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CHROMATOGRAPHIC FRACTIONATION OF HUMAN RETICULOCYTES AFTER UPTAKE OF DOUBLY LABELLED [^{59}Fe, ^{125}I]TRANSFERRIN

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SUMMARY

1. After exposure to [^{59}Fe, ^{125}I]transferrin, human reticulocytes were separated into membrane (stroma) and water-soluble (cytosol) fractions.

2. The cytosol yielded two ^{59}Fe-bearing components on Sephadex G-200 columns: haemoglobin and a higher molecular weight component which was not further analysed.

3. Membrane solubilised in Triton X-100 was separated by sequential chromatography into three ^{59}Fe-bearing components called A, B1 and B2. Sepharose 2B excluded component A which was thus of high particle size.

4. Passage through Sepharose 2B was an obligatory step in the separation of B1 and B2 from A. Prior to this step, B1 and B2 were not retarded by a Sepharose 4B column whereas after Sepharose 2B both were retarded in Sepharose 4B and also in Sepharose 6B, by which they were more clearly separated. Thus, Sepharose 2B appears to play an active part in the detachment and separation of these components.

5. Component B1 had a molecular weight of the order of 10^6 and was associated with the main membrane protein.

6. Component B2 had a molecular weight of 230000 and was associated with all the membrane ^{125}I.

7. We conclude that component B2 is a complex of iron–transferrin and a membrane binding site. The binding site has a molecular weight about 150000, assuming a 1:1 molar ratio.

INTRODUCTION

It has been shown that immature red cells including reticulocytes obtain their main supply of iron from plasma transferrin (Jandl et al. [1], Pollycove and Mortimer [2]). Reticulocytes but not mature erythrocytes bind iron–transferrin to the surface and return apotransferrin to the medium [3,4]. The principal destination of this iron is its incorporation into haem in mitochondria (Sano et al. [5]). Attempts have been made to define more closely the process of transport between iron uptake at the membrane surface and its incorporation into haem. Allen and Jandl [6] identified a soluble protein iron carrier. Similar soluble intermediates have been studied by several other workers. Greenough et al. [7], using IRC-50 chromato-

graphic analysis described an iron–protein intermediate which they called Fraction 1. In later work [8] other soluble fractions were identified including ferritin and a low-molecular-weight iron component derived from Fraction 1. Mazur and Carlton [9] demonstrated the formation of ferritin in reticulocytes and concluded that it was an intermediate in haem synthesis. Zail et al. [10] also paid special attention to ferritin in the soluble fraction of reticulocytes exposed to [^{59}Fe] transferrin and concluded that ferritin was formed only when more iron enters the cell than can be utilised for haemoglobin synthesis, in other words, ferritin was not an intermediate in haem synthesis. The problem of accounting for the disappearance of ferritin from the reticulocyte during maturation was not resolved.

There have been few attempts to characterise intermediates associated with membrane fractions of reticulocytes. Allen and Jandl [6] used differential centrifugation to fractionate stroma; Falbe-Hansen and Lothe [11,12] subjected homogenised reticulocyte ghosts to starch electrophoresis. They identified three labelled fractions, one of which resembled ferritin. Garrett et al. [13] solubilised reticulocyte membrane with surface active agents and organic solvents, followed by fractionation on Sephadex G-200 and Bio Gel A-15m. They identified an iron-binding macromolecule and free transferrin.

In previous work (Fielding et al. [14], Edwards and Fielding [15]) it was shown that sulphydryl inhibitors in low concentration, inhibited the uptake of iron into reticulocytes, but did not inhibit the binding of iron transferrin to the reticulocyte surface. We have used this blocking system to investigate membrane and intracellular transport of iron after the binding of iron transferrin (Fielding, J. and Speyer, B. E., unpublished).

We report here techniques of solubilisation and chromatography which have separated whole reticulocytes, both soluble and membrane fractions, into clearly defined iron-bearing components.

MATERIALS AND METHODS

^{59}Fe, ^{125}I-Labelled transferrin

Purified transferrin was labelled as previously described (Edwards and Fielding [15]) except that a solution of $^{59}FeCl_3$ of higher activity (3–30 Ci/g Fe) was used in order to give adequate counting rates after fractionation. Iodination followed the method of Katz [16] using iodine in $Na^{125}I$ solution.

