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Role of Plasma Membrane Phospholipids in the Uptake and Release of Transferrin and its Iron by Reticulocytes

Deeporn Hemmaplardh*, Reginald G.H. Morgan, and Evan H. Morgan

Department of Physiology, University of Western Australia, Nedlands, Western Australia. 6009

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Summary. The involvement of membrane phospholipids in the utilization of transferrin-bound iron by reticulocytes was investigated using [^{59}Fe]- and [^{125}I]-labelled transferrin and rabbit reticulocytes which had been incubated with phospholipase A. Transferrin and iron uptake and release were all inhibited by phospholipase A which produced a marked decrease in the relative abundance of phosphatidylcholine and phosphatidylethanolamine and equivalent increases in their lyso-compounds in the reticulocyte plasma membrane. There was a close correlation between the iron uptake rate and the rate and amount of transferrin uptake and the amount of the lysophospholipids in the membrane. Incubation of the cells with exogenous lysophosphatidylethanolamine or lysophosphatidylcholine also produced inhibition of iron and transferrin uptake. The reduced uptake produced by phospholipase A could be reversed if the lyso-compounds were removed by fatty acid-free bovine serum albumin or by reincubation in medium 199. Treatment with phospholipase A was shown to increase the amount of transferrin bound by specific receptors on the reticulocyte membrane but to inhibit the entry of transferrin into the cells.

The present investigation provides evidence that the phospholipid composition of the cell membrane influences the interaction of transferrin with its receptors, the processes of endocytosis and exocytosis whereby transferrin enters and leaves the cells, and the mechanism by which iron is mobilized between its binding to transferrin and incorporation into heme. In addition, the results indicate that phosphatidylethanolamine is present in the outer half of the lipid bilayer of reticulocyte membrane.

Transferrin, the iron-carrying protein of blood plasma, is taken up by erythropoietic cells prior to the transfer of its iron to the cells for hemoglobin synthesis (Walsh *et al.*, 1949, Jandl *et al.*, 1959). The mecha-

* *Present address:* Department of Medicine, University of Washington School of Medicine, Seattle, Washington.

nism of this uptake involves an initial rapid adsorption of the protein to the cell membrane followed by a slower energy-dependent stage of progressive uptake (Morgan, 1964). Subsequently the iron is removed from transferrin before the protein is released from the cell. Various lines of evidence suggest that transferrin molecules bind to specific receptors on the reticulocyte plasma membrane prior to entering the cells by endocytosis during the stage of progressive uptake (Morgan & Appleton, 1969; Appleton, Morgan & Baker, 1971; Fielding & Speyer, 1974; Hemmaplardh & Morgan, 1976*a*). The exact mechanism of interaction between transferrin and its receptors and the changes which occur in the cell membrane subsequent to this interaction have not been fully elucidated. The present work was aimed at investigating the importance of membrane phospholipids in these processes by studying the effects of treatment of reticulocytes with phospholipase A (EC 3.1.1.4) on the uptake and release of transferrin and of iron.

Materials and Methods

Chemicals

Phospholipase A (from bee venom, 1219–1515 U/mg), bovine serum albumin (Fraction V) and butylated hydroxy toluene were purchased from Sigma Chemical Co., St. Louis, Mo. The phospholipase A was tested for proteolytic activity by the method of Murata, Satake & Suzuki (1963). No activity was detectable using casein as substrate and phospholipase at a concentration of 30 μg/ml. The albumin was washed free of fatty acid by the method of Chen (1967) before use. Reference phospholipids (phospholipid kit, natural) and synthetic oleyl lysophosphatidylethanolamine ($C_{18:1}$) were obtained from Serdary Research Laboratories, London, Ontario, Canada. Lysolecithin from egg white was obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England; silica gel H from E. Merck Ag, Darmstadt, Germany; and 1-amino-2-naphthol-4-sulphonic acid from B.D.H. Chemicals Ltd., Poole, England. Medium 199 (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) was kindly supplied by the Royal Perth Hospital, Western Australia. Teric 12A9, a nonionic detergent, was a gift from I.C.I. Research Laboratories, Chesire, England. The radioisotopes, ^{59}Fe as $FeCl_3$ (specific activity 10–30 μCi/μg Fe) and ^{125}I (NaI, carrier-free), were obtained from The Radiochemical Centre, Amersham, Bucks, England. All organic solvents and chemical reagents used in the present experiments were of analytical grade.

Rabbit Reticulocytes

Reticulocytosis was induced in rabbits by daily injections of phenylhydrazine as described in earlier investigations (Morgan & Baker, 1969; Hemmaplardh & Morgan, 1974). The cells were washed three times with 0.15 M NaCl and once with balanced salt solution (Hanks & Wallace, 1949) before use. The white, buffy cell layer was aspirated after each wash.

Purification and Labelling of Rabbit Transferrin

Rabbit serum transferrin was isolated as described previously (Baker, Shaw & Morgan, 1968; Baker & Morgan, 1971) and purified by repeated recrystallization. The protein was labelled with ^{125}I by the iodine monochloride method of McFarlane (1963).

