

CELLULAR UPTAKE OF SOLUBLE AND AGGREGATED FERRITIN: DISTINCTION BETWEEN PINOCYTOSIS AND PHAGOCYTOSIS

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SUMMARY

Cellular uptake of ferritin amounting to 0.5 µg/mg cell protein or more can be measured colorimetrically on the basis of ferritin-iron content. ¹³¹I-serum albumin, soluble ferritin and aggregated ferritin used in equimolar concentrations are taken up differently by Sarcoma S180 cells in culture. The net uptakes in 2 h at 37 °C are 0.065, 4.3 and 24.7 µg/mg cell protein or 0.93, 8.0 and 45.7 µmol, respectively. Albumin uptake is not inhibited by a 26-fold molar ferritin excess but is significantly inhibited by a 43-fold excess.

The transport mechanism of the ferritins differs from that of albumin in that it is significantly inhibitable by 2×10^{-4} M monoiodoacetate. Soluble ferritin contains small aggregates which are removed by filtration through Millipore membranes of 0.05, 0.1 and 0.22 µm. When the 0.1-µm filtrate is re-examined, uptake is no longer inhibited by iodoacetate. Since it can be inferred from other work that albumin is taken up by pinocytosis and ferritin aggregates by phagocytosis, the difference in susceptibility to inhibition is proposed as a way to distinguish pinocytosis from phagocytosis.

Ferritin may form larger visible aggregates in culture medium. The transport mechanism of this aggregated ferritin differs from that of soluble unfiltered ferritin in that it causes concomitant enhancement of albumin uptake. Albumin transported by virtue of this effect becomes partially susceptible to iodoacetate. Thus, in addition to a distinction between pinocytosis and phagocytosis, our data single out 2 forms of albumin transport and 3 forms of ferritin transport.

INTRODUCTION

The use of ferritin and peroxidase as tracers in electron microscopy has firmly established that proteins can be transported into mammalian cells by the process of pinocytosis (Farquhar & Palade, 1961; Friend & Farquhar, 1967; Graham & Karnovsky, 1966; Ryser, Caulfield & Aub, 1962). The basic characteristics of this transport process, its relevance and possible functions have been described and reviewed (Ryser, 1963, 1968*a*). While ferritin has been used extensively as a probe in ultrastructural studies, and shown to penetrate into cells of Sarcoma S180 and Ehrlich carcinoma cells (Caulfield, 1963; Ryser *et al.* 1962), there are no quantitative data on ferritin uptake by tumour cells. Little is known about the relative uptake of different proteins in the same cell system, and about selectivity of uptake in general. Previous work using ¹³¹I-albumin as a tracer has shown that the cellular uptake of protein by

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tumour cells can be measured and modified (Ryser, 1963; Ryser & Hancock, 1965). The present study compares, under similar conditions, the uptake of albumin and 2 forms of ferritin by cells of Sarcoma S180 grown in monolayers. It also analyses the effect which the 2 proteins exert upon each other's uptake when present simultaneously in the experimental medium.

Several molecular parameters are known to influence the rate of uptake of macromolecules. Basic protein, and other polycations, for instance, are taken up more readily than albumin (Ryser, 1968*a*). It has been suggested also that, within limits, an increase in molecular size may determine an increase in cellular uptake (Ryser, 1967*b*). The relative importance of the factor of size is examined with special attention in this study. It is known that mammalian cells can take up macromolecules in a considerable size range. The availability of ferritin in aggregated form has extended the scope of our study. While albumin is taken up by pinocytosis, it can be assumed that aggregates of ferritin are taken up by phagocytosis. Consequently, this paper focuses on the distinction ordinarily drawn between the 2 processes. It is known from earlier data that albumin uptake is not decreased by metabolic inhibitors (Ryser & Hancock, 1965; Ryser, 1970). Several studies carried out on other cells show, however, that the same metabolic inhibitors decrease phagocytosis (Casley-Smith, 1969; Gordon & King, 1960; Roberts & Quastel, 1963). The present study compares both processes in the same cell system and attempts to explain this difference.

Previous work described procedures which enhance the uptake of macromolecules by mammalian cells. It has been shown, for instance, that histones, basic polyamino acids and DEAE-Dextran can stimulate the cellular uptake of albumin up to 20-fold (Ryser & Hancock, 1965). It has been shown also that this enhancing effect increases with the molecular size of the enhancer (Ryser, 1967*b*). The present study describes a new type of enhancement which is related to the state of aggregation of the enhancer. The current study thus describes different forms of protein uptake as well as different forms of uptake enhancement.

MATERIALS AND METHODS

Cells

All experiments were carried out with monolayers of Sarcoma S180 II, an established mouse tumour line obtained from the American Type Culture Collection (Rockville, Maryland).

Medium

Ingredients used to prepare the tissue culture medium (Eagle's Minimal Essential Medium) were obtained from Grand Island Biological Company (Grand Island, N.Y.). The trypsin preparation used to detach monolayers was Bacto-Trypsin 1:250 (Difco), diluted with medium to 0.125% final concentration.

Albumin

¹³¹I-labelled human serum albumin was obtained from Abbott Laboratories (North Chicago Ill.) as a sterile aqueous solution containing 10–15 mg albumin/ml, in the average 1 mCi/ml and an initial specific activity of 75–100 μCi/mg. All shipments were checked by paper chroma-

tography for contamination with free ^{131}I , which in all cases was 3% or less. Non-radioactive human serum albumin used to adjust final albumin concentration was Cohn's fraction V, purchased from Pentex (Kankakee, Ill.).

