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OXIDATIVE EFFECTS OF IRON ON ERYTHROCYTES

CATHERINE RICE-EVANS, E. BAYSAL, G.J. KONTOGHIORGHES†, D.M. FLYNN†† and A.V. HOFFBRAND*

Department of Biochemistry, Department of Haematology†, Department of Paediatrics††

Royal Free Hospital and School of Medicine, Rowland Hill Street, London. NW3 2PF.

In this work we have investigated the effects of iron-induced free radical formation in normal human erythrocytes in vitro, as a model system for studying iron damage, and in erythrocytes from patients with β-thalassaemia major. The resulting oxidative effects were measured in terms of methaemoglobin formation and reduced glutathione loss. The effects of desferrioxamine, an iron-chelating agent, were also investigated.

The results show that the increased methaemoglobin formation after iron-induced oxidative stress is consistent with a decline in the intracellular glutathione levels and that this process is inhibited by desferrioxamine. Similar treatment of red cell haemolysates produces less methaemoglobin. This suggests that, on exposure of intact erythrocytes to iron-induced free radical effects, the red cell membrane exacerbates the breakdown of the antioxidant defences of the cell and the oxidation of haemoglobin.

Key words: erythrocytes; thalassaemia; iron; methaemoglobin

INTRODUCTION

The mechanism of iron-induced free radical production may have an important role in pathological conditions such as iron-overload in thalassaemia[1] and excess iron in the synovial fluid of rheumatoid arthritis patients[2]. Free radical formation catalysed by transition metal ions is well-documented according to the Fenton[3] and Haber-Weiss reactions[4].

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + OH^- \text{ FENTON}$$

$$O_2^{\cdot -} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe salt}} O_2 + {\cdot}OH + OH^- \text{ HABER-WEISS}$$

In conditions such as thalassaemia the transferrin-iron saturation in the plasma

may be exceeded and the excess iron may cause free radical damage to cells[5] and tissues[6].

The purpose of this project is to compare a model system for excess iron with its effects on erythrocytes in such a pathological disorder. The free radical effects are monitored by measuring methaemoglobin formation and cellular glutathione oxidation which under normal conditions are prevented by the antioxidant defences of the cell. Ascorbate is included in this model system since it is given as well as desferrioxamine to thalassaemic patients to aid iron excretion[7]. However, it has been reported that ascorbate may enhance tissue damage e.g. deterioration of cardiac function in the patients[8]. Oral ascorbate (200 mg daily) has been shown to double iron excretion in most patients[9]. Normal physiological ascorbate concentrations in the blood of healthy individuals range from 0.006–0.017 mM up to 0.08 mM when the human blood is fully saturated. Ascorbate in blood is normally present in the plasma in the reduced form.

MATERIALS AND METHODS

Fresh human erythrocytes were obtained from normal, healthy donors and from splenectomised patients with β-thalassaemia major. After centrifugation, the plasma and the buffy coat were removed and the red cells washed three times with isotonic phosphate buffer, pH 7.4.

Erythrocytes at a 5% suspension in isotonic buffer were incubated with iron (II) sulphate (100 μM), or iron (III) chloride (100 μM), L-ascorbic acid (Sigma) (1 mM), hydrogen peroxide (200 μM) in the presence and absence of desferrioxamine mesylate (CIBA) (400 μM). Methaemoglobin formation was measured according to the method in[10] involving a spectrophotometric assay at 620 nm. Reduced glutathione levels were determined by the Beutler assay[11] involving the spectrophotometric measurement of complexes with 5, 5′- dithio-bis-(2-nitrobenzoic acid) at 412 nm and using reduced glutathione (Sigma) as standard.

In the experiments involving the haemolysates the original red cells were lysed in 20 volumes of ice-cold distilled water (comparable with the intact cell concentrations) and treated as in the intact cell experiments.

Haemolysis was monitored according to the method of Brownlee *et al*[12].

