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IRON TRANSPORT INTERMEDIATES IN HUMAN RETICULOCYTES AND THE MEMBRANE BINDING SITE OF IRON-TRANSFERRIN

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SUMMARY

1. The iron-bearing components obtained by chromatographic fractionation of human reticulocytes have been further studied in relation to their role in intracellular iron transport.

2. In timed and chaser experiments membrane component A, like haemoglobin, behaved as an end product, while membrane component B1 and cytosol component C behaved as intermediates.

3. We confirm that component B2, molecular weight 230 000, behaves as a complex of iron–transferrin and its membrane binding site.

4. Sulphydryl inhibition with *p*-hydroxymercuribenzoate does not affect iron–transferrin binding, but blocks the pathway between components B2 and B1 leading to an accumulation of the iron–transferrin–receptor complex.

5. The results suggest that initially iron moves from the iron–transferrin–receptor complex (B2) to membrane component B1 from which it may diverge into membrane component A or follow the main pathway through cytosol component C to haemoglobin.

INTRODUCTION

In a previous communication (Speyer and Fielding [1]) we described the chromatographic fractionation of reticulocytes after incubation with doubly labelled [^{59}Fe, ^{125}I]–transferrin into four iron-bearing components in addition to haemoglobin. Three of these components were associated with membrane and were called A, B1 and B2. It was postulated that the B2 membrane component of molecular weight about 230 000 comprised the complex of iron–transferrin and its binding site of molecular weight about 150 000. In the cytosol two components were recognised, haemoglobin and component C of higher molecular weight.

We report here the dynamic inter-relationships of these components. These have been investigated by studying the effect of varying incubation time of reticulocytes with labelled transferrin, and by chaser experiments observing the effect of further incubation with non-radioactive iron–transferrin.

It has been shown (Fielding et al. [2], Edwards and Fielding [3]) that sulphy-

dryl inhibitors such as *p*-hydroxymercuribenzoate in low concentration inhibit reticulocyte uptake of iron, although the binding of iron–transferrin to the cell surface is unaffected. The effect of *p*-hydroxymercuribenzoate inhibition on the distribution of ^{59}Fe in the iron-bearing membrane and cytosol components was therefore also studied.

MATERIALS AND METHODS

The chromatographic fractionation of human reticulocytes after uptake of doubly-labelled transferrin has already been described in detail (Speyer and Fielding [1]). Briefly, the water soluble (cytosol) fractions were chromatographed on Sephadex G-200 and produced two ^{59}Fe components, haemoglobin and component C in the void volume. The membrane (stroma) fractions were solubilised in Triton X-100 and separated by sequential chromatography first through Sepharose 2B and then through Sepharose 6B to produce three iron bearing components A, B1 and B2. Primary chromatography through Sepharose 2B was an obligatory step in this procedure.

Varying incubation time with [^{59}Fe, ^{125}I]–transferrin

The details of incubation of reticulocytes with labelled transferrin have been described [1]. Incubation was continued for varying intervals of time from 5–64 min, arranged so that all incubation times were completed together and the tubes chilled in ice-cold water until processed with the minimum of delay.

Chaser experiments

One vol. of a 50 % (v/v) suspension of reticulocyte-rich erythrocytes in Hank's solution was incubated with one fifth its volume of labelled transferrin solution at 37 °C in each of several tubes. After incubation for 15 min the tubes were chilled in ice-cold water, and two vol. of ice-cold Hank's solution was added to each. The suspensions were centrifuged at 1000 × *g* for 4 min, the supernatants removed, and the cells washed 3 times with eight vol. of ice-cold Hank's solution, centrifuging at 1000 × *g* for 4 min at each washing. The tubes were then placed in ice-cold water. At several timed intervals from 0–60 min one of the tubes was warmed to 37 °C and half a vol. of Hank's solution followed by one fifth vol. of non-radioactive iron–transferrin was added. After 60 min, incubation was stopped in all tubes by chilling in ice-cold water and adding two vol. ice-cold 310 imosM sodium phosphate buffer, pH 7.4. Subsequent washing, haemolysis and fractionation were as previously described.