The [^{125}I]-labelled transferrin has been shown to have the same biological properties as unlabelled transferrin (Hemmaplardh & Morgan, 1976*b*). The methods used to remove iron from transferrin and to label it with ^{59}Fe were described in an earlier publication (Hemmaplardh & Morgan, 1974).

Measurement of Transferrin and Iron Uptake

Washed reticulocytes were preincubated with phospholipase A dissolved in Hanks and Wallace balanced salt solution at 37 °C for a specific period of time. The cells were washed 3 times with 0.15 M ice-cold NaCl before aliquots were taken for the study of the transferrin and iron uptake, and for lipid analysis. Controls were treated similarly but without the enzymes. The procedures used for the present investigation for incubating the cells with labelled proteins, washing them and measuring radioactivity were as described previously (Baker & Morgan, 1969). Iron uptake was linear with respect to time and could be expressed as a single uptake rate. Transferrin uptake, however, occurred in three steps as described previously (Morgan, 1964); an initial adsorption, followed by a slower phase of progressive uptake, to reach a plateau level after 15 to 30 min incubation at 37°. The results for transferrin uptake will be given for adsorption, rate or transferrin uptake during the initial part of the progressive phase of uptake, and total uptake after the plateau level had been reached.

Measurement of Transferrin and Iron Release

Reticulocytes were first incubated with [^{59}Fe]-[^{125}I]-labelled transferrin, followed by 3 washes with 0.15 M ice-cold NaCl. The cells were then resuspended in Hanks and Wallace balanced salt solution containing phospholipase A at the required concentration. For the controls, no enzyme was added to the Hanks and Wallace balanced salt solution. The procedures for reincubation at 37 °C, sampling at different time intervals, separation of the supernatant solution and cells, and counting of the radioactivity in these two parts of the mixture were the same as in earlier work (Hemmaplardh & Morgan, 1974).

Preparation of Reticulocyte Ghosts

The method of Dodge, Mitchell & Hanahan (1963) was used for the preparation of unsealed reticulocyte ghosts from phospholipase A treated and washed cells or from control cells.

Lipid Extraction and Analysis

Lipid was extracted from reticulocyte ghosts using chloroform-methanol containing butylated hydroxy toluene as an antioxidant (Nelson, 1972). Phospholipids in the unwashed extract were separated by two-dimensional thin-layer chromatography according to the method of Turner & Rouser (1970). It was found in the present study that improved fractionation could be achieved by running the solvent system twice for each dimension. The different phospholipids separated on the plate were detected by the use of the modified

spray of Vaskovsky & Kostetsky (1968). After visualization, each phospholipid component was quantitatively determined by phosphorus assay (Bartlett, 1959; Parker & Peterson, 1965).

Extraction of Reticulocyte Ghosts with Teric 12A9

Reticulocyte ghosts were extracted with 4 vols ice-cold 1% Teric 12A9 in 20mOSM sodium phosphate buffer, pH 7.4 for 30 min and were then centrifuged at $40,000 \times g$ for 30 min to separate residue from solubilized extract.

Transmission and Scanning Electron Microscopy and Electron Microscope Autoradiography

The methods for transmission and scanning electron microscopy employed in the present study were as reported in a previous paper (Hemmaplardh & Morgan, 1976). Electron microscope autoradiography of reticulocytes incubated with [^{125}I]-labelled transferrin was performed using methods described previously (Morgan & Appleton, 1969).

Analytical Methods

Reticulocytes were counted on dry smears after staining with new methylene blue. The hematocrit was measured using a Hawksley microhematocrit centrifuge (Hawksley Co., London, England). Heme was extracted from cells using the ethyl acetate-acetic acid method of Thunell (1965). Determination of iron was by the procedure recommended by the International Committee for Standardization in Hematology (1971). Plasma transferrin was determined by measuring its total iron binding capacity (Morgan & Carter, 1960). Radioactivity was measured in a Packard Autogamma 3-Channel well-type scintillation counter (Packard Instruments Co., Downers Grove, Ill.).

Results

Transferrin and Iron Uptake by Reticulocytes Treated with Phospholipase A

Treatment with phospholipase A caused an inhibition of the rates of iron and transferrin uptake and the total amount of transferrin uptake (Fig. 1). The higher the concentration of the enzyme used, the greater was the inhibition. Treatment with phospholipase A produced only about a 5% decrease in total cell phospholipids but there was a marked change in the relative abundance of phosphatidylcholine and phosphatidylethanolamine and their lyso-compounds (Fig. 2) without any change in the other components. As the concentration of the enzyme was increased the concentrations of phosphatidylcholine and phosphatidylethanolamine in the cells declined while those of the corresponding lyso-compounds increased. As shown in Fig. 3 the degree of inhibition of the rate of iron uptake was closely related to the amount of lysophospholipids in

