

# Biochemical and physiological correlates of deer mouse $\alpha$ -chain hemoglobin polymorphisms

(hemoglobin oxygen affinity/high-altitude adaptation/maximum oxygen consumption/cold exposure exercise)

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**ABSTRACT** The  $\alpha$ -hemoglobin chains in adult deer mice are usually encoded by two tightly linked loci. Because of strong linkage disequilibrium, almost all  $\alpha$ -globin haplotypes fall into just two classes. The  $a^0c^0$  class predominates in high-altitude populations, whereas the  $a^1c^1$  class is generally fixed in low-altitude populations. Here we show that the  $\alpha$ -globin genotype has effects at both the biochemical level [on blood oxygen affinity ( $P_{50}$ )] and at the level of whole-animal physiology [on maximum rate of oxygen consumption ( $\dot{V}_{O_{2max}}$ ) during both exercise and cold exposure]. The  $a^1c^1/a^1c^1$  genotype mice have the highest  $P_{50}$  values and show the highest  $\dot{V}_{O_{2max}}$  values at low altitude (340 m) but the lowest  $\dot{V}_{O_{2max}}$  values at high altitude (3800 m). The  $a^0c^0/a^0c^0$  mice have the lowest  $P_{50}$ , and show the highest  $\dot{V}_{O_{2max}}$  at high altitude but usually have the lowest  $\dot{V}_{O_{2max}}$  at low altitude. The  $a^0c^0/a^1c^1$  heterozygotes have an intermediate  $P_{50}$  and are generally intermediate in  $\dot{V}_{O_{2max}}$  at both altitudes. Since high  $\dot{V}_{O_{2max}}$  is advantageous for aerobic exercise and thermogenesis, the physiological data provide a potential explanation for the correlation of haplotype frequencies with altitude.

For almost two decades, a central question in population genetics has concerned the extent to which protein polymorphisms in natural populations are subject to selection. There is now a sizable list of protein polymorphisms known to be differentiated at the biochemical level (1). Much rarer are protein polymorphisms that have been shown to have differential effects on the level of whole-animal physiology in a way that influences Darwinian fitness (1–12).

The deer mouse *Peromyscus maniculatus* provides an opportunity to assess the effects of a specific selective force (high-altitude hypoxia) on discrete genetic elements ( $\alpha$ -chain hemoglobin haplotypes). The species inhabits one of the broadest altitudinal ranges of any North American mammal, from sea level to above 4300 m. At 4300 m, the partial pressure of oxygen ( $PO_2$ ) is only 55% of the sea-level value, and that may severely limit aerobic metabolism. Deer mice also exhibit one of the most complex and extensive polymorphisms for hemoglobin of any mammal (13–15). In most individuals, the adult  $\alpha$ -chains of hemoglobin are encoded by two tightly linked loci, *Hba* and *Hbc* (15). At both loci, the multiple structural alleles can be classified into two groups ( $a^0$  versus  $a^1$  and  $c^0$  versus  $c^1$ ) on the basis of similarity of isoelectric points. There is very strong linkage disequilibrium between the two loci, such that  $\alpha$ -globin haplotypes are almost always composed of alleles of like superscript (ref. 16; unpublished data). “Recombinant” haplotypes (i.e., either  $a^0c^1$  or  $a^1c^0$ ) are quite rare; their average population frequency is estimated at 0.015. In populations in western North America, there is a highly significant negative correlation between  $a^1c^1$  haplotype frequency and the altitude of the collection site (ref. 17; unpublished data). That distribu-

tion provides circumstantial evidence for evolutionary adaptation of  $\alpha$ -globin polymorphisms to altitude, but other interpretations not involving natural selection—e.g., stochastic biogeographical processes—cannot be ruled out. To resolve those two possibilities, independent criteria are needed to assess whether the haplotype classes are in fact differentially adapted to altitude.

Here we examine the effects of the common  $\alpha$ -globin genotypes on the oxygen affinity ( $P_{50}$ ) of whole blood and also on an important parameter of whole-animal physiology, maximum rate of oxygen consumption ( $\dot{V}_{O_{2max}}$ ), during both cold exposure and exercise.