RESULTS

Exposure of normal human erythrocytes to iron-induced free radical formation involving the 100 μM iron (II)/1 mM ascorbate/200 μM hydrogen peroxide system for varying time intervals induces a progressive increase in the oxidation of haemoglobin to methaemoglobin up to ten hours and this rate continues steadily up to 24 hours (Figure 1). Curve (g) of Figure 1 shows the same profile whether or not the oxidising system includes the 200 μM hydrogen peroxide suggesting that the presence of hydrogen peroxide in these systems makes no additional positive contribution to the oxidative effects on haemoglobin. The iron-chelating agent desferrioxamine (400 μM) delays the formation of methaemoglobin but does not totally inhibit the oxidation. The rate of the decline in the reduced glutathione levels on incubation of the erythrocytes is greatly increased in the presence of the iron/ascorbate/hydrogen

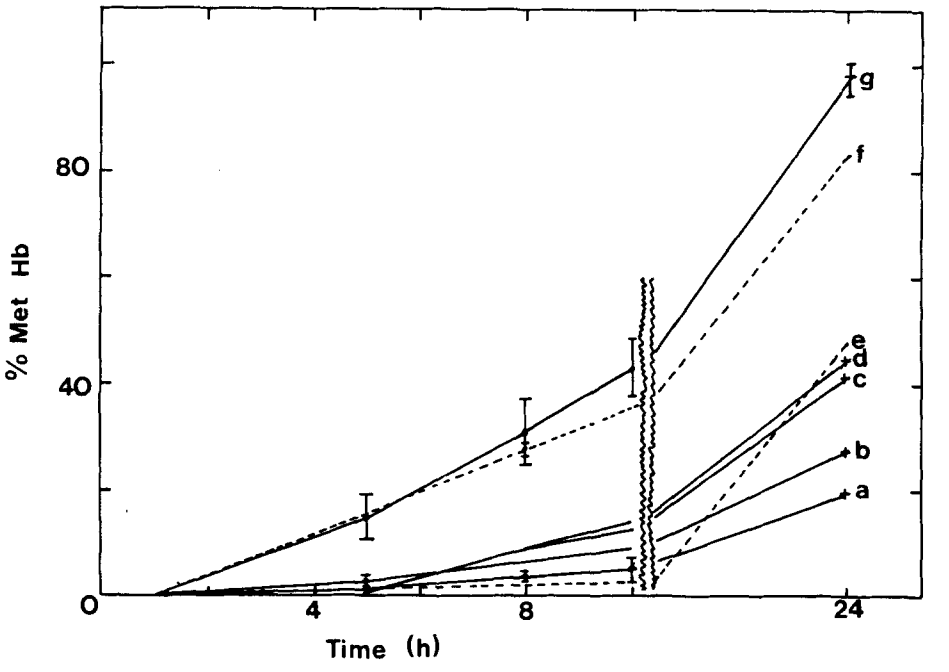

FIGURE 1 The effects of the iron (II)/hydrogen peroxide/ascorbate — free radical system on methaemoglobin production in normal human erythrocytes ± desferrioxamine.

a. control erythrocytes
b. 400 μM desferrioxamine — treatment
c. 100 μM iron (II)/1 mM ascorbate/200 μM hydrogen peroxide/400 μM desferrioxamine — treatment
d. 100 μM iron (II)/1 mM ascorbate/400 μM desferrioxamine — treatment
e. 100 μM iron (III)/1 mM ascorbate/200 μM hydrogen peroxide/400 μM desferrioxamine — treatment
f. 100 μM iron (III)/1 mM ascorbate/ ± 200 μM hydrogen peroxide
g. 100 μM iron (II)/1 mM ascorbate/ ± 200 μM hydrogen peroxide

peroxide system (Figure 2). The half-life of reduced glutathione is ca. 6 hours in control erythrocytes, ca. 2 hours in the iron (II)/ascorbate/hydrogen peroxide system, ca. 3 hours with desferrioxamine included in the experimental system. Desferrioxamine does not totally inhibit the iron-induced free radical effects.

The degree of haemolysis of the intact cells with time of incubation varies little up to a ten-hour incubation in any of these systems (8%) and reaches 12% after a 24 hour treatment.

Iron (III) in this normal erythrocyte model system is comparable with iron (II) in its oxidative effects on haemoglobin (Figure 1).