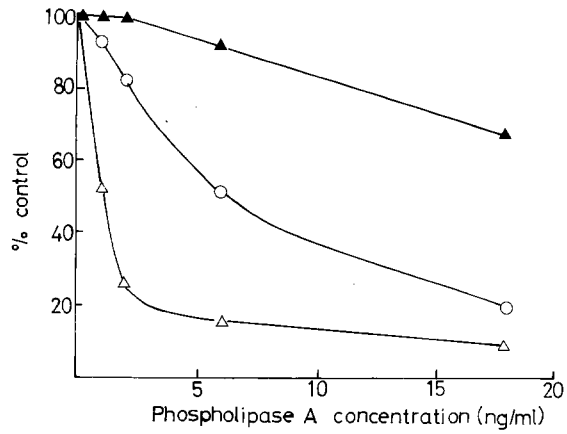

Fig. 1. Effect of phospholipase A on transferrin and iron uptake by reticulocytes. The cells (50% reticulocytes) were incubated for 20 min at 37 °C with Hanks and Wallace balanced salt solution containing 0,1,2,6 or 18 μg/ml of phospholipase A. They were then washed 3 times and reincubated with transferrin labelled with ^{125}I and ^{59}Fe (transferrin concentration; 2.5 mg/ml; iron concentration, 2.25 μg/ml). ○, transferrin uptake rate; ▲, total transferrin uptake; △, iron uptake rate

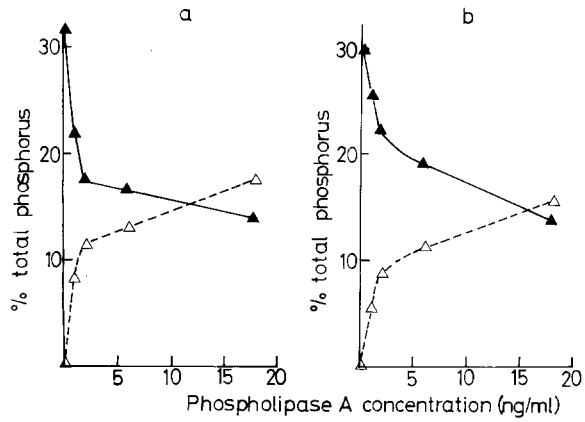

Fig. 2. Effect of phospholipase A on the phospholipid composition of reticulocytes. The cells were preincubated for 20 min at 37 °C with Hanks and Wallace solution containing a specified concentration of phospholipase A. After washing 3 times, ghosts were prepared prior to lipid extraction and analysis. (*a*): phosphatidylethanolamine; (*b*): phosphatidylcholine. ▲, phospholipid; △, lysophospholipid

the membrane (correlation coefficient greater than 0.9). Similar correlations were found between the rate of uptake of transferrin and the total amount of transferrin uptake and the membrane lysophospholipid levels.

The percentage of the ^{59}Fe taken up by the cells which was incorporated into heme in the cells treated with phospholipase A (83%) was greater than that in the controls (74%).

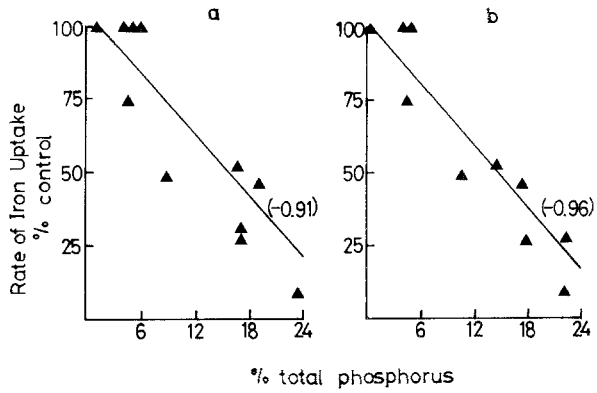

Fig. 3. Correlation between iron uptake by reticulocytes and lysophospholipid levels of the cell membrane. Data was obtained from 4 experiments. The rate of iron uptake expressed as a percent of the control value was plotted against the amount of lysophosphatidylethanolamine (*a*) or lysophosphatidylcholine (*b*) on the membrane expressed as the percent of total phosphorus. The correlation coefficients are shown in brackets

Recovery of Function in Cells Treated with Phospholipase A

When reticulocytes which had been treated with phospholipase A were reincubated in medium 199 for 3 hr at 37 °C in the absence of the enzyme there was a partial reversal of the inhibition of transferrin and iron uptake induced by treatment with the enzyme (Table 1). Recovery was more complete for transferrin than for iron. Lipid analysis of the reticulocyte membranes showed that reincubation in medium 199 resulted in a reduction of both lysophosphatidylethanolamine and lysophosphatidylcholine and a rise in phosphatidylethanolamine and phosphatidylcholine (Table 1).

A second method was also used to alter the concentration of lysophospholipids in the membranes of reticulocytes incubated with phospholipase A. This was to add fatty acid-free bovine serum albumin to the incubation mixture containing the phospholipase A. The albumin was used to remove lysophospholipids formed during the incubation. The results are shown in Table 2. The values for the uptake of transferrin and iron were greater in the cells incubated with phospholipase A in the presence of albumin than in those where no albumin was used. Analysis of the cells for phospholipids showed that the concentration of lysophosphatidylcholine was lower and that of phosphatidylcholine higher in the albumin treated cells. Little change occurred in the concentrations of lysophosphatidylethanolamine and phosphatidylethanolamine. The use of albumin in concentrations above 5 mg/ml was associated with lysis of the cells as has been described previously (Gul & Smith, 1974).

Table 1. Recovery of phospholipase A treated reticulocytes.

