sis revealed active spermatogenesis when testis indices were large (>1.8) and arrest of spermatogenesis when testis indices fell below 1.0.

The present results agree with and expand upon the findings of Tamarkin *et al.* (*6*), who demonstrated the efficacy of injecting melatonin once and thrice daily in intact and pinealectomized hamsters, respectively. My results indicate that either pattern of injections produces regression in both pinealectomized and intact animals; pinealectomy alters only the time course of the effect. It is not known whether the responsiveness of pinealectomized hamsters to single injections fluctuates with time of day.

In contrast to intact and pinealectomized hamsters, animals whose testes have regressed and spontaneously recrudesced fail to respond within 7 weeks to either regimen of melatonin injections. The difference between these animals and those subjected to pinealectomy refutes the hypothesis that recrudescence and subsequent refractoriness to short days result from pineal exhaustion. The fact that the gonads of hamsters initially induced to regress by daily melatonin treatments spontaneously recover despite continuation of such injections further strengthens the target insensitivity model. There is no need to posit any change in pineal secretory activity during the course of recrudescence. The desensitization of the pineal's target, indicated by these experiments, is adequate to account for both spontaneous recrudescence and the ensuing refractoriness to short days.

There are at least two ways in which such insensitivity might develop. It is possible that the locus at which the pineal hormone acts actually ceases to take up the substance or stops responding to it intracellularly. Alternatively, recovery from suppression of gonadotropin secretion might be a general property of the neuroendocrine system. Both puberty and spontaneous recrudescence may partially reflect spontaneous shifts in steroid feedback threshold from hypersensitive levels (*14*).

Long days might terminate refractoriness by inducing the functional equivalent of pinealectomy. This would be the case if the absence of some pineal principle, perhaps the antigonadotropin itself, were required for the target to regenerate. Alternatively, secretion of melatonin at a phase appropriate to long photoperiods might be involved in restoration of sensitivity to short days. Still another possibility is that the photoperiodic time measurement mechanism might operate directly on the pineal's target to restore its responsiveness.

The present results establish that melatonin loses its ability to suppress reproductive activity during that phase of the breeding cycle characterized by unresponsiveness to short photoperiods. Furthermore, single daily injections of melatonin induce gonadal regression and recrudescence along a time course similar to that elicited by exposure to short days. These findings strengthen the argument that melatonin is a pineal antigonadotropic hormone (*15*) and indicate that target tissue insensitivity, or some other alternative to pineal exhaustion, accounts for refractoriness to short photoperiods.

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Sickling Rates of Human AS Red Cells Infected in vitro with *Plasmodium falciparum* Malaria

Abstract. *The kinetics of sickling of malaria-infected red cells from humans with sickle cell trait were studied in vitro in an attempt to obtain direct experimental evidence for a selective advantage of the hemoglobin S heterozygote in a malarious region. The sickling rates of cells infected with* Plasmodium falciparum *and of noninfected cells were studied both in the total absence of oxygen (by dithionite addition) and at several different concentrations of oxyhemoglobin which might obtain in vivo. In all cases, red cells containing small plasmodium parasite forms (ring forms) sickled approximately eight times as readily as uninfected cells. Cells containing large parasitic forms (trophozoites and schizonts) appeared to sickle less readily than uninfected cells, by light microscopy criteria, but electron micrographs demonstrated the presence of polymerized deoxyhemoglobin S with a high frequency. It is concluded that enhanced sickling of plasmodium-infected AS cells may be one mechanism whereby the hemoglobin S polymorphism is balanced in favor of the heterozygote.*

Epidemiological evidence suggests that hemoglobin S (Hb S) and other red cell polymorphisms protect against the lethal effects of malaria caused by *Plasmodium falciparum*. The overlap of the high gene frequency of Hb S and the geographical location of malaria, as well as the lower parasite counts and decreased mortality in individuals heterozygous for Hb S (*1–3*) (that is, possessing normal hemoglobin, Hb A, as well as Hb S), have been interpreted as proof of this relationship. On the other hand, experimental data to support the notion that Hb S offers protection against malaria are scarce. Perhaps the best experimen-

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tal evidence to date is a study by Luzzatto *et al.* (*4*) on the rates of sickling of parasitized AS red cells obtained from acutely ill patients living in an endemic area. By inducing sickling in vitro, it was determined that parasitized red cells sickled two to eight times faster than uninfected cells. These results led to the conclusion that the presence of Hb S accelerated the clearance of infected cells from the circulation and thereby constituted the main advantage of this abnormal hemoglobin in endemic areas.

