### 1. Title and Subtitle

ASSESSMENT OF FACTORS POSSIBLY CONTRIBUTING TO THE SUSCEPTIBILITY OF SICKLE TRAIT ERYTHROCYTES TO MILD HYPOXIA: I. DESIGN CONSIDERATIONS AND RESEARCH PROTOCOL

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### 16. Abstract

This report continues a review (see FAA-AM-76-15) of the evidence for mixed dominance of the HbS gene in people with the sickle cell trait. These individuals, whose erythrocytes contain a mixture of hemoglobins (HbA/HbS), are healthy and have a normal life expectancy. They are tolerant to moderate altitudes; their erythrocytes become sickled only at oxygen tensions that would be hazardous to any person. However, there is a possibility that other debilitating factors (e.g., alcoholism, pulmonary disorders) can, in a small fraction of those with the trait, produce an abnormal susceptibility to hypoxia. Those so debilitated would not be medically qualified to serve as air crewmembers. This report presents an experimental plan for estimating the proportion of such individuals in a population of young people with the sickle trait and outlines methods to be used in the study.

### 17. Key Words

Stress Physiology, Hemoglobin, Aviation Personnel, Sickle Cell Disease, Sickle Cell Trait, and Medical Certification

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INTRODUCTION

In 1976, we reviewed the published evidence for and against the medical opinion prevalent at that time that people with sickle trait (genotype AS) are abnormally susceptible to moderate hypoxia. Our conclusions, based on in vitro studies and controlled experiments in which type AS individuals were exposed to moderate altitudes, were: (1) most people of the AS genotype are tolerant of hypoxia; and (2) there may exist in certain individuals a combination of conditions (including the sickle trait) that renders them abnormally susceptible to lowered oxygen tensions. Our report cited a number of medical and other factors that might contribute to a sickling crises in type AS individuals and suggested possible pathogenic mechanisms.

The present report is the result of a study made during 1977 to identify practical techniques for detecting the factors that may contribute to susceptibility to hypoxia in a population of young people of the AS genotype.

We will review recent evidence that certain organ and physiological systems can play a role in the genesis of sickle crises. This is intended to augment the evidence considered in the earlier, more extensive review. Also, techniques to be used in the population survey are outlined and the rationale for their selection is discussed.

CONDITIONS POSSIBLY CONTRIBUTING TO SUSCEPTIBILITY TO HYPOXIA IN PEOPLE WITH THE SICKLE CELL TRAIT

It is now widely accepted that the pathologic sequelae of sickling are due to an inability of the rigid sickled erythrocytes (RBC) to flow through channels that are smaller than the "normal" RBC diameter. Normal RBC suspended in Eagle-albumin solution will pass through polycarbonate pores of 3μm diameter, but after hardening with acetaldehyde, they will not pass through pores as large as 6.8 μm. The presence of Hbs in polymerized form confers a similar loss of filterability that contributes to a vicious cycle: increased blood viscosity and decreased flow, leading to increased hypoxemia and further sickling.

Any factor that may contribute to the sickling process, to localized or general hypoxia, to the stiffness of RBC, or to a reduction in blood vessel diameter may also contribute to pathologic changes that may be indistinguishable from those changes seen during sickle crises or, in chronic conditions, in sickle cell disease (SCD).

The AS condition may be one of mixed or incomplete dominance; thus, there may be heterozygous (AS) individuals who are clinically more like those with SCD than are most individuals of the AS genotype. This review considers factors that may be expressions of the HbS gene, as well as factors that are not genetically related.
Blood Factors. We previously\(^1\) cited a number of blood components that may affect the genesis of the sickling crises; these include fibrinogen, fetal hemoglobin, and other hemoglobins. There are also other factors that may be considered.