Cell	Time in medium 199	% control				% total			
		Iron	Transferrin			Lipid phosphorus			
		Rate	Adsorption	Rate	Amount	PE	LPE	PC	LPC
Control	0	100	100	100	100	28	4	36	0
	3 hr	100	100	100	100	30	6	33	0
Treated	0	31	94	59	73	11	17	15	22
	3 hr	74	108	90	93	26	5	27	12

Rabbit reticulocytes were incubated with phospholipase A (33 μg/ml) at 37 °C for 30 min, washed, and samples were used immediately for transferrin and iron uptake study and for lipid extraction. Controls were treated similarly but without the enzyme. Additional samples of phospholipase A-treated or control cells were incubated in medium 199 at 37 °C for 1.5 hr and in a fresh sample of the medium for a further 1.5 hr, and then washed 3 times with 0.15 M ice-cold NaCl before use in uptake studies and for lipid extraction. The rate of iron uptake and the initial adsorption of transferrin to the cells, the rate of transferrin uptake during the stage of progressive uptake and the total amount of transferrin taken up by the cells were all measured. These results are expressed as percent of the corresponding controls. Reticulocyte count was, 90%; iron concentration, 1.5 μg/ml; transferrin concentration, 1.8 mg/ml. Abbreviations: PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

Table 2. Removal of lysophospholipids from reticulocytes treated with phospholipase A by bovine serum albumin

	Control	Treated		
		Bovine serum albumin (mg/ml)		
		0	3	5
Phospholipids (% total phosphorus)				
Phosphatidylethanolamine	28.5	10.2	11.5	11.2
Lysophosphatidylethanolamine	0.7	18.2	17.0	17.2
Phosphatidylcholine	31.6	14.0	18.2	19.2
Lysophosphatidylcholine	0.8	18.1	13.6	12.7
Uptake (% control)				
Iron uptake rate	100	34.8	62.4	77.2
Transferrin uptake rate	100	50.6	75.3	75.3
Transferrin uptake plateau	100	59.9	89.5	112.7

Reticulocyte suspensions were incubated in the absence or presence of phospholipase A (30 μg/ml) at 37 °C for 30 min. Bovine serum albumin, at varying concentrations, was also present simultaneously with the enzyme. The cells were washed 3 times in 0.15 M ice-cold NaCl before samples were used for lipid extraction and analysis and for the measurement of transferrin and iron uptake. Reticulocyte count, 60%; iron concentration, 1.4 μg/ml; transferrin concentration, 1.25 mg/ml.

Effect of Exogenous Lysophosphatidylethanolamine and Lysophosphatidylcholine

When reticulocytes were incubated at 37 °C for 30 min with lysophosphatidylethanolamine or lysophosphatidylcholine dissolved in Hanks and Wallace solution there was an increase in the concentrations of these phospholipids in the cells (Table 3). This was associated with a 9–20% reduction in the rates of uptake of both transferrin and iron (Table 3). Incubation of the cells with concentrations of the lysophospholipids above those shown in Table 3, caused the cells to hemolyze. Hence, it was not possible to determine the effects of higher concentrations of exogenous lyso-phospholipids on transferrin and iron uptake.

Gel Filtration of Extracts of Reticulocyte Ghosts

Untreated reticulocyte ghosts were incubated with [^{125}I]-labelled transferrin, washed and extracted with Teric 12A9. When the supernatant solution obtained after centrifugation was fractionated by gel filtration on Sephadex G-200 radioactive transferrin was eluted in two peaks. The first peak emerged just after the void volume and the second peak at a volume corresponding to that of free transferrin (Fig. 4). This two-peak elution profile of transferrin extracted from reticulocytes with Teric 12A9 has been described previously (van Bockxmeer, Hemmaplardh &

Table 3. Loading of reticulocytes with exogenous lysophosphatidylethanolamine or lysophosphatidylcholine

	% control			% total lipid	
	Iron Rate	Transferrin		Phosphorus	
		Rate	Amount	LPE	LPC
Control	100	100	100	0.7	0.8
LPE (0.33 mg/ml)	80	91	120	6.5	1.0
LPC (0.4 mg/ml)	85	86	103	0.9	2.3

Reticulocytes were pretreated with lysophosphatidylethanolamine (LPE, 0.33 mg/ml) or lysophosphatidylcholine (LPC, 0.4 mg/ml) dissolved in Hanks and Wallace balanced salt solution at 37 °C for 30 min. Control cells were incubated in Hanks and Wallace solution, but without the addition of the lysophospholipids. After washing 3 times in 0.15 M ice-cold NaCl, aliquots of the cells were taken for the measurement of transferrin and iron uptake and for lipid extraction and analysis. Reticulocyte count was 50%; iron concentration 1.9 μg/ml; transferrin concentration 2.1 mg/ml.

