Mons, B. (1990) Blood Cells 16, 299–312
Wolowyk, M. (1982) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 1–11, Academic Press, London and New York
Uney, J. B., Marchbanks, R. M. & Marsh, A. (1985) J. Neurol. Neurosurg. Psych. 48, 229–233
Tucker, E. M. & Young, J. D. (1980) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 31–41, Academic Press, London and New York

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Saturable and non-saturable components of choline transport in *Plasmodium*-infected mammalian erythrocytes: possible role of experimental conditions

Studies on choline transport in normal erythrocytes were initiated by Askari [1]. He revealed the presence of two distinct mechanisms for the uptake of choline in normal erythrocytes: a facilitated uptake mechanism, which becomes saturated at low external choline concentration (K_m 20 μM), and a simple diffusion mechanism in which the uptake rate is proportional to the concentration detected at very high concentrations (in the millimolar range). Thorough studies were then carried out which led to characterization of the saturable component. It is mediated by an asymmetric carrier operating according to a cyclic model whose limiting step is the re-orientation of the free carrier inside the membrane (for review see [2]).

Until recently little attention has been paid to choline transport across the membrane of malaria-infected erythrocytes. However, two papers were published in 1991 which led to different conclusions concerning the nature of choline transport after malaria infection. Kirk *et al.* [3] found that human erythrocytes infected *in vitro* with *Plasmodium falciparum* incorporated choline mainly via a component differing kinetically and pharmacologically from the endogenous transporter and which did not saturate at choline concentrations up to 0.5 mM, whereas only the saturable component was observed in normal cultured erythrocytes (K_m 11 μM). By contrast, Ancelin *et al.* [4] found that *P. knowlesi*-infected simian erythrocytes showed an increased rate of choline transport via a pathway with the same kinetic properties as the endogenous choline transporter (K_m 9 μM), except that the $V_{max.}$ was more than 10-fold higher than that of uninfected erythrocytes. Non-saturable permeability pathways were not detectable at physiological choline concentration, *i.e.* up to 24 μM. In fact, we observed a non-saturable component both for infected and uninfected erythrocytes detected from 1.2 to 1.7 mM (our unpublished work), that is about 50–100-fold the physiological concentrations [5].

Considering that the transport measurements in the two studies were carried out using essentially similar techniques, Kirk *et al.* (1992) (the preceding Letter) investigated a possible effect of cell age on normal red blood cell choline transport and showed that the $V_{max.}$ for choline influx decreased with cell age. Similar results have already been demonstrated for other transport systems such as nucleosides [6,7], amino acids and sugars [8]. Nevertheless, Kirk *et al.* (1992) attempt to benefit from this difference by suggesting that the wide difference in the $V_{max.}$ we observed in cells from malaria-infected and uninfected monkeys might have been due to reticulocyte contaminants in our parasite preparation.

The presence of reticulocytes can be ruled out. First, monkey splenectomies were performed 4–7 years before and could not reasonably have been involved in an increased proportion of circulating reticulocytes. Secondly, in parasites like *P. knowlesi* there is not any real restriction for reticulocytes as observed in *P. vivax* ([9] and B. Mons, personal communication). Furthermore, since reticulocytes comprise only 1–3 % of the total red blood cell population at any given moment, most rings of the non-restricted parasites will end up in mature cells (B. Mons, personal communication). In our experiments, parasitaemia was 10–30 % (late stages) on days 6–9. A simple calculation reveals that the majority of these parasites cannot conceptually be anywhere else than in mature cells. Lastly, we never observed induced reticulocytosis during infection (our observations) and reticulocyte-restricted parasites can never provide such fulminating parasitaemia without first inducing reticulocytosis (B. Mons, personal communication). The last possibility involving contamination by uninfected reticulocytes of the Percoll parasite-enriched fraction can be easily ruled out since smears always showed parasitaemia of more than 95 %, which seriously reduces the possibility of any reticulocyte involvement in the 10-fold increase in $V_{max.}$ of choline influx in the *P. knowlesi*-enriched fraction.

On the other hand, several technical differences in the transport measurements between the two studies should be detailed. They notably concern the method of obtaining infected and uninfected erythrocytes, the composition of washing, incubation and stopping media, the mode of stopping the influx reaction, and nonspecific determination.

Concerning the *P. falciparum* measurements [3], human erythrocytes were sorted for 1–5 days before being infected by *P. falciparum*, then maintained in culture, synchronized and later enriched (parasitaemia 46–93 %). Noninfected control cells were obtained from the same donor and cultured at the same time. The culture medium consisted of RPMI 1640, whose choline concentration is 21 μM [10], plus 10 % human serum. The washing and incubation media were phosphate- and Hepes-buffered saline and the medium for stopping the reaction was Mops-buffered $MgCl_2$ solution.