It is known that HbS polymerizes and becomes insoluble when it is deoxygenated. On the other hand, a susceptibility of HbS to oxidation has also been reported. Asakura et al.\(^3\) reported that this hemoglobin becomes insoluble in the oxygenated state when it is agitated mechanically. Bensinger and Beutler\(^4\) confirmed these observations. They diluted hemoglobin solutions obtained from the RBC of individuals of the SS, AS, SC, AC, and AA genotypes to concentrations of 10 percent and exposed these to air by manual inversion. After these solutions were further diluted with 17 percent isopropanol buffer, they were observed for the turbidity that developed over the next 40 min. Only those tubes containing HbS developed a significant turbidity. The precipitate was composed principally of \(\beta_S\)-globulin chains. This finding raises the possibility that Heinz body hemolytic states may be an important cause of anemia in SCD.

Changes in the blood complement may also be related to the sickling phenomenon. In 1976, Wilson, Hughes, and Lachmann\(^5\) reported a deficiency of complement factors B(C3 proactivator) and D(C3 proactivator convertase) in patients with SCD; these authors suggested that complement deficiencies could be an important factor contributing to the well-known susceptibility to infection in patients with SCD. The authors did not propose a specific mechanism for the reduction of complement, but indicated that it could be the result of reduced synthesis, increased degradation (chronic activation), or inhibition. We have found no direct evidence in the literature that complement activity is depressed in the heterozygous state, but it has been reported that the malarial parasite, Babesia rodhaini, cannot parasitize human RBC in the absence of complement.\(^6\) This finding, in the light of the low malarial death rate in type AS individuals,\(^1\) is consistent with a hypothesis that complement levels are low in sickle trait. The report by Shultz and Arnold\(^7\) that cyanate, an ion that offers some protection against sickling, also inactivates complement, suggests that complement may play a role in the sickling process.

Friedman's recent report\(^8\) of in vitro studies seems to contradict the hypothesis that a lower infection rate, possibly as a result of complement deficiency, is a reason for the low morbidity from malaria in those with sickle trait. Friedman's results showed a strong inverse correlation between sickling rate in HbS-containing RBC that were cultured with *Plasmodium falciparum* and the survival of the malarial organism within the cell. Sickling, and reduced survival of the organism, resulted when the oxygen level was lowered to 1 to 5 percent. The rates of infection, the proportion of infected RBC, seemed to be no different in cells containing HbS and those containing only HbA. Friedman concluded that the mechanism for resistance to malaria in HbS-containing RBC is due only to intracellular conditions that discourage the maturation of the parasite. It is not clear, however, that his culture medium contained an intact complement system, the only source for this system being human serum at a concentration of 10 percent.
Because some of its components are highly labile it is possible that complement was deficient in these studies and that, therefore, its effects were not detected. The maximum rates of infection recorded were below 40 parasitized cells per thousand. Perhaps an intact complement system would have augmented the rate considerably.

The sickling process seems to be related to changes in the permeability of the RBC membrane. Tosteson et al.\textsuperscript{9} were among the first to report an association between the sickling process and the flow of ions across the RBC membrane. They observed a net loss of $K^+$ and a net gain of $Na^+$ in the cell during sickling. This flux was reversible if reoxygenation was carried out early. If the sickling response to hypoxia was prevented by pretreating the blood with carbon monoxide (CO), the $K^+$ loss and $Na^+$ gain response was also prevented. Palek\textsuperscript{10} reported in 1974 that RBC accumulate Ca\textsuperscript{++} during sickling. The phenomenon was also prevented by CO but was not reversed by reoxygenation, a finding that may have been due to a delay in reexposure to an oxygen environment. Palek also found Ca\textsuperscript{++} changes in the sickling RBC of people with sickle trait.\textsuperscript{11}