Using different techniques designed to eliminate potential artifacts, we have demonstrated the increased sickling propensity of red cells infected with parasites under conditions of total and partial deoxygenation.

Heparinized blood was obtained by venipuncture from patients heterozygous for Hb S (informed consent was obtained). Evidence for the presence of Hb S included clinical evaluation; family studies; electrophoresis on starch gel, *p*H 8.6; and on citrate agar medium, *p*H 6.4; solubility in concentrated phosphate-dithionite buffers; sickling preparations with dithionite. The erythrocytes were stored in CPD (citrate-phosphate-dextrose) solution and used for the growth of *P. falciparum* as described (*5*). Parasitized samples contained between 3 and 15 percent parasitized cells in culture medium at a packed cell volume of 6 percent. Control cells that were not infected were also maintained in the same medium under the same conditions, and sickling rates were also obtained for these cells.

The cells were diluted 1:20 with a buffered saline solution containing 0.1*M* $NaHPO_4$, *p*H 7.35, and NaCl 1.25 g/liter. Ten milliliters of this cell suspension were drawn into an airtight 20-ml glass syringe fitted with an 18-gauge needle on a three-way stopcock valve. In a second syringe of the same type, the same buffer with sodium dithionite was prepared as follows. The buffered saline was deoxygenated for 1 hour with moist 100 percent N_2 and was then allowed to flow anaerobically into a N_2-filled flask containing dry sodium dithionite. The second syringe was flushed several times with this solution, and finally 10 ml without bubbles was drawn into the syringe which was fitted to the three-way stopcock of the syringe containing the cell suspension. At zero time, the dithionite buffer was rapidly transferred to the syringe with the cell suspension and the experiment was begun. The final concentration of dithionite was 0.3 g/dl. At time intervals ranging from 2 to 50 minutes, approximately 2-ml portions of the deoxygenated cells were expressed into tubes containing 3.8 percent formaldehyde in an isotonic buffered saline, *p*H 7.35. All experiments were performed at 24°C. The final *p*H after the cell suspension was mixed with the dithionite buffer was 7.27, and the final osmolality was 280 to 290 milliosmols as determined on a Fiske osmometer. After fixation overnight, the cells were washed in unbuffered isotonic sodium chloride and smears were made; these were fixed in 100 percent methanol for 5 minutes and stained with Giemsa stain for 8 minutes more. We found that fixation in formalin decreased the staining time with Giemsa from 50 minutes to approximately 8 minutes. The smear for each time point was evaluated for percentages of parasitemia, sickling of uninfected cells, sickling of cells with large parasites (trophozoites and schizonts), and sickling of cells with small parasites (ring forms). We counted 100 to 200 cells of each type for each time point. In addition, an oxygenated portion of cells was also counted to ensure that no prior deformity of cells had occurred and to compare the parasitemia in these cells with that in the deoxygenated cells. The treatment with dithionite and the subsequent handling did not alter significantly the percentage of infected cells. Ten experiments were done on different portions of cultured cells from four different AS patients. Of these, five experiments were differentiated into those with sickling of cells containing large or small parasites.

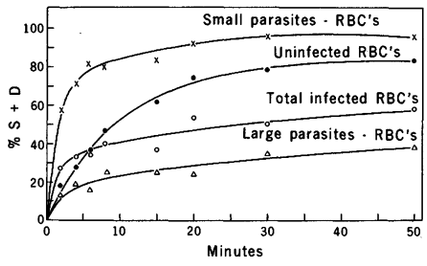

Fig. 1. Sickling rates in infected and noninfected AS cells. The percentage of sickled plus deformed cells (*S* + *D*) is plotted against time.