Ferritin

Cadmium-free horse spleen ferritin, twice crystallized, was purchased from Pentex (Kankakee, Ill.). It was obtained in aqueous solution of 100 mg/ml which contained not less than 20% iron. The ferritin shipments were characterized as Lots No. 11, 12 and 13. As discussed under Results, Lot No. 11 differed from the others in terms of its stability in solutions. When diluted in culture medium or saline, this ferritin formed aggregates. Aggregation caused turbidity which could be followed photometrically and levelled off after 2 h at 36 °C. With ferritin at 7 mg/ml, aggregation almost doubled the optical density of an Eagle's medium-ferritin solution in the course of 5 h; aggregation was partially prevented by the addition of albumin to the culture medium (Fig. 1, p. 146). The aggregates represented 20% of the total ferritin content. They sedimented during a 30-min centrifugation (3000 g) in a table centrifuge leaving a clear supernatant. Comparable ferritin dilutions of Lots No. 12 and 13 did not cause aggregation, did not yield detectable sediment upon comparable centrifugation, and did not change their optical density upon addition of albumin. Aggregated and non-aggregated ferritin differed markedly in their cellular uptake and are therefore compared extensively in this paper (see Results). Ferritin of Lot No. 11 is referred to in the text as 'unstable' or 'insoluble', while Lots No. 12 and 13 are referred to as 'stable' or 'soluble'. The Pentex Company, from which these lots were obtained, could offer no enlightening comments regarding the peculiar physiochemical properties of Lot No. 11. Although this lot was in all respects an odd one, its use led to results which, although fortuitous, cannot be ignored. Upon closer examination, the stable ferritin of Lots 12 and 13 contained smaller aggregates which did not cause measurable turbidity, but which could be demonstrated by filtration through Millipore membranes of small pore sizes, i.e., 0.05, 0.1 and 0.22 μm . This second type of aggregate is also associated with distinct characteristics of ferritin uptake. Soluble ferritin was therefore compared before and after Millipore filtration through a membrane of 0.1- μm pore size (see Results). Filtrations were carried out in volumes of 10 ml of 5 mg/ml soluble ferritin with the help of a Swinney Adapter.

Cell cultures and incubation

The sarcoma cells were grown as monolayers on glass in Eagle's MEM containing 10% horse serum and penicillin-streptomycin, 50 U/ml each. Culture flasks of 15 cm² area (*t*-Flasks, Bellco, Vineland, N.J.) were seeded with 4×10^5 cells in 2 ml, closed with silicon stoppers and grown to confluency (3×10^6 cells) with daily medium changes starting on day 2. Experiments used 20–40 such cultures divided into groups of 3–5 flasks. After microscopic inspection, the monolayers were rinsed once with serum-free medium and incubated with 2.2 ml serum-free experimental medium. In most experiments, incubation lasted 2 h. After this – or other periods of time – the culture was rinsed once with serum-free culture medium and detached by trypsinization. The trypsin preparation added at a final concentration of 0.125% to serum-free medium detached the cells within 2–5 min. The detached cells were centrifuged at low speed and washed twice with 3 ml ice-cold Tyrode's saline. Duplicate 0.2-ml aliquots of the last resuspension were taken for protein determination, according to Lowry, Rosebrough, Farr & Randall (1951). The remaining cells were processed for the measurement of cellular uptake of albumin and ferritin.

Measurement of albumin uptake

In experiments measuring albumin uptake, the experimental medium contained 45–100 μg /ml ^{131}I -serum albumin, corresponding to $4\text{--}10 \times 10^6$ cpm/ml. For easier comparison, the media of all experiments were corrected to a standard activity of 5×10^6 cpm/ml. Different final albumin concentrations, ranging from 0.05 to 5 mg/ml were achieved by isotopic dilution with non-radioactive human serum albumin. The resulting specific activities of the albumin in the

medium ranged from 0.5 to 60 $\mu\text{Ci}/\text{mg}$. At the end of incubation, the cells were detached and washed as described above. They were resuspended in 3 ml saline and killed by the addition of 0.25 ml 60% ice-cold trichloroacetic acid (5% final concentration). After 1 h at room temperature, the insoluble cell fraction was collected on a 22-mm diameter Millipore membrane, 0.45- μm pore size, and washed 3 times on the filter with 3 ml of 5% trichloroacetic acid (TCA). The filters were counted on a low background gas flow β -counter (Nuclear Chicago, C115, counting efficiency 45%, background 2 to 3 dpm). The relatively strong β emission of ^{131}I and the low protein content of the protein film (< 1 mg per filter) made corrections for auto-absorption unnecessary. The results obtained in cpm/mg cell protein were converted to μg albumin/mg or μmol albumin/mg. Albumin binding measured after exposures of 2–3 min is a reliable expression of albumin adsorption to the cell surface (Ryser, 1963). Subtracting this value from total binding measured after exposure of cells to albumin for x min gives an expression for the net uptake occurring between 1 and x min. This correction for albumin adsorption was carried out throughout in expressing results on albumin uptake. In experiments studying the effect of ferritin on albumin uptake, the incubation media contained up to 7.5 mg/ml of stable or unstable ferritin, to obtain ratios of ferritin to albumin ranging from 0.5 to 43.