Zinc is another ionic species that may alter the susceptibility of RBC to sickling. Attention was first drawn to this metal when Serjeant, Galloway, and Gueri\textsuperscript{12} reported that orally administered ZnSO\textsubscript{4} promoted the healing of leg ulcers in patients with SCD. It was later reported by Karayalcin et al.\textsuperscript{13} and other groups of investigators that serum zinc levels are depressed in SCD, but it was pointed out by Kapu et al.\textsuperscript{14} that serum zinc is lower than normal in many Africans—even those of type AA—who may suffer from chronic inflammation, poor diet, and/or parasitism. Schoomaker et al.\textsuperscript{15} have demonstrated that Zn\textsuperscript{++} prevents some of the loss of elasticity of RBC during hypoxia and decreases the retention of Ca\textsuperscript{++} by the RBC membrane. This Ca\textsuperscript{++} effect may be associated with the reduction in the number of irreversibly sickled cells in patients given Zn\textsuperscript{++}.\textsuperscript{16} It has not been shown that zinc deficiency promotes the sickling crisis, nor, for that matter, that zinc is depleted as a result of repeated crises. Certainly, there may be other ions that play a role in promoting or attenuating the clinical intensity of SCD. In that regard, Kapu et al.\textsuperscript{17} have reported that plasma copper is elevated in SCD.

Renal Factors. In our earlier review, we cited hyposthenuria (HPU) as a finding common to both the AS and SS (homozygous) conditions and proposed a mechanism by which dehydration (induced by alcoholism, for example) might trigger a sickling crisis in those with HPU. Probably HPU exists to a variable degree throughout those of the AS genotype. Even in type SS individuals, there may be a variation of expression. Keitel et al.\textsuperscript{18} reported that HPU can be relieved in young children with SCD by transfusion with normal RBC. They pointed out that HPU is probably unrelated to the degree of anemia because HPU is also present in those with the trait who are not anemic.

It seems reasonable to assume that the degree of expression of the HB\textsuperscript{S} gene is reflected, at least to some extent, in the degree of HPU and that measurement of osmotic clearance in the dehydrated subject would be useful in assessing the degree of gene expression. This may not be practical, however,
without using unacceptable invasive techniques, since HPU may be confined to unilateral renal defects as in the unilateral hematuria seen in those with SCD and with the sickle trait.\textsuperscript{19,20}

There are other conditions, unrelated to pathological changes in the vasa recta, that may aggravate HPU and thus promote sickling (see reference 1 for a review of the evidence). Alcohol and other diuretics may contribute in this way to sickling crises, as may some normal physiological responses to exercise and emotional stress. For example, Raizs et al.\textsuperscript{21} found that heavy exercise antagonizes the renal concentrating mechanism and Del Greco et al.\textsuperscript{22} produced the same result with hexamethonium. Catecholamine injections cause an increase in urine flow.\textsuperscript{23,24} These findings are interesting because of the association of alcohol abuse, emotional trauma, and severe exercise with sudden unexpected deaths of people with the sickle trait. Considering these and other findings, one can construct a hypothesis that the kidney is the site of initial sickling in certain sickle crises that lead to sudden unexpected death and that varying degrees of pathological changes in the vasa recta confer varying degrees of susceptibility in those with sickle trait. Thus, an evaluation of maximum renal osmolar clearance could be an important part of a test battery for detecting those type AS individuals who are unusually susceptible to hypoxia.

It seems appropriate to mention that in persons with sickle trait renal pathology and immunological defects may be interrelated. Evidence of this was reported by Ozawa et al.\textsuperscript{25} who found complexes of renal tubular antigen and its autologous antibody in the serum of a child suffering from proteinuria. The patient also had the sickle trait. Although immune deposit nephritides associated with SCD had been reported earlier, Ozawa et al. were first to report an autoimmune renal condition in the heterozygous state. This finding indicates that immune and renal deficiencies may be a complex manifestation of the Hb\textsubscript{S} gene in the heterozygote and that varying degrees of autoimmune disease may be related to varying degrees of renal pathology, including mild HPU and frank hematuria.

EXPERIMENTAL APPROACHES USING HUMAN SUBJECTS

The most important consideration in a study of any group of human subjects is that of risk of harm to the subjects. Although it has been concluded that most people with the sickle trait are at no greater risk than are those without it, there are other conditions that can, in combination with the trait, produce a dangerous susceptibility to sickle crises.\textsuperscript{1} One is obliged, therefore, to take precautions to ensure that these conditions do not coincide in experimental groups. This is best accomplished by carefully screening prospective subjects; thus, subjects will be chosen from a population of healthy young men. Each subject will visit the laboratory on two occasions. On the first visit, a careful medical examination will be made and a thorough history taken. In addition, a blood sample will be taken for quantitative analysis of hemoglobin types and for assessment of RBC filterability. When any of the suspected contributing factors is detected in an applicant, the subject will be fully informed of the findings and referred to his own physician. The subject will be allowed to continue in the study if he wishes, but will not participate in any experiment involving \textit{in vivo} physiological stress.
This study has two goals: (1) to make an estimate of the proportion of those of the AS genotype who may be medically unqualified to serve as air crew members, and (2) to estimate the degrees of expression of the Hb\textsubscript{B} gene in the heterozygous population.

