Effect of sulphydryl inhibition on the uptake of transferrin-bound iron by reticulocytes

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SYNOPSIS Iron-transferrin, the highly stable carrier of plasma iron, may be dissociated, under physiological conditions, by sodium thioglycollate, a reaction which involves the formation of iron thioglycollate complexes. This suggested that sulphydryl radicals, which are the active groups in thioglycollate and related compounds, may play a part in the uptake of iron by immature red cells from iron transferrin. If this were so, it could be predicted that the sulphydryl inhibitor, *p*-hydroxymercuribenzoate, would depress the uptake of iron by reticulocytes. It is demonstrated here that inhibition of uptake of transferrin-bound iron by reticulocytes, which have been exposed to micromolar concentration of *p*-hydroxymercuribenzoate, is virtually complete. It is suggested that sulphydryl-containing compounds are intimately involved in the process of iron uptake by immature red cells.

It is well established that the source of iron for haem synthesis in immature red cells of the marrow and in reticulocytes is transferrin-bound iron. Jandl and Katz (1963) have demonstrated that the complex first adheres to the surface of the cell membrane and is here dissociated, resulting in uptake of iron by the cell and the release of iron-free transferrin from the cell surface to the surrounding medium.

Although the complex has a very high stability constant, 10^{31} under physiological conditions, the activity of the developing red cell in removing iron presented in this form is highly efficient, since in a hyperplastic marrow virtually all transferrin-bound iron may be cleared from the blood during a single passage through the marrow.

We have been interested in reactions which lead to the release of transferrin-bound iron in connexion with the assay of serum iron. Many techniques *in vitro* are described to achieve dissociation of the complex, most of which require *pH* conditions far from physiological and the use of various reducing agents such as ascorbic acid, sodium sulphite, or thioglycollic acid. The role of the reducing agent has been unclear. It certainly acts in the transformation of Fe(III) to Fe(II) as a necessary intermediate for complexing with chromogens before colorimetric estimation. We have shown that dissociation of the iron transferrin may be achieved under physiological conditions of *pH* by the use of

sodium thioglycollate. Ferric complexes of thioglycollate (Leussing and Kolthoff, 1953) and of cysteine (Tanaka, Kolthoff, and Stricks, 1955) are known, both of which spontaneously reduce to the ferrous complex. Leussing and Kolthoff (1953) demonstrate that the ferrous thioglycollate results from the dual properties of the sulphydryl radical acting both as reducing agent and complexing group.

These observations suggested to us the possibility that sulphydryl groups in the immature red cell surface membrane may play a part in the uptake of transferrin-bound iron. We therefore investigated the effect of the sulphydryl inhibitor, *p*-hydroxymercuribenzoate (PMB), on the uptake of transferrin-bound iron by reticulocytes. This inhibitor does not penetrate the cell and is known to act only at the cell surface (Jacob and Jandl, 1962a).

MATERIALS AND METHODS

Peripheral blood was taken into heparin from two normal subjects and patients with reticulocytosis due to various causes. The red cells were washed three times in physiological saline and made up to 50% concentration in Hanks balanced salt solution (Burroughs Wellcome & Co. Ltd). Aliquots (1.5 to 3.0 ml) of these suspensions were incubated at 37°C for one hour with an equal volume of *p*-hydroxymercuribenzoate solution to give a final concentration varying from 1 to 10 μ M per ml red cells. After incubation the red cells were again washed three times and re-suspended to 50% concentration in

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Hanks solution. p-Hydroxymercuribenzoate was obtained from Sigma Chemicals as its sodium salt.

Human plasma was obtained from normal volunteers of blood groups compatible with the red cells under test. Twenty μCi $^{59}\text{FeCl}_3$ (containing 2 μg Fe) was added to 2 ml plasma and incubated for one hour at 37°C. After incubation it was demonstrated that all the ^{59}Fe was transferrin-bound as shown by electrophoresis and subsequent autoradiography.

Twenty μl of plasma containing 0.2 μCi ^{59}Fe transferrin was added to aliquots of red cell suspension, untreated and treated in various concentrations of p-hydroxymercuribenzoate and incubated for two hours. After incubation the red cells were washed four times to remove supernatant radioactivity and the residual activity of the red cells was counted in a well type scintillation counter.

