

Detection of neutral amino acid substitutions in proteins

(biochemical genetics/*Mus musculus* hemoglobins/ α -globin locus/immobilized gradient isoelectric focusing/mutation screening)

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Communicated by Elizabeth S. Russell, May 20, 1985

ABSTRACT The field of biochemical genetics relies heavily upon the detection by electrophoresis of genetically determined variants of proteins. Most of these variants differ by substitutions that involve charged amino acids. Genetic variants of another large class, ones that involve substitutions among neutral amino acids, are not easily detected and are often ignored. Ampholyte isoelectric focusing in some cases can separate proteins indistinguishable by standard electrophoresis, including genetic variants of mouse hemoglobins that differ only by neutral amino acid substitutions. A revolutionary variation of isoelectric focusing, in which gradients covering a small pH range are fixed into place in a polyacrylamide gel, provides greater resolution of these nearly identical proteins. Mouse hemoglobin tetramers that differ only by the substitution of alanine for glycine in the α -globin chains are resolved by several millimeters with the new technique; by comparison, these tetramers are imperfectly resolved on a standard pH 7-9 isoelectric focusing gel. This improved technique of isoelectric focusing was used to identify a variety of previously unreported genetic variants of mouse hemoglobin α chains. Immobilized gradients tailored to the requirements of the proteins being analyzed will extend greatly the ranges of protein variations that can be easily recognized for diverse applications, including genetic quality-control analyses and in studies of genetics, mutagenesis, and evolution.

Standard electrophoretic techniques are often suitable for differentiating among proteins that differ from one another by the substitution of charged amino acids but generally are not capable of separating proteins that differ only by neutral amino acid substitutions. We have shown previously (1) that isoelectric focusing—a method for the separation of proteins based upon differences in their isoelectric points—can resolve at least some proteins that differ from one another only by neutral amino acid substitutions. That demonstration relied upon the availability of a variety of mouse hemoglobins (2-14) of known amino acid sequence (15-20) [and now-known gene sequence (21, 22)] that differ from one another only by one to four substitutions of neutral amino acids (valine, isoleucine, serine, threonine, asparagine, glycine, and alanine). Although several additional α -globin genotypes have been discovered since then (23-26), all of the naturally occurring variant chains subsequently characterized by protein sequence analysis also have differed in neutral amino acids only (Table 1). That no charge variation among mouse α -globins has been found in nature is consistent with the deleterious effect that charged amino acid substitutions generally have in human hemoglobin α chains. Charge variations among mouse β -globin chains are easily characterized by standard electrophoretic methods.

It is now possible to perform isoelectric focusing in narrow pH gradients that are immobilized to a polyacrylamide gel

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Table 1. Known amino acid substitutions in the mouse α -globins

Chain number	Residues at α -globin chain position				
	25	62	68	78	89
5' (mutant)	Gly	Val	Asn	Ala	Leu
5	Gly	Val	Asn	Ala	His
1	Gly	Val	Asn	Gly	His
6	Not yet characterized				
2	Gly	Val	Ser	Gly	His
4	Val	Ile	Ser	Gly	His
3	Gly	Val	Thr	Gly	His

Positions not indicated are presumed to have the same amino acid in all chains. Chains are listed in the order of their hemoglobins' pIs, basic to acidic. Table 2 catalogs which haplotypes specify particular α -globins.

matrix (27, 28). The immobilized pH gradients offer a number of advantages compared to standard ampholyte-generated gradients. Particularly important are the elimination of "cathodal drift" and the possibility of creating gradients of essentially any desired pH span. Immobilized gradients of a small pH range produce notably higher resolutions of proteins than do gradients normally obtained with wider-range diffusible ampholytes.

Up to 1977, five genetic variants of mouse hemoglobin α chains were known from studies of hemoglobin solubility and polypeptide sequence analyses (1-20). None of these genetic variants is distinguishable from any other by standard electrophoretic analyses because substitution of neutral amino acids for other neutral amino acids is involved. Four structurally distinct α -globin chains were characterized among mice of these five *Hba* (hemoglobin α) genotypes or haplotypes [alleles of a complex locus (1)]. Some inbred, therefore homozygous, mice have two structurally different α -globin chains, consistent with the presence of a duplicated gene locus as found in man. Through the use of isoelectric focusing on standard ampholyte-containing pH 7-9 polyacrylamide gels, several additional *Hba* haplotypes were discovered in mice of other inbred strains (23-25). We describe here the analyses of these variants and several additional *Hba* variants discovered among mice of noninbred exotic stocks derived from mice captured worldwide. We also compare to these normal variants a mutant isolated after chemical treatment of a DBA/2J mouse (29).