Treatment of red cell haemolysates under the same conditions as the intact erythrocytes (Figure 3) has no significant effect up to a ten-hour incubation. After 24

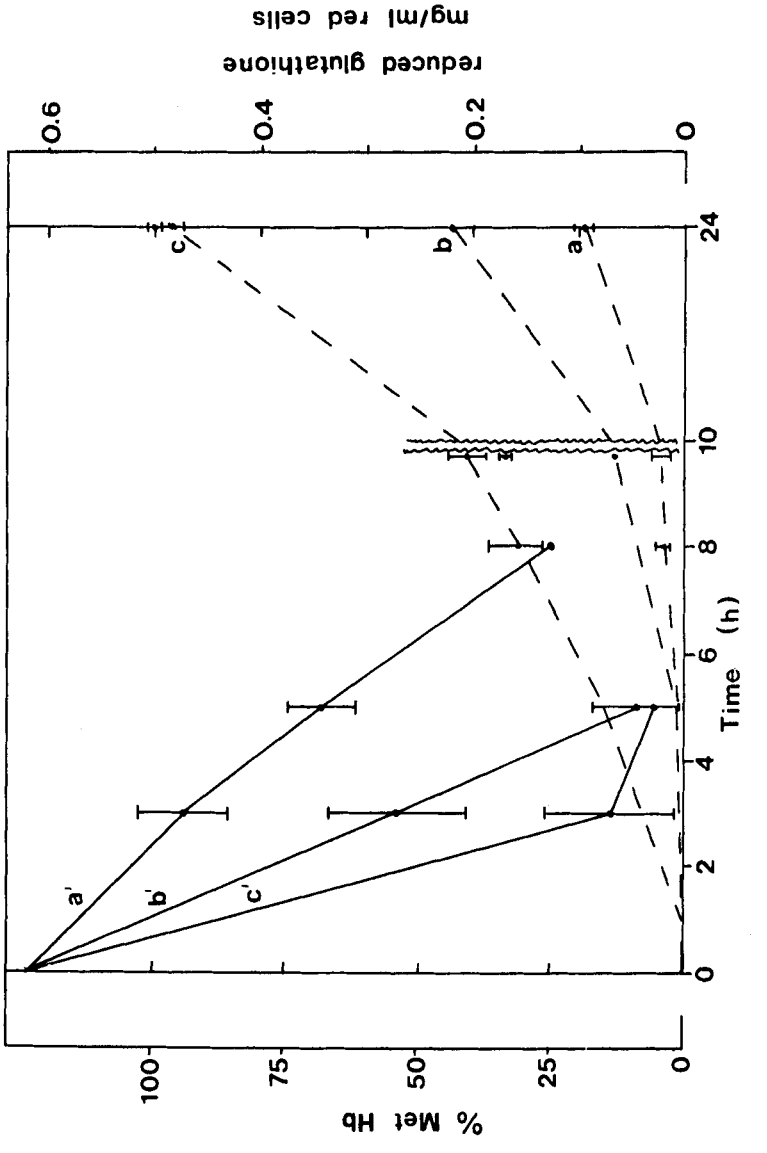

FIGURE 2 The effects of the iron (II)/hydrogen peroxide/ascorbate — free radical system on reduced glutathione (–) and methaemoglobin levels (--) in normal human erythrocytes.

a,a′ control erythrocytes
b,b′ 100 μM iron (II)/1 mM ascorbate/400 μM desferrioxamine — treatment
c,c′ 100 μM iron (II)/1 mM ascorbate/ ± 200 μM hydrogen peroxide — treatment

hours increased oxidation of methaemoglobin occurs in the iron/ascorbate/hydrogen peroxide system, but this is only 30% of that of the intact erythrocyte system (Figure 1). In the presence of desferrioxamine (Figure 4) the effect of the Iron (II) — free radical system on methaemoglobin formation in the haemolysates is not highly significantly different from that in the absence of this iron-chelator.

All these effects in the intact normal erythrocytes are more pronounced in the red cells from some patients with β-thalassaemia major (Figure 5) in that a higher level of

