Considering all of the pitfalls that can be encountered in the determination of transport parameters, the existence and significance of carrier-mediated choline transport at physiological concentrations in infected erythrocytes must be seriously considered [4]. Moreover, the experiments of Kirk *et al.* [3] and (from the same group) Elford *et al.* [19] also revealed that the straight lines drawn for the infected cell uptake rate as a function of high choline concentrations did not pass through the origin (see Fig. 2 in [3] and Fig. 1 in [19]). In fact, the line intercepted the ordinate, thus indicating the presence of a different relationship between the external concentration and the uptake rate at low choline concentrations, as demonstrated by Askari [1]. Indeed, it is important that the concentration range should extend on either side of the K_m value. If only concentrations above K_m are used, the K_m value is unreliable [15].

In summary, the study of Kirk *et al.* (1992) concerning cell aging, although consistent, does not account for the discrepancies in V_{max} of choline influx between *P. falciparum*- and *P. knowlesi*-infected erythrocytes. Indeed, our preparation showed 95–100 % infected erythrocytes and conditions were such that parasites were essentially present in mature cells and not in reticulocytes. Controls were uninfected erythrocytes from the same infected monkey as well as noninfected erythrocytes from a healthy monkey, treated under the same conditions, since both possessed the same kinetic characteristics [4]. Nevertheless, these results are very compatible with our hypothesis [4], suggesting that choline influx in immature red blood cells could be higher than in mature erythrocytes due to a higher number of active choline carriers. In this context, the large increase in V_{max} after parasite invasion could have resulted from demasking of remnant sites which would have been masked during maturation of the normal erythrocyte.

In our view, in the experiments of Kirk *et al.* [3], facilitated diffusion was superseded by passive diffusion that has been increased due to experimental conditions. In our experiments using fresh erythrocytes, passive diffusion was also detected (our unpublished work) but at a low level, as also noted by Askari [1]. The reasons for this discrepancy could be due to the use of cultured erythrocytes, the cationic imbalance, the means of stopping the influx reaction and the absence of any evaluation of the nonspecific part. All of these parameters were indeed shown to greatly modify the transport kinetic characteristics which led to erroneous interpretations, with a notable increased evaluation of the non-saturable component in various transport systems. Caution must also be taken for flux determinations in malaria-infected erythrocytes due to the fragility of infected erythrocytes. Hence, when discrepancies are observed, the first question should be: are the experimental conditions as close as possible to physiological conditions and are controls adequately treated?

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Effect of mild oxidants on glycolysis in human erythrocytes

In a recent article [1], Harrison *et al.* reported that mild oxidants such as potassium ferricyanide, diamide and hydrogen peroxide elevated glycolytic rates in human erythrocytes. They suggested that the oxidants stimulated the tyrosine phosphorylation of the glyceraldehyde-3-phosphate-dehydrogenase-binding site on band 3 protein and thereby activated the enzyme through blockage of its inhibitory interaction with the plasma membrane.

Their paper reported that ferricyanide (2 mM), diamide (25 μ M), and hydrogen peroxide (1 mM) enhanced erythrocyte lactate production by 75 %, 54 %, and 88 %, respectively. For the assay of glycolysis, Harrison *et al.* preincubated washed erythrocytes at 37 °C for 1 h in Ringer's buffer (pH 7.4) containing 5 mM-glucose and 30 mM-Hepes, incubated the cell suspension (50 % haematocrit) in the presence or absence of oxidant for 10 min, stopped the incubation by adding HClO_4 , and determined the amount of lactate. We examined the effect of the oxidants on erythrocyte glycolysis under identical conditions. Since it was not clear in their paper whether an oxidant was present or not during the preincubation period, we performed the experiments both in the presence and absence of oxidant during the preincubation period. Whether the preincubation was performed in the presence or absence of oxidant, diamide and hydrogen peroxide only slightly increased erythrocyte lactate production, whereas ferricyanide rather decreased it by about 20 % (Table 1). Furthermore, the paper in question stated that other workers [2,3] had also reported an increase in erythrocyte glycolysis by ferricyanide. There are, however, no such data in [2] and [3].

Harrison *et al.* [1] also examined the changes in the concentrations of various glycolytic intermediates in erythrocytes as a function of exposure to ferricyanide to determine which steps in glycolysis are affected by the treatment. They described that intermediates (glucose, glucose 6-phosphate, and glyceraldehyde 3-phosphate) preceding the glyceraldehyde-3-phosphate dehydrogenase step in glycolysis were severely decreased in erythrocytes treated with 0.4 mM-ferricyanide. Since

Table 1. Effect of mild oxidants on erythrocyte lactate production

Washed human erythrocytes were preincubated at 37 °C for 1 h in the presence or absence of oxidant in the medium described in [1]. After preincubation, the cells were washed once with the same medium warmed to 37 °C, resuspended to a haematocrit value of 50 %, and then incubated for 10 min in the presence or absence of oxidant. In the control experiment, no oxidant was present in either the preincubation or incubation period. After incubation, 4 ml of ice-cold 1 M-HClO₄ was added to each of the suspensions (2 ml). The supernatants obtained by centrifugation were neutralized with 3 M-K₂CO₃ and again centrifuged. Lactate in neutral supernatants was assayed as described previously [5]. Values are expressed as means $\pm$ S.D. with the number of experiments in parentheses. The control values in experiments in which the cells were preincubated with and without oxidant were 99.1 and 98.3 nmol/10 min per 100 mg of haemoglobin, respectively. **P* < 0.001, †*P* < 0.02 versus control by Student's *t* test.