MATERIALS AND METHODS

Mice. Mice used for this study were bred from a variety of progenitors obtained from The Jackson Laboratory (the *Hba*^a, *Hba*^b, *Hba*^c, *Hba*^d, *Hba*^e, *Hba*^f, *Hba*^g, and *Hba*^h haplotypes), from Michael Potter of the National Cancer Institute (the *Hba*ⁱ, *Hba*^{w1}, *Hba*^{w2}, *Hba*^{w3}, *Hba*^{w4}, and *Hba*^{w5} haplotypes), from Susan Lewis of the Research Triangle Institute

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[the *Hba*^{s2} haplotype (29), and the mouse β -thalassemia (30)], and from the Charles River Breeding Laboratories (the "Swiss" CDI-derived *Hba*^h haplotype). The Laboratory Animal Resources of the Medical College of Georgia are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Immobilized Gradient Isoelectric Focusing. Immobilized-pH gradient gels 0.5-mm thick on Gelbond polyacrylamide gel (PAG) films were prepared essentially as described (28) with the LKB gradient gel kit. Sample wells in the gel were formed during acrylamide polymerization by 5-mm squares of Dymo tape aligned 5 mm apart on the glass facing-plate 3 cm from the open (future cathodal) edge. Solutions of acrylamide/methylenebisacrylamide (Bio-Rad) were deionized by using Amberlite MB-1. For a pH 7.2–7.55 gel, the dense solution contained 863 μ l of LKB Immobiline pK 7.0 and 350 μ l of Immobiline pK 3.6 per 15 ml, and the light solution contained 863 μ l of Immobiline pK 7.0 and 150 μ l of Immobiline pK 3.6 per 15 ml. After polymerization at 50°C, the gels were washed with several changes of deionized water over 5–24 hr. The gel was allowed to lie in a horizontal position at room temperature to dry for 55–60 min. Parallel troughs approximately 2 mm wide were prepared to separate samples in adjacent lanes.

Hemolysates for Immobiline isoelectric focusing were prepared by mixing two drops of whole blood collected into a heparinized microhematocrit tube by retroorbital puncture into 200 μ l of deionized water; samples of 7 μ l, containing about 0.15 mg of hemoglobin, were applied to each lane. Focusing at 10°C on the LKB Multiphor apparatus was initiated at not more than 1 W or 2 mA. Within 30 min the gel was covered with a sheet of polyester film. Focusing was continued overnight at 200 V/cm at 10°C. The proteins were fixed and stained in a dilute solution of Coomassie brilliant blue (1). The stained 0.5-mm gels dry rapidly at room temperature and can be kept for a permanent record; alternatively, the fresh unstained gels can be Xerox-copied.

RESULTS

The figures present immobilized gradient isoelectric focusing patterns of hemoglobins from mice with a variety of *Hba* haplotypes. Most of the mice are homozygous for the "single" hemoglobin β genotype (*Hbb*^s) because the variant *Hba* haplotypes have been placed on the C57BL/6 background. Each group also includes an artificial mixture of six structurally different hemoglobins for comparison. For reference to previously published isoelectric focusing patterns (1), it should be noted that the distance between lane centers here is also 1 cm.

In Fig. 1 are shown the focusing patterns of the hemoglobins of mice of a variety of genotypes, each of which specifies the α -globin known as chain 1, either alone or in combination with a second α -globin (Table 2). Lane 10 in Fig. 1 shows a mixture of hemoglobins with α -globin chains 5', 5, 1, 6, 2, and 3. The hemoglobin in lane 1, from a C57BL/6J (*Hba*^a) mouse, contains α -globin chain 1 alone. In the other samples the quantity of the second chain present is characteristic of the particular haplotype. The sample in lane 2 is from a mouse of the *Hba*^c genotype and contains the hemoglobin with α -globin chain 1 (upper, cathodal, band), which aligned with the chain 1-containing band in lane 1, and more of a second hemoglobin that contains α -globin chain 4. In lane 3 is hemoglobin from a mouse with the *Hba*^d haplotype; the second hemoglobin, present in greater amount, contains chain 2. Lane 4 contains hemoglobin specified by the *Hba*^s haplotype; approximately half contains α -globin chain 1 and half or more contains α -globin chain 5. Chain 5, initially discovered by isoelectric focusing (1), was subsequently found to differ from chain 1 by a novel glycine-for-alanine