Measurement of ferritin uptake

All experiments measuring ferritin uptake were carried out in incubation media containing 2–15 mg/ml ferritin. Because of a time-dependent formation of ferritin aggregates in media containing the unstable form of ferritin, all ferritin-containing incubation media were pre-incubated for 90 min at 30 °C before the start of the experiment. At the end of incubation, the rinsed cells were detached and washed as described above and processed for colorimetric measurement of their ferritin content, using a modification of the Reissman assay for iron (Reissman & Dietrich, 1956). Cells from 2 monolayers totalling 1.2–2 mg protein were pooled, resuspended in 2 ml saline and incubated for 24 h, at 45 °C with 0.5 ml 6 N HCl, in sealed test tubes. Protein denaturation was then completed by adding 0.22 ml 60% TCA (final concentration, 5%). After 1 h at room temperature, the precipitate was centrifuged for 3 min at 3000 g in a conventional table centrifuge. Two millilitres of iron-containing supernatant were then mixed with 0.7 ml saturated potassium acetate, 0.15 ml of freshly prepared 3% hydroquinone and 0.25 ml of 0.1% orthophenanthroline. The developing red colour was measured after 1 h of equilibration at room temperature at 510 nm in a spectrophotometer (Model Beckman DB or Hitachi 124). Standard curves were prepared for each experiment with ferritin solutions of known concentrations. The concentrations were determined by protein assay. The standard curves are linear up to a concentration of 20 μg ferritin. The method measured accurately quantities of 0.5 μg . The cellular ferritin uptake was expressed in $\mu\text{g}/\text{mg}$ cell protein or $\mu\text{mol}/\text{mg}$ cell protein. It ranged from 1 to 30 $\mu\text{g}/\text{mg}$. Ferritin adsorption to the cell surface was measured as ferritin bound after 2 min of exposure. In most experiments, the amount was less than 0.5 μg per pooled sample and thus not accurately measurable by the method available. When measurable, adsorption was small; it represented in the average only $\frac{1}{18}$ of the ferritin binding measured after 120 min and was therefore deemed negligible. In several experiments, the uptakes of ferritin and albumin were measured on the same cell sample. In these cases the protein precipitate resulting from treatment with HCl and TCA was collected for radioactivity measurement, while the supernatant was used for colorimetric measurements. In experiments measuring the effect of albumin on ferritin uptake, the incubation medium contained up to 5 mg/ml non-radioactive albumin, and the ferritin:albumin ratios ranged from 5 to 0.19. Metabolic inhibitors used to influence the uptake of ferritin were added as freshly prepared solutions to the incubation medium.

Test of cellular integrity

The lack of cellular toxicity of the ferritin-containing media was ascertained in a dye-exclusion test. For that purpose, cells detached by trypsinization at the end of an exposure were incubated for 10 min at 37 °C in complete medium containing 1 mg/ml nigrosin. The number of cells stained by nigrosin in a population of 500–800 cells was within the normal range of 1–2%.

RESULTS

Uptake of albumin, ferritin and ferritin aggregates

The basic characteristics of cellular uptake were found to be comparable for albumin and ferritin. Uptake increases with time during a 2-h incubation at 37 °C, and this increment is inhibited at lower temperatures. Under the experimental conditions used, i.e., 2 cell washings following trypsinization, the net uptake of albumin in 2 h obtained by subtracting adsorption from total binding is, on average,

Table 1. *Cellular uptake of albumin, soluble- and aggregated ferritin*

	Uptake, $\mu\text{g}/\text{mg}$ cell protein	Relative uptake	Uptake, $\mu\text{mol} \times 10^6/\text{mg}$ cell protein	Relative uptake
Albumin (4)	0.065 ± 0.012	1	0.93 ± 0.17	1
Soluble ferritin (5)	4.3 ± 2.0	66	8.00 ± 3.7	8.6
Aggregated ferritin (6)	24.7 ± 13.8	386	45.7 ± 25.6	49.1

Cells were incubated for 2 h at 37 °C in serum-free Eagle's medium containing 1×10^{-5} M ^{131}I -labelled albumin or ferritin. The figures in parentheses give the numbers of measurements. For the expression of uptake in molar terms, molecular weights of 70000 and 540000 have been used for albumin and ferritin, respectively.

4 times greater than albumin adsorption. Ferritin adsorption is not measurable in all experiments. When measurable, the ratio between net uptake and adsorption is greater than 10. The uptake of ferritin increases with concentration within the tested range of from 2 to 15 mg/ml. For the purpose of quantitative comparison, albumin, ferritin and aggregated ferritin were presented to cells separately in identical molar concentrations. Table 1 shows that under comparable conditions sarcoma cells take up distinctly more ferritin than albumin. It shows, in addition, that aggregated ferritin is taken up more readily than non-aggregated ferritin. The values obtained for net uptake over a period of 2 h are 0.065, 4.3 and 24.7 $\mu\text{g}/\text{mg}$ for albumin, soluble and aggregated ferritin, respectively. Table 1 shows that these marked differences are seen even when uptake is expressed on a molar basis. In this case, the uptake ratios for albumin : soluble ferritin : aggregated ferritin are roughly 1 : 9 : 50. It is known that cell damage markedly increases the non-specific cellular binding of proteins (Ryser, 1967a) and that cadmium-containing ferritin is toxic to mammalian cells *in vitro* (Eybl & Ryser, 1964). The ferritin used in these experiments, however, is cadmium-free, hence non-toxic, and rigorous testing of cellular integrity carried out in parallel experiments failed to show any signs of cell damage.

The difference in the uptake of soluble and aggregated ferritin was analysed in further experiments. Nephelometric measurements showed that aggregates of ferritin form as a function of time when unstable ferritin is added to the incubation medium. This is shown by the upper curve of Fig. 1, while the lower curves indicate that this process of aggregation is markedly inhibited by addition of increasing concentrations of serum albumin. This simple means of decreasing aggregation was used to compare

the uptake of ferritin present in identical concentrations but different states of aggregation. Table 2 shows that amounts of albumin which inhibit aggregation also inhibit uptake. The 66% inhibition of uptake caused by 5 mg/ml albumin in a molar ratio of ferritin : albumin of 0.19 seen in Table 2 can be related to the 60 to 70% inhibition of aggregation caused, in Fig. 1, by 6 mg/ml albumin in a molar ratio of 0.15. The effect of albumin on the uptake of unstable ferritin is seen also in Fig. 2, A. The right

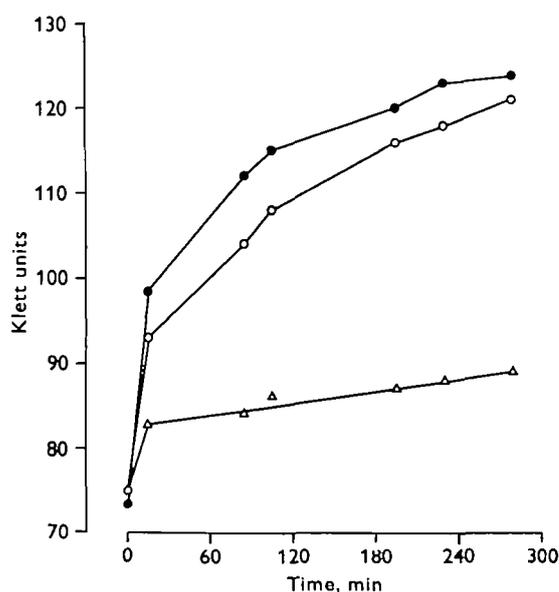


Fig. 1. Formation of aggregates when 7 mg/ml of unstable ferritin are added to Eagle's medium at 37 °C. Aggregation is inhibited by presence in the medium of 0.1 and 6 mg/ml serum albumin, ○ and △, respectively; control without albumin, ●. Aggregation was followed as a function of time by measuring optical density in a Klett photometer.

Table 2. *Effect of increasing albumin concentrations on cellular penetration of aggregated ferritin*

Concentration of added albumin, mg/ml	F/A	Ferritin uptake, % of control
0	∞	100
0.18	5.20	120
0.50	1.94	66
1.0	0.96	41
1.5	0.64	38
5.0	0.19	34

Incubation conditions: 150 min at 37 °C in serum-free medium containing 7.5 mg/ml unstable ferritin and human serum albumin in increasing amounts. F/A gives the molar ratios of ferritin/albumin. Uptake is expressed as % of value measured in absence of albumin.

part of this figure (B) shows in addition that the same concentration of albumin has no inhibitory effect on the smaller uptake of the stable form of ferritin. Thus, at a ferritin : albumin ratio close to unity, there is no evidence of competition between albumin and soluble ferritin for uptake. This remains true when the molar concentration of albumin

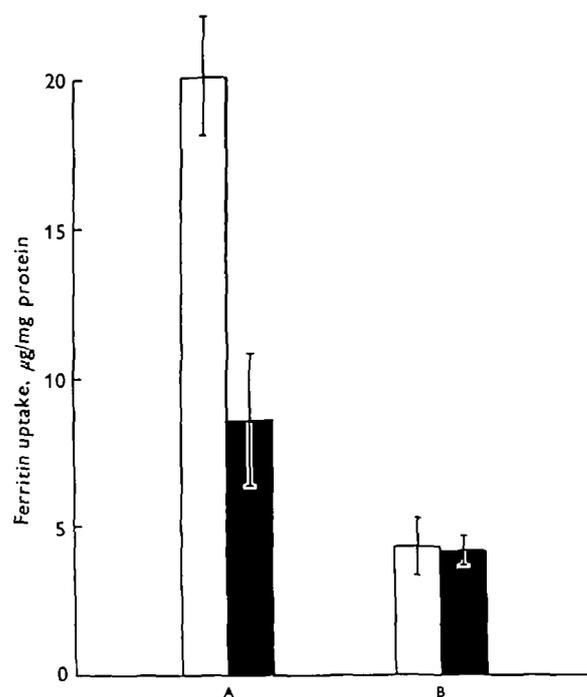


Fig. 2. Effect of albumin on the cellular uptake of stable and unstable ferritin. A, aggregated ferritin; B, soluble ferritin; □, controls, ■, with albumin. Incubation conditions: 2 h at 37 °C in serum-free medium containing 7.5 mg/ml stable or unstable ferritin with or without addition of 1.0 mg/ml serum albumin. When albumin was added (dark columns) the molar ratio of ferritin to albumin was 0.96. The effect of albumin on the uptake of unstable ferritin (A) is significant ($P < 0.02$). A and B are means of 4 and 8 measurements, respectively.

is raised 2-fold over that of ferritin. Solutions of stable ferritin are clear and their optical density is not modified by added albumin. It can be concluded that, in this case, albumin acts solely by decreasing the aggregation of unstable ferritin. The relation between the state of aggregation and uptake is illustrated by 2 further observations. On the one hand it was found that aggregation of unstable ferritin is more pronounced in Earle's than in Ringer's salt solutions. Measurements carried out in these 2 solutions showed a 2.5-fold greater uptake of unstable ferritin in Earle's solution; the uptake of stable ferritin showed no such difference. On the other hand, the aggregates of unstable ferritin that form in culture medium can be easily eliminated by centrifugation, in which case the supernatant behaves like a solution of stable ferritin.

Effect of monoiodoacetate on the penetration of ferritin

The comparison of albumin and ferritin has revealed a major difference in the susceptibility of the uptake process to inhibition by iodoacetate. As shown by the left side of Fig. 3, the uptake of albumin is not inhibited by 1×10^{-4} M iodoacetate. Higher concentration of iodoacetate and the use of other conventional inhibitors such as NaF, sodium azide, dinitrophenol and KCN also failed to inhibit albumin uptake when tested either alone or in combination. Increasing the rate of albumin

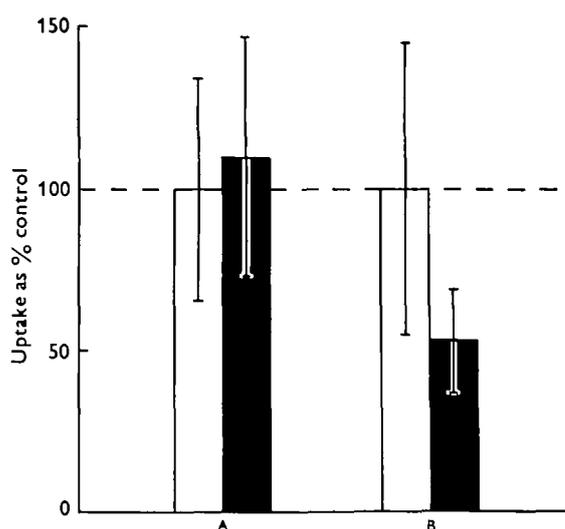


Fig. 3. Effect of monoiodoacetate on cellular uptake of albumin and ferritin, A and B, respectively. Incubation conditions: 150 min at 37 °C in serum-free medium containing 45 μ g/ml 131 I-albumin (A) or 5 mg/ml soluble ferritin (B). The uptake in the presence of 1×10^{-4} M iodoacetate (■) is expressed as % of control uptake, measured in absence of inhibitor (□ and dashed horizontal line). Each column gives the mean of 4 experiments, with 3-5 measurements each. The inhibition of ferritin uptake is highly significant ($P < 0.01$), but the change in uptake of 131 I-albumin is not ($P = 0.35$).

uptake either by increasing the albumin concentration or adding enhancers of uptake such as basic polyamino acids, did not render the process susceptible to inhibition. By contrast, as seen in Fig. 3, B, the uptake of soluble ferritin is significantly inhibited by 1×10^{-4} M iodoacetate. Comparable results are obtained with aggregated ferritin. This finding has been analysed in further experiments. Culture medium containing 5 mg/ml soluble ferritin was filtered through a Millipore membrane of 100-nm pore size. Unfiltered and filtered medium were used for separate measurements of ferritin uptake in the presence and absence of 2×10^{-4} M iodoacetate. Fig. 4, A, confirms that the uptake of ferritin can be significantly inhibited by iodoacetate. However, Fig. 4, B, demonstrates that the uptake of filtered ferritin is no longer susceptible to iodoacetate. This finding suggests: even stable, soluble ferritin forms aggregates in

Eagle's medium; aggregates are removed by filtration; and the susceptibility of ferritin uptake is related to the presence of ferritin aggregates.

An attempt was made to define the sizes and amount of such aggregates by differential filtration. Samples of 10 ml Eagle's medium containing 5 mg/ml ferritin, pre-incubated for 150 min at 37 °C, were filtered successively through Millipore filters of 3 decreasing pore sizes, namely 220, 100 and 50 nm. The amount of ferritin retained by each filtration was determined by measuring the protein content before and after this treatment. Retention at each step was expressed as a percentage of the initial ferritin content. The results of 4 experiments are as follows: a small fraction (1.5%) was retained by the 220-nm membrane; this fraction must contain mostly

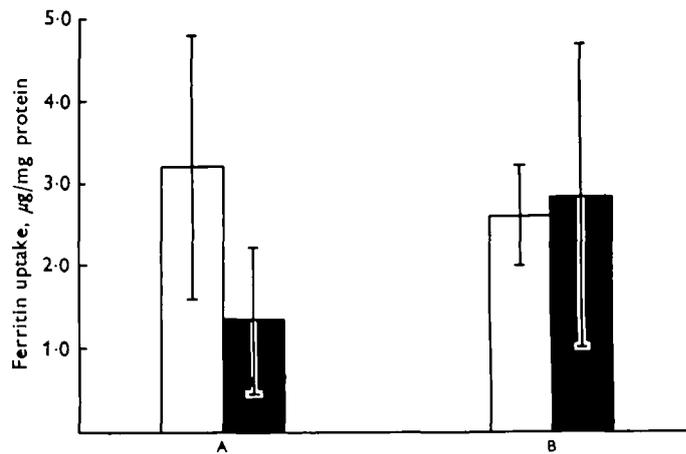


Fig. 4. Effect of monoiodoacetate on the penetration of unfiltered (A) and filtered (B) ferritin. Incubation conditions: 150 min, 37 °C, in serum-free medium containing 5 mg/ml of ferritin. Dark columns represent uptake in presence of 2×10^{-4} M iodoacetate. The uptake of unfiltered ferritin is significantly inhibited by iodoacetate ($P < 0.001$). The uptake of filtered ferritin is not inhibited. Each column gives mean of 5 experiments with 3-5 measurements each. Filtration through 0.1- μ m pore size Millipore membrane.

aggregates of average diameter larger than 220 nm. The 100-nm membrane retained an additional 3.7% which must correspond mostly to particles ranging from 100 to 220 nm. The 50-nm membrane retained another 17.2%, corresponding to sizes from 50 to 100 nm. The remaining 77.2% which passed through this last membrane contains particles smaller than 50 nm.

These estimates of sizes require some qualifications. The relationship between pore size and particle size is not a rigorous one and it is known that Millipore membranes may retain particles of smaller average diameter than the given pore size. As a rule, however, filtrates never contain particles larger than the pore size. It can be concluded that the filtered ferritin referred to in Fig. 4 contained no aggregates larger than 0.1 μ m, and that the aggregates eliminated by filtration represented roughly 5% of the initial ferritin content.

Effect of ferritin on albumin uptake

Since ferritin in both its stable and aggregated form is taken up more readily than albumin, it was of interest to test whether this uptake modified in any way the uptake of albumin when both proteins were present in the medium. Fig. 5 shows that at a

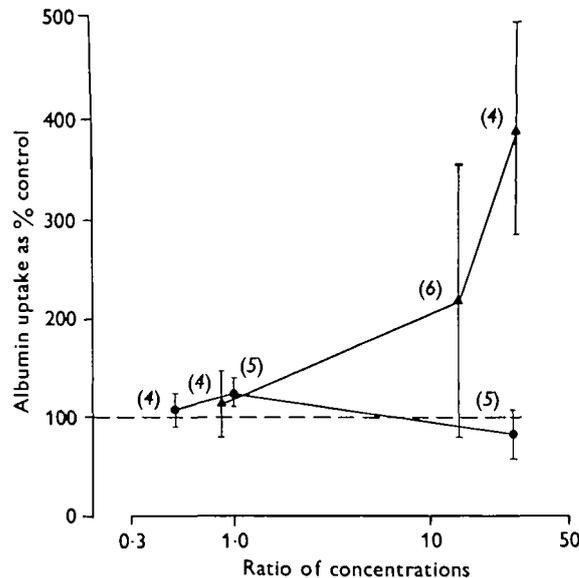


Fig. 5. Effect of soluble (●) and aggregated (▲) ferritin on the cellular uptake of ^{125}I -albumin. The concentrations of the 2 ferritins are given on a log scale (abscissa) as molar ferritin:albumin ratios. The albumin uptake is expressed as % of the control, measured in the absence of ferritin (dashed horizontal line). Incubation conditions: 2 h, 37 °C in serum-free medium containing 5.0–7.5 mg/ml ferritin and ^{125}I -albumin concentrations varying from 0.027 to 1.3 mg/ml. Each point gives the mean from the number of experiments given in parentheses. The small inhibitory effect of soluble ferritin measured between 1 and 26 is significant ($P < 0.02$). The enhancing effects of aggregated ferritin measured between 14 and 26 and between 0.85 and 26 are significant ($P < 0.05$ and < 0.025 , respectively).

molar ratio of ferritin : albumin of 1 : 1, stable ferritin causes a moderate but significant enhancement of albumin uptake of the order of 25 %. This effect, however, disappears with increasing ratios. At the ratio of 26 : 1, ferritin inhibits albumin uptake slightly (13 %). The reversal of effect observed between 1 : 1 and 26 : 1 is statistically significant. At 43 : 1, the inhibition reaches 50 %, as shown by Fig. 6. Thus, while low concentrations of soluble ferritin slightly enhance albumin uptake, high concentrations do inhibit it. The nature of this interference has not been studied further.

Very different results are obtained with aggregated ferritin. As shown in the upper curve of Fig. 5, the slight enhancement of albumin uptake observed at a ratio of 0.85, becomes more pronounced as the proportion of ferritin increases. At a ratio of 26 : 1, aggregated ferritin causes 4-fold enhancement of albumin uptake. This effect is abolished when aggregates are eliminated by centrifugation or by filtration. In the

light of the data of Fig. 1 (p. 146), the inverse relationship between effect and relative albumin concentration can be attributed to the waning stabilizing effect of albumin and the increased occurrence of aggregates. These observations thus lead to the conclusion that larger ferritin aggregates present in the medium have a marked enhancing effect on albumin uptake and that this effect increases with concentration. Thus, the 2 forms of ferritin differ in their rate of uptake as well as in their effect on albumin transport.

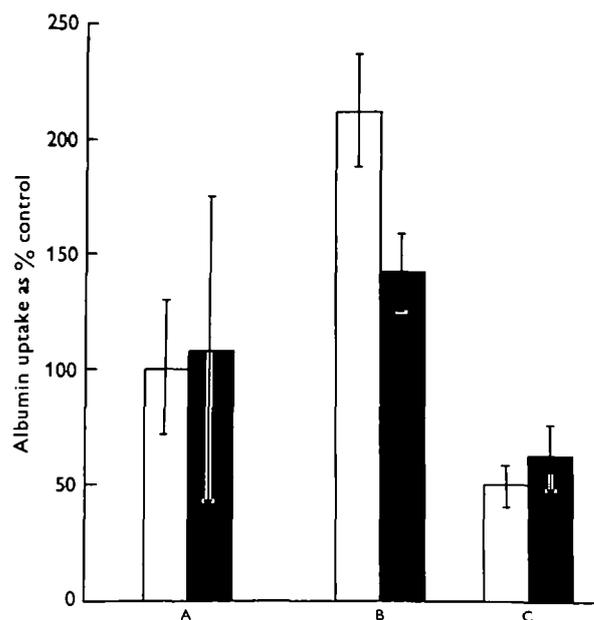


Fig. 6. Effect of monoiodoacetate on albumin uptake, in the absence of ferritin (A), in the presence of aggregated ferritin (B) and in the presence of soluble ferritin (C). Incubation conditions: 2 h, 37 °C in serum-free medium containing ^{125}I -albumin with or without 5 mg/ml ferritin. The molar ferritin:albumin ratios were 43 and 15 for soluble and aggregated ferritins, respectively. The effect of 2×10^{-4} M iodoacetate (■) is inhibitory only when albumin uptake is enhanced by ferritin aggregates. This effect is significant ($P < 0.01$). All values are means of 5 or 6 measurements.

It was shown in Fig. 4 that the susceptibility of ferritin to metabolic inhibition is related to the presence of ferritin aggregates. It was of interest, therefore, to test whether the enhancement of albumin uptake, caused by ferritin aggregates, would be susceptible to such inhibition as well. The results of this test are given in Fig. 6. Confirming the result of Fig. 3, Fig. 6 shows that iodoacetate has no effect on albumin uptake (A), even in the presence of an excess of soluble ferritin (C). Iodoacetate, however, reduces the additional uptake caused by ferritin aggregates (B). This additional uptake therefore is different in nature from non-enhanced albumin uptake.

DISCUSSION

The time, concentration and temperature-dependence of protein uptake described previously for albumin is confirmed by the current experiments and is shown to obtain also for ferritin. The net uptake measured in a medium containing 1×10^{-6} M albumin (0.7 mg/ml) is of the order of 0.9×10^5 molecules/cell in 2 h. This agrees with the previous value of 1.2×10^5 molecules per cell in 2 h, obtained under comparable conditions (Ryser, 1963). The net ferritin uptake measured in the same system and same molar concentration amounts to 8×10^5 molecules/cell in 2 h. These values may represent less than total uptake since intracellular degradation of ingested albumin begins while the uptake is being measured (Gabathuler & Ryser, 1969; Ryser, 1968*b*), but losses during that period are small and probably comparable for albumin and ferritin (Crichton, 1971). The quantitative differences in cell-bound albumin and ferritin reflect, therefore, true differences in uptake.

The molecular basis of this difference is not known. Our prior work has singled out 2 factors which favour the uptake of macromolecules, namely, multiple charges and molecular size (Ryser, 1968*a*). Since albumin and ferritin have nearly identical isoelectric points but different molecular weights, it is likely that the molecular size is a contributing factor in this case. Obviously, parameters other than size and charge play a role in determining rates of uptake. The 2 optical isomers of polylysine, for instance, are taken up differently by tumour cells (Ryser, 1968*a*). Specific proteins are taken up preferentially by different tissues, for instance, transferrin by rat reticulocytes (Baker & Morgan, 1971); heterologous immunoglobulin by polymorphonuclear leucocytes (Bona & Ghyka, 1968); asialoproteins by liver tissue *in vivo* (Morell *et al.* 1971). Human fibroblasts in culture differ in their ability to take up *N*-acetyl- β -hexoseaminidase depending upon the source of the enzyme (Hickman, Shapiro & Neufeld, 1974). Selectivity in these cases may be determined by subtle differences in protein structure or membrane receptors.

The availability of an unstable ferritin which formed aggregates when added to culture medium led to the fortuitous finding that aggregated ferritin is taken up 6 times more readily than soluble ferritin. These aggregates have not been analysed for size distribution, but a large size can be inferred from the fact that they cause measurable turbidity and sediment at 3000 g in a table centrifuge. Decreasing the amount of aggregation causes a corresponding decrease of uptake. The notion that uptake of a macromolecule should be favoured by aggregation, i.e., that uptake should increase with size, appears paradoxical at first. It is consistent, however, with several published observations. Thus, L-cells in culture ingest soluble DNA to a lesser extent than aggregated DNA (Bensch & King, 1961). Spleen cells take up ferritin more readily when the protein is ingested as a ferritin-antibody complex (Patterson *et al.* 1965). Electron microscopy has shown that ascites carcinoma cells take up more ferritin in the form of ferritin-antibody complexes (Easton, Goldberg & Green, 1962). Caulfield (1963) has described in Ehrlich ascites carcinoma cells 2 forms of ferritin uptake which differ in their morphology. Single ferritin molecules are taken up by pinocytosis without evidence of much membrane movement, while cell debris laden with

ferritin evokes distinct membrane activity associated with uptake of complexed ferritin.

The increase in absolute uptake of ferritin, when aggregated, is not necessarily due to a higher rate of uptake. The magnitude of enhancement (6-fold) must be related, here, to the size of aggregates. Sedimentation and filtration data indicate that the aggregates contain many more than six ferritin molecules. If the particles of soluble and aggregated ferritin were taken up at the same rate over a period of 2 h, the increase in uptake of the aggregated form would be much greater than 6-fold. When expressed as number of particles in 2 h, the rate of uptake of aggregates is therefore considerably less than that of soluble ferritin. The aggregates are of a size that must be taken up by phagocytosis, rather than pinocytosis. While sarcoma cells may engage in both processes, it can be deduced from the preceding argument that over a period of 2 h phagocytosis occurs at a slower overall rate than pinocytosis. This does not preclude that short bursts of phagocytosis might occur at higher rates.