Inhibition of ^{59}Fe uptake by p-hydroxymercuribenzoate

Sodium *p*-hydroxymercuribenzoate (Sigma Chemical Co.) was dissolved in 0.145 M NaCl by warming to 50 °C to give a solution twice the final concentration required. One volume of a 50 % (v/v) suspension of reticulocytes in Hank's solution was warmed to 37 °C and one vol. *p*-hydroxymercuribenzoate solution at 37 °C added. A control suspension received one vol. of 0.145 M NaCl instead of *p*-hydroxymercuribenzoate solution. Incubation at 37 °C was continued for 15 min. The tubes were centrifuged at 1000 × *g* for 4 min, and the cells washed four times with ice-cold Hank's solution, centrifuging at 1000 × *g* for 4 min each time. One half volume of

Hank's solution was added, the cell suspension was warmed to 37 °C and one fifth vol. of doubly labelled transferrin solution was added. Incubation was continued for 30 min, and the cells were washed and fractionated as previous described.

^{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 were derived.

Reticulocyte suspensions

In addition to the clinical sources previously described, [1], some patients with severe iron deficiency anaemia with or without acute blood loss, responding to iron therapy, were included.

RESULTS

Effect of varying time of incubation with transferrin on ^{59}Fe and ^{125}I content of components

The effect of varying the time of incubation of reticulocyte suspension with labelled transferrin on the relative amounts of the iron-bearing membrane components A, B1 and B2 and the cytosol components C and haemoglobin, is shown (Figs 1 and 2). The amounts of all components increase with time but at different rates. The total counts comprising each peak are plotted in Fig. 3. It is seen that the ^{59}Fe content of membrane component A increased linearly with time, resembling in behaviour the haemoglobin component of the cytosol. However, the amounts of ^{59}Fe in membrane component B and in cytosol component C tend to a maximum with time. The increase in component C precedes that of haemoglobin (Fig. 2).

The effects on sub-components B1 and B2 are shown in Fig. 4. All membrane

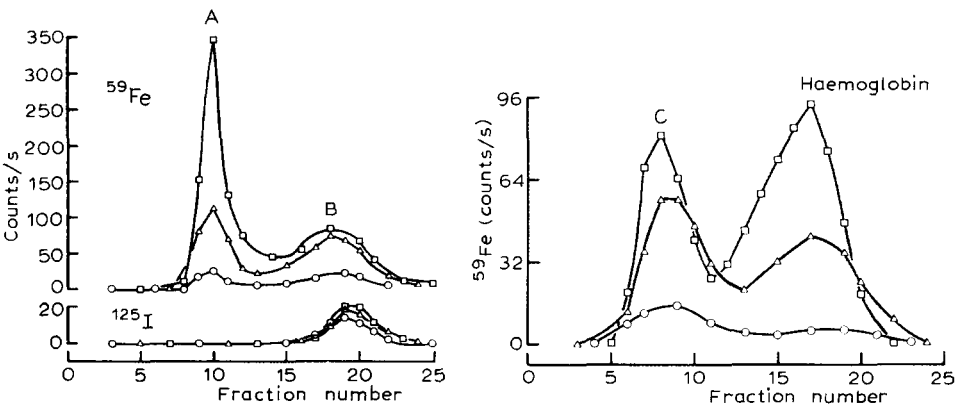
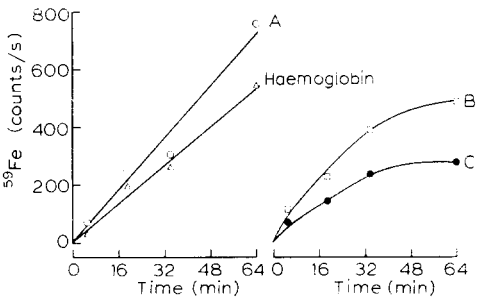


Fig. 1. Fractionation of reticulocyte membrane on Sepharose 2B. Effect of time of incubation with transferrin on ^{59}Fe and ^{125}I content. ○-○, 5 min; △-△, 34 min; □-□, 64 min.

Fig. 2. Fractionation of reticulocyte cytosol on Sephadex G-200. Effect of time of incubation with transferrin on ^{59}Fe and ^{125}I content. ○-○, 5 min; △-△, 34 min; □-□, 64 min.



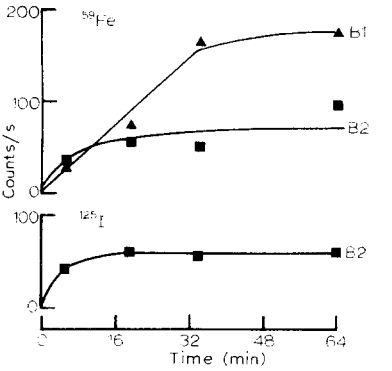


Fig. 3. Effect of time of incubation on ^{59}Fe content of reticulocyte components. ○, component A; △, haemoglobin; □, component B; ●, component C.