Reticulocyte suspensions

Human reticulocyte suspensions were prepared as previously described [15]. The main clinical sources were patients with pernicious anaemia responding to vitamin B12 therapy and patients with acute haemolytic anaemia.

Sephadex and Sepharose gels were supplied by Pharmacia Ltd; $^{59}FeCl_3$ and $Na^{125}I$ solutions from Radiochemical Centre, Amersham; horse-spleen ferritin from Koch-Light Laboratories Ltd; catalase and yeast alcohol dehydrogenase from Sigma Chemical Co. Ltd. Glass chromatographic columns were used, since Triton X-100 was observed to attack some plastic columns; glass columns of 150 cm were supplied by Wright Scientific Ltd and collodion bags for concentrating fractions by V. A. Howe and Co Ltd.

^{59}Fe and ^{125}I activities were counted in a Packard autogamma spectrometer; ^{125}I required correction for ^{59}Fe counts in the ^{125}I channel. In plotting ^{59}Fe and ^{125}I in the figures, the actual counts were corrected to a standard by which the counts of ^{59}Fe and ^{125}I in the original labelled transferrin have been equalised. Thus the counts of either ^{59}Fe or of ^{125}I are comparably related to the original transferrin from which they are derived.

Protein was estimated by the method of Lowry et al. [17]. In material containing Triton X-100 a yellow precipitate formed during the colour-producing step. This was centrifuged and the extinction of the clear supernatant compared with standards to which Triton had been added at the start of the estimation. The presence of Triton did not interfere with the assay method, but it is important to note that standards containing Triton must be used under these conditions.

Experimental procedures

A 50% (v/v) suspension of reticulocyte-rich erythrocytes in Hank's solution was warmed to 37 °C and 1/5th its volume of labelled transferrin solution added. The final concentration of transferrin protein was 1.4 mg/ml. The transferrin–reticulocyte mixtures were incubated for 30 min, except where the effect of incubation time as a variable was being investigated. Immediately after incubation, the tubes were chilled in ice-cold water and 2 vol. of ice-cold 310 ideal-milli-osmolar (imosM) [18] sodium phosphate buffer, pH 7.4, was added. The suspension was centrifuged at $1000 \times g$ for 4 min, the supernatant removed and the cells washed four times with 8 vol. of ice-cold 310 imosM buffer centrifuging at $1000 \times g$ for 4 min at each washing.

Separation of soluble fraction ("cytosol") from deposit ("membrane") after haemolysing the cell suspension followed the method of Dodge et al. [18]. The washed cells, in a volume of 0.5–2 ml, were haemolysed by dropwise addition to 10 ml ice-cold 20 imosM sodium phosphate buffer, pH 7.4, with continual shaking. Tube and pipette were washed out with a further 4 ml of buffer. After mixing, the haemolysate was centrifuged at $20\,000 \times g$ for 20 min at 4 °C. The supernatant was separated as far as possible without disturbing the deposit and the latter washed 4 times with 14 ml of 20 imosM buffer. After the final suspension, a small clump of fibrous material was often present in the tube and was removed with a fine glass rod. These small amounts of material contained detectable amounts of ^{125}I and of ^{59}Fe, were insoluble in Triton X-100 and are probably derived from leukocytes and nucleated red cells [19]. At this stage cytosol and membrane material were stored at 4 °C before chromatography. Time of storage did not affect the pattern of subsequent chromatography.

In preliminary experiments sodium dodecylsulphate was also used to solubilise membranes. We found, however, that dodecylsulphate splits iron from iron–transferrin and also splits ^{59}Fe from membrane material (Speyer, B. E., and Fielding, J., unpublished). These effects were not seen with Triton X-100.