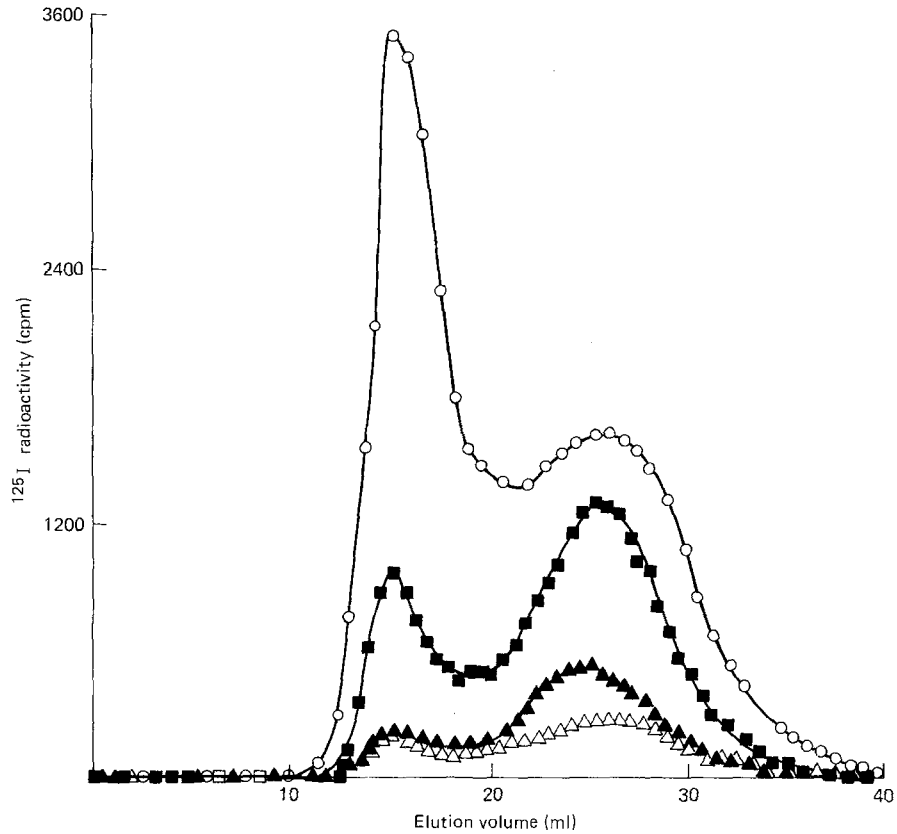

Fig. 4. Sephadex G-200 gel filtration of Teric 12A9 extracts prepared from control and phospholipase A-treated reticulocyte ghosts which had been preincubated with or without unlabelled transferrin followed by incubation with [^{125}I]-labelled transferrin. Rabbit reticulocyte ghosts (80% reticulocytes) were incubated for 21 min at 37 °C in the absence of phospholipase A (control; ▲, ■) or with phospholipase A (4 μg/ml; △, ○). The ghosts were then washed 3 times, reincubated with (▲, △) or without (■, ○) unlabelled iron-saturated transferrin (6.25 mg/ml) at 37 °C for 3 min, followed by incubation with [^{125}I]-labelled transferrin (0.125 mg/ml) at 37 °C for 12 min, then rewashed and extracted with Teric as described in the text

Morgan, 1975). Treatment of reticulocyte ghosts with phospholipase A prior to incubation with [^{125}I]-labelled transferrin resulted in a 60% increase in the uptake of the protein compared with uptake by untreated ghosts (Table 4). When Teric extracts of the phospholipase A treated ghosts were fractionated by gel filtration it was found that most of the additional radioactivity appeared in the first elution peak (Fig. 4). Samples of the control and phospholipase A-treated ghosts were also incubated with unlabelled transferrin in the presence of the [^{125}I]-labelled transferrin, with unlabelled transferrin present at 50 times the

Table 4. Transferrin uptake by reticulocyte ghosts and distribution of labelled transferrin between the two gel filtration elution peaks

Treatment of cells	Incubation with unlabelled transferrin	Transferrin uptake (μg/ml cell)	(% total)	
			First peak	Second peak
Control	+	2.99	16	84
Control	−	7.25	30	70
Phospholipase A	+	1.56	20	80
Phospholipase A	−	11.53	55	45

Control and phospholipase A-treated reticulocyte ghosts were incubated with [^{125}I]-labelled transferrin, extracted with Teric 12A9 and chromatographed on Sephadex G-200 as described for Fig. 4. The ^{125}I radioactivity in the two elution peaks were measured and expressed as percentages of the total radioactivity.

concentration of the labelled protein. This resulted in a marked reduction in the uptake of [^{125}I]-labelled transferrin by the ghosts in both samples of ghosts, with a greater reduction in those cells treated with phospholipase A. This reduction was relatively greater for the transferrin present in the first gel filtration peak than for that in the second peak (Fig. 4, Table 4).

Release of Transferrin and Iron by Cells Treated with Phospholipase A

Studies were made on the effects of incubation with phospholipase A on the release of transferrin and iron from reticulocytes which had taken up transferrin labelled with ^{59}Fe and ^{125}I during incubation at 37 °C for 20 min prior to washing and reincubation with the enzymes. As shown in Fig. 5, the release of transferrin from the cells immediately after addition of the reincubation solution and during the first 2–5 min of incubation at 37 °C was unaffected by the presence of phospholipase A (8 and 33 μg/ml). Prolongation of the period of reincubation was accompanied by a small reduction in the rate of release of the protein in the presence of the lower concentration of the enzyme but by almost complete inhibition of further release of transferrin when the higher enzyme concentration was used. No hemolysis and no release of ^{59}Fe above that found with the control cells was observed during incubation with phospholipase A at any concentration used.

