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Inheritance of Multiple Hemoglobins in Two Species of Woodrats, Genus *Neotoma* (Rodentia: Cricetidae)

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ABSTRACT

Hemoglobin samples of 171 woodrats of the species *Neotoma floridana* and *N. micropus,* and laboratory-bred hybrids of these two species, were studied by horizontal starch-gel electrophoresis. The patterns observed were grouped into seven categories or phenotypes. One hemoglobin phenotype was common to both species, three were present in *N. floridana* but not in *N. micropus,* two seen in *N. micropus* were not present in *N. floridana,* and one phenotype was unique to hybrids. Results of electrophoresing globins in urea-veronal starch demonstrated that all the variation observed resulted from differences in beta chains. Study of hemoglobins through as many as three generations of woodrats indicate that at least three beta loci are involved in the genetic control of woodrat hemoglobins. Tentative hypotheses are proposed to explain the inheritance patterns observed and a possible sequence for the evolution of multiple beta loci in woodrats.

INTRODUCTION

The woodrat species *Neotoma floridana* and *Neotoma micropus* are mostly allopatric in distribution and apparently behave in nature as good biological species, although they readily hybridize in the laboratory. *N. floridana* occurs from the eastern United States westward into the Central Great Plains, whereas *N. micropus* ranges from San Luis Potosí, México, northward as far as southwestern Kansas and adjacent Colorado (Hall and Kelson, 1959).

In the course of an extensive study to better elucidate the relationships of these two woodrats, it was found that both species are polymorphic in the ionographic properties of their hemoglobins as determined by horizontal starch-gel electrophoresis. Intraspecific variation of hemoglobins has long

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been known in humans and more recently has been reported in a variety of mammalian species including several of the rodent genus *Peromyscus* (Foreman, 1960, 1966; Rasmussen *et al.*, 1968). Because published information on the hemoglobins of *Neotoma* is not available and because we had a number of laboratory-bred woodrats of known parentage, we felt it worthwhile to study the inheritance of the observed polymorphisms.

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MATERIALS AND METHODS

Original stocks of woodrats were captured at the following localities (here specified only to county): *Neotoma floridana*—Cherry and Rock counties, Nebraska, and Douglas, Ellsworth, Ellis, Logan, and Finney counties, Kansas; *N. micropus*—Baca and Prowers counties, Colorado, and Barber, Meade, and Haskell counties, Kansas. Although individuals of three subspecies of *N. floridana* and two subspecies of *N. micropus* were studied, we are concerned herein only with variation and inheritance at the specific and interspecific levels. Animals were housed indoors in individual cages. Because breeding experiments were conducted for another purpose, matings were made without regard to hemoglobin phenotype. The woodrats used in this study later were prepared as museum specimens and cataloged in the Museum of Natural History, The University of Kansas. Thus, hemoglobin phenotypes can be correlated with individual specimens.

Hemoglobin samples were not studied from woodrats less than two months of age and no ontogenetic changes in hemoglobins were detected. Approximately 0.5 cc of whole blood was obtained by cardiac puncture, from an incision at the base of the tail, or from the suborbital canthal sinus. Blood was suspended immediately in 3 cc of 3.2% solution of trisodium citrate, pH 7.0, to prevent coagulation. After centrifugation, the citrate solution was

removed by aspiration and the cells washed three times in phosphate buffered saline, pH 7.0, lysed in about three times their volume of deionized water, and the hemoglobin solution separated from cell membranes by centrifugation.

Phenotypes of hemoglobins were determined by horizontal starch-gel electrophoresis. Gels were prepared using 16 grams of hydrolyzed starch in 125 ml of buffer that consisted of 0.2 M Tris and 0.025 M citric acid, pH 7.6. Samples were inserted into gels on Whatman number 3 filter paper. Buffer in the electrode reservoir consisted of 0.3 M sodium borate, pH 8.6. Electrophoresis was conducted at 25v/cm at room temperature for 90 minutes, the gels removed, sliced, and both halves stained in a saturated solution of amido black in water-methanol-glacial acetic acid (5:5:1). At the beginning of the study, half the preparations were stained by the benzidine method (50 ml of water, 0.1 ml of 30% hydrogen peroxide, 0.25 ml acetic acid, and 100 mg benzidine dihydrochloride), but this procedure was discontinued when it became evident that both techniques demonstrated the same pattern. The iodoacetimide method described by Riggs (1965) was used to investigate the possibility that some or all of the hemoglobin patterns observed were artifacts resulting from polymerization.