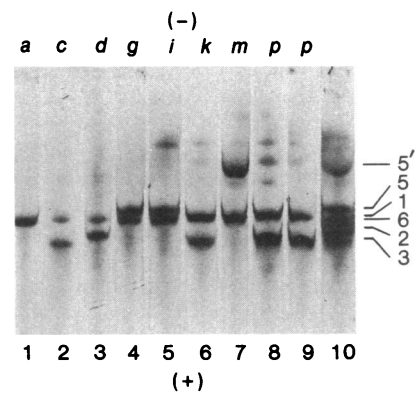


FIG. 1. Isoelectric focusing in an immobilized gradient of pH range 7.20–7.55 of hemoglobins of mice known or presumed to have α -globin chain 1 alone or accompanied by other α -globin chains. The samples were applied near the cathode, and current was applied for 12 hr or more. The distance between the centers of adjacent lanes is approximately 1 cm. Origins of the *Hba* haplotypes (genotypes): a, C57BL/6J; c, PosA; d, SM/J; g, DBA/2J; i, *Mus musculus molossinus*; k, "Centerville light" Maryland *Mus domesticus* (*Hba*^{w2}); m, induced mutation in DBA/2J; p (left lane), "Skive" Danish *Mus musculus* (*Hba*^{w5}); p (right lane), *Mus musculus brevisrostris* (*Hba*^{w5}). All mice used are congenic with C57BL/6J and therefore have the C57BL/6J hemoglobin β chain (*Hba*^a) type, except the PosA strain which has a presumed-to-be-identical *Hbb*^s haplotype from C57BL/Ks, and the Skive Danish-derived mouse that probably carries *Hbb*^d in heterozygous state.

substitution at position 78 of the 141-residue polypeptide (26). In lane 5 is hemoglobin specified by the *M. m. molossinus* *Hba*ⁱ haplotype. As confirmed by protein sequence analysis (26), the α -globins of mice of this haplotype are structurally identical to those with the DBA/2J (*Hba*^g) haplotype (chains 1 and 5), though they are present in different relative amounts. It is evident that this difference in the relative expressions of these closely linked structural genes is controlled by the *Hba* locus itself, but it remains to be defined whether the known DNA-level difference between these mice (31) is involved and whether control is exerted at transcription of the different mRNAs for the two α -globin chains or at their translation, or later.

The *Hba*^k (*Hba*^{w2}) haplotype (lane 6 in Fig. 1) specifies chains 1 and 3, confirmed by sequence analysis (data to be presented elsewhere). This haplotype has been found in feral ("wild") mice from England (24), Maryland (25), and Canada but has not yet been found in any standard inbred strain. This haplotype is unique in that it is the only one discovered thus far that determines appreciably more of a cathodal-group hemoglobin than of an anodal-group hemoglobin (chains 2, 3, or 4). The mutant haplotype (*Hba*^m or *Hba*^{s2}) derived from the DBA/2J *Hba*^g haplotype by an induced mutation, still specifies chain 1 but no longer specifies chain 5; instead, a new hemoglobin band with a much higher isoelectric point was found that must contain the derivative α -globin chain (29), here denoted chain 5'. Despite the structural change, the relative expression of chain 1 vs. the variant chain 5' seemed to be similar to that seen in standard *Hba*^g mice (lane 4). The shape of the chain 5' band was often different from that of the bands with other chains, as can be seen more clearly in the artificial mixture samples.