In order to study sickling as it might occur under physiological circumstances, in several experiments we induced sickling at different partial pressures of oxygen in the absence of sodium dithionite or metabisulfite. For these experiments, the cell suspension was adjusted to the desired percentage of oxyhemoglobin content and maintained at 37°C for 3 minutes in an Imai apparatus (*6*). The entire contents of the chamber were then removed into 3.8 percent buffered formalin and processed for counting as described above. Each oxygen saturation point represents a fresh portion of cell suspension. In this manner partial deoxygenation was produced and no dithionite or metabisulfite were employed. The red cells were suspended in phosphate-buffered isotonic saline (*7*), *p*H 7.35, with 10 m*M* glucose. For elec-

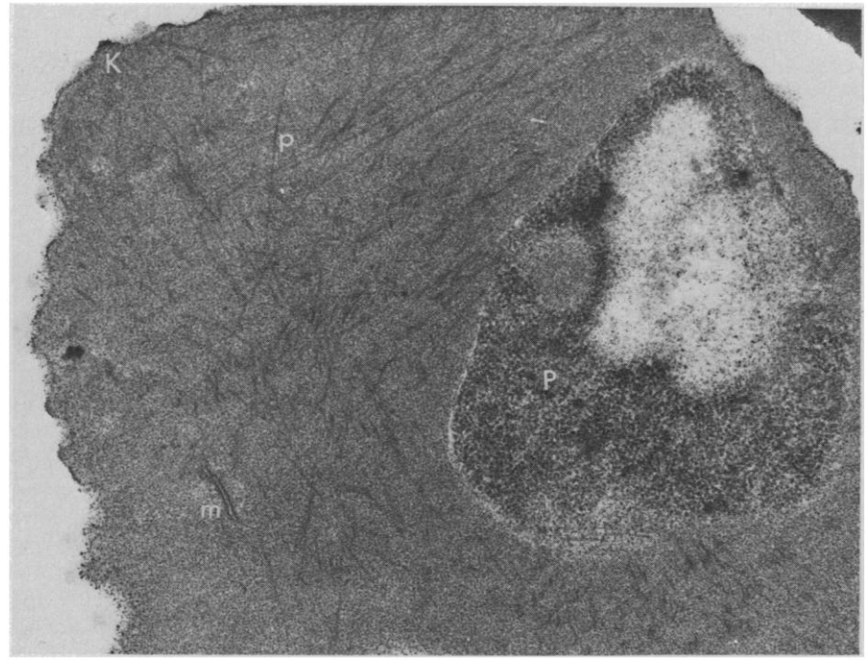

Fig. 2. Electron micrograph of a sickle trait (AS) red cell (deoxygenated) containing a trophozoite of *P. falciparum*. Abbreviations: *P*, parasite; *p*, Hb S polymer; *K*, red cell membrane knobs characteristic of erythrocytes containing schizonts and trophozoites of *P. falciparum*; and *m*, Mauer's clefts (×20,000).

tron microscopy, blood cell pellets were fixed in 5 percent glutaraldehyde in phosphate buffer (0.067*M*). The cells were fixed for 12 hours and then transferred to Dalton's fixative (*8*) for 90 minutes. The samples were dehydrated in a series of graded alcohols. Ultrathin sections were obtained with diamond knives in a Porter-Blum ultramicrotome. The electron micrographs were taken in an Elmiskop IA electron microscope.

Figure 1 shows a typical result obtained with *P. falciparum*-infected AS erythrocytes. Sickling of red cells containing small parasites occurred so very rapidly that an accurate estimate of the time needed to sickle 50 percent (T_{50}) of the cells is not possible by this technique. The T_{50} appears to be less than 1 minute ($N = 5$). In contrast, uninfected cells from the same culture sickled more slowly so that a mean T_{50} of 7.1 minutes (range 5.0 to 9.3 minutes) ($N = 10$) was obtained. The cells containing large parasites appeared by light microscopy to sickle so slowly that no T_{50} could be determined. Therefore, when data for both large and small parasites were combined, a T_{50} of 24.8 minutes (range 14 to 45 minutes) ($N = 10$) was obtained. When a portion of cells was carried through the conditions of the culture but without infection, the sickling curve resembled that of the uninfected cells very closely (data not shown). When the sickling rates of cells with small parasites are compared to the rates in uninfected cells, the results show approximately an eightfold faster sickling rate for infected cells, which agrees well with the estimates of Luzzatto *et al.* (*4*).