## MATERIALS AND METHODS

**Genetic Strains and Population Samples.** Strains of deer mice were developed that carried distinct  $\alpha$ -globin haplotypes in identical-by-descent (IBD) condition, arrayed against contrasting  $\alpha$ -globin haplotypes chosen at random from their original wild population. To develop an IBD strain for an  $a^1c^1$  haplotype, a single  $a^1c^1/a^0c^0$  heterozygote was mated to several unrelated  $a^0c^0/a^0c^0$  mice from the source population. Progeny carrying the  $a^1c^1$  haplotype (all copies of which are identical, barring mutation) were mated to unrelated  $a^0c^0/a^0c^0$  mice. After one or more generations of such outcrossing,  $a^1c^1/a^0c^0$  mice within the strain were mated to produce all three genotype classes ( $a^1c^1/a^1c^1$ ,  $a^1c^1/a^0c^0$ , and  $a^0c^0/a^0c^0$ ). Progeny of all three genotypes were chosen from several such matings to create groups of 10–14 mice matched for age, sex, parentage, and developmental history, and differing (as far as could be controlled) only in  $\alpha$ -globin genotype.

Nine IBD strains were developed from four subspecies (Table 1). In addition, groups of the three genotype classes were assembled from large samples of three highly polymorphic populations from subspecies *sonoriensis* from California and Utah and subspecies *rufinus* from Colorado. All genotype groups contained approximately equal numbers of males and females.

**Blood Oxygen Affinity.** Blood gas and hematological measurements were carried out on 5 ml of fresh blood pooled from the mice within a genotype group. (Pooling was necessary to obtain sufficient sample volume.) Equal volumes of the blood sample were equilibrated at 37°C against gases with identical  $PCO_2$  (pressure of  $CO_2$ ) values but containing either 30% or 0%  $O_2$ . The oxygenated and deoxygenated aliquots were mixed anaerobically to obtain blood at 50% oxygen saturation (18). The pH and  $PCO_2$  of the blood were varied by using three pairs of equilibration gases with different  $PCO_2$  values. The following parameters were determined:  $P_{50(7.4)}$ , the partial pressure of  $O_2$  in half-saturated blood at

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Abbreviations: IBD, identical-by-descent;  $PO_2$ , partial pressure of  $O_2$ ;  $P_{50}$ , partial pressure of oxygen at which hemoglobin is half-saturated;  $P_{50(7.4)}$ ,  $P_{50}$  at blood pH 7.4;  $PCO_{2(7.4)}$ , partial pressure of carbon dioxide in blood at pH 7.4 and 50% oxygen saturation;  $\dot{V}_{O_2}$ , rate of oxygen consumption;  $\dot{V}_{O_{2max}}$ , maximum  $\dot{V}_{O_2}$ .

Table 1.  $P_{50(7.4)}$  as a function of  $\alpha$ -globin genotype in nine IBD strains and three population samples of *Peromyscus maniculatus*