The 6 M urea-0.1 M veronal method of Chernoff and Pettit (1964) was used to determine whether variation was in the alpha (α) or beta (β) chains of hemoglobin molecules. Globins were prepared by cold acid-acetone precipitation, and redissolved in the urea-veronal buffer containing mercaptoethanol. Horizontal electrophoresis was run at 4° C for 22 hrs at 25v/cm. Urea-veronal buffer, pH 8.0, was also used in electrode reservoirs. Following electrophoresis, gels were sliced and both halves stained with amido black. In all cases, gels were destained in repeated rinses of the stain solvent.

RESULTS

The hemoglobin phenotypes of 171 woodrats, including members of both species and their hybrids, were determined. These animals were involved in 35 matings in which the phenotypes of both parents were known and 18 matings in which the phenotype of only one parent was known (Table 1). Hemoglobin patterns were grouped into seven categories or phenotypes, designated A through G (Fig. 1). Groupings were made according to the number and position of major bands without consideration of diffuse zones and minor bands. For the sake of completeness, the status of diffuse zones and minor bands is included in the description of each phenotype; their patterns and characteristics generally were consistent in the hemoglobin pattern of an individual and probably represent genetic differences, but because there was considerable variation in these characters when all animals were considered, interpretation was not possible. Major bands of four different migra-

