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Uptake of 26-Al and 67-Ga into brain and other tissues of normal and hypotransferrinaemic mice

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Aluminium uptake from blood into tissues of control and homozygous hypotransferrinaemic (*hpx/hpx*) mice, following continuous intravenous infusion of ^{26}Al and ^{67}Ga, has been compared with that of gallium, a proposed tracer for aluminium. ^{26}Al uptake into tissues of control (*hpx*/+ and +/+) mice occurred in the order (expressed as a space): bone 464.7 ml 100 g^{-1}; renal cortex 102.9 ml 100 g^{-1}; liver 13.0 ml 100 g^{-1}; spleen 8.4 ml 100 g^{-1} and brain 0.8 ml 100 g^{-1}. ^{67}Ga uptakes were similar in liver, spleen and brain, but smaller in the renal cortex and bone, at one-third and one-fifth of the values for ^{26}Al, respectively. In the hypotransferrinaemic mice, uptake of ^{67}Ga into all tissues was increased, especially in renal cortex (ninefold) and bone (twentyfold) as compared with the controls. Increases in ^{67}Ga uptakes into cerebral hemisphere, cerebellum and brain stem of the hypotransferrinaemic mice were 3.8, 4.2 and 2.8 fold, respectively. ^{26}Al uptake into tissues of the hypotransferrinaemic mice was similar to control values except in bone where it was three times greater. Pre-treatment of control animals with the anti-transferrin receptor antibody, RI7 208, enhanced ^{67}Ga uptake in all tissues, the effect being greatest in renal cortex (tenfold) and bone (ninefold). ^{67}Ga uptakes into cerebral hemisphere, cerebellum and brain stem in the mice pre-treated with RI7 208 were 6.4, 6 and 10 times greater than in untreated mice, respectively. No influence of antibody on ^{26}Al uptake into mouse tissues was observed except in spleen where it was three times greater than in untreated mice. Hence, transport of aluminium and gallium into mouse tissues is not similar under all conditions. Non-transferrin mediated transport of each metal can occur into all tissues, especially in renal cortex and bone, where gallium may be a suitable marker for aluminium.

Keywords: aluminium, gallium, hypotransferrinaemia, transferrin, transferrin receptor

Introduction

Aluminium is known to be a causative agent of dialysis dementia (Alfrey *et al.* 1976) and it has also been implicated in certain neurodegenerations (Crapper *et al.* 1976, Perl *et al.* 1982, Candy *et al.* 1986). However, the mechanism of transport of aluminium into the brain remains unclear. Most of the aluminium in blood is bound to transferrin (Harris & Sheldon 1989) and transferrin receptors would therefore seem necessary for the uptake of aluminium into tissues. There are indications that transport of aluminium into brain is associated with transferrin receptor mediated endocytosis (Morris *et al.* 1989). Aluminium does also enter other cells and tissues, and indeed can cause anaemia and osteodystrophy (Kerr *et al.* 1992).

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In this study, the role of transferrin and transferrin receptors in the transport of aluminium into brain, liver, spleen, renal cortex and bone of the mouse has been investigated. The laboratory mouse was chosen for a number of reasons. It is of a size which allowed use of a small dose of expensive ^{26}Al; techniques have already been developed for the study of ^{59}Fe-transferrin uptake from blood into the brain and other tissues of this species (Ueda *et al.* 1993, Bradbury *et al.* 1994); the availability of genetically hypotransferrinaemic mice (Bernstein 1987) permitted investigation of aluminium transport in the near absence of plasma transferrin; and finally we have experience of using the IgM anti-mouse antibody against transferrin receptors, RI7 208, which is effective against both transferrin-mediated iron uptake and growth in cultured mouse cells (Trowbridge *et al.* 1987) and iron uptake into several mouse tissues *in vivo* (Ueda *et al.* 1993, Bradbury *et al.* 1994).

In addition, direct comparisons have been made between tissue uptakes of ^{26}Al and ^{67}Ga in the same animals and under identical experimental conditions. In the past, in the absence of a suitable radioisotope of aluminium, ^{67}Ga has been used as an alternative for investigating aluminium transport (Farrar *et al.* 1990, Pullen *et al.* 1990). Although gallium has very similar physico-chemical properties to aluminium, there is little direct evidence that it behaves identically in biological systems. It was therefore anticipated that this study would allow a direct comparison of transport mechanisms of the two metals.

