

The preparation of ^{59}Fe -labelled transferrin for ferrokinetic studies

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SUMMARY Serum labelled with ^{59}Fe for ferrokinetic studies should contain only [^{59}Fe] transferrin. [^{59}Fe] ferric citrate was incubated at 37°C for up to 24 hours with sera having an excess latent iron-binding capacity. The results showed that unbound [^{59}Fe] ferric citrate may be present for several hours. An anion exchange resin (IRA 400 Cl^-) column removed all the [^{59}Fe] ferric citrate and left only [^{59}Fe] transferrin in the eluate. It is suggested that all ^{59}Fe -labelled preparations for ferrokinetic studies should be passed through a resin column before injection.

Iron in the extracellular body fluids is maintained in solution bound mainly to transferrin (Bearn and Parker, 1966) although other forms may exist (Aisen and Leibman, 1968). Ferrokinetic studies are concerned with the behaviour of transferrin bound iron, and the importance of ensuring that other soluble forms of iron are excluded from a tracer dose has been recognized (Finch, Deubelbeiss, Cook, Eschbach, Harker, Funk, Marsaglia, Hillman, Slichter, Adamson, Ganzoni, and Giblett, 1970). The two iron-binding sites of transferrin have extremely high affinities for ferric iron (Aasa, Malmström, Saltman, and Vänngård, 1963) and it has generally been assumed that iron bound to other ligands, such as citrate, would rapidly and completely bind to any unsaturated transferrin. In preparing [^{59}Fe] transferrin a latent iron-binding capacity in serum greater than the amount of added iron should produce complete binding of the tracer to the transferrin. Studies *in vivo* have thrown doubt on this assumption. When [^{59}Fe] ferric citrate was incubated at 37°C for 30 min with plasma having an excess binding capacity and then injected intradermally up to 40% of the ^{59}Fe was cleared rapidly as unbound ferric citrate (Cavill and Jacobs, 1970). The preparation of [^{59}Fe] transferrin has been examined to see in what circumstances unbound iron remains in serum and how this can be prevented.

Methods and Results

^{59}Fe ferric iron is commercially available as either ferric chloride in 0.1N HCl or as ferric citrate in

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1% (w/v) sodium citrate pH 6.8 (Radiochemical Centre, Amersham). Before being used to label transferrin the ferric chloride is generally brought to neutral pH with sodium citrate (Dacie and Lewis, 1968). The result is a ferric citrate solution. In the commercial [^{59}Fe] ferric citrate preparation the citrate:iron ratio is approximately 250:1 but this may be even higher if neutralized [^{59}Fe] ferric chloride is used.

Standard haematological techniques (Dacie and Lewis, 1968) were used where appropriate. Serum iron concentration and total iron-binding capacity was measured by the method of Young and Hicks (1965) and latent iron-binding capacity per 5 ml serum was calculated from the difference.

SEPARATION OF [^{59}Fe] TRANSFERRIN AND [^{59}Fe] FERRIC CITRATE

Sephadex gel filtration (G 75) of an aliquot of 5 ml serum (latent iron-binding capacity $1.4 \mu\text{g}$ per 5 ml) incubated with 0.1 ml [^{59}Fe] ferric citrate, containing $1.2 \mu\text{g}$ iron, at 37°C for 30 min showed that a significant proportion of the activity was eluted as a small molecular weight fraction (Fig. 1). Unbound ferric citrate was still present after incubation with an excess of iron-free transferrin. Gel filtration was not, however, suitable for the routine removal of unbound ferric citrate.