Cytosol fractionation

2-ml portions of cytosol were passed by downward flow through columns of Sephadex G-200, 30 cm × 1.5 cm diameter, previously equilibrated with 50 mM sodium phosphate buffer, pH 7.4, and followed with the same buffer. Fractions

were collected in 2.4-ml volumes at a flow rate of 14ml/h. Recoveries of ^{59}Fe from the column were close to 100%, although traces of ^{59}Fe retained on the column tended to interfere with subsequent runs, unless special care was taken. After each run, the column was washed with 150 ml of 50 mM citric acid which removed retained ^{59}Fe, and the column was then again equilibrated by 200 ml 50 mM sodium phosphate buffer.

Primary membrane fractionation

All columns used for fractionation of membrane prepa rations were equilibrated with 5 imosM sodium phosphate buffer at pH 7.4 containing 1% (v/v) Triton X-100 and the same buffer was used for the flow system. The low salt concentration used, approximately 2 mM had been found by Miller [20] to aid solubilisation of the erythrocyte membrane by Triton.

The washed membrane preparation suspended in 1–2 ml of phosphate buffer was solubilised by the addition of one quarter its volume of 5% (v/v) Triton X-100 and incubated at 37 °C for 15 min. A water-clear very pale yellow solution was obtained. The solubilised preparation was then passed by upward flow through a column of Sepharose 2B, 30 cm × 1.5 cm diameter. Fractions of 2.4 ml volume were collected at a flow rate of 3.6 ml/h in a Chromofrac fractionator (Baird and Tatlock Ltd).

Secondary membrane fractionation

Sepharose 6B, 4B and Sephadex G-200 columns were used for further analysis of pooled fractions derived from the Sepharose 2B column. The pooled fractions were concentrated to approximately 1 ml by vacuum ultra-filtration through collodion bags. In Sepharose 6B the concentrate was passed by upward flow through columns 150 cm × 1.6 cm at a flow rate of 6 ml/h and 2.4 ml fractions collected. In Sepharose 4B columns 50 cm × 1.5 cm were used at a flow rate of 6 ml/h, and 2.4 ml fractions collected. In Sephadex G-200 columns 90 cm × 1.5 cm diameter were used at a flow rate of 2.4 ml/h and 2.4-ml fractions collected.

Recoveries of ^{59}Fe from columns

Recoveries of ^{59}Fe from Sepharose 2B and Sephadex G-200 were close to 100% (mean 100.4%, S. D. 7.6%). Recoveries from Sepharose 6B (that is, of pooled and concentrated fractions from Sepharose 2B columns) were more variable and ranged from 55 to 77%. The precise explanation for this loss of iron in Sepharose 6B under these conditions was not determined but since iodine recoveries were 100% and iodine was invariably associated with fraction B2 (see below) such loss of iron as occurred in this column appeared to have come from fraction B1.

Column markers

Markers were run through the columns under the same conditions as the cell preparations. Transferrin, ferritin, catalase and yeast alcohol dehydrogenase were incubated in Triton–sodium phosphate buffer for 15 min at 37 °C before passing through the columns.

RESULTS

Fractionation of cytosol preparation on Sephadex G-200

In all experiments ^{59}Fe in the cytosol preparation was eluted from Sephadex G-200 as two well defined peaks (Fig. 1). The first peak in the void volume was subsequently named component C. The second peak comprised the haemoglobin of cytosol. Component C appears to be the same as fraction 1 described by Greenough et al. [7] and by Zail et al. [10] since it is the only peak in addition to that of haemoglobin derived from cytosol.

A very small amount of ^{125}I was eluted as a single peak from the cytosol preparation emerging just in advance of haemoglobin and in the same position as marker transferrin. This small iodine peak presumably represents free transferrin associated with the cell at the end of the 30 min reticulocyte incubation period.

Fractionation of membrane preparation on Sepharose and on Sephadex G-200

The behaviour of the membrane preparation on Sepharose columns was unusual. Fig. 2 shows that when passed through Sephadex G-200 or Sepharose 4B, ^{59}Fe was eluted as a single peak in the void volume. From 4B a slight shoulder is seen behind the main void volume peak. However, when passed through Sepharose 2B, it eluted as two peaks, referred to as component A and component B. When component B was re-run in Sepharose 4B, all ^{59}Fe was retarded by the column