Fig. 4. Effect of time of incubation on ^{59}Fe and ^{125}I content of sub-components B1 and B2. ▲, component B1; ■, component B2.

^{125}I is associated with B2 and rapidly reaches a maximum in 15–20 min, resembling the pattern in whole cells. The ^{59}Fe content of component B2 increases with time approximately in parallel with ^{125}I. Component B1 reaches a maximum after component B2, in about 30 min.

Effect of chase with non-radioactive iron-transferrin

The redistribution of ^{59}Fe among the components after further incubation with non-radioactive iron–transferrin was studied in five experiments (Table 1). In all experiments the ^{59}Fe in components B and C decreased rapidly during the early part of the chase, suggesting that these components are metabolic intermediates. The haemoglobin component increased over the whole period. However the behaviour of ^{59}Fe in component A varied. It showed a rapid increase in one experiment but a minor decrease in three and a minor increase in one. In three experiments the increase in haemoglobin was greater than the fall in component C, indicating a transfer from

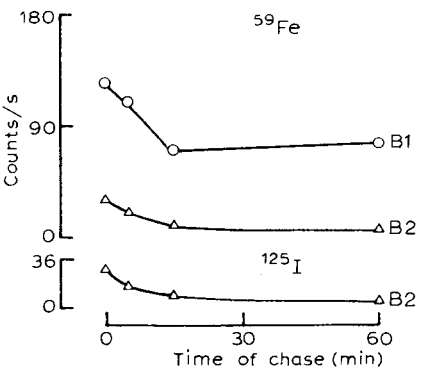


Fig. 5. Effect of chase with non-radioactive iron–transferrin on ^{59}Fe and ^{125}I content of sub-components B1 and B2. ○–○, component B1; △–△, component B2.

TABLE I

REDISTRIBUTION OF ^{59}Fe IN RETICULOCYTE COMPONENTS DURING CHASE WITH NON-RADIOACTIVE IRON-TRANSFERRIN

Time of chase (min)	Total ^{59}Fe membrane plus cytosol (counts/s)	Component A		B		C		Haemoglobin		Total ^{59}Fe in all components (counts/s)	Total column recovery (%)
		a*	b**	a	b	a	b	a	b		
Experiment 1											
0	173	41	(25)	60	(37)	16	(9)	48	(29)	165	95.4
15	173	..				17		59		..	
30	156	36	(24)	36	(25)	18	(12)	59	(39)	149	95.5
60	162	36	(24)	41	(28)	2	(2)	69	(47)	149	92.0
Experiment 2											
0	274	35	(13)	67	(24)	27	(10)	145	(53)	275	100.4
90	263	41	(16)	44	(17)	14	(5)	166	(63)	264	100.4
210	270	48	(16)	42	(14)	13	(4)	188	(65)	291	108.1
Experiment 3											
0	737	296	(47)	161	(25)	45	(7)	134	(21)	636	86.3
15	623	275	(53)	79	(15)	40	(8)	130	(25)	525	84.3
60	589	257	(48)	91	(17)	26	(5)	165	(31)	539	91.5
Experiment 4											
0	1405	346	(24)	345	(24)	336	(24)	402	(28)	1429	101.7
40	1269	307	(24)	280	(22)	188	(14)	523	(40)	1298	102.3
Experiment 5											
0	749	108	(16)	234	(35)	147	(22)	190	(28)	679	90.7
15	704	138	(21)	198	(30)	131	(20)	200	(30)	667	94.7
30	673	202	(31)	132	(20)	125	(19)	193	(30)	651	96.7
60	648	193	(30)	133	(21)	86	(13)	235	(36)	647	99.8

* Counts/s recovered in each component.

** Numbers in parentheses indicate percent of total ^{59}Fe recovered.

membrane to cytosol. It is clear that membrane component B transfers ^{59}Fe to the cytosol components during the chase, and also transfers a variable amount to component A. The behaviour of the sub-components B1 and B2 during the chase is shown in Fig. 5. As in the incubation-timed experiment all the ^{125}I of component B is found in sub-component B2, and it decreases progressively during the chase. This decrease parallels the loss of ^{125}I from whole cells during the chase. Thus the loss of ^{125}I from component B2 represents loss of labelled membrane-associated transferrin into the medium during final incubation, during which time some loss of ^{59}Fe into the medium also occurs (Table I).