RESULTS

EFFECT OF P-HYDROXYMERCURIBENZOATE CONCENTRATION The Figure shows the increasing inhibition of ^{59}Fe uptake by the cell suspensions with increasing concentration of p-hydroxymercuribenzoate in the pre-incubation medium. Iron uptake decreases sharply with small amounts of added p-hydroxymercuribenzoate and falls to a minimum at a concentration of 5×10^{-6} μM p-hydroxymercuribenzoate per millilitre red cells.

EFFECT OF RETICULOCYTE CONCENTRATION The Table shows the effect of p-hydroxymercuribenzoate on uptake of iron by red cell suspensions containing varying proportions of reticulocytes. Where re-

FIG. 1. The inhibition by p-hydroxymercuribenzoate of the uptake of transferrin-bound ^{59}Fe by reticulocytes.

TABLE

EFFECT OF PRE-INCUBATION WITH P-HYDROXYMERCURIBENZOATE (PMB) ON THE UPTAKE OF TRANSFERRIN-BOUND ^{59}Fe BY RETICULOCYTES

Source of Red Cells	Reticulocytes (%)	PMB (μM per ml red cells)	Uptake of ^{59}Fe (counts/100 sec)
Acute haemolytic anaemia	40	0	25,606
		1	16,756
		5	1,871
		10	1,759
Acute haemolytic anaemia	30	0	13,449
		1	8,084
		5	506
		10	394
Chronic haemolytic anaemia	27	0	18,020
		4	3,617
Recovery from haemorrhage under oral Fe therapy	13	0	20,182
		4	350
Fe-deficiency anaemia under treatment	1	0	1,102
		4	403
Normal subject	< 1	0	375
		1	312
		5	191
		10	175
Normal subject	< 1	0	1,126
		1	997
		5	1,013
		10	441

ticulocyte counts are high the uptake of ^{59}Fe by untreated cell suspensions resulted in net counts of the order of 20,000 per 100 seconds; after incubation with p-hydroxymercuribenzoate uptakes fell to as little as 300 per 100 seconds.

In general the higher the reticulocyte count, the higher the initial uptake, but the inhibition by p-hydroxymercuribenzoate is clearly seen in cell suspensions with as few as 1% or less reticulocytes.

DISCUSSION

These results show a profound decrease in uptake of transferrin-bound iron by reticulocytes exposed to the action of the sulphhydryl inhibitor, p-hydroxymercuribenzoate. It has been demonstrated that in contrast with other sulphhydryl inhibitors, p-hydroxymercuribenzoate acts only on the cell surface (Jacob and Jandl, 1962a). Since the uptake of iron from transferrin is also a membrane activity it is clear that sulphhydryl groups located in the cell membrane are involved in the process of iron uptake by reticulocytes.

p-Hydroxymercuribenzoate is known to affect other membrane functions such as glucose transport (Dawson and Widdas, 1963; Vansteveninck, Weed, and Rothstein, 1965); it has also been shown to disrupt the cation gradient across the red cell membrane *in vitro*: red cells so treated accumulate sodium and are rapidly sequestered in the splee

after re-injection (Jacob and Jandl, 1962b). It is notable that these effects are produced by concentrations of p-hydroxymercuribenzoate similar to those effective in the inhibition of iron uptake. However, these effects occur in mature red cells whose capacity to take up iron from transferrin is virtually non-existent, and therefore iron uptake inhibited by p-hydroxymercuribenzoate must affect other mechanisms than those affected in mature cells.

The uptake of iron, which is a function of immature red cells, may therefore be dependent on different sulphydryl-containing compounds from those involved in the maintenance of cation gradients, or iron uptake requires additional factors present only in immature cells. These alternatives are not mutually exclusive.

Many intensive studies have been made on active membrane transport of substances such as sugars and cations, but the chemical nature of the carriers involved has not been demonstrated. It has been assumed that in these processes inhibition by p-hydroxymercuribenzoate has acted indirectly by affecting sulphydryl groups, essential to the integrity of the cell membrane.

The dual properties of thioglycollate sulphydryl groups in the reduction and complexing of transferrin-bound iron *in vitro* suggested a direct involvement of sulphydryl groups in iron uptake *in vivo*, and the present demonstration of inhibition by p-hydroxymercuribenzoate of iron uptake lends support to this hypothesis.

The inhibition of iron uptake could be due to blocking of specific receptor sites by p-hydroxymercuribenzoate, or to interference with dissociation of the transferrin iron complex after its adhesion to the cell surface; it is also possible that both activities are intimately linked. Further work is proceeding to explore these alternative mechanisms.

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