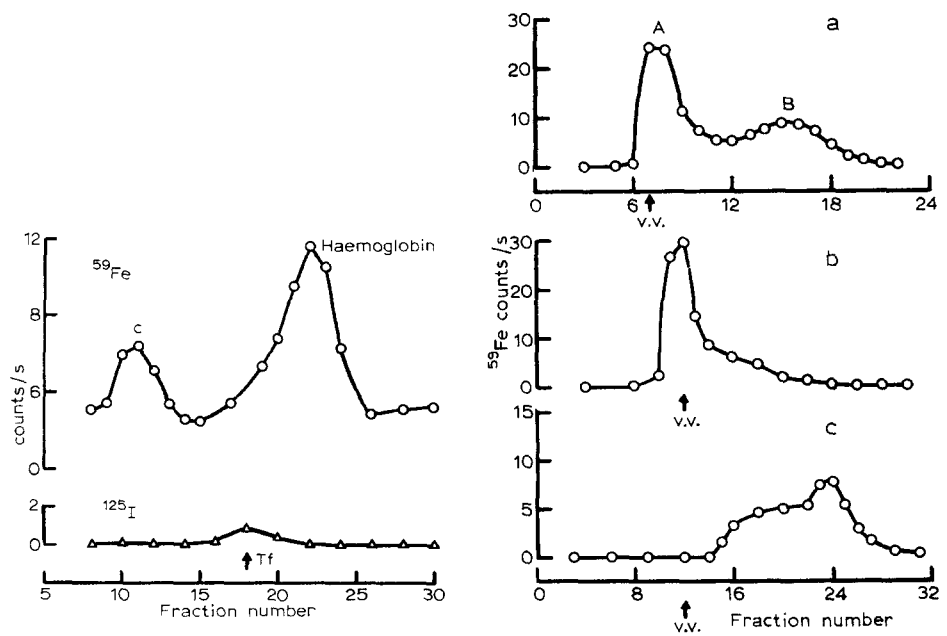

Fig. 1. Fractionation of reticulocyte cytosol on Sephadex G-200, Tf, transferrin marker; ○, ^{59}Fe (counts/s); △, ^{125}I (counts/s).

Fig. 2. Breakdown of membranes by Sepharose 2B. (a) Primary chromatography through Sepharose 2B. (b) Primary chromatography through Sepharose 4B. (c) Secondary passage of peak B through Sepharose 4 B. v/v, void volume.

and none appeared in the void volume. Since first passage through Sepharose 4B did not lead to the emergence of component B, even after exposure to Triton X-100 for as long as 24 h before chromatography, it appears that passage through Sepharose 2B is an obligatory step in the separation of component B from the membrane preparation in our method. Thus, component B studied as described below, was necessarily always prepared by initial passage through Sepharose 2B.

Repassage of component A through Sepharose 2B gave no alteration in its distribution (Fig. 3). There was no breakdown during the second passage through the column and thus component A and component B are qualitatively distinct.

The ^{125}I label, representing transferrin protein in the membrane preparation, was eluted in component B (Fig. 4). Most of the membrane protein as assayed by Lowry's method, also emerged in the region of peak B. There was little protein associated with component A.

Component A was also studied by centrifugation. After $500 \times g$ for 15 min, 90% of ^{59}Fe remained in the supernatant; after $95000 \times g$ for 60 min a scanty deposit was seen which contained 80% of the ^{59}Fe of component A. From this finding and its position in the void volume on Sepharose 2B, it is evident that this component is of high particle size.

Secondary fractionation of component B on Sepharose 6B

The interrelation of ^{59}Fe, ^{125}I and of protein in membrane component B was further studied by fractionation in Sepharose 6B. Inspection of component B derived from Sepharose 2B columns showed that the peak of ^{125}I consistently

