published by other workers). This represents a definite throwback to the kind of study done many years ago with mice strains (Hutton et al., 1962) which did not make a substantial or reliable contribution to the investigation of mouse hemoglobin.

The remainder of this survey is based largely on the compilation of Dayhoff and Eck (1968) and concerns those other mammals for which analyses of the amino acid compositions of hemoglobin peptides have been completed.

Horse and Donkey. Definitive studies of the alpha chains of hemoglobins from horse (fast and slow components) and from donkey have been reported (Braunitzer and Matsuda, 1963; Kilmartin and Clegg, 1967). There is a single difference in the alpha chains of horse hemoglobin at position 60 where glutamine occurs in the fast component, lysine in the slow component. This is according to Kilmartin and Clegg (1967), although Braunitzer et al. (1964) had recorded the same alpha chains and different beta chains. In horse alpha chains (both fast and slow hemoglobins) histidine occupies position 20, phenylalanine and/or

tyrosine position 24 (ambiguity?). The alpha chain of donkey hemoglobin is characterized by asparagine at position 20, phenylalanine at position 24, and lysine at position 60.

A complete sequence for a single beta chain of horse hemoglobin is given in the "Atlas" (Dayhoff and Eck, 1968) on the basis of peptide compositions reported by Smith (1964) and Smith (1967). Tryptic peptides were aligned by homology with beta chain of adult human hemoglobin.

Pig. The entire sequence of amino acids has been determined for alpha chain of pig hemoglobin (Yamaguchi et al., 1965; Braunitzer and Kohler, 1966). Eight differences between the compositions determined in the two laboratories may be attributed to structural differences between two strains of domestic pig. Braunitzer and Kohler (1966) also reported the compositions of tryptic peptides of pig beta chain, and a sequence has been inferred from them.

Cow. The complete sequences are probably identical for bovine fetal and adult alpha chains of hemoglobin from Holstein cow as reported by Schroeder et al. (1967). There are 28 differences between the amino acid compositions of the alpha tryptic peptides for adult bovine hemoglobin as determined by Schroeder et al. (1967) and by earlier workers (Satake and Sasakawa, 1963). Schroeder et al. (1967) have also reported two different sequences for beta chains of bovine hemoglobins A and B, although the sequence of the former is not complete. The beta chains of bovine B hemoglobin and bovine fetal hemoglobin, for which an exact amino acid sequence has been reported (Babin et al., 1966), both consist

of 145 amino acids due to an apparent deletion at the position penultimate to the N-terminal of the chain.

Sheep and Goat. The last species for which definitive information is available at this time from amino acid compositions of their polymorphic hemoglobins are sheep and goat. Beale (1966) reported that two allelic adult sheep hemoglobins A and B have similar alpha chains but differ in their beta chains. The alpha chains of hemoglobin A from two breeds (Soay and Clan) of sheep have the same tryptic peptide amino acid compositions and probably the same sequence of 141 amino acids (Beale, 1967).

Beale (1966) reported differences in the beta chains of ovine hemoglobins A and B as follows: peptide 14, arginine in place of lysine, respectively; peptide 5, alanine and serine in place of proline and asparagine, respectively. Independent analyses by Huisman and coworkers (Wilson et al., 1966) and by Naughton and coworkers (Boyer et al., 1967) for beta chains of sheep hemoglobins A and B, each from a different breed of sheep (Rambouillet and Dorset), are in general agreement (Dayhoff and Eck, 1968).

The beta chains of the hemoglobin of adult goat, A-beta, and of adult sheep, A-beta (but not B-beta), are replaced by another type of beta chain, C-beta, during severe anemia from blood loss. The same general composition and sequence of the beta chain of this hemoglobin C has been determined for two breeds of sheep by Boyer et al. (1967) and by Wilson et al. (1966). There are at least 7 differences between the amino acid sequences of sheep A- and B-beta chains, at least 16

differences between the sequences of sheep A- and C-beta chains and at least 21 differences between the sequences of sheep B- and C-beta chains.

Hemoglobins C from both sheep and goat lack 4 residues in the N-terminal portion of the beta chain and consequently have only 141 amino acids. Sheep hemoglobin C-beta chain is different from goat hemoglobin C-beta chain with threonine at position 45 instead of serine (Huisman et al., 1967). These authors also give the analyses for peptides of the beta chain for the single normal adult goat hemoglobin. Analyses of peptides for the alpha chain from goat hemoglobin have apparently not been reported.

Heterogeneity of Hemoglobins.

Mammalian hemoglobins which have been isolated by fractionation procedures and shown to be homogeneous by a variety of physical-chemical techniques appear generally to have precisely unique chemical structures. That is, they conform to the law of multiple proportions in terms of the fixed integral numbers of their constituent amino acid monomers.

Examination of hemoglobins from different animals (either within the same species or between two and more species) according to physical-chemical criteria reveals heterogeneity which is thought to have a genetic origin. Table 1 presents a classification of genetic heterogeneity for mammalian hemoglobins in terms of the physical criterion, electrophoresis, and the criterion of chemical composition, as well as in terms of biosynthetic capability and its origin in the chromosome. The categories of heterogeneity suggested at the electrophoretic level

Table 1. Heterogeneity of Mammalian Hemoglobins.
(Referred to Experimental and to Theoretical Levels of Study)

Electrophoretic Patterns	Chemical Composition	Synthesis	Chromosome
I. Single Components	A. One kind of single tetramer	1. Homozygotes	Single Genes e.g. for β_4^A
Different single components with same mobility.		2. Variable translation during biosynthesis.	Multiple (non-allelic) genes e.g. for $\alpha_2\beta_2$
Different single components with different mobilities	B. Two (or more) kinds of single tetramers:		
	(1) with same charge and: -common α or β chains. -neither α nor β chains in common.		
	(2) with different charges and: -common α or β chains. -neither α nor β chains in common.		

Table 1 (Cont.). Heterogeneity of Mammalian Hemoglobins.
(Referred to Experimental and to Theoretical Levels of Study)

Electrophoretic Patterns	Chemical Composition	Synthesis	Chromosome
II. Multiple Components	A. Two (or more) kinds of tetramers with different charges and:	1. Heterozygotes	Multiple (allelic) genes e.g. for $\alpha_2^A\beta_2^A$ and $\alpha_2^A\beta_2^S$, etc.[b]
Different components with different mobilities.		2. Maturation Fetus Adult	Multiple (non-allelic) genes e.g. for $\alpha_2^A\gamma_2^F$
-components are resolved.	-common α or β chains. -neither α nor β chains in common.		for $\alpha_2^A\beta_2^A$, $\alpha_2^A\delta_2^A$ [c]
	B. Double or higher polymeric tetramers.		
-components are overlapping[a] or "diffuse."			

[a]Overlapping may result from closely similar structures or from interactions e.g. n Monomer $\rightleftarrows$ $(\text{Polymer})_n$.

[b]Molar ratio of gene products is approximately 1.

[c]Molar ratio of gene products may be highly variable depending upon the age and the rate of evolution of gene duplication EXCEPT in cases such as $\alpha_2\beta_2$ where synthesis of equal amounts of polypeptides appears to be imposed by mechanisms unrelated to structural genes.

are not all mutually exclusive, e.g. it may be desired to compare a single individual component from a multiple component class with one component from a single component class. Furthermore, two (or more) different hemoglobins may coincide to form a "single" electrophoresis pattern or a "multiple" pattern which may be imperfectly resolved (overlapping, "diffuse") or well-resolved.

HEMOGLOBINS OF THE LABORATORY MOUSE.

Because of significant recent developments in biochemical genetics derived from intensive studies of the hemoglobins of laboratory mouse and rabbit, and because of a measure of relatedness anticipated between the laboratory mouse and the Microtus discussed in this thesis, the hemoglobins of the two laboratory species are set apart from the other hemoglobins for special consideration here.

Single and Multiple Mouse Hemoglobins.

Certain inbred laboratory white mice, e.g. strains[1] C57BL (and related strains), SEC and NB, appear to have one component hemoglobin each as judged by electrophoresis. This homogeneous aspect of the electrophoretic pattern was referred to as "single" by Ranney and Gluecksohn-Waelsh (1955).

Other strains of inbred laboratory white mice, e.g. BALB/cJ, AKR,

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[1] "Laboratory Animals. Part II. Animals for Research." A report of the Institute of Laboratory Animal Resources, 6th ed., Publication 1413, National Academy of Sciences - National Research Council, Washington, D.C., 1966.

DBA/2J, have two or more component hemoglobins. To this author's knowledge, none of the electrophoretic patterns from these strains of mice show clearly resolved, discrete multiple components. Rather, two or more hemoglobin components, though rather well-separated from each other, are joined by intervals which are blurred by the continuous presence of hemoglobin. This heterogeneous aspect of the electrophoretic pattern was referred to as "diffuse" by Ranney and Gluecksohn-Waelsh (1955).

Riggs (1965) and Bonaventura and Riggs (1967) have demonstrated that one of three hemoglobin components from white mouse strain BALB/cJ is polymeric. If the polymeric component is removed or prevented from forming, two blurred components still remain in the electrophoretic pattern.[2]

Popp (1965a) discussed hemoglobin variants in inbred strains of laboratory mice on the basis of his work of five years and more. He noted that the major and minor components of the multiple (diffuse) hemoglobins have different ionic charges; the electrophoretic mobilities of the multiple hemoglobins accordingly distinguish them not only from the single hemoglobins but also from each other.

Popp detected differences in various strains of laboratory mice which have single hemoglobins by their different solubilities (and

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[2]Hemoglobin from a strain of white mouse from the animal colony of the Institute of Arctic Biology was examined and found to have multiple (probably two), "diffuse" components but no polymeric component.

crystal size) in phosphate buffers. The single hemoglobins of various strains appear to have the same effective ionic charges and are not distinguished by their electrophoretic mobilities.

Mice with a single hemoglobin, strain C57BL, were mated to mice with multiple hemoglobins, BALB/cJ. The progeny (F_1) from these matings were test crossed, and the numbers of mice among the test progeny (F_2) with hemoglobins which had the electrophoretic properties (single or multiple hemoglobins) of parental or F_1 type suggested that these traits are controlled by allelic genes (Popp, 1962b). The locus was identified by the symbol Hb and later Hb b. This hemoglobin locus governs the variant electrophoretic patterns of mouse hemoglobins. Tests showed the linkage of the Hb locus with albinism.

Mice with a single hemoglobin, strain C57BL, were mated to SEC strain mice which also have a single hemoglobin (Popp, 1962a). The progeny (F_1) from these matings were test crossed, and the numbers of mice among the test progeny (F_2) with hemoglobin which had the solubility properties of parental or F_1 type suggested that the solubilities of these hemoglobins are controlled by allelic genes. It was also observed, in a mouse strain with single hemoglobin, that the genes for chinchilla (an allele of albinism) and for solubility segregated independently. Thus a solubility locus was defined by the symbol Sol and, subsequently, by the symbol Hb a. (It was established further that genes which control the distinguishable electrophoretic patterns, i.e. multiple or diffuse hemoglobins, do not control solubility properties.)

The designations Hb a and Hb b were derived from evidence that the

structures of the alpha and beta chains of mouse hemoglobin are controlled by genes at these two loci (controlling respectively solubility and electrophoretic properties) which segregate independently. This is to say that solubility, the criterion for Popp's detecting differences between single hemoglobins from mice of different strains, is controlled by genes at the same locus as that which determines differences in structure of the alpha chain. Electrophoretic mobility, an obvious criterion for distinguishing the multiple hemoglobins, is controlled by genes at the same locus as that which determines differences in structure of the beta chain.

Comparative Composition of the Single and Multiple Mouse Hemoglobins.

Subsequent peptide mapping of mouse hemoglobins (especially of the separate alpha and beta chains), supported by amino acid analysis of the peptides, showed conclusively that:

1. The major component of the multiple hemoglobins of strain BALB/cJ had a different map pattern of beta chain tryptic peptides from that of the single hemoglobin of strain C57BL (Popp, 1962b).

2. The single hemoglobins of strains SEC and C57BL had the same beta chain tryptic peptides by mapping and by composition (Rifkin et al., 1966c). The same beta chains were also expected to be present in the strains C57BL/6 and NB each of which have single hemoglobins.

3.a. Different strains of mice with single hemoglobins, NB and C57BL, had different map patterns of alpha chain tryptic peptides which reflected differences in solubility (Popp, 1962c).

3.b. The map patterns of alpha chain tryptic peptides were also

different between the single hemoglobin of strain NB and the major component of the multiple hemoglobins of strain BALB/cJ, hemoglobins which had different solubilities (Popp, 1962c).

4.a. The strains SEC and C57BL which have single hemoglobins of different solubilities showed the same map pattern of alpha chain tryptic peptides (Hutton et al., 1962), but analyses of the isolated tryptic peptides showed one difference in amino acid composition (Rifkin et al., 1966a).

4.b. The major component of the hemoglobins from strain BALB/cJ and the single hemoglobin from strain C57BL, hemoglobins with different solubility properties, differed in the composition of one alpha chain tryptic peptide although there was no apparent difference in the overall map pattern of alpha chain tryptic peptides (Popp, 1962b). The single hemoglobin of strain SEC and the major hemoglobin component of strain BALB/c appeared to share common alpha chains.

The genetic and biochemical situation can be summarized in terms of the older nomenclature used by Popp (1962b) to describe the different strains of inbred laboratory mouse, that is: C57BL/Cum ($Hb^1/Hb^1;Sol^1/Sol^1$), BALB/cJ ($Hb^2/Hb^2;Sol^2/Sol^2$), NB ($Hb^1/Hb^1;Sol^3/Sol^3$), SEC ($Hb^1/Hb^1;Sol^2/Sol^2$).

There appear to have been no reports thus far in which minor components of the multiple mouse hemoglobins have been separated and examined in detail with the following two exceptions.

Hutton et al. (1962) prepared the peptide maps for the separate alpha and beta chains of the single hemoblobins from the mouse strains

Sec and C57BL. They compared the resultant peptide locations and peptide staining properties with those from maps of the separate alpha and beta chains from the separated major and minor hemoglobins from each of the mouse strains AKR and FL. Some of their conclusions have subsequently been shown to be incorrect. But their inference of common beta chains in the minor component hemoglobins (beta-AKR-1 and beta-FL-1) and common beta chains in the major component hemoglobins (beta-AKR-2 and beta-FL-2) of the two multiple (diffuse) hemoglobins has apparently not been examined further.

Bonaventura and Riggs (1967) have recently reported a partial characterization of the beta chain of a minor polymeric hemoglobin component, one of the three components of the multiple hemoglobins from mouse strain BALB/cJ. (Strains DBA/2J and C3H/HeJ were also observed to possess polymeric hemoglobins in a diffuse pattern.) There is one amino acid in the beta-T2 peptide which gives rise to the formation of the polymeric component in the multiple hemoglobin electrophoretic pattern if the amino acid is not chemically blocked by iodoacetamide during lysis of red cells. The presence of this amino acid distinguishes this beta chain from that characterized for the single hemoglobins from strains SEC and C57BL. Bonaventura and Riggs (1967) "have not yet isolated the (two other) different components of BALB/cJ mouse hemoglobin." However, they have evidence for heterogeneity in four of the beta peptides of the polymeric component, i.e. beta-T2b, beta-T7, beta-T11, and beta-T14.

The separation and amino acid composition of the tryptic peptides

from the beta chain of hemoglobin of C57BL/6 mice were reported by Rifkin et al. (1966b). A total of 146 amino acids is present in the beta chain like those from human and from a number of other mammals which have been tabulated (Dayhoff and Eck, 1968). The minimal number of amino acid differences between human and C57BL/6 beta chains is 22. The beta peptides of the mouse hemoglobin were designated beta-T1, beta-T2, etc. on the basis of their compositional homology with the corresponding human beta peptides numbered from the N-terminal of the polypeptide chain. Dayhoff and Eck (1968) assigned the amino acids to a particular sequence by homology with human beta chain. A probable error in this sequence for peptide beta-T2 has been pointed out by Bonaventura and Riggs (1967) who determined a sequence for the N-terminal amino acids of the corresponding peptide from the polymeric BALB/cJ component by a chemical procedure.

Several characteristics were considered by Rifkin et al. (1966b) to be of immediate significance with regard to the composition of C57BL/6 hemoglobin beta chain:

1. There is a single cysteinyl residue, hence a single sulfhydryl function, in this beta chain as compared with two in the human hemoglobin beta chain. The introduction of a second cysteinyl residue at a new location in a certain BALB/cJ hemoglobin chain, as found by Bonaventura and Riggs (1967) leads to a new chemical property of cross-linkage between those BALB/cJ beta chains.

2. There are only three prolines in C57BL/6 hemoglobin beta chain as compared with seven in human hemoglobin beta chain. Because of the

angular linkage of this amino acid the conformation of the beta chain could readily be affected by its substitution unless there are certain groups of other amino acids which can compensate in the absence of proline.

3. The C-terminal dipeptide, which is identical in mouse and human hemoglobin beta chains, has been shown to be essential in maintaining certain physiological properties of the hemoglobin molecule (Antonini et al., 1961).

Rifkin et al. (1966c) have determined that the beta chains of hemoglobins from strains SEC and C57BL/6 are identical, as mentioned earlier; it is considered likely that the beta chain of the single hemoglobin from NB strain is also identical with them.

The separation and amino acid composition of the tryptic peptides from the alpha chain of hemoglobin from C57BL mice was reported by Popp (1965b). There are 141 amino acids in this alpha chain, like those from human hemoglobin and from hemoglobins of several other mammals that have been tabulated (Dayhoff and Eck, 1968). A minimum of 16 amino acid differences between C57BL and human hemoglobin alpha chains was suggested by the compositional data. Subsequently Popp (1967) determined a complete sequence for the alpha chain of the single hemoglobin of strain C57BL by the use of proteolysis, chemical (Edman) degradation following the separation of peptides, and amino acid analysis. Assignment of 23 positions was based on homology with human hemoglobin. Differences were found at 17 positions between the alpha chains of human and C57BL mouse hemoglobins.

AMBIGUITIES IN HEMOGLOBIN SYNTHESIS.

An entirely new phase in the investigation of the amino acid sequences of mammalian hemoglobins was opened almost simultaneously with the studies of the alpha chain of rabbit hemoglobin by von Ehrenstein (1966) and coworkers, with the studies of the alpha chain of SEC mouse hemoglobin by Rifkin et al. (1966a), and with the studies of the alpha chains of NB and BALB/c mouse hemoglobin by Popp (1967). In each study it was found by careful peptide separation followed by sequential Edman degradation and/or amino acid analysis that there are one or more positions in these alpha chains which are unique. At these positions there are fractional proportions of two different amino acids which reflect the simultaneous presence in the hemoglobin of the same animal of two alpha chains which differ at that one (or more than one) specific position.

The alpha chain of NB hemoglobin was found to differ from alpha chain of C57BL hemoglobin by complete substitutions at three positions: alpha-T4^{25}, glycine (G) → valine (V); alpha-T9^{62}, valine (V) → isoleucine (I); and alpha-T9^{68}, asparagine (N) → serine (S). The alpha chains of SEC and BALB/c hemoglobins differ from that of C57BL only at the 68 position in alpha-T9, but the substitution of asparagine (N) involves approximately equal proportions of threonine (T) and serine (S).

Of a total of 141 amino acid positions in the alpha chain of rabbit hemoglobin at least six are occupied by more than one amino acid. Four positions are occupied by amino acids in approximately 50:50 molar proportions, i.e., position 29, valine (V) and leucine (L); position 48,

leucine (L) and phenylalanine (F); position 49, serine (S) and threonine (T); position 80, serine (S) and leucine (L). The ratios of amino acids in the other two positions are clearly different from 1 which suggests the presence of more than two alpha chains, i.e., position 70, V 0.8 and T 0.2; position 76, L 0.8 and V 0.2 (from one individual rabbit).

These replacements of neutrally charged amino acids account for the similar electrophoretic properties of the variant tryptic peptides. (However, separation of two alpha-T4 peptides from rabbit hemoglobin was realized on paper, the peptides containing positions 29 V or L.)

The origins of these ambiguities are not known, but several arguments have been advanced to account for them:

1. Rifkin et al. (1966c) rule out contamination of mouse peptide alpha-T9 since analysis of peptide alpha-T(8-9), which they were also able to isolate, independent of alpha-T9, furnished the identical results from Edman degradation.

2. Double substitutions at the same site, reported for human heart cytochrome c (Matsubara and Smith, 1962) and for bovine carboxypeptidase (Bargetzi et al., 1964) have been explained to arise from a heterozygous population. This explanation is considered very unlikely in the case of strains of mice which have been inbred for nearly 100 generations.

von Ehrenstein (1966) considers it unlikely that the valine-leucine duplicity in alpha-T4 peptide of rabbit hemoglobin is due to a simple heterozygosity for two alpha chain genes, because from four matings of rabbits all 20 offspring had the duplication of the peptide alpha-T4.

3. Unequal crossing-over and subsequent translocation have been

suggested by Ingram (1961) as a basis for the presence in man of the chemically similar polypeptide chains beta and delta which differ by six amino acids. Linkage of the beta and delta genes in man is well established; at present there is no evidence for alpha chain duplication in man. According to Popp (1967) the presence in strain NB hemoglobin alpha chain of amino acid replacements which are complete argues against the origin by gene duplication of the two amino acids at position 68 in BALB/c hemoglobin alpha chain.

4. Somatic mutations of a single alpha chain gene, i.e. the existence of different multiple templates for a particular protein, are considered by von Ehrenstein (1966) to be unlikely to explain the observed alpha chain multiplicities in rabbit hemoglobin. Although duplicities in four positions of rabbit hemoglobin alpha chain can be explained by single nucleotide changes (in the triplet codon), the valine-threonine shift at position 70 can definitely not be explained by a single nucleotide change according to the present genetic code. In addition the leucine-serine shift observed at the position 80 appears to exclude a single nucleotide change because of the nature of the leucine transfer-RNA which was shown to serve that position.

5. The favored explanation seems to be that these amino acid sequence variations can be caused by (in vivo) variable translation of a unique template. This could be due to the messenger-ribosome complex, to the mutational alteration of the aminoacyl-tRNA, or perhaps to mutation of an aminoacyl-RNA synthetase.

Rifkin et al. (1966a) are attempting to distinguish between the

influence of the ribosome complex and of the aminoacyl-tRNA upon the duplicity at position 68 in the alpha-T9 peptide of hemoglobin of mouse strain SEC. They are using cell-free reticulocyte systems to synthesize hemoglobin in vitro; ribosomes and aminoacyl-tRNA's (labeled with H^3-serine and C^{14}-threonine) are being isolated from the two strains of mice SEC and C57BL for use in the amino acid incorporation studies.

The earlier work of von Ehrenstein and coworkers (Weisblum et al., 1965), which first directed their attention to the possibility of variable translation, was concerned with leucine transfer experiments in the rabbit reticulocyte ribosomal system using different transfer RNA fractions separated from E. coli. They found that a minor fraction, IIB, of sRNA directed C^{14}-labeled leucine to only one position (48) in the alpha chain of rabbit hemoglobin synthesized in vitro. The other positions were served by a major fraction, I, of sRNA and were detected in terms of the incorporated H^3-labeled leucine furnished by this RNA. These observations are consistent with the expectation that the two different sRNA's should respond to different codons.

Close analysis of peptide alpha-T6 of rabbit hemoglobin revealed that position 48 was not only exceptional in its response to the minor leucine transfer RNA, but also that the alpha chains from hemoglobin of a single rabbit showed approximately 50% leucine (L) and 50% phenylalanine (F) at position 48, as mentioned above, and similar proportions of serine (S) and threonine (T) in the adjacent position 49. Ratios from 4/10 to 9/10 were subsequently observed for the residue pairs L-S/F-T at positions 48-49 of alpha chains of hemoglobins from other

individual New Zealand white rabbits.

If the rabbit ribosome complex represents a unique template containing special codons which can be served more or less randomly by either of two (special) tRNA fractions then the occurrence of the sequence permutations L-T and F-S would also be expected. An indication of the F-S combination was obtained in the amount of 10% of that of the L-S combination. Thus it seems that there may be ambiguous codons served by either of two adaptors.

SIGNIFICANCE FOR THE PRESENT STUDY OF THE RESULTS OF INVESTIGATIONS OF THE HEMOGLOBINS OF HUMAN AND OF THE LABORATORY MOUSE AND RABBIT.

It is fortunate for the present study that several groups of workers have devoted themselves to such intensive investigation of the hemoglobins of laboratory mouse and rabbit. A close relationship between the hemoglobins of Microtus and white mice (both members of the same rodent suborder, the Myomorpha) may be anticipated. The relationship of hemoglobins of Microtus to human hemoglobin (or to rabbit hemoglobin) may be nearly as close; it was noted above that there are a rather small number of amino acid differences between hemoglobins of mouse and human.

The complete compositions and amino acid sequences of all of the peptides of alpha and beta chains of rabbit hemoglobin have been reported (Braunitzer et al., 1966; von Ehrenstein, 1966). The beta chain of rabbit differs from that of human in 14 positions, one of which involves the replacement of a cysteine (human) by isoleucine in rabbit.

The alpha chain of rabbit hemoglobin differs from that of human hemoglobin in at least 24 positions.

Of particular significance to the present thesis are the following points from the works concerning the nature of the single and multiple mouse hemoglobins, translational variation in hemoglobins, and amino acid compositions of the peptides of human, of mouse and of rabbit hemoglobins:

1. The compositions of all the tryptic peptides are established for the hemoglobins of several strains of laboratory mice and for domestic rabbit as well as for human. Thus a fruitful comparison with peptides of Microtus hemoglobins is possible. Both mouse and rabbit hemoglobin beta chains contain isoleucine rather than the cysteine which occurs at position 112 of the corresponding human hemoglobin beta chain. This could be of importance in assessing the results of tryptic hydrolyses of Microtus hemoglobins.

2. Non-integral ratios of amino acids have been found at certain positions in the alpha chains of both mouse and rabbit hemoglobins. This alerts one to the possibility of ambiguity in the alpha chains of Microtus hemoglobins; despite the substitution of neutral amino acid residues for one another, dual peptides may be found to be separated on peptide maps.

There has been no discussion of the possible significance of ambiguity confined to the alpha chains of mouse and of rabbit hemoglobins, but Bonaventura and Riggs (1967) allude to the possibility of translational errors also in the beta chain of BALB/cJ mouse hemoglobin.

3. Single and multiple hemoglobins of inbred laboratory mice are distinguished from one another principally by their electrophoretic characteristics which have been demonstrated to arise from differences in their beta chains. It will be of interest to determine if single and multiple hemoglobin character in species of Microtus might also be related to differences in the beta chain peptides.

4. Also to be expected from Popp's studies is that the multiple components of diffuse mouse hemoglobins (with different electrophoretic mobilities) should be distinguished especially by differences in their beta chains. This prediction from Popp's work has not been tested for the non-polymeric components of the hemoglobin of laboratory mouse. Bonaventura and Riggs (1967) have found beta chain differences(s) between the slow polymeric component of the multiple BALB/cJ hemoglobins and the single hemoglobins of other strains. It will be of interest to determine the nature and origin of the amino acid difference(s) which give rise to multiple components of Microtus hemoglobin.

5. When amino acid differences have been observed between single hemoglobins from mice of different strains, the differences have been detected in the alpha chains (and reflect differences of solubility). In the present study only one species, Microtus pennsylvanicus, has a single hemoglobin. However, its electrophoretic mobility (at pH 8.6) is indistinguishable from that of the fast (major) components of the two hemoglobins from other species of Microtus, and differences in alpha chains between these single and multiple hemoglobins from Microtus species may be postulated. As suggested above, differences in amino

acid compositions of beta chains are also expected between the single and multiple hemoglobins.

MATERIALS AND METHODS

MICROTUS.

Animals used in this work were obtained from the animal colony of the Institute of Arctic Biology. The colony has been established from wild stock during the past five years. The taxonomic designations for the several species are given in Table 2 according to Hall and Kelson (1959), and the geographic origins of the several species in Alaska are included. Rausch (1953) did not differentiate the central Alaskan subspecies M. oeconomus macfarlani from that subspecies of the Arctic Slope but designated all animals common to this extensive area as M. oeconomus endoecus.

Laboratory-bred animals are indicated in Table 2 by the Letter "I" in conjunction with the species code and the number assigned to each individual animal. Animal 5813AA-183 was "wild-caught", not laboratory-bred. Other members of the Microtinae (Dicrostonyx, Clethrionomys, and Lemmus) were not considered experimentally in the present investigation.

PREPARATION OF HEMOGLOBIN AND GLOBIN.

Hemoglobin was isolated and characterized from the blood of each of the single animals listed above, not from pooled samples. The individual Microtus (voles, "wild mice") were decapitated, and blood was collected in small plastic dishes with heparin and in heparinized capillary tubes. Volumes of red blood cells from 0.2 ml to 1.0 ml were

Table 2. Species and Geographic Origins of *Microtus* from Which Hemoglobin Was Obtained.

Species Code	Species	Geographic Area
5811AA-I-426	*Microtus pennsylvanicus tananaensis*	Fairbanks (Tanana Valley)
5812BA-I-281	*Microtus miurus muriei*	Umiat
5813AA-183	*Microtus oeconomus macfarlani*	Fairbanks
5818AA-I-25	*Microtus abbreviatus fisheri*	St. Mathew Island

obtained in this way depending upon the size of the mouse. In several instances small volumes of blood were obtained for analytical purposes by capillary puncture behind the eye without sacrificing the animal and with no apparent damage to the animal. The blood was diluted with 0.85% saline immediately after its collection, and the red blood cells were washed three times each in ten volumes of 0.85% saline. The washed cells were lysed with five volumes of water, and the well-mixed hemolysates were stored frozen. Prior to their use, hemolysates from fresh or frozen samples were centrifuged for 60 min at 2500 rpm at 0°C in the refrigerated high speed centrifuge Servall RC-2. Supernates were removed to 2 ml capped, plastic storage vessels and were refrigerated.

Gel Electrophoresis.

Analytical gel electrophoresis was at first performed with acrylamide gel by the method of disc electrophoresis of Ornstein and Davis (1962). Subsequently the vertical gel sheet apparatus of Raymond (1962) was used.[1] Use of the gel sheet permitted comparison of eight samples run simultaneously on the same sheet. The sheets were stained for 30 min in 0.1% amido black (10B, electrophoretic grade, Harleco) in 7% acetic acid, then were destained in portions of fresh 7% acetic acid. The sheets could then be stored in sealed envelopes of clear Mylar (K and E, 193103).

The following conditions were used for electrophoresis with the

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[1]A commercial model of the vertical gel sheet apparatus is available from E-C Apparatus Corp., 222 South 40th Street, University City, Philadelphia, Pa., 19104; Technical Bulletin 141.

Raymond apparatus. A stock solution for pH 8.6 buffer was prepared according to Boyer et al. (1963): 54.5 g TRIS (Tris-hydroxymethyl-aminomethane), 15.5 g boric acid, and 3.4 g Na_2EDTA (Ethylenediaminetetraacetic acid, disodium salt) were dissolved in water and made up to a total volume of 1 liter. For use as the electrolyte in electrophoresis, 1 volume of stock solution was diluted to 10 volumes (approximately 2 liters of electrolyte were required for the apparatus).

A stock solution of acrylamide was prepared according to Peterson (1963): 70 g acrylamide, 2.0 g BIS (N,N'-Methylenebisacrylamide), 1.4 ml TEMED (N,N,N',N'-Tetramethylethylenediamine), and 110 ml of the stock solution for pH 8.6 buffer were made up to a total volume of 1 liter. For preparation of the gel sheet, 300 mg of ammonium persulfate were dissolved in 200 ml of acrylamide stock solution with vigorous mixing and avoiding aeration. The solution was immediately poured into the assembled apparatus where the gel set within 5 minutes.

Hemoglobin samples for analytical electrophoresis were treated with sufficient sucrose to form a 20% solution whose density allowed the sample to be layered easily in the starting slots. Aliquots of 5-10 microliters of approximately 5% solutions of hemoglobin were used.

Preparative disc gel electrophoresis was performed using the technique of Jovin et al. (1964) and the commercial Canalco Prep-Disc Electrophoresis Apparatus.[2] The largest glass column PD2/320 was

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[2]Canal Industrial Corporation, 5635 Fisher Lane, Rockville, Md., 20852

employed with a short annular column of separating gel (see below) approximately 3 cm tall (7.5 ml). It was found necessary to provide the glass column near the bottom with four small inward projections to support the gel which flexed like a sensitive diaphragm in response to changes in the hydrostatic head. Hemoglobin samples of 0.5 ml, 20% in sucrose, were layered on the gel. Separations could be effected within 2 to 3 hours, and the gels were routinely used for three or four successive separations of aliquots of the same hemoglobin.

During the operation of preparative gel electrophoresis an optimal flow rate of "eluting" buffer of approximately 1 ml/min was used, and an optimal spacing was set between the annular gel column and the "flow disc" which forms the bottom of the flow chamber. A special procedure had to be adopted to achieve the isolation of the two hemoglobin components from M. oeconomus, M. abbreviatus and M. miurus. As the fast (major) component of these hemoglobins moved off the gel column (downward), some of it, because of charge or density effects, deposited in a conical pile on the flow disc beneath the (upward) eluting tube located in the center of the gel ring. This pile was removed slowly by the eluting buffer but not before the disappearance of the major component from the gel and the imminent elution of the minor component. At this point, therefore, electrophoresis was discontinued until the deposit of major component was cleaned up. Afterwards electrophoresis was resumed, and the minor component was brought off. The net effect on the record of optical density was an exaggerated appearance of separation of the component hemoglobins. The loss of hemoglobin by

this delayed elution was aggravated by slight misalignment of the gel face, out of parallel with the flow disc.

A linear, volumetric fractionator and ultra-violet absorption meter (280 nm) was used to collect and to monitor eluate in these and other fractionations.[3] A record of the optical density was obtained using an Esterline-Angus recorder (Graphic Ammeter).

Pooled fractions of hemoglobins in TRIS-HCl buffer, obtained by the above procedure, were concentrated approximately 10-fold by electrophoresis as described by Jovin et al. (1964) or by the following procedure derived from their method. The PD2/320 column was loaded with approximately 4 ml of the concentration gel formulation (see below) which provided a rather rigid gel column about 1.5 cm high. A cellulose membrane was attached securely over the face of the gel. Electrophoresis was performed with the usual upper and lower buffers, and 2 ml aliquots of hemoglobin solution, 20% in sucrose, were layered on the gel at approximately 1/2 hour intervals. As hemoglobin passed through the gel and then into the space below the gel, confined by cellulose, there occurred a small osmotic dilution, and the resultant pressure forced the concentrated eluate upwards through the elution tube into a receiving vessel. Approximately 16 ml of pooled fractions could be concentrated and collected in 6-8 hours of continuous operation in this way. Fresh buffers were replaced in the apparatus after this time and before continued use of the gel, because deterioration of the buffer with extended

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[3]Gilson Medical Electronics, Middleton, Wisconsin.

electrophoresis led to the evolution of chlorine and the consequent denaturation and precipitation of hemoglobin.

The single gel for preparative electrophoresis (lower gel, TRIS system; Jovin et al., 1964) was prepared just prior to loading the column by combining equal volumes of fresh ammonium persulfate solution (70 mg AP/50 ml) and an acrylamide stock solution which contained per 200 ml: 30.0 g acrylamide, 0.8 g BIS, 18.15 g TRIS, 0.23 ml TEMED, and 24.0 ml of N HCL.

The TRIS concentration gel (Jovin et al., 1964) was prepared by mixing 0.1 ml of fresh ammonium persulfate solution (175 mg AP/ml) with 10 ml of an acrylamide stock solution which contained per 400 ml: 15.0 g acrylamide, 0.4 g BIS, 2.85 g TRIS, 0.23 ml TEMED, and 12.8 ml of M H_3PO_4.

The buffers used in both preparative gel and concentration gel electrophoresis were prepared as follows:

1. For the upper column (cathode at pH 8), 2.88 g glycine and 0.6 g TRIS per liter.

2. For the lower column (anode at pH 8) and eluting buffer, 60.0 ml of N HCl was diluted to 900 ml, and solid TRIS was added to bring the final 1000 ml of solution to pH 8.1.

Reagent organic chemicals were obtained from Eastman Kodak and Matheson Coleman and Bell.

Preparation of Globin.

Hemoglobin solution, 1 to 5%, in water or TRIS-HCl elution buffer, was added dropwise with efficient stirring to 20 volumes of 2% acid-

acetone (2 ml concentrated HCl in 100 ml acetone) at approximately -20°C (Clegg et al., 1966) in a well-stirred methanol bath cooled with snow,[4] with liquid nitrogen, or with dry ice. The precipitated globin was allowed to settle at -20°C until the supernate could be removed by suction. The residue was washed once with acid-acetone, twice with acetone and twice with ether. These liquids were all cooled initially to -20°C. The residues were dried under nitrogen at room temperature or were dissolved in water and recovered as a glassy film by evaporation in the vacuum desiccator.

Conversion of Globin into S-(Beta-Aminoethyl) Derivatives.

This procedure was derived from the work of Jones (1964) and of Clegg et al. (1966). Ten grams of a mixed bed deionizing resin (Bio-Rad, AG 501-X8 (D), 20-50 mesh) were washed repeatedly with water by decantation. A small volume of water, e.g. 10 ml, was added to the resin, and 48 g of urea were then added in increments with gentle heating and efficient stirring. Some dilution with water was required to bring the final volume, which included the resin, to 100 ml. The urea solution was filtered using a Buchner funnel; to the filtrate were added 12.11 g TRIS and 15 mg EDTA, and the pH was adjusted from 11.2 to 9 with additions of concentrated HCl. The resultant solution had a volume of approximately 100 ml and was M in TRIS and 8M in urea.

Aliquots of 7.5 ml of the deionized urea solution were used to

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[4] "Handbook of Chemistry and Physics," 43rd ed., Chemical Rubber Publishing Co., Cleveland, Ohio. 1961. p. 2345.

dissolve globin samples of 10-20 mg (5-150 mg protein is suggested by Jones, 1964) which were contained in 10 ml vials or flasks. The globin solutions were stirred efficiently with magnetic bars in a covered beaker which was flushed continuously with prepurified nitrogen gas (Matheson). Each sample was treated with 0.1 ml of 2-mercaptoethanol (Aldrich), and stirring was continued 45-60 min. After this the samples were handled one at a time by the following procedure.

A volume of 0.2 ml of ethylenimine (Matheson Coleman and Bell) was added to one sample, and reaction was allowed to proceed for 30 min with stirring under nitrogen. The reaction mixture was next adjusted to pH 3 with concentrated HCL and was layered on a column of G-25 Sephadex (Pharmacia), 2 X 25 cm, equilibrated with 0.5% formic acid. The protein was eluted with 0.5% formic acid at a rate of 1-2 ml/min; 4 ml fractions were collected with the GME fractionator, and a record of the ultra-violet absorption was obtained. There was a clean separation of protein from the urea-HCl solution which was monitored by tests for turbidity of silver chloride.

Other globin samples were reacted singly with ethylenimine as the G-25 column was washed free of the previous sample. Pooled eluates of derivatized globin were frozen and evaporated to dryness repeatedly in vacuo prior to tryptic digestion.

DIGESTION OF GLOBIN WITH TRYPSIN OR CHYMOTRYPSIN.

Dried residues of approximately 10 mg of globin were treated in 10 ml vials with 3 ml of fresh 1% NH_4HCO_3 solution (Clegg et al., 1966).

The vials were furnished with small magnetic stirring bars and were placed in a covered beaker which was flushed with a rapid flow of nitrogen gas. A 0.1 ml aliquot of a fresh solution of trypsin, 10 mg/10 ml 0.001N HCL (Worthington trypsin, bovine pancreas, TRL 100S, 3Xcrystallized, salt free, lyophilized, sterile), was added to each globin suspension, and reaction was allowed to proceed up to 4 hours. A temperature of 30°C was attained due to the heat of operation of the magnetic stirrer on which the digestions took place. The pH of the digests changed during 4 hours from about 8 to 8.7.

The digests were transferred quantitatively to 50 ml beakers with portions of 5-10 ml water in which they were frozen and evaporated to dryness in vacuo. Dried residues from the digests were rubbed up in portions of 3 ml water which were transferred to test tubes for centrifugation, 30 min at 6000 rpm in the high speed centrifuge. Supernates were withdrawn and were evaporated to dryness in 20 ml beakers. Insoluble material which remained following the digestion was washed three times with water, and the first two washes were added to dried residues from the previous supernate. The supernate fraction was evaporated to dryness a third time to insure removal of volatile salts. The supernate fraction was then ready for mapping (see below).

Washed, insoluble material from tryptic digestion of hemoglobin was transferred to 10 ml vials with portions of 3 ml of fresh 1% NH_4HCO_3 solution. Digestion with chymotrypsin, 0.1 ml of 10 mg/10 ml 0.001N HCL (Worthington alpha-chymotrypsin, CD 100S, lyophilized, sterile), was conducted in a manner analagous to that described above for tryptic

digestion. A final pH of 8.9 was attained. This soluble digest was frozen, evaporated to dryness in vacuo, and re-evaporated twice after resolution in water. The dried residue from the chymotryptic digestion was then ready for mapping (see below).

A number of early tryptic digestions of globin samples larger than 10 mg were conducted in unbuffered aqueous solution according to the experience of Ingram (1958). A 1:100 weight ratio of trypsin to protein was used, and the initial pH was brought to 8.5 by the manual addition of 0.1N NaOH from a Gilmont Micrometer Buret (Cole-Parmer), 2.000 ml capacity. A pH of 8.5 was maintained during 90 minutes' digestion of a single sample by continuous monitoring with the Radiometer pH Meter 4 and by continuous manual additions of alkali. There seemed to be no particular merit in conducting the digestions in this way as compared with the "automatic" digestion of multiple samples in NH_4HCO_3 buffer which consequently superceded.

THE SEPARATION OF PEPTIDES FROM PROTEOLYSATES.

The proteolytic action of trypsin or chymotrypsin on globin produces a partial hydrolysate composed of polypeptides of varying lengths and compositions; in the case of tryptic action each peptide terminates generally in a lysine or an arginine residue. Peptides were displayed in a two-dimensional array or "map" by consecutive electrophoresis and chromatography on paper. The classical methods of Ingram (1958), modified by experiences of Clegg et al. (1966), were adapted with experiences in this laboratory.

High Voltage Electrophoresis.

Sheets of Whatman 3MM chromatography paper 46 X 57 cm (approximately 18 X 22 in) were laid out for cutting with the "machine direction" oriented in the length of the paper. This arrangement for the peptides to migrate electrophoretically towards the cathode in the machine direction (MD) appeared to influence markedly the quality of their separation. The sheet was marked and cut according to the diagram in Figure 1. Rectangles were cut out to leave wicks of 1 in X 1 1/2 in and 5 in X 1 1/2 in at the bottom or anode end, and a single wick of 1 in X 3 1/2 in at the top or cathode end. Penciled lines, shown in Figure 1 dotted parallel to the edge of the paper and 1/2 in and 2 in from it, were used as guides for folding prior to chromatography. The origin, at which sample was applied, was located 5 in from the bottom edge and 3 in from the right edge of the paper.

In prepartion for electrophoresis two such papers were superimposed, and the top left wick was cut back 2 in from the underlying paper to prevent siphoning. The two papers were then sandwiched between two full sheets of clean 3MM paper, and the sandwich was saturated by dipping it in 1 liter of volatile buffer (see below). (Considerable care is required to prevent tearing of the wet papers during this and subsequent handling.) The saturated quartet of papers was blotted between two other dry sheets of 3MM paper, and the 6-paper sandwich was separated so that the lucite support rack could be interposed beneath the two prepared papers as shown in Figure 2 and as described below.

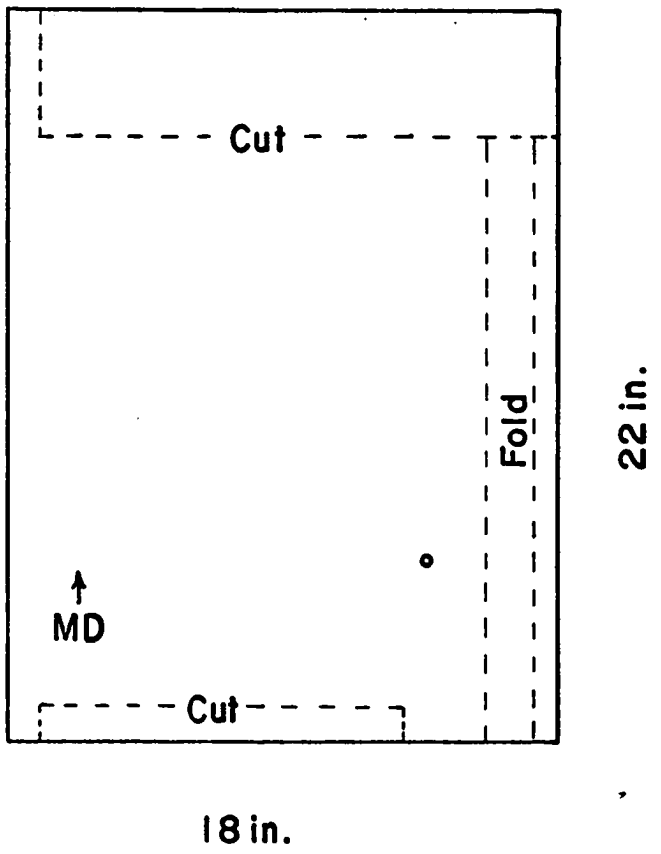

Figure 1. Preparation of Chromatography Paper for Peptide Mapping.

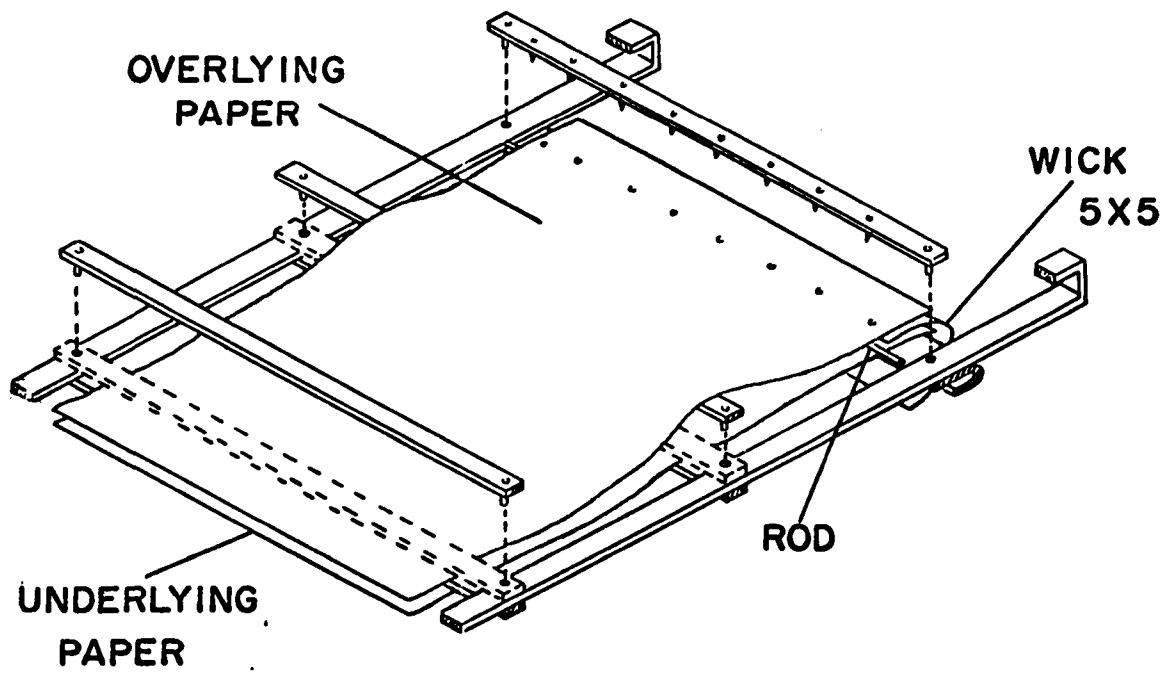

Figure 2. Arrangement of Chromatography Paper for Electrophoresis.

The support rack was laid on a clean surface, and the three removable, crosswise retaining bars were set aside. The two analytical papers were centered lengthwise on the rack, otherwise keeping the sandwiching papers in place above and below the rack. The important point in this handling of the wet papers was to keep them from drying. The top papers of the sandwich were turned back from their bottom edges so that the middle of the three retaining bars could be installed between the two analytical papers. The sandwich was then closed, and the two analytical papers were exposed at their upper ends. Before the papers were clamped in place with the upper, spiked retaining bar, a piece of 3MM paper 5 X 5 in, saturated with buffer, was interposed between the two papers at the upper right end so that a wick was produced 5 in wide by 3 1/2 in long. The overlying analytical paper was withdrawn about 1/2 in from coincidence with the upper edge of the underlying paper to adjust for a decrease in its effective length due to the support arrangement. The papers were clamped in place with the spiked retaining bar.

With satisfactory adjustments (carried out in heady pyridine vapors) the lower wicks of the two papers nearly reached the bottom of the legs of the support rack, and the origins of the two analytical papers were in contact with each other. Proteolysates (approximately 10 mg) were taken up in 200-400 microliters water, and a total of 50-100 microliters were placed at the origin of each paper in several applications. The uppermost blotting paper in the sandwich was treated with 25 ml of buffer from a pipet to maintain the saturated environment. A glass rod

was pushed through the sandwich between the analytical papers and just next to the middle retaining bar. The rod was worked upwards to a position just next to the upper retaining bar and was fastened in place with small loops of plastic tubing in which position it separated the two analytical papers.

With the samples loaded, the covering 3MM papers were removed, the support rack was placed upright, and the lower edges of the analytical papers were separated by the bottom, fixed retaining bar. The removable bottom bar was put in place, the upper wicks were moistened and caused to cling to the curved shoulder near the top of the rack, and the rack was immersed in the electrophoresis tank. The electrophoresis tank was patterned after that described by Katz et al. (1959).[5]

The volatile buffer generally employed for high voltage electrophoresis and for the preparation of papers described above was composed of pyridine: acetic acid: water in the proportions 500:20:4500 by volume, pH 6.4 (Michl, 1951, cited in Ingram, 1958). Approximately 6-8 liters of this buffer covered the floor of the tank and immersed the anode and the bottom wicks of the papers. (If the entire bottom edges of the papers are immersed in the buffer, poor electrical characteristics, high current and low voltage, are the result.) The cathode and the top wicks of the papers were immersed in approximately 4 liters of buffer contained in separate elevated compartments between which electrical

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[5]The lucite tank and support rack were constructed by Mr. Otto Hiller, P.O. Box 1294, Madison, Wisconsin, 53701.

contact was made by a sandwich of 10-12 pieces of 3MM paper 7 X 18 in.

The tank was nearly filled with about 75 liters of Chevron 325 petroleum thinner purchased from the local distributor for Standard Oil of California; the thinner was recommended as the equivalent of the eastern S. O. petroleum product called Varsol which is frequently cited in the technical literature as the dielectric and cooling medium used in high voltage electrophoresis. Cooling was produced by pumping ice-water through a stainless steel coil which induced an efficient convection in the petroleum thinner in which the coil was immersed in the upper part of the tank. An average temperature of 20°C could be maintained in the tank during the typical electrophoresis which was continued for 3 hours at 1500 v and current changing from 125 to 160 ma.[6]

Chromatography.

Following electrophoresis the sheets of 3MM paper were dried thoroughly at room temperature. Sets of from 2-8 dried papers, carefully marked for identification, were folded along the penciled lines described above and were placed in a Chromatocab (Research Specialties Co.) in preparation for descending chromatography. The papers were equilibrated 6-12 hours in the closed Chromatocab in vapors of the developer which was composed of 1-butanol: acetic acid: water: pyridine in the proportions 300:60:240:200 by volume (Waley and Watson, 1953, cited in Clegg et al., 1966). The papers were developed 16 hours and were again allowed

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[6]The DC power supply, rated at 5000 v and 200 ma was manufactured by the Canadian Research Institute, Don Mills, Ontario, Canada.

to dry completely at room temperature.

The positions of peptides were revealed in the finished two-dimensional peptide maps by dipping the papers in solutions of ninhydrin (Pierce Chemical Co.) in acetone. Maps for reference use only were stained in 0.2% solutions of ninhydrin in acetone; those maps from which peptides were to be isolated were stained more lightly by dipping them in 0.02% solutions. After being dipped, the papers were placed in a chromatography oven (New Brunswick Scientific Co.) heated at 60°C. Excess acetone vapors were driven off with the door open, and the papers were then heated in the closed oven at 60°C for two 5 min periods which were separated by a brief ventilation of the oven. (It is important that an oven properly constructed for chromatography be used to avoid the possibility of igniting the acetone-air mixtures.)

Peptide spots reached optimal definition in 6-24 hours after staining, and the maps could be marked for identification purposes and traced. Selected duplicate maps were stained further to obtain evidence for the presence of tryptophan in certain peptides. The ninhydrin colors were bleached with age upon storage or were bleached by dipping the maps in a solution of N HCl:acetone, 1:4 by volume (Baglioni, 1961). Bleached, dry maps were dipped in a fresh solution of 1% p-dimethylaminobenzaldehyde (Eastman Kodak) in a mixture of 9 volumes acetone and 1 volume concentrated HCl (Ehrlich's reagent; Smith, 1953). A purple-colored spot, which appeared at room temperature shortly after the maps were dipped, was taken as a positive qualitative indication for the presence of tryptophan in the underlying peptide. These maps were dried

and marked for tryptophan.

Elution of Peptides for Hydrolysis and for N-Terminal Analysis.

The peptide spots on maps were outlined in pencil on a light box according to the shape and intensity of the ninhydrin-stained areas. The spots were given identifying numbers, and the maps were traced on separate sheets of paper for reference purposes. Peptide spots were cut from maps stained in 0.02% ninhydrin in acetone. The excised spots were provided with small pointed tips on one side to which elution could be directed; the opposite side was cut off square to fit against the square end of a strip of 3MM paper retained between two microscope slides. The arrangement was derived from that of Sanger and Tuppy (1951), but dilute HCl (fresh reagent concentrated HCl, approximately 12N, and water, 1:1 by volume) was used in place of water as the eluting liquid (Clegg et al., 1966). An arrangement for producing a gentle siphon action was provided as shown in Figure 3. Approximately 100 microliters of eluates from peptide spots were collected in capillary tubes (Scientific Products, diSPo Micro Pipets). A support was improvised to accommodate ten simultaneous elutions. The filled capillary tubes were sealed carefully in an oxygen-gas flame to exclude as much air as possible. The labeled tubes were heated at 105°C for 24 hours. The resulting hydrolysates were washed into 3-in test tubes and evaporated to dryness in the vacuum desiccator prior to amino acid analysis to be described below. The vacuum desiccator was provided for routine evaporations with a beaker of concentrated sulfuric acid and a dish of sodium hydroxide flakes as desiccants; the desiccator was housed in a

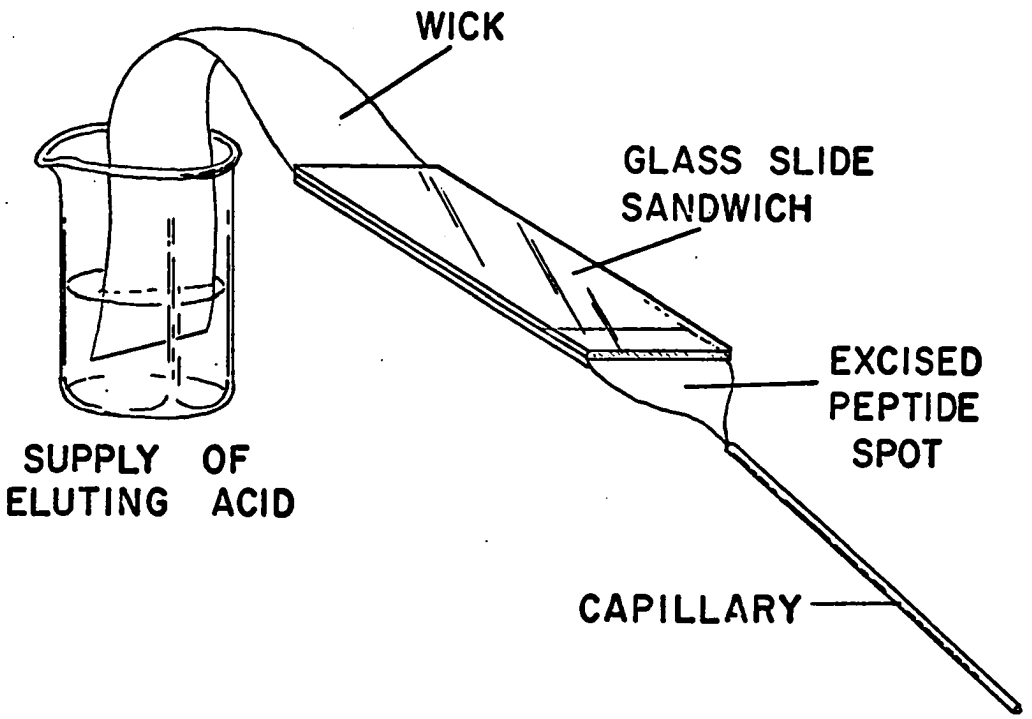

Figure 3. Arrangement for Elution of Peptides from Peptide Maps.

Fisher Desiguard and could be evacuated to pressures of 5 mm Hg or less with a Welch 1400B Duo Seal vacuum pump.

Selected peptides were eluted from paper spots with water, derivatized with 2,4-dinitrofluorobenzene (FDNB, Pierce Chemical Co.) in trimethylamine, hydrolyzed and finally chromatographed in the tert-amyl alcohol-phthalate system (Fraenkel-Conrat et al., 1955).

AMINO ACID ANALYSIS OF PEPTIDE HYDROLYSATES.

Dried residues from peptide hydrolysates were largely soluble in 0.25 ml of an aqueous solution containing 0.5 μmole/ml each of cysteic acid and norleucine. These two amino acids were included as internal references for the analyses. An entire sample of hydrolysate was loaded on one of the five 3 mm X 130 cm columns of cation exchange resin (Technicon Chromo-Beads, Type A, Lot No. 111A) employed in a Technicon Auto-Analyzer[7] for multiple column analysis of amino acids. The columns were operated at 60°C and were developed with a gradient of pH and ionic strength derived from the works of Piez and Morris (1960) and of Hamilton (1963). One analysis required approximately 6 1/2 hours. From peptide hydrolysates chromatograms were obtained which displayed the constituent amino acids in terms of the absorbance at 570 nm and 440 nm of their products from reaction with ninhydrin as a function of the time of their elution. A record of the elution of the amino acids was also obtained on punched tape in terms of percent transmittance.

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[7]Technicon Chromatography Corp., Chauncey, New York.

The analytical system was calibrated by allowing it to analyze "standard" samples containing 0.1 μmole of each of the 17 common amino acids (excluding tryptophan) and ammonia to which 0.1 μmole amounts of cysteic acid and norleucine were added as internal references. Standard mixtures containing 2.5 μmole/ml of each of the amino acids were obtained from Technicon Chromatography Corp. and were also prepared in this Laboratory from reagent grade amino acids supplied by Nutritional Biochemicals Corp.

Because tryptophan was destroyed during acid hydrolysis of peptides it was not detected by ordinary amino acid analysis; tryptophan was detected qualitatively in certain intact peptides as described above.

Cysteine was estimated in peptide hydrolysates by ordinary amino acid analysis as its S-beta-aminoethyl derivative which resulted from the previous treatment of globin with ethylenimine. A standard sample of S-beta-aminoethylcysteine was prepared by adding 62 mg of L-cysteine (free base; Nutritional Biochemicals Corp.) to a 10-fold excess of ethylenimine (approximately 200 mg) in 10 ml of water magnetically stirred and deaerated by bubbling with nitrogen gas. After 15 minutes the solution was diluted to 1 liter, and aliquots were evaporated to dryness prior to amino acid analysis.

CALCULATION OF THE DATA.

Single amino acid analyses (chromatograms) of the hydrolysates of individual peptides were reduced to numbers of μmoles of the amino acids using the areas of the absorbance peaks calculated by hand and by

means of an automated computer program. The standard areas shown in Table 3 and obtained from analyses of the 0.1 μmolar amino acid mixtures mentioned above were used for the hand reductions.

Amounts of peptides found in the single analyses of their hydrolysates were in the range of 0.004 to 0.050 μmole. The raw micromolar data were fitted empirically to "the best" near-integral proportions by using a selected divisor. A range of variation of ± 0.3 amino acid was allowed in the estimation of the integral number of amino acids in a peptide; thus numbers in the range of 0.7 to 1.3, 1.7 to 2.3, etc. have been evaluated as whole numbers 1, 2, etc. of amino acids. (Consult Tables 4 ff.) It will be noted that the majority of the data is well inside of these ranges.

Departures of numbers from these near-integral ranges have occurred, and they may be attributed to properties of the amino acids and their peptides and to conditions encountered during peptide mapping and amino acid analysis. The presence of non-integral numbers of amino acids may reflect otherwise unsuspected information about peptide structure.

EVALUATION OF NON-INTEGRAL DATA FOR AMINO ACID COMPOSITIONS: THE LOSS OF AMINO ACIDS DURING PEPTIDE MAPPING AND AMINO ACID ANALYSIS.

An adequate interpretation of the amino acid compositions of the tryptic peptides of hemoglobin requires that non-integral numbers of amino acids which appear there be explained. The bulk of the data shows clearly proportions of essentially integral numbers of the amino acids constituting the individual polypeptides and represents conformance to

Table 3. Standard Areas (Color Values) Obtained from the Analyses of Reference Mixtures 0.1 μmolar in Each of the Amino Acids.

AA[a]	C^{b}_{1711}	C^{c}_{2037}	AA[a]	C^{b}_{1711}	C^{c}_{2037}
B(D,N)	3.18	3.78	I	3.18	3.70
T	3.66	4.27	L	4.28	5.23
S	3.84	4.44	O	4.56	5.28
Z(E,Q)	3.94	3.88	F	4.55	5.26
P	1.16	0.98	NH_3	1.25	1.39
G	5.40	6.55	K	4.84	4.51
A	3.26	3.96	H	5.46	5.84
V	3.00	3.64	R	4.05	4.70
C		3.70			
M	4.72	5.26			

[a]See p. 72 for explanation of abbreviations.

[b]C-values (color-values per 0.1 μmole of amino acid) from standard analysis 1711 were used in the calculations of analyses 1700 to 2000.

[c]C-values from standard analysis 2037 were used in the calculations of analyses 2000 to 2300. The C-value for cysteine (S-beta-aminoethyl derivative) was obtained from standard analysis 2382.

the fundamental chemical law of multiple proportions in terms of combining molecular subunits.

The experimental procedures adapted to and developed for the research reported in this dissertation were applied to human hemoglobin as well as to Microtus hemoglobin. To the extent that the outcome of the analyses of the tryptic peptides of human hemoglobin does not exactly represent new information, certain aspects of the "results" from the analyses are evaluated here rather than in the Results or Discussion section of the dissertation. The peptide maps and the amino acid analyses obtained for human hemoglobin comprise invaluable references for the evaluation and subsequent interpretation of the data from Microtus hemoglobins.

The Response of Amino Acids to Conditions of Acid Hydrolysis.

There is a large literature concerning the effects of the conditions of hydrolysis upon the resulting amino acid analyses of proteins and polypeptides; a recent general discussion of the problem was given by Tristram (1963).

Tryptophan. The amino acid tryptophan is invariably destroyed completely (relative to the ninhydrin method of detection) by the generally recommended methods of acid hydrolysis as employed in this research and as described earlier in this dissertation. Tryptophan was detected qualitatively in the present research in the two intact peptides alpha-T3 and beta-T2 by staining peptide maps with Ehrlich's reagent, p-dimethylaminobenzaldehyde.

Tyrosine. The amino acid tyrosine possesses a phenolic side chain

and suffers partial destruction during acid hydrolysis. The six peptides concerned in this work were: alpha-T4, alpha-T5, alpha-T6, alpha-T9, alpha-T14 and beta-T15. Numbers of tyrosines from 0.3 to 0.8 were obtained experimentally from peptides of normal human hemoglobin which are reported to possess one tyrosine residue per peptide. Numbers from 0.2 to 0.8 tyrosine, obtained in amino acid analyses of Microtus hemoglobin peptides, are considered to represent single tyrosines in each of the peptides.

Methionine. The amino acid methionine possesses the thio-ether group in its side chain, $-CH_2-CH_2-S-CH_3$, and suffers partial destruction during acid hydrolysis of polypeptides. The three peptides concerned are: alpha-T5, alpha-T9 and beta-T5. Numbers from 0.2 to 0.6 methionine are considered to represent one methionine in each of these peptides.

One route of "destruction" of methionine follows its oxidation at the sulfur atom to the sulfone and sulfoxide. The properties of methionine and oxidized methionine are sufficiently different that a single methionine-containing peptide may be split into two components with different chromatographic mobilities according to the oxidation state of the methionine. This may account for the appearance of the slower peptides which were designated as A5' in the maps of M. abbreviatus Hb-f and M. miurus Hb-f and which had amino acid compositions essentially the same as peptides alpha-T5. Differences in the chromatographic mobilities between peptides beta-T5 of M. pennsylvanicus hemoglobin and of the other Microtus may also be related to this oxidation.

The properties of the oxidized methionine bring about its elution

prior to aspartic acid during quantitative amino acid chromatography. Figure 29 shows a small oxidized methionine component from which methionine was estimated in the chromatogram for peptide beta-T5 from M. pennsylvanicus hemoglobin.

Cysteine. The amino acid cysteine, containing the mercaptomethyl side chain, may be completely destroyed under ordinary conditions of acid hydrolysis. Its oxidized dimer, cystine, 2 $-CH_2-SH \rightleftarrows -CH_2-S-S-CH_2-$ is subject to similar destruction during acid hydrolysis. There is a large literature concerned with the determination of these amino acids (see e.g. Cecil and McPhee, 1959). It has been recommended that cysteine-cystine be determined 1) as cysteine in intact proteins by independent chemical analysis, 2) as the relatively stable cysteic acid derivative in an independent amino acid analysis, 3) as one of various relatively stable thio-ether derivatives of cysteine which can usually be determined during the course of ordinary amino acid analytical procedures. For the purposes of the present research the porphyrin prosthetic groups were removed from hemoglobins, and the resulting globins were treated with ethylenimine. Amino acid analyses of hydrolysates of peptides alpha-T12a, beta-T10 and beta-T12 were obtained in which the aminoethylcysteine could be estimated in amounts from TRACE to 0.9. Single residues of cysteine are considered to be present in these peptides. The fragmentation of peptides alpha-T12 and beta-T12 is independent evidence for the presence in the proteins of aminoethylcysteine residues at which tryptic cleavage occurred. In certain analyses in which poor performance of the analytical system was observed,

very high background absorbances in the terminal ("basic") region of the analyses helped to obscure the absorbance of the cysteine derivative. This can be seen in the sequence of chromatograms given in Figure 22.

Threonine and Serine. Corrections to the numbers of these amino acids have frequently been included in reports in the literature to account for their destruction during the acid hydrolysis of proteins. Special corrections or interpretations of the data obtained for threonine and for serine from acid hydrolysates of peptides did not in general appear to be necessary in the present research. In occasional analyses, fractions of 0.4-0.6 serine or threonine were considered to represent whole numbers of these amino acids.

Other Amino Acids. Less than integral numbers of certain amino acids have been observed in acid hydrolysates of proteins not because of any particular destruction but rather because of their incomplete hydrolysis from peptide fragments. The amino acids with aliphatic side chains, valine, leucine and isoleucine, have been noted to participate in resistant peptide bonds which may not be completely hydrolyzed during the course of an ordinary hydrolysis. Homopeptides of these amino acids, e.g. the dipeptide valylvaline which occurs frequently in hemoglobin, may be particularly resistant to hydrolysis.

The Response of Amino Acids and of Their Peptides to Reaction with Ninhydrin.

Amino Acids. In their chemical reaction with ninhydrin the amino acids suffer oxidative deamination and are thereby destroyed. The ninhydrin product which results (with its characteristic color, Ruhemann's

purple), although not specific for a given amino acid, provides an excellent quantitative measure of the amounts of individual amino acids which can be separated in a known sequence by chromatographic methods. The amino acid proline differs in its reaction with ninhydrin and yields a yellow-colored product which can be quantitated less satisfactorily.

The yield of ninhydrin product (color value or "color yield") per mole of amino acid varies somewhat with the particular amino acid as well as with the conditions under which the reaction occurs. Two groups of "color yields" or color calibration values were used in the present research and were listed in Table 3. It is possible that some of the small fluctuations about integral numbers of amino acids which are recorded in Tables 4 ff. are due to fluctuations in "color yields."

Peptides. Peptides react with ninhydrin to furnish products similar in color to those produced with amino acids. This property of peptides is used to locate them on peptide maps after the maps are dipped in a weak ninhydrin solution. There appears to have been little study of the nature of the reaction between ninhydrin and peptides although partial destruction of some amino acids must be consequent upon this reaction. It is very likely that the reaction of a peptide with ninhydrin should involve the exposed terminal amino group as it does in the case of the free amino acids. Data from human hemoglobin peptides obtained in the present research have been observed frequently to contain low non-integral numbers of those amino acids which are known to occupy N-terminal (amino terminal) positions in their respective peptides.

It is proposed to infer from the data of this research that certain amino acids are N-terminal in their respective Microtus peptides on the basis of non-integral numbers obtained for them in the total amino acid analyses of the peptides. The basis for such an inference and the consequent estimate of the N-terminal amino acid is well illustrated in the comparison of the data for Microtus hemoglobin peptides alpha-beta-T7 and alpha-beta-T(7-8) with those from human hemoglobin. Data taken from Table 12 for human hemoglobin peptides alpha-T7 and beta-T7 are compared first with the established amino acid sequences for the two peptides:

	Human alpha-T7 G H G K	Human beta-T7 A H G K
K	1.14	1.08
H	1.14	0.91
G	1.78	1.00
A		0.82

Data from Tables 11 and 12 for the unresolved peptides alpha-T7 and beta-T7 of M. abbreviatus Hb-f are next compared with their proposed sequences:

	Microtus alpha-beta-T7		Microtus alpha-beta-T(7-8)	
	alpha-T7 A.H.G.K	beta-T7 A.H.G.K	alpha-T(7-8) A.H.G.K.K	beta-T(7-8) A.H.G.K.K
K	2.00		4.20	
H	1.92		2.10	
G	2.12		2.34	
A	1.54		1.38	

Data obtained for Microtus hemoglobin peptides beta-T2 are indicative of N-terminal alanine rather than N-terminal serine as (the latter) might be inferred from the human hemoglobin peptide beta-T2. The non-integral data for alanine in this peptide may also be low as a consequence of incomplete hydrolysis for an N-terminal homodipeptide, alanylalanine sequence.

When a labile amino acid such as methionine occupies the N-terminal position in a peptide it appears to be recovered in even lower proportions. See for example peptide alpha-T5.

Aberrations in Amino Acid Data Originating with the Automatic Apparatus for Amino Acid Analysis.

The general performance of the automatic Technicon apparatus for amino acid analysis was exceptionally good and must be credited in large measure to those individuals who were responsible for its operation. At the same time, over the months of continuous operation several operating characteristics were noted on a regular or an irregular basis which could affect adversely the determination of the amino acids.

It was mentioned above that a poorly controlled and excessively high base line accompanying the elution of the aminoethyl derivative of cysteine helped to obscure this amino acid. Rigorous exclusion of ammonia and possibly of volatile amines from the make-up water used in the analytical reagents should control the amount of background elution of ammonia in this region and hence control the base line level.

Instances are documented in Figures 25 and 32 in which abrupt failures of short duration in the chromatographic base line in the

region of leucine contributed to low apparent absorbances and hence to low results for leucine.

Nauman (1968) noted the apparent formation of the gamma methyl ester of glutamic acid from this amino acid on the ion exchange columns used in chromatography at approximately 60°C. The equilibrium amount of ester, approximately 25%, was formed rapidly in the time during which glutamic acid remained on the column in the presence of a 10% v/v methanol-containing buffer regularly used early in gradient elution. The suspected methyl ester differed in its elution properties from those of free glutamic acid. Not only was the amount of eluted glutamic acid diminished by the departure of the ester, but the ester eluted together with glycine. Corrections for this behavior did not seem to be required in the calculations of the data from the amino acid analyses of peptides in this research. Esterification was subsequently circumvented by the use of tert-butyl alcohol.

RESULTS

The tryptic peptides from each of the six hemoglobins, five Microtus and one human, were separated from one another in the two-dimensional arrays on paper called peptide maps. All possible peptides, detected by ninhydrin staining, were eluted from each map, hydrolyzed and analyzed for amino acid composition.

From a total of 127 peptides which were analyzed from five Microtus hemoglobins it was possible to correlate 110 peptides with specific homologous peptide segments of the alpha and beta proteins of human hemoglobin. Tryptic peptides of the Microtus hemoglobins were not found which could be correlated with the three peptides of the alpha and beta proteins of human hemoglobin: alpha-T9, alpha-T13 and beta-T12. On the other hand, there were 12 peptides of two classes from the five Microtus hemoglobins which were not assigned to specific alpha or beta proteins but which could represent fragments of the peptides homologous with the alpha-T9 and beta-T12 peptides of human hemoglobin.

Several scientific papers and compilations of data referring to the amino acid composition of hemoglobins were of especial assistance in the task of making specific identifications of peptides in terms of their map positions and their amino acid compositions, and, subsequently, of making inferences concerning possible amino acid sequences in the Microtus peptides: Clegg et al. (1966); Dayhoff and Eck (1968); Braunitzer et al. (1964); Baglioni (1961).

PEPTIDE MAPS FROM TRYPTIC HYDROLYSATES OF THE HEMOGLOBINS.[1]

Individual Peptide Maps.

Tracings of maps of the separated tryptic peptides of hemoglobins from human and from Alaskan *Microtus* are shown in Figures 4 through 9. Definite similarities and definite differences between the human map and those of *Microtus* are evident in terms of the arrangement of spots which are numbered according to the peptides of the alpha and beta chains which they represent.

Composite Peptide Maps.

The relative positions of homologous peptides from the several maps are compared by superposition in Figures 10 and 11. Figure 10 shows composite "spots" which are defined for alpha peptides by the centers of the individual peptides in successive superpositions of

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[1]Abbreviations and symbols used in Figures 4 through 11 are:

Hb	Hemoglobin
HA	Normal Human Hb-A
MP	*M. pennsylvanicus* Hb
MAf	*M. abbreviatus* Hb-f (fast component of two)
MAs	*M. abbreviatus* Hb-s (slow component of two)
MMf	*M. miurus* Hb-f
MOf	*M. oeconomus* Hb-f

A1, B2, etc. are alternative designations for the tryptic peptides alpha-T1, beta-T2, etc. and describe the situation of the tryptic peptides in the alpha or beta protein chains, first, second, etc. removed from the N-terminal of the respective chain.

Similarly, B4fc refers to the fourth tryptic peptide from the N-terminal of the beta chain of hemoglobin; in this case only a fragment, f, of the C-terminal, c, of this peptide was recovered. Fragments of some other peptides are designated by the letters a and b and fn. *Microtus* peptides which have not been identified (uid = unidentified) as belonging specifically to the alpha or beta chain are simply designated by numbers, 16 (or uid 16), 16', 17, etc.

The origins for electrophoresis are shown in the lower left corners of the figures together with the sign of the electric field.

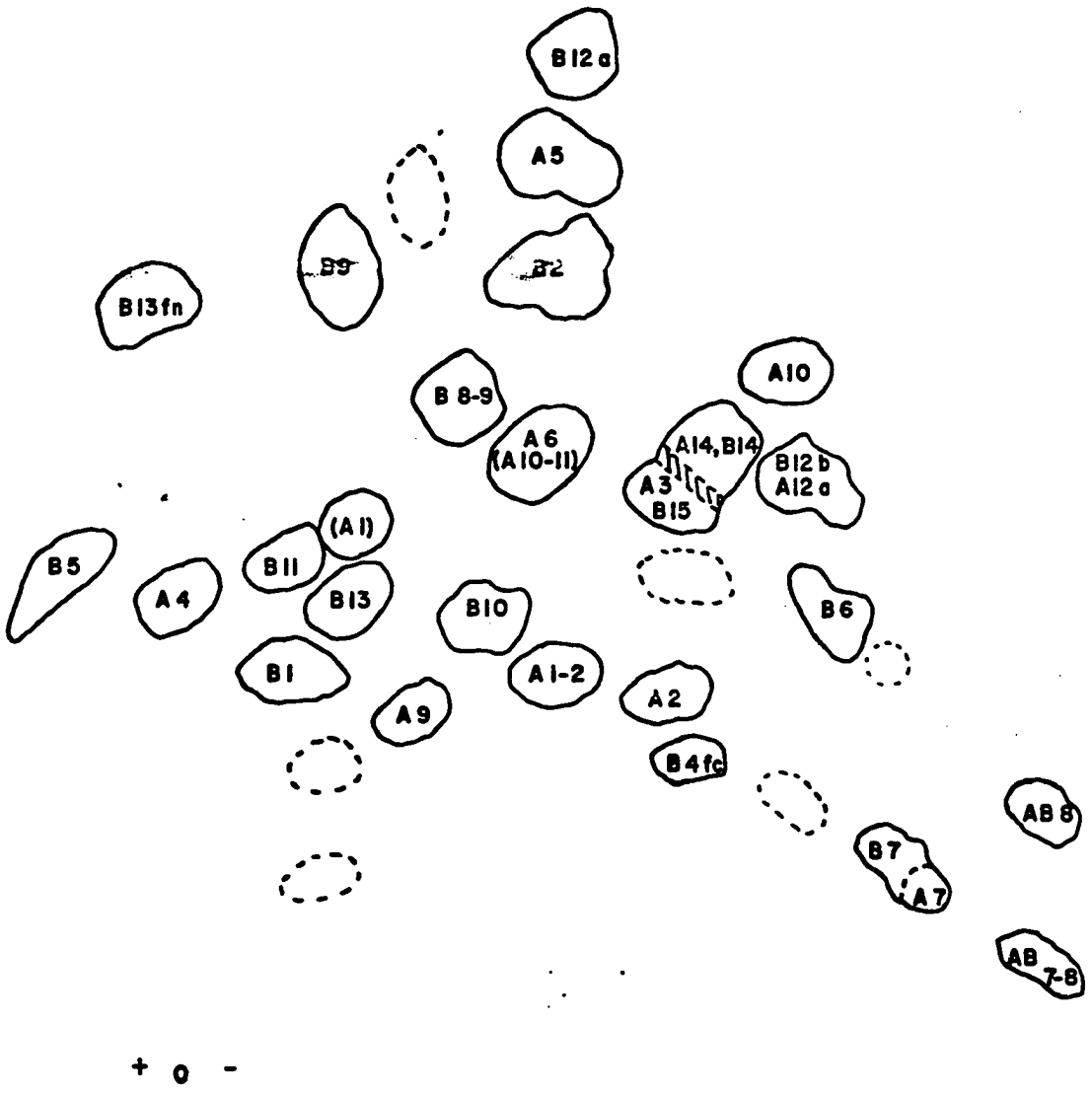

Figure 4. Map of Tryptic Peptides from Human Hemoglobin.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

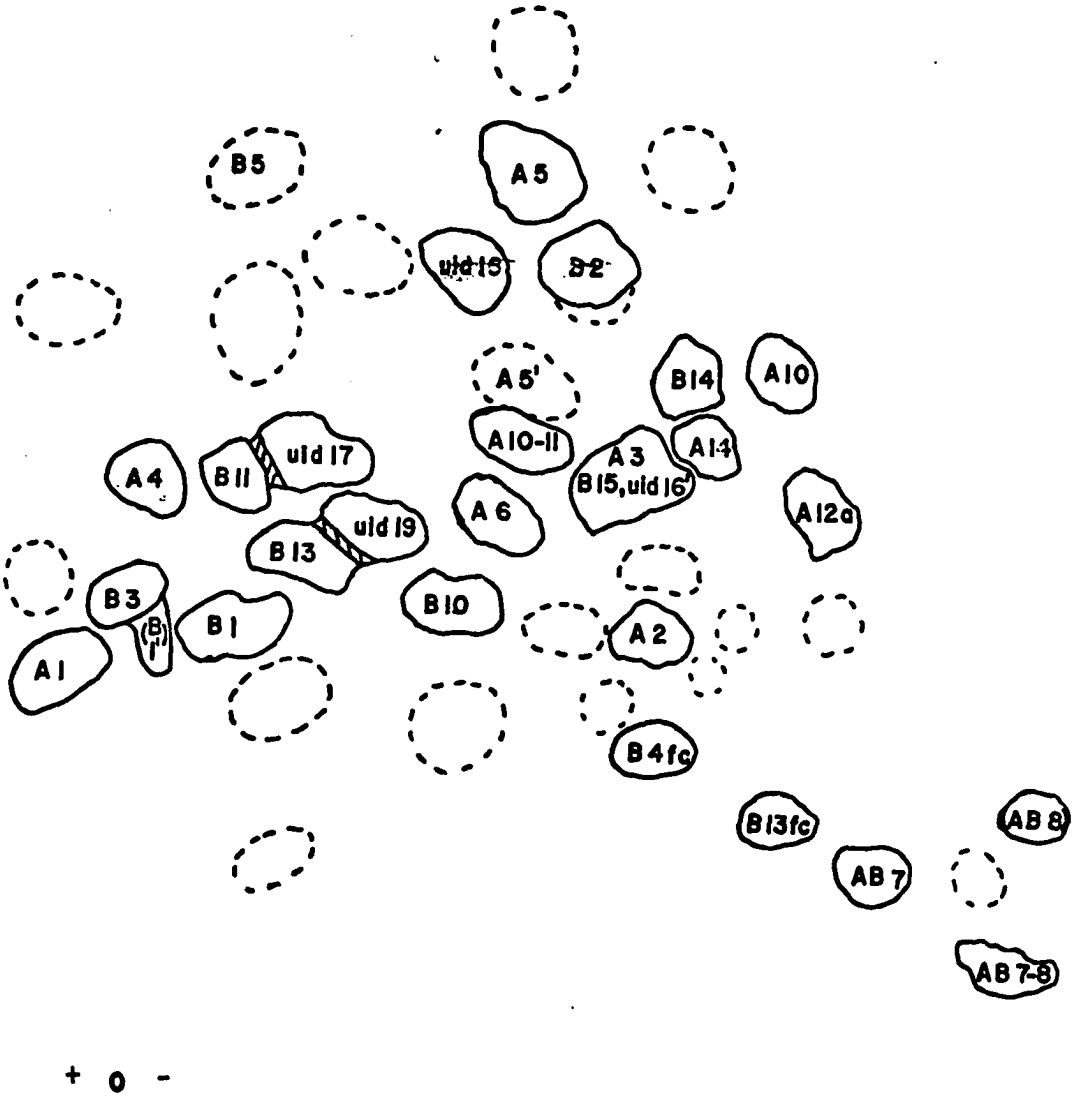

Figure 5. Map of Tryptic Peptides from Hemoglobin of Microtus pennsylvanicus.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

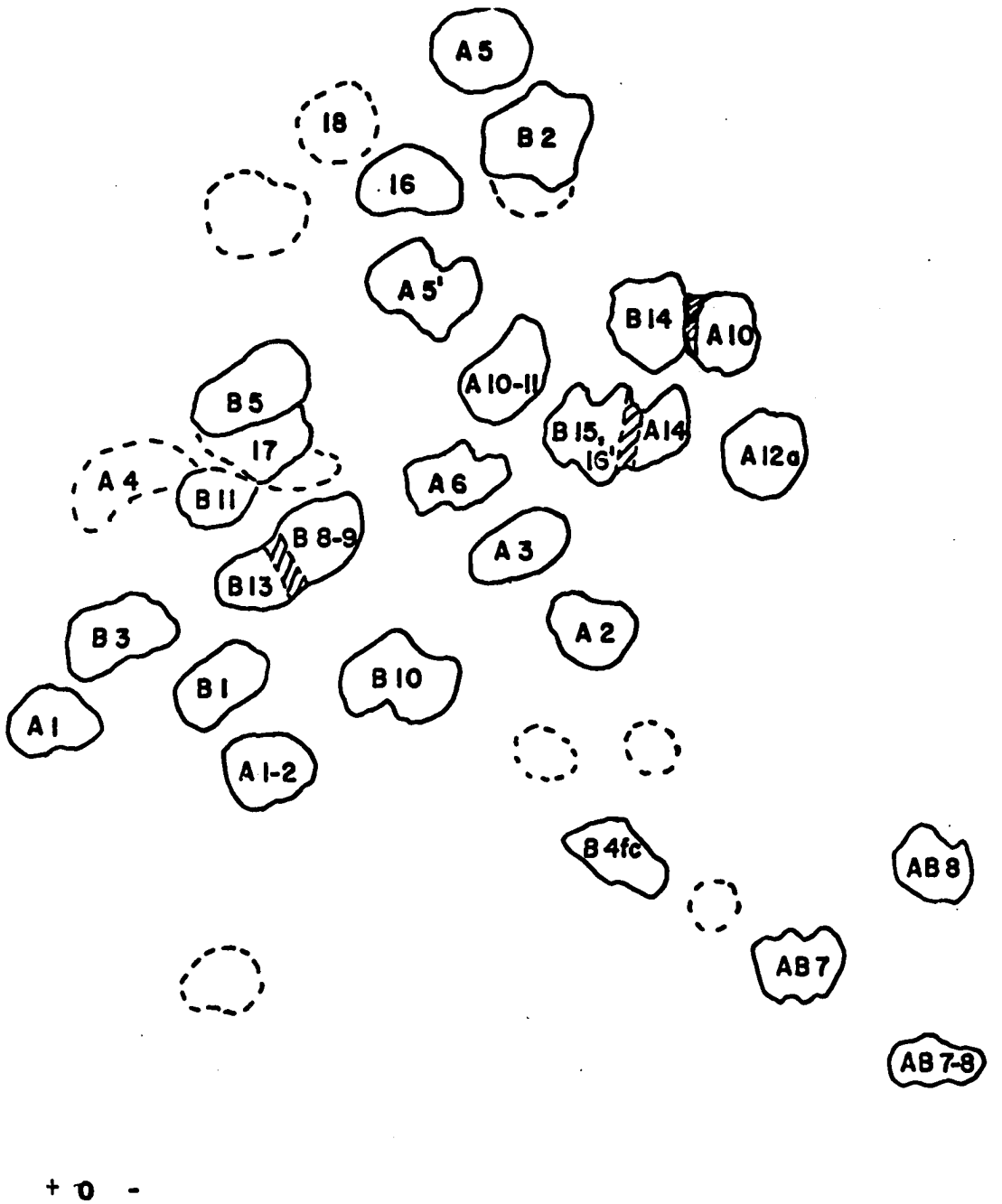

Figure 6. Map of Tryptic Peptides from Hemoglobin-f of Microtus abbreviatus.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

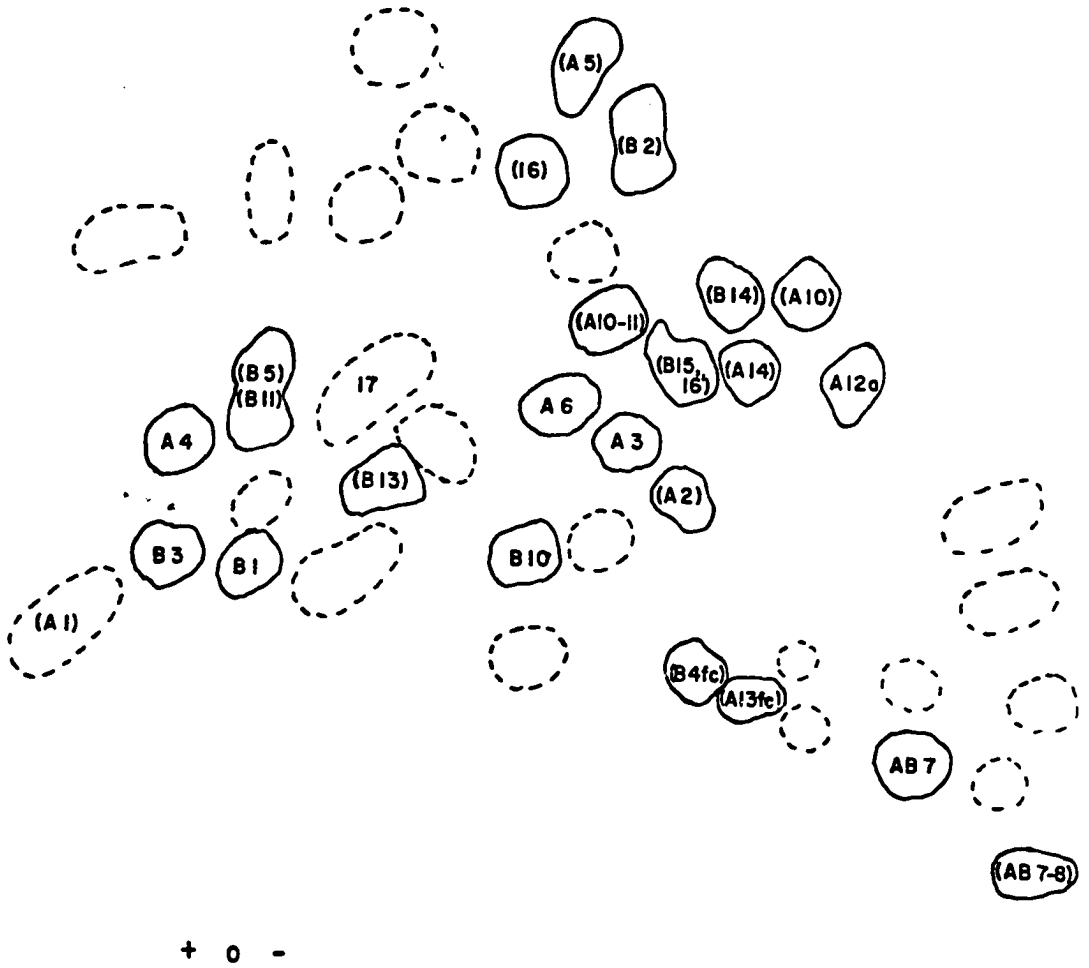

Figure 7. Map of Tryptic Peptides from Hemoglobin-s of Microtus abbreviatus.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

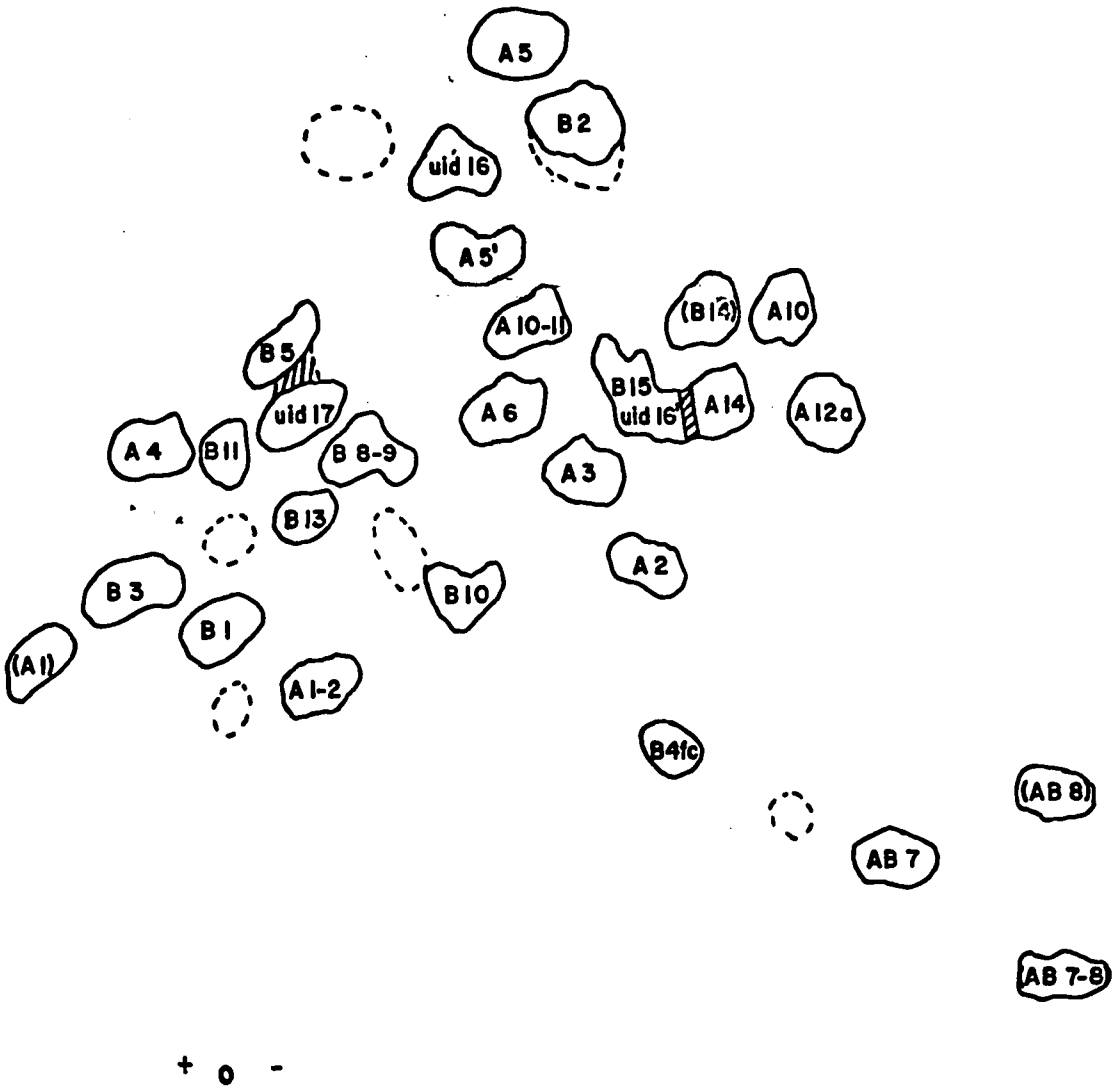

Figure 8. Map of Tryptic Peptides from Hemoglobin-f of Microtus miurus.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

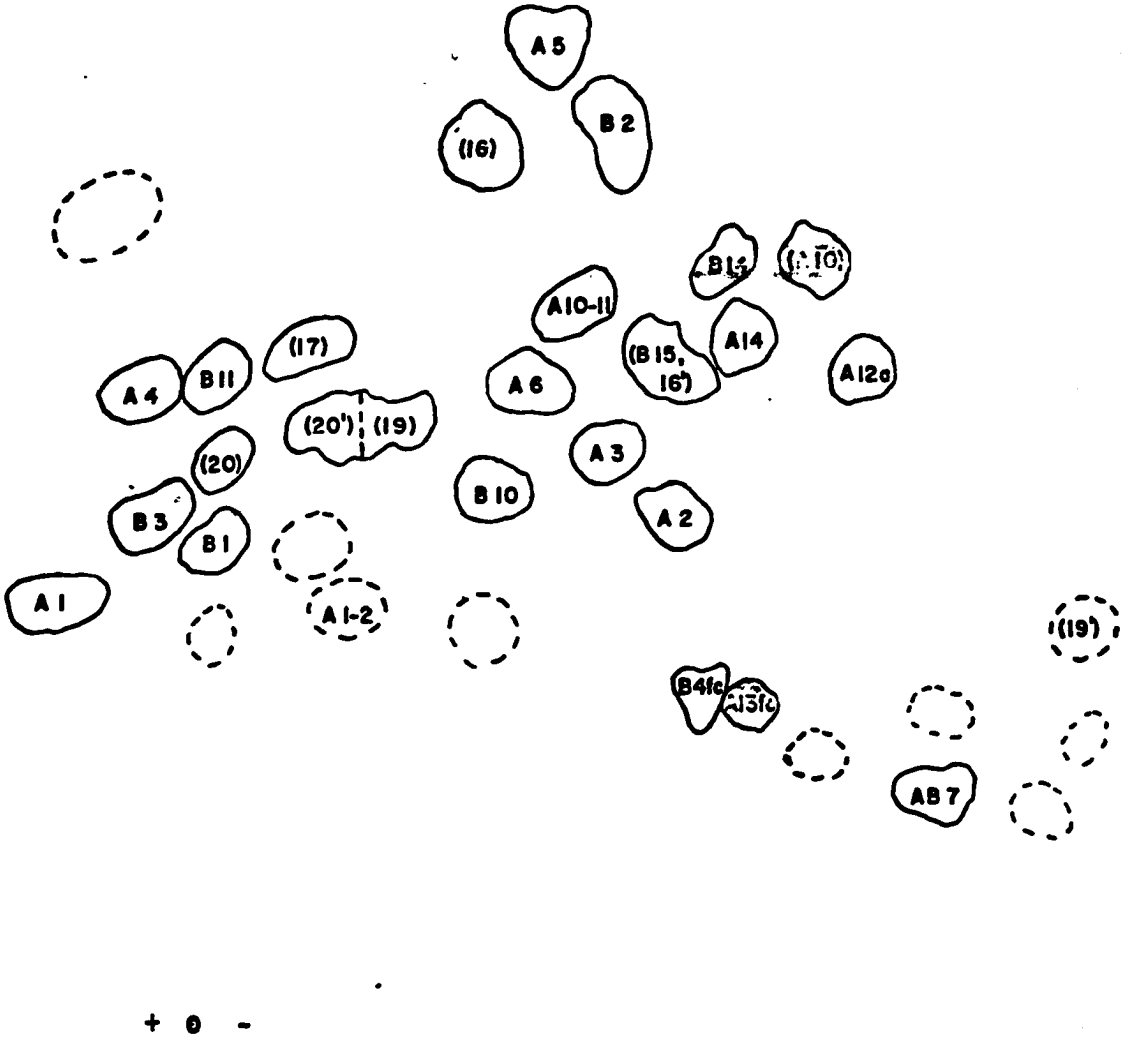

Figure 9. Map of Tryptic Peptides from Hemoglobin-f of Microtus oeconomus.

Tracing is reduced approximately X 1/2.
Refer to text for abbreviations.

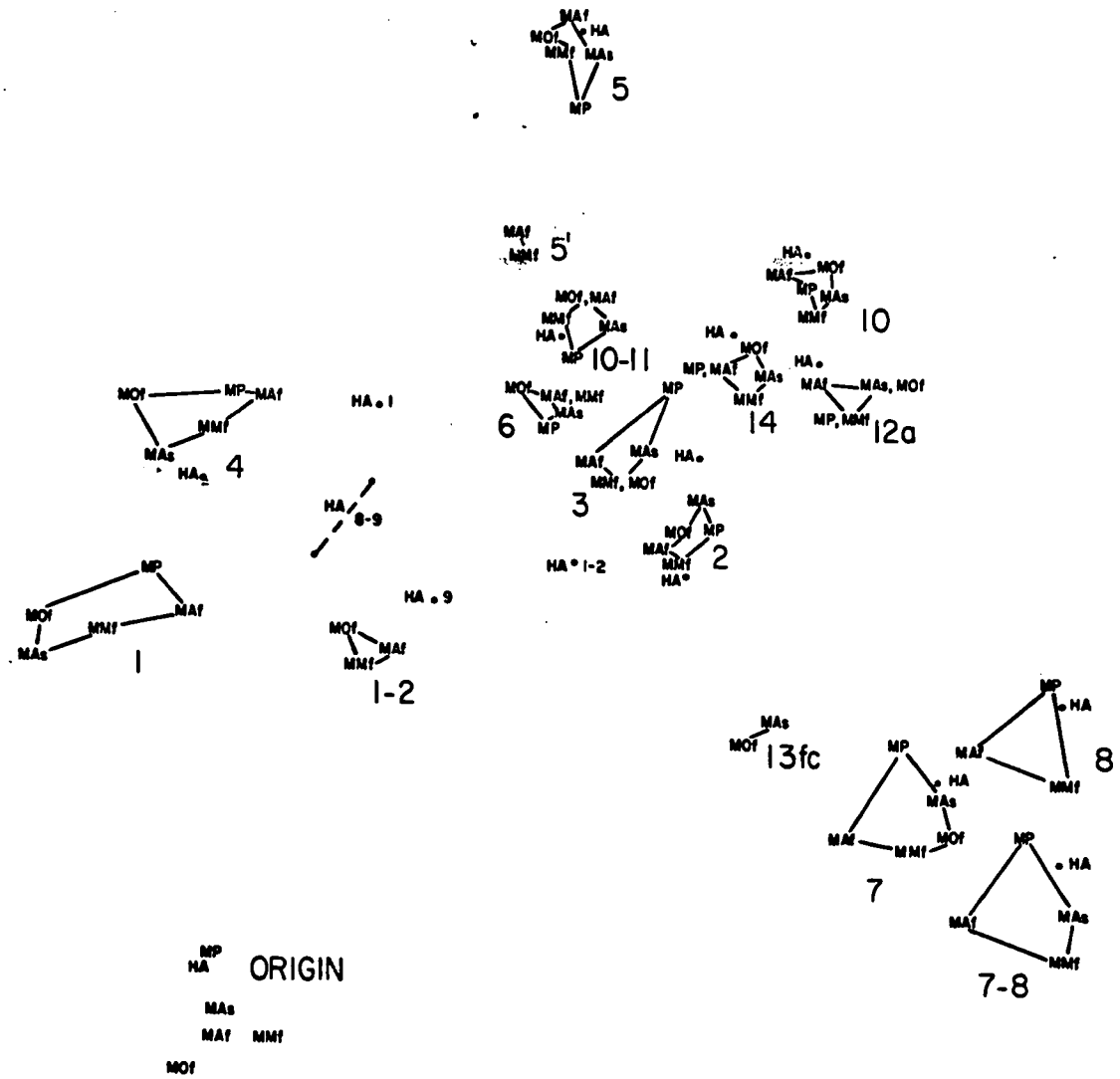

Figure 10. Composite Map of the Tryptic α Peptides from the Hemoglobins of:

Ha	Human
MP	Microtus pennsylvanicus
MAf	Microtus abbreviatus (Hb-f)
MAs	Microtus abbreviatus (Hb-s)
MMf	Microtus miurus (Hb-f)
MOf	Microtus oeconomus (Hb-f)

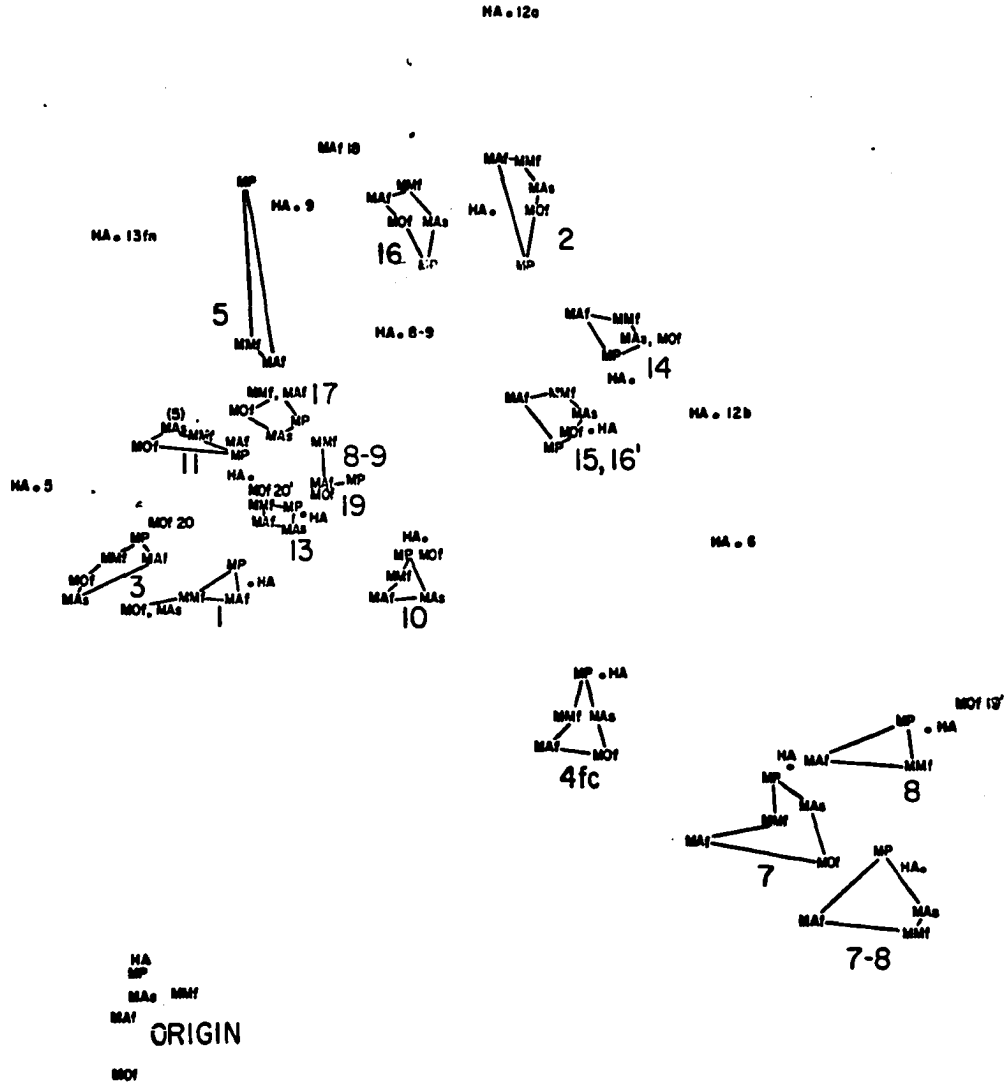

Figure 11. Composite Map of the Tryptic β Peptides from the Hemoglobins of-

HA	Human
MP	Microtus pennsylvanicus
MAf	Microtus abbreviatus (Hb-f)
MAs	Microtus abbreviatus (Hb-s)
MMf	Microtus miurus (Hb-f)
MOf	Microtus oeconomus (Hb-f)

the several peptide maps shown in Figures 4 through 9. The peptide centers are marked by the positions of the abbreviations MP, etc., for the respective species of Microtus. The composite "spots" are numbered according to the common peptides of the alpha chains which they represent.

The centers of the respective human peptides, superimposed and identified as HA relative to the Microtus "spots," are numbered independently when they differ markedly in position from the corresponding Microtus peptides.

Figure 11 shows composite "spots" for beta peptides.

AMINO ACID COMPOSITION OF PEPTIDES SEPARATED FROM TRYPTIC HYDROLYSATES OF HEMOGLOBIN.[2]

The results from the analyses of the amino acid compositions of

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[2]Single letter abbreviations are used in Tables 4 through 38 and in Figures 12 through 40 to designate the amino acids according to Dayhoff and Eck (1968):

K	Lysine	P	Proline
H	Histidine	G	Glycine
R	Arginine	A	Alanine
D	Aspartic Acid	V	Valine
N	Asparagine	M	Methionine
B	Either D or N, not distinguished	I	Isoleucine
T	Threonine	L	Leucine
S	Serine	O	Tyrosine
E	Glutamic Acid	F	Phenylalanine
Q	Glutamine	C	(Aminoethyl-)Cysteine
Z	Either E or Q, not distinguished	W	Tryptophan

The abbreviation nd is used to signify that "no data" was obtained.

The symbol + or the word TRACE signifies that qualitative evidence was observed for the presence of the amino acid.

In the tables the identifying number for the single amino acid analysis is given at the bottom of each column of data to which it pertains together with the total micromoles of peptide recovered in that analysis.

The other abbreviations are as given in Footnote 1, p. 63.

the tryptic peptides, taken from the peptide maps described above, are summarized in Figures 12ff and in Tables 4ff. Included in Tables 4 through 33 is REFERENCE data for the integral numbers of amino acid residues in the respective peptides from hemoglobins of human and of laboratory white mouse.

Reference data for Human Hb are based on the work of Braunitzer et al. (1961), Hill and Konigsberg (1962), and Schroeder et al. (1963) and were taken from the tabulated sequences given in "The Atlas of Protein Sequence and Structure, 1967-1968" (Dayhoff and Eck, 1968).

Reference data for White Mouse Hb are based on the work of Popp (1965b) and of Rifkin et al. (1966c) and were taken from the compilation in the latter paper for C57BL strain.

Tryptic Peptides of the Alpha Chain of Hemoglobin.

Figures 12 through 24 reproduce chromatograms which were obtained for tryptic peptides of alpha chains of hemoglobin. The chromatograms in each Figure generally compare alpha peptide compositions from Human Hb, from M. pennsylvanicus Hb, and from M. abbreviatus Hb-f. The alpha peptide compositions of M. abbreviatus Hb-f have been found to be representative of most of the alpha peptides from the other species: M. oeconomus Hb-f, M. miurus Hb-f, and M. abbreviatus Hb-s. The three large unlabeled spikes which appear regularly in all chromatograms are due to cysteic acid, norleucine, and ammonia, respectively, from left to right in the chromatograms.

Peptides Alpha-T1 and Alpha-T2.

See Tables 4-6; Figures 12 and 13.

Table 4.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT1 (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human[b]	Microtus				Human	White
AA		MP	MAf	MAs	MMf[b]	MOf		Mouse
K		0.91	0.68	1.17		1.13	1	1.00
H								
R								
B		2.17	2.18	1.78		1.85	1D	1.00
T								
S		1.06	0.90	0.88		0.99	1	0.95
Z								1.02
P							1	
G		1.09	1.04	1.15		0.98		0.99
A							1	
C								
V		1.08	0.66	0.92		0.70	1	0.99
M								
I								
L		0.96	0.88	0.55		0.89	1	0.99
O								
F								
W								
Peptide-								
μMol:	nd	0.020	0.024	0.004	nd	0.009		
Anal:		1992	2041	2197		2254		

[a]Compare Figure 12.

[b]Peptide not analyzed.

Table 5.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT(1-2) (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human	Microtus				Human	White
AA		MP[b]	MAf	MAs[b]	MMf	MOf		Mouse
K	1.63		1.73		1.66	1.94	2	
H								
R								
B	2.02		3.23		3.22	2.52	1D 1N	
T	1.08		1.12		0.98	0.95	1	
S	1.11		1.15		0.98	1.38	1	
Z								
P	1.24						1	
G			1.03		0.90	1.15		
A	1.20						1	
C								
V	1.75		0.94		0.86	1.08	2	
M								
I			0.83		0.79	1.14		
L	1.28		0.86		0.92	1.08	1	
O								
F								
W								
Peptide-								
μMol:	0.014	nd	0.017	nd	0.012	0.004		
Anal:	1948		2035		2075	2158		

[a]Compare Figure 12.

[b]Peptide not found.

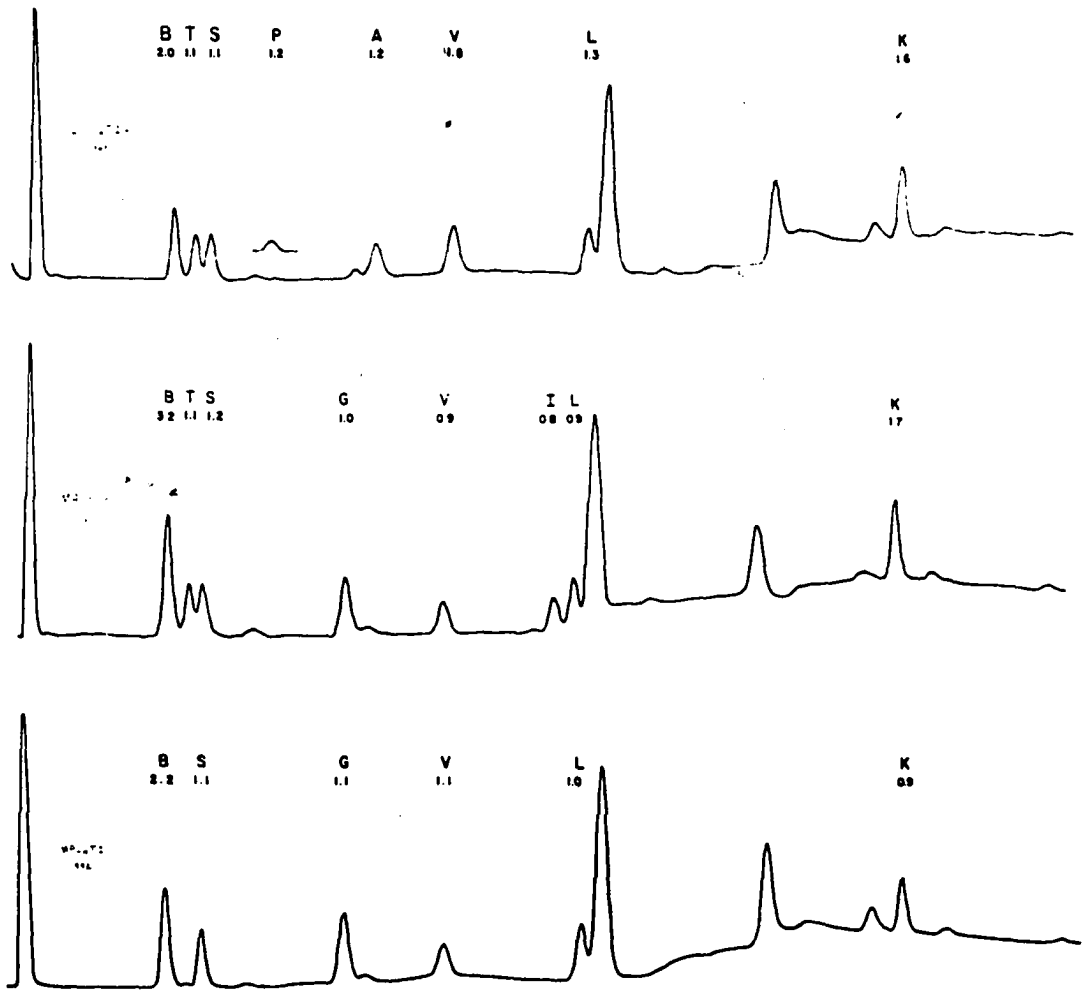


Figure 12. Chromatograms of Peptides αT1 and αT(1-2).

TOP: Human Hemoglobin, αT(1-2).

CENTER: M. abbreviatus Hb-f, αT(1-2).

BOTTOM: M. pennsylvanicus Hb, αT1.

Table 6.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT2 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	0.92	0.86	0.89	1.22	1.06	1.31	1	0.96
H								
R								
B	1.01	1.08	1.20	1.04	1.10	1.00	1N	1.09
T	0.81		0.69	0.58	0.58	0.59	1	
S		0.79						0.85
Z								
P								
G								(0.21)
A								
C								
V	1.21						1	
M								
I		1.02	0.94	0.81	1.01	1.00		0.95
L								
O								
F								
W								
Peptide-								
μMol:	0.028	0.028	0.025	0.008	0.017	0.010		
Anal:	1988	2003	2044	2125	2259	2273		

[a]Compare Figure 13.

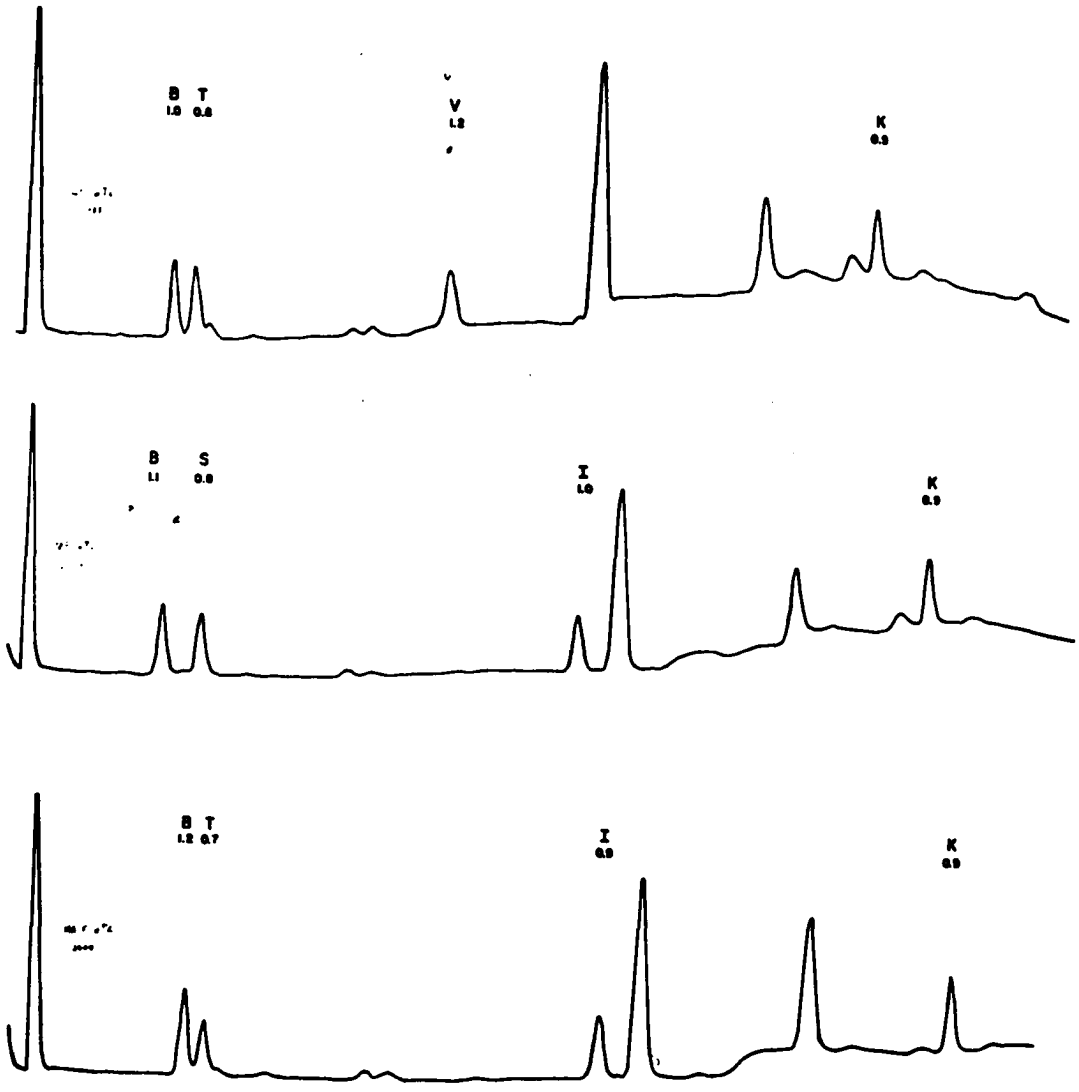


Figure 13. Chromatograms of Peptides αT2.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

The first two tryptic peptides, numbering from the N-terminal of the alpha chains of hemoglobin from human, from white mouse and from Microtus, are the lysine-containing peptides alpha-T1 and alpha-T2 which are made up of 7 and 4 amino acids respectively. The peptide maps of human hemoglobin and of M. abbreviatus Hb-f showed also the third composite peptide alpha-T(1-2) which did not appear in the map of M. pennsylvanicus Hb.

The human peptides show the compositions anticipated by the reference integers in Tables 4, 5, and 6. Relative to the human alpha-T1 peptide there appears to be a substitution for proline and alanine by glycine and free aspartic acid in Microtus: the Microtus alpha-T1 is displaced towards the anode compared to the human alpha-T1 (Figures 4-9). Relative to the white mouse hemoglobin peptide alpha-T1, there appears to be one less free glutamic acid and one more free aspartic acid in Microtus (Tables 4 and 5).

Peptides alpha-T2 of Microtus and of human hemoglobin occupy similar map positions. Peptide alpha-T2 of M. pennsylvanicus is distinguished by the presence of serine and isoleucine and by the absence of valine and threonine as found in human alpha-T2. To this extent it resembles peptide alpha-T2 from the white mouse strain (Table 5).

Peptide alpha-T2 of M. abbreviatus Hb-f is different from either alpha-T2 from human or from M. pennsylvanicus Hb and has isoleucine and threonine in its composition.

Peptides Alpha-T3.

See Table 7; Figure 14.

Table 7.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT3 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP[c]	MAf	MAs	MMf	MOf		Mouse
K	1.05	1.8/2 (1.8)	0.88	1.16	1.08	1.00	1	1.04
H	(1.1)	(3.3)						
R								
B		(1.2)	0.84	0.93	0.72	0.62		
T		2.3/2						
S								
Z								
P								
G	0.97	2.0/2 (1.2)	1.06	1.01	1.02	0.86	1	1.03
A	1.97	2.0/2 (2.3)	1.18	0.84	1.08	1.02	2	1.94
C								
V		(0.6)						
M								
I		(0.5)						
L		(1.1)						
O	(0.6)	(1.0)						
F		(0.6)						
W	+	+	+	+	+	+	1	1.00
Peptide-								
μMol:	0.032	0.020	0.033	0.010	0.026	0.016		
Anal:	1970	2007	2043	2224	2139	2147		

[a]Compare Figure 14.

[b,c]Numbers in parentheses pertain to [b]coincident peptide βT15 and [c]coincident peptides βT15 and 16'.

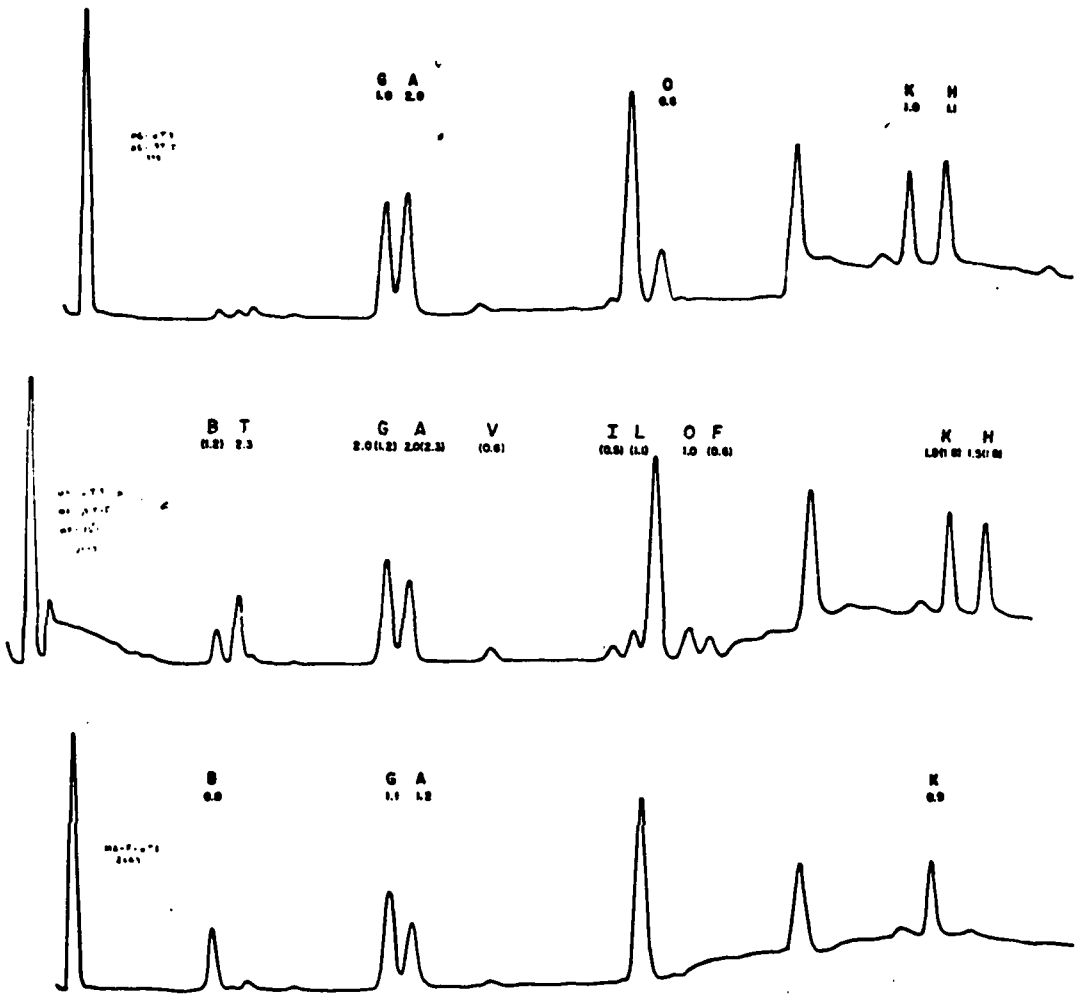


Figure 14. Chromatograms of Peptides αT3.

TOP: Human Hemoglobin (βT15 present).

CENTER: M. pennsylvanicus Hb (βT15, 16' present).

BOTTOM: M. abbreviatus Hb-f.

Peptide alpha-T3 from M. abbreviatus Hb-f occupies a map position different from that observed for the alpha-T3 peptide from human hemoglobin or from M. pennsylvanicus hemoglobin (Figures 4-9). Both of the latter peptides are located at sites in common with the beta-T15 peptide.

Among the 5 amino acids of the alpha-T3 peptides is one tryptophan (W) which does not survive acid hydrolysis. Hence only 4 amino acids appear in the amino acid analyses for these peptides. Qualitative evidence for the presence of tryptophan in peptides alpha-T3 was obtained by specific staining of the intact peptides for tryptophan on separate peptide maps.

The amino acid analysis of the M. abbreviatus Hb-f peptide alpha-T3 shows one additional aspartic acid (probably the amide since all alpha-T3 peptides had similar electrophoretic positions) and one less alanine relative to the corresponding human or white mouse peptide alpha-T3. A third peptide, unidentified and designated 16', is associated with beta-T15 in the M. abbreviatus Hb-f map (Figure 36) and with beta-T15 and alpha-T3 in the M. pennsylvanicus map (Figure 14). If the amino acid components of the unidentified peptide 16' from M. abbreviatus Hb-f are subtracted proportionately from the same components in the mixed M. pennsylvanicus peptide, after allowing a correction for the presence of beta-T15, there remain the components alanine, glycine, threonine and lysine in approximately equimolar proportions from the analysis of the M. pennsylvanicus peptide. It can be inferred that this represents a unique alpha-T3 peptide in which there is one additional

threonine and one less alanine relative to the corresponding human or white mouse hemoglobin peptide alpha-T3.

The situation can be summarized in terms of the four independent analyses which were useful in extricating the results for M. pennsylvanicus:

Hb Analysis	Peptides		
	Alpha-T3	Beta-T15	16'
Human (Fig. 14)	K,G,A,A,W	O,H	(Possibly related to Beta-T12)
M. abbreviatus-f (Fig. 14)	K,G,A,N,W		
M. abbreviatus-f (Fig. 36)		O,H	2K,2H,B,G,2A,V,I,L,F
M. pennsylvanicus (Fig. 14)	K,G,A,T,W	O,H	2K,2H,B,G,2A,V,I,L,F

Peptide alpha-T3 of Microtus pennsylvanicus hemoglobin appears to differ in composition from the peptides alpha-T3 of hemoglobins from human, from white mouse strain C57BL, and from the other species of Microtus.

Peptides Alpha-T4.

See Table 8; Figure 15.

Like the corresponding human hemoglobin peptide, Microtus hemoglobin peptides alpha-T4 consist of 15 amino acids, and they occupy map positions similar to that occupied by human alpha-T4. But peptide alpha-T4 of M. pennsylvanicus hemoglobin appears to differ from the corresponding peptides of human hemoglobin (one more isoleucine and one less valine), of white mouse hemoglobin (one more alanine and one less glycine), and of the other Microtus hemoglobins (one more alanine and one less glycine). The analysis for the M. pennsylvanicus peptide alpha-T4 contains a small proportion of another arginine peptide, beta-T11.

Table 8.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT4 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP[b]	MAf	MAs	MMf	MOf		Mouse
K								
H	0.86	1.08	1.01	0.95	1.06	0.90	1	0.97
R	0.86	1.24	1.04	0.72	1.05	0.86	1	1.03
B		(0.8)						
T								
S								
Z	2.46	3.20	2.96	3.30	2.96	2.86	3E	2.70
P								
G	3.30	3.20	3.70	3.76	4.00	4.06	3	4.07
A	4.30	3.80	3.48	2.68	3.12	3.05	4	3.25
C								
V	1.11	(0.3)					1	
M								
I		0.74	0.88	0.39	0.70	0.81		0.94
L	1.09	1.34	1.10	0.66	1.01	0.98	1	0.97
O	0.42	0.57	0.64	0.21	0.67	0.16	1	1.07
F		(0.2)						
W								
Peptide								
μMol:	0.047	0.028	0.032	0.010	0.030	0.016		
Anal:	1966	2030	2034	2188	2247	2113		

[a]Compare Figure 15.

[b]Numbers in parentheses pertain to coincident peptide βT11.

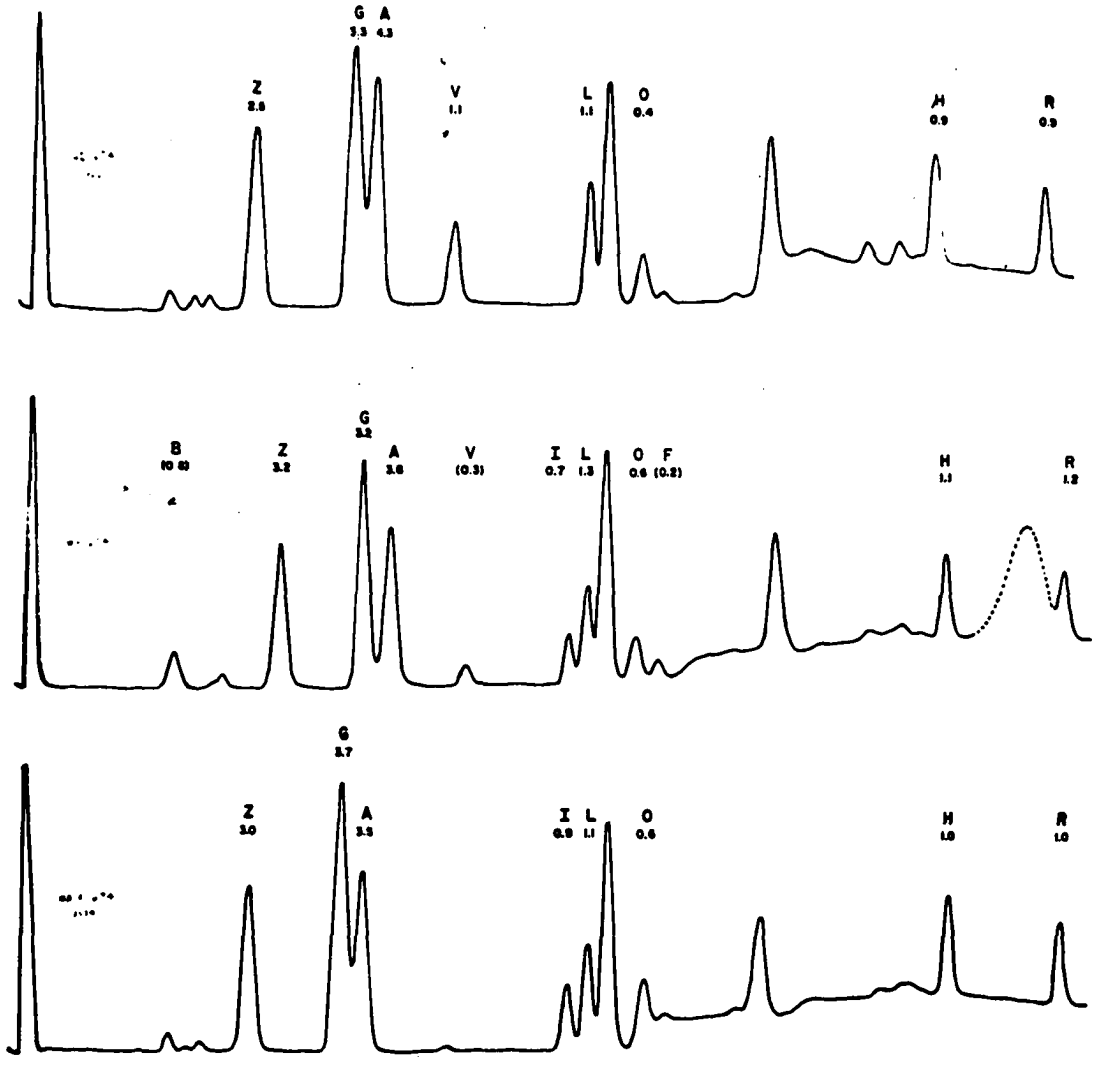


Figure 15. Chromatograms of Peptides αT4.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Peptides Alpha-T5.

See Table 9; Figure 16.

Human peptide alpha-T5 was not completely separated in the present system from its faster and slower neighbors migrating in the chromatographic direction. Although three discrete spots are present on the peptide map, the alpha-T5 spot is contaminated by its faster neighbor beta-T12a. Peptide alpha-T5 sometimes occurred as a considerable contaminant with its slower neighbor beta-T2. It is possible to "resolve" the overlapping peptides in terms of their relative molar quantities, and these are given in Table 9. An analysis for the 9 amino acids of the human alpha-T5 is obtained.

From tryptic digests of Microtus hemoglobins, fast-moving alpha-T5 peptides composed of the same 9 amino acids appear to be cleanly separated from neighboring peptides; in these cases there are no peptides moving faster than alpha-T5. All alpha-T5 peptides observed have similar map positions.

From tryptic digests of M. abbreviatus Hb-f and of M. miurus Hb-f additional peptides were isolated which have a composition consistent with alpha-T5 but which moved more slowly than beta-T2 in the chromatographic direction. These peptides are designated alpha-T5' (Table 10).

The Microtus hemoglobin alpha-T5 peptides possess single valine, phenylalanine and tyrosine in their amino acid composition rather than the one serine and the two phenylalanines found in the alpha-T5 peptide of white mouse. The Microtus hemoglobin alpha-T5 peptides possess the amino acids valine, tyrosine and alanine which are not found in the

Table 9.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT5 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP[c]	MAf	MAs[c]	MMf[c]	MOf		Mouse
K	1.02	0.87	1.09	1.01	0.84	0.98	1	1.08
H				(0.5)				
R								
B	(0.5)							
T	2.00	2.04	1.95	1.70	1.75	1.87	2	1.83
S	1.30	(0.4)			(0.4)		1	0.95
Z								
P	1.33	1.05	0.94	+	1.21	0.61	1	1.09
G	(0.6)	(0.1)			(0.3)			
A		1.22	1.24	1.01	1.09	1.14		0.95
C	(0.6)							
V	(1.2)	1.15	1.17	0.88	1.12	0.96		
M	0.21	0.16	0.27	+	+	+	1	0.75
I								
L	0.8 (1.4)	(0.3)					1	
O		0.62	0.79	+	0.76	nd		
F	1.65	0.80	1.20	+	0.87	0.74	2	1.96
W								
Peptide-								
μMol:	0.020	0.018	0.016	0.007	0.012	0.010		
Anal:	1989	2036	2065	2106	2219	2153		

[a]Compare Figure 16.

[b,c]Numbers in parentheses pertain to [b]coincident peptide βT12a and [c]amino acids not belonging to peptides αT5.

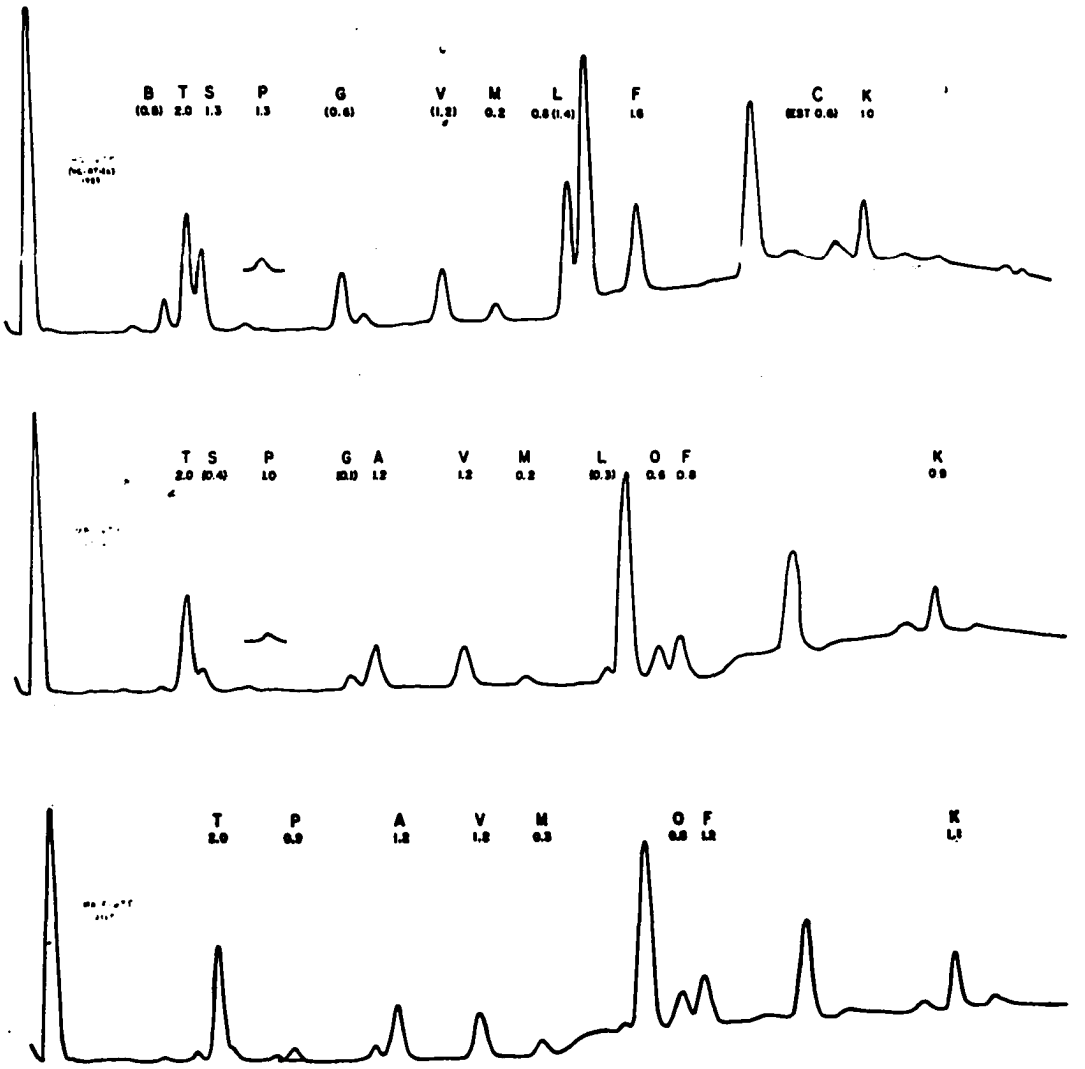


Figure 16. Chromatograms of Peptides αT5.

TOP: Human Hemoglobin (βT12a present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 10.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT5' (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human	Microtus				Human	White
AA		MP[c]	MAf[b]	MAs[c]	MMf[b]	MOf[c]		Mouse
K			0.84		0.92		
H							
R							
B							
T			2.02		1.77		
S			(0.5)				
Z							
P			0.74		1.00		
G			(0.2)		(0.2)		
A			1.20		1.10		
C							
V			1.01		1.05		
M			TRACE		0.35		
I							
L							
O			0.56		0.65		
F			0.84		0.93		
W							
Peptide-							
μMol:			0.010		0.012		
Anal:			2060		2213		

[a]Compare Figure 16.

[b]Numbers in parentheses pertain to amino acids not belonging to peptides αT5'.

[c]Corresponding peptides were not found for these Microtus.

corresponding human peptide in which leucine, phenylalanine and serine are present.

Peptides Alpha-T6.

See Table 11; Figure 17.

Human hemoglobin peptide alpha-T6 was found to be composed of the expected 16 amino acids after correction for the presence of the coincident arginine-lysine peptide alpha-T(10-11).

Microtus hemoglobin peptides alpha-T6 were identified on the basis of their map positions and their compositions which are similar to those of the human hemoglobin peptide alpha-T6. The Microtus hemoglobin peptides are well-resolved from the neighboring peptide alpha-T(10-11). Like peptide alpha-T6 of white mouse hemoglobin, the Microtus hemoglobin peptides alpha-T6 contain a second valine but not the leucine of the corresponding human peptide. The amino acid tyrosine was poorly recovered from all these peptides. Although tyrosine does not appear in the chromatogram for the M. oeconomus hemoglobin peptide, it is inferred to complete the complement of 16 amino acids in the peptide.

Peptides Alpha-T7, Alpha-T8 and Alpha-T(7-8).

See Tables 12 and 13; Figures 18 and 19.

Two closely neighboring peptides, obtained from the digests of human hemoglobin, are identified from their compositions as alpha-T7 and beta-T7 with 4 amino acids each. The corresponding Microtus peptides coincide in their map position and are analyzed as alpha-beta-T7. All Microtus hemoglobin peptides alpha-beta-T7 show the same composition of 8 amino acids as illustrated for M. miurus Hb-f. The coincidence of the

Table 11.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT6 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	0.7 (0.3)	1.00	0.86	1.17	1.03	1.24	1	0.91
H	1.74	2.07	2.06	2.60	2.08	1.97	2	1.90
R	(0.3)							
B	1.0 (0.6)	1.21	1.08	1.15	0.90	1.07	1D	1.02
T	0.92	0.92	1.08	0.42	0.57	0.68	1	0.63
S	1.92	2.10	2.02	1.70	1.97	2.12	2	2.11
Z	0.90	1.10	1.03	1.22	1.13	1.15	1Q	1.08
P	0.9 (0.3)	0.92	1.12	1.12	0.98	1.30	1	0.97
G	1.15	1.10	1.14	1.17	1.19	1.13	1	1.33
A	1.33	1.19	1.12	1.00	1.16	1.13	1	1.06
C								
V	1.2 (0.6)	2.02	1.93	1.96	2.10	2.08	1	1.99
M								
I								
L	1.2 (0.3)						1	
O	0.34	0.16	0.62	0.68	0.72	nd	1	0.90
F	1.7 (0.3)	1.67	1.95	1.78	1.94	1.75	2	1.90
W								
Peptide-								
μMol:	0.040	0.016	0.031	0.008	0.030	0.012		
Anal:	1940	2009	2053	2230	2243	2143		

[a] Compare Figure 17.

[b] Numbers in parentheses pertain to amino acids belonging to coincident peptide αT(10-11).

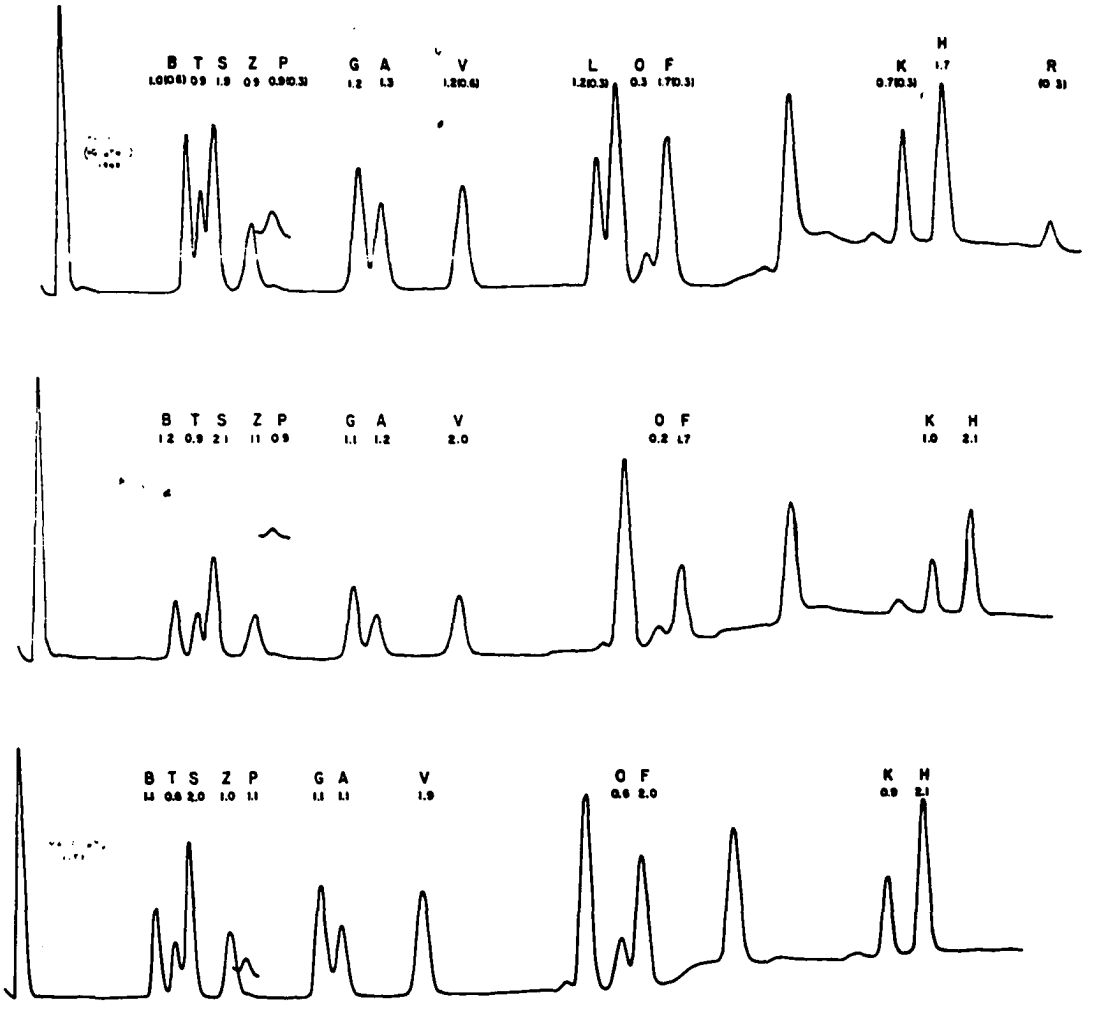


Figure 17. Chromatograms of Peptides αT6.

TOP: Human Hemoglobin (αT(10-11) present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 12.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT7 (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human	Microtus[b]				Human	White
AA		MP[c]	MAf	MAs	MMf	MOf		Mouse
K	1.14		2.00	2.20	1.96	2.24	1	0.94
H	1.14		1.92	2.02	2.22	1.96	1	1.11
R								
B								
T								
S								
Z								
P								
G	1.78		2.12	2.22	2.08	1.90	2	1.95
A			1.54	1.31	1.55	1.08		
C								
V								
M								
I								
L								
O								
F								
W								
Peptide-								
μMol:	0.015	nd	0.092	0.012	0.038	0.032		
Anal:	1971		2199	2229	2068	2267		

[a]Compare Figure 18.

[b]Numbers for Microtus pertain to amino acids belonging to coincident peptides αβT7.

[c]No data were obtained for this peptide.

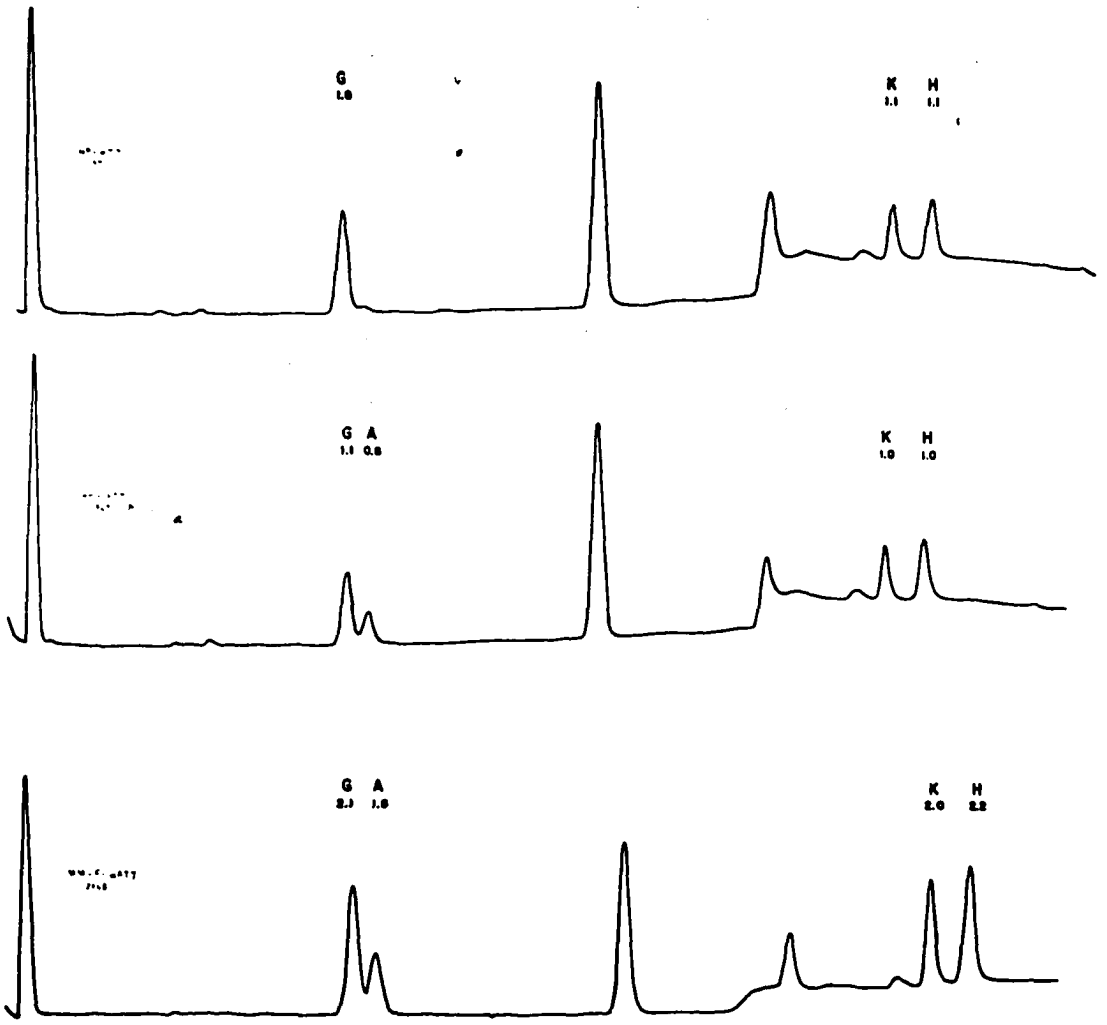


Figure 18. Chromatograms of Peptides αT7 and βT7.

TOP: Human Hemoglobin, αT7.

CENTER: Human Hemoglobin, βT7.

BOTTOM: M. miurus Hb-f, αβT7.

Table 13.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αβT(7-8) (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human[b]	Microtus				Human	White
AA		MP	MAf	MAs	MMf[b]	MOf[b]		Mouse
K		4.04	4.20	3.97			4	4
H		2.29	2.10	2.28			2	2
R								
B								
T								
S								
Z								
P								
G		1.90	2.34	1.86			3	3
A		1.55	1.38	0.84			1	1
C								
V								
M								
I								
L								
O								
F								
W								
Peptide-								
μMol:	nd	0.010	0.025	0.004	nd	nd		
Anal:		2069	2180	2100				

[a]Compare Figure 19.

[b]No data were obtained for these peptides.

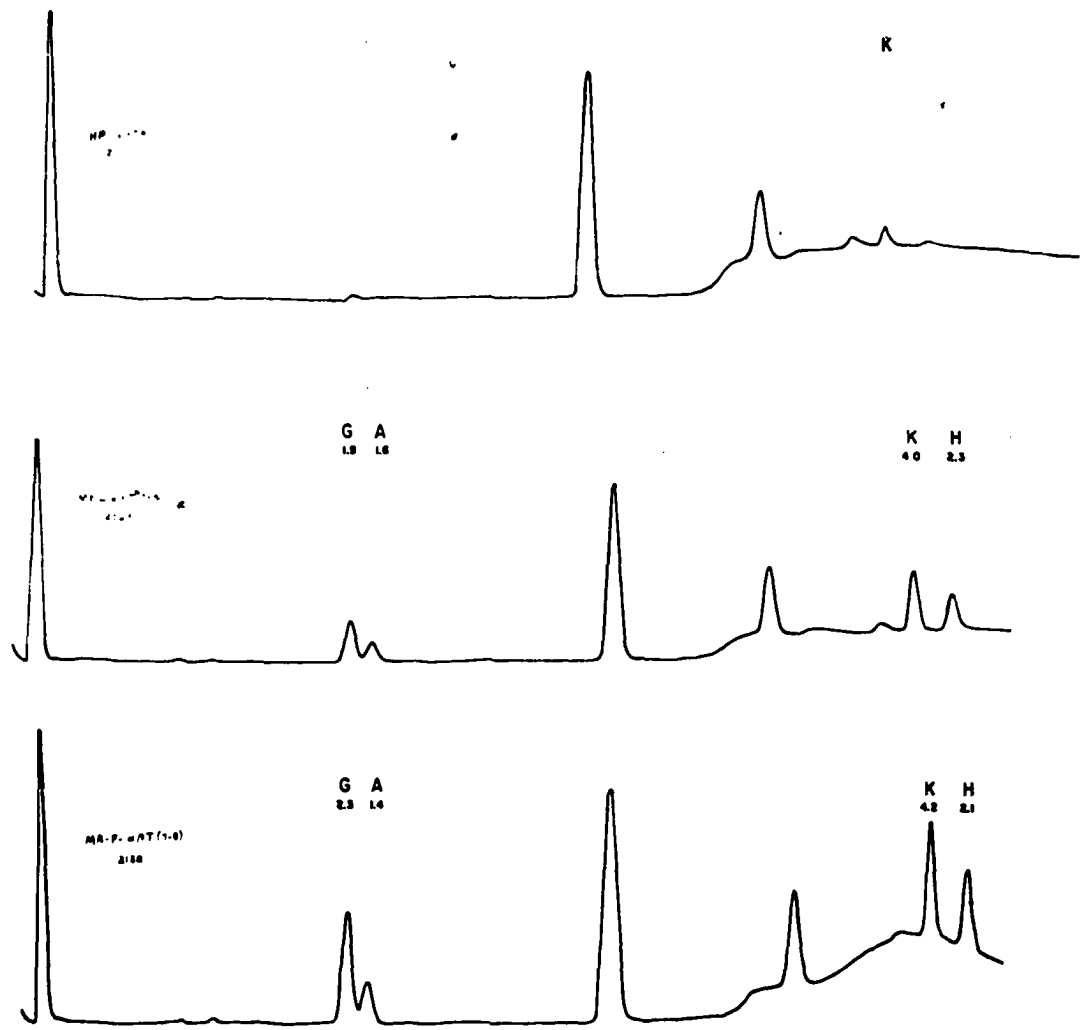


Figure 19. Chromatograms of Peptides αβT8 and αβT(7-8).

TOP: Human Hemoglobin, αβT8.

CENTER: M. pennsylvanicus Hb, αβT(7-8).

BOTTOM: M. abbreviatus Hb-f, αβT(7-8).

T7 peptides of Microtus hemoglobins appears to be related to the presence, in the peptides, of two glycines and two alanines rather than the three glycines and one alanine of the combined T7 peptides of human hemoglobin.

Free lysine was obtained among the peptides from digests of all the hemoglobins examined. It is attributed to the "peptides" alpha-T8 and beta-T8 in each case. Another peptide found in tryptic digests of all the hemoglobins examined is considered to be a composite of the T7 and T8 peptides and is shown as alpha-beta-T(7-8).

Peptides Alpha-T9 and Alpha-T(8-9).

See Table 14; Figures 25 and 34.

The alpha-T9 peptide, the largest peptide to be anticipated in tryptic digests of human hemoglobin, comprises 29 amino acids or 30 in the case that alpha-T8, lysine, is associated with it. A minor peptide of human hemoglobin was isolated and classified as alpha-T9 according to its analysis. Other peptides which were isolated together with peptides beta-T1 and beta-T13 were classified as alpha-T(8-9) and alpha-T9 following calculations of the results from the analyses of these peptides.

Peptides alpha-T9 were not identified in tryptic hydrolysates of Microtus hemoglobins. They will be considered further in the Discussion in terms of certain unidentified peptides which were obtained from Microtus hemoglobins.

Peptides Alpha-T10.

See Table 15; Figure 20.

Table 14.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT9 (in Residues of AA per Peptide).

	EXPERIMENTAL				REFERENCE	
	Human	Human[b]	Human[c]	Microtus[d]	Human	White Mouse
AA						
K	1.34	1.87	0.79		1	0.96
H	2.66	2.61	2.29		3	2.77
R						
B	5.95	6.30	7.18		4D 2N	4.88
T	1.10	0.89	1.34		1	
S	2.18	2.61	3.04		2	1.86
Z	(0.5)					
P	TRACE	0.89	1.20		1	1.05
G	(0.6)					2.04
A	6.67	6.60	6.76		7	8.30
C						
V	2.82	2.95	2.74		3	1.01
M	0.45	0.33	TRACE		1	
I						
L	nd	3.69	4.21		4	6.18
O						
F						
W						
Peptide-						
μMol:	0.049	0.010	0.011			
Anal:	1947	1960	1961			

[a]Compare Figures 25 and 34.

[b,c]Numbers represent [b]peptide αT(8-9) taken from data combined with peptide βT1; [c]peptide αT9 taken from data combined with peptide βT13.

[d]These peptides were not identified in terms of the available analyses.

Table 15.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT10 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K								
H								
R	1.03	1.10	1.24	0.89	1.00	+	1	1.03
B								
T								
S								
Z								
P								
G								
A								
C								
V								
M								
I								
L	0.83	0.81	0.55	0.19	0.36	+	1	0.97
O								
F								
W								
Peptide-								
μMol:	0.019	0.017	0.023	0.010	0.016	<0.004		
Anal:	1949	1999	2094	2235	2091	2169		

[a]Compare Figure 20.

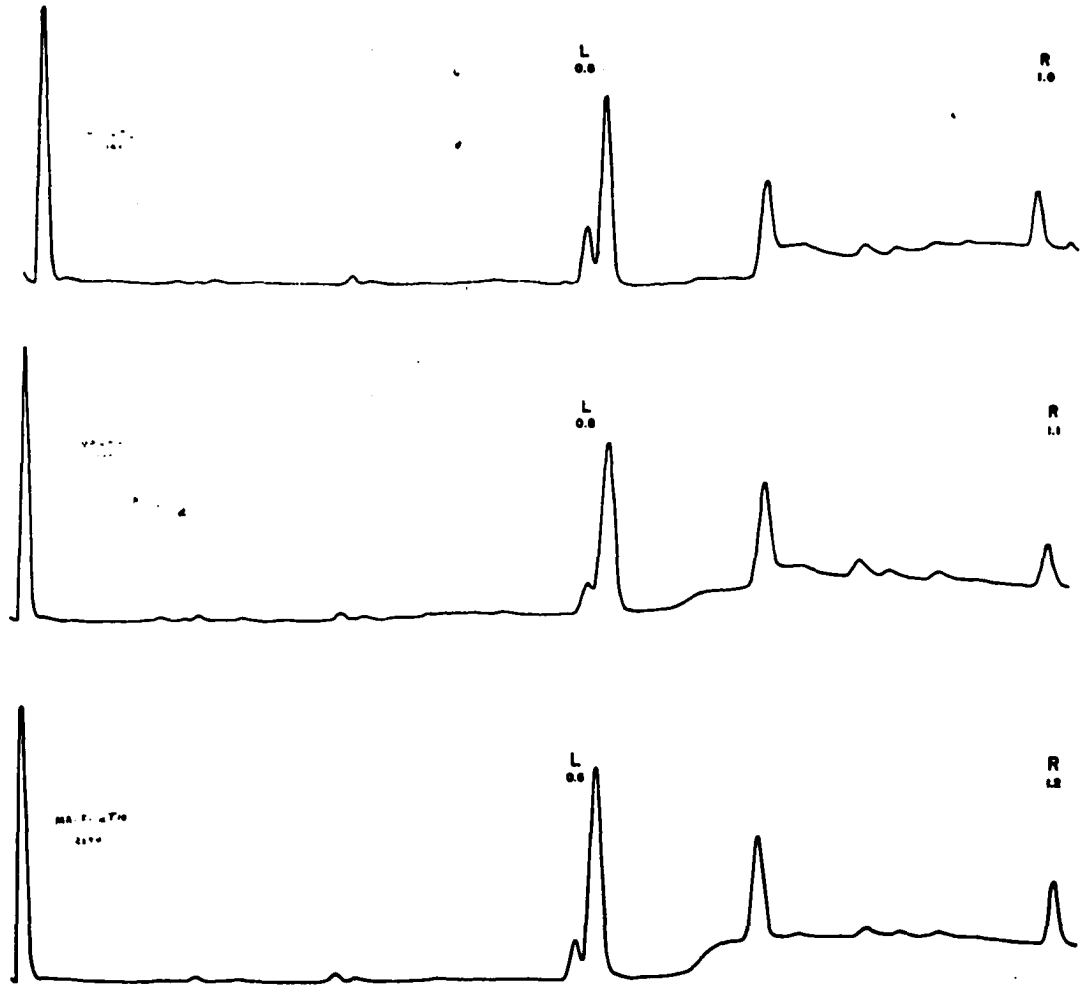


Figure 20. Chromatograms of Peptides αT10.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

The dipeptide alpha-T10 was found to occupy a similar map position and to have the same composition when isolated from tryptic digests of human or of Microtus hemoglobins.

Peptides Alpha-T11 and Alpha-T(10-11).

See Table 16; Figure 21.

The peptide alpha-T11, recognized first in tryptic digests of M. abbreviatus Hb-f, was identified only in combination with peptide alpha-T10 as an arginine- and lysine-containing peptide. Subsequently, peptide alpha-T(10-11) of human hemoglobin was detected in the presence of the human peptide alpha-T6 described above (Figure 17).

All peptides alpha-T(10-11) examined appeared to have the same 9 amino acids in their composition although the peptides from M. pennsylvanicus Hb and from M. abbreviatus Hb-s were recovered in only small amounts. The same amino acid composition is shared by the hemoglobin peptides alpha-T(10-11) from white mouse.

Peptides Alpha-T12: Alpha-T12a and Alpha-T12b.

See Tables 17 and 18; Figures 22 and 23.

Complete peptides alpha-T12 were not identified in the soluble portion of tryptic digests of human or of Microtus hemoglobins. However, peptides were obtained which represent the same N-terminal fragments of peptide alpha-T12 in each of the hemoglobin digests. The peptide fragments, designated alpha-T12a, occupy similar map positions and possess identical compositions of 5 amino acids. The composition of human hemoglobin peptide alpha-T12a was determined together with that of peptide beta-T12b which occupied the same spot on the peptide map.

Table 16.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT(10-11) (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP[c]	MAf	MAs	MMf[c]	MOf		Mouse
K	1.0	1.30	1.23	+	0.94	1.29	1	0.94
H		(0.6)						
R	1.0	0.90	0.87	+	0.66	0.81	1	1.03
B	2.0	2.03	2.22	+	2.13	1.82	1D 1N	1.83
T								
S		(1.1)			(0.2)			
Z								
P	1.0	TRACE	1.24	nd	0.78	TRACE	1	0.98
G								
A								
C								
V	2.0	1.79	1.96	+	2.09	1.95	2	2.23
M								
I								
L	1.0	0.96	0.65	+	0.62	0.47	1	0.97
O								
F	1.0	1.23	0.92	+	0.89	1.03	1	1.02
W								
Peptide-								
μMol:	0.030	0.007	0.010	<0.004	0.009	0.006		
Anal:	1940	2027	2054	2107	2138	2121		

[a]Compare Figure 21.

[b]Determined in the presence of peptide αT6.

[c]Numbers in parentheses pertain to amino acids not belonging to peptides αT(10-11).

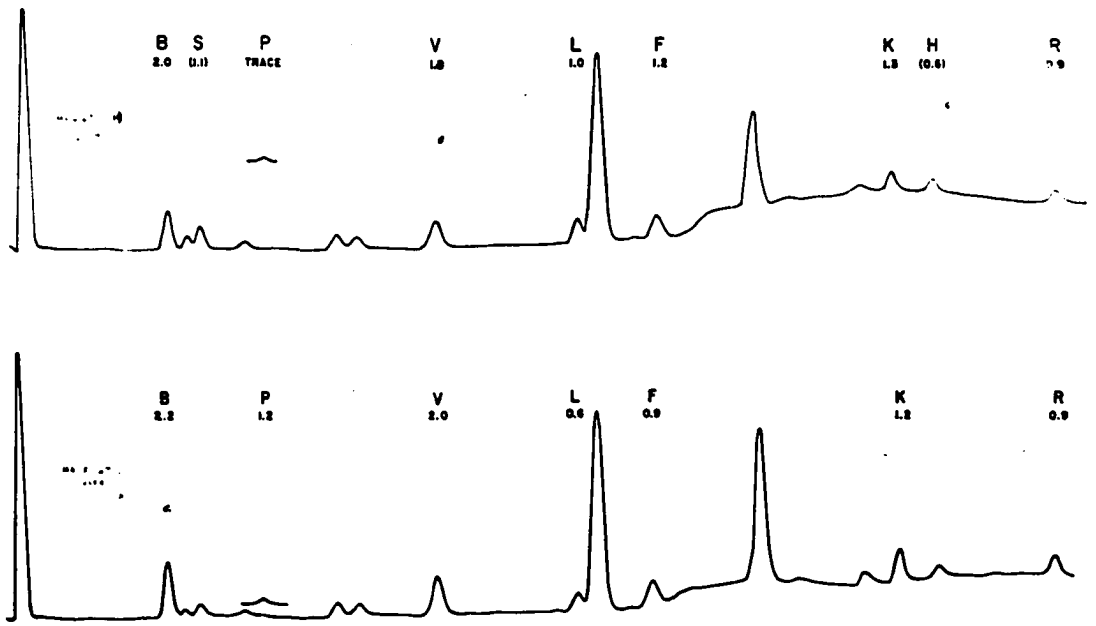


Figure 21. Chromatograms of Peptides αT(10-11).[a]

TOP: M. pennsylvanicus Hb.

CENTER: M. abbreviatus Hb-f.

[a]See Figure 17 for Peptide αT(10-11) of Human Hemoglobin.

Table 17.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT12a (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE[c]	
	Human[b]	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf	12a+12b	Mouse
K							+1	1.
H	1.14	1.03	1.16	1.16	1.14	1.24	1+2	4.
R								
B							+1D	2.
T							+2	2.
S	0.98	1.12	1.12	1.08	1.06	1.29	1+1	3.
Z							+1E	
P							+2	2.
G								
A							+5	4.
C	0.69	0.25	TRACE	TRACE	nd	nd	1	1.
V							+2	2.
M								
I								
L	1.85	1.78	1.76	1.64	1.77	1.65	2+5	6.
O								
F							+1	1.
W								
Peptide-								
μMol:	0.021	0.014	0.015	0.005	0.010	0.006		
Anal:	1973	1990	2176	2201	2198	2182		

[a]Compare Figure 22.

[b]Determined in the presence of coincident peptide βT12b.

[c]Numbers pertain to total peptide, αT12a+αT12b.

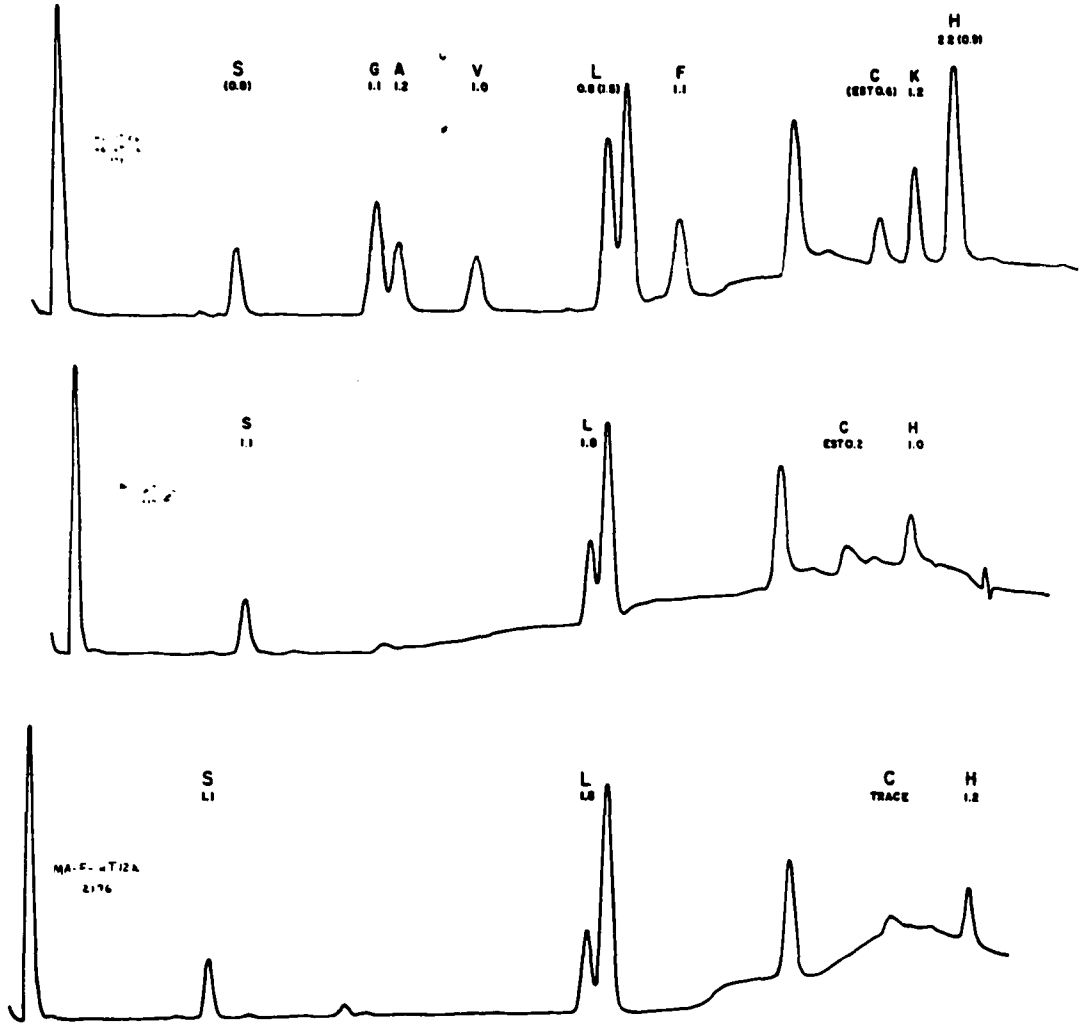


Figure 22. Chromatograms of Peptides αT12a.

TOP: Human Hemoglobin (βT12b present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 18.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT(12b-13) (in Residues AA per Peptide).

	EXPERIMENTAL[b]						REFERENCE[c]	
	MP	MP	MP	MP	MAs	MOf	Human	White
AA	12b(2)	12b(3)	12b-13fn	13-fc	13-fc	13-fc		Mouse
K			0.82	0.95	1.11	0.84	1	1.22
H	0.99	1.04						(0.23)
R								
B			1.25					
T		0.46		0.38	0.70	0.62	2	2.
S			0.90	0.92	1.18	0.99	3	2.83
Z	1.05							
P	1.04	1.30						(0.33)
G								(0.37)
A	2.05	1.27	0.54				1	1.12
C								
V		1.09					2	1.93
M								
I								(0.15)
L	1.05		0.98				2	1.97
O								
F	0.88		0.90				1	1.02
W								
Peptide-								
μMol:	0.011	0.011	0.010	0.010	0.008	0.010		
Anal:	2039	2057	2045	2278	2109	2118		

[a]Compare Figure 23.

[b]Numbers pertain to peptide fragments as indicated.

[c]Numbers pertain to total peptides αT13.

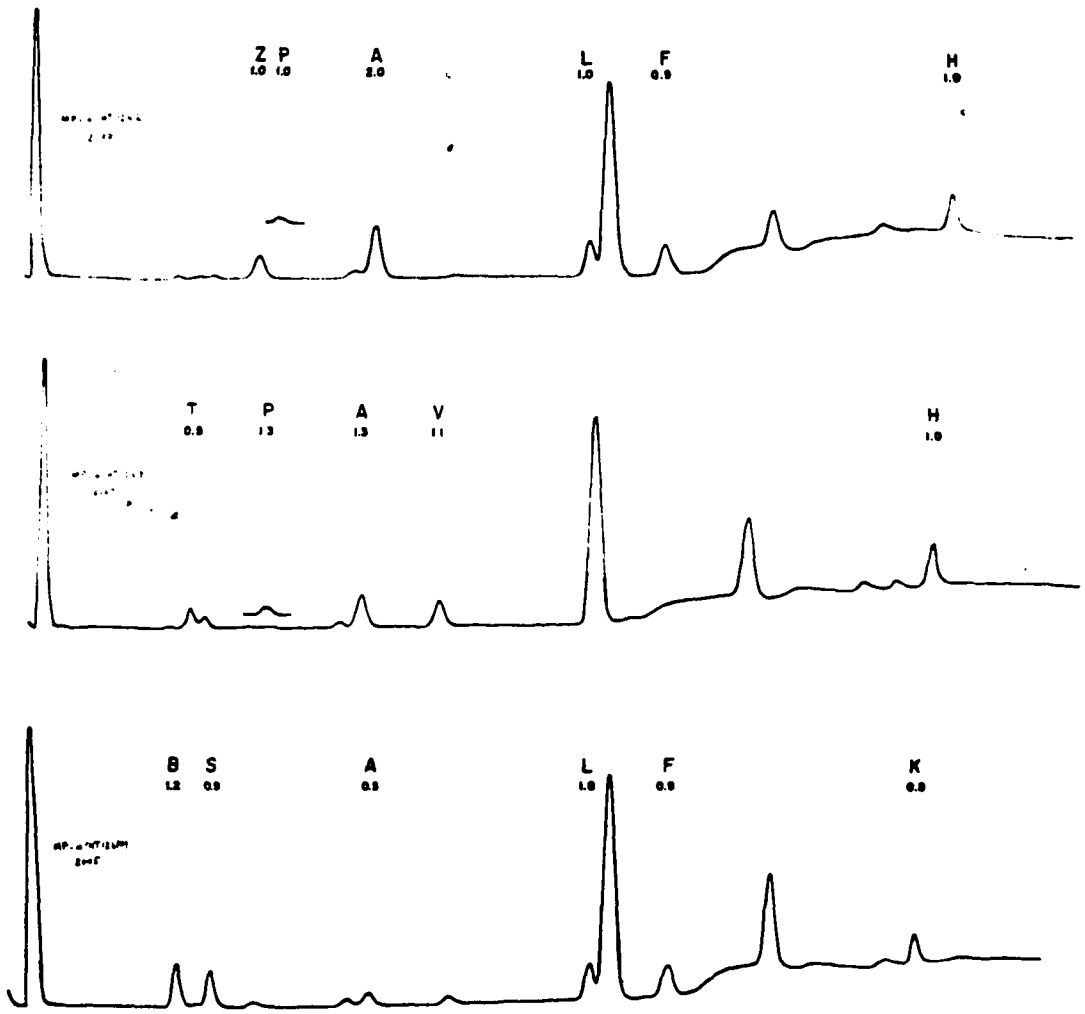


Figure 23. Chromatograms of Peptide αT(12b-13), Consecutive Chymotryptic Fragments from M. pennsylvanicus Hb.

TOP: αT12b(2).

CENTER: αT12b(3).

BOTTOM: αT(12b-13fn).

C-terminal fragments alpha-T12b were not observed in the soluble portion of tryptic digests.

Insoluble residues from a tryptic digest of M. pennsylvanicus hemoglobin were digested with chymotrypsin. Several chymotryptic peptides which were isolated following peptide mapping were identified as fragments of peptides alpha-T12b and alpha-T13. These chymotryptic fragments are designated 12b(2), 12b(3), 12b-13fn, and 13fc in Table 18. The compositions of these fragments represent approximately half of the expected 33 amino acids from peptides alpha-T12b and alpha-T13. The fragments appear to have the same composition as specific sections of the human hemoglobin alpha-T12b and alpha-T13 peptides. No results were obtained from chymotryptic digests of tryptic residues from other Microtus hemoglobins or from human hemoglobin.

Peptides Alpha-T13.

See Table 18.

Complete peptides alpha-T13 were not identified. No evidence was found for this peptide in tryptic digests of human or of Microtus hemoglobins with three exceptions. A peptide designated alpha-T13fc, which was obtained from tryptic digests of M. abbreviatus Hb-s and of M. oeconomus Hb-f, could represent a soluble C-terminal fragment of alpha-T13. Two chymotryptic peptides obtained from the tryptic residues from M. pennsylvanicus hemoglobin were estimated as the fragments alpha-T(12b-13fn) and alpha-T13fc.

Peptides Alpha-T14.

See Table 19; Figures 24 and 35.

Table 19.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT14 (in Residues AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K								
H								
R	1.12	1.00	0.90	1.17	1.06	0.99	1	1.04
B								
T								
S								
Z								
P								
G								
A								
C								
V								
M								
I								
L								
O	0.84	0.59	0.43	+	0.43	+	1	0.96
F								
W								
Peptide-								
μMol:	0.014	0.028	0.034	0.010	0.040	0.020		
Anal:	1974	2001	2051	2244	2189	2157		

[a]Compare Figures 24 and 35.

[b]Determined in the presence of coincident peptide βT14.

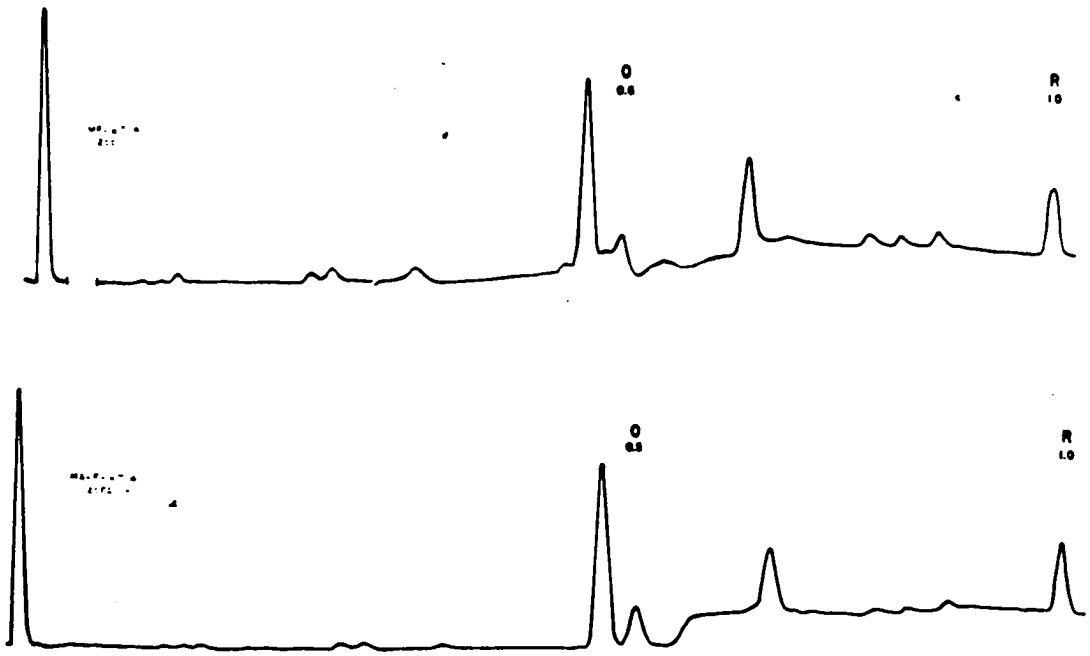


Figure 24. Chromatograms of Peptides αT14.[a]

TOP: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

[a]See Figure 35 for Peptide αT14 of Human Hemoglobin.

The dipeptides alpha-T14 show similar map positions and the same amino acid composition when obtained from human or from Microtus hemoglobins. Human hemoglobin peptide alpha-T14 was determined in the presence of peptide beta-T14 from which it was not clearly separated on the peptide map. The same amino acid composition is shared by the hemoglobin peptide alpha-T14 from white mouse.

Tryptic Peptides of the Beta Chain of Hemoglobin.

Figures 25 through 36 reproduce chromatograms which were obtained for tryptic peptides of beta chains of hemoglobin. Figures 36 and 37 reproduce also chromatograms obtained for the unidentified tryptic peptides 16, 16', 17, and 18 from Microtus hemoglobins. The usual three chromatograms in each Figure compare beta-peptide compositions for hemoglobins of human, of M. pennsylvanicus, and of M. abbreviatus Hb-f. Beta-peptide compositions for M. abbreviatus Hb-f have been found to be generally representative of the corresponding beta-peptides of M. oeconomus Hb-f, of M. miurus Hb-f, and of M. abbreviatus Hb-s. The three large unlabeled spikes which appear regularly in all chromatograms are due to cysteic acid, norleucine and ammonia, respectively, from left to right in the chromatograms.

Peptides Beta-T1.

See Table 20; Figure 25.

The same relative map positions are occupied by the beta-T1 peptides which are the N-terminal peptides of the beta chains of human and of Microtus hemoglobins. Peptides beta-T1 from the hemoglobins of the several species of Microtus appear to be composed of the same 8 amino

Table 20.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT1 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP	MAf	MAs[c]	MMf[c]	MOf[c]		Mouse
K	0.7 (0.4)	1.06	1.03	1.21	1.09	1.04	1	1.01
H	0.7 (0.5)	0.83	0.86	0.96	1.00	0.76	1	1.03
R								
B	(1.3)	1.08	1.20	1.16	1.00	1.32		1.00
T	1.1 (0.2)	0.92	1.10	0.94	0.88	0.90	1	0.96
S	(0.5)			(0.26)				
Z	2.05	1.12	1.06	1.20	0.92	1.24	2E	1.00
P	1.1 (0.2)						1	
G	(0.2)			(0.23)	(0.09)	(0.11)		
A	(1.34)	1.11	1.24	1.16	1.06	1.11		1.04
C								
V	0.7 (0.6)	0.73	0.69	0.67	0.69	0.65	1	0.84
M								
I								
L	1.0 (0.8)	0.52	1.14	1.02	1.10	1.05	1	1.02
O								
F								
W								
Peptide-μMol:	0.052	0.018	0.032	0.011	0.029	0.015		
Anal:	1960	2005	2061	2206	2208	2152		

[a]Compare Figure 25.

[b]Numbers in parentheses pertain to coincident peptide αT(8-9).

[c]Numbers in parentheses pertain to amino acids not belonging to peptide βT1.

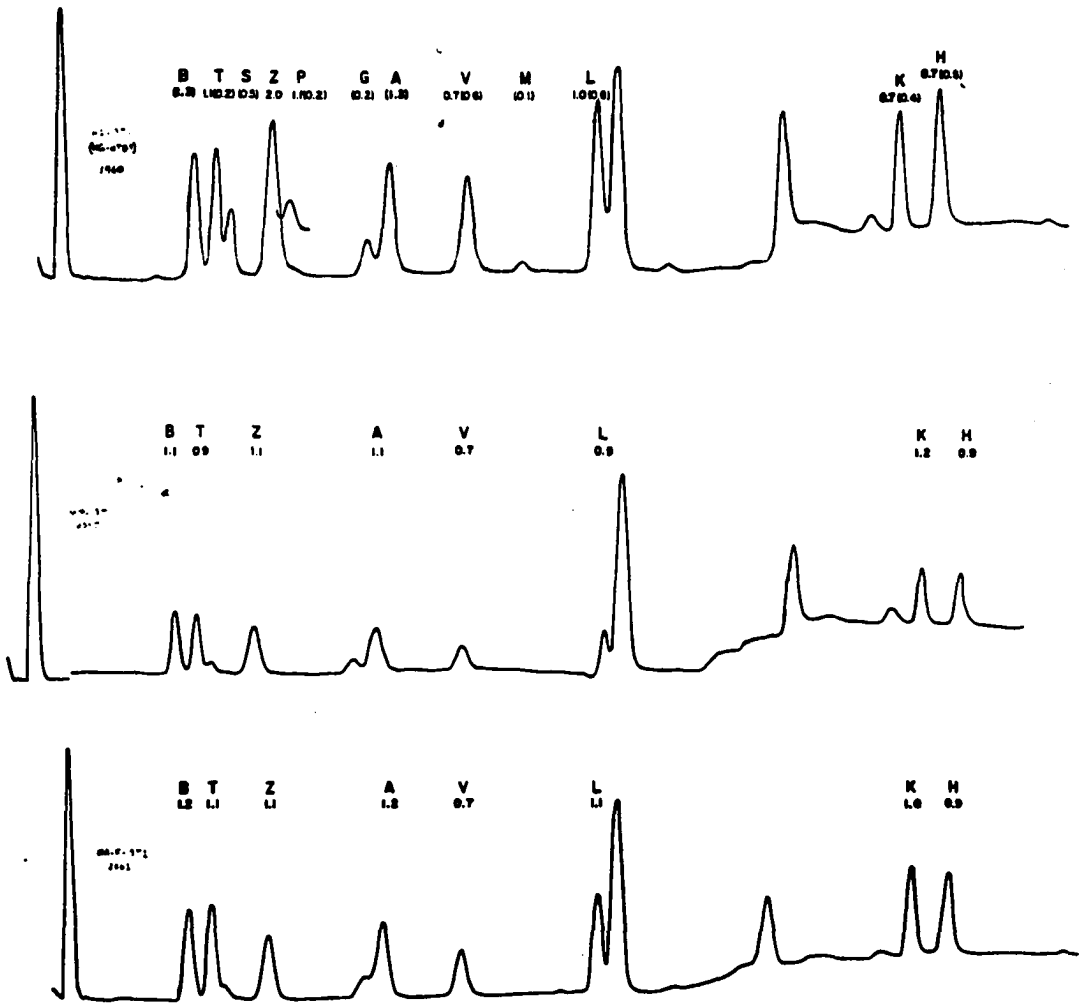


Figure 25. Chromatograms of Peptides βT1.

TOP: Human Hemoglobin (αT(8-9) present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

acids among themselves but to differ from those peptides reported for white mouse strain C57BL/6 hemoglobin and for human hemoglobin. Relative to the same human hemoglobin peptide the Microtus peptides beta-T1 have one more each of alanine and of aspartic acid, one less each of proline and of glutamic acid. Relative to the same white mouse hemoglobin peptide each Microtus beta-T1 peptide has one more aspartic acid and one less glutamic acid.

Peptides Beta-T2.

See Table 21; Figure 26.

Peptides beta-T2 from the hemoglobins of the several species of Microtus occupy similar map positions and appear to be composed of the same 9 amino acids among themselves but to differ in composition from those hemoglobin peptides reported for white mouse and for human. Independent qualitative evidence for the presence of tryptophan (W) in peptides beta-T2 was obtained by specific staining of the intact peptides on separate peptide maps. Relative to the same human hemoglobin peptide the Microtus peptides beta-T2 have one more each of glycine and isoleucine, one less each of threonine and valine. Relative to the same white mouse hemoglobin peptide the Microtus beta-T2 peptides have one more isoleucine and one less valine.

Peptides Beta-T3.

See Table 22; Figure 27.

The peptide beta-T3 could not be found on the peptide map of the tryptic hydrolysate of human hemoglobin.

Tryptic peptides beta-T3 from the hemoglobins of the several species

Table 21.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT2 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	1.10	0.82	1.04	1.40	1.01	1.06	1	1.00
H								
R								
B								
T	1.00						1	
S	0.84	0.92	1.08	1.19	1.06	0.98	1	0.92
Z								
P								
G	0.96	1.97	2.12	2.00	2.24	1.92	1	2.24
A	2.01	1.56	1.69	1.49	1.66	1.78	2	2.21
C								
V	0.86						1	1.05
M								
I		0.96	1.13	0.60	1.20	1.12		
L	1.00	1.07	1.13	+	1.14	1.00	1	0.98
O								
F								
W	+	+	+	+	+	+	1	1.00
Peptide-								
μMol:	0.012	0.018	0.026	0.005	0.015	0.010		
Anal:	1767	2038	2059	2231	2128	2146		

[a]Compare Figure 26.

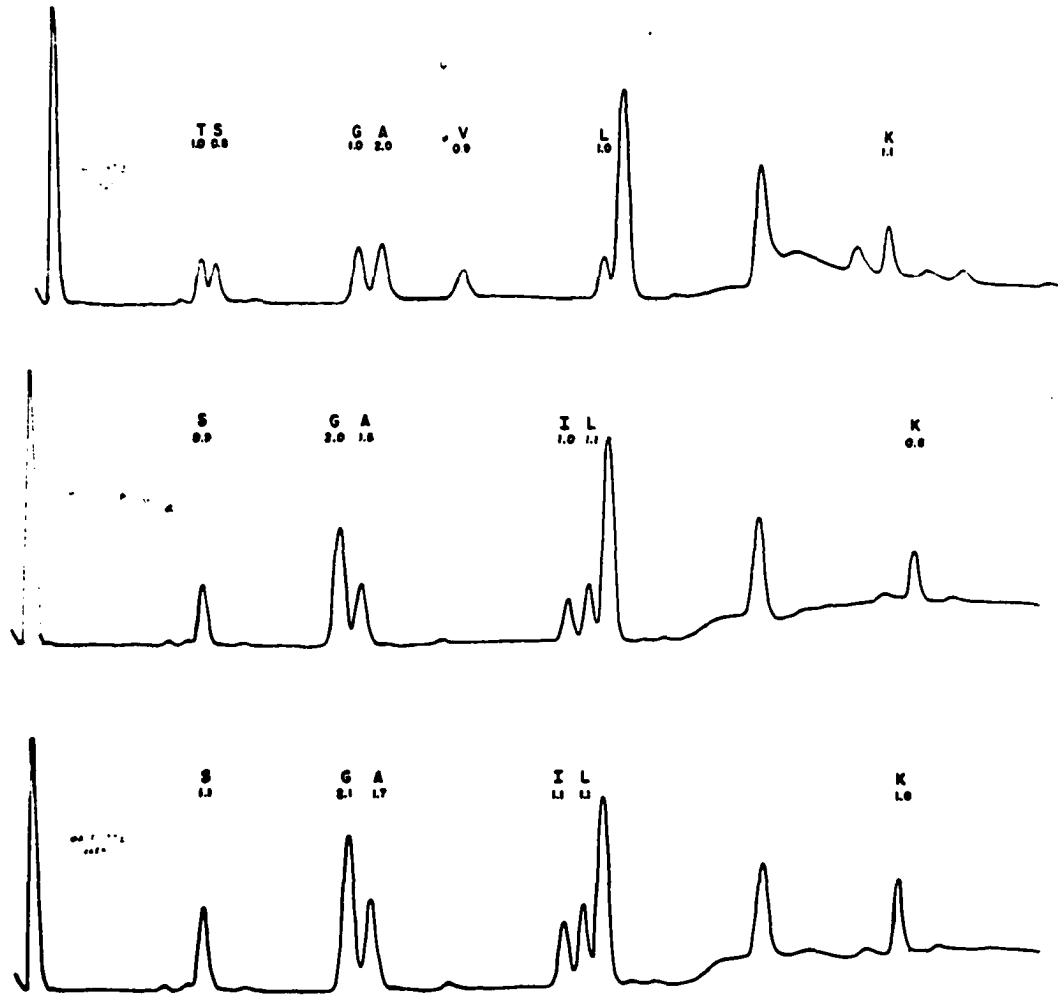


Figure 26. Chromatograms of Peptides βT2.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 22.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT3 (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human[b]	Microtus				Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K								
H								
R		0.87	1.00	0.94	1.04	0.89	1	1.00
B		2.11	2.22	2.16	2.16	2.05	1D 1N	2.10
T								
S			(0.10)					
Z		1.12	1.14	1.28	1.28	1.00	2E	2.03
P								
G		1.79	1.96	1.90	2.05	1.91	3	3.02
A		4.76	4.62	4.80	4.75	4.62	1	2.20
C								
V		1.03	1.11	0.94	1.02	1.03	3	1.95
M								
I								
L		1.03	1.28	0.98	1.06	0.79	1	1.00
O								
F								
W								
Peptide-								
μMol:	nd	0.015	0.048	0.012	0.039	0.020		
Anal:		2023	2033	2104	2233	2088		

[a]Compare Figure 27.

[b]This peptide was not found.

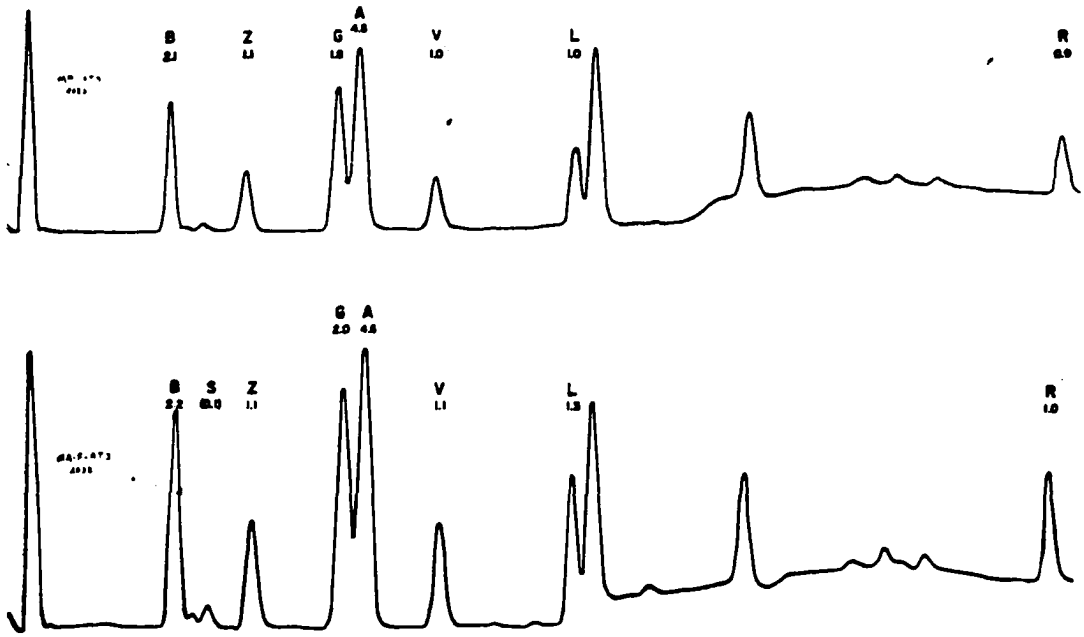


Figure 27. Chromatograms of Peptides βT3.

TOP: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

of Microtus are composed of the same 13 amino acids among themselves. Peptides beta-T3 of Microtus hemoglobins were recovered from the anode region of the maps, indicating that they migrated little during electrophoresis. This suggests a possible difference in charge from that of the human hemoglobin peptide beta-T3. In fact the amino acid composition found for Microtus peptide beta-T3 contains one less glutamic acid than that reported for the corresponding peptide of human hemoglobin. One or more of the three remaining acidic amino acids may be present as the neutral amides asparagine or glutamine which would further reduce the negative charge on the Microtus hemoglobin peptide.

Relative to the same human hemoglobin peptide the Microtus peptides beta-T3 have four more alanines, two less valines, one less glutamic acid and one less glycine. Relative to the same white mouse hemoglobin peptide the Microtus beta-T3 peptides have three more alanines and one less each of valine, glutamic acid and glycine.

Peptides Beta-T4.

See Table 23; Figure 28.

A peptide of human hemoglobin was not found which furnished the amino acid analysis for an entire beta-T4 peptide. However, an arginine-containing peptide fragment, designated beta-T4fc, was found which furnished an analysis consistent with a C-terminal peptide from beta-T4. Similar fragments at similar map positions and with the same compositions were detected among the peptides of all digests of Microtus hemoglobins which were examined. Entire beta-T4 peptides of 10 amino acids were not detected for Microtus hemoglobins.

Table 23.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT4 (in Residues of AA per Peptide).

	EXPERIMENTAL[b]						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K								
H								
R	1.10	0.96	1.12	1.00	1.06	1.00	1	1.00
B								
T	0.87	0.76	0.69	0.55	0.56	0.64	1	0.80
S				(0.4)				(0.18)
Z	0.94	1.14	1.14	1.27	1.20	1.05	1Q	1.16
P							1	0.82
G								
A								
C								
V							2	2.04
M								
I								
L							2	1.90
O							1	0.87
F								
W							1	1.00
Peptide-								
μMol:	0.028	0.022	0.036	0.015	0.023	0.020		
Anal:	1981	2025	2063	2226	2141	2120		

[a]Compare Figure 28.

[b]Only C-terminal fragments were detected for all hemoglobins examined.

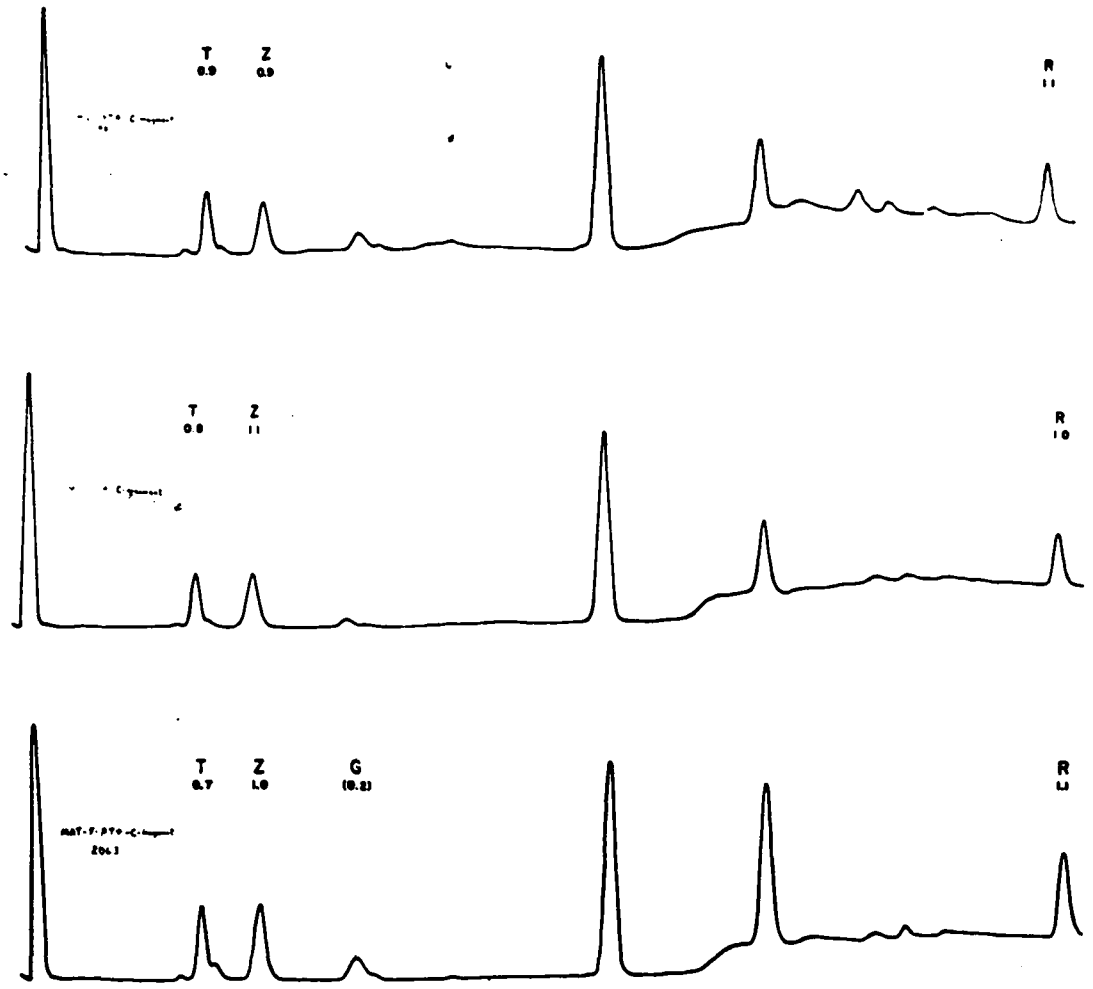


Figure 28. Chromatograms of Peptides βT4, C-Terminal Fragments βT4fc.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

The N-terminal fragments of the beta-T4 peptides could not be located on peptide maps by staining with ninhydrin. However, faint positive tests for tryptophan using Ehrlich's reagent were observed in two maps near the farthest edges in the chromatographic direction. It is possible that the N-terminal fragment of beta-T4 with its high proportion of aliphatic and aromatic functional groups moved so rapidly during chromatography that it left the paper and was lost from the maps. It is noteworthy that intact beta-T4 peptide of human hemoglobin was reported to move farther in the chromatographic direction than any of the other tryptic peptides by Baglioni (1961). In the present system the human peptide fragment beta-T12a was originally suspected to be beta-T4 because of its map position, but there was no fast peptide in this position in the maps of Microtus hemoglobin.

Peptides Beta-T5.

See Table 24; Figure 29.

Tryptic peptides beta-T5, each containing 19 amino acids, were identified for human hemoglobin and for the Microtus hemoglobins except M. abbreviatus Hb-s and M. oeconomus Hb-f. The proposed beta-T5 peptide occupies a map position displaced toward the cathode relative to the map position of the beta-T5 peptide of human hemoglobin (Figures 4, 5, and 11). This is consistent with the loss of one or more negative charges or the addition of at least one positive charge to the Microtus hemoglobin beta-T5 peptide, possibly due to the side chain imidazole of the basic amino acid histidine.

Relative to the same human hemoglobin peptide the M. pennsylvanicus

Table 24.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT5 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs[b]	MMf	MOf[c]		Mouse
K	0.82	1.03	0.79	+	0.80		1	1.00
H		1.21	1.22	+	1.21			
R								
B	3.20	1.83	2.00	+	2.19		2D 1N	3.20
T	1.11		0.52	+	0.65		1	
S	1.97	2.57	1.92	+	2.09		2	3.84
Z	1.06	1.34	1.05	+	1.08		1E	
P	2.00 (0.76)		TRACE	(+)			2	
G	2.05	2.19	1.68	+	1.72		2	2.00
A	1.25	2.82	3.02	+	3.12		1	3.00
C								
V	0.98	1.82	1.52	+	1.68		1	
M	0.65	0.61	0.28	+	0.16		1	1.00
I								0.92
L	1.06	1.02	1.93	+	2.16		1	0.96
O								0.88
F	2.49	2.10	1.29	+	1.20		3	2.00
W								
Peptide-								
μMol:	0.017	0.005	0.055	<0.004	0.017	nd		
Anal:	1951	1923	2211	2237	2140			

[a]Compare Figure 29.

[b]Qualitative evidence for coincident peptides βT11 and βT5.

[c]No data were obtained for this peptide.

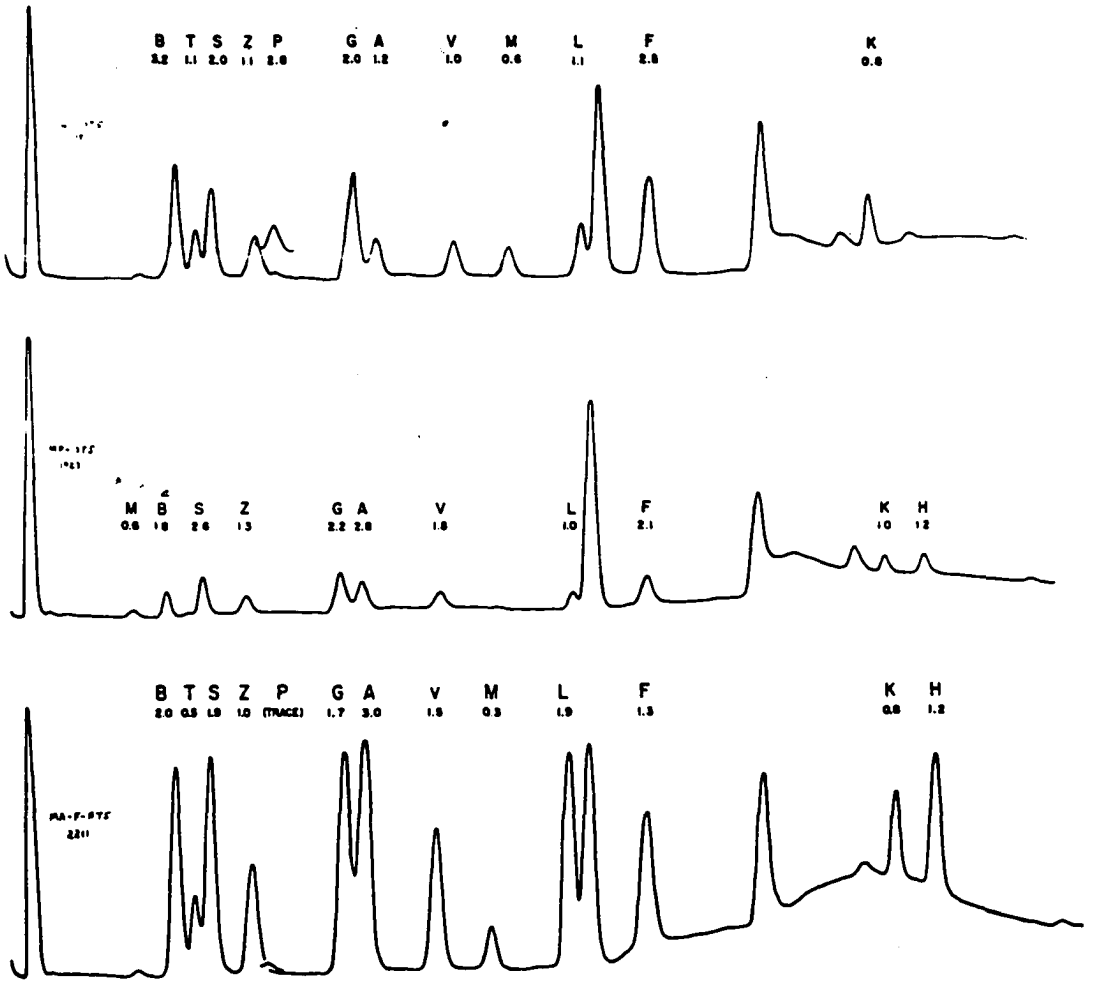


Figure 29. Chromatograms of Peptides βT5.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

peptide beta-T5 has one more each of histidine, valine, and serine, and two more alanines; one less each of aspartic acid, phenylalanine, and threonine, and two less prolines. Relative to the same white mouse hemoglobin peptide the M. pennsylvanicus beta-T5 peptide appears to have two more valines and one more each of histidine and glutamic acid; one less each of serine, aspartic acid, isoleucine and tyrosine.

The beta-T5 peptide of M. pennsylvanicus hemoglobin differed from that of the other Microtus in having one more each of serine and of phenylalanine and one less each of threonine and of leucine.

Peptides Beta-T6.

See Table 25; Figure 33.

The dipeptide beta-T6 was identified from tryptic digests of human hemoglobin. A corresponding peptide was not identified alone in the tryptic digests of any of the Microtus hemoglobins.

Peptides Beta-T7, Beta-T8 and Beta-T(7-8).

See Table 26; Figures 18 and 19..

These peptides were described above in conjunction with the corresponding alpha-peptides from which they were not separated (with the exception of the human peptides as described above).

Peptides Beta-T9 and Beta-T(8-9).

See Table 27; Figure 30.

Peptides beta-T9 and beta-T(8-9) were identified in tryptic hydrolysates of human hemoglobin. The peptides beta-T(8-9) were also identified for M. abbreviatus Hb-f and for M. miurus Hb-f. Unidentified peptides of M. pennsylvanicus Hb and of M. oeconomus Hb-f, which were designated

Table 25.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT6 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus[b]					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	0.87						1	1.03
H								
R								
B								
T								
S								
Z								
P								
G								
A								
C								
V	1.00						1	1.11
M								
I								
L								
O								
F								
W								

Peptide-
μMol: 0.049
Anal: 1936

[a]Compare Figure 33.

[b]Free peptides βT6 were absent from maps of the tryptic hemoglobin peptides of Microtus.

Table 26.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT7 (in Residues of AA per Peptide).

	EXPERIMENTAL					REFERENCE	
	Human	Microtus[b]				Human	White
AA		MP[c]	MAf	MAs	MMf	MOf		Mouse
K	1.08		2.00	2.20	1.96	2.24	1	0.95
H	0.91		1.92	2.02	2.22	1.96	1	1.02
R								
B								
T								
S								
Z								
P								
G	1.00		2.12	2.22	2.08	1.90	1	1.03
A	0.82		1.54	1.31	1.55	1.08	1	0.95
C								
V								
M								
I								
L								
O								
F								
W								
Peptide-								
μMol:	0.018	nd	0.092	0.012	0.038	0.032		
Anal:	1967		2199	2229	2068	2267		

[a]Compare Figure 18.

[b]Numbers for Microtus pertain to amino acids belonging to coincident peptides αβT7.

[c]No data were obtained for this peptide.

Table 27.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT9 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP[b]	MAf[c]	MAs[d]	MMf[c]	MOf[b]		Mouse
K	1.01		1.81		1.51		1	1.00
H	1.00		1.20		1.30		1	1.12
R								
B	3.04		4.00		3.88		2D 1N	4.00
T			0.63		0.80			0.97
S	0.96		0.64		0.82		1	1.16
Z								(0.07)
P								
G	2.04		0.53		0.66		2	1.11
A	2.30		1.72		1.96		2	1.20
C								
V	1.05		0.52		0.62		1	0.86
M								
I								0.84
L	3.84		4.16		4.32		4	3.00
O								
F	0.95		(0.2)				1	0.89
W								
Peptide-								
μMol:	0.018		0.027	nd	0.022			
Anal:	1953		2142		2240			

[a]Compare Figure 30.

[b]Peptides βT9 were not identified in terms of the available analyses.

[c]Numbers for these Microtus may pertain to peptide βT(8-9).

[d]No data were obtained for this peptide.

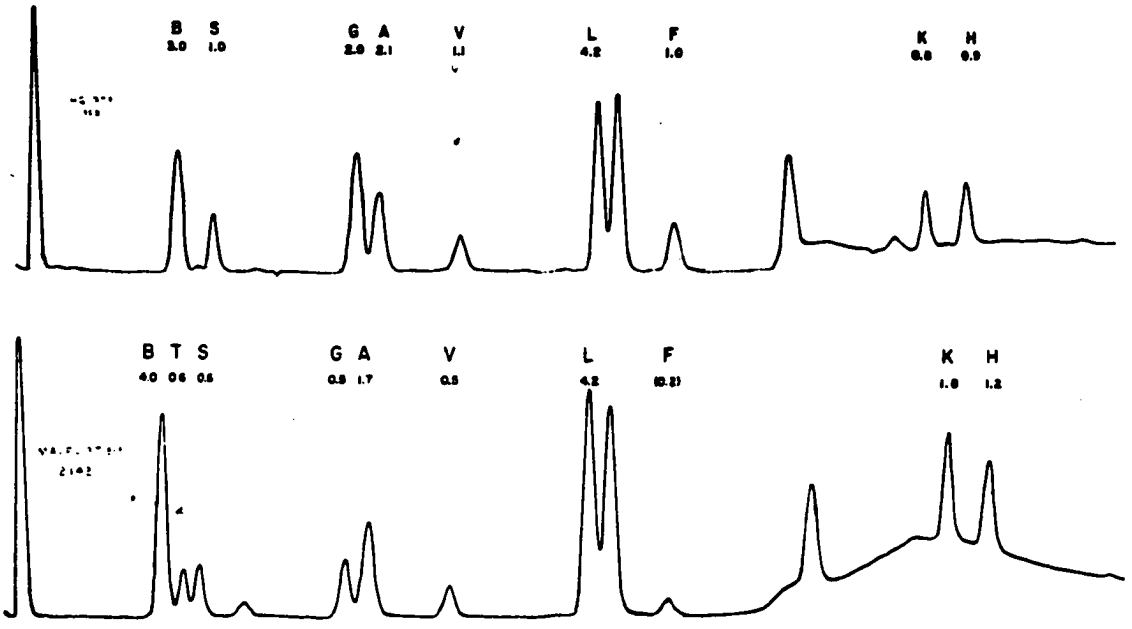


Figure 30. Chromatograms of Peptides βT9.

TOP: Human Hemoglobin.

BOTTOM: M. abbreviatus Hb-f, βT(8-9).

peptides 19, occupy map positions similar to that observed for beta-T(8-9) in the maps of the other Microtus. But their compositions cannot be reconciled with that of a complete beta-T9 peptide. The amino acid composition of peptide 19 of M. pennsylvanicus Hb appears to lack aspartic acid, alanine, leucine and possibly a lysine relative to the composition obtained for M. abbreviatus Hb-f peptide beta-T(8-9).

Relative to the same human hemoglobin peptide the M. abbreviatus Hb-f peptide beta-T(8-9) appears to have two differences among the 17 amino acids: one more each of aspartic acid and of threonine, and one less each of glycine and of phenylalanine. Relative to the same white mouse hemoglobin peptide the M. abbreviatus Hb-f peptide beta-T(8-9) appears to have one less each of phenylalanine and isoleucine, and one more each of alanine and leucine.

Peptides Beta-T10.

See Table 28; Figure 31.

The human hemoglobin peptide beta-T10 contains, among 13 amino acids, one alanine, one serine and two threonines. Differences in composition between the same peptides of the several hemoglobins examined occur only in these three amino acids.

The peptides beta-T10 of M. pennsylvanicus Hb and of M. oeconomus Hb-f contain one alanine, two serines and one threonine; their total composition is identical to that reported for the corresponding peptide of hemoglobin of white mouse strain C57BL/6.

Peptides beta-T10 of the other Microtus differ from all of the other peptides beta-T10 in that they lack alanine and contain three serines and one threonine.

Table 28.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT10 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	1.00	0.88	1.11	1.28	1.02	1.11	1	1.00
H	1.03	0.93	1.07	1.03	1.07	0.98	1	1.00
R								
B	1.13	1.20	1.24	1.30	1.24	1.07	1D	1.14
T	2.04	0.98	1.04	1.17	0.96	0.86	2	0.90
S	1.14	1.94	2.76	3.02	2.85	1.86	1	1.98
Z	0.98	1.13	1.28	1.26	1.11	1.24	1E	1.12
P								
G	0.72	0.79	0.54	0.57	0.63	0.52	1	1.10
A	1.18	1.14				1.01	1	1.04
C	0.88	0.52	0.44	+	0.74	+	1	1.00
V								
M								
I								
L	2.00	1.82	1.89	1.72	1.76	1.72	2	1.85
O								
F	0.90	0.85	0.89	0.91	0.82	1.11	1	0.88
W								
Peptide-								
μMol:	0.030	0.021	0.026	0.004	0.011	0.006		
Anal:	1964	1991	2079	2086	2076	2177		

[a]Compare Figure 31.

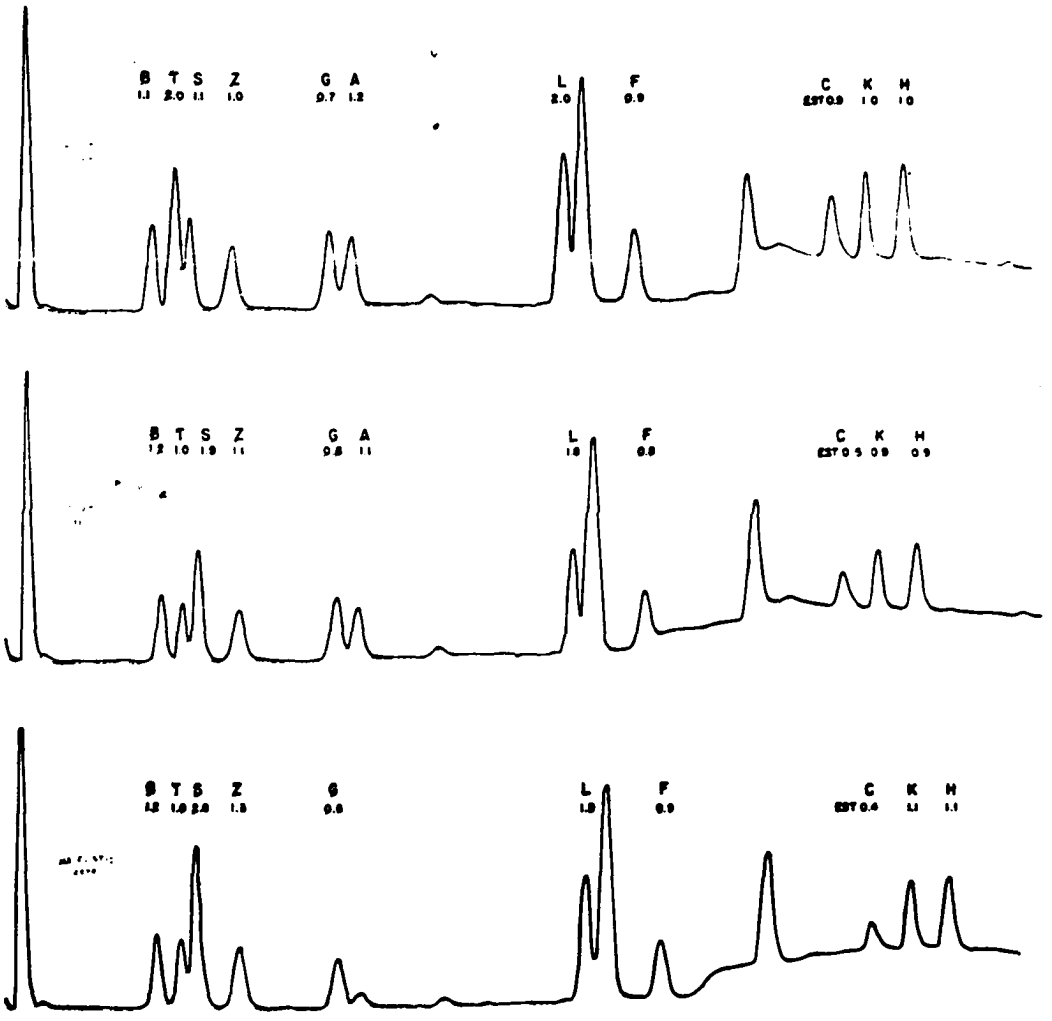


Figure 31. Chromatograms of Peptides βT10.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Peptides Beta-T11.

See Table 29; Figure 32.

Peptides beta-T11 were isolated from tryptic digests of human hemoglobin and of Microtus hemoglobins in similar map positions. All of these peptides are composed of the same 9 amino acids, and the same composition has been reported for peptide beta-T11 of hemoglobin from white mouse strain C57BL/6. Conspicuous in each of the analyses are additional amounts of glycine and alanine which are not attributed to the beta-T11 peptide.

Peptides Beta-T12: Beta-T12a and Beta-T12b.

See Table 30; Figures 22 and 33.

Peptide fragments beta-T12a and beta-T12b were recognized easily in the human hemoglobin peptide map on the basis of their amino acid analyses even though fragments beta-T12b and alpha-T12a were coincident in the map. The sum of the compositions of the two fragments beta-T12a and beta-T12b accounts for the expected 16 amino acids composing the entire peptide.

Peptide beta-T12 or its fragments were not identified in the soluble portion of tryptic digests of Microtus hemoglobins. The absence of beta-T12 fragments suggests that there is a difference in composition from that of the corresponding human hemoglobin peptide. Peptide beta-T12 fragments were not identified in a chymotryptic digest of insoluble tryptic residues from M. pennsylvanicus hemoglobin. Peptides beta-T12 will be considered further in the Discussion in terms of certain unidentified peptides which were obtained from Microtus hemoglobins.

Table 29.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT11 (in Residues of AA per Peptide).

	EXPERIMENTAL[b]						REFERENCE	
	Human	Microtus					Human	White
AA		MP	MAf	MAs[c]	MMf	MOf		Mouse
K	(0.2)			(+)	(0.4)			(0.20)
H	1.06	1.11	0.96	+	0.94	1.31	1	1.04
R	0.90	0.86	0.76	nd	0.50	0.99	1	1.16
B	2.21	2.21	1.88	+	2.16	2.12	1D 1N	2.18
T				(+)	(0.4)			
S	(0.2)		(0.4)	(+)	(0.6)			
Z	0.98	1.16	1.01	+	0.96	0.74	1E	1.16
P	1.27	1.06	0.88	+	0.61	+	1	0.90
G	(0.3)	(0.3)	(0.7)	(+)	(0.7)	(1.0)		(0.18)
A	(0.6)	(0.7)	(0.9)	(+)	(1.3)	(1.4)		
C								
V	1.18	1.03	0.92	+	1.08	1.15	1	0.94
M				(+)				
I								
L	0.70	0.86	0.86	+	1.24	1.20	1	1.02
O								
F	0.93	0.82	0.82	+	0.67	0.55	1	1.00
W								
Peptide-								
μMol:	0.031	0.015	0.029	<0.004	0.025	0.006		
Anal:	1969	2029	2187	2237	2137	2154		

[a]Compare Figure 32.

[b]Numbers in parentheses pertain to amino acids not considered to belong to peptides βT11.

[c]Qualitative evidence for coincident peptides βT11 and βT5.

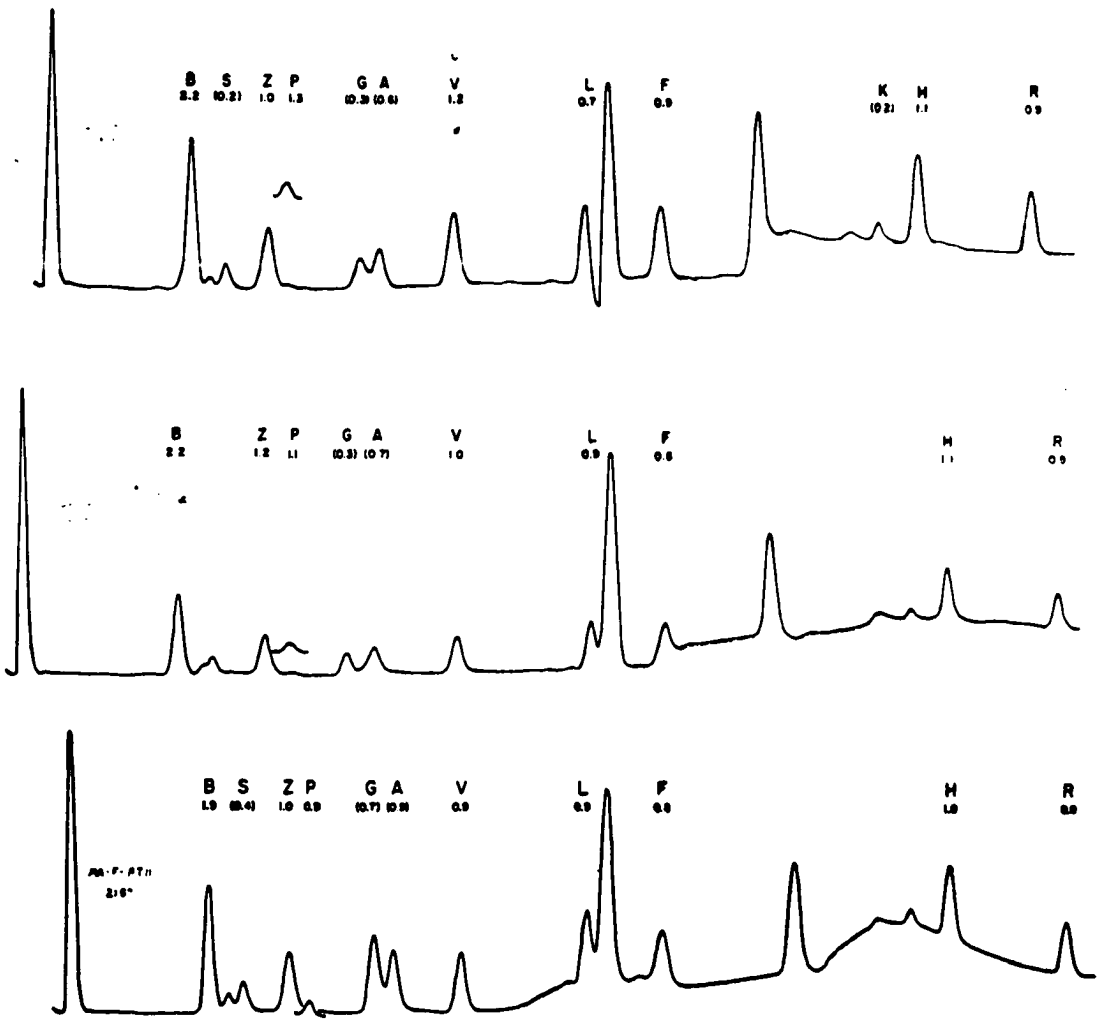


Figure 32. Chromatograms of Peptides βT11.

TOP: Human Hemoglobin.

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 30.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT12 (in Residues of AA per Peptide).

	EXPERIMENTAL			REFERENCE	
	Human[b]	Human[c]	Microtus[d]	Human	White Mouse
AA					
K		1.19		1	0.96
H		2.20 (0.91)		2	1.91
R					
B	1.00			1N	1.17
T					
S		(0.78)			
Z					
P					
G	1.09	1.15		2	3.12
A		1.22		1	
C	0.48	(0.55)		1	
V	1.99	1.03		3	1.83
M					0.90
I					1.78
L	2.70	0.80 (1.48)		4	4.13
O					
F		1.07		1	
W					
Peptide-					
μMol:	0.020	0.027			
Anal:	1985	1973			

[a] Compare Figures 22 and 33.

[b] Numbers refer to the N-terminal fragment βT12a.

[c] Numbers refer to the C-terminal fragment βT12b coincident with (numbers shown in parentheses) the N-terminal fragment αT12a.

[d] Peptides βT12 were not identified in terms of the available analyses.

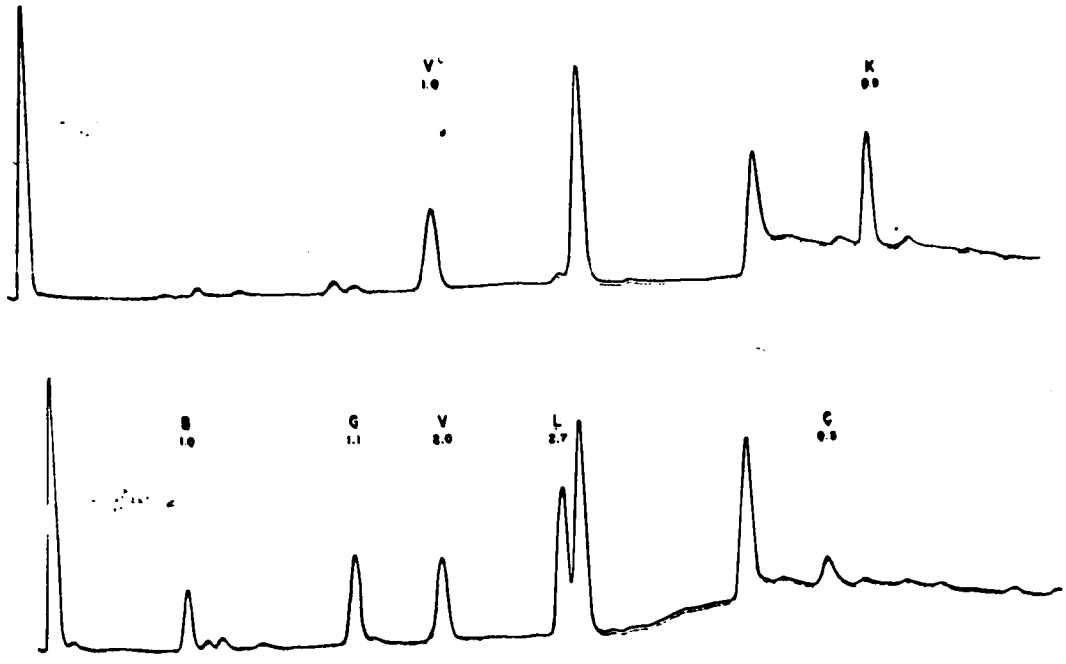


Figure 33. Chromatograms of Peptides βT6 and βT12a of Human Hemoglobin.[a]

TOP: βT6.

BOTTOM: βT12a.

[a]See Figure 22 for Peptide βT12b of Human Hemoglobin.

Peptides Beta-T13.

See Table 31; Figure 34.

The two peptides beta-T13 and alpha-T9 were isolated from one spot in the human hemoglobin peptide map. Peptides beta-T13 of Microtus and of human hemoglobins occupy the same relative positions on their respective peptide maps, but the Microtus peptides beta-T13 are free from other peptides. There is an apparent substitution of an aspartic acid of the human hemoglobin peptide for a glutamic acid in the Microtus peptides without a change in the net charge of the peptides. Peptide beta-T13 was not obtained for M. oeconomus Hb-f.

Peptide beta-T13 from M. abbreviatus Hb-f is composed essentially of the same 12 amino acids as reported for the corresponding peptide from hemoglobin of white mouse strain C57BL/6. Relative to the corresponding human hemoglobin peptide beta-T13, the M. abbreviatus Hb-f peptide has two more alanines and one more each of phenylalanine and aspartic acid; one less each of glutamic acid, proline, valine and tyrosine.

The beta-T13 peptide of M. pennsylvanicus Hb appears to differ from that of M. abbreviatus Hb-f by one alanine and in its fractional numbers of serine, glutamic acid, proline, leucine and phenylalanine.

Peptides Beta-T14.

See Table 32; Figure 35.

Peptides beta-T14 of Microtus hemoglobin occupy the same map positions relative to the beta-T14 peptide of human hemoglobin, but

Table 31.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT13 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus[c]					Human	White
AA		MP	MAf	MAs	MMf	MOf[d]		Mouse
K	1.10 (0.21)	1.20	1.09	1.00 (0.91)	1.11		1	1.00
H	(0.61)				(0.34)			
R								
B	(1.91)	1.14	0.91	1.42	1.03			0.92
T	1.00 (0.35)	1.22	1.17	1.10	0.92		1	1.01
S	(0.81)	0.60	(0.31)	0.92	0.50			
Z	3.08	1.63	2.19	1.53	1.68		1E 2Q	2.06
P	2.20 (0.32)	0.62	1.14	+	nd		2	0.94
G	(0.54)	(0.38)	(0.45)	(1.10)	(0.50)			
A	1.80 (1.80)	3.06	4.05	2.82	3.08		2	4.04
C								
V	1.10 (0.76)	(0.31)					1	
M	(TRACE)							
I								
L	(1.12)	0.58	(0.45)	0.97	0.78			
O	0.30						1	
F	1.00	1.30	1.82	0.94	1.33		1	2.06
W								
Peptide-µMol:	0.038	0.018	0.018	0.005	0.010			
Anal:	1961	2031	2200	2252	2242			

[a]Compare Figure 34.

[b]Numbers in parentheses pertain to coincident peptide αT9.

[c]Numbers in parentheses pertain to amino acids not considered to belong to peptides βT13.

[d]Peptide βT13 was not identified in terms of available analyses.

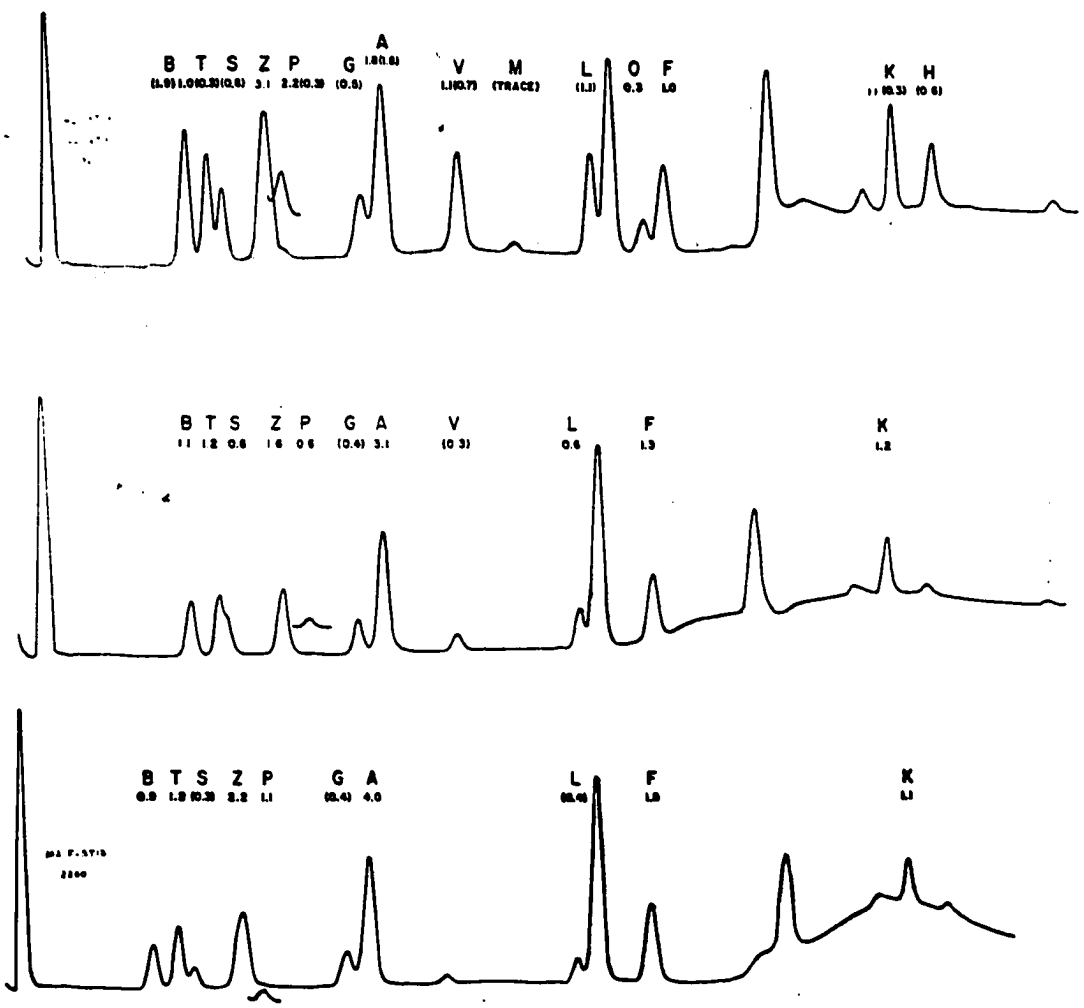


Figure 34. Chromatograms of Peptides βT13.

TOP: Human Hemoglobin (αT9 present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

Table 32.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT14 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus					Human	White
AA		MP	MAf	MAs	MMf	MOf		Mouse
K	1.02	1.21	1.04	+	0.76	1.03	1	1.07
H	1.14	1.28	1.14	+	1.35	1.32	1	0.95
R	(0.56)							
B	1.13						1N	
T								
S		1.09	1.07	+	0.89	1.00		
Z								
P								
G	1.09	1.13	1.08	+	0.84	1.13	1	1.17
A	4.30	4.28	4.40	+	4.37	4.29	4	5.20
C								
V	2.54	2.48	2.28	+	2.09	2.12	3	2.90
M								
I								
L	1.09	1.12	1.15	+	0.87	0.86	1	1.12
O	(0.42)							
F								(0.17)
W								
Peptide-								
μMol:	0.025	0.017	0.026	<0.004	0.024	0.006		
Anal:	1974	2000	2184	2190	2096	2119		

[a]Compare Figure 35.

[b]Numbers in parentheses pertain to coincident peptide αT14.

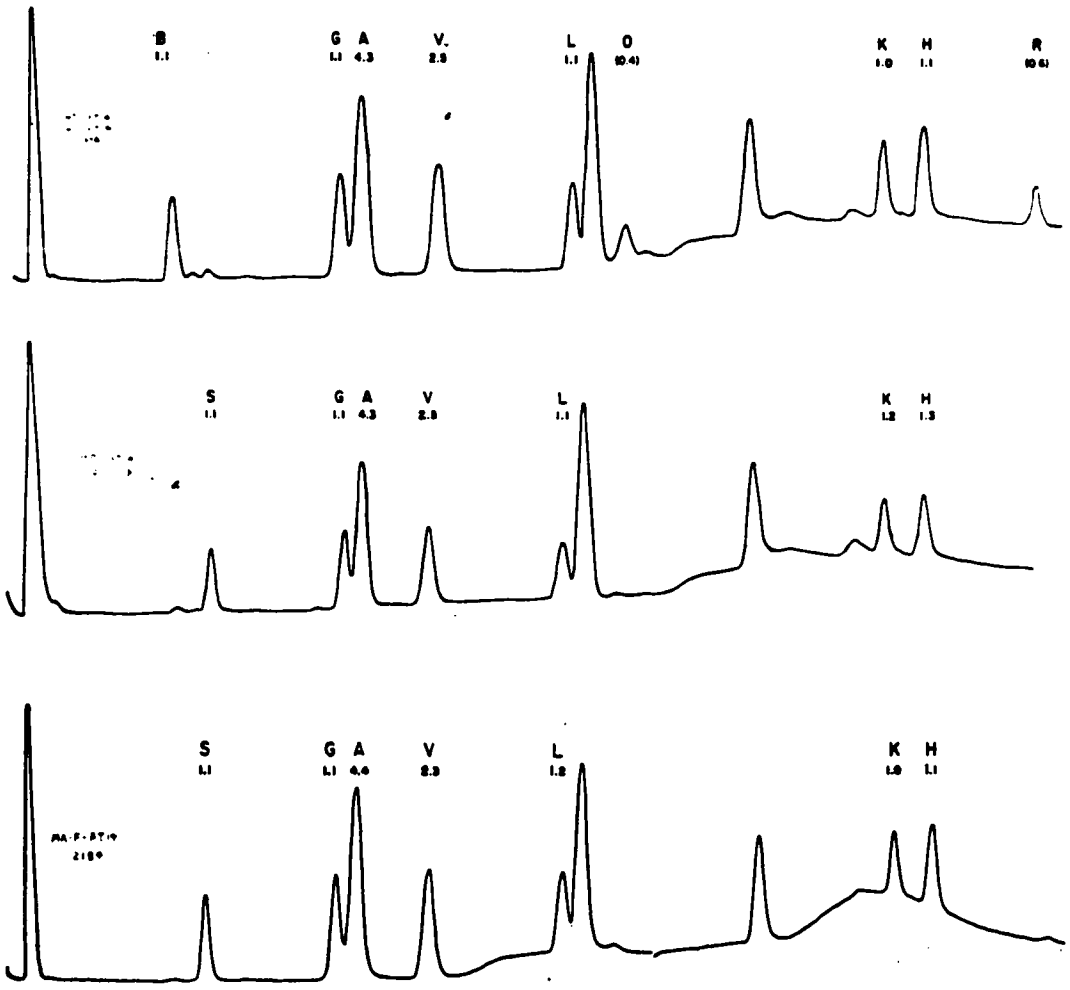


Figure 35. Chromatograms of Peptides βT14.

TOP: Human Hemoglobin (αT14 present).

CENTER: M. pennsylvanicus Hb.

BOTTOM: M. abbreviatus Hb-f.

unlike the human peptide, the Microtus peptides beta-T14 are well-resolved from the adjacent alpha-T14 peptides.

All Microtus peptides beta-T14 are composed of the same 12 amino acids, but they differ in composition from the corresponding peptides of both human and white mouse hemoglobins. The Microtus peptides contain: relative to the human hemoglobin peptide beta-T14, one less aspartic acid and one more serine; relative to the white mouse hemoglobin peptide beta-T14, one less alanine and one more serine.

Peptides Beta-T15.

See Table 33; Figures 14 and 36.

The same dipeptide beta-T15 can be attributed to each of the hemoglobins which were investigated. In each case, peptides beta-T15 were analyzed in the presence of one or both of the other peptides, alpha-T3 and 16'.

Unidentified Peptides.

A number of peptide spots taken from maps of tryptic digests of human and of Microtus hemoglobins yielded amounts of amino acids which were insufficient for identification purposes. However several analyses were obtained from Microtus peptide materials which were present in sufficient amounts that relative proportions of amino acids could be calculated.

Peptides 16 and 16'.

See Tables 34 and 35; Figures 14 and 36.

Two unidentified peptides obtained from tryptic hydrolysates of

Table 33.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT15 (in Residues of AA per Peptide).

	EXPERIMENTAL						REFERENCE	
	Human[b]	Microtus[c]					Human	White
AA		MP[d]	MAf	MAs	MMf	MOf		Mouse
K								
H	1.09	1.0	1.0	+	0.8	+	1	1.08
R								
B								
T								
S								
Z								
P								
G								
A								
C								
V								
M								
I								
L								
O	0.58	0.7	0.6	+	0.4	nd	1	1.00
F								
W								
Peptide-								
μMol:	0.032	0.020	0.018	<0.004	0.008	<0.004		
Anal:	1970	2007	2049	2103	2209	2116		

[a]Compare Figures 14 and 36.

[b]Numbers were taken from data for coincident peptides αT3 and βT15.

[c]Numbers taken from data for coincident peptides 16' and βT15.

[d]Numbers taken from data for coincident peptides 16', αT3 and βT15.

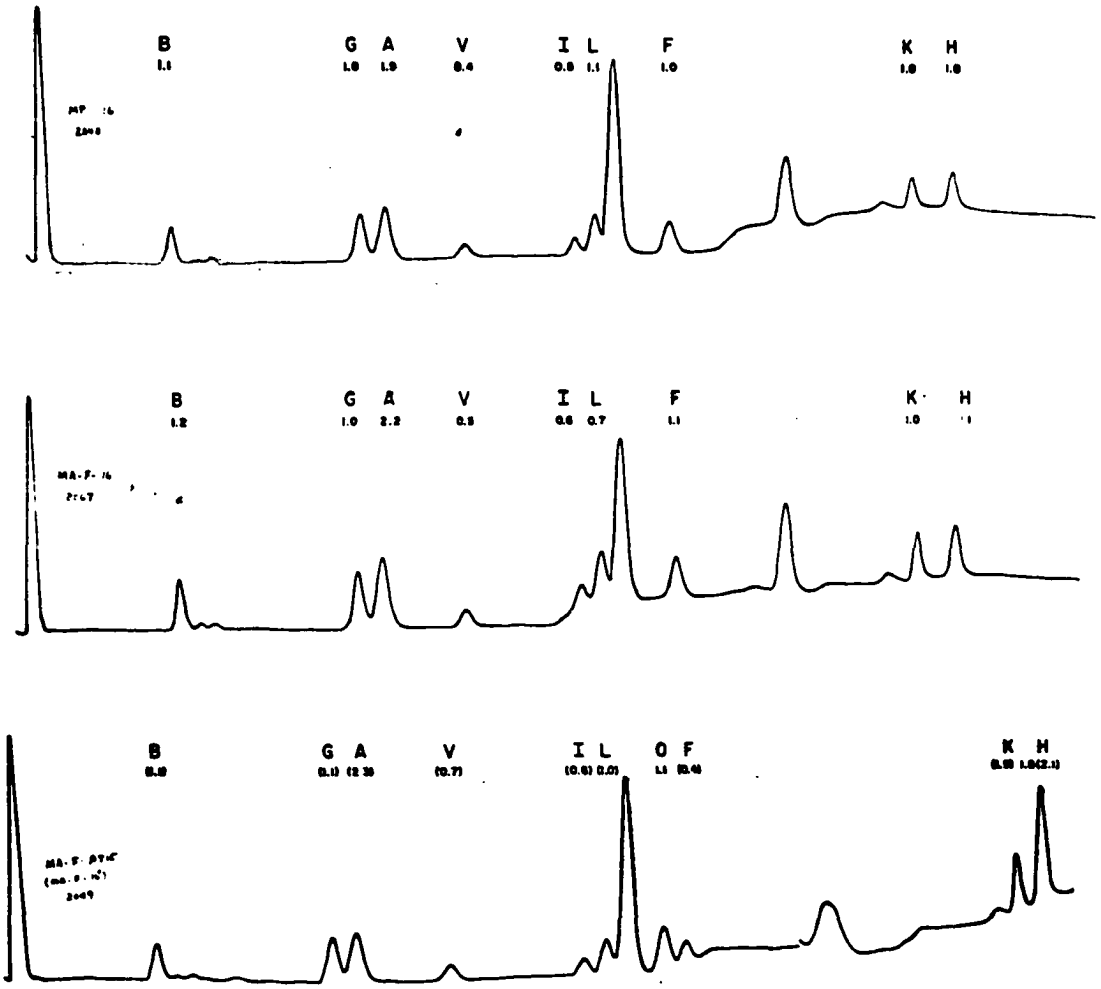


Figure 36. Chromatograms of Peptides 16, 16' and βT15.[a]

TOP: M. pennsylvanicus, 16.

CENTER: M. abbreviatus Hb-f, 16.

BOTTOM: M. abbreviatus, βT15 (16' present).

[a]See Figure 14 for Peptide βT15 of Human Hemoglobin and Peptides βT15 and 16' of M. pennsylvanicus Hb.

Table 34.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides 16 (in Residues of AA per Peptide).

	EXPERIMENTAL				
	Microtus				
AA	MP	MAf	MAs	MMf	MOf
K	0.95	0.98	+	0.89	1.22
H	0.99	1.06	+	0.98	0.97
R					
B	1.13	1.18	+	1.12	1.02
T					
S					
Z					
P					
G	0.98	1.02	+	1.17	1.01
A	1.92	2.23	+	2.18	2.06
C					
V	0.45	0.46	+	0.40	0.47
M					
I	0.50	0.63	+	0.73	0.18
L	1.11	0.74	+	0.87	0.68
O					
F	1.00	1.08	+	0.96	1.06
W					
Peptide-					
µMol:	0.012	0.016	<0.004	0.005	0.007
Anal:	2040	2067	2196	2126	2204

[a]Compare Figure 36.

Table 35.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides 16' (in Residues of AA per Peptide).

	EXPERIMENTAL				
	Microtus				
AA	MP[b]	MAf[c]	MAs	MMf[c]	MOf
K	1.8 (1.8)	1.91	+	1.55	+
H	1.8 (1.5)	2.1 (1.8)	+	1.5 (0.8)	+
R					
B	1.16	1.14	+	0.90	+
T	(2.3)				
S					
Z					
P					
G	1.2 (2.0)	1.13	+	1.16	+
A	2.3 (2.0)	2.30	+	2.20	+
C					
V	0.65	0.69	+	0.58	+
M					
I	0.54	0.57	+	0.49	+
L	1.06	1.03	+	0.92	+
O	(1.0)	(1.08)	(+)	(0.43)	nd
F	0.60	0.42	+	1.22	+
W					
Peptide-					
µMol:	0.010	0.011	<0.004	0.010	0.004
Anal:	2007	2049	2103	2209	2116

[a]Compare Figures 14 and 36.

[b]Numbers in parentheses pertain to coincident peptides αT3 and βT15.

[c]Numbers in parentheses pertain to the coincident peptide βT15.

each of the Microtus hemoglobins are designated peptides 16 and 16'. Peptides 16 occur on peptide maps as isolated entities whereas peptides 16' occur in the presence of the other peptides beta-T15 and, as has been described above, in the presence of the presumed peptide alpha-T3 from M. pennsylvanicus Hb. Although the two peptides are rather widely separated on peptide maps, they have amino acid compositions which are very similar, within the same species and between the different species. Peptide 16 appears to have a total of 9 amino acids if the fractional proportions of isoleucine and valine are combined. Peptide 16' appears to have a total of 11 amino acids including one additional residue each of lysine and histidine and fractional proportions of isoleucine and phenylalanine.

Peptides 17.

See Table 36; Figure 37.

Other unidentified peptides, designated 17, are located in the same region of the maps of each of the species of Microtus, near the arginine-containing peptide beta-T11. If the possibility of other overlapping peptides is neglected, peptide 17 of M. abbreviatus Hb-f could be composed of 17 amino acids. Also neglecting the possibility of other overlapping peptides, peptide 17 of M. pennsylvanicus Hb appears to be composed of 22 amino acids including one more of each of the amino acids aspartic acid, proline, valine, lysine and possibly phenylalanine than observed in the same peptide 17 of M. abbreviatus Hb-f.

Peptide 18.

See Table 37; Figure 37.

Table 36.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides 17 (in Residues of AA per Peptide).

	EXPERIMENTAL				
	Microtus				
AA	MP	MAf	MAs	MMf	MOf[b]
K	1.91	1.04	1.20	0.82	
H	1.04	1.01	0.90	0.84	
R	(0.41)	(0.21)	(0.45)	(0.36)	
B	4.07	2.80	2.64	2.82	
T	0.78	0.54		0.39	
S	0.89	0.99	0.72	0.67	
Z	0.60	0.56	0.71	0.67	
P	2.09	1.00	1.22	1.04	
G	0.62	0.86	0.54	0.60	
A	1.81	1.89	0.96	1.30	
C					
V	3.20	2.04	1.80	1.89	
M		TRACE			
I					
L	1.71	1.69	0.94	1.21	
O					
F	1.51	1.15	0.92	0.98	
W					
Peptide-					
µMol:	0.012	0.028	0.010	0.020	nd
Anal:	2010	2218	2234	2127	2272

[a]Compare Figure 37.

[b]Data were not obtained from the analysis of this peptide.

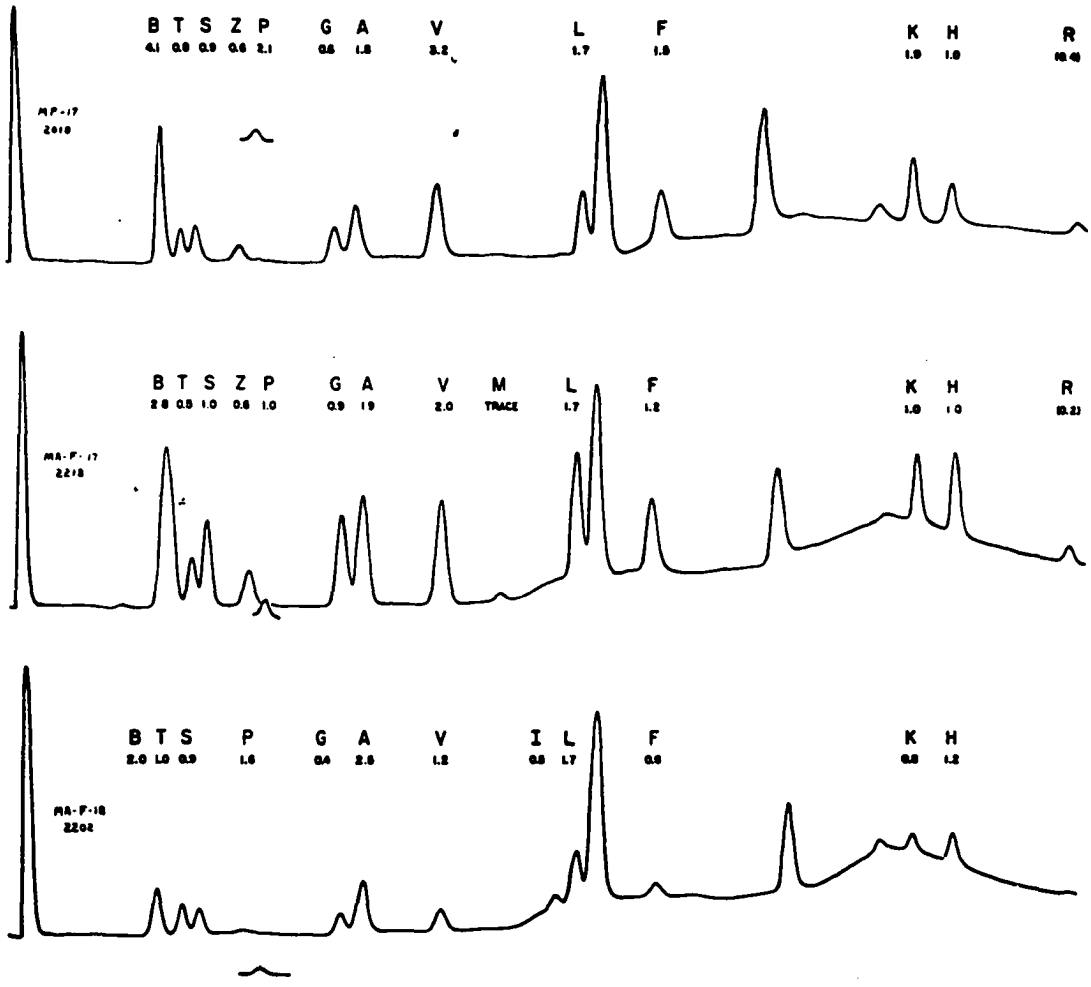


Figure 37. Chromatograms of Peptides 17 and 18.

TOP: M. pennsylvanicus Hb, 17.

CENTER: M. abbreviatus Hb-f, 17.

BOTTOM: M. abbreviatus Hb-f, 18.

Table 37.[a] Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides 18, 19, 19', 20 and 20' (in Residues of AA per Peptide).

	EXPERIMENTAL					
	Microtus[b]					
AA	MAf-18	MP-19	MOf-19	MOf-19'	MOf-20	MOf-20'
K	0.84	1.23	2.22	1.11	1.07	0.86
H	1.17	1.12	1.41	0.46	0.57	
R						
B	1.98	3.17	3.08	2.06	1.65	1.13
T	1.05	0.35	0.39		0.79	0.29
S	0.87	0.49	0.53		0.68	
Z		(0.16)	0.54		1.33	1.13
P	1.63					
G	0.42	0.48	0.56	0.38	0.74	
A	2.54	1.10	1.78		2.46	1.91
C						
V	1.20	0.62	0.54			
M						
I	0.52					
L	1.71	3.22	4.12	2.05	1.35	0.73
O						
F	0.55	0.23	0.34		0.74	0.89
W						
Peptide-						
µMol:	0.010	0.019	0.006	0.010	0.006	0.009
Anal:	2202	2006	2274	2181	2117	2155

[a]Compare Figure 37.

[b]Respective peptides are as designated.

An unidentified peptide, obtained only from the map of M. abbreviatus Hb-f, appears to be constituted of perhaps 15 amino acids and is designated as peptide 18.

Peptides 19 and 19'.

See Table 37.

Peptides isolated from maps of M. pennsylvanicus Hb and M. oeconomus Hb-f, from a region adjacent to peptide beta-T13, could not be identified and are designated as peptides 19. In the maps from other species of Microtus, peptides in this same region have been correlated with peptide beta-T9. Peptide 19 of M. pennsylvanicus Hb appears to be composed of 10-12 amino acids.

A peptide which was located on the far side of the map from 19 was observed only in the map of M. oeconomus Hb-f. This peptide, which showed an amino acid analysis similar in some respects to that of peptide 19, is designated peptide 19'.

Peptides 20 and 20'.

See Table 37.

Two unidentified peptides, which were observed only in the map of M. oeconomus Hb-f, appear to be of related compositions and are designated peptides 20 and 20'. They are located in a region of the map common to peptide beta-T13, but their compositions could not be reconciled with that of a beta-T13 peptide.

DISCUSSION

More than 30 differences in amino acid composition have been discovered between the hemoglobins of human and Microtus, and these differences affect 16 of the 31 expected tryptic hemoglobin peptides. More than 20 differences in amino acid composition between the hemoglobins of Microtus and white mouse have been observed to affect 12 of the expected 31 tryptic peptides of the alpha and beta chains. Several amino acid substitutions not cataloged heretofore for hemoglobins (Dayhoff and Eck, 1968) were observed in Microtus hemoglobins. Amino acid analyses compiled from hydrolysates of tryptic peptides of M. pennsylvanicus hemoglobin and from M. abbreviatus Hb-f account for 90 percent of the amino acids expected from the alpha and beta chains. Special properties seem to pertain to each of three elusive peptides and to each of the fragments of three other peptides which were not identified.

The comparative study of the hemoglobins of both human and Microtus by the methods described earlier proved invaluable in the reciprocal identification of peptides from the several species. In this Discussion sequences of amino acids have been postulated for the alpha and beta chains of Microtus hemoglobins on the basis of the well-documented sequences for human hemoglobin taken together with the properties observed for the Microtus peptides.

THE COMPARATIVE AMINO ACID COMPOSITION OF HEMOGLOBINS FROM HUMAN, FROM MICROTUS PENNSYLVANICUS AND FROM WHITE MOUSE.

M. pennsylvanicus possesses a single, apparently homogeneous (by electrophoresis) hemoglobin in contrast to the other species of Microtus to be discussed later, each of which possesses major and minor component hemoglobins of different electrophoretic mobilities. Major effort in this research was directed to establishing the amino acid compositions for all possible tryptic peptides of Microtus pennsylvanicus hemoglobin. The amino acid compositions were related to specific alpha and beta tryptic peptides using published, tabular data for human hemoglobin (especially Braunitzer et al., 1964) together with the comparative map positions and amino acid compositions of human tryptic hemoglobin peptides obtained experimentally in this laboratory. Subsequent analyses of hemoglobins of other species of Microtus could then be related to those of M. pennsylvanicus by means of map positions and amino acid analyses of the respective tryptic peptides. In fact, subsequent studies of the tryptic peptides of M. abbreviatus Hb-f furnished some information which aided in the interpretation of the peptide analyses obtained from both human and M. pennsylvanicus hemoglobins.

Figures 4 and 5 show several "obvious" (now) differences between the peptide maps of human and M. pennsylvanicus hemoglobins. It must be noted that a degree of confidence in the apparent map differences was only obtained with the detailed amino acid analyses of peptides from each of the maps. On the basis of amino acid analyses it became evident that the relatively simple tryptic human hemoglobin dipeptide

beta-T6 (V K) was not among the Microtus peptides. Also absent from the Microtus map were the other outlying peptides beta-T12a and beta-T12b as seen in the human peptide map. Conversely, the tryptic peptide beta-T3 was not found among the peptides obtained from human hemoglobin by the methods employed in this research. These and other differences observed between the tryptic peptides of human and M. pennsylvanicus hemoglobins will be discussed in detail below.

Tryptic Peptides of Identical Composition from Hemoglobins of M. pennsylvanicus, of Human and of White Mouse Strain C57BL.

Nine tryptic peptides of the alpha and beta hemoglobin chains from M. pennsylvanicus and from human were found to have the same amino acid compositions. The peptides are represented here in terms of the possible homologous sequences of amino acids for the M. pennsylvanicus peptides inferred relative to those sequences of human and of white mouse hemoglobin peptides (Dayhoff and Eck, 1968). Various residues of aspartic acid and glutamic acid are inferred to be present as aspartic acid with a free beta carboxyl group (D), the amide of aspartic acid (asparagine, N), glutamic acid with a free gamma carboxyl group (E), or the amide of glutamic acid (glutamine, Q), on the basis of the relative mobilities observed for the respective human and Microtus peptides. That the sequence of amino acids for Microtus hemoglobin peptides has been inferred is indicated by the periods interposed between the letter designations for the amino acids. The letter designations B and Z are used for aspartic acid and glutamic acid, respectively, when there is no information about the nature of the side-chain carboxyls.

	αT8	αT10	αT11	αT12a	αT14
MP	K	L.R	V.(D).P.V.(N).F.K	L.L.S.H.C	O.R
HA	K	L R	V D P V N F K	L L S H C	O R
C57BL	K	L R	V D P V N F K	L L S H C	O R

	βT7	βT8	βT11	βT15
MP	A.H.G.K	K	L.H.V.(D).P.(E).(N).F.R	O.H
HA	A H G K	K	L H V D P E N F R	O H
C57BL	A.H.G.K	K	L.H.V. B. P. Z. B. F.R	O.H

Analyses were also obtained for 21 amino acids in peptides produced by chymotrypsin acting upon insoluble residues remaining after the initial trypsin digestion of M. pennsylvanicus globin. The 21 amino acids were correlated with an identical 21 of 35 amino acids expected in human hemoglobin peptides alpha-T12b and alpha-T13.

	αT12b | αT13
MP	A.H.L.P.A.Z.F.T.P.A.V.H.A.S.L.B.K.F T.S.K
HA	LLVTLAA H L P A E F T P A V H A S L D K FLASVSTVLT S K
C57BL	LLVTLAS H H P A D F T P A V H A S L D K FLASVSTVLT S K

Differences in the sequence of alpha-T12b between white mouse and human hemoglobins are indicated by the underlined letters in the mouse sequence. There is only a small probability that the 14 amino acids remaining unidentified in Microtus hemoglobin are different from those found in the corresponding human peptides, for there appears to be a

larger degree of conservation of primary structure in the C-terminal portion than elsewhere in the alpha chains of various hemoglobins (Dayhoff and Eck, 1968). White mouse hemoglobin has exactly the same sequence (hence composition) as human hemoglobin for all but three of fifty amino acid residues in the C-terminal portion of the alpha chain.

Identical fragments were obtained from the C-terminal end of peptides beta-T4 from human and from Microtus hemoglobins. It is probable that the entire compositions are the same in view of the similarity in amino acid compositions of all beta-T4 peptides that have been tabulated for hemoglobin (Dayhoff and Eck, 1968). The tripeptide fragments probably resulted from weak residual chymotryptic activity in the trypsin enzyme acting at the susceptible tryptophan-threonine (W T) peptide bond. Positive tryptophan tests corroborated hemoglobin peptides alpha-T3 and beta-T2 in which there is a tryptophan-glycine (W G) bond.

	βT4
MP	T.(Q).R
HA	L L V V O P W T Q R
C57BL	L.L.V.V.O.P.W.T. Z. R

Tryptic Alpha Peptides of Different Composition from Hemoglobins of M. pennsylvanicus, of Human and of White Mouse Strain C57BL.

Peptides Alpha-T1 and Alpha-T2. The relative compositions are represented in the following sequences inferred for Microtus hemoglobin

peptides alpha-T1 and alpha-T2. Positions of possible differences are underlined.

	αT1	αT2
MP	V.L.S.G.(D).(D).K.S.(N).I.K	
HA	V L S P A D K T N V K	
C57BL	V L S G E D K S N I K	

The presence of some intact human alpha-T(1-2) hemoglobin peptide suggests that this lysylthreonine (K T) bond possesses a resistance to tryptic hydrolysis which is not shared by the lysylserine (K S) bond (in the same environment). Differences in the trypsin susceptibilities of these two bonds has apparently not been noted before.

Peptides Alpha-T3. The following sequence inferred for M. pennsylvanicus hemoglobin peptide alpha-T3 became evident only after the definition of peptide 16' in the peptide maps from other Microtus species to be discussed later.

	αT3
MP	T.A.W.G.K
HA	A A W G K
C57BL	A A W G K

The N-terminal arrangement might equally well be A.T. There was no evidence for the presence of linked alpha-T(2-3) peptides that might

have resulted from a trypsin-resistant lysylthreonine peptide bond (K T) at this position in the peptide chain.

Peptides Alpha-T4. The composition of the Microtus hemoglobin peptide alpha-T4 is expressed in the following sequence inferred relative to those sequences known for human and for white mouse hemoglobins.

	αT4
MP	I.G.A.H.A.G.(E).O.G.A.(E).A.L.(E).R
HA	V G A H A G E O G A E A L E R
C57BL	I G G H A G E O G A E A L E R

Peptides Alpha-T5. Differences observed in amino acid compositions of peptides alpha-T5 are summarized with a sequence of amino acids inferred for M. pennsylvanicus hemoglobin peptide alpha-T5 relative to the other hemoglobin peptides alpha-T5.

	αT5
MP	M.F.V.A.O.P.T.T.K
HA	M F L S F P T T K
C57BL	M F A S F P T T K

Peptides Alpha-T6. Two differences in composition observed between the hemoglobin peptides alpha-T6 from Microtus and those from human and white mouse are recorded in the following sequences.

	αT6
MP	T.O.F.P.H.F.(D).V.S.H.G.S.A.(Q).V.K
HA	T O F P H F D L S H G S A Q V K
C57BL	T O F P H F D V S H G S A E V K

Peptides Alpha-Beta-T7. Differences in amino acid composition were observed between hemoglobin peptides alpha-T7 of Microtus and those of human and of white mouse. It had been anticipated from the work of Baglioni (1961) that the similar human hemoglobin peptides alpha-T7 and beta-T7 would be unresolved in peptide maps. However, the present system of peptide mapping resolved a distinctly grey area from which the human hemoglobin peptide alpha-T7 could be isolated. By contrast, only one peptide spot from the Microtus hemoglobin map furnished a tetrapeptide analysis; coincident peptides, designated alpha-beta-T7, which are considered to be present in this case, showed essentially the same integral numbers of the four different amino acids. Thus, in Table 12 and in Figure 18, relative proportions of amino acids are designated as 2:2:2:2 in order to satisfy two independent peptides. It is believed that the amino acid sequence of both Microtus hemoglobin peptides alpha-T7 and beta-T7 is that inferred below.

	αT7
MP	A.H.G.K
HA	G H G K
C57BL	G H G K

Arguments bearing upon this sequence were presented earlier in the Methods section of this dissertation.

Peptides Alpha-Beta-T(7-8). Additional evidence for peptides alpha-T7 and beta-T7 as well as for alpha-T8 and beta-T8 was obtained from the well-resolved composite peptides alpha-beta-T(7-8) observed in peptide maps of both human and Microtus hemoglobins.

	αT7 | αT8
MP	A.H.G.K.K
HA	G H G K K
C57BL	G H G K K

Peptide Alpha-T9. Peptide alpha-T9 of human hemoglobin was detected on the peptide map in three positions in one of which it appeared to occur as the composite peptide alpha-T(8-9). Location of this large peptide, which may comprise as many as 30 amino acids, in maps of Microtus hemoglobin has presented a special problem which will be discussed in greater detail below.

The remaining tryptic peptides of the alpha chains of hemoglobin from Microtus pennsylvanicus, from human and from white mouse strain C57BL which were presented above possessed identical amino acid compositions as far as could be determined, with the exception of the three differences in composition of the mouse hemoglobin peptide alpha-T12b.

Tryptic Beta Peptides of Different Composition from Hemoglobins of M. pennsylvanicus, of Human and of White Mouse Strain C57BL/6.

There has been no published report yet of the experimental establishment of the exact amino acid sequence for the beta chain of white mouse hemoglobin although compositions were reported for strains C57BL/6 and SEC (Rifkin et al., 1966b). The following amino acid sequences inferred for white mouse strain C57BL/6 beta chain were taken from Dayhoff and Eck (1968). Aspartic acid and glutamic acid have been designated for white mouse hemoglobin by the noncommital letters B and Z respectively since there has been no report yet of the experimental establishment of the free acid or amide forms. Information from peptide maps of human hemoglobin and of Microtus hemoglobin from the present research has been used as a basis to suggest possible charged or uncharged forms of the dicarboxylic amino acids, aspartic acid and glutamic acid, in the Microtus hemoglobin peptides. Again, periods interposed between the letter designations for the amino acids indicate that the suggested sequences have been inferred and have not been established by specific chemical sequencing.

Peptides Beta-T1. Bonaventura and Riggs (1967) reported a chemically determined sequence for hemoglobin peptide beta-T1 from white mouse strain BALB/cJ, and this sequence is included below together with the sequence inferred for peptide beta-T1 from M. pennsylvanicus Hb.

	βT1
MP	V.H.L.T.A.(E).(D).K
HA	V H L T P E E K
C57BL/6	V.H.L.T.A. Z. B. K
BALB/cJ	V H L T B Z A K

Peptides Beta-T2. Numerous differences in the amino acid composition of homologous peptides from human and from Microtus hemoglobins are presented in this dissertation on the basis of determinations of their gross amino acid compositions. That the sequence of amino acids in homologous peptides from different animals can affect the number of amino acid differences derived is well illustrated by a comparison of the beta-T2 peptides. Dayhoff and Eck (1968) inferred the following amino acid sequence for peptides beta-T2 of C57BL/6 mouse hemoglobin by homology with the established amino acid sequence for peptide beta-T1 of human hemoglobin. Bonaventura and Riggs (1967) reported the sequence of the first 5 amino acids in peptide beta-T2 of BALB/cJ mouse hemoglobin resulting from a chemical determination. Alternative inferred sequences are given below using the experimental results for Microtus and the data for C57BL/6 white mouse hemoglobin.

	βT2	
MP	A.A.I.S.G.L.W.G.K	by homology with BALB/cJ.
MP	S.A.I.G.A.L.W.G.K	by homology with human.
HA	S A V T A L W G K	
BABL/cJ	A A V S C L.W.A/G.K	
C57BL/6	S.A.V.G.A.L.W.G.K	by homology with human.
C57BL/6	A.A.V.S.G.L.W.G.K	by homology with BABL/cJ.

On the basis of the data for total amino acid composition (Table 21) two differences are indicated between peptides beta-T2 of human and of Microtus hemoglobin. This conforms to the two differences in the sequence of amino acids for the Microtus hemoglobin peptide beta-T2 inferred above by homology with the corresponding human hemoglobin peptide beta-T2.

By contrast, if the amino acid sequence for Microtus hemoglobin peptide beta-T2 is inferred by homology with the corresponding BALB/cJ hemoglobin peptide beta-T2, then there are four differences in sequence, hence four differences in composition, between the Microtus and the human hemoglobin peptides beta-T2. The transpositions of an alanine (A), a serine (S), and a glycine (G) reduce the apparent number of differences in composition to two.

Data from this research which suggest the first sequence inferred above for the Microtus peptide to be more likely were presented earlier in the Methods section of this dissertation in connection with amino acid destruction in peptide mapping and amino acid analysis.

Peptides Beta-T3. Tryptic peptide beta-T3 of normal human hemoglobin was not observed in peptide maps prepared by the techniques described in this research. A possible explanation for the absence of this peptide may be found in the already established amino acid composition of the peptide shown in the sequence below. The peptide contains among a total of 13 amino acids one aspartic acid (D) and two glutamic acids (E) which have ionizable side-chain carboxyls. The negative charge

conferred on the peptide by these side-chain groups at the pH of the electrophoresis could have led to the migration of the beta-T3 peptide off the paper in the direction of the anode with its consequent loss from the map.

A possible sequence inferred for the amino acids in M. pennsylvanicus hemoglobin peptide beta-T3 is shown below; there is a striking difference between the numbers of alanine (A) present in the several peptides beta-T3.

	βT3
MP	A.B.V.B.A.A.G.A.Z.A.L.G.R
HA	V N V D E V G G E A L G R
C57BL/6	V.B.V.B.Z.A.G.G.Z.A.L.G.R

The glycine (G) at position 24 from the N-terminal of the beta chain had been reported to be invariant in all hemoglobin peptides (including myoglobin, Dayhoff and Eck, 1968) until Popp (1967) determined that valine was substituted at this position in hemoglobin of white mouse strain NB. The homologous position in the alpha chain is position number 25 which occurs in tryptic peptide alpha-T4.

Peptides Beta-T4. These peptides were presented above in the discussion of tryptic hemoglobin peptides of identical amino acid composition from the several species.

Peptides Beta-T5 and Beta-T6. It was noted already that the independent tryptic dipeptide beta-T6 (V K in human hemoglobin) was absent from the peptide maps of Microtus hemoglobin and from the results of the comprehensive amino acid analyses of Microtus peptides. No peptides were found which could be reconciled with composite peptides beta-T(5-6) or beta-T(6-7), each of which would be expected to show a relative proportion of two lysines (K) among the constituent amino acids.

It became apparent from the eventual comparative examination of the amino acid analysis for the human hemoglobin beta-T5 peptide and the amino acid analysis for the proposed Microtus hemoglobin beta-T5 peptide that the Microtus peptide differed at least from the human peptide in having one additional residue each of valine and histidine. Such a difference could account for the inclusion of the V K dipeptide in the beta-T5 peptide if the beta-T5 C-terminal amino acid lysine (K), as found in human hemoglobin, had been substituted by a histidine (H) at this position in the Microtus hemoglobin. In this case the two beta-T5 peptides of human and of Microtus hemoglobin would be expected to possess a total of 19 and 21 amino acids respectively.

The largest number of amino acids that could be accounted for in the Microtus hemoglobin peptide beta-T5 was at best 19, taking into consideration possible losses of amino acids due to peptide mapping and amino acid analysis procedures as considered earlier in the Methods section. There is at least one explanation of how the union of two peptides of 19 and 2 amino acids could lead to a peptide containing 19

amino acids. Two deletions of amino acids could have occurred in the beta-T5 region of the beta chain; that is, two amino acids could have been "substituted without a replacement." Some support for this explanation may be found in the absence particularly of one aspartic acid from the Microtus peptide relative to both human and white mouse hemoglobin peptides beta-T5. Perhaps significant also could be the absence of isoleucine from the Microtus peptide relative to white mouse hemoglobin peptide beta-T5, or the absence of a third valine from the Microtus peptide relative to human hemoglobin peptide beta-T5.

In order to achieve the maximum degree of homology between alpha and beta hemoglobin chains, Braunitzer et al. (1965) first suggested that five amino acids could be considered to have been deleted from the alpha chains of all hemoglobins in a region found in the tryptic peptide alpha-T6. It is precisely in the homologous region of the beta chain that the deletions may be proposed for the Microtus beta chain. It may also be noteworthy that the alpha-T6 peptide is longer by two amino acids because of the presence of the segment Q V K in place of the homologous amino acids K V K of the beta chain.

In the context of the new research of this dissertation it is proposed that there are two deletions of amino acids in the beta-T5 peptide of Microtus hemoglobin. Positions for these deletions are suggested in the sequence of amino acids inferred below for the Microtus hemoglobin beta-T5(6) peptide. The alpha-T6 peptides of human hemoglobin and of Microtus hemoglobin are also shown for comparison. The alignment of the alpha and beta peptides for maximum homology introduces another

"Braunitzer gap" in the alpha-T6 peptides.

	βT5	βT6 (HA etc.)
MP	V.F.Z.S.F.G.B.L.S.S.A._.A._.M.G.B.A.H.V.K	
HA	F F E S F G D L S I P D A V M G N P K V K	
C57BL/6	O.F.S.S.F.G.B.L.S.S.A.B.A.I.M.G.B.A.K.V.K	

	αT6
MP	T.O.F.P.H.F._.(D).V.S.H._._._._._.G.S.A.(Q).V.K
HA	T O F P H F _ D L S H _ _ _ _ _ G S A Q V K
C57BL	T O F P H F _ D V S H _ _ _ _ _ G S A E V K

The phenylalanine (F) at position 42 from the N-terminal of the beta chain (second from N-terminal of the peptide beta-T5 above) is invariant in all hemoglobin peptides which have been reported to date. Both phenylalanines at positions 42 and 45 in the beta chain (positions 43 and 46 in the alpha chain) are implicated in the binding of heme to globin in accordance with the observations of Kendrew (1962) on myoglobin (Braunitzer et al., 1964). It is not to be anticipated that the deletion of 2 amino acids in this region of the beta chain should necessarily affect the functioning of the Microtus hemoglobin since alpha chains are functional in the absence of 5 amino acids in this region.

The effect of two deletions in the beta-T5(6) peptide of Microtus hemoglobin should be to reduce the total number of amino acids in the beta chain to 144 (barring insertions or deletions of other amino acids

elsewhere in the chain). The difference in lengths of alpha and beta chains of human hemoglobin, 141 and 146 amino acids respectively, can be attributed in part to the 5 deletions in the alpha chain.

The effect of the fusion of the Microtus beta-T5 and beta-T6 peptides is to reduce the number of expected tryptic peptides from 15 to 14 (barring other substitutions of lysine or arginine elsewhere in the beta chain) relative to the beta chain of human hemoglobin. There are a number of instances documented in which the presence of additional lysine or arginine in a hemoglobin chain increases the possible number of tryptic peptides beyond the 29 expected from human hemoglobin. A decrease in the possible number of tryptic peptides of hemoglobin due to the absence of one or more of the 27 lysines and arginines (expected from human hemoglobin) is not believed to have been reported previously.

Peptides Beta-T7 and Beta-T8. Peptides beta-T7, beta-T8 and the composite peptide beta-T(7-8) were referred to in the preceding discussion of peptides alpha-beta-T7 and alpha-beta-T(7-8). The proposed sequence of tryptic peptides beta-T7 and beta-T8 for Microtus hemoglobin is given below. The histidine (H) is invariant in all alpha-T7 and beta-T7 hemoglobin peptides reported to date; it is the so-called distal histidine at the sixth coordination position of the iron in hemoglobin.

	βT7 | βT8
MP	A.H.G.K.K
HA	A H G K K
C57BL/6	A.H.G.K.K

Peptides Beta-T9. Tryptic hemoglobin peptides beta-T9 or beta-T(8-9) have not been positively identified for M. pennsylvanicus, but the unidentified peptide 19 occupies a map position similar to that occupied by beta-T(8-9) identified in the maps of M. abbreviatus Hb-f and M. miurus Hb-f.

The amino acid composition of M. abbreviatus Hb-f beta-T(8-9) is represented in the following sequence inferred relative to that sequence which has been established for human hemoglobin peptide beta-T(8-9) and relative to that sequence which has been inferred for white mouse hemoglobin peptide beta-T(8-9).

	βT8 | βT9
MAf	K.V.L.B.T.L.S.B.G.A.A.H.L.B.B.L.K
HA	K V L G A F S D G L A H L D N L K
C57BL/6	K.V.L.B.T.F.S.B.G.I.A.H.L.B.B.L.K

Further consideration will be given to peptides beta-T9 and 19 later in this Discussion.

Peptides Beta-T10. Two of the 13 constituent amino acids of this peptide, leucine (L) at position 88 and histidine (H) at position 92 relative to human beta chain, have proven to be invariant in all hemoglobins reported to date. The homologous positions in the human alpha-T9 peptide are L-83 and H-87. It is these histidines which are coordinated (proximal) to the iron porphyryns of the alpha and beta chains of hemoglobin.

Human hemoglobin contains cysteine (C) in each of the three tryptic peptides alpha-T12, beta-T10, and beta-T12. Both human and Microtus hemoglobins were reacted with ethylenimine in the present studies prior to peptide mapping procedures as described in the Methods section. The reaction of ethylenimine with cysteine is expected to produce a cysteine derivative with a basic side chain analagous to that of lysine:

Aminoethylcysteine Side Chain $-CH_2-S-CH_2-CH_2-NH_2$

Aminobutyl Side Chain of Lysine $-CH_2-CH_2-CH_2-CH_2-NH_2$

Trypsin was successfully induced in this way to cleave peptide bonds at the C-termini of the cysteine residues in peptides alpha-T12 and beta-T12 of human hemoglobin; cleavage of the peptide beta-T10 failed to take place, and this behavior accorded with that reported by Jones (1964).

The three peptides of Microtus hemoglobin, alpha-T12, beta-T10, and beta-T12 each responded to tryptic digestion differently, one from another. Microtus hemoglobin peptide alpha-T12 was cleaved to produce a soluble fragment alpha-T12a which was recovered in the tryptic peptide map and an insoluble fragment alpha-T12b which was largely recovered in a map of the chymotryptic digest of the residues from the tryptic digestion. Peptide beta-T10 was recovered intact without cleavage, and aminoethylcysteine was observed in the amino acid analysis. Precise identification of peptide beta-T12 or of its fragments was not obtained as will be discussed presently.

The sequence of amino acids inferred for Microtus hemoglobin peptide beta-T10 is shown here in relationship to the sequences of the

corresponding human and white mouse hemoglobin peptides beta-T10.

	βT10
MP	G.T.F.A.S.L.S.(E).L.H.C.(D).K
HA	G T F A T L S E L H C D K
C57BL/6	G.T.F.A.S.L.S. Z. L.H.C. B. K

Peptides Beta-T11. All hemoglobin peptides beta-T11 possess the same amino acid composition which was presented earlier together with other peptides of identical composition.

Peptides Beta-T12. Microtus peptides beta-T12 have not been identified with certainty. The absence of peptide fragments beta-T12a and beta-T12b from the maps of Microtus hemoglobins points to 1) a failure of trypsin to cleave the peptide beta-T12 at an aminoethylcysteine or 2) the absence of a cysteine from beta-T12. Cysteine has been reported to be absent from the beta-T12 peptide from hemoglobins of white mouse strains C57BL/6 and SEC (Rifkin et al., 1966b). The problem of the Microtus beta-T12 peptides will be considered below in relationship to the amino acid analyses for certain unidentified Microtus peptides.

Peptides Beta-T13. A sequence for M. pennsylvanicus hemoglobin peptide beta-T13 is proposed relative to the sequences for the same peptides of human and white mouse hemoglobins. The L/F designation is intended to suggest that the amino acid may not be uniquely determined

at this position. Additional consideration is given to this point in the next section.

	βT13
MP	(E).F.T.P.A.A.(Q).A.S.(L/F).(N).K
HA	E F T P P V Q A A O Q K
C57BL/6	B. F.T.P.A.A. Z. A.A. F . Z. K

Peptides Beta-T14. The proposed sequence of amino acids for this peptide of M. pennsylvanicus hemoglobin differs at one position from those beta-T14 peptides of hemoglobins of human and white mouse.

	βT14
MP	V.V.A.G.V.A.S.A.L.A.H.K
HA	V V A G V A N A L A H K
C57BL/6	V.V.A.G.V.A.B.A.L.A.H.K

Peptides Beta-T15. Peptides beta-T15 were found to be the same in all species of hemoglobin, as presented earlier. The tyrosine (O) at position 145 of the human beta chain (and in the homologous position 140, peptide alpha-T14, of the human alpha chain) is invariant in all hemoglobins reported to date.

THE COMPARATIVE AMINO ACID COMPOSITION OF HEMOGLOBINS OF DIFFERENT SPECIES OF ALASKAN MICROTUS: M. PENNSYLVANICUS, M. ABBREVIATUS Hb-f AND Hb-s, M. MIURUS Hb-f AND M. OECONOMUS Hb-f.

Some of the most interesting results of the present research have come from the comparison of the amino acid composition of tryptic peptides established for M. pennsylvanicus hemoglobin with the composition of tryptic peptides of hemoglobins determined for other species of Microtus. There was no a priori reason to anticipate that differences in numbers of charged amino acids should be found between the single hemoglobin of M. pennsylvanicus and the fast, major component of the hemoglobins (Hb-f) of the other Microtus species: all of these hemoglobins showed the same electrophoretic mobilities in acrylamide gel at pH 8.6.

However, as stated in the Introduction to this dissertation, Popp had observed that there were differences in the neutral amino acid composition between the alpha chains of the single hemoglobin of strain NB and the major component of the multiple hemoglobins of strain BALB/cJ. Differences in the neutral amino acid composition were detected in the present research between the alpha chains of M. pennsylvanicus hemoglobin and the alpha chains of the major components of the multiple hemoglobins of the other Microtus species.

Popp had also concluded that the fundamentally different character of single and multiple hemoglobins of different strains of inbred laboratory mice resulted from differences in amino acid compositions between the different beta chains. An extension of this argument postulates that the differences of electrophoretic behavior observed between the multiple hemoglobin components from the same strain of mouse should be related to differences in amino acid composition

between their beta chains.

In the present research, amino acid differences have in fact been detected in the beta chains between the single hemoglobin of M. pennsylvanicus and the major components of the two hemoglobins of the other species of Microtus which were examined. However, no differences could be distinguished between the amino acid compositions of Hb-f and Hb-s of M. abbreviatus, the only Microtus for which analysis of the slow, minor component (Hb-s) was attempted. To this author's knowledge there has been no adequate verification yet for the prediction from Popp's work that multiple hemoglobins with different electrophoretic mobilities from the same mouse should show differences in amino acid composition between their beta chains.

Tryptic Alpha Peptides of Different Composition from Hemoglobins of M. pennsylvanicus, M. abbreviatus Hb-f and Hb-s, M. miurus Hb-f, and M. oeconomus Hb-f.

Peptides Alpha-T2. The amino acid sequences inferred for the several Microtus hemoglobin peptides alpha-T2 are presented together with the reference sequences for the human and white mouse hemoglobin peptides. As stated earlier, the finding of the composite alpha-T(1-2) peptide in peptide maps for human hemoglobin, for M. abbreviatus Hb-f and for M. miurus Hb-f is considered to support the inference of the N-terminal threonine for the Microtus alpha-T2 peptides. N-Terminal serine (S) in the alpha-T2 peptide of M. pennsylvanicus hemoglobin represents the substitution of an amino acid with the same neutral

functional group -OH in its side chain but without the additional methyl group of threonine (T):

(S) -CHOH-H ; (T) -CHOH-CH_3 .

	αT2
MP	S.N.I.K
MAf	T.N.I.K
MAs	T.N.I.K
MMf	T.N.I.K
MOf	T.N.I.K
HA	T N V K
C57BL	S N I K

The presence of serine or threonine in this peptide bond with lysine joining peptides alpha-T1 and alpha-T2 appears to affect the susceptibility of this peptide bond to the attack of the proteolytic enzyme trypsin.

That the several peptides alpha-T2 had essentially the same electrophoretic mobilities, supports the inference of asparagine (N) residues with their uncharged side chain amide groups:

(N) -CH_2-CO-NH_2 .

Peptides Alpha-T3. The amino acid sequences inferred for the several Microtus hemoglobin peptides alpha-T3 are shown together with the same peptides from human and white mouse. All peptides alpha-T3 of the Microtus hemoglobins contained the smaller proportions of alanine relative to the human hemoglobin peptide which leads to the inference that an alanine was substituted by a threonine or an aspartic

acid. The electrophoretic mobilities of all of the hemoglobin peptides alpha-T3 were essentially the same which suggest that aspartic acid is present as asparagine with its neutral amide side chain.

	αT3
MP	T.A.W.G.K
MAf	N.A.W.G.K
MAs	N.A.W.G.K
MMf	N.A.W.G.K
MOf	N.A.W.G.K
HA	A A W G K
C57BL	A A W G K

The peptides alpha-T3 of human and of M. pennsylvanicus hemoglobin moved slightly farther in the chromatographic direction than the alpha-T3 peptides of the other Microtus hemoglobins, presumably reflecting differences between the residues of alanine (A) or of threonine (T) with methyl groups in their side chains and residues of asparagine (N) with their amide side chains:

(A) $-CH_3$; (T) $-CHOH-CH_3$; (N) $-CH_2-CO-NH_2$.

Peptides Alpha-T4. The amino acid sequences inferred for the several Microtus hemoglobin peptides alpha-T4 are:

	αT4
MP	I.G.A.H.A.G.E.O.G.A.E.A.L.E.R
MAf	I.G.G.H.A.G.E.O.G.A.E.A.L.E.R
MAs	I.G.G.H.A.G.E.O.G.A.E.A.L.E.R
MMf	I.G.G.H.A.G.E.O.G.A.E.A.L.E.R
MOf	I.G.G.H.A.G.E.O.G.A.E.A.L.E.R
HA	V G A H A G E O G A E A L E R
C57BL	I G G H A G E O G A E A L E R

The only difference in amino acid composition between peptides alpha-T4 of Microtus hemoglobins appeared to be in the substitution of an alanine in M. pennsylvanicus by a glycine in the other Microtus hemoglobin alpha-T4 peptides. The position of this substitution as well as that of isoleucine relative to human hemoglobin alpha-T4 was inferred from the amino acid sequence of the white mouse C57BL strain hemoglobin peptide alpha-T4. The substitution involves the difference of a methyl group in the side chains of glycine and of alanine:

(G) -H ; (A) $-CH_3$.

Tryptic Beta Peptides of Different Composition from Hemoglobins of M. pennsylvanicus, M. abbreviatus Hb-f and Hb-s, M. miurus Hb-f, and M. oeconomus Hb-f.

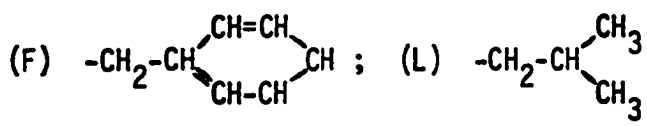
Amino acid differences were found in three beta peptides among the hemoglobins of the several Microtus species.

Peptides Beta-T5(6). The peptide beta-T5 of M. pennsylvanicus was discussed in some detail above. The fusion of the tryptic peptides beta-T5 and beta-T6 which was postulated for M. pennsylvanicus hemoglobin is also proposed to apply to peptides of the other species of Microtus in view of their similar amino acid compositions and the absence of the dipeptide VK. Apparent substitutions not common to all species of Microtus are underlined below in sequences inferred relative to that of human hemoglobin peptides beta-T5 and beta-T6. One substitution inferred here between peptides beta-T5(6) of the Microtus hemoglobins again involves a serine in M. pennsylvanicus hemoglobin

substituted by a threonine in the peptides beta-T5(6) of the other species of Microtus from which quantitative data were obtained.

	βT5(6)
MP	V.F.Z.S.F.G.B.L.S.S.A._.A._.M.G.B.A.H.V.K
MAf	V.F.Z.S.L.G.B.L.S.T.A._.A._.M.G.B.A.H.V.K
MMf	V.F.Z.S.L.G.B.L.S.T.A._.A._.M.G.B.A.H.V.K
HA	F F E S F G D L S T P D A V M G N P K V K
C57BL/6	O.F.S.S.F.G.B.L.S.S.A.B.A.I.M.G.B.A.K.V.K

The replacement of a phenylalanine in the beta-T5(6) peptides of M. abbreviatus Hb-f and M. miurus Hb-f is another unique feature of these peptides. The phenylalanines of the beta-T5 peptides are implicated in heme-protein binding (see later section on Significance of Primary Structure in a Functional Context). Only one other such replacement has been reported for hemoglobins: the substitution of the phenylalanine in the homologous position of the alpha chain of carp hemoglobin by a tryptophan (Hilse and Braunitzer, 1968). The replacement of a phenylalanine (F) by a leucine (L) is attended by the following change in the side chain:



The map position of the M. pennsylvanicus hemoglobin peptide, displaced to a greater distance in the chromatographic direction relative to the other Microtus peptides beta-T5(6), may be a difference resulting from the oxidation state of the methionine.

Peptides Beta-T10. The amino acid sequences inferred for the several Microtus hemoglobin peptides beta-T10 are shown below.

	βT10
MP	G.T.F.A.S.L.S.E.L.H.C.D.K
MAf	G.T.F.S.S.L.S.E.L.H.C.D.K
MAs	G.T.F.S.S.L.S.E.L.H.C.D.K
MMf	G.T.F.S.S.L.S.E.L.H.C.D.K
MOf	G.T.F.A.S.L.S.E.L.H.C.D.K
HA	G T F A T L S E L H C D K
C57BL/6	G.T.F.A.S.L.S.Z.L.H.C.B.K

The inferred substitutions involve the methyl side chain of alanine (A) and the hydroxymethyl side chain of serine (S): (S) $-CH_2OH$; (A) $-CH_3$. It is of interest to note for the first time that a peptide of M. pennsylvanicus hemoglobin, beta-T10, appears to have the same amino acid composition as one other Microtus peptide, beta-T10 of M. oeconomus Hb-f, and at the same time it differs in amino acid composition from the peptides beta-T10 of the other three Microtus hemoglobins. The increases in serine between the beta-T10 peptides of human hemoglobin, of M. pennsylvanicus Hb and of M. abbreviatus Hb-f are strikingly shown in Figure 31.

Peptides Beta-T13. The beta-T13 peptides present differences in amino acid compositions between the Microtus species, but the differences are less well defined than those for other peptides discussed earlier. Peptide beta-T13 of M. abbreviatus Hb-f appears to have

additional alanine and phenylalanine and less serine and leucine than the beta-T13 of the other Microtus hemoglobins. It is inferred that there are substitutions between serine and alanine residues and between leucine and phenylalanine residues in the sequences given below. Peptide beta-T13 of M. oeconomus Hb-f was not identified.

	βT13
MP	(E).F.T.P.A.A.(Q).A.S.(L/F).(N).K
MAf	(D).F.T.P.A.A.(Q).A.A. F. (Q).K
MAs	(E).F.T.P.A.A.(Q).A.S. L. (N).K
MMf	(E).F.T.P.A.A.(Q).A.S. L. (N).K
HA	E F T P P V Q A A O Q K
C57BL/6	B. F.T.P.A.A. Z. A.A. F. Z. K

The inference of N-terminal aspartic acid (D) or glutamic acid (E) for the several Microtus peptides follows from the nature of the departures from integral numbers seen in the data for these two amino acids. A suggestion that these non-integral data are meaningful was presented in the Materials section of this dissertation.

The data suggest the possibility that there might be a mixture of two peptides beta-T13 of M. pennsylvanicus Hb, one peptide with leucine and the other peptide with phenylalanine substituted at the same position in the peptide chain. There remains the possibility, presented in the Introduction, that certain fractional proportions of different amino acids in a peptide may be real and may reflect the simultaneous presence in the hemoglobin of the same animal of two alpha or of two beta chains

which differ in composition at one (or more than one) specific position in the peptide. Such substitutions, occurring simultaneously within the hemoglobin of a single animal, have been termed ambiguities or translational variations.

The first evidence for such internal substitutions was reported only very recently (von Ehrenstein, 1966; Rifkin et al., 1966a; Popp, 1967) and is still subject to careful substantiation. The ambiguities presented by these authors appeared to be confined to the alpha chains of hemoglobins of rabbits and of white mice from various strains. The substitutions at the ambiguous positions involved five amino acids, the similar pair serine and threonine and the similar trio valine, leucine and phenylalanine. von Ehrenstein (1966) noted substitutions in rabbit hemoglobin peptides which involved pairs of the above five amino acids including valine-threonine and leucine-serine substitutions. Non-integral numbers for threonine and for serine were reported by Popp (1967) for peptide alpha-T9 from a white mouse of the SEC strain. Bonaventura and Riggs (1967) suggested a possible ambiguity in the beta-T2 peptide of white mouse hemoglobin, strain BALB/cJ, involving glycine and alanine.

Data from the present research do not support much conjecture about the possibility of ambiguities in Microtus hemoglobin peptides. The substitutions of amino acids that have been detected between similar peptides from hemoglobins of different Microtus species involve only the neutral amino acids serine, threonine, alanine, glycine, and probably leucine and phenylalanine. Data from the one peptide beta-T13 of M. pennsylvanicus hemoglobin suggest the possibility of an ambiguity

involving leucine and phenylalanine. There is a relatively clear indication that only leucine or phenylalanine may occupy the suggested position of ambiguity in the beta-T13 peptides of the other Microtus hemoglobins.

Data for the unidentified peptides 16, 16' and 18 contain some suggestive non-integral numbers for the amino acids valine, leucine, isoleucine and phenylalanine.

Unidentified Microtus Hemoglobin Peptides.

Fragments of the Microtus (and of the human) peptides alpha-T12b, alpha-T13, and beta-T4 were not identified in this research; as has already been presented above, there seems to be only a small probability that unique amino acid substitutions might be found in these unidentified fragments.

The case of the missing Microtus hemoglobin beta-T6 peptide has been discussed in some detail above in connection with the identification of the beta-T5 peptide.

Peptide beta-T9 of M. pennsylvanicus Hb (and of M. oeconomus Hb-f) was not found although the unidentified peptide 19 seems to bear some relationship to beta-T9 in both map position and in amino acid analysis. An unidentified peptide 19' obtained from M. oeconomus Hb-f may also be related to beta-T9.

Unidentified peptides 20 and 20' were obtained from the map of M. oeconomus hemoglobin and may be related to the peptide beta-T13 which was not found for this species.

The two major Microtus hemoglobin peptides which have not been accounted for are alpha-T9 and beta-T12. Three prominent unidentified peptides, 16, 16' and 17 (in addition to 18 which was obtained only from the map of M. abbreviatus Hb-f) may be related to Microtus peptides alpha-T9 and beta-T12.

Peptides Alpha-T9 and Beta-T12. Alpha-T9 is the largest single peptide expected to be recovered from tryptic digests of human hemoglobin. Intact human alpha-T9, which contains 29 amino acid residues, was recovered in this research in the soluble fraction of tryptic digests. (Other, "multiple," tryptic peptides, e.g. alpha-T(8-9) or alpha-T(12-13), contain a greater number of amino acids than alpha-T9; alpha-T(12-13) appeared largely in the insoluble fraction of tryptic digests.)

The largest unidentified peptide, M. pennsylvanicus peptide 17, may have 22 amino acids although the other Microtus peptides 17 appear to have about 17 amino acids (Table 36). These numbers of amino acids in the peptides 17 are clearly insufficient to account for the expected total number of amino acids in intact alpha-T9. The presence of proline (P) in the analyses of peptides 17 is suggestive of alpha-T9. The replacement of an amino acid residue within the region of Microtus hemoglobin which is homologous with the alpha-T9 peptide of human hemoglobin could lead to a tryptic splitting of this nominal Microtus peptide alpha-T9 into smaller fragments. It is of interest to consider, as shown below: the established amino acid sequence of peptides alpha-T9

of human and of white mouse hemoglobins; the established homologous sequence of amino acids in the beta-T(9-10) peptides of human hemoglobin beta chain; additional information which has been reported concerning the substitution of lysine (or arginine) residues in homologous regions of the alpha and beta chains of hemoglobins of other species (Dayhoff and Eck, 1968).

The basis for the homology of the alpha-T9 peptides and of the beta-T(9-10) peptide is clearly seen in terms of the identical residues at the same positions in the 29 member polypeptide chains. Numbering from the N-terminal of the respective peptides, the identical residues and positions are: V1, A4, A10, H11, D13, L22, S23, L25, H26, K29.

Human αT9

V A D A L T N A V A H V D D M P N A L S A L S D L H A H K
1 5 10 15 20 25

Human βT9 | Human βT10

V L G A F S D G L A H L D N L K G T F A T L S E L H C D K
1 5 10 15 20 25

C57BL αT9

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
1 5 10 15 20 25

Lysine (K) at position 16 in the beta chain represents a substitution for proline (P) at the equivalent position in the alpha chain. Substitutions of lysine (K) at the position equivalent to 7 in the human alpha chain have been reported in the alpha chains of sheep, llama, pig and rabbit hemoglobins. Substitutions of lysine (K) at the position equivalent to 10 in the human beta chain have been reported in

the beta chains of pig, camel, sheep and bovine hemoglobins. A substitution of arginine (R) at the position equivalent to 4 in the human beta chain has been reported in the beta chain of camel hemoglobin.

The amino acid sequence for peptide alpha-T9 of white mouse C57BL hemoglobin is seen to be similar to alpha-T9 of human hemoglobin and to differ from it in only 5 places.

The difficulty in speculating on a reasonable substitution(s) of lysine in the alpha-T9 peptide of Microtus hemoglobin which might fit the peptide 17 data seems to be increased by the lack of uniformity between the compositions of the peptides 17 from the different species of Microtus. The presence of arginine (R) in each of the analyses suggests the presence of a second peptide coincident with 17, perhaps an overlapping of the neighboring peptide beta-T11 (Figures 5-9) which is an arginine peptide.

Peptides beta-T12 of human and of white mouse hemoglobins contain 16 amino acids:

	βT12
Human	L L G N V L V C V L A H H F G K
C57BL/6	L.L.G.B.V.L.I.I.V.L.G.H.H.M.G.K

Superficially the unidentified peptides 16 and 16' resemble the beta-T12 peptides in the absence from them of threonine (T), serine (S), glutamic acid (Z) and proline (P) and in the presence of isoleucine (I). Peptides 17 are unlike the peptides 16, 16' and beta-T12 in terms of these same amino acids. Peptide 18 looks superficially like some hybrid of 16 and

17.

There is a striking resemblance between the amino acid compositions of the unidentified peptides 16 and 16' which were recovered from widely separated positions in the Microtus peptide maps (Figures 5-9 and 11). An outstanding difference between the two peptides is observed in the single additional residues each of lysine and of histidine in peptide 16'. The presence of the additional basic residues in peptide 16' is consistent with its map position which is displaced toward the cathode relative to peptide 16.

The apparent numbers of amino acids in peptides 16 and 16', perhaps 9 and 11 amino acids respectively, are not sufficient to account for the expected amino acid composition of intact beta-T12 peptides as found in human hemoglobin.

THE PRIMARY STRUCTURE INFERRED FOR MICROTUS HEMOGLOBIN.

The precise linear sequence of amino acids in polypeptide linkage, the primary structure, for the alpha and beta proteins of Microtus hemoglobin has not been determined directly in this research. Yet there are compelling reasons for considering the results of the amino acid analyses of the various Microtus hemoglobin tryptic peptides in terms of 1) the probable origin of the peptides in specific portions of the alpha and beta proteins and 2) the probable linear sequence of amino acids within those peptides.

Although the ordering of amino acids within proteins is random according to the best contemporary estimates, yet proteins have unique

structures. The unique character of hemoglobin protein has been shown to reside not only in differences from other kinds of proteins (including differences in amino acid composition and sequence as well as functional differences) but by persistant elements of structural similarity between hemoglobins originating in a wide range of animal species. In the foregoing presentation, points of difference between the hemoglobins of human and other animal species have frequently been stressed, but at least equal stress should be placed upon the remarkable similarities in structure which are also observed. Popp (1967) found amino acid differences at 17 positions between the alpha chains of human and C57BL mouse hemoglobins. That is to say that at 124 positions (23 of which, admittedly, themselves were based on homology with human hemoglobin) the identical amino acids were present in alpha chains from both human and mouse hemoglobins.

If all possible hemoglobins are considered for which primary structures have been determined, the number of invariant amino acids, that is the number of specific amino acids which persist at the same homologous positions in all globin chains, dwindles to six or less out of approximately 150 amino acids per chain. Yet despite the extensive amino acid replacements possible in the primary structures of the normal hemoglobins of various species, the uniqueness of hemoglobin is preserved in terms of the functional properties and more especially in the recognized higher orders of hemoglobin structure. Several current lines of evidence strongly suggest that the three-dimensional structure of proteins is completely determined by the primary structure.

A unique feature of the higher order hemoglobin structure is the very great content of alpha-helical regions; nearly 80% of normal (horse) hemoglobin protein is compactly coiled in regions of alpha-helix which is sometimes referred to as secondary structure. Superimposed upon this is unique tertiary structure, the folding of the coiled polypeptide chains which thereby provides "pockets" for the essential heme groups. Finally, a fourth level of structure is attained by the association of four folded, coiled polypeptide chains as the active oxygen-carrying tetramer $\alpha_2\beta_2$.

At the other end of the possible range of amino acid substitutions, the studies of Ingram and others on the primary structures of abnormal hemoglobins have indicated that only a single amino acid substitution in the normal hemoglobin primary structure can result in a molecule that is both structurally and functionally distinct from the normal one.

The above considerations both support the concept of the primary structural control of higher-order structures for proteins and demonstrate a sensitive relationship between biological function and primary structure (Mahler and Cordes, 1966).

Significance of Inferred Primary Structure for Microtus Hemoglobin in a Functional Context.

There are no data from this research which bear upon the detailed functional properties of the Microtus hemoglobins, and what has been proposed here concerning the primary structure of the Microtus hemoglobins has been pieced together by inference. Accordingly discussion

in the context of structure and function must be restricted to a few generalizations, these cautiously advanced.

It is probably safe to assume that the normal Microtus hemoglobins in vivo are perfectly functional; that their molecules are equivalent in size and in three-dimensional structure to the tetrameric $\alpha_2\beta_2$ molecules which have been observed generally in mammalian blood; and that iron(II)-heme prosthetic groups of the hemoglobins are involved centrally in the physiologically important reaction of oxygen binding. Two features of the interaction of the ferroheme prosthetic group and the native globin protein are of especial importance for the reversible combination with oxygen: 1) the presence of the ligand to the heme iron in the fifth coordination position and 2) the environment of the heme group.

The data presented in this dissertation concerning the amino acid composition of the Microtus hemoglobins point clearly to the presence of a histidine in peptide beta-T10, a so-called proximal histidine which is supposed to occupy the fifth coordination position in the same peptide of the human and other hemoglobins on the basis of X-ray data obtained from horse hemoglobin and sperm whale myoglobin. (See Figure 39.) It has not been possible to specify a proximal histidine for the alpha chain of Microtus hemoglobin because the pertinent peptide alpha-T9 is not accounted for. (See Figure 38.) The so-called distal histidines in the sixth coordination position of the heme iron can be accounted for by the Microtus data; see Figures 38 and 39.

Antonini (1965) emphasizes repeatedly the greater extent to which

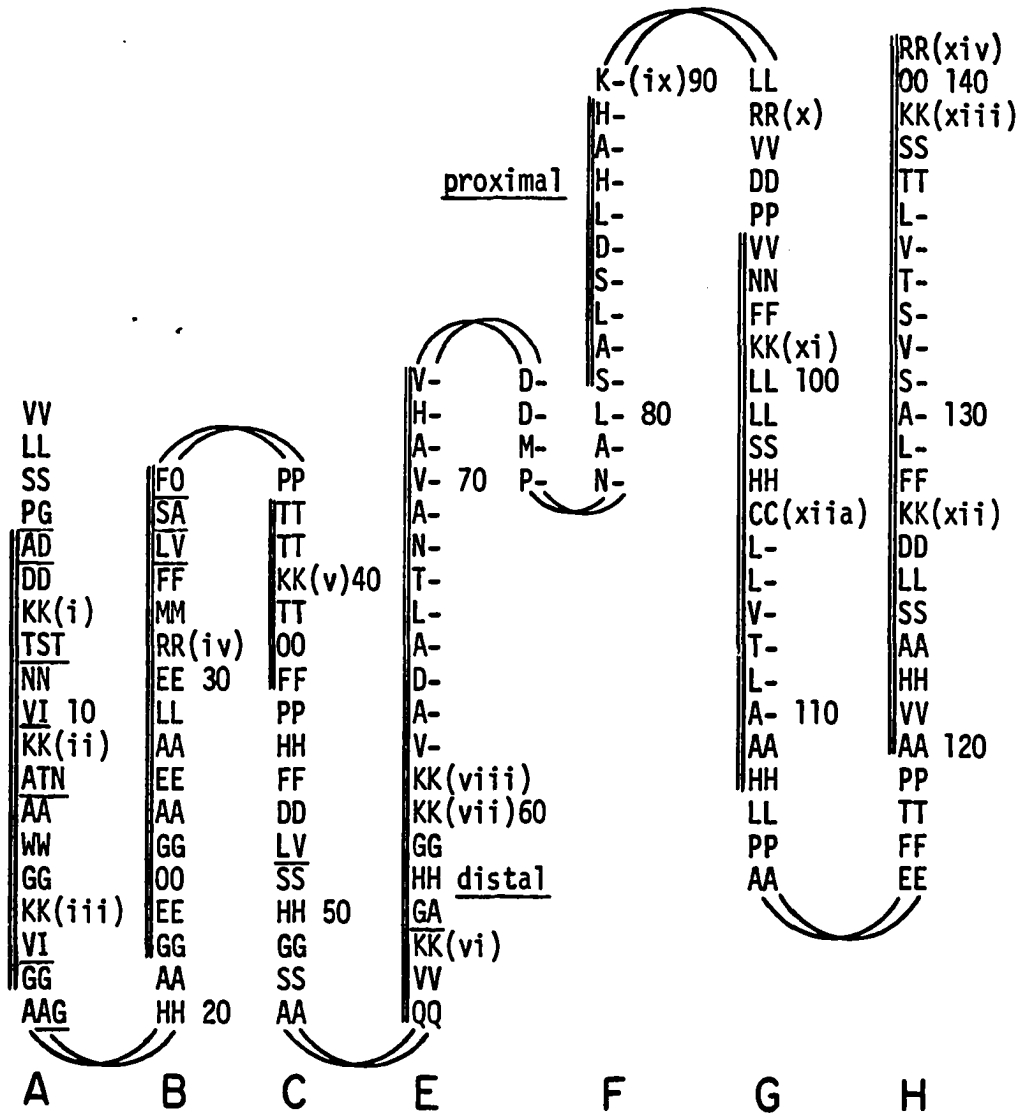


Figure 38. Primary and Secondary (Helical) Structure of Human Hemoglobin α Chain Compared with the Inferred Microtus Hemoglobin α Chain.

(Letters to the left in each column refer to human hemoglobin, to the right, Microtus hemoglobin.)

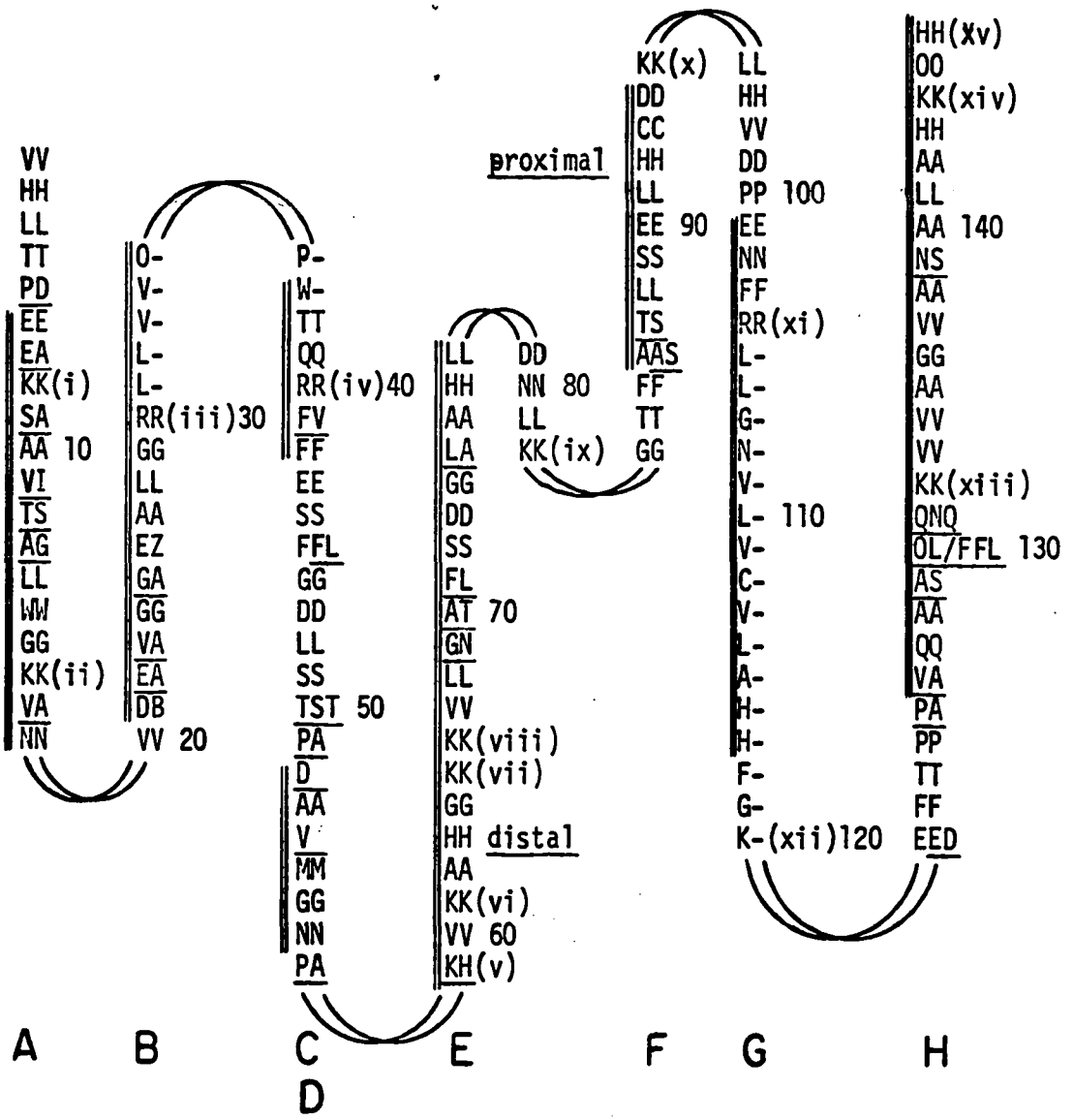


Figure 39. Primary and Secondary (Helical) Structure of Human Hemoglobin β Chain Compared with the Inferred Microtus Hemoglobin β Chain.

(Letters to the left in each column refer to human hemoglobin, to the right, Microtus hemoglobin.)

the stability of the heme-protein complex depends upon the linkages of the protein with the porphyrin part of the heme group rather than upon the iron-protein bond. Evidence has been adduced from chemical and X-ray studies that the region of the globin polypeptide chains around the heme group is particularly rich in aromatic side chains which can provide a hydrophobic environment for the two buried pyrrole rings of the heme. Perutz (1960) referred to the heme group as lying in a "pocket" on the surface of the hemoglobin molecule, a pocket formed by the folds (tertiary structure) of the polypeptide chain which appears to make contact with the heme group at four different points at least. Kendrew (1962) suggested the possible stabilization of the heme group of myoglobin by phenylalanine residues which, in the hemoglobin molecule, correspond to the two phenylalanines, 43 and 46 in the peptide alpha-T6, 42 and 45 in the peptide beta-T5. It has been noted that the hemoglobins of M. abbreviatus Hb-f and M. miurus Hb-f appear to possess a substitution of leucine for one of these phenylalanines. See Figures 38 and 39 (Braunitzer et al., 1964; Schroeder, 1963).

The interaction of ferroheme and globin is especially relevant to a consideration of the function of the monomeric myoglobin protein and to the monomeric alpha and beta proteins of hemoglobin. In addition to this type of interaction, one must consider for hemoglobin the effects of the additional molecular organization resulting from the presence of four protein chains of two kinds in the molecule. The increased structural complexity of the tetrameric protein leads to important modifications of its physiological function which have been related to so-called

"heme-heme" interactions. Whatever their mechanism, Ingram (1961) has suggested that the heme-heme interactions resulting from the combination of hemoglobin monomers gave such improved properties of oxygenation and deoxygenation that once the mutation was incorporated in the genome it would never be lost.

Significance of Inferred Primary Structure for Microtus Hemoglobin in an Evolutionary Context.

Aspects of the evolutionary picture for hemoglobin as presented by Ingram (1963), Zuckerkandl and Pauling (1962) and others are reflected in the data of this dissertation collected for Microtus hemoglobins.

Evolution proceeds through natural selection acting on spontaneous variations. A particular amino acid alteration in a protein, to be selected favorably, must eventually produce an increase in the number of progeny of its possessor. Of the potentially large number of mutations that can occur, most of them will be deleterious, and they either will not be observed at all or will eventually be eliminated. A rather large number of reciprocal amino acid substitutions occur between the hemoglobins of Microtus, human and white mouse, and the respective organisms are all viable. Evidence from other contemporary studies of proteins suggests that large parts of protein molecules are not connected with any of their known specific functions but that these "silent" parts may be involved in the stabilization of tertiary structure or may be random surviving structures that were useful during earlier evolutionary stages. Two aspects of Microtus structure may be singled out for brief

discussion in an evolutionary context.

Conservation of Amino Acid Composition and Primary Structure. With respect to the amino acid proline, Rifkin et al. (1966b) remarked at the fact that there are only 3 prolines in the C57BL/6 hemoglobin beta chain as compared with 7 in the corresponding beta chain of human hemoglobin. There are 6 prolines in the white mouse hemoglobin alpha chain, 7 prolines in the human hemoglobin alpha chain. Evidently the same number of prolines are observed in the Microtus hemoglobin chains as in the white mouse hemoglobin chains although this assumes that one proline from each chain of Microtus is missing together with the peptides beta-T4 and alpha-T9 (the presence of proline in the Microtus peptides 17 suggests their origin in a polypeptide segment related to alpha-T9 of human hemoglobin).

The replacement of the angular prolines, at least in the beta chains of Microtus and of white mouse hemoglobins, suggests that proline is not crucial in the primary structure of hemoglobin to account for corners observed in its tertiary (folded) structure.

The apparent conservatism shown in the substitution of prolines among alpha chains of the hemoglobins of the several species probably reflects a greater overall conservation of common alpha chain primary structure among hemoglobins. Conservation of primary structure among alpha hemoglobin chains from different species may follow from several considerations in an evolutionary context.

The alpha hemoglobin chains are believed to be older on the

evolutionary scale, and they may have retained an as yet unexplained structural stability characteristic of the "original," probably monomeric, protein. The beta, delta and gamma hemoglobin chains are considered to have evolved from and to have varied more extensively from their alpha-like precursors.

From a purely structural standpoint it may be noted that alpha hemoglobin chains are common to both adult and fetal hemoglobins which differ from one another in the amino acid composition of their respective beta and gamma chains. To the extent that alpha chains are required to fit with two different partner chains, beta and gamma, less variation in the alpha chain structure is allowed and the alpha protein of the tetramer becomes "conservative."

Alternatively, to the extent that the gene for alpha protein has a controlling influence upon fetal hemoglobin, less extensive mutational alterations may be allowed to survive because of a greater presumed vulnerability of the fetal organism to such alterations.

Overall, 28 of the observed 39 differences in amino acid composition between the hemoglobins of Microtus pennsylvanicus and human occurred in the beta chain, only 11 in the alpha chain.

Homology Between Alpha and Beta Peptide Chains. The probability of two deletions in the Microtus hemoglobin peptides beta-T5 bears upon another aspect of evolutionary mechanisms. Alpha and beta chains of the same hemoglobin are similar in amino acid sequence at the ends of the chains, yet they differ in chain length. Braunitzer first suggested

(see Braunitzer et al., 1965) that the maximum similarity between the alpha and beta chains could be realized by the judicious introduction of "gaps" in the respective sequences such as are depicted in Figure 40 in which the postulated primary structures for alpha and beta chains of Microtus hemoglobin are compared. The question immediately arises whether the absence of amino acid partners between the two different chains means a loss or an addition of amino acids and, in turn, reflects the introduction or deletion of nucleotides in the chromosomal DNA which "codes" for the protein. It does not seem to be possible at present to point to any preferred mode of action of evolution operating to lengthen or to shorten polypeptide chains.

Significance of Inferred Primary Structure for Microtus Hemoglobin in a Genetic Context.

The reciprocal amino acid substitutions which have been suggested between hemoglobins of human and Microtus (Figures 38 and 39), and the hemoglobin of white mouse, can be "tested" in terms of the genetic code. The genetic code is currently visualized to consist in all possible triplet combinations (64) of the four nucleotides of adenine, A; guanine, G; cytosine, C; and uracil, U (or thymine, T). Through the unparalleled recent successes resulting from the intensive and ingeneous theoretical and experimental work of Crick, of Nirenberg et al., of Ochoa et al., of Khorana et al., of Yanofsky et al. and others (see Crick, 1967), the precise linear sequences of nucleotide triplets which are expected to code for the individual amino acids in protein have been determined. More than one of the 64 coding triplets (codons), as many

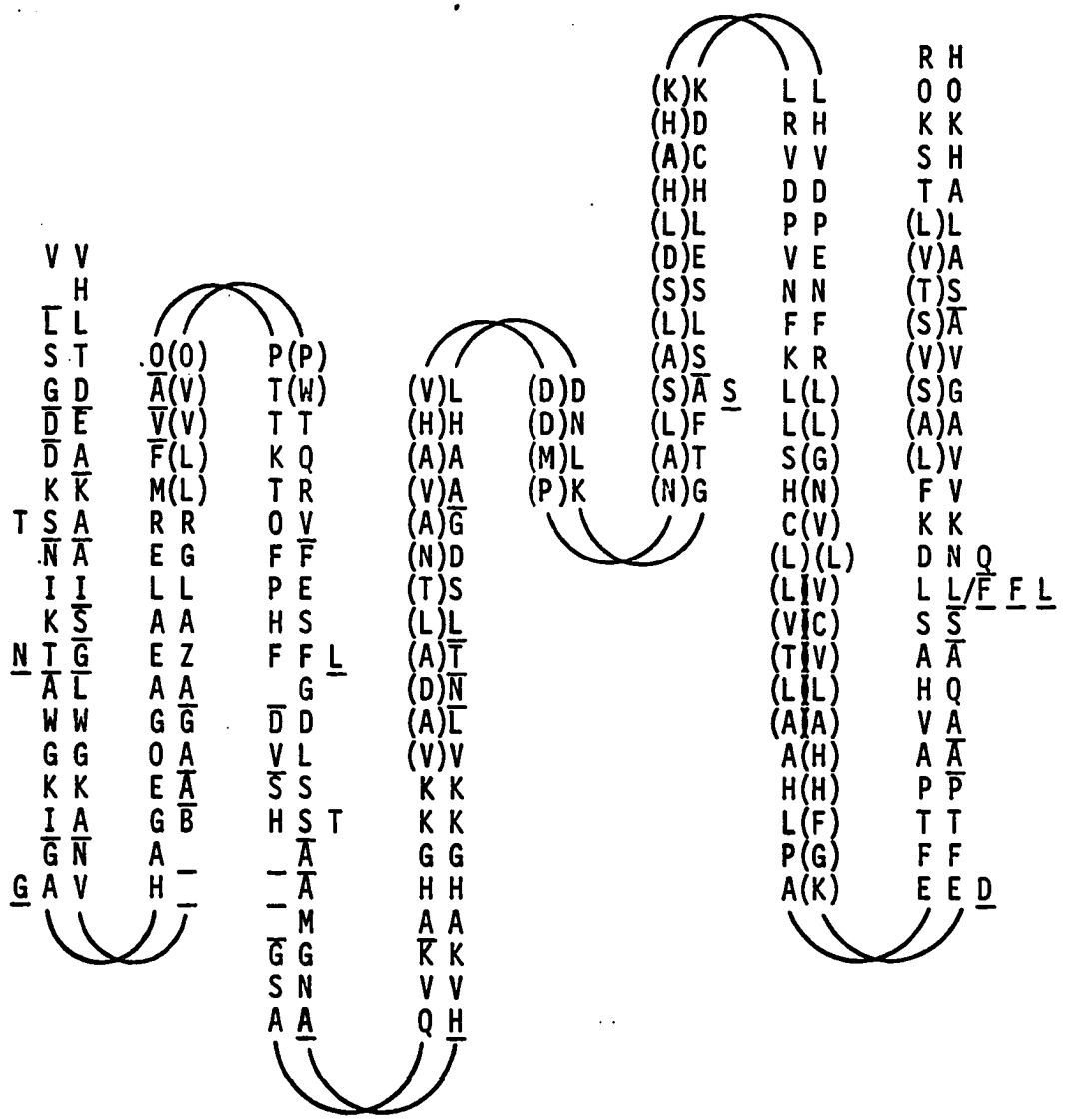


Figure 40. Comparison of Inferred Primary Structures for α and β Chains of Microtus Hemoglobins.

(Letters to the left in each column refer to α chain, to the right β chain. Letters in parentheses refer to amino acids of the respective human chains.)

as six, may code for the same one of the 20 different amino acids common to protein, and the code is thus said to be degenerate.

In Table 38 the reciprocal amino acid substitutions which are proposed to occur between the hemoglobins of human, Microtus pennsylvanicus and Microtus abbreviatus Hb-f, and white mouse are tabulated in terms of the possible codons for each amino acid. In the light of current thinking, those replacements of amino acids which correspond to a single point mutation (a change in one nucleotide pair of the triplet) can be tentatively validated. For example, all substitutions of amino acids which have been established in the abnormal human hemoglobins can be shown to correspond to point mutations. Reasons for considering point mutations most likely are:

1. The average tempo with which natural mutations (amino acid substitutions) have been selected and have become established in hemoglobin has been estimated statistically to be of the order of one amino acid substitution every ten million years (Zuckerkandl and Pauling, 1962).

2. The probability that two (or more) successive mutations should occur at the same position in the hemoglobin protein is statistically less than the probability of a single mutation at the given position.

Table 38 contains 6 instances in which two point mutations appear to intervene between amino acid substitutions. Amino acid substitutions which resulted from more than two mutations in the corresponding genetic material presumably would not be detected because alternative codons which exist for each amino acid can be equated by changes in only two nucleotides.

Table 38. Inferred Amino Acid Substitutions in Microtus Hemoglobin, Relative to Human Hemoglobin,[a] Expressed as Nucleotide Changes in the Genetic Codon.

Peptide	Chain Position	HA AA:NNN	MP (MAf)[b] AA:NNN	C57BL AA:NNN
αT1	4	P:CC- ←2→	G:GG-	G
	5	A:GC	D:GAC/U	E:GAA/G
αT2	8	T:AC-	S:UC- S:AGC/U	S
	10	V:GU-	I:AUA/C/U	I
αT3	12	A:GC- ←2→	T:AC- (N:AAC/U) ←2→	A
αT4	17	V:GU-	I:AUA/C/U	I
	19	A:GC-	A:GC- (G:GG-)	G:GG-
αT5	34	L:CU- L:UUA/G	V:GU-	A:GC-
	35	S:UC- S:AGC/U	A:GC-	S
	36	F:UUC/U	O:UAC/U	F
αT6	48	L:CU- L:UUA/G	V:GU-	V
	54	Q:CAA/G	Q:CAA/G	E:GAA/G
αT7	57	G:GG-	A:GC-	G
αT12b	111	A:GC-	A:GC-	S:UC-
	113	L:CU-	L:CU-	H:CAC/U
	116	E:GAA/G	Z	D:GAC/U
βT1	5	P:CC-	A:GC-	B[c]
	7	E:GAA/G	D:GAC/U	A:GC-[c]

Table 38. (Continued) Inferred Amino Acid Substitutions in Microtus Hemoglobin, Relative to Human Hemoglobin and White Mouse Hemoglobin, Expressed as Nucleotide Changes in the Genetic Codon.[a]

Peptide	Chain Position	HA AA:NNN	MP(MAf)[b] AA:NNN	C57BL AA:NNN
βT2	9	S:UC-	A:CG-	A
	11	V:GU-	I:AUA/C/U	V
	12	T:AC-	S:UC-	S
	13	A:GC-	G:GG-	C:UGC/U[c]
βT3	18	V:GU-	A:GC-	V
	22	E:GAA/G	A:GC-	Z
	23	V:GU-	A:GC-	A
	25	G:GG-	A:GC-	G
βT5(6)	41	F:UUC/U	V:GU- ←2→	O:UAC/U
	43	E:GAA/G	Z ←2→	S:UC- S:AGC/U
	45	F:UUC/U	F:UUC/U (L:CU-)	F
	50	T:AC-	S:UC- (T:AC-)	S
	51	P:CC-	A:GC-	A
	52	D:GAC/U	Delete	B
	54	V:GU-	Delete	I:AUA/C/U
	58	P:CC-	A:GC-	A
	59	K:AAA/G ←2→	H:CAC/U ←2→	K
βT9	69	G:GG	B (D:GAC/U)	B
		←2→	(N:AAC/U)	
	70	A:GC-	T:AC-	T
	71	F:UUC/U	L:CU- L:UUA/G	F
	75	L:CU- L:UUA/G ←2→	A:GC- ←2→	I:AUA/C/U

Table 38. (Continued) Inferred Amino Acid Substitutions in Microtus Hemoglobin, Relative to Human Hemoglobin and White Mouse Hemoglobin, Expressed as Nucleotide Changes in the Genetic Codon.[a]

Peptide	Chain Position	HA AA:NNN	MP(MAf)[b] AA:NNN	C57BL AA:NNN
βT10	86	A:GC-	A:GC- (S:UC-)	A
	87	T:AC-	S:UC-	S
βT13	121	E:GAA/G	E:GAA/G (D:GAC/U)	B
	125	P:CC-	A:GC-	A
	126	V:GU-	A:GC-	A
	129	A:GC-	S:UC-	A
	130	O:UAC/U	L:UUA/G (F:UUC/U)	F:UUC/U
	131	Q:CAA/G ←2→	N:AAC/U ←2→	Z

[a]Abbreviations and symbols for species and for amino acids (AA) are as given earlier (pp. 63 and 72-73). The triplet codons (NNN) are constituted of the nucleotides of the bases adenine (A), guanine (G), cytosine (C), or uracil (U). Alternative bases in the third position are designated by N/N. Any one of the four bases may occupy the unfilled position NN-.

[b]Symbols in parentheses refer to AA:NNN substitutions for M. abbreviatus Hb-f.

[c]White mouse strain BALB/cJ.

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