Ferric citrate has an overall charge of -1 (Helbock and Saltman, 1967) and binds strongly to the anion exchange resin IRA 400 in the Cl^- form. The iron-transferrin complex at neutral pH also carries a negative charge but does not exchange readily with the resin. A serum and [^{59}Fe] ferric citrate mixture was passed through an IRA 400

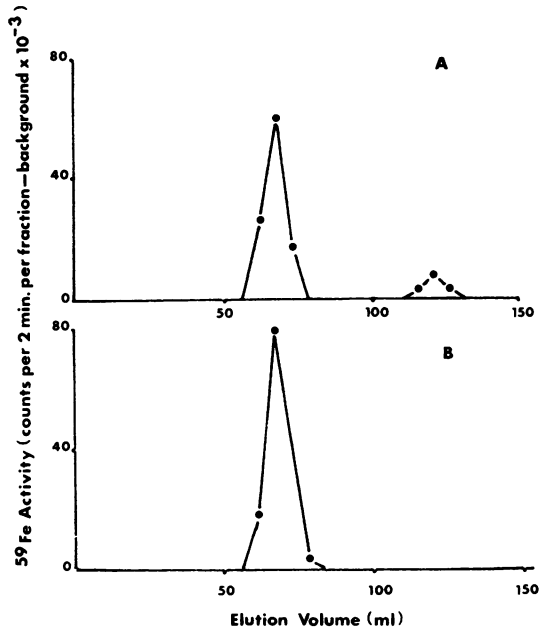


Fig. 1 Gel filtration on Sephadex G 75 of serum incubated with ^{59}Fe ferric citrate for 30 min at 37°C . A Before and B After passage through IRA 400 column.

Cl^- column and the eluate applied to a G 75 column. The elution pattern from the G 75 column showed that all the ^{59}Fe was associated with a large molecular weight, transferrin, fraction (Fig. 1).

A short resin column, contained in the barrel

of a 1 ml plastic Mantoux syringe, removed all of the ^{59}Fe ferric citrate from a mixture of 0.5 ml ferric citrate, $6\ \mu\text{g}$ iron, and 5 ml of either saline or serum that had previously been saturated with iron. Repeated passage of ^{59}Fe -labelled transferrin through fresh resin columns failed to remove any ^{59}Fe activity. Two ml ^{59}Fe ferric citrate ($8.6\ \mu\text{g}$ per ml) was incubated with 20 ml of five separate sera (latent iron-binding capacity 10-310 μg per 100 ml) at 37°C for six hours. The percentage ^{59}Fe binding was estimated in quadruplicate for each serum and showed a mean coefficient of variation of 3% of the estimated percentage binding. The short IRA 400 Cl^- resin column was a quick and effective method of separating ^{59}Fe bound to transferrin or chelated to citrate.

^{59}Fe -TRANSFERRIN BINDING AND TIME

Sera, with varying iron-binding capacities, were mixed with ^{59}Fe ferric citrate and incubated at 37°C . Samples were removed at intervals and passed through a short IRA 400 Cl^- column. The percentage ^{59}Fe bound at each instant is shown in Figure 2. A latent iron-binding capacity two to six times greater than the amount of iron added (A and B) produced a rapid initial rate of binding although in one case the maximum binding was achieved only after two hours' incubation. Binding was slower when the latent iron-binding capacity was only twice the amount of iron added and 24 hours were required to achieve a maximum (C and D). When the iron added was 25 times the latent iron-binding capacity then ^{59}Fe transferrin binding, although only 6%, was virtually instantaneous (E). These results show that it is impossible to assume

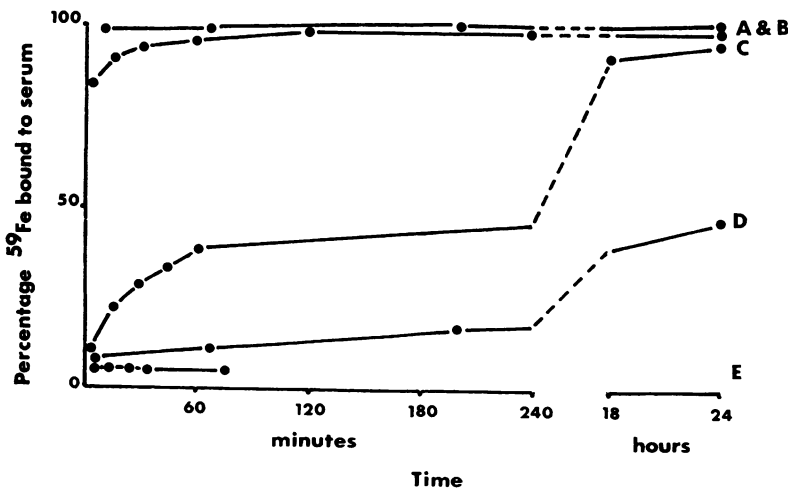


Fig. 2 The effect of time on the percentage ^{59}Fe bound to five sera incubated with ^{59}Fe ferric citrate at 37°C .