The small amounts of ^{125}I found in the cytosol fraction, previously shown to be free transferrin (Speyer and Fielding [1]) also decreased as the chase proceeded. There is no evidence here that transferrin protein enters the reticulocyte beyond binding to the membrane.

Effect of p-hydroxymercuribenzoate inhibited iron uptake

The effect of pre-treatment of the reticulocyte suspensions with varying concentrations of *p*-hydroxymercuribenzoate on the incorporation of ^{59}Fe into components A, B, C and haemoglobin is shown in Fig. 6.

It is seen that the incorporation of ^{59}Fe into components A, C and haemoglobin falls sharply with *p*-hydroxymercuribenzoate pre-treatment, whereas incorporation into component B as a whole is unaffected. As has been previously shown (Edwards and Fielding [3]) about 50% inhibition of uptake is produced by as little as 0.2 mM *p*-hydroxymercuribenzoate.

While the incorporation of ^{59}Fe into component B as a whole is unaffected by *p*-hydroxymercuribenzoate inhibition, its distribution between sub-components B1 and B2 is markedly modified, reversing the proportions of these sub-components. Fig. 7 shows component B separated into B1 and B2 by chromatography on Se-

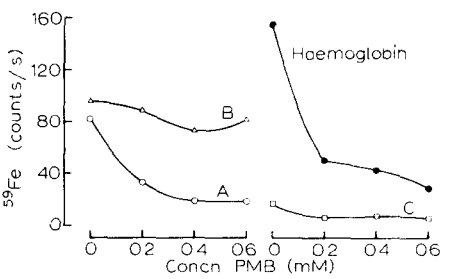


Fig. 6. Effect of pre-treatment with *p*-hydroxymercuribenzoate on ^{59}Fe content of reticulocyte components. ○–○, component A; △–△, component B; □–□, component C; ●–●, haemoglobin.

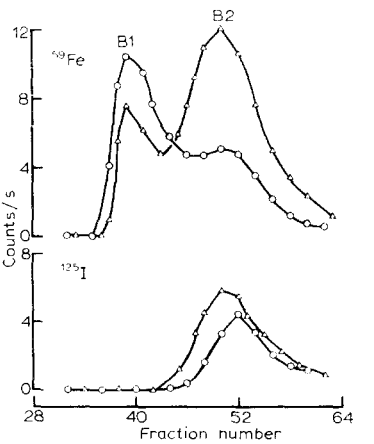


Fig. 7. Secondary fractionation of component B on Sepharose 6B. Effect of pre-treatment with *p*-hydroxymercuribenzoate on ^{59}Fe and ^{125}I in sub-components B1 and B2. ○–○, no *p*-hydroxymercuribenzoate; △–△, 0.3 mM *p*-hydroxymercuribenzoate.

TABLE II

DISTRIBUTION OF ^{59}FE IN RETICULOCYTE COMPONENTS

	Component	Control		*p*-hydroxymercuribenzoate Inhibited		Inhibited counts/s Control counts/s
		Counts/s	(% total)	Counts/s	(% total)	
	A	346	(29)	169	(22)	0.49
Membrane	B1	93	(8)	61	(8)	0.66
	B2	67	(6)	172	(23)	2.57
Cytosol	C	312	(26)	121	(16)	0.39
	Haemoglobin	374	(31)	238	(31)	0.64
Total in all components		1192	(100)	761	(100)	0.64
Total in whole cells		1538		929		0.60
Recovery		78 %		82 %		

* The final concentration of *p*-hydroxymercuribenzoate was 0.3 mM.

pharose 6B. Pre-treatment with 0.2–0.3 mM *p*-hydroxymercuribenzoate consistently caused diminution of ^{59}Fe in component B1 and an increase in both ^{59}Fe and ^{125}I of component B2. Here again it is notable that all the ^{125}I is associated with component B2. The ratio ^{59}Fe : ^{125}I in component B2 under these conditions of *p*-hydroxymercuribenzoate inhibition was greater in all experiments than the ratio in the labelled transferrin used.

Table II shows the quantitative changes in the distribution of ^{59}Fe produced by *p*-hydroxymercuribenzoate inhibition. Total uptake by inhibited reticulocytes is 60 % of the control preparation. The amount of uptake incorporated in component B2 is increased 2.57 times, which represents an increase of about fourfold in percent of total uptake. It is evident that *p*-hydroxymercuribenzoate inhibition acts by blocking onward transport from component B2. The amount of ^{59}Fe incorporated in haemoglobin is proportional to the total uptake by inhibited and by control reticulocytes.

