In vivo Testing of the Fletcher–Huehns Hypothesis of Functional Differences of Iron Atoms bound by Transferrin

RECENTLY Fletcher and Huehns[1–3] proposed an interesting hypothesis on the possible functional difference between the two iron binding sites of the iron transport protein, transferrin. They presented evidence from *in vitro* studies that iron uptake by the two binding sites is random, while the release of iron to erythroid precursors occurs preferentially from one site, which they arbitrarily designated the A site. This site is also postulated to be important in the transfer of iron to the placenta of pregnant animals. The other site, designated B, is somehow concerned with delivery of iron to storage depots. Complete saturation of both sites with iron produces greater transfer to erythroid precursors and/or storage depots. The concept of selective release of iron from functionally different binding sites on the transferrin molecule is potentially of great importance and warranted repetition of Fletcher and Huehns's *in vitro* experiments and the exploration of their hypothesis *in vivo*.

The procedures for collecting blood enriched with reticulocytes and incubating them with labelled transferrin were those described originally by Jandl and co-workers[4,5] and modified by Kornfeld[6]. Purified, iron-free, human transferrin (Behringwerke) was used in all experiments.

Transferrin, 100 per cent saturated with ^{59}Fe, was preincubated with rabbit reticulocytes until 30 to 50 per cent of the ^{59}Fe was removed from solution. The transferrin solution was freed of reticulocytes by centrifugation and then incubated with fresh reticulocytes for 60 min at 37° C. The uptake of iron from the predominantly B site binding of this transferrin solution was diminished by 70 per cent compared with the uptake from a control preparation of ^{59}Fe-transferrin of equal saturation that had ^{59}Fe bound randomly to A and B sites. Furthermore, when ^{59}Fe was added back to the preincubated transferrin to restore 100 per cent saturation, ^{59}Fe uptake by fresh reticulocytes was restored to the control levels, confirming the original observations of Fletcher and Huehns.

To test *in vivo* the hypothesis of selective iron distribution from the different binding sites of transferrin, double-labelled transferrin was prepared. Transferrin was totally saturated with ^{59}Fe and then incubated with rabbit reticulocytes until 30 to 50 per cent of the ^{59}Fe had been removed from the transferrin solution (presumably most of the ^{59}Fe was removed from site A). Complete saturation was restored by adding ^{55}Fe to achieve selective labelling of site A while site B retained its original ^{59}Fe. Alternatively, the transferrin was first saturated with ^{55}Fe and the ^{59}Fe was added to replace the iron removed by incubation with reticulocytes. The transferrin solutions with selective tagging of A and B sites were injected intravenously into rats. The 2 h uptake of radioactivity, expressed as a percentage of the dose administered, was calculated for selected organs which were analysed for ^{55}Fe and ^{59}Fe simultaneously by the method of Katz and co-workers[7]. In seven experiments, pregnant rats were studied for evidence of selective placental radio iron transfer (Table 1).

A significant difference in the tissue distribution of radioactive iron was observed in only two instances in these rats injected *in vivo*. Selective labelling of site B produced enhanced uptake by the foetuses of pregnant rats which is contrary to the prediction of the Fletcher–Huehns hypothesis. Diminished radio iron in the blood of rats injected with site A labelled transferrin in comparison with uniformly labelled transferrin but not with site B labelled transferrin seems to have statistical rather than biological significance. Most noteworthy is the lack of selective uptake of radio iron of a specific type by bone marrow, liver or spleen, receptor tissues with expected polarity for selectively labelled transferrin.

We conclude from these studies, which were designed to test *in vivo* the release of iron from selectively labelled transferrin, that the differences of iron transfer predicted by *in vitro* studies are not demonstrated and that a re-examination of Fletcher and Huehns's attractive hypothesis is necessary.

Table 1. PERCENTAGE UPTAKE OF RADIO IRON (MEAN ± SD)

Tissue	Equal labelling of transferrin sites A and B (9)	Predominant labelling of transferrin site B (20)	Predominant labelling of transferrin site A (13)	Significance Student's t test
Bone marrow	44·1 ± 10·1	44·6 ± 18·1	47·8 ± 9·7	N.S.
Blood	16·2* ± 1·1	15·4 ± 12·4	11·4* ± 5·1	$0{\cdot}05 > t > 0{\cdot}025$
Liver	18·2 ± 6·3	16·9 ± 12·0	14·2 ± 7·1	N.S.
Spleen	2·2 ± 0·6	3·4 ± 2·6	3·3 ± 2·7	N.S.
Maternal placenta		(4) 0·2 ± 0·16	(3) 0·2 ± 0·13	N.S.
Foetal placenta		1·9 ± 0·81	1·9 ± 0·46	N.S.
Foetus		2·7* ± 2·09	0·91* ± 0·48	$0{\cdot}05 > t > 0{\cdot}025$

Rats (250–380 g) were injected intravenously with transferrin totally labelled with radio iron on selected iron-binding sites. Tissue distribution of ^{59}Fe and ^{55}Fe expressed as per cent of injected radioactivity was measured at 2 h. The number of experiments is given in parentheses and significant differences are shown by *. Bone marrow mass is calculated as 2 per cent of body weight, blood volume as 5 per cent of body weight. Four to ten foetuses in the last third of gestation were analysed for each pregnant rat.

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1 Fletcher, J., and Huehns, E. R., *Nature*, **215**, 584 (1967).
2 Fletcher, J., and Huehns, E. R., *Nature*, **218**, 1211 (1968).
3 Fletcher, J., *Clin. Sci.*, **37**, 273 (1969).
4 Jandl, J. H., Inman, J. K., Simmons, R. L., and Allen, D. W., *J. Clin. Invest.*, **38**, 161 (1959).
5 Katz, J. H., and Jandl, J. H., in *Iron Metabolism* (edit. by Gross, F.), 103 (Springer-Verlag, Berlin, 1964).
6 Kornfeld, S., *Biochemistry*, **7**, 945 (1968).
7 Katz, J. H., Zoukis, M., Hart, W. L., and Dern, R. J., *J. Lab. Clin. Med.*, **63**, 885 (1964).

Crystalline Fc prepared in High Yield from Normal Human IgG

CRYSTALLINE rabbit Fc fragment is simple to prepare in high yield from the corresponding IgG by cysteine-activated papain digestion[1]. Although crystalline products can be obtained from human myeloma globulins, only one report[2] has hitherto appeared describing an analogous substance from normal human IgG. The conditions for digestion of normal rabbit or human IgG were markedly different. One-tenth the ratio of enzyme: antibody was used, and the reaction time was reduced from 16 h to 30 min for the human material. In our hands, although a solid human Fc was obtained by the method of Hershgold *et al.*, the material did not crystallize, and the yield was low. We therefore decided to re-