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.

Ichitomo MIWA, Hideo FUKATSU and Jun OKUDA

Department of Clinical Biochemistry, Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, Japan

1. Harrison, M. L., Rathinavelu, P., Arese, P., Geahlen, R. L. & Low, P. S. (1991) *J. Biol. Chem.* **266**, 4106–4111
2. Mishra, R. K. & Passow, H. (1969) *J. Membr. Biol.* **1**, 214–224
3. Arese, P., Bosia, A., Pescarmona, G. P. & Till, U. (1977) *Acta Biol. Med. Germ.* **36**, 481–490
4. Elbrink, J. & Bihler, I. (1975) *Science* **188**, 1177–1184
5. Miwa, I., Fukatsu, H., Toyoda, Y. & Okuda, J. (1990) *Biochem. Biophys. Res. Commun.* **173**, 201–207

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-

cytes is 250 times faster than its utilization. Reference [3] does indeed state that glucose transport in erythrocytes exceeds glucose utilization, and they cite [8] as a source of this information. However, the studies in [8] only measured glucose penetration in fresh blood while another paper was cited regarding the rate of glucose utilization. The authors in [8] state that glucose permeation depended on such variables as pH and temperature, and they cite another study which reported that the glucose permeation rate dropped in cell suspensions kept at 20 °C. How all these previous studies relate to blood stored at 4 °C for several days to a few weeks and assayed under completely different conditions is totally unclear. Our data would suggest that in stored blood assayed under our conditions [2], glucose transport cannot compensate for the rapid depletion of glucose caused by incubating red cells with ferricyanide over a 10 min period. In addition, findings similar to those we reported (Fig. 4 of [2]) regarding the effect of ferricyanide on the concentration of the various intermediates in glycolysis have been previously reported [9]. Furthermore, one of us (P. Arese, unpublished work) has also observed that diamide causes a similar change in the concentrations of the various glycolytic intermediates, except that in fresh blood no net lactate production was observed.

Finally, the apparent discrepancy between Miwa *et al.* and ourselves concerning the amount of lactate production observed in control cells may be reconciled by two observations. First, as mentioned above, our studies were conducted on stored blood. Depending on the length of storage in high-phosphate, glucose- and inosine-containing medium, erythrocytes will conduct glycolysis at vastly different rates. This observation is mentioned as a footnote in our paper [2]. Second, the data presented in Tables II–IV of [2] report the total concentration of lactate at the end of the incubation period, and not the rate of lactate production during the incubation. Because prior to oxidant stimulation all cells within an experiment contained the same lactate concentration, the increases in lactate concentration at the end of the 10 min stimulation period were interpreted to reflect the stimulation of lactate production by the oxidants.

In conclusion, we would like to state that the data we reported [2] are correct. As discussed above, related observations have been reported by others [4–6]. We believe that the failure of Miwa *et al.* [1] to repeat our observations may reflect differences in the age or storage conditions of the red cells.

Marietta L. HARRISON*, Prema RATHINAVELU†, Paolo ARESE‡, Robert L. GEAHLEN* and Philip S. LOW†

* Department of Medicinal Chemistry and Pharmacognosy and the † Department of Chemistry, Purdue University, West Lafayette, IN 47907, U.S.A. and ‡ Department of Genetics, Biology and Medicinal Chemistry, University of Turin, Turin, Italy

1. Miwa, I., Fukatsu, H. & Okuda, J. (1992) *Biochem. J.* **283**, 621–622
2. Harrison, M. L., Rathinavelu, P., Arese, P., Geahlen, R. L. & Low, P. S. (1991) *J. Biol. Chem.* **266**, 4106–4111
3. Elbrink, J. & Bihler, I. (1975) *Science* **188**, 1177–1184
4. Mishra, R. K. & Passow, H. (1969) *J. Membr. Biol.* **1**, 214–224
5. Arese, P., Bosia, G. P., Pescarmona, G. P. & Till, U. (1977) *Acta Biol. Med. Germ.* **36**, 481–490
6. Manyai, S. & Szekely, M. (1954) *Acta Physiol. Acad. Sci. Hung.* **5**, 7–18
7. Magnani, M., Rossi, L., Cucchiari, L., Stocchi, V. & Fornaini, G. (1988) *Cell Biochem. Function* **6**, 175–182
8. Widdas, W. F. (1954) *J. Physiol. (London)* **125**, 163
9. Arese, P., Bosia, A. & Pescarmona, G. P. (1975) VII. Internationales Symposium über Struktur und Funktion der Erythrozyten (Rapport, S. & Jung, F., eds.), pp. 91–102, Akademie Verlag, Berlin

The error in the Michaelis–Menten equation when substrate depletion by binding to the enzyme is not taken into account

In deriving equations for interpreting equilibrium binding or enzymic assays it is convenient and often justified to neglect depletion of the substrate by binding to the enzyme. This leads to considerable simplification of the final formulae. The present letter provides a handy rule for deciding whether the approximation is justified. Consider enzymic mono-substrate conversion according to the mechanism:

where A, E, Q, EA and EQ are the substrate, enzyme, product, enzyme–substrate and enzyme–product complex, respectively. In the absence of product, the observed relative reaction rate r is given by:

$$r = v/V = u/(K_m + u) \quad (2)$$

where v and V are the initial and maximum reaction rate, respectively, u is the concentration of unbound substrate and K_m the Michaelis constant. The substrate-concentration balance is:

$$A_t = u + E_t \cdot u/(K_m + u) \quad (3)$$

where the terms on the right-hand side represent the unbound and enzyme-bound substrate, respectively, and A_t and E_t are the total concentrations of substrate and enzyme, respectively. Methods are available for determining the system parameters when the term for enzyme-bound substrate cannot be neglected in the concentration balance [1, 2]. The kinetic equation has been analysed in some detail by Reiner [3].

Neglect of the enzyme-bound substrate in eqn. 3 implies interpretation of the observed reaction rates r in terms of the model:

$$r_{\text{approx.}} = \tilde{u}/(K_m + \tilde{u}) \quad (4)$$

$$\tilde{u} = A_t \quad (5)$$

where $\tilde{u}$ is an approximate value for the total concentration of unbound substrate u . The relative error:

$$\epsilon \equiv (r_{\text{approx.}} - r)/r \quad (6)$$

has been discussed by Cha [4] in terms of a square-root function of the substrate concentration A_t . It can easily be shown that ϵ can either be expressed as a unique function of A_t/K_m and E_t/K_m :

$$\epsilon = F_1(A_t/K_m; E_t/K_m) \quad \text{square-root function} \quad (7)$$

or, equivalently, as:

$$\epsilon = F_2(r; E_t/K_m) \quad (8)$$

For deciding whether assays performed over a suitable range of r values (e.g. $0.1 \leq r \leq 0.9$) can be interpreted by the approximate model or not, the second choice (eqn. 8) is the more practical. The explicit form of eqn. 8 is mathematically simpler than that of eqn. 7. Further, the values of r being available, only an estimate of the ratio E_t/K_m , not of the individual values of E_t and K_m , is needed for evaluating the error.

The function F_2 is obtained as follows: $u = K_m \cdot r/(1-r)$, obtained from eqn. 2, is substituted into eqn. 3 yielding A_t as a function of r . Further making use of eqns. 4 and 5 one obtains $r_{\text{approx.}}$ and, finally, from eqn. 6:

$$\epsilon = (E_t/K_m) \cdot \frac{(1-r)^2}{1 + (E_t/K_m) \cdot (1-r) \cdot r} \quad (9)$$