Methods and materials

Animals

Mice (Balb/C), 6–8 weeks old, of either sex and of about 20 g body weight were used. The homozygous hypotransferrinaemic (*hpx/hpx*) mice had serum transferrin of less than 0.07 mg ml^{-1} (Simpson *et al.* 1991), compared with 3.31 ± 0.29 mg ml^{-1} and 1.74 ± 0.14 mg ml^{-1} in wild-type (+/+) and heterozygous (*hpx*/+) mice, respectively. *Hpx/hpx* mice were maintained by weekly intraperitoneal (i.p.) injections of mouse serum (~ 0.2–1.0 mg transferrin; Simpson *et al.* 1991). The experiments were performed one week after the last maintenance injection of transferrin. All procedures for the care and use of animals were performed within the terms of the appropriate licenses from the United Kingdom Home Office.

Animal preparation

Mice were anaesthetised with a combination of 3.3 μl per 10 g of fentanyl/fluanisone (Janssen Pharmaceutical, Grove, Oxford, UK) and 50 μg per 10 g of diazepam (Roche Products, Welwyn Garden City, Hertfordshire, UK) i.p. The right external jugular vein was used for infusion whereas blood samples were collected from the left external iliac artery, there being no tracheostomy.

Intravenous infusion and sampling procedure

Mice were infused with a total of 50 Bq (1.4 nCi) of ^{26}Al (Los Alamos National Laboratories, USA) (2.8 nmol of total Al), 3.7×10^5 Bq (10 μCi) of ^{67}Ga (Amersham International) (0.24 pmol of total Ga) and 1.5 μmol of citrate (from ^{67}Ga and ^{26}Al preparations) in isotonic NaCl over two hours. The infusions were performed at a diminishing rate with a Harvard syringe pump, the objective being to maintain a fairly constant serum level of ^{26}Al and ^{67}Ga. The infusion rate was 36.3 μl min^{-1} over the first 2 min and was gradually reduced to 9 μl min^{-1} during 20–120 min. The uptake of ^{26}Al and ^{67}Ga into mouse tissues was performed in the absence and presence of a monoclonal rat anti-mouse antibody against transferrin receptors, IgM RI7 208 (Dr I.S. Trowbridge, Salk Institute, San Diego, USA). RI7 208 was administered as a single intravenous injection (1 mg per mouse) 10 min before the start of the infusion. Previous data have shown that this dose is adequate to inhibit transferrin receptor mediated transport of iron (Ueda *et al.* 1993).

During each experiment, five arterial blood samples of approximately 40 μl were collected periodically into capillary tubes, and spun immediately. At the end of the infusion period, the vascular system was washed out with 1 mM EDTA–saline, immediately prior to the decapitation, as has been described in detail by Bradbury & Deane (1986). This served to remove any residual blood and any ^{26}Al or ^{67}Ga that may have adhered to the luminal surface of the tissue capillaries. Tissues were sampled, placed in preweighed scintillation vials and immediately reweighed.

^{67}Ga analysis

For analysis of ^{67}Ga, samples placed in the scintillation vials were counted in a Canberra Packard Cobra II Autogamma counter. Counts were corrected for background and isotope decay.

^{26}Al analysis

After decay of ^{67}Ga, ^{26}Al was analysed by high energy accelerator mass spectrometry (AMS) at 150 MeV in the tandem Van de Graaff accelerator (acceleration potential ~ 17 MV) and high resolution magnetic spectrometer at the SERC Laboratory (Daresbury, Warrington, UK). At

the energy of 150 MeV fully stripped ^{26}Al ions are generated, and completely separated from potentially interfering isobar ^{26}Mg ions by a magnetic spectrometer (Barker *et al.* 1990).

The ^{26}Al concentrations in samples were estimated indirectly by measuring the isotope ratios of $^{26}Al/^{27}Al$ following addition of a known large amount of stable ^{27}Al to facilitate the extraction of the tracer amounts of ^{26}Al. The known macroscopic quantity of ^{27}Al ensures that $^{26}Al/^{27}Al$ ratios are in the appropriate range for AMS measurements. The optimum range is $10^{-12} - 10^{-8}$ with a precision of measurements *c.* 2% (Barker *et al.* 1990). The ^{26}Al concentration in the sample is then calculated as the product of the isotopic ratio and the known concentration of ^{27}Al.

Calculation and statistics

The uptake of ^{26}Al and ^{67}Ga into tissues was expressed as a space, ml 100 g^{-1}; the tissue concentration, or counts per min per g (C_{tis}) at the end of experimental time, T, being related to the serum concentration, or counts per min per ml (C_{ser}) integrated over time, as

$$\text{UPTAKE SPACE} = \frac{C_{tis} \cdot T}{\int_0^T C_{ser} \cdot dt}$$

Results are represented as means ± SEM. Differences in the uptake into different tissues were tested for statistical significance by *t*-test (two-tailed) unless otherwise stated.