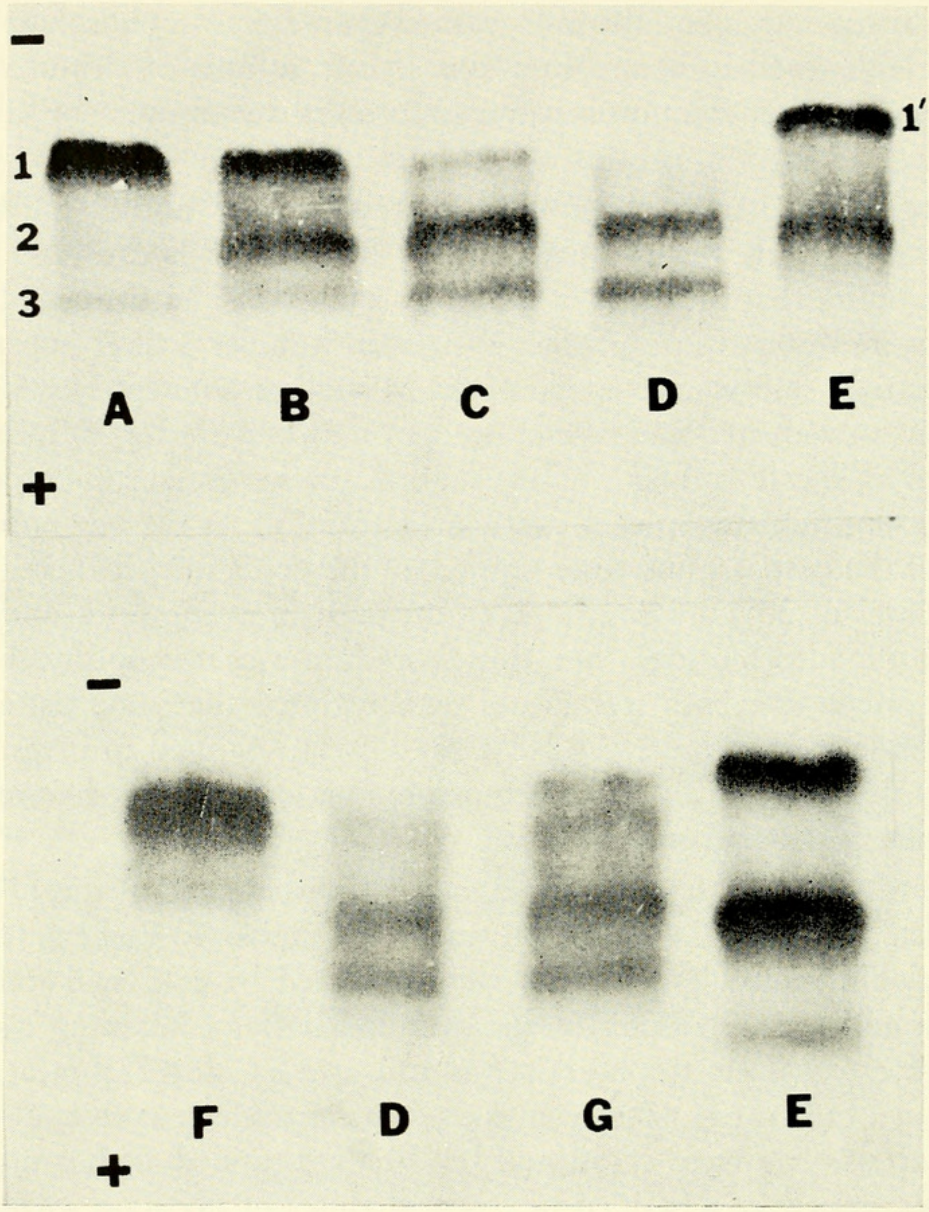

FIG. 1. Starch gels showing electrophoretic patterns of woodrat hemoglobins. Phenotypes are indicated by letter, cathode by a minus sign, and anode by a plus sign. The points of insertion of the samples are out of view at the cathodal end of each gel.

tion distances were observed. These were labeled 1′, 1, 2, and 3, with 1′ having the slowest and 3 the fastest rate of migration. Minor bands were observed in some individuals of both species at position 3 and another faint band was seen in some *N. micropus* and some hybrids near the leading edge of the diffuse zone at a position designated 4.

Phenotype A consisted of band 1 and a leading diffuse zone that terminated in a minor band at position 3. Only five *N. micropus* demonstrated this pattern. Phenotype B, observable as major bands 1 and 2 preceded by a diffuse zone, was the most common phenotype in *N. floridana* and was not uncommon in *N. micropus* and hybrids. The diffuse zone was slightly longer in *N. micropus* than in *N. floridana* and a minor band sometimes occupied position 3 (Fig. 1B). A minor band also was present occasionally at

position 4 in the B phenotype of *N. micropus* and hybrids. Phenotype C had major bands at positions 1, 2, and 3, whereas D had only bands 2 and 3 and a trailing diffuse zone that terminated at the level of position 1 in *N. floridana* but extended to position 1′ in some hybrids. Position 3 was never occupied by a major band in the phenotypes of *N. micropus,* thus limiting phenotypes C and D to *N. floridana* and hybrids. Phenotype E consisted of bands 1′ and 2 with a long leading diffuse zone that usually contained a minor band at position 4. It was difficult in a few cases to identify a sample either as E or B, but an attempt to establish an intermediate category was unsuccessful. This problem and its ramifications will be discussed below in more detail. Because band 1′ was never found in *N. floridana,* phenotype E occurred only in *N. micropus,* where it was the most common phenotype observed. Two *N. floridana,* neither of which was involved in laboratory matings, were the only animals having phenotype F. This phenotype consisted of a single broad band and a small leading diffuse zone. The proteins that formed the band migrated slightly more slowly than proteins normally forming band 1, but not so slow as those of band 1′. Assignment of this band to category 1 is open to question; however, because only two individuals of the phenotype were observed and because no other *N. floridana* had band 1′, it seems more parsimonious to consider it as band 1. Phenotype G, which was seen only in four hybrids, sometimes appeared to have all four major bands. On other occasions a hemoglobin sample from the same individual rats would show only bands 1′, 2, and 3.

Phenotypic patterns of samples treated with iodoacetamide and electrophoresed in the Tris-borate system did not differ from those of untreated samples. Globins electrophoresed in the urea-veronal system showed no variation in the cathodal fractions, but variation was present in anodal fractions (Fig. 2).

DISCUSSION

Chernoff and Pettit (1964) summarized the technique of electrophoresing globins in a urea-veronal system containing mercaptoethanol. Urea dissociates the polypeptide chains of hemoglobin and mercaptoethanol prevents disulfide bridging of reactive -SH groups. In this system, human alpha polypeptides migrate toward the cathode and human beta polypeptides migrate toward the anode. If it is assumed that hemoglobins of woodrats behave in a similar manner, our results indicate that variation observed in the hemoglobins of *Neotoma* is in the beta chains. This is generally consistent with situations in other mammals, including humans (see Ingram, 1963, for review), where variation is known in α-chains but to a lesser extent than in β-chains. Variation in both α and β chains also has been reported for orangutans, genus *Pongo* (Sullivan and Nute, 1968). House mice of the genus *Mus*