The intraerythrocytic phase of growth of *P. falciparum* begins with a parasite that is small in relation to the size of the red cell. These red cells continue to circulate and represent the forms observed by Luzzatto *et al.* (*4*) in blood obtained by venipunctures from infected AS patients. The larger forms no longer circulate and are sequestered in the capillaries. All forms are seen in synchronous growing cultures. The presence of large parasite forms represents one of the differences between our study and that of Luzzatto *et al.* Despite our failure to observe a high degree of morphological sickling by light microscopy, the electron micrograph (Fig. 2) of a deoxygenated AS cell containing a large parasite clearly shows the presence of polymerized hemoglobin. These polymers are found with a frequency higher than that suggested by the morphological changes observed by light microscopy. We conclude that light microscopy criteria for sickling alone may be misleading under conditions where the membrane of the cell is not free to deform. In previous studies (*4*), total deoxygenation was accomplished with sodium metabisulfite or dithionite. This method raises the question of potential artifacts introduced by the lowering of *p*H which is characteristic of dithionite. The reduction in *p*H could affect infected and uninfected cells in a quantitatively different manner. To remove this possibility, we experimented with red cells deoxygenated with N_2 alone. Red cells were equilibrated at several different levels of oxygen saturation (Table 1). At each percentage of oxyhemoglobin, the extent of sickling in red cells containing small parasites always exceeded the extent of sickling of uninfected cells. This difference was greater at higher oxygen saturations and tended to disappear as total deoxygenation was approached. In addition to answering negatively the question of potential artifacts derived from chemical deoxygenation, this study demonstrates that under conditions which are likely to obtain in vivo, the enhanced sickling propensity of infected red cells is apparent. Hence, the physiological significance of this finding is now clearly established.

Table 1. The effect of different oxygen saturations on sickling of AS red cells infected with *Plasmodium falciparum*. The red cells with a 10 to 15 percent parasitemia were suspended in isotonic phosphate-buffered saline, *p*H 7.35, with 10 m*M* glucose and partially deoxygenated to a fixed oxygen saturation and maintained for 3 minutes before fixation in buffered formalin. After Giemsa staining, the percentages of sickled and deformed cells were evaluated for both infected and uninfected red cells. Results are expressed as the percentage of sickled and deformed cells in a 200-cell count.

Oxygen saturation (% oxyhemoglobin)	Sickling of uninfected red cells (%)	Sickling of red cells with small parasites (%)	Sickling of red cells with large parasites (%)
	Patient No. 1		
49	44	84	24
31	54	87	25
20	67	95	32
1	89	96	39
	Patient No. 2		
48.5	62	90	23
29.5	62	91	31
18.5	84	98	40
0	92	98	38

The results of this study support the conclusion of Luzzatto *et al.* (*4*) that sickling is enhanced by the presence of the *P. falciparum* parasite under circumstances likely to occur in vivo. A lowering of the intracellular *p*H or an increase in the mean corpuscular hemoglobin concentration of red cells could account for this effect. While growth and multiplication of the parasite are normal in AS cells when they are fully oxygenated, death results from prolonged sickling (*5*). This condition might obtain during sequestration of parasitized cells, particularly cells with large parasites, in a low-oxygen environment. It is not clear which mechanism—increased sickling of cells with small parasites or disrupted parasite growth in sickled cells—is the predominant factor that enhances fitness in individuals heterozygous for Hb S. Our studies lend support to the concept that heterozygotes for Hb S in a malarious region may have improved fitness for survival which, in turn, maintains a balanced polymorphism for the Hb S gene.

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