Results

Levels of ^{67}Ga and ^{26}Al in mouse serum

The serum profile of ^{67}Ga during a 2 h intravenous infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl in control mice, mice pre-treated with antibody RI7 208 and in hypotransferrinaemic mice is shown in Figure 1. The serum level of ^{67}Ga in control mice increased rapidly in the first 15–30 min to reach 450 cpm μl^{-1} serum and thereafter remained fairly constant (integrated serum level of ^{67}Ga was 443.3 ± 163.7 cpm μl^{-1}). The serum ^{67}Ga in both hypotransferrinaemic mice and animals pre-treated with antibody was also maintained at a near constant level, but at a level much lower than in control mice despite the same infusion rate over 2 h. In hypotransferrinaemic mice the serum level of ^{67}Ga integrated over 2 h was 57.3 ± 19.2 cpm μl^{-1}, whereas for mice pre-treated with antibody it was 55.5 ± 6.4 cpm μl^{-}.

Corresponding individual serum concentrations of ^{26}Al integrated over 2 h are shown in Table 1.

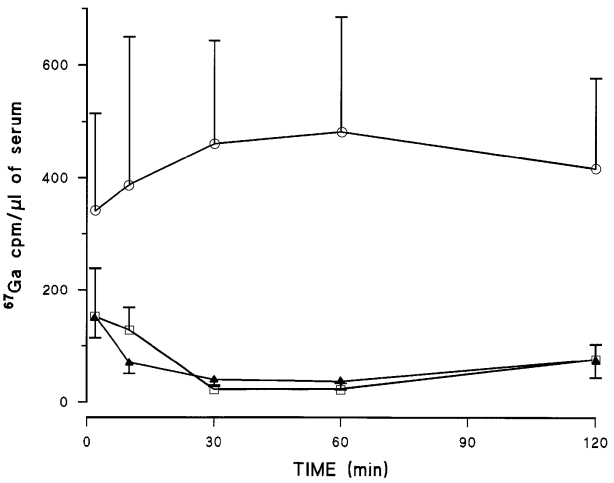

Figure 1. Serum profile for ^{67}Ga during a 2 h intravenous infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl: control mice (○–○); mice pre-treated with monoclonal antibody against transferrin receptor (RI7 208) (□–□); hypotransferrinaemic mice (▲–▲). Vertical bars represent SEM, $n = 3$.

Table 1. Integrated concentrations of ^{26}Al in serum over time following a 2 h infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl in control mice, mice pre-treated with anti-transferrin receptor antibody (RI7 208) and hypotransferrinaemic mice. Results shown are individual integrated concentrations for two experiments in each group

Treatment	^{26}Al (ng ml^{-1})	
Control	0.341	0.436
With RI7 208 antibody	0.343	0.280
Hypotransferrinaemia	0.341	0.192

Neither the prior injection of RI7 208 nor hypotransferrinaemia influenced the level of ^{26}Al in serum. The amounts of ^{26}Al infused (3.64 ng Al per g of mouse) were comparable with the aluminium content in the blood, as inferred from the rat (Radunović *et al.* 1993), but were negligible compared with the total aluminium in the body.

Uptake of ^{67}Ga and ^{26}Al into mouse tissues and effect of RI7 208

The closure of AMS facilities at SERC Laboratory in Daresbury unfortunately prevented complete analysis of ^{26}Al. Consequently ^{26}Al uptake spaces are only represented as the individual values in each group of mice. Statistical differences for individual tissues between different treatment groups could not

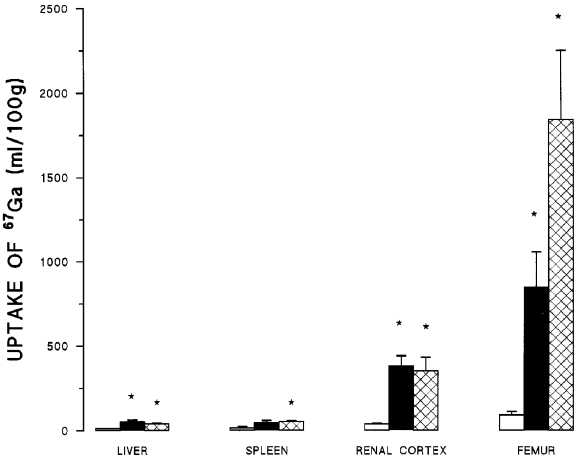

Figure 2. Uptake of ^{67}Ga as a space (ml 100 g^{-1}) into liver, spleen, renal cortex and femur of control mice (open bars), mice pre-treated with monoclonal antibody RI7 208 (closed bars) and hypotransferrinaemic mice (hatched bars) following a 2 h infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl. The results are represented as means ± SEM, $n = 3$. Differs from control ^{67}Ga spaces by *t*-test (two-tailed), ★ $P < 0.05$.