Strain	Sub-species*	Collection site	IBD haplotype(s) <sup>†</sup>	Test altitude, m	$P_{50(7.4)}$ of genotypes <sup>‡</sup>		
					$a^0c^0/a^0c^0$	$a^0c^0/a^1c^1$	$a^1c^1/a^1c^1$
764	<i>son</i>	Mono Co., CA	$a^1c^1$	340	31.35 (31.22–31.48)	33.04 (32.77–33.31)	33.87 (33.60–34.15)
946	<i>son</i>	Mono Co., CA	$a^1c^1$	340	31.91 (31.79–32.03)	33.42 (33.20–33.65)	34.58 (34.27–34.89)
4246B	<i>son</i>	Mono Co., CA	$a^1c^1$ $a^0c^0$	340	31.05 (30.78–31.33)	32.54 (32.20–32.89)	34.16 (33.72–34.60)
				3800	31.79 (31.49–32.10)	32.70 (32.42–32.98)	34.50 (33.07–36.00)
4246A	<i>son</i>	Mono Co., CA	$a^1c^1$ $a^0c^0$	340	31.25 (30.78–31.33)	32.76 (32.22–33.32)	33.85 (33.62–34.08)
				3800	31.32 (31.18–31.46)	32.71 (32.18–33.23)	34.30 (34.16–34.43)
4229	<i>son</i>	Mono Co., CA	$a^0c^0$	340	30.97 (30.72–31.23)	32.31 (32.13–32.48)	33.03 (32.97–33.28)
5113	<i>son</i>	Mono Co., CA	$a^0c^0$	340	32.57 (32.12–33.02)	33.53 (33.19–33.88)	35.65 (35.33–35.96)
1136	<i>neb</i>	Mesa Co., CO	$a^1c^1$	340	31.47 (31.35–31.60)	32.98 (32.85–33.11)	34.31 (34.19–34.43)
1801	<i>neb</i>	Mesa Co., CO	$a^1c^1$	340	31.08 (30.74–31.43)	31.85 (31.40–32.20)	32.45 (32.23–32.67)
				3800	31.32 (31.07–31.57)	32.46 (32.15–32.78)	32.90 (32.73–33.07)
4988	hyb	—	$a^1c^1$	340	30.87 (30.71–31.03)	32.97 (32.53–33.42)	34.04 (33.55–34.52)
—	<i>son</i>	Mono Co., CA	—	340	30.43 (29.67–31.21)	32.36 (32.16–32.55)	33.20 (32.88–33.53)
—	<i>son</i>	Kane Co., UT	—	340	31.75 (31.39–32.12)	32.99 (32.76–33.22)	34.09 (33.79–34.38)
—	<i>ruf</i>	Wasatch Co., UT	—	340	32.02 (31.84–32.19)	32.39 (32.27–32.51)	32.96 (32.68–33.23)

\*Subspecies: *neb*, *nebrascensis*; *ruf*, *rufinus*; *son*, *sonoriensis*; hyb, hybrid between subspecies *gambelii* from Riverside Co., CA, and *sonoriensis* from Mono Co., CA.

<sup>†</sup>In strains 4246A and 4246B, both the  $a^0c^0$  and  $a^1c^1$  haplotypes were IBD.

<sup>‡</sup> $P_{50(7.4)}$  (in units of torr; 1 torr = 133 Pa) was determined by interpolation of the regression of log  $P_{50}$  on pH; 95% confidence limits of the interpolated values are shown in parentheses.

pH 7.4;  $PCO_{2(7.4)}$ , the partial pressure of  $CO_2$  at 50% oxygen saturation and pH 7.4;  $CO_2$ -Bohr effect, the regression coefficient of log  $P_{50}$  on pH; and blood buffering capacity, the regression of log  $PCO_2$  on pH. Complete procedural details have been published (19).

**Maximum Oxygen Consumption.**  $\dot{V}_{O_{2max}}$  was determined for both exercise and cold exposure by open-circuit respirometry. Untrained mice were exercised for a single 6-min session on an enclosed treadmill (effective volume,  $\approx 1.2$  liters) at air temperatures of 17–24°C. The treadmill speed (0.5–0.7 m/sec) was set slightly above the maximum aerobic running speed of deer mice; higher speeds did not elicit a higher rate of  $O_2$  consumption ( $\dot{V}_{O_2}$ ). Attainment of  $\dot{V}_{O_{2max}}$  was judged by evident exhaustion of the mice during and after runs, post-run oxygen debt, and respiratory exchange ratios  $>1.2$  during the run. Mice were subjected to acute cold exposure (0–5°C) in a closed-loop wind tunnel at wind speeds of 4.5–5 m/sec. A mouse was left in the wind tunnel until  $\dot{V}_{O_2}$  fell rapidly, as the animal became hypothermic (usually within 15–20 min). Airflow (1.2 liters/min) through the treadmill and wind tunnel was regulated within 1% by mass flow controllers (Applied Materials AFC-550), and gas exchange was monitored with  $O_2$  and  $CO_2$  analyzers (Applied Electrochemistry S-3A and CD-3A, respectively) interfaced with a computer. The computer continually calculated “instantaneous”  $\dot{V}_{O_2}$  using the washout times of the chambers (20), thereby providing enhanced resolution.

The  $\dot{V}_{O_2}$  for a given test was calculated by having the computer search the stored record for the highest average  $\dot{V}_{O_2}$  sustained over various time intervals (1, 2, and 5 min for

exercise; 2, 5, and 8 min for cold exposure). The resulting values were compiled for all individuals within a particular IBD strain or population, and the performances of the three genotype classes were compared using one-way analysis of variance.