DISCUSSION

The object of the experiments described here was to define the inter-relationships between the reticulocyte iron-bearing components described in our previous report (Speyer and Fielding [1]).

Cytosol components

The haemoglobin component increased linearly with time of incubation of reticulocytes with iron–transferrin. It continued to increase in amount in the chaser experiments during incubation with unlabelled iron–transferrin.

Component C tended to a maximum value in about 30 min of incubation with iron–transferrin, taking rather longer to reach its maximum than the binding of iron–transferrin to the surface which occurred in about 20 min. During increasing time of incubation with iron–transferrin, component C increased in advance of haemoglobin (Fig. 2). On the other hand, during chaser experiments component C decreased with time while haemoglobin increased and there was a reciprocal relationship between the

two. The amount of component C also decreased as a result of inhibition of uptake with *p*-hydroxymercuribenzoate, and its proportionate decrease (61 %) was far greater than that of haemoglobin (36 %). All these findings characterise component C as an iron transport intermediate on the haemoglobin pathway. However since it is recovered in the void volume it is not necessarily homogeneous. Component C is possibly identical to the cytosol intermediate of Allen and Jandl [4] and to fraction 1 of Greenough et al. [5] and Zail et al. [6].

We have found no evidence that transferrin as such moves beyond the membrane binding site: in addition to its presence in component B2 only small quantities of free transferrin were found in the cytosol, while the ^{125}I content of the membrane decreased rapidly during the chase experiments.

Membrane components

Component A. It has already been pointed out that this component is of large particle size since on centrifugation of the Triton X-100 solution as much as 80 % is deposited at 95 000 × *g* for 60 min. Nevertheless, it appears to be an integral part of the membrane and not a contamination by cytosol organelles; when the washed membrane deposit before solubilisation in Triton was centrifuged, component A behaved uniformly with component B; 46 % of the ^{59}Fe in the 500 × *g* deposit and 52 % of the ^{59}Fe in the 20 000 × *g* deposit were found in component A and the remainder in component B. In addition, the ratio of ^{59}Fe to ^{125}I in the deposits at the two speeds of centrifugation remained constant, also indicating (since all ^{125}I is associated with B) that the proportion of component A to component B was constant in the deposits.

This component behaved in the kinetic experiments as an end product. It did not tend to a maximum, but like haemoglobin it increased linearly with time of incubation with iron–transferrin. When cell uptake was inhibited by *p*-hydroxymercuribenzoate there was a sharp fall in the amount of this component. In all experiments, the behaviour of component A did not parallel that of the soluble components, either separately or in combination, so that component A is kinetically a distinct entity. In the chase experiments component A varied in its behaviour, unlike the other components. This variability was unrelated to the experimental variables studied. However, it appeared that component A behaviour was related to the clinical origin of the reticulocyte suspension. Where reticulocytes were derived from a state of iron repletion, for example in pernicious anaemia under Vitamin B_{12} therapy, a greater proportion of ^{59}Fe (mean 26 %, range 21–38 %) was incorporated into component A than where the state was of iron deficiency (mean 15 %, range 11–18 %).

Component B2. In our previous communication we showed that membrane component B, which was retarded on Sepharose 2B, could then be split into component B1 and B2 on passing through Sepharose 6B. We postulated that B2 was a complex consisting of iron–transferrin bound to its membrane binding site; all the membrane ^{125}I was associated with this fraction which thus contained all the membrane–associated transferrin, yet the molecular weight of the complex was demonstrably higher (230 000) than that of transferrin. The present experiments confirm this view. In all the timed and chaser experiments and those involving blocking of iron uptake by *p*-hydroxymercuribenzoate, all membrane ^{125}I was associated only with component B2. As time of incubation with labelled transferrin increased, the ^{59}Fe

content of component B2 increased to a maximum simultaneously with its ^{125}I content. In chaser experiments, the ^{59}Fe and ^{125}I content of component B2 falls to zero values. The experiments with *p*-hydroxymercuribenzoate inhibition also confirm this conclusion. As the uptake of iron by reticulocytes as a whole was inhibited, the relative and absolute amount of component B2 increased, and this was the only component to increase in amount. In particular, the relationship between components B1 and B2 varied inversely. Table II shows that the uptake of ^{59}Fe in *p*-hydroxymercuribenzoate-inhibited reticulocytes compared with the control decreases for all components except component B2. Incorporation into component B2 increased 2.57 times, and taking into consideration the reduced total cell uptake (60 % of control values) the proportion of component B2 in *p*-hydroxymercuribenzoate inhibited cells is increased four fold.