Condition	Lactate production (% of control)	
	Preincubation with oxidant	Preincubation without oxidant
Control	100 $\pm$ 4.6 (9)	100 $\pm$ 3.6 (6)
Ferricyanide (2 mM)	83.4 $\pm$ 4.4* (8)	80.9 $\pm$ 4.6* (6)
Diamide (25 μ M)	107.4 $\pm$ 5.4† (7)	109.5 $\pm$ 3.5† (6)
Hydrogen peroxide (1 mM)	115.2 $\pm$ 3.2* (6)	116.3 $\pm$ 3.8* (6)

glucose transport into human erythrocytes is about 250 times faster than its utilization [4], the intracellular glucose concentration is unlikely to change appreciably even if glucose utilization is markedly altered. It seems, therefore, that at least a part of their data is unreliable. As far as we analysed the levels of glycolytic intermediates, the concentrations of glucose, glucose 6-phosphate, and fructose 6-phosphate were not affected at all by incubation of erythrocytes in the presence of 2 mM-ferricyanide for 10 min.

Another inscrutable riddle is that the amount of lactate production is quite different between their and our experiments. From the data in Table II of [1], the control value of lactate production can be calculated to be 6.76 μ mol/10 min per 100 mg of haemoglobin. In our experiments, however, the corresponding value is 0.10 μ mol/10 min per 100 mg of haemoglobin.

We are compelled to question the validity of the experiments on erythrocyte glycolysis in [1]. Those experiments are crucial for the authors to deduce their conclusions. The controversy discussed here will be cleared up in the future, but at present it seems necessary to carefully assess their conclusions.

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The effect of mild oxidants on glycolysis in human erythrocytes

In the preceding article, Miwa *et al.* [1] question the validity of the experiments we reported in our paper [2]. In particular, they report an inability to repeat our finding that the mild oxidants, ferricyanide, diamide and hydrogen peroxide increase glycolysis in red blood cells. Moreover, they state that we incorrectly cited two papers [4,5] that previously reported that ferricyanide increased glycolysis. Additionally, they state that it was unclear from our paper whether mild oxidants were present during the 1 h preincubation period.

Regarding the last relatively trivial point, we state in the Materials and methods section that red cells were incubated for 10 min with the exogenous agents (mild oxidants) after a 1 h preincubation in Ringer's buffer. Now, articles [4] and [5] do indeed report an increase in intracellular ATP synthesis as a result of ferricyanide treatment. This has also been reported by Manyai & Szekely [6]. Since glycolysis is the only known mechanism for ATP synthesis in the red cell [7], a reasonable interpretation of these data is that ferricyanide stimulates red cell glycolysis. In fact, both papers [4,5] discuss the possibility that ferricyanide is acting by increasing the activity of glyceraldehyde-3-phosphate dehydrogenase. This hypothesis is repeated in our paper [2], but we did not wish to imply that we were proposing an original idea, since in fact this possibility had been publicly presented by others.

The reason for the failure of Miwa *et al.* [1] to obtain an increase in lactate content as a result of treating red cells with oxidants is unclear. A possible explanation is that they used fresh blood (the source of their blood is not reported), and as clearly stated in our paper, our experiments were performed on stored blood purchased from a local blood bank. In our hands, ferricyanide showed a donor-dependent effect on glycolytic rates in fresh blood, repeatedly enhancing glycolysis in blood from certain donors but reproducibly exerting no effect on blood from others. Further, we now believe that the elevation of glycolysis induced by ferricyanide is a phenomenon seen only under conditions where glyceraldehyde-3-phosphate dehydrogenase is rate limiting. Such conditions arise, for example, when the intracellular pH increases slightly [5] or when ribose-containing compounds such as inosine are used as substrates. The studies we reported were conducted between January 1989 and April 1990 and were repeated thirteen times in an effort to refine solution conditions. In all thirteen studies, treatment with the mild oxidants increased lactate production. However, we have also observed recently that some stored blood samples are not stimulated by mild oxidants. In these instances, the storage medium for the blood was changed. We are currently assessing the various agents used in collecting and storing blood for their ability to promote the stimulation of glycolysis by ferricyanide. In this regard, we have found that inosine, a metabolite added to blood packs during the period of the experiments in question, restores ferricyanide stimulation of glycolysis to most of the blood samples that do not otherwise respond. We should also mention that in blood packs where ferricyanide fails to stimulate glycolysis, it also fails to stimulate band 3 tyrosine phosphorylation, which is totally consistent with the major conclusion of our paper.

Miwa *et al.* [1] further assert that our data are unreliable because we report that glucose levels are significantly lowered in cells treated for 10 min with ferricyanide in comparison with untreated cells. They argue that glucose transport into cells would compensate for any increase in glucose utilization and they cite a paper [3] reporting that glucose transport in erythro-