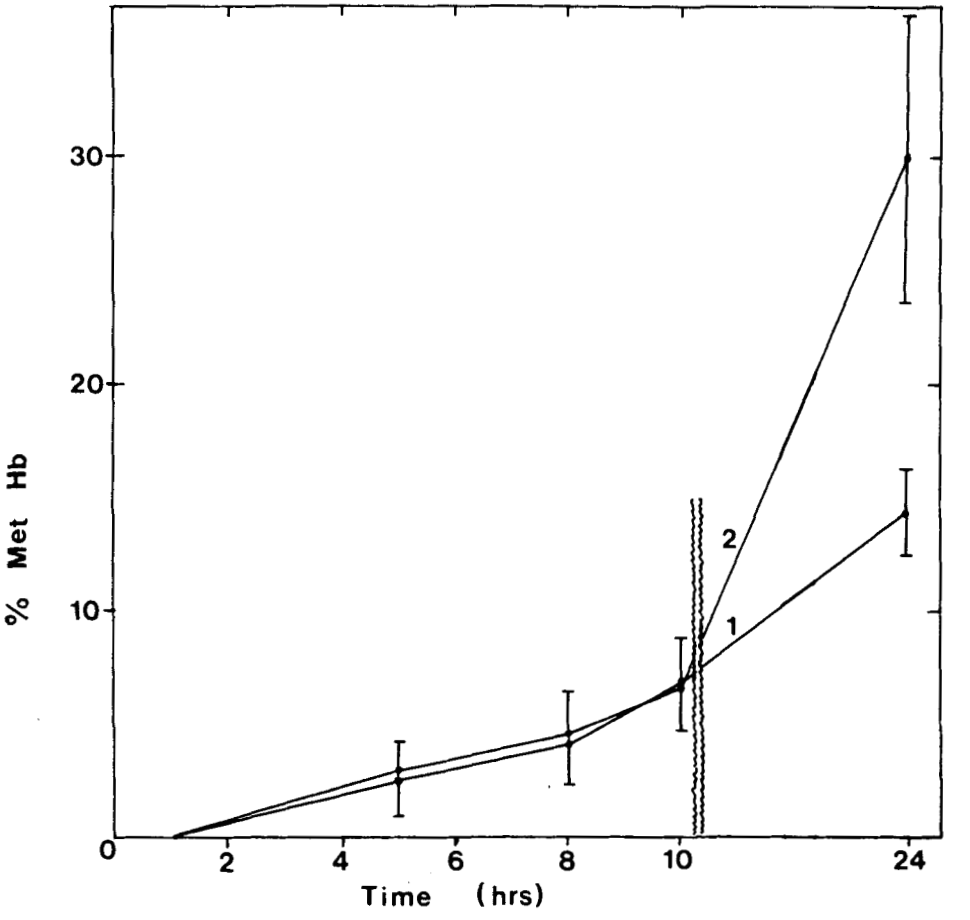

FIGURE 3 Methaemoglobin production in normal red cell haemolysates after oxidative stress with iron (II)/ascorbate/hydrogen peroxide — free radical system.

1. control erythrocytes
2. 100 μM iron (II)/1 mM ascorbate/ ±200 μM hydrogen peroxide.