Concerning the *P. knowlesi* experiments [4], fresh *P. knowlesi*-infected simian erythrocyte suspensions and noninfected erythrocytes from a healthy monkey were removed from the leukocyte contaminants by using a cellulose-powder column. The highly enriched parasite fractions (parasitaemia > 95 %) were separated from uninfected erythrocytes by using a Percoll gradient. All fractions (containing infected and uninfected cells from the same monkey, or noninfected cells from a healthy monkey) were then treated in parallel, under the same conditions, i.e. extensively washed (for 2–3 h) with large volumes of choline-free RPMI 1640. This notably excludes any possible transactivation by the few uninfected cells present in the parasitized preparation in contributing to the $V_{max.}$ increase, as suggested by Kirk *et al.* for whom preincubation at 37 °C for 2 h, in phosphate-buffered saline supplemented with 14 mM-Hepes and 10 mM-glucose, was sufficient to deplete cells of endogenous choline (see Fig. 1 of Kirk *et al.*, 1992). Choline-free RPMI 1640 was also used for the choline influx measurements and to stop the reaction.

Blood storage preservation and culture conditions have been reported to be responsible for various modifications of several transport systems. Thus, the nucleoside transporter has kinetic properties consistent with a symmetrical carrier mechanism in fresh erythrocytes, but is asymmetrical in erythrocytes from outdated blood [11,12]. The difference between human and guinea pig erythrocytes in the degree of mobility of the empty and uridine-loaded carrier is highly accentuated during blood storage [13]. Storage of red cells in blood bank conditions or cultivation of the cells for 4–5 days in medium required for the propagation of *P. falciparum* parasites were demonstrated to increase (up to 5-fold) the non-saturable component of tryptophan transport (L system), and also the saturable component (T system) without any modification of K_m. Culture conditions

also reduce the susceptibility of these transport systems to nonspecific inhibitors while virtually unaltering the effect of competitive inhibitors [14].

Another major point should be mentioned concerning the cationic composition of the various media used in the studies. Askari [1] and Ancelin *et al.* [4] used fresh blood cells, and the same medium for washings, incubations or stopping reactions with similar physiological cationic concentrations, whereas Kirk *et al.* [3] used three quite different media (Table 1). Hence, after cultivation a significant depletion in Ca^{2+} and Mg^{2+} was induced, and the transfer of cells to stop the influx reaction was accompanied by a very high depletion of Na^{+} and K^{+} ($\sim 85\,\%$), whereas the Mg^{2+} concentration was greatly and unexplainably increased (from 0 to 91 mM). The extracellular cation concentration can affect the transfer rate of various substrates, with alterations in the K_m values [15]. Martin showed that cation gradients can determine whether erythrocytes accumulate or extrude choline [16]. Choline accumulation is increased when external Na^{+} is replaced by Mg^{2+} due to a very rapid (within a matter of minutes) and marked decrease of choline efflux. Modifying the extracellular concentrations of K^{+} and Na^{+} to concentrations close to those in the intracellular water resulted in choline being passively distributed across the cell membrane [16]. Finally, changes in the ionic balance of the external medium is one of the processes (along with temperature reduction or metabolic inhibitors) that can be used to eliminate the saturable component (carrier-mediated) when measuring only the linear uptake component [15]. If, moreover, permeability to the cations differs according to particular conditions (fresh blood or cultured cells) and between infected and normal erythrocytes, it would also cause additional choline movement differences in each cell type.

Finally, at the end of the incubation period the cells were treated differently according to whether they were infected or not [3]. Infected cell suspensions were layered over dibutyl phthalate then centrifuged, lysed, precipitated with trichloroacetic acid and finally counted for radioactivity, whereas noninfected cells were washed four times before being processed for scintillation counting. Under these extensive washing conditions in a choline-free medium, part of the choline that could have entered the erythrocyte would be able to leave the cell, even at 4 °C (our observations).

These points needed to be emphasized because when alterations of transport functions are investigated, one must be aware of changes which might occur due to mere exposure of the cells to *in vitro* culture conditions or to modifications in the cationic composition of the medium. Kirk *et al.* [3] admit that the relative magnitudes of the malaria-induced choline fluxes which they observed were highly variable between cell cultures and they also suggested possible variations in specific properties of cells from different donors.