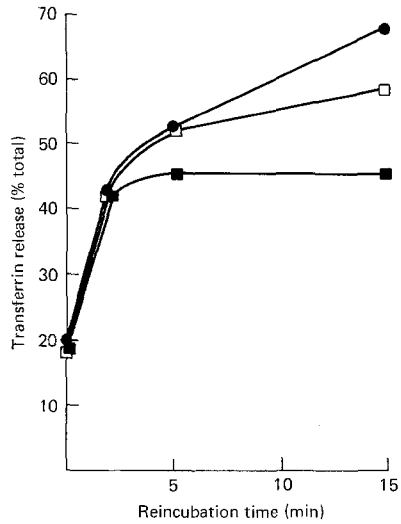

Fig. 5. Effect of incubation with phospholipase A on the release of [^{125}I]-labelled transferrin from rabbit reticulocytes. The cells (40% reticulocytes) were incubated with transferrin labelled with ^{125}I and ^{59}Fe for 20 min at 37 °C. They were then washed with 0.15 M ice-cold NaCl and reincubated at 37 °C in Hanks and Wallace solution in the absence (●) or presence of phospholipase A at 8.3 (□) or 33 (■) μg/ml

Morphology and Autoradiography of Treated Cells

Cells treated with phospholipase A were examined by scanning and transmission electron microscopy. The cells showed an echinocytic appearance. When reticulocytes were incubated with phospholipase A, washed, incubated with [^{125}I]-labelled transferrin, rewashed and the cells examined by electron microscope autoradiography it was found that the silver grains corresponding to transferrin molecules were confined to the outer cell membrane (Fig. 6*b*). In control cells the grains were found both within the cells and on the membrane (Fig. 6*a*) and many of the grains within the cells were close to intracellular vesicles. This is in agreement with the concept that transferrin enters the cells by endocytosis (Appleton *et al.*, 1971).

Discussion

Evidence is provided in the present study for the involvement of membrane phospholipids in the process of transferrin and iron uptake by reticulocytes. The decrease in transferrin and iron uptake which

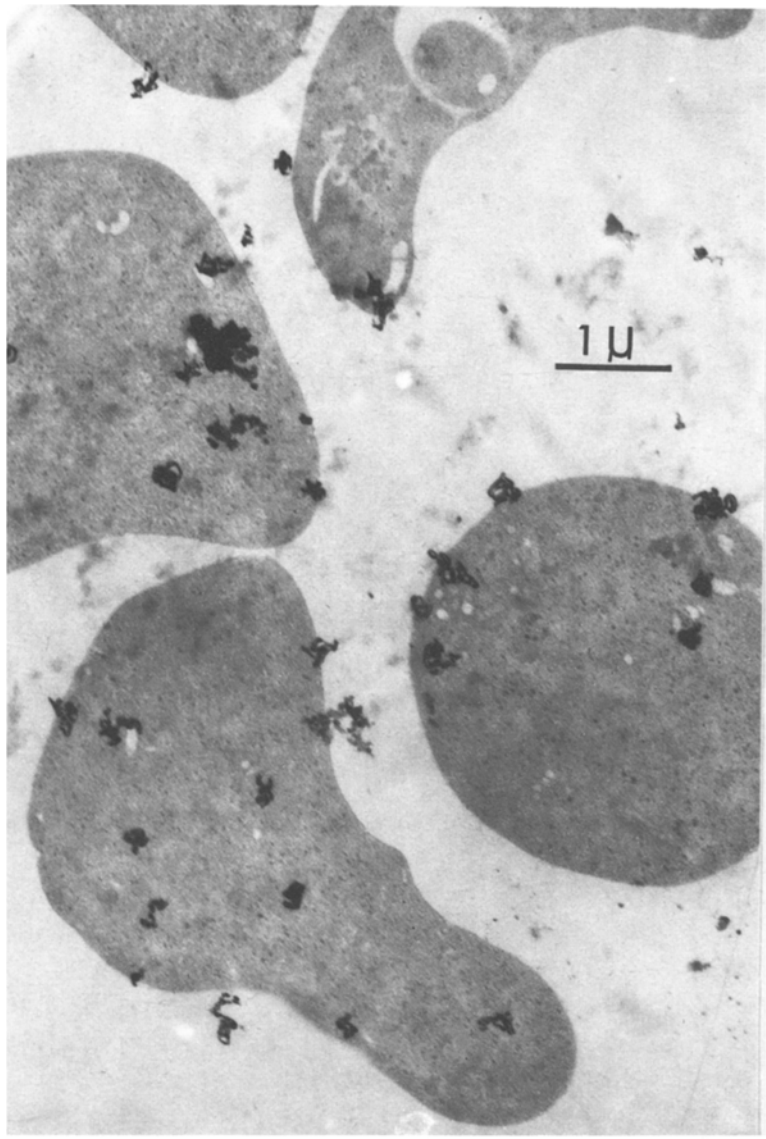

Fig. 6. Autoradiographs of control reticulocytes and reticulocytes treated with phospholipase A (30 μg/ml) for 30 min which were washed then incubated with [^{125}I]-labelled transferrin for 20 min. In the cells treated with phospholipase A (*B*) autoradiographic grains corresponding to transferrin molecules were confined to the cell periphery but in the control cells (*A*) the grains were also present inside the cells, often in close relation to endocytotic vesicles

resulted from treatment of the cells with phospholipase A could have been due to the decrease of phosphatidylethanolamine and phosphatidylcholine, the two predominant phospholipids of the plasma membrane