Descendants of feral mice from Europe, maintained by M. Potter, are the source of the hemoglobins analyzed in lanes 8 and 9 in Fig. 1. These isoelectric focusing patterns resemble that shown in lane 2, known to represent chains 1 and 4; however, when the solubility of hemoglobins with these α -globin chains and "single" β -globin chain (as in lane 9) was tested by the Dintzis spot-elution method (1), it was clear that these mice have a different haplotype, here called *Hba*^{w5} or

Table 2. Strain distribution pattern of *Hba* and *Hbb* genotypes

<i>Hba</i> haplotype*	α -Globin(s) [†]	Strains/stocks with <i>Hbb</i> ^s	Strains/stocks with <i>Hbb</i> ^d
<i>a</i>	1	C57BL/6J, C57BR, C57L	129
<i>b</i>	3 < 2	C58, SEC/1Re, LP	BALB/c
<i>c</i>	1 < 4	SWR, SJL, GRS	C3H, ST/b, WB/Re
<i>d</i>	1 < 2	SM/J	CBA, NZB
<i>e</i>	4		
<i>f</i>	5	CE/J, F/St, WK/Re	AKR, PL, RF, NZW
<i>g</i>	5 = 1	STAR/N	A, DBA/2, DBA/1
<i>g2</i>	5' = 1	(derived from chain 5 of DBA/2J)	
<i>h</i>	5 < 4		P/J, BDP, SEA
<i>i</i>	5 < 1		Some <i>M. m. molossinus</i>
<i>j</i> (<i>w1</i>)	6	<i>M. spretus</i>	(<i>M. hortulanus</i>)
<i>k</i> (<i>w2</i>)	1 > 3	Various <i>M. domesticus</i>	Various <i>M. domesticus</i>
<i>l</i> (<i>w3</i>)	2		Skive <i>M. musculus</i>
<i>n</i> (<i>w4</i>)	3, 4	Czech <i>M. musculus</i>	
<i>p</i> (<i>w5</i>)	1 < 3		<i>M. m. brevisrostris</i>
<i>b2</i> (<i>th</i>)	None [‡]		
<i>b3</i> (<i>th</i>)	None [§]		
<i>th-J</i>	None [¶]		

α -Globins specified by the various haplotypes are presented along with important strains (or noninbred source stocks) carrying those haplotypes. The amino acid differences among the chains are given in Table 1.

*The α -globins of haplotypes *j*, *k*, *l*, and *p* have not been sequenced, so chain assignments are tentative.

Nomenclature changes proposed for *j-p* haplotypes are shown in parentheses.

[†]The α -globin chain(s) (1-6) that is present is indicated by number; when more than one are present, the relative amounts are indicated when known.

[‡]352HB thalassemia mutation.

[§]27HB thalassemia mutation.

[¶]Thalassemia mutation.

Hba^p, that encodes hemoglobins, of which at least one is of far lower solubility than that characteristic of mice with the true *Hba*^c haplotype. Sequencing studies will be necessary to determine whether these mice have chain 3 or a previously uncharacterized mouse α -globin and whether the α -globin chains of the two *Hba*^{w5} haplotypes are actually identical to one another.

The minor, more-cathodal hemoglobins evident in lane 8 of Fig. 1 contain the minor β -globin chain specified by the *Hbb*^d haplotype. The other minor bands (for example, in lanes 5-7) were not always evident (compare with Fig. 3), especially in unstained or lightly stained gels, but when present they seemed to mirror the multibandedness of the major bands. They may be purely artifactual or they may contain uncharacterized minor non- α -globins expressed at very low levels in normal adult mice. They were sometimes observed as well in ampholyte-focusing gels in samples from *Hbb*^s homozygous as well as *Hbb*^d mice. Some hemoglobin also generally migrated to a position near the electrodes on these narrow-range gels, and its nature is also not currently known.

In Fig. 2 are shown the hemoglobins of mice known or presumed to have α -globin chains 2 or 6. The sample in lanes 2 and 6 is from a mouse with the *Hba*^b haplotype that is characteristic of inbred strains BALB/c and SEC/1Re. Protein sequence analysis defined the α -globins of these hemoglobins as chain 2 (upper, cathodal band) and chain 3. In lane 3 is hemoglobin from a mouse of the C57BL/6J.SM-*Hba*^d congenic strain with chains 1 and 2. The sample in lane 4 is from a mouse homozygous for an *Hba* haplotype that we designate *Hba*^{w3} or *Hba*^l, derived from Danish feral mice; isoelectric focusing seemed to define a single α -globin in this hemoglobin, presumed to be chain 2 alone. Lane 5 contains hemoglobins from a heterozygote of *Hba*^a and *Hba*^{w3}; such heterozygotes consistently have somewhat more hemoglobin with chain 2 than with the chain 1 specified by the opposing *Hba*^a haplotype. This is the only obvious exception to the codominant, equal expression of *Hba* haplotypes in normal