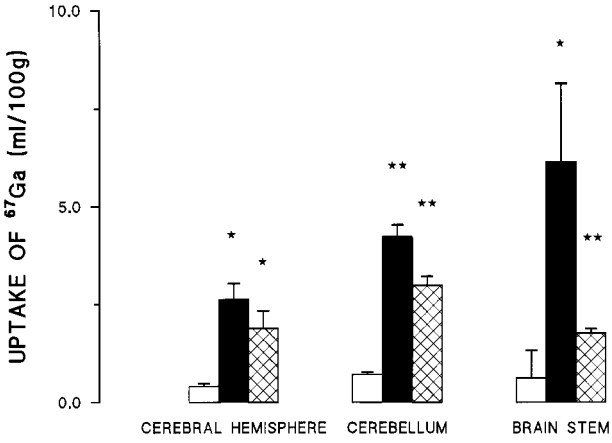

Figure 3. Uptake of ^{67}Ga as a space (ml 100 g^{-1}) into cerebral hemisphere, cerebellum and brain stem of control mice (open bars), mice pre-treated with monoclonal antibody RI7 208 (closed bars) and hypotransferrinaemic mice (hatched bars) following a 2 h infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl. The results are represented as means ± SEM, $n = 3$. Differs from control ^{67}Ga spaces by *t*-test (two-tailed), ★ $P < 0.05$; ★★ $P < 0.01$.

be tested, nor could differences between ^{26}Al and ^{67}Ga uptake spaces. Uptake of ^{26}Al into tissues of control mice occurred in the order: femur > renal cortex >> liver/spleen > brain. ^{67}Ga uptake spaces increased in a similar order (Table 2). It was possible to analyse correlation between ^{26}Al and ^{67}Ga uptake spaces across all tissues from control mice. A significant correlation was seen ($P < 0.02$), suggesting some relationship between ^{26}Al and ^{67}Ga uptake. However, the slope of the regression line (0.16 ± 0.05, $n = 10$) differed significantly from 1 ($P < 0.001$).

Prior treatment of mice with RI7 208 significantly increased uptake of ^{67}Ga into liver, renal cortex, femur and brain, whilst the enhanced uptake of ^{67}Ga into spleen did not reach statistical difference due to the large variability in data (Figure 2, Table 2). The mean uptakes of ^{67}Ga into cerebral hemisphere, cerebellum and brain stem in the presence of RI7 208 were about 6.4, 6 and 10 times greater than mean control values, respectively (Figure 3). In contrast to ^{67}Ga, the uptake of ^{26}Al into mouse tissues appeared relatively insensitive to the effect of anti-

Table 2. Uptake of ^{67}Ga and ^{26}Al as a space (ml 100 g^{-1}) into tissues of control mice, mice pre-treated with monoclonal antibody RI7 208 and hypotransferrinaemic (*hpx/hpx*) mice after a 2 h infusion of tracer amounts of ^{67}Ga and ^{26}Al in isotonic NaCl. ^{67}Ga spaces are represented as the means ± SEM, $n = 3$ whereas ^{26}Al spaces are shown as individual values for each experiment. [a]Differs from control spaces by *t*-test (two-tailed), $P < 0.05$

	Control		RI7 208		*hpx/hpx*	
	^{67}Ga	^{26}Al	^{67}Ga	^{26}Al	^{67}Ga	^{26}Al
Liver	11.6 (± 2.0)	15.3; 10.6	53.7 (± 7.5)[a]	15.5; 9.6	39.5 (± 4.1)[a]	13.4; 21.7
Spleen	14.7 (± 7.8)	7.5; 9.3	46.8 (± 11.9)	20.0; 26.8	52.2 (± 4.0)[a]	5.4; 8.7
Renal cortex	37.5 (± 4.3)	123.5; 82.3	381.9 (± 59.7)[a]	83.1; 120.9	351.3 (± 82.2)[a]	109.7; 127.9
Femur	91.1 (± 19.7)	435.9; 493.5	848.2 (± 208.9)[a]	585.1; 457.0	1843.4 (± 407.2)[a]	1271.0; 1362.1
Brain	0.48 (± 0.06)	0.86; 0.77	3.29 (± 0.57)[a]	0.86	2.00 (± 0.32)[a]	0.90; 1.39

body except in spleen where it was threefold higher than in control mice (Table 2).