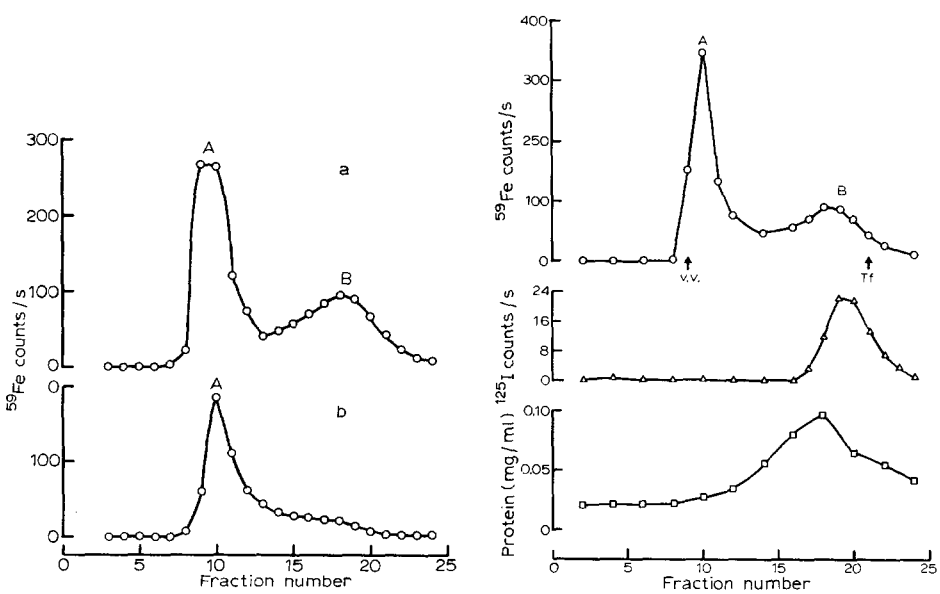

Fig. 3. (a) Primary chromatography of membrane through Sepharose 2B. (b) Re-chromatography of pooled fractions comprising peak A showing absence of peak B.

Fig. 4. Fractionation of reticulocyte membrane on Sepharose 2B. v.v, void volume; Tf, transferrin marker; ○, ^{59}Fe (counts/s); △, ^{125}I (counts/s); □, protein (mg/ml).

emerged from the column slightly later than the peak of ^{59}Fe (Fig. 4). This separation of the isotopes suggested that component B comprised two sub-components. The fractions comprising the component B from Sepharose 2B were therefore pooled, concentrated by ultrafiltration and passed through a 150 cm × 1.6 cm column of Sepharose 6B. Two well-separated peaks of ^{59}Fe were eluted (Fig. 5). These are referred to as component B1 and component B2. B1 was associated with most of the membrane protein and had a particle size larger than that of a ferritin marker, i.e. more than 470000. Two peaks were also observed on secondary passage of component B through both Sepharose 4B and through G-200 but the clearest separation was obtained in the 150 cm column of Sepharose 6B. There was no ^{125}I associated with B1.

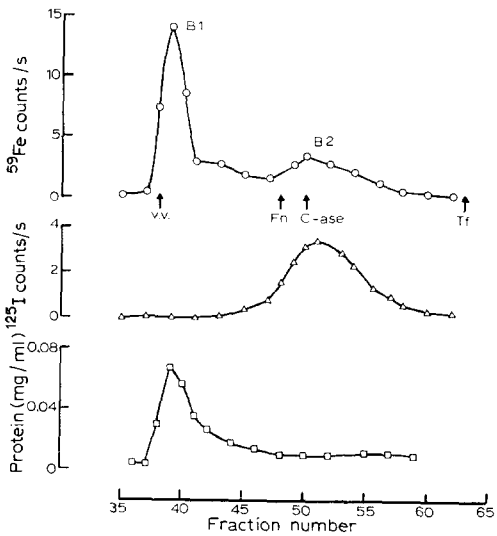

Fig. 5. Chromatography of peak B from Sepharose 2B on a column of Sepharose 6B. v.v, void volume; Fn, ferritin marker; C-ase, catalase marker; Tf, transferrin marker; ○, ^{59}Fe (counts/s; △, ^{125}I (counts/s); □, protein (mg/ml).

Component B2 had a far smaller content of ^{59}Fe than B1, but it contained all the ^{125}I of the membrane preparation. It was eluted from the column in the same fractions as a catalase marker indicating a molecular weight of about 230000 (Schroeder et al. [21]), well in advance of a transferrin marker. Since component B2 contained all the membrane ^{125}I and is associated with ^{59}Fe, it is reasonable to assume that iron-transferrin is present in this component. Since the molecular weight of iron transferrin is about 80000, these findings suggest that B2 comprises a complex of iron–transferrin bound to a component of the membrane.