**Acclimation to Altitude.** Whenever possible, groups of mice were tested at both low altitude (340 m, at Riverside, CA) and high altitude (3800 m, at the Barcroft Laboratory of the White Mountain Research Station), with at least 2 months allowed for acclimation to local altitude before testing. Animal care was identical at both altitudes, except that at 3800 m the room temperature varied between 12°C and 22°C. All  $\dot{V}_{O_{2max}}$  measurements at a given altitude were completed before the animals were bled for oxygen-affinity studies.

## RESULTS

**Blood Oxygen Affinity.** In all nine IBD strains and all three polymorphic populations, the  $P_{50(7.4)}$  values of the genotype classes were well differentiated (as indicated by minimal or no overlap of 95% confidence intervals) and the ranking of genotypes was identical:  $a^0c^0/a^0c^0 < a^0c^0/a^1c^1 < a^1c^1/a^1c^1$  (Table 1). The probability that the same ranking would be observed in all 12 strains and populations by chance alone is  $(1/6)^{12}$ , or  $4.6 \times 10^{-10}$ . Genotypic effects on the other blood parameters [ $PCO_{2(7.4)}$ ,  $CO_2$ -Bohr effect, blood buffering capacity, hematocrit, and hemoglobin concentration] and on body weight were evaluated with one-way analysis of variance, but no significant effects were found in any of the strains or populations (data not shown). The differences in

$P_{50(7.4)}$  between genotypes could stem from differences in the erythrocyte concentration of 2,3-diphosphoglycerate, an allosteric effector of hemoglobin (22). However, 2,3-diphosphoglycerate levels were assayed in five IBD strains and no significant differences were found (data not shown).

In three IBD strains,  $P_{50(7.4)}$  was determined after acclimation to both low and high altitude. The values changed only negligibly between altitudes (Table 1), and the relative rankings of the genotypes remained the same. Those results corroborate other data indicating that, whereas other parameters of hematology, acid-base status, and blood-gas transport change markedly in deer mice during acclimation to altitude,  $P_{50(7.4)}$  does not (34).

**Maximum Oxygen Consumption.** Analyses of variance were determined on all the  $\dot{V}_{O_2}$  data to assess whether the averaging interval used to calculate  $\dot{V}_{O_{2max}}$  affected the magnitude of differences observed between genotypes. In general, slightly greater differentiation between genotypes was observed using the 2-min interval for both exercise and cold exposure; hence, those data are reported here. However, the choice of interval was not critical, because all intervals yielded qualitatively similar results.

Fig. 1 shows the relationship between  $\alpha$ -globin genotype and  $\dot{V}_{O_{2max}}$ , for both exercise and cold exposure at both low and high altitude. Genotypic effects on  $\dot{V}_{O_{2max}}$  were not entirely consistent among the strains and populations, as was the case for  $P_{50(7.4)}$ . Nevertheless, for each of the four experimental situations the ranking of genotypes was generally similar among the groups of mice tested. The greatest

variability was observed in the eight strains and populations exercised at low altitude (Fig. 1). Even so, in five of the groups the rankings of genotypes were the same, and in four of those the genotypic differences were statistically significant.

Fig. 1 also shows the data pooled from all strains and populations for a given experimental situation. The pooling was statistically legitimate, as indicated by normality of data within, and homogeneity of variances among, the pooled genotype groups. Analyses of variance revealed highly significant differences between genotypes (largest  $P$  value, 0.0005). Furthermore, the results from both exercise and cold exposure were generally consistent, both in the ranking of the genotypes and in absolute value. Under both conditions, the  $a^1c^1/a^1c^1$  genotypes showed the highest relative  $\dot{V}_{O_{2max}}$  at low altitude and the lowest relative  $\dot{V}_{O_{2max}}$  at high altitude. The  $a^0c^0/a^0c^0$  genotypes generally showed the highest  $\dot{V}_{O_{2max}}$  at high altitude and the lowest  $\dot{V}_{O_{2max}}$  at low altitude, except for cold exposure at low altitude, where the  $a^0c^0/a^1c^1$  heterozygotes showed the lowest mean value.