Tissue uptake of ^{67}Ga and ^{26}Al in the hpx/hpx *mice*

The ^{67}Ga uptake was significantly higher in all of the studied tissues of the hypotransferrinaemic mice than in those of the control mice (Figures 2 and 3, Table 2). The ^{67}Ga uptake increased from 3.5 (liver, spleen), 4 (brain), 9 (renal cortex) to 20 (femur) fold as compared with control values.

There was no evidence of a difference in the uptake of ^{26}Al into tissues of hypotransferrinaemic and control mice, except for femur where the uptake was about three times greater in hypotransferrinaemic mice (Table 2).

Discussion

Tracers in serum

The levels of ^{67}Ga and ^{26}Al attained in the serum allowed fairly precise tissue uptakes to be measured for each isotope by the respective analytical method used. In the case of ^{67}Ga, the relative depressions of serum concentrations in hypotransferrinaemia and after antibody administration conformed well to the greater tissue uptakes in these conditions. We were not able to determine the chemical speciation of the two isotopes in the circulation, but the very high formation constants of gallium–transferrin (Harris & Pecoraro 1983, Martin *et al.* 1987), together with the enhanced ^{67}Ga uptakes into tissues of hypotransferrinaemic and anti-transferrin receptor antibody treated mice, suggest that much of the ^{67}Ga in control mice was bound to transferrin. Despite its ready binding to transferrin, dialysis experiments *in vitro* show that ^{67}Ga in plasma can, in the presence of 0.1 M citrate, readily exchange across a membrane, impermeable to protein, as a low molecular weight species, presumably as a citrate complex (Vallabhajosula *et al.* 1980, Raijmakers *et al.* 1992).

For reasons converse to those applied to ^{67}Ga, we are less certain that most of the ^{26}Al present in control mouse serum is in combination with transferrin. The formation constants for aluminium–transferrin are much lower (Martin *et al.* 1987, Harris & Sheldon 1989) and tissue uptakes are remarkably insensitive to both hypotransferrinaemia and presence of the antibody. It is noteworthy that even in the steady state, at least 5% of aluminium in human serum is present as citrate complexes rather than in combination with transferrin (Harris & Sheldon 1989, Fatemi *et al.* 1991).

Tissue uptakes in control mice

These experiments allowed a direct comparison between tissue uptakes of ^{26}Al and ^{67}Ga, and also a comparison between these uptakes and that of ^{59}Fe in similar groups of control and hypotransferrinaemic mice. In the latter case, the radioisotope was infused as ^{59}Fe-ferrous chloride in the presence of ascorbate and was shown to be 99% associated with transferrin *in vivo* (Ueda *et al.* 1993). Whilst statistical tests cannot be applied to compare ^{26}Al uptake with that of the other two isotopes, the consistency within the paired ^{26}Al values encourages us to believe that any large differences are real.

Except in the cases of renal cortex and bone, uptakes of ^{67}Ga and ^{26}Al are rather low and of similar size. Not only are uptakes of both isotopes into the renal cortex and bone higher than into other tissues, but that of ^{26}Al is nearly three times that of ^{67}Ga into renal cortex and five times that of ^{67}Ga into bone. ^{26}Al transport into renal cortex is also 6.5 times higher than that of ^{59}Fe; bone uptake of ^{59}Fe was unfortunately not measured (Bradbury *et al.* 1994). Since ^{67}Ga uptake into renal cortex and bone is also high and markedly enhanced by infusion of citrate (A. Radunović, H.T. Delves and M.W.B. Bradbury, unpublished), these findings indicate that transport of ^{67}Ga and ^{26}Al into renal cortex and bone occurs mainly as non-transferrin bound species, probably as citrate complexes, under our experimental conditions.

The transferrin-mediated transport of ^{59}Fe into brain and liver (Ueda *et al.* 1993, Bradbury *et al.* 1994) was greater than measured uptakes of ^{67}Ga and ^{26}Al in the present study. Transport of ^{59}Fe into spleen *in vivo* has been considered by ourselves to be an index of transferrin-mediated transport since it is large and almost totally inhibited by antibodies against the transferrin receptor *in vivo* in both the mouse and rat (Ueda *et al.* 1993, Bradbury *et al.* 1994). In the mouse, spleen ^{59}Fe uptake is 16.5 times that of ^{67}Ga and 29 times that of ^{26}Al.