Serum	Latent Iron-binding Capacity per 5 ml Serum (μg)	Iron Added (μg)
A	40.0	8.6
B	30.0	4.6
C	4.0	1.8
D	14.0	8.6
E	0.5	12.4

that an excess latent iron-binding capacity and incubation at 37°C will produce complete ^{59}Fe binding to transferrin in the presence of a high citrate concentration.

A METHOD FOR PREPARING [^{59}Fe] TRANSFERRIN
Depyrogenized IRA 400 (Amberlite) is prepared in the Cl^- form by soaking the resin in 11N HCl for one hr and is then washed with saline until acid free. The barrel of a 1 ml plastic Mantoux syringe is filled up to the 0.8-1.0 ml mark with moist resin.

Twelve ml venous blood is defibrinated by mixing with depyrogenized glass beads for 15 minutes. Five ml serum is added to not more than 0.5 ml [^{59}Fe] ferric citrate and allowed to stand at room temperature for five minutes. Sufficient ^{59}Fe activity for ferrokinetic studies is usually bound to the transferrin in this time. Of the solution, 0.5 ml is added to the column and the eluate discarded. The remainder is then added and the eluate, containing only transferrin bound ^{59}Fe , is sterilized by passing it through a 0.45 μm Millipore filter. The percentage ^{59}Fe binding is calculated by counting the activity in an aliquot of solution before and after it has been through the column.

^{59}Fe BINDING TO VARIOUS SERA AND TO PURIFIED HUMAN TRANSFERRIN

The percentage ^{59}Fe binding to the sera of 17 normal subjects, in which the latent iron-binding capacity per 5 ml serum was 2-15 (mean, 3) times greater than the amount of iron added, was between 80 and 100%. The mean value (90%) was significantly less than 100% ($t = 5.4913$, $P < 0.001$). Sera from seven iron-deficient patients, in whom the latent iron-binding capacity per 5 ml serum was between 2 and 26 (mean, 8) times the added iron bound between 89 and 100% of the ^{59}Fe . The mean binding (94%) was significantly less than 100% ($t = 3.6207$, $P < 0.02$) but was not significantly greater than in normal subjects. In five patients with an iron overload, as a result of haemochromatosis or transfusion siderosis, the iron added to their sera was four to 20 times its latent iron-binding capacity per 5 ml serum and ^{59}Fe binding was between 4 and 8%.

Twenty mg purified human transferrin (Behringwerke, A. G. Marburg/Lahn) was dissolved in

0.4 ml [^{59}Fe] ferric citrate, containing 3.7 μg iron. After incubation for one hr at 37°C only 78% of the ^{59}Fe was transferrin bound. This was considerably less than would be expected and suggests that the iron-binding capacity of the transferrin had been impaired during the purification process.

Although the ratio of the serum latent iron-binding capacity to the iron added is an important factor governing the percentage ^{59}Fe binding it seems likely that a high citrate:iron ratio may also have an effect (Bates, Billups, and Saltman, 1967). These results show that an excess latent iron-binding capacity, which may occur in both normal and iron-deficient patients, does not ensure complete ^{59}Fe binding in the presence of a high citrate concentration. In order to ensure that only ^{59}Fe bound to transferrin is used as a tracer in ferrokinetic studies all preparations should be passed through an anion exchange column before use to remove any unbound ferric citrate. This is especially important in patients with a reduced latent iron-binding capacity. The presence of unbound ^{59}Fe in 'labelled' serum or plasma may lead to aberrant results in studies of plasma clearance and tissue distribution.

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