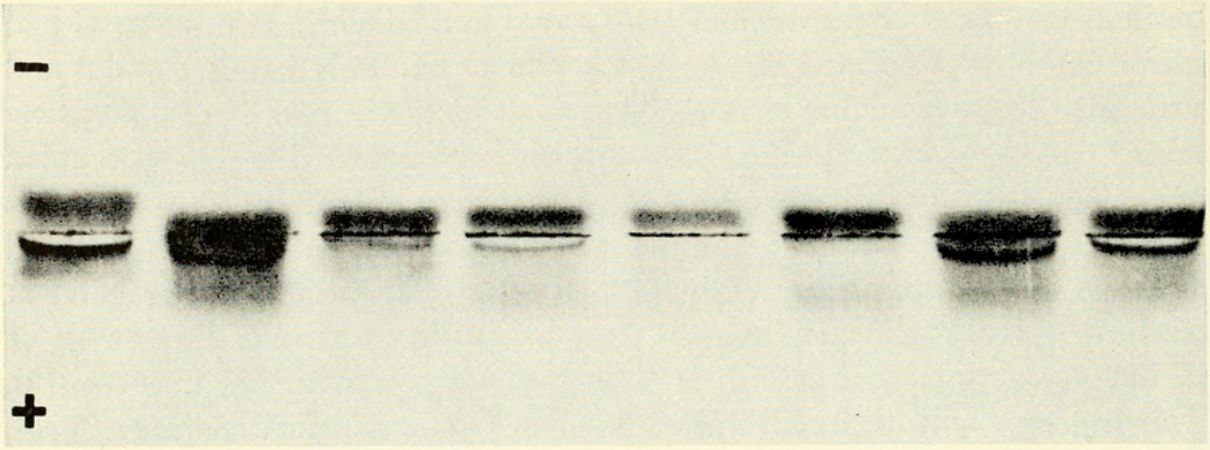

FIG. 2. Electrophoretic patterns of globins in urea-veronal starch. The sample to the left is human globin, probably human A. Reading left to right, subsequent samples are woodrat globins from animals having phenotypes C (*N. floridana*), B (*N. floridana*), B (*N. micropus*), A (*N. micropus*), E (*N. micropus*), C (*N. floridana*), and B (*N. floridana*). The cathode is indicated by a minus sign and the anode by a plus sign.

are known to have at least two β-chain variants that affect electrophoretic patterns of hemoglobin (see Lush, 1967, for review) and two α-chain variants that affect solubility (Hutton *et al.*, 1964).

Glucksohn-Waelsch (1960) proposed that the beta locus of *Mus* has undergone duplication to produce two or more closely linked beta loci. We found that when all phenotypes of *Neotoma* were considered there was no position consistently occupied by a band. It therefore appears that the same hypothesis may be valid for the control of hemoglobin production in woodrats. Foreman (1966) discussed variation in hemoglobins of *Peromyscus gossypinus* in terms of an animal being either homozygous or heterozygous for a given multibanded phenotype. In attempting to interpret our data in a similar fashion, we noted that all matings of phenotype B $\times$ B resulted only in progeny of phenotype B. This indicated that B might represent a homozygous condition. It was thought that D might be the other homozygote in *N. floridana* with C representing the heterozygous condition. No D $\times$ D matings were available for study, but results of B $\times$ C and B $\times$ D matings indicate that B does not always represent a homozygous condition because one D progeny resulted from a cross of the former (only B and C progeny should have been observed if B is always homozygous) and five progeny of phenotype B and two of phenotype D were observed in 15 offspring from the latter cross. Furthermore, such a hypothesis does not account for the presence in natural populations of phenotypes A and F, which lack band 2, and E and G, which have band 1′. It is necessary, therefore, to consider genetic control of hemoglobin production in woodrats in terms of the alleles controlling each electrophoretic band.

In the two species studied, variation in major bands can be explained in terms of either two or three beta loci for *N. micropus* and three beta loci for

TABLE 1. Summary of hemoglobin phenotypes of parental woodrats *(Neotoma)* and their laboratory-born progeny.