Overall, our data indicate that transport of ^{67}Ga and of ^{26}Al is relatively low in those tissues where transferrin-mediated transport of iron is well established, i.e. spleen, liver and brain (Ueda *et al.* 1993, Bradbury *et al.* 1994), and high in the renal cortex and bone where other mechanisms operate for these two metals. The findings suggest that gallium is a better marker for aluminium in tissues where non-transferrin mediated transport is predominant.

Tissue uptakes in the hpx/hpx *mouse*

Since *hpx/hpx* mice have little circulating transferrin, appreciable tissue uptake of metals, which are normally transferrin-bound, is likely to be due to transport into tissues of one or more species which are of low molecular weight. During infusion of ^{59}Fe-ferrous chloride into such mice, tissue uptake of this radiotracer was increased from about 14 times in the spleen and to about 700 times in the liver and pancreas as compared with control values (Ueda *et al.* 1993, Bradbury *et al.* 1994). The increases in ^{67}Ga uptake, observed here in the *hpx/hpx* mice, are much less than those seen with Fe^{2+}. Since gallium does not readily exist in divalent form and was infused as the Ga(III)–citrate complex, the most parsimonious explanation is that we are seeing direct tissue entry of this infused complex in the *hpx/hpx* mice. Hence, since hypotransferrinaemia does not cause a non-specific increase in permeability (Ueda *et al.* 1993), effective permeability between blood and tissues must be generally greater for the gallium–citrate complex than for gallium–transferrin. It is noteworthy that the highest uptakes of ^{67}Ga in the *hpx/hpx* mouse were in the renal cortex and bone, the very two tissues which took up the largest amounts of gallium in the normal rat, when this metal was infused with higher concentrations of citrate (A. Radunović, H.T. Delves and M.W.B. Bradbury, unpublished).

The situation with ^{26}Al infused into the *hpx/hpx* mouse was strikingly different. There was no obvious increase in the ^{26}Al space in any tissue in the *hpx/hpx* mouse over that in the control mouse, except in the case of bone where the space was about three times greater. If we neglect the bone results, the conclusion must be either that transport of unaltered aluminium–citrate complex is occurring in both control and *hpx/hpx* mice, or that the sum of transferrin- and citrate-mediated transport in the controls fortuitously equals citrate-mediated transport in the *hpx/hpx* animals. The simplest conclusion is the first and hence is to be preferred on grounds of parsimony. However, if we include the bone results in the argument, they indicate that the less easily transported ^{26}Al–transferrin may play a small role in the controls.

Effects of the antibody against transferrin receptor

The finding of marked increases in gallium transport in all tissues studied in the presence of the anti-transferrin receptor antibody was quite unexpected since only inhibitory effects against ^{59}Fe transport have been observed *in vivo* (Ueda *et al.* 1993, Bradbury *et al.* 1994) or in cultured cerebral endothelial cells; in fact ^{67}Ga–transferrin uptake into such cells was also inhibited *in vitro* (Egleton *et al.* 1996). The increases in gallium uptake observed were generally of comparable magnitude to those occurring in the *hpx/hpx* mice, except that transport into bone was somewhat less. Similar increases in gallium uptake into rat tissues occur in the presence of anti-rat antibody against transferrin receptor OX-26 (A. Radunović, H.T. Delves and M.W.B. Bradbury, unpublished). We have no direct evidence about the mechanism of these *in vivo* changes, but the similarity of the increases seen in the *hpx/hpx* mice, together with arguments made about non-transferrin dependent transport into control tissues, give strong circumstantial support for the view that they are associated with large rises in non-transferrin bound gallium in serum, probably as citrate complexes. Such a shift in the chemical state of gallium in serum in the presence of antibody might be related either to increased saturation of serum transferrin with iron (this is already high in the mouse at 75%), or to a failure in recycling of apo-transferrin from cells into extracellular fluids, or to both causes. It is known that the antibody–transferrin receptor complex can be endocytosed into erythroblasts, but is trapped in the early endosome compartment (Killisch *et al.* 1992).

The minimal effect of the antibody on ^{26}Al uptake into any of the tissues confirms the arguments from the *hpx/hpx* mice that there is little transferrin-mediated transport under our experimental conditions in any of the mice.

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