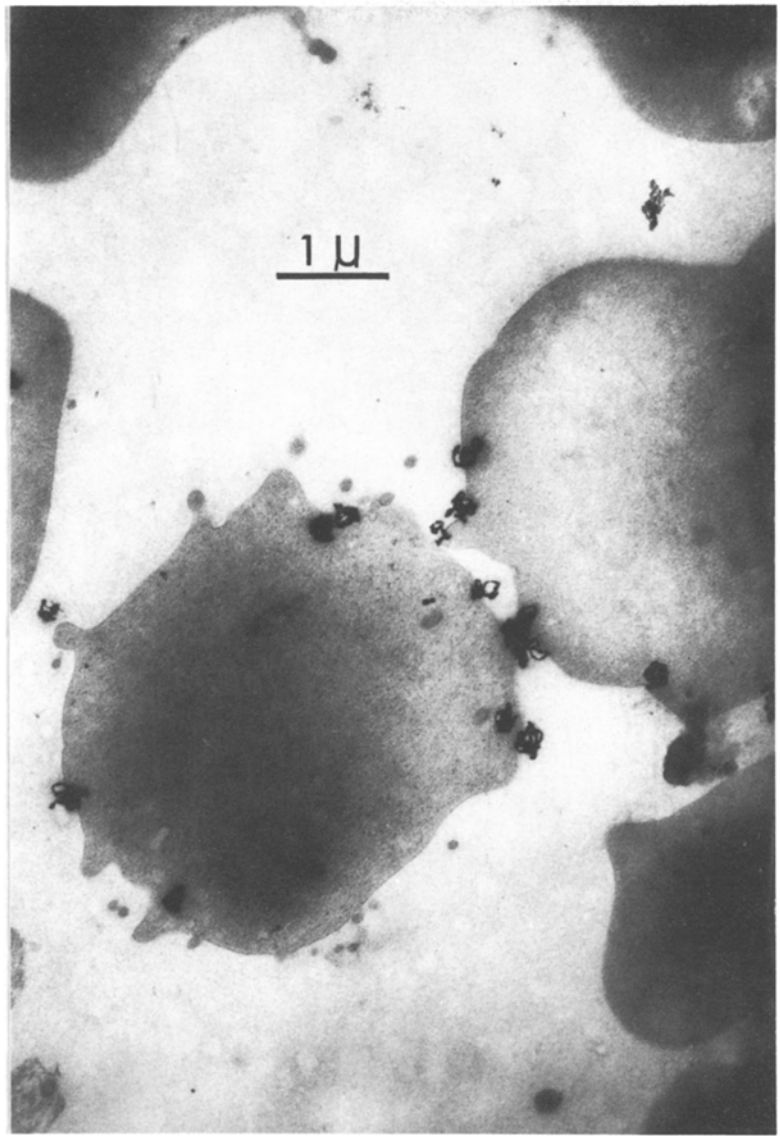

Fig. 6B

or to the increase in the membrane of their lysocompound and/or free fatty acids which are the products of digestion of the phospholipids by the enzyme. The results obtained with the cells loaded with exogenous lysophosphatidylethanolamine and lysophosphatidylcholine (Table 3) suggest that the presence of the lysophospholipids, which are normally present only in low concentrations in the cells, is the direct cause of the reduction of transferrin uptake. The high correlation found between

the degree of inhibition of transferrin and iron uptake and the concentrations of lysophospholipids in the cells supports this concept. It is also supported by the results of the experiment in which albumin was included during incubation with phospholipase A. However, membrane phosphatidylcholine levels increased during incubation, and albumin would also have been expected to remove free fatty acids. Hence, it is possible that transferrin uptake is also affected by the concentration of phosphatidylcholine and of free fatty acids in the membrane.

Changes in membrane phospholipid composition could have affected transferrin uptake in at least two ways, by altering the interaction of transferrin with specific receptors on the cell or by changing the processes which occur subsequent to the binding of transferrin to its receptors. The results presented above indicate that both mechanisms of action were operative.