For both exercise and cold stress, all genotypes showed lower mean  $\dot{V}_{O_{2max}}$  at 3800 m. The substantial depression of  $\dot{V}_{O_{2max}}$  confirms that aerobic performance is limited by ambient  $PO_2$  at 3800 m.

## DISCUSSION

The results reported here match *a priori* expectations based on the strong correlation between  $\alpha$ -globin haplotype frequency and native altitude (ref. 17; unpublished data). In re-

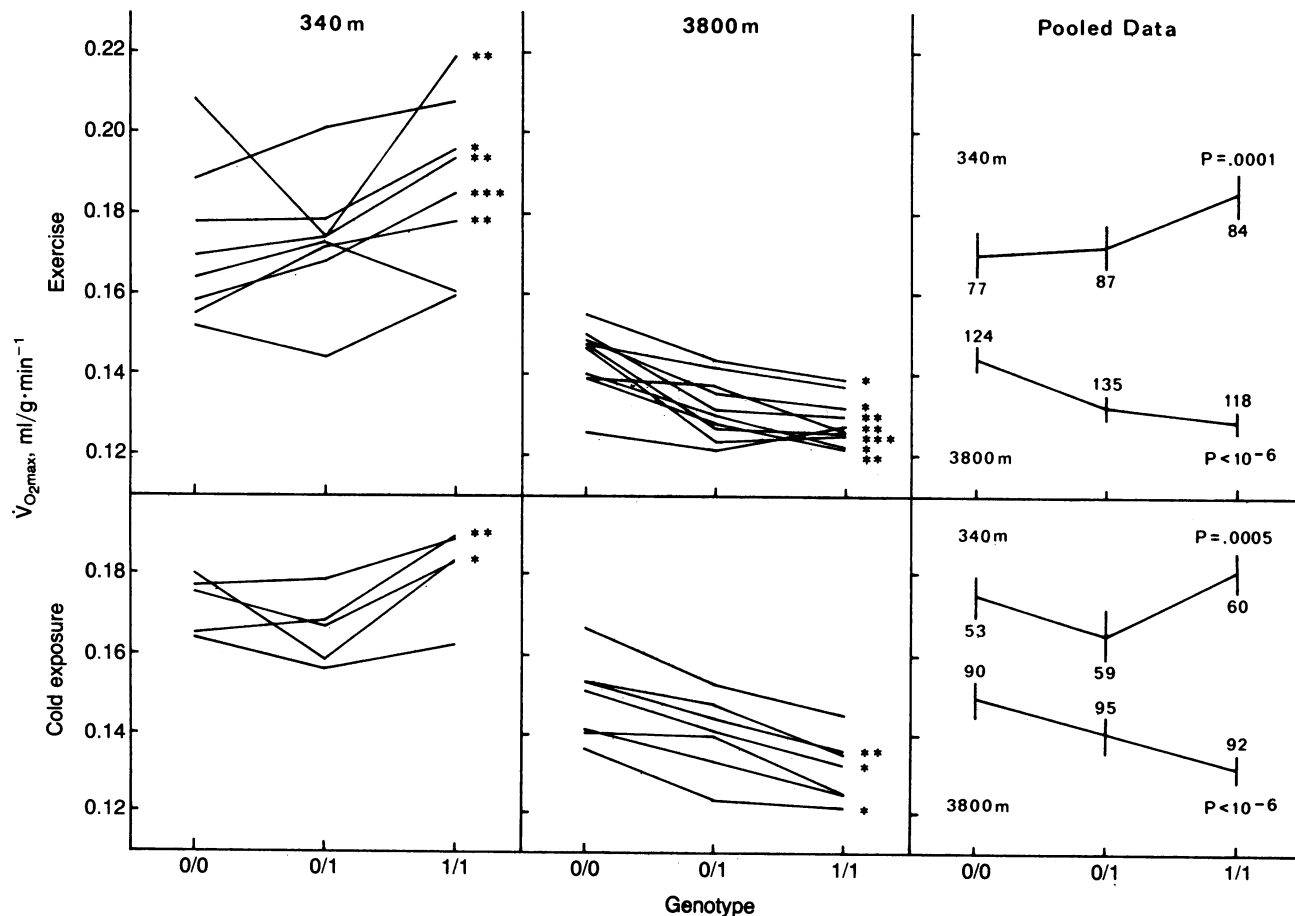


Fig. 1. Maximum  $\dot{V}_{O_2}$  (averaged over a 2-min interval) as a function of  $\alpha$ -globin genotype, during treadmill exercise and cold exposure at low and high altitude. The asterisks denote strains or populations for which analysis of variance indicated a significant genotypic effect. Probabilities are as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Genotypes: 0/0,  $a^0c^0/a^0c^0$ ; 0/1,  $a^0c^0/a^1c^1$ ; 1/1,  $a^1c^1/a^1c^1$ . The right-hand column shows the data pooled from all lines and populations at a given altitude and test condition. The vertical bars indicate the 95% confidence limits of the means. The numbers by the bars are the number of mice tested.

gions below  $\approx 1750$  m average altitude, populations are generally fixed for  $a^1c^1$  haplotypes, whereas in regions above  $\approx 2750$  m average altitude, the populations are close to fixation for  $a^0c^0$  haplotypes. In regions of intermediate average altitude, populations tend to be highly polymorphic for haplotype class. Furthermore, high- and low-altitude populations are genetically differentiated with regard to average blood-oxygen affinity (19), such that low-altitude populations show higher  $P_{50(7.4)}$ . These two correlations suggest that  $\alpha$ -globin genotype affects  $P_{50}$ , with  $a^0c^0$  haplotypes resulting in lower values. The results reported here confirm that prediction and corroborate independently determined preliminary results (17).