heterozygous mice of which we are aware. In lane 7 is hemoglobin from a mouse with the *Hba*^{w1} or *Hba*^j haplotype derived from mice of the related mouse species *M. spretus*. This hemoglobin focused anodal to the hemoglobin that contains chain 1 but cathodal to the hemoglobin with chain 2; we conclude that it must contain a novel α -globin chain not detected within the species *M. musculus*, which we call α -globin chain 6. Lane 8 contains a sample from a heterozygote with *Hba*^a and *Hba*^{w1}. Definition of the structure of this α -globin chain 6 awaits sequence analysis. Reasonably closely related to these *M. spretus* (that will hybridize with inbred mice in the laboratory) is *M. hortulanus*, the mound-building mouse (32-34). A sample

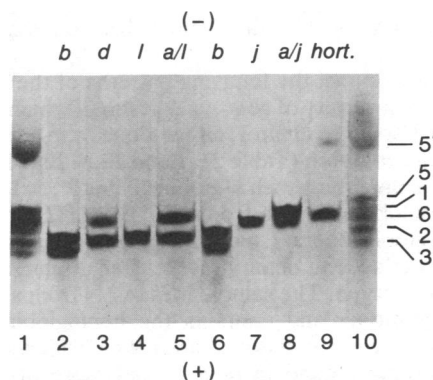


FIG. 2. Isoelectric focusing of hemoglobins of mice known or presumed to have α -globin chain 2 or 6 alone or accompanied by other α -globin chains. Origins of the *Hba* haplotypes (genotypes): *b*, SEC/1Re; *d*, SM/J; *l*, Skive Danish *M. musculus* (*Hba*^{w3}); *j*, *Mus spretus* (*Hba*^{w1}); "hort," *Mus hortulanus*. All mice used are congenic with C57BL/6J and therefore have the C57BL/6J hemoglobin β chain (*Hbb*^s) type, except the *M. hortulanus*, which has the *M. hortulanus* "diffuse" β -globin genes.

from a pure noninbred *M. hortulanus* mouse is shown in lane 9. All other *M. hortulanus* tested also had this *Hba* haplotype, which appears to be identical to the *M. spretus* haplotype *Hba*^{wl}, although breeding and protein sequence analyses have not been carried out. The minor more-cathodal hemoglobin band in this sample contains the minor β -globin chain that is also specified by the *Hbb*^d (or similar) haplotype of the *M. hortulanus* species.

The focusing patterns depicted in Fig. 3 are from mice that have α -globin chains that are or that resemble chains 3 or 4. The samples in lanes 2, 3, 6, 8, and 9 are as described above. In lane 4 is hemoglobin with α -globin chains encoded by the *Hba* haplotype of the BDP/J inbred strain, here defined as *Hba*^h (though previously presumed to be *Hba*^c before the existence of chain 5 was suspected). That the α -globin chain of the anodal band is truly chain 4 is indicated from its earlier tryptic peptide characterization (12). The α -globin chain of the more cathodal band appeared to be chain 5 by the criteria of isoelectric point and relatively high overall hemoglobin solubility. The hemolysate of lane 5 is from a mouse with an *Hba* haplotype that was discovered in a few noninbred Swiss mice (32). The hemoglobins appeared to have chains 5 and 4 in the same ratios as found in the C57BL/6J.BDP-*Hba*^h mice, so it was given the same haplotype designation. Whether the haplotypes are identical by descent or arose independently by crossover is unknown. The sample in lane 7 is from a C57BL congenic mouse homozygous for an *Hba* haplotype extracted by repeated backcross from a stock derived from mice originally captured in Czechoslovakia, here designated *Hba*^{ms} or *Hba*ⁿ. From peptide analyses it is known that these mice have two different α -globins, chains 3 and 4, consistent with the intermediate solubility of these hemoglobins and their failure to separate completely in our current isoelectric focusing system.