These observations not only support the concept of component B2 as the binding site–transferrin complex, but also demonstrate that the effect of *p*-hydroxymercuribenzoate inhibition is to create a metabolic block to the onward passage of ^{59}Fe from component B2. In preliminary experiments we have demonstrated transferrin in component B2 harvested after *p*-hydroxymercuribenzoate inhibition, by precipitation with anti-transferrin serum.

If membrane-bound transferrin accounts for all the ^{59}Fe and ^{125}I of component B2, then the ratio $^{59}Fe : {}^{125}I$ would be expected to be equal to one or less than one, compared with that of the labelled transferrin used in the experiment. In experiments with clear separation of component B2 from component B1 ratios of 1.53, 1.07, 0.83, 1.05 and 0.42 were observed at the peak of the component B2 fractions. On the other hand, when uptake was inhibited with *p*-hydroxymercuribenzoate and thus the outflow of iron from component B2 diminished, the ratio was invariably greater than 1.0 and the highest ratio observed was 2.03 when the corresponding uninhibited control ratio was 1.07. A ratio greater than 1 would suggest that component B2 consisting of iron transferrin and its membrane receptor may include iron already detached from iron transferrin (as well as the intact iron–transferrin molecule) whose onward pathway is blocked by *p*-hydroxymercuribenzoate. If significance may be attached to the fact that the highest ratio is virtually exactly 2, it could be hypothesised that the membrane receptor itself receives iron from iron–transferrin as the first step in membrane transport, while still retaining the capacity to accept a further iron–transferrin molecule. This would imply that the receptor as described here can accommodate twice as much iron as transferrin protein. At least two models would fit these facts: (a) the receptor consists of two sub-units, one of which binds iron–transferrin, while the other sub-unit accepts iron from the first; (b) the receptor has two iron–transferrin binding sites, only one of which is positioned for binding at a given time: on accepting iron a conformational change or spacial reorientation would bring the second binding site into place. In either model, *p*-hydroxymercuribenzoate inhibition would be considered to operate on the process of detachment of iron from the receptor rather than from iron–transferrin.

Component B1. This component was shown to have a molecular weight of the order of 10^6, to contain no associated ^{125}I and to be associated with most of the membrane protein [1]. On incubation of reticulocytes with iron–transferrin, component B1 tended to a maximum in about 30 min, which suggested that it acted as an intermediate rather than an end product. It should be noted however that during a

chase this component does not reach zero levels but tends to a constant value, suggesting that it may not be homogeneously reactive. The chaser experiments confirmed the view of B1 as an intermediate since it decreased with time of chase, and the redistribution of ^{59}Fe among the components showed (Table I) that it transferred ^{59}Fe to either or to both the components A and C.

After inhibition of uptake with *p*-hydroxymercuribenzoate, component B1 fell sharply as component B2 accumulated. Thus since component B1 and component B2 are membrane components and are reciprocally related in inhibition experiments, and component B1 is clearly an intermediate, it is highly probable that component B1 represents the iron-bearing intermediate immediately following the receptor. If this is so, it follows that it contains the sulphydryl groups whose inhibition leads to loss of iron uptake.

We have given no direct evidence on the nature of the components described other than haemoglobin and the binding-site–transferrin complex B2. However their relation to ferritin is of considerable interest Using a radioimmunoassay (kindly carried out by Dr M. Worwood) membrane-associated ferritin was identified and assayed in the fractions derived from Triton X-100 solution of membrane on Sepharose 2B. A single peak of ferritin was found which was co-incident with the peak ^{59}Fe activity of component B. It was not however determined whether the ferritin was labelled. Clearly no part of component B2 could be ferritin on grounds of molecular weight. It is therefore possible that component B1 includes ferritin bound to a membrane component, but this requires further investigation.

The results of these experiments show that of the three membrane components A behaves as an end product while component B2 represents the binding-site–iron–transferrin complex. Component B1 includes a membrane iron transport intermediate but it is possible that this component is not metabolically homogeneous. In the cytosol the non-haemoglobin component C is a transport intermediate on the pathway to haemoglobin. The evidence suggests that the transport pathway moves from component B2 to component B1 and thereafter diverges into two pathways, one leading to component A in the membrane and the other leading through component C to haemoglobin.

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