If the  $\alpha$ -globin polymorphisms are indeed subject to natural selection and represent a genetic adaptation to high altitude, then the population genetic data and the  $P_{50}$  data indicate that hemoglobins with low  $P_{50}$  values are favored at high altitude. This conclusion appears consistent with theoretical models (23, 24), which indicate that for optimal oxygen transport,  $P_{50}$  should increase up to moderately high altitudes and then decrease and fall below the sea-level value at very high altitudes. Unfortunately, for any given species it is difficult to specify the altitude above which a decrease in  $P_{50}$  is advantageous. Furthermore, the extent to which animal species conform to the theoretical models remains unclear. Various species show different responses to high altitude and/or hypoxia, and as a result there has been considerable controversy over what characteristics of hemoglobin are truly adaptive at high altitude (25, 26, 34).

The results on maximum oxygen consumption reported here provide independent evidence that the  $\alpha$ -globin haplotypes are differentially favored as a function of altitude, and hence that low  $P_{50}$  values are in fact adaptive at high altitude, at least in deer mice. For both exercise and cold exposure, the genotypes that predominate at low altitude ( $a^1c^1/a^1c^1$ ) had the highest  $\dot{V}_{O_{2,max}}$  at 340 m, whereas the genotypes that predominate at high altitude ( $a^0c^0/a^0c^0$ ) had the highest  $\dot{V}_{O_{2,max}}$  at 3800 m. At both altitudes, the  $a^0c^0/a^1c^1$  heterozygotes tended to be intermediate. These results make sense, because there is good reason to believe that an ability to sustain high levels of aerobic exercise or thermogenesis is directly relevant to Darwinian fitness. A mouse capable of attaining a higher  $\dot{V}_{O_{2,max}}$  can exercise more vigorously without incurring debilitating oxygen debt and/or it can maintain body temperature by means of aerobic thermogenesis at lower ambient temperatures. Many behavior patterns of deer mice, including foraging, courtship, territorial defense, and predator avoidance probably necessitate substantial exertion (27). At least in captivity, deer mice exhibit a marked penchant for vigorous exercise. Given the opportunity, the mice typically spend 80%–90% of their active time running on an exercise wheel (28, 29).

Because deer mice do not hibernate, they must produce considerable metabolic heat in the winter, especially in cold, windy alpine habitats (30). Even in the summer, deer mice at high altitude are subjected to significant cold exposure. Measurements of  $\dot{V}_{O_2}$  in summer-acclimated deer mice at various combinations of temperature and wind speed indicate that  $\dot{V}_{O_{2,max}}$  is elicited at about  $-10^\circ\text{C}$  in unstirred air and at  $0$ – $5^\circ\text{C}$  at a wind speed of 3–4 m/sec (31). Nighttime air temperatures near freezing are common in summer at high altitude, and the additional thermal effects of wind and radiative heat loss can interact to produce thermal stress near the tolerance limits of deer mice (ref. 31; unpublished data). Given a joint necessity for exercise and thermoregulation during their nocturnal activity period, mice incapable of sustaining high  $\dot{V}_{O_2}$  may be doubly at risk. In small mammals, the costs of exercise and thermoregulation are additive rather than substitutive, so high rates of metabolic heat production severely restrict the portion of the metabolic "scope" (the dif-

ference between basal and maximal  $\dot{V}_{O_2}$ ) available for locomotion or other activity (32, 33).

The effect of the  $\alpha$ -globin genotypes on  $\dot{V}_{O_{2,max}}$  may be a direct consequence of their effect on  $P_{50}$ . We observed significant genotypic effects only on  $P_{50}$  and  $\dot{V}_{O_{2,max}}$ , and not on any of the other parameters tested [ $\text{CO}_2$ -Bohr effect,  $\text{PCO}_{2(7.4)}$ , blood buffer slope, erythrocyte 2,3-diphosphoglycerate concentration, hematocrit, hemoglobin concentration, and body weight]. Although there was not a strict correspondence between  $P_{50}$  and  $\dot{V}_{O_{2,max}}$  in the individual strains and populations, that is not surprising, given the high variability in the physiological data. It remains to be seen whether there are haplotypes that have a significant effect on  $\dot{V}_{O_{2,max}}$  but not on  $P_{50}$ , or vice versa. The genotypic effects on  $\dot{V}_{O_{2,max}}$  may also be mediated by the shape and slope of the hemoglobin-oxygen dissociation curve, because these parameters are also critical determinants of oxygen transport (23, 24).

The effects on  $P_{50}$  and  $\dot{V}_{O_{2,max}}$  could stem either from the  $\alpha$ -globin *per se* or from closely linked genetic loci. In the latter case, the linked loci would have to be polymorphic and in consistent linkage disequilibrium with the  $\alpha$ -globins in essentially all populations. Such strong linkage disequilibrium maintained over a broad geographic area would be difficult to explain without postulating stringent selection on all the loci held in association. If the  $\alpha$ -globin polymorphisms were selectively neutral and merely "hitchhiking" with a selected locus, then any fortuitous linkage disequilibrium should be comparatively transient and local in distribution.

We stress that the comparisons of performance reported here are not between mice native to high versus low altitude, but between hemoglobin genotypes characteristic of high-versus low-altitude populations but derived primarily from populations collected at intermediate altitudes. Each set of genotypic groups was assembled from a single IBD strain or from a single population. All mice within a particular IBD strain had the same genetic background, derived from wild-caught mice from a single collection site.

The data presented here meet a rarely satisfied set of criteria: (i) Polymorphic proteins are shown to exert an effect at the level of whole-animal physiology. (ii) The physiological effects appear to be specific and predictable manifestations of biochemical differences observed *in vitro*. (iii) The physiological effects can reasonably be related to Darwinian fitness. (iv) The relative fitnesses indicated by the physiological effects appear to explain the maintenance of a polymorphism. It is not sufficient to demonstrate physiological differences between genotypes. Rather, it must be shown that the relative advantages and disadvantages of the genotypes are balanced in some fashion to account for the existence of a polymorphism and the observed geographical patterns of gene frequencies. In this case, the physiological data indicate that the  $a^1c^1/a^1c^1$  genotypes are superior at low altitude but are at a disadvantage at high altitude, whereas the converse is true of the  $a^0c^0/a^0c^0$  genotypes. At both extremes of altitude, the  $a^0c^0/a^1c^1$  heterozygotes tend to be intermediate in performance. Additional studies are under way to examine the relative performances of the three genotype classes at intermediate altitudes. It may be that the heterozygotes are at an advantage over a fairly broad range of intermediate altitudes. Such a fitness scheme, in conjunction with gene flow between populations, would help to explain the otherwise puzzling observation that, within regions of highly variable topography and/or along particularly steep altitudinal gradients, the high- and low-altitude populations are not strongly differentiated with regard to either  $\alpha$ -globin haplotype frequency or average  $P_{50}$  (refs. 17 and 19; unpublished data).

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