DISCUSSION

Standard electrophoresis does not resolve mouse hemoglobins with any of the naturally occurring *Hba* variants. All of the major bands shown (except that with chain 5') appear identical by cellulose acetate, starch gel, or polyacrylamide gel electrophoresis of the intact hemoglobins. Isoelectric focusing with standard ampholytes was found to be capable

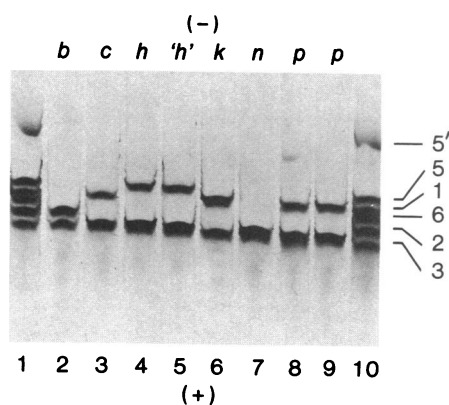


FIG. 3. Isoelectric focusing of hemoglobins of mice known or presumed to have α -globin chain 3 or 4 accompanied by other α -globin chains. Origins of the *Hba* haplotypes (genotypes): *b*, SEC/1Re; *c*, PosA; *h*, BDP/J; "h", noninbred "Swiss" CD1; *k*, Centerville light Maryland *M. domesticus* (*Hba*^{ms}); *n*, Czechoslovakian *M. musculus* (*Hba*^{ms}); *p* (left lane), Skive Danish *M. musculus* (*Hba*^{ms}); *p* (right lane), *M. m. brevisrostris* (*Hba*^{ms}). All mice used are congenic with C57BL/6J and therefore have the C57BL/6J hemoglobin β chain (*Hbb*^s) type, except the PosA and "Swiss-h," which have *Hbb*^s haplotypes of other derivations, and the Skive sample as described above.

of resolving some of these hemoglobins by distances as great as 2–3 mm (1). However, the resolution of chain 5 hemoglobin from chain 1 hemoglobin was incomplete at best, and accurate discrimination among the *Hba*^a, *Hba*^g, *Hba*ⁱ, and *Hba*^f (chain 5 alone) hemoglobins, especially in heterozygous combinations, was frequently difficult. Likewise, the resolution of hemoglobins with chains 2 and 3 or 4 was also incomplete. Commercial ampholyte preparations that nominally covered a narrower pH range proved not to give significantly better results. The cathodal drift problem inherent in the unbound-ampholyte isoelectric focusing method precluded the application of sufficiently high voltages that might have provided sharper bands distinguishable from one another. Nonetheless, until now, ampholyte isoelectric focusing remained the only feasible method for discriminating among these virtually identical proteins.

The introduction of the LKB "Immobilines," buffering compounds that can be incorporated into the support matrix in a defined way during the polymerization of a polyacrylamide gel, has provided a straightforward way to create isoelectric focusing gels of defined narrow ranges. Primarily because of these narrower ranges, it is now possible via isoelectric focusing to obtain complete separations of proteins that differ only among neutral amino acids (see Table 1). The pH 7.20–7.55 system described here has been tailored to provide good and complete resolution between the mouse hemoglobins with α -globin chains 5 and 1 or with chains 2 and 3—discriminations that are especially important in assessing the phenotypes commonly found in laboratory mice. It also provides adequate resolution between chain 6 hemoglobin and hemoglobins with chains 1 or 2, which has allowed transfer of the hemoglobin α -chain gene region from an entirely different species of mouse [*M. spretus* (31, 33–38)] into the standard C57BL/6J inbred genetic background. This pH range is also useful because it assures the retention on the gel of the hemoglobins with the mutant α -globin chain 5' and the minor β -globin chain of *Hbb*^d mice. The patterns of subbanding characteristic of α -globin variation that are seen in the single or major β -globin primary bands are also seen in weaker form in the minor β -globin-containing, more cathodal, bands. Nonetheless, the present system cannot resolve the mouse hemoglobin with α -globin chain 3 from that with chain 4. The application of higher voltages and the use of smaller pH ranges may provide this resolution, though it is conceivable that the substitutions involved (see Table 1) so closely offset one another that this separation will not be feasible in this system.