DISCUSSION

Most previous studies on iron-labelled components of reticulocytes have dealt either with membrane (stroma) or with cytosol (supernatant) but not with both simultaneously. However, the distinction between membrane and cytosol

is somewhat arbitrary and depends on technical detail of separation. For instance, membrane components other than those "intrinsic" to the membrane [22] may be extracted into the cytosol, and on the other hand, whole or fragmented organelles may be included in membrane components, depending on speed of centrifugation and other conditions. Clearly, procedures which deal with whole reticulocytes in as quantitative a manner as possible are to be preferred in transport pathway studies.

With the techniques described here, whole reticulocytes after incubation with doubly labelled transferrin, were cleanly and reproducibly separated into five distinct labelled components. No labelled reticulocyte material was discarded. Our method for fractionation was found to be quantitative for ^{59}Fe in membrane component A and B, and in cytosol component C and haemoglobin; the further separation of component B into its two sub-components B1 and B2 was semi-quantitative. The method is therefore suitable for kinetic and inhibition studies on the movement of iron between components within the reticulocyte, and studies of this type have been carried out (Fielding, J., and Speyer, B. E., unpublished).

The role of the various Sepharose gels in the separation of the ^{59}Fe-binding membrane components in our method is of some interest. Initial passage of the membrane preparation in Triton X-100 through Sephadex G-200 or Sepharose 4B even after prolonged contact with Triton, yielded a single labelled peak in the void volume, whereas initial passage through Sepharose 2B yielded two well defined peaks. Once this procedure of primary passage through Sepharose 2B had been followed, peak B could be re-chromatographed on Sepharose 4B and now yielded two retarded components B1 and B2. Furthermore, these separate peaks were now consistently evident in eluates from Sepharose 6B and Sephadex G-200. Thus passage through Sepharose 2B plays an important role in the detachment and solubilisation of the B complex from the membrane. Such an effect due to Sepharose has not to our knowledge been previously described.

We have not further studied component C or subjected it to further fractionation. This soluble fraction appears to be similar to that isolated by other workers [6–8]. From its behaviour in Sepharose 2B columns and on centrifugation, it is clear that component A is of large particle size. It is therefore possible that it is a particle associated with the cytosol or one of the cytoplasmic organelles. However, in experiments not reported here (Fielding, J. and Speyer, B. E. unpublished) it was found that the ratio of component A to component B in the pre-solubilised material spun at $500 \times g$ was the same as the ratio after centrifugation at $20000 \times g$. The membrane is thus homogeneous with respect to the two components and despite its large particle size, component A would appear to be membrane associated.

Component B1 was recovered in the same fractions as most of the membrane protein and its position on the column suggests a molecular weight of the order of 10^6. Garrett et al. [13] have recently studied the effect of solubilising agents on reticulocyte stroma after incubation with doubly labelled transferrin. They treated the stroma with 1% deoxycholate, centrifuged at $100000 \times g$ and discarded the deposit which contained 60% of the stromal ^{59}Fe. They obtained from the solubilised material two peaks on Sephadex G-200, one of which ran with marker transferrin and the other was a macro-molecule of molecular weight 350000–700000 containing both ^{59}Fe and ^{125}I. The latter may bear some resemblance to our B1 fraction,

although it is seen to contain some ^{125}I whereas B1 was free from this isotope. Their second eluted peak is however free transferrin and thus not similar to our B2 component.

Component B2 contains all the ^{125}I of the membrane. It is clear therefore that all the membrane-associated transferrin is contained in this fraction. However, it is eluted from the column at a point which indicates a molecular weight of 230000 (shown by a catalase marker) and thus the transferrin (molecular weight about 80000) is present bound to a membrane component. Furthermore since ^{59}Fe is associated with this fraction, it would appear that the iron–transferrin with which the reticulocytes were incubated, is here bound with its membrane binding site. If there is a mole–mole relationship between iron transferrin and its binding site, this would give a molecular weight of approximately 150000 to the receptor.

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