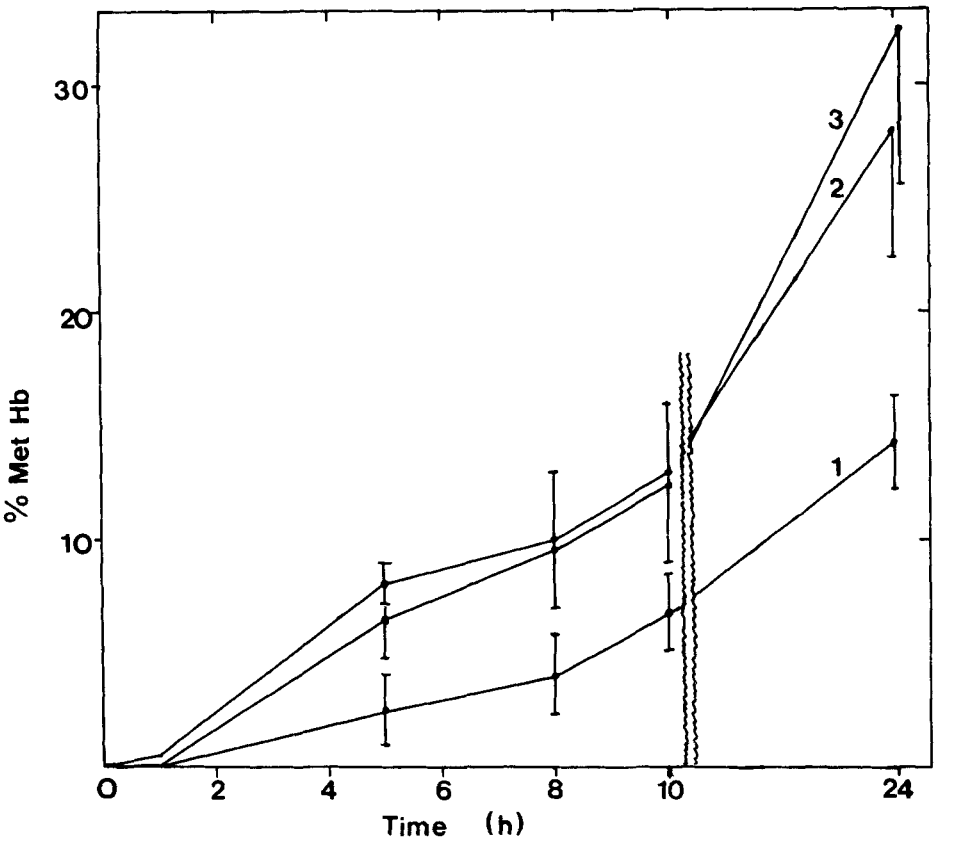

FIGURE 4 The effects of desferrioxamine (400 μM) on methaemoglobin production in normal red cell haemolysates during oxidative stress with iron (II)/ascorbate/hydrogen peroxide — free radical system.

1. control erythrocytes
2. 100 μM iron (II)/1 mM ascorbate/200 μM hydrogen peroxide
3. 100 μM iron (II)/1 mM ascorbate

methaemoglobin is produced in the one group of patients after 5 hours, in comparison with normal cells. The other group corresponds more closely with erythrocytes from normal subjects which may be anticipated since the majority of blood cells circulating in these patients are transfused (Table I). Desferrioxamine considerably inhibits methaemoglobin formation in these systems. The serun. ferritin levels do not correlate directly with the propensity for haemoglobin oxidation in this number of patients (Table I).

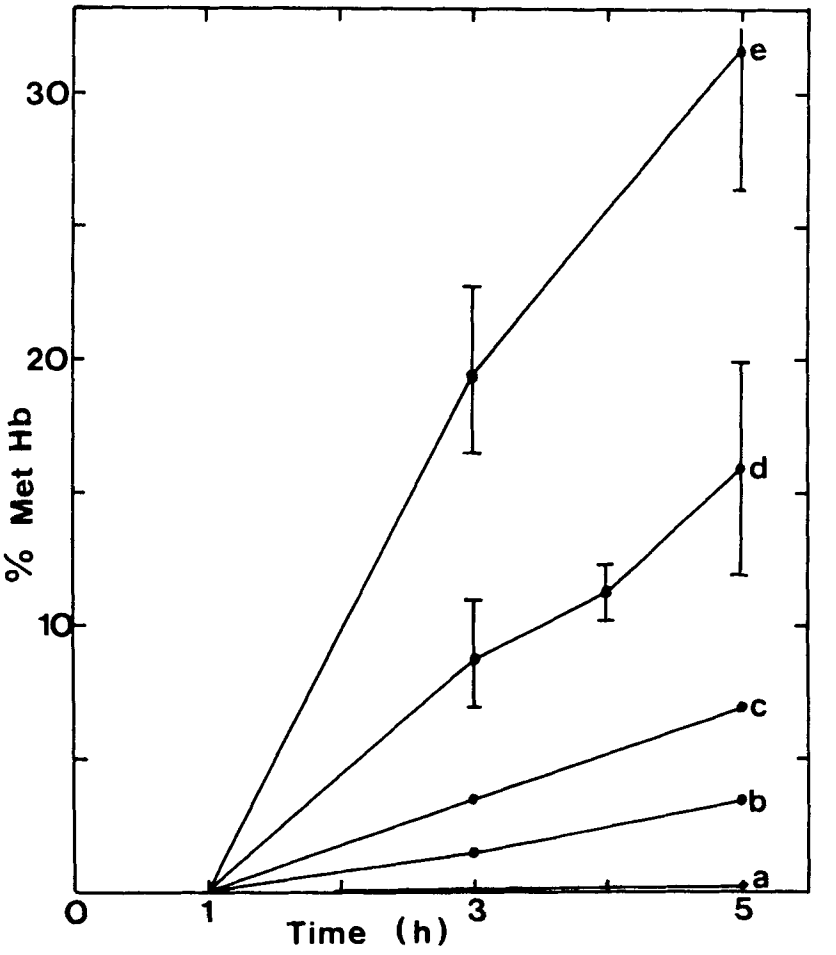

FIGURE 5 The effects of the iron (II)/hydrogen peroxide/ascorbate — free radical system on methaemoglobin production in erythrocytes from patients with β-thalassaemia major.