The analytical system described here is also suitable for the characterization of some of the human fetal hemoglobins. Both of the human fetal hemoglobins have a pI similar to that of mouse hemoglobin with α -globin chain 2 and the "single" β -globin chain. Although attempts to separate the human fetal hemoglobin with glycine at γ -globin position 136 from that with alanine at the same position have not succeeded with this system, the normal polymorphic substitution of threonine for isoleucine at position 75 can readily be detected.

The potential significance of a method capable of separating virtually identical proteins is enhanced by the fact that substitutions of amino acids by other similar amino acids are relatively much more common than might be expected (39, 40). Severely deleterious mutations not only are selected against, but also they occur infrequently. Several factors minimize the likelihood of the occurrence of the most "drastic" mutations (39)—those that generate an untranslatable codon from a translatable codon or substitutions of hydrophobic amino acids for hydrophilic amino acids or vice versa. Consequently, substitutions of amino acids by other similar amino acids are free to occur relatively frequently. Righetti (40) has estimated that at least 60% of the possible

mutations will fall into this electrophoretically "silent" class. Furthermore, such substitutions, having occurred, would have a less dramatic effect upon the function of the protein involved and, therefore, would be less deleterious and more likely to persist in populations. Because such substitutions are often not detected by standard electrophoresis, very many genetic variants of this type go unnoticed in biochemical-genetic surveys (40, 41). Immobilized gradient isoelectric focusing should also be especially valuable for mutagenesis screening.

The variations among the mouse hemoglobin α chains provide a prime example of the silent substitution effect: numerous studies of wild and laboratory normal mice have characterized electrophoretic variation determined by the hemoglobin β -globin chain locus, but no natural α -globin differences are detected by standard electrophoresis alone, in spite of the multiplicity of genetic variations that we now know exist. As the power of immobilized-gradient focusing becomes better recognized, its application to the analysis of a variety of other proteins will undoubtedly uncover many genetic variants among other polypeptides, perhaps even more than have been discovered by using standard electrophoresis alone.

In applications such as genetic monitoring or quality-control programs, this use of immobilized gradient isoelectric focusing for *Hba* and *Hbb* typing will permit rapid, inexpensive, unequivocal discrimination of mice of almost all of the commonly used inbred strains. The mice currently most widely used for biomedical research are of the A, AKR, BALB/c, C57BL, C57BR, C57L, C58, CBA, C3H, DBA/2, GRS, LP, RF, SJL, SM, ST/b, SWR, NZB, NZW, and 129 inbred strains. Among the agouti-color mice, isoelectric focusing readily distinguishes among CBA, C3H, agouti SM, and LP mice. Among the black mice, isoelectric focusing readily distinguishes among C57BL and the related C58, NZB, and black SM. Among nonagouti brown mice, C57BR, SEC, and BRSUNT are readily identified. Albino mice present a major need for biochemical genetic monitoring because of the lack of readily apparent differences among them (42). The most widely used albino inbred strains—A, BALB/c, AKR, and SJL—are readily distinguished by immobilized-gradient isoelectric focusing, even though A, BALB/c, and AKR mice all have "diffuse" hemoglobins. Contamination of any albino inbred strain would almost always be detected by immobilized-gradient isoelectric focusing by the appearance of uncharacteristic *Hba* or *Hbb* genotypes, generally in heterozygous state, assuming that several individuals were to be tested.

Righetti (40) has considered the resolutions of the mouse hemoglobin tetramers that differ only by neutral amino acid substitutions and has concluded that progressively lower isoelectric points probably are the result of increasing hydrophobicity conferred by the amino acids substituted (especially the asparagine, serine, and threonine residues that occupy position 68). This hydrophobicity would have an effect upon the ionization of one or more charged amino acids situated in the vicinity of the neutral amino acids in question. If neutral amino acid substitutions in other proteins prove as easy to detect by immobilized-gradient isoelectric focusing as are the variations in the mouse α -globins, then this method—with pH gradients tailored to the requirements of particular proteins or other amphoteric substances—should find widespread analytical and preparative applications in a number of fields.

We thank Dr. Michael Potter for his generous gifts of mice of various exotic origins and Dr. Susan Lewis for her gift of the *Hba*

mutant mouse and its offspring. We thank Dr. Titus H. J. Huisman for supplying the samples of human hemoglobin. This work was supported by National Institutes of Health Grant AM 31104. This is contribution number 0851 from the Department of Cell and Molecular Biology, Medical College of Georgia, Augusta.

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