The interaction of transferrin with its receptors was studied in the experiment in which reticulocyte ghosts were incubated with [^{125}I]-labelled transferrin, extracted with Teric 12A9 and the extract fractionated by gel filtration. Evidence has been presented previously that the addition of unlabelled transferrin to an incubation mixture of reticulocytes ghosts and [^{125}I]-labelled transferrin inhibits the binding of labelled transferrin to specific receptors but not nonspecific binding to the membranes (van Bockxmeer *et al.*, 1975). In addition the transferrin eluted in the first peak during gel filtration on Sephadex G-200 of extracts of reticulocytes prepared with Teric 12A9 represents transferrin bound to its receptor (van Bockxmeer *et al.*, 1975). The results of the present experiments therefore indicate that the binding of transferrin to specific receptors on the reticulocyte membrane increases after incubation of the membrane with phospholipase A (Table 5). A greater proportion of the transferrin in the ghosts treated with phospholipase A was eluted in the first gel filtration peak than was the case with control ghosts (Table 4 and Fig. 4). This confirms that treatment with the enzyme increased the binding of transferrin by its receptors. These results indicate that digestion of reticulocyte cell membrane phospholipids with phospholipase A leads to an unmasking of transferrin receptors in the membrane, leading either to an increase in the number of available receptors or to an increase in their affinity for transferrin. A similar unmasking of insulin receptors in fat cell and liver cell membranes has been observed following incubation of the membranes with phospholipase A and phospholipase C (Cuatrecasas, 1971).

The increase in binding of transferrin to its receptors which was

caused by treatment with phospholipase A could not, by itself, have produced the observed decrease in the rate of uptake of transferrin by intact cells and in the total amount taken up when equilibrium was achieved (Fig. 1). Hence, a second result of the enzymatic digestion was to inhibit those steps in the uptake of transferrin which occur subsequent to its binding to the membrane receptors. The evidence from morphological studies indicates that these steps involve uptake of the protein into the cells by endocytosis (Morgan & Appleton, 1969; Appleton *et al.*, 1971). Alteration in the reticulocyte membrane phospholipid composition may inhibit endocytosis. The autoradiographs obtained in the present work, which showed no inhibition of transferrin binding to the outer cell membrane but inhibition of the entry of transferrin into the cell (Fig. 6), support this concept. The release of transferrin from reticulocytes is believed to occur by exocytosis (Morgan, 1974), which is probably dependent on similar membrane properties to those required for endocytosis. Treatment of the reticulocytes with phospholipase A was found to inhibit release of transferrin (Fig. 5). The results obtained with phospholipase A contrast with those found when reticulocytes or their ghosts were incubated with proteolytic enzymes. The latter treatment inhibited the binding of transferrin to its receptors but there was no evidence of inhibition of endocytosis (Hemmaplardh & Morgan, 1976*a*).

The changes in transferrin uptake and release which were produced by phospholipase A were accompanied by alterations in cell shape, leading to an echinocytic appearance. Several workers have previously presented evidence for the importance of the phospholipid composition of the cell membrane for cell shape, deformability and membrane vesiculation (Deuticke, 1968; Weed, LaCelle & Udkow, 1974). In addition, Sheetz and Singer (1974) have proposed that biological membranes behave as bilayer couples, in that the two halves can respond differently to a perturbation. They also reported that substances which produce crenation in erythrocytes inhibit antibody-induced endocytosis in lymphocytes. It is possible that the echinocytic change produced in reticulocytes by the action of phospholipase A inhibits the endocytic uptake of transferrin-iron and that this shape transformation is due to the accumulation of lysophosphatides in the cell membrane.

The degree of inhibition of the rate of iron uptake was greater than that of transferrin uptake at each concentration of phospholipase which was used (Fig. 1). This implies that treatment with the enzyme inhibited the mechanisms by which iron is removed from transferrin and/or those by which it is subsequently metabolized by the cell in addition to inhibit-

ing the initial stage of iron uptake which is dependent on transferrin uptake. The proportion of the iron taken up by the cell which was incorporated into heme was not reduced by phospholipase. Hence, it is likely that treatment with the enzyme resulted in inhibition of iron release from transferrin and/or of intracellular transport of iron from the site of release to ferrochaelatase (EC 4.99.1.1), on the inner mitochondrial membrane (Jones & Jones, 1968), where it is incorporated into heme.

The changes in membrane phospholipid composition and the inhibition of transferrin and iron uptake produced by pre-treatment of reticulocytes with phospholipase A were reversible (Table 2). These results are in agreement with the finding that reticulocytes contain an enzyme system capable of phospholipid biosynthesis (Percy *et al.*, 1973; Ballas & Burke, 1974).

It is generally accepted that phosphatidylcholine and sphingomyelin are located chiefly in the outer half of the lipid bilayer of the cell membrane, while phosphatidylethanolamine and phosphatidylserine are located on the inner (cytoplasmic) half of the bilayer (Bretscher, 1972). However, the results of the present investigation demonstrate that, in rabbit reticulocytes, phosphatidylethanolamine was accessible to phospholipase A. It has been shown in synthetic vesicles that "flip-flop" migration of phospholipids from one side of the bilayer to the other occurs at an extremely slow rate (Kornberg & McConnell, 1971), but it has been suggested that little such migration takes place in the erythrocyte membrane (Bretscher, 1972). If this is true the present results suggest that in the rabbit reticulocyte phosphatidylethanolamine exists in the outer half of the lipid bilayer. However, as pointed out by Martin *et al.* (1975), the action of phospholipase A on intact erythrocytes is complex and such factors as animal species and conditions of incubation may alter the susceptibility of different phospholipids to hydrolysis by the enzyme. Hence, any conclusion as to the distribution of phospholipid classes in the rabbit reticulocyte must be treated with caution until further investigations are performed.

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