a control erythrocytes
b,c 100 μM iron (II)/1 mM ascorbate/400 μM desferrioxamine — treatment of cells from Group 2 and Group 1 patients respectively
d,e 100 μM iron (II)/1 mM ascorbate/ ± 200 μM hydrogen peroxide — treatment of Group 2 and Group 1 patients respectively.

DISCUSSION

Haemoglobin in normal red cell haemolysates is less susceptible to oxidation by iron-induced free radical effects than haemoglobin in intact erythrocytes. This work demonstrates that the human erythrocyte membrane has an important role in enhancing the free radical damage initiated by the addition of iron(II)/ascorbate/hydrogen peroxide to red cells possibly through the formation of lipid hydroperoxides and their reactive intermediates.

TABLE I
Serum ferritin levels and time interval since transfusion for thalassaemic patients

Patient	Group	Serum Ferritin (μg/l) (on the day of expt.)	Time interval since last transfusion	Age
A.L.	2	2000	4 weeks	15
A.L.	2	1760	3 weeks	15
A.H.	1	2520	5 weeks	14
C.K.	1	1800	5 weeks	23
S.A.	2	1380	11 weeks	22
M.S.	2	2790	8 weeks	14

In the intact erythrocyte system, desferrioxamine is relatively less effective in partially suppressing the oxidation of reduced glutathione than the oxidation of haemoglobin. However, desferrioxamine has no effect on free radical-induced methaemoglobin formation in treated haemolysates compared with its inhibitory action in the intact erythrocyte system. This observation suggests that desferrioxamine is acting through an inhibition of the membrane-mediated free radical effects on the haemoglobin.

This supports the idea that the site of action of desferrioxamine is mainly extracellular. Further evidence for this suggestion comes from the finding[13] that desferrioxamine-iron(III) complexes do not permeate the red cell membrane.

Acknowledgements

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References

1. Modell, B. and Beck, J., *Ann. N.Y. Acad. Sci.*, **232**, 201–210, (1974).
2. Gutteridge, J.M.C., Rowley, D.A. and Halliwell, B., *Biochem. J.*, **199**, 263–265, (1981).
3. Walling, C., in *Oxidases and Related Redox Systems*, (King, T.E., Mason, H.S. and Morrison, M., Eds.,) pp. 85–97., Pergamon Press, Oxford (1982).
4. Haber, F. and Weiss, J., *Proc. Roy. Soc. London Ser. A.*, **147**, 332–351, (1934).
5. Jacobs, A., *British Journal of Haematology* **34**, 1–4, (1976).
6. Rachmilewitz, E.A., Lubin, B.H. and Shohet, S.B. Blood **47**, 495–505, (1976).
7. Cook, J., Watson, S.S., Simpson, K.M., Lipschitz, D.A. and Skikne, B.S., *Blood* **64**, 721–726, (1984).
8. Nienhuis, A.W., Griffith, P., Strawczynski, H., Henry, W., Borer, J., Leon, M. and Anderson, F.W., *Annals. N.Y. Acad. Sci.*, **344**, 384–396.
9. Modell, B. and Berdoukas, V., in *The Clinical Approach to Thalassaemia*, p. 222–223, Grune and Stratton (1984).
10. Harley, J.D. and Mauer, A.M., *Blood* **16**, 1722–1735, (1960).
11. Beutler, E., Duron, O. and Kelly, B.M., *J. Lab. Clin. Med.* **61**, 882–888, (1963).
12. Brownlee, N.R., Huttner, J.J., Panganamala, R.V. and Cornwell, D.G., *Journal of Lipid Research* **18**, 635–644, (1977).
13. Kontoghiorghes, G., "The Design of Orally Active Iron Chelators for the Treatment of Thalassaemia", PhD. Thesis., Essex University, 1982.

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