Biochimica et Biophysica Acta, 469 (1977) 281—291

BBA 77796

MOBILE CARRIER IONOPHORES FOR Fe(II)

STEPHEN P. YOUNG [a] and BASTIEN D. GOMPERTS [b]

Departments of [a] *Clinical Haematology and* [b] *Experimental Pathology, University College Hospital Medical School, London WC1E 6JJ (U.K.)*

(Received January 7th, 1977)

Summary

A23187 and certain other carboxylate ionophores are capable of transferring Fe(II) but not Fe(III) * across phospholipid bilayers (liposomes) and red cell membranes. A23187 is able to transfer Fe(II) from ferritin loaded liposomes when allied with a suitable redox couple and sink. The affinity of A23187 for Fe(II) is approximately five orders of magnitude greater than for Ca^{2+}, as judged by two phase extraction techniques.

Introduction

Since the advent of ionophore A23187, a large number of experiments have been conducted in which its ability to transfer Ca^{2+} across membrane barriers has been exploited (for review, see refs. 1, 2 and 3). It has been apparent from the first that its specificity among divalent cations is low, and it has been shown that as well as the cations of the alkaline earth series, A23187 is also able to transfer the divalent cations of a number of transition metals into bulk organic phases [4].

Ionophores capable of carrying iron would be useful in the investigation of iron metabolism and a specific carrier for Fe(II) could have clinical application in certain disorders in which there is an excess of iron in the tissues or failure to mobilise stored iron [5,6].

It has been reported on a previous occasion that ionophores A23187 and X537A are able to carry iron across both artificial and biological membranes [7]. Here, the ability of A23187 (and a number of other substances known to

* The expressions FE(II) and Fe(III) are used to describe the oxidation states of iron. The expression Fe^{2+} is used only when it is clear that a simple ionised form of Fe(II) is involved.
Abbreviations: ferrozine, 3(2-pyridyl)-5,6 diphenyl 1,2,4 triazine-p,p'-disulphonic acid disodium salt trihydrate; PMS, phenazinemethosulphate; TMPD, tetramethyl phenylenediamine; Et_4AmCl and Et_4AmOH, tetraethylammonium chloride and hydroxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid; bis-tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane.

have ionophoric properties) to act as carriers for Fe(II) is further examined by bulk phase extraction and transport studies using liposomes and red blood cells.

Materials

Chemicals. Egg lecithin grade I was obtained from Lipid Products, Surrey. Horse spleen ferritin (2 × crystallised) and stearylamine from Koch-Light Laboratories, Bucks. Ferrozine was from Aldrich Chemical Co. Ltd., Dorset. PMS and TMPD were obtained from Sigma London Ltd. $^{59}FeCl_3$ and $^{45}CaCl_2$ were supplied by the Radiochemical Centre, Amersham, Bucks. Chelex 100 chelating resin was from Bio-Rad Laboratories Ltd., Kent, and IRA410 anion exchanger from BDH Ltd. All other chemicals were of the highest grade commercially available.

Ionophores A23187, monensin, dianemycin, A204, and A 28695A and B were the gift of Dr. Bob Hamill, of Eli Lilly Research Laboratories, Indianapolis. X537A and its bromo-, nitro-, and acetyl-derivatives, nigericin, X206 and lysocellin were the gift of Dr. Julius Berger of Hoffman La Roche, Nutley, N.J. Gramicidin D was from Sigma; valinomycin, nystatin and amphotericin B were from Calbiochem.

Methods

Red cell experiments. Human red cells were washed in Et_4AmCl (0.15 M) three times and suspended at 10% packed cell volume in a solution containing Et_4AmCl (0.12 M), HEPES (20 mM), ascorbic acid (10 mM), $(NH_4)_2Fe(SO_4)_2$ (1 mM) at pH 7.5. A trace of ^{59}Fe as $FeCl_3$ was added so that the activity of the solution was about 1 μCi ml^{-1}. The suspension was incubated at 37°C in a shaking water bath. Ionophores dissolved in ethanol were added to give a final concentration of 50 μM. Duplicate samples (50 μl) were removed at intervals and the red cells rapidly separated from the supernatant solution by centrifuging through silicone oil for 1 min at 12 000 × g on a Beckman Microfuge (model 152). The supernatant was aspirated together with some of the oil and the tube above the oil was washed to remove residual supernatant solution. The microcentrifuge tube containing the red cell pellet was placed in the γ-counter for measurement of the intracellular radioactivity.