Phenotypes of parents	Identification of parents	Number of matings	Number of progeny	Number of progeny of each phenotype						
				A	B	C	D	E	F	G
B × B	floridana × floridana	3	8	0	8	0	0	0	0	0
	floridana × micropus	3	6	0	6	0	0	0	0	0
	micropus × micropus	2	2	0	2	0	0	0	0	0
	hybrid × hybrid	1	2	0	2	0	0	0	0	0
	Totals	9	18	0	18	0	0	0	0	0
B × C	floridana × floridana	2	8	0	5	3	0	0	0	0
	floridana × micropus	1	2	0	1	0	1	0	0	0
	Totals	3	10	0	6	3	1	0	0	0
B × D	floridana × floridana	4	9	0	3	5	1	0	0	0
	floridana × hybrid	1	2	0	1	1	0	0	0	0
	micropus × hybrid	1	1	0	0	0	1	0	0	0
	hybrid × hybrid	1	3	0	1	2	0	0	0	0
	Totals	7	15	0	5	8	2	0	0	0
B × E	micropus × micropus	2	5	0	2	0	0	3	0	0
C × C	floridana × floridana	3	8	0	2	2	4	0	0	0
C × D	floridana × floridana	4	13	0	0	10	3	0	0	0
C × E	floridana × micropus	1	1	0	0	0	0	0	0	1
D × E	floridana × micropus	3	8	0	0	0	3	2	0	3
	micropus × hybrid	1	1	0	0	0	1	0	0	0
	hybrid × hybrid	2	4	0	0	0	0	4	0	0
	Totals	6	13	0	0	0	4	6	0	3
? × B	floridana × floridana	3	4	0	4	0	0	0	0	0
	floridana × micropus	1	2	0	2	0	0	0	0	0
	micropus × micropus	2	5	0	3	0	0	2	0	0
	micropus × hybrid	1	2	0	2	0	0	0	0	0
	Totals	7	13	0	11	0	0	2	0	0
? × C	floridana × floridana	2	4	0	1	2	1	0	0	0
? × D	floridana × floridana	2	3	0	0	1	2	0	0	0
? × E	micropus × micropus	7	18	5	0	0	0	13	0	0
Grand totals		53	121	5	45	26	16	25	0	4

N. floridana. Both species apparently have separate loci producing the proteins that form bands 1 and 2, and *N. floridana* has a third locus for production of the β-chain of the protein that forms band 3. Whether or not the minor band formed at this position by some *N. micropus* implies that the same locus is present in this species is not known. If the same locus is involved, the protein produced is clearly in lower concentration in *N. micropus* than in *N. floridana*. The presence of a minor band at position 4 in some *N. micropus* indicates that this species has another locus not present in *N. floridana*. Bands 1′ and 1 either may be alleles of each other or band 1′

may be controlled by a locus not present in *N. floridana;* if allelic, the 1′ allele occurs naturally only in *N. micropus.*

In several matings (see Table 1) it was noted that one or more progeny lacked a band that was formed by the hemoglobin of both parents, but any position occupied by a band in the progeny was always occupied in at least one parent. Our data are not conclusive, but they do indicate that alleles we have termed β^0 (no peptide produced) are involved in the hemoglobin inheritance of *Neotoma.* Such a non-functional "allele," which may be either a deleted area on the chromosome as discussed below or an area that is physically present but for some reason (being under the control of modifier genes, for example) does not contribute a peptide chain, was proposed and designated $H\beta\ I^0$ to explain inheritance of hemoglobins in *Peromyscus maniculatus* (Rasmussen *et al.,* 1968). Harris *et al.* (1959) suggested that a similar allele, Hp^0, might exist in association with normal haptoglobin alleles in a human family. A "silent" gene also has been proposed (see Heyworth and Firth, 1967) to explain the absence of serum-cholinesterase in children of parents that both produced the enzyme; Ashton (1958) and Gahne (1964) explained the absence of slow-α 2 plasma protein in cattle with a "silent" S^0 allele. In *Neotoma,* β^0 alleles seem to occur at all of the beta loci, but such a hypothesis makes possible three hypothetical phenotypes for *N. floridana* (bands 2 and 3 singly observed, and bands 1 and 3 together) and two for *N. micropus* (band 1′ alone and band 2 alone) that were not observed in this study.

In some cases, at least, gene duplication probably results from small unequal cross-overs of homologous chromosomes so that one chromosome contains both loci and the other no longer has the locus in question (see Fig. 3). In the case of *Neotoma,* the chromosome without a beta locus may be lethal and thus lost from the population, or it may remain to act as a β^0 in the inheritance of hemoglobin. Certainly two such chromosomes would be lethal for an individual, but one conceivably could be carried in association with a functional homologue. In any event, it is the chromosome bearing the duplicated locus that is of most interest because it now would contain a beta locus free to mutate with a high degree of impunity. Even if altered in a manner that would be advantageous to the population, however, the chromosome capable of stimulating production of two forms of polypeptide chains would probably take a considerable period of time to become fixed in a population or species. During this period, woodrats having one "new" or altered chromosome paired with one of the unaltered form would be heterozygous for the new beta peptide. If duplications and mutations of this type have occurred twice during the evolution of these two species of woodrats, the variation and inheritance patterns of the major electrophoretic bands can be explained.

Table 2 shows a series of combinations of alleles that will explain the

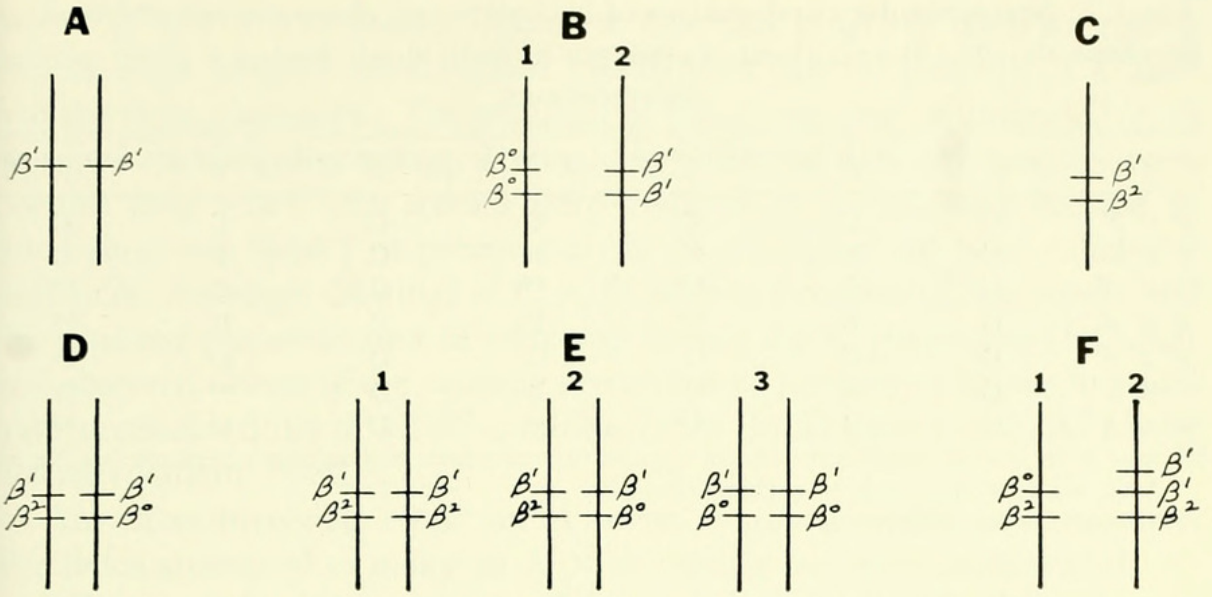

FIG. 3. Hypothetical sequence showing evolution of beta loci in *Neotoma*. Vertical lines are diagramatic representations of the chromosomal portions supporting beta loci, which are depicted by horizontal lines. A, "original" diploid condition of an individual having unduplicated loci; B, possible arrangements of beta loci after a small unequal crossover; C, condition after one locus of chromosome 2 mutates; D, the "new" chromosome pairs with an unaltered chromosome resulting in woodrats heterozygous for β^2; E, three diploid conditions of progeny resulting from a mating of two heterozygotes; F, possible arrangement of beta loci after another crossover.

hemoglobin variation seen in *N. floridana* together with the phenotypes of animals having each pair of chromosomes. The relative frequencies of phenotypes in natural populations does not approach that seen in the table. This suggests that some of the hypothetical combinations do not exist, or that some combinations exist in higher frequencies than others. Most likely both situations are true. A similar interpretation of possible chromosomal combinations can be made for *N. micropus,* except that in this species locus number three is absent and locus number one either has three alleles (β^0, $\beta^{1'}$, and β^1) or there is another locus controlling band 1′.

As indicated above, the relationship between $\beta^{1'}$ and β^1 is not clear. When two forms of hemoglobin molecules having mobilities nearly the same are present, it is possible that they usually migrate together with the faster form either being held back to the speed of the slower or "dragging" the slower along with it. The occasional occurrence of both bands in some runs of phenotype G hemoglobin suggests (1) that a woodrat can produce both peptides, (2) that these can separate during electrophoresis, and (3) that when they do not separate 1′ is present and band 1 is absent. Three intraspecific matings of *N. micropus* with one B parent and one E parent (Table 1) failed to help answer this question because the genotypes of the parents were unknown. In one case both progeny were B, in another all three progeny were E, and in the third litter one offspring was B and two were E. However, interspecific matings (Table 1) involving individuals of *N. floridana* (which

TABLE 2. Seven possible combinations of beta alleles on chromosomes of *Neotoma floridana* showing the resultant phenotypes of individuals having a given pair of chromosomes.

	$\beta^1\ \beta^2\ \beta^3$	$\beta^1\ \beta^2\ \beta^0$	$*\beta^1\ \beta^0\ \beta^3$	$\beta^1\ \beta^0\ \beta^0$	$\beta^0\ \beta^2\ \beta^3$	$\beta^0\ \beta^2\ \beta^0$	$*\beta^0\ \beta^0\ \beta^3$
$\beta^1\ \beta^2\ \beta^3$	C	C	C	C	C	C	C
$\beta^1\ \beta^2\ \beta^0$	C	B	C	B	C	B	C
$*\beta^1\ \beta^0\ \beta^3$	C	C	**	**	C	C	**
$\beta^1\ \beta^0\ \beta^0$	C	B	**	F	C	B	**
$\beta^0\ \beta^2\ \beta^3$	C	C	C	C	D	D	D
$\beta^0\ \beta^2\ \beta^0$	C	B	C	B	D	**	D
$*\beta^0\ \beta^0\ \beta^3$	C	C	**	**	D	D	**

* No evidence that such a combination exists.

** Phenotype not observed.

never have the $\beta^{1'}$ allele) and those of *N. micropus* with phenotype E frequently resulted in progeny having band 1′. These animals necessarily were heterozygous, having received the $\beta^{1'}$ gene from the *micropus* parent and either a β^1 or β^0 from the *floridana* parent. This clearly demonstrates that animals having band 1′ can be heterozygous, and suggests that the $\beta^{1'}$ allele usually acts as a dominant, either by influencing the mobility of molecules containing polypeptides produced at the β^1 locus or by inhibiting the production of such polypeptides.

Only the G phenotype was unique to hybrids of the two species. This pattern resulted only when one parent, either a *micropus* or hybrid, had band 1′ and the other, either a *floridana* or hybrid, had band 3. Observation of a "hybrid" phenotype having bands 1′, 2, and 3 was expected. However, as discussed above, we had not predicted that band 1 would occur in association with 1′.

The three B × C matings (Table 1) are of special interest. Six of the 10 progeny from these matings were phenotype B, three were C, and one was D. The fact that more were phenotype B than C possibly can be attributed to the small sample, but the occurrence of a D individual necessitates that the C parent was heterozygous for band number 1 and that a chromosome (one of the pair in the B parent, which was a *micropus*) exists with the combination of alleles $\beta^0\ \beta^2\ \beta^0$. This combination in the homozygous condition would yield a phenotype having a single major band occurring in the number 2 position. No individual having such a phenotype was observed. Other evidence for the existence of the $\beta^0\ \beta^2\ \beta^0$ chromosome can be derived from the six D × E matings studied. It was expected from the model that most of the progeny would be either phenotype C or G. Phenotype C, however, was not observed in the 13 progeny and only three were of phenotype G. The six E progeny could result only if the D parent was genotypically $\beta^0\ \beta^2\ \beta^3$ and $\beta^0\ \beta^2\ \beta^0$. The E parent of the four offspring of phenotype D must have been $\beta^{1'}\ \beta^2\ \beta^0$

and β^0 β^2 β^0. It is of further interest to note that in five of these six crosses two or more progeny were studied and in each case all progeny of a litter had the same phenotype. The meaning of this is not clear and more D × E crosses would be enlightening. It should be reiterated that such matings were possible only when both species were involved in the ancestry because in non-hybrid rats band 1′ is present only in *N. micropus* and band number 3 only in *N. floridana*. Matings of C × D rats were conducted four times and the expected preponderance of offspring having the C phenotype (10C:3D) was observed. None of the progeny demonstrated phenotype B, which would have necessitated the β^0 β^2 β^0 combination in the D parent and a C parent heterozygous for band 3.

Any cross involving either an A or an F parent would be instructive. We twice attempted to make an A × A mating but were unsuccessful.

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