In addition, certain considerations concerning facilitated and nonfacilitated diffusion should also be detailed. In many transport processes passive diffusion and carrier mediated transport may occur together, especially detectable at high substrate concentrations. It is crucial to be able to correct for this when accurate studies on carrier mediated transport are required [17,18]. Conversely, when diffusion is studied, the saturable component should be eliminated, usually by working at substrate concentrations sufficiently high to saturate any saturable pathway present. For the saturable component, the kinetic parameters can be evaluated after determining the time at which initial rate conditions are observed. The validity of this measurement is also based on the assumption that the amount of substrate which has accumulated in the cell is small enough for outward transfer to be ignored [15]. This is why our preliminary experiments concerning time courses for choline influx were carried out at 0.9 μM-choline concentration, for which the contribution of any non-saturable transport pathway to the total influx is very small. In no case, as claimed by Kirk *et al.* (1992), were these results used to rule out the presence of a new malaria permeability pathway, but rather to determine initial rate conditions. Incidentally, Kirk *et al.* (1992) also used a low choline concentration (2 μM) to verify such linearity over time. The experiments in which we demonstrate the carrier-mediated component of choline influx in infected cells concerned: temperature-dependence, saturability, usually over 0–24 μM-choline, complete inhibition by specific pharmacological agents based on analogy to choline (dimethylethanolamine, decamethonium and dodecyltrimethylammonium), irreversible inactivation by the thiol reagent *N*-ethylmaleimide and transactivation by ethanolamine [4].

In normal erythrocytes, for choline concentrations that are assumed to be physiological, a carrier-mediated transport process with saturation kinetics predominates [1]. At much higher concentrations the transport is increasingly superseded by simple diffusion. The nonfacilitated influx rate constant found by Askari [1] was $k = 0.003\ h^{-1}$, i.e. 4.5×10^{-5} nmol/min per μM of external choline per ml of cells, as described by Domin & Mahory [18]. At physiological choline concentrations, nonfacilitated diffusion is thus estimated to contribute less than 1 % of the total influx rate of choline, (when compared with $V_{max.} = 0.23$ nmol/min per ml of cells). Under our conditions, the contribution of non-carrier-mediated choline uptake (estimated by the addition of 1.2 mM-choline) to our measurement of basal uptake was also low (usually less than 8 %). In the experiments of Kirk *et al.* [3], no details were given concerning the nonspecific evaluation.

Table 1. Cationic composition of the various media used for choline influx measurements

The values were obtained from data published by Askari [1], Ancelin *et al.* [4] and Kirk *et al.* [3].

Medium for	[Cations] (mM)				Reference
	Na^{+}	K^{+}	Mg^{2+}	Ca^{2+}	
Washing, incubation and stopping	145	5.3	1.3	1.3	[1]
Washing, incubation and stopping	138	5.4	0.4	0.4	[4]
Culture*	138	5.4	0.4	0.4	[3]
Washing and incubation	150	4.2	0	0	[3]
Stopping†	0 (23)	0 (0.6)	107 (90.6)	0 (0)	[3]

* Corresponds to RPMI medium without taking into account the 10 % serum supplement.

† 800 μl of the stopping medium was added to 145 μl of incubation medium to stop the reaction. The final ionic concentration is noted in parentheses.

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 erronous 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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1. Askari, A. (1966) J. Gen. Physiol. **49**, 1147–1160
2. Krupka, R. M. & Devés, R. (1986) Biochem. Cell Biol. **64**, 1099–1107
3. Kirk, K., Wong, H. Y., Elford, B. C., Newbold, C. I. & Ellory, J. C. (1991) Biochem. J. **278**, 521–525
4. Ancelin, M. L., Parant, M., Thuet, M., Philippot, J. R. & Vial, H. J. (1991) Biochem. J. **273**, 701–709
5. Das, J., De Belleroche, J., Moore, C. J. & Rose, F. C. (1986) Anal. Biochem. **152**, 178–182
6. Blostein, R. & Grafova, E. (1987) Biochem. Cell. Biol. **65**, 869–875
7. Jarvis, S. M. & Young, J. D. (1982) J. Physiol. (London) **324**, 47–66
8. Tucker, E. M. & Young, J. D. (1982) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 31–41, Academic Press, London and New York
9. Mons, B. (1990) Blood Cells **16**, 299–312
10. Moore, G. E., Gerner, R. E. & Franklin, H. A. (1967) J. Am. Med. Assoc. **199**, 87–92
11. Jarvis, S. M., Hammond, J. R., Paterson, A. R. P. & Clanachan, A. S. (1983) Biochem. J. **210**, 457–461
12. Plagemann, P. G. W. & Wohlhueter, R. M. (1984) Biochim. Biophys. Acta **778**, 176–184
13. Jarvis, S. M. (1988) in Adenosine Receptors, pp. 113–123, Alan R. Liss, Inc.
14. Ginsburg, G. & Krugliak, M. (1983) Biochim. Biophys. Acta **729**, 97–103
15. Neame, K. D. & Richards, T. G. (1972) in Elementary Kinetics of Membrane Carrier Transport, Blackwell Scientific Publications, Oxford
16. Martin, K. (1972) J. Gen. Physiol. **224**, 207–230
17. Halestrap, A. P. & McGivan, J. D. (1979) Techniques Metabol. Res. **B206**, 1–23
18. Domin, B. A. & Mahory, W. B. (1988) J. Biol. Chem. **263**, 9276–9284
19. Elford, B. C., Pinches, R. A., Newbold, C. I. & Ellory, J. C. (1990) Blood Cells **16**, 433–436

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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 μM), 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