The first goal can be accomplished through medical examination and history-taking in a large population of young people who are most likely candidates for aeromedical certification. The criterion for qualification, based on our earlier conclusions, is a simple one; those who have no history or signs of \textit{in vivo} sickling, who possess none of the suspected and known contributing factors (other abnormal hemoglobins, alcoholism, overweight, pulmonary disorders, etc.), and whose RBC remain filterable at oxygen tensions above 40 mmHg will be passed as "qualified" and will be invited to participate in the physiological stress experiments. Those who are not qualified will return to the laboratory, but only to give blood. Because blood samples will be taken from all subjects, adequate hematological data will be available and will contribute toward the second goal of estimating the degree of expression of the Hb\textsubscript{B} gene in RBC.

Responses to exercise and the degree of HPU will be measured in only the medically qualified subjects. In the exercise test, both hematologic and neuroendocrine responses will be assessed. The exercise test is included in the battery because of the possibility that physical exertion may produce an increased susceptibility to hypoxia in the RBC of type AS individuals.

Because subjects will be deprived of water for over 15 hr, it will be possible to estimate the degree of HPU by measuring osmotic clearance under increasing degrees of dehydration: $C_{OSM} = U_{OSM} \frac{V}{P_{OSM}}$, where $U$ and $P$ are the osmolarities of urine and plasma and $V$ is urine volume. In normal individuals during progressive dehydration, the urine becomes concentrated and its osmolarity rises above that of the plasma as urine flow decreases. The volume of solute-free water, $T_{H2O}^C = C_{OSM} - V$, approaches a maximum value, $T_{H2O}^C$, that is characteristic of the individual.\textsuperscript{26} This maximum is approximately 5 ml/min per deciliter of glomerular filtrate. In those with defective water reabsorption, this level is not reached, even under conditions of water deprivation. Smith states that $T_{H2O}^C$ is reached after 12 to 18 hr of deprivation; it is not enhanced by antidiuretic hormone, by mannitol infusion, or by mercurial diuretics.\textsuperscript{26}

**Basic Protocol for the Proposed Study.** On the second visit to the laboratory, each subject will report at 0830 in a fasted state; he will not have consumed any fluids after 2200 on the previous night. At 0900, a blood sample will be taken for analysis of \textit{in vitro} filterability and other hematological variables. Immediately after this, the subject, if qualified, will begin a treadmill exercise test. This test will continue until the heart rate reaches 160 to 180 bpm. At this time another blood sample will be taken and the subject will remain in the laboratory, abstaining from food and liquids until 1400 when another blood sample will be taken. The subject will then be given fluids and allowed to leave the laboratory. Urine samples will be taken throughout the experimental period.
Hematological and Biochemical Measurements. Urine will be analyzed for osmolarity, catecholamines, 17-ketogenic steroids, protein, plasma hemoglobin, total protein and creatinine. Blood measurements will include osmolarity, complement, plasma hemoglobin, RBC and plasma cholinesterases, $K^+$, $Na^+$, $Ca^{++}$, $Mg^{++}$, and $Zn^{++}$. These ions will also be measured in the urine for clearance estimates. Also the RBC will be examined for filterability at graded oxygen tensions by using published techniques, for resistance to oxidative damage, for $O_2$-hemoglobin dissociation, and for reversibility of sickling. In addition, if a suitable technique can be developed, we will also measure the sickling time, which has been reported to vary in different genotypes and may be an important factor in establishing the relative susceptibility to sickle crises.
REFERENCES