Our results show that ferritin, even when it does not form visible aggregates in culture medium forms small aggregates of different sizes. Thus, 1.3% of a solution which appeared clear at inspection is retained by a Millipore membrane of 220-nm pore size and as much as 22% is retained by pores of 50-nm. This observation is in agreement with the findings of Harrison & Gregory (1965) and Suran & Tarver (1965) who have detected stable polymers of different sizes in solutions of purified ferritin. Our data show therefore that sarcoma cells in culture are able to take up ferritin in 3 different forms, namely in molecular solution, as small aggregates, and as large aggregates. More importantly, they show that these 3 forms of uptake can be distinguished from each other. On the one hand, there is a 6-fold difference in the amount of ferritin taken up as small versus large aggregates; only the large aggregates cause enhancement of albumin uptake, so there must be a size limit beyond which aggregates evoke concomitant transport of other macromolecules. On the other hand, ferritin, when filtered through a Millipore membrane of $0.1 \mu\text{m}$, is taken up by a process that cannot be inhibited by iodoacetate; it behaves in this case like albumin. By contrast, soluble ferritin-containing aggregates retained by a $0.1\text{-}\mu\text{m}$ Millipore membrane are taken up by a process that can be partially inhibited by iodoacetate. There must be a lower size limit beyond which endocytosis differs with regard to its energetic requirements.

Could the susceptibility to inhibition be related to the rate of uptake rather than the size of aggregates? Our data on enhanced uptakes neither exclude nor support this view. It was shown that a 20-fold enhancement of net albumin uptake by basic polyamino acids does not render the process sensitive to inhibition (Ryser, 1970). Since the molar uptake of albumin and soluble ferritin differ only by a factor of 9 (Table 1), it is obvious that a parameter other than overall rate of uptake must be involved. Phagocytosis, however, might be discontinuous, with periods of high activity followed by periods of rest. Uptake during bursts of activity could conceivably occur at very high rates requiring more metabolic energy. In either case, ferritin aggregates larger than $0.1 \mu\text{m}$ and albumin trigger a different process, characterized by different susceptibilities to metabolic inhibitors. These findings can be related to the

ultrastructural study of Casley-Smith (1969) who compared the effect of metabolic inhibitors on the uptake of probe molecules and particles of different sizes. He found that the formation of large endocytic vesicles containing bacteria, polystyrene beads or ferritin *en masse* is significantly decreased by 10^{-3} M iodoacetate, and other inhibitors, while the formation of small vesicles containing single ferritin molecules is unchanged. His term ferritin *en masse* appears to correspond, in our study, to ferritin aggregates larger than $0.1 \mu\text{m}$.

The presence of visible aggregates in the culture medium causes a concurrent enhancement of albumin uptake. This observation is reminiscent of prior data on the effect of copolymers of amino acids. Poly (L-lysine:L-tyrosine) rendered partially water insoluble by a content of 50–75% tyrosine, strongly enhances albumin uptake (Ryser, 1970). This enhancement, which can reach 60-fold, is abolished by filtration of the copolymer solution through a Millipore membrane of $1.2\text{-}\mu\text{m}$ pore size (Ryser, 1970). It was concluded that, in this case, the enhancement is related to the presence of large aggregates of insoluble poly(L-lysine:L-tyrosine). Since filtration also abolished the enhancing effect of ferritin aggregates, the observations are comparable even though the aggregates are chemically different and have little in common except their supramolecular size. The enhancement caused by either aggregates is partially inhibited by iodoacetate, while the enhancement caused by poly-L-ornithine is not. Our data do not explain these differences; they only point out that there must be different types of enhancement of albumin uptake.

That molecular size plays a role in the enhancement phenomenon has been recognized previously (Ryser, 1967*b*). When studying the enhancing effect of basic poly-amino acids and DEAE-Dextran, it was found that enhancement increases linearly with the molecular weight of the polymer in the range of 4×10^3 to 1×10^7 (Ryser, 1967*b*). We suggested that size acts in concert with positive charge to increase the number of simultaneous contacts which a macromolecule can make with the cell surface (Ryser, 1967*b*). The new data on ferritin show that a similar effect is observed when ferritin goes from a soluble to an aggregated state. Although the protein is not polycationic in character at neutral pH, it is likely that aggregation increases the number of possible contacts which ferritin can make at one time with the cell surface.

It can be postulated that aggregates are taken up by phagocytosis while soluble macromolecules and their oligomers are taken up by pinocytosis. Our data suggest therefore that, in spite of many similarities, these 2 forms of endocytosis may be fundamentally different. The criterion proposed here as a basis for distinction is a difference in energy requirement. It is known since the work of Sbarra & Karnovsky (1959) that phagocytosis elicits a metabolic response characterized by increased utilization of glucose. It was shown by Gordon & King (1960) that phagocytosis of carmine particles by L-cells and its concomitant metabolic effects are diminished by several metabolic inhibitors. Comparable results were obtained with both leukocytes and tumour cells by Roberts & Quastel (1963). Casley-Smith (1969) showed on thin sections of macrophages that uptake of particles measuring $0.2 \mu\text{m}$ is inhibited by metabolic inhibitors, while the uptake of probes smaller than $0.04 \mu\text{m}$ is not influenced. Our data confirm that phagocytosis is susceptible to metabolic inhibition. In

agreement with Casley-Smith's conclusions, they show that cellular uptake of oligomeric ferritin and of albumin is not subject to such limitations, and suggest that this difference can be used to distinguish pinocytosis from phagocytosis. Why the uptake of monomeric ferritin and aggregated ferritin should differ so markedly in their energy requirement is not yet understood. It is conceivable that the difference is a quantitative one and that a total depletion of cellular energy stores (ATP and other energy-rich compounds) would inhibit the uptake of albumin and filtered ferritin as well. A recent study shows that ingestion of (unfiltered) horseradish peroxidase by L-cells in culture can be decreased when cells are exposed to high concentrations of inhibitors before as well as during the period of measurement (Steinman, Silver & Cohn, 1974). Regardless of its ultimate nature, this difference may prove useful in characterizing the metabolic basis of the transport of macromolecules and aggregates of different sizes.

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