Fe(II)-liposomes. Egg lecithin, cholesterol and stearylamine were mixed in organic solvent in a molar ratio 7 : 2 : 1 and the solvent was evaporated under a stream of N_2. The dry lipid film was hydrated by vigorous shaking in a small volume of an aqueous solution containing $FeSO_4$ (25 mM). Ascorbic acid (0.1 M), adjusted to the required pH with Bis-Tris, was then added. The buffer had been gassed with N_2 to minimise oxidation of Fe(II). Non-entrapped Fe(II) was removed by the addition of a few beads of the cation exchange resin Chelex 100 which had previously been equilibrated with the buffer.

Liposomes were suspended at a concentration of 1 mM total lipid in a solution containing ferrozine (0.5 mM) and ascorbic acid (0.1 M) buffered with Bis-Tris at either pH 5.5 or 6.5. Test compounds dissolved in ethanol were added to give a concentration of 25 μM. Changes in the absorbance at 562 nm due to

the formation of the Fe(II)-ferrozine complex in the extra-liposomal solution were monitored.

Fe(III)-liposomes. To dried lipids prepared as above, a solution of $FeCl_3$ (25 mM) and Na_3 citrate (62.5 mM) at pH 5 was added and the liposomes prepared by shaking. Extraliposomal Fe as the anionic dicitrate complex was removed by addition of IRA410 resin previously equilibrated with the buffer.

The Fe(III) liposomes were then treated as for the Fe(II) liposomes described above.

Ferritin-liposomes. To dried lipids prepared as above was added a solution containing ferritin (50 mg · ml^{-1}) and liposomes generated by shaking as described. Most of the non-entrapped ferritin was removed by centrifuging the liposomes at 10 000 × *g* for 30 min. Any external ferritin iron remaining after this was removed by dialysis against ascorbate buffer (0.1 M, pH 5.5). In this way, the external ferritin-Fe(III) was reduced to Fe(II) and liberated to the dialysate. Dialysis was continued until no Fe(II) was detectable in the dialysate by optical determination using ferrozine (limit of detection 50 nM). In order to check the stability of liposomes under the varied conditions of the experiments, we added β-methyl umbelliferyl phosphate (5 mM) to the ferritin solution used to hydrate the lipid. The appearance of this substance on the exterior of the liposomes could be detected by the addition of alkaline phosphatase to the suspending medium [8]. This results in the generation of the intensely fluorescent product β-methyl umbelliferone upon subsequent alkalinization.

Solvent extractions. Duplicate samples containing ionophores (0.1 mM) dissolved in 0.2 ml toluene : butanol (3 : 1) and 0.2 ml aqueous buffer (ascorbic acid, 0.1 M and Bis-Tris, 50 mM) containing ^{59}Fe (1—5 μCi · ml^{-1}) in $FeSO_4$ or ^{45}Ca (1—5 μCi · ml^{-1}) in $CaCl_2$ were pipetted into Beckman polypropylene microfuge tubes. With all Fe(II) solutions, care was taken to maintain an atmosphere of N_2. The pH of the aqueous phases was adjusted with Et_4AmOH. In certain experiments where a concentration of 0.5 M Fe(II) was used, the ascorbic acid concentration in the aqueous phase was raised to 1 M and the ^{59}Fe to 15 μCi · ml^{-1}. The tubes were shaken vigorously for 5 min and then centrifuged to separate the phases. 0.1 ml of the organic phase was removed for counting and the pH of the aqueous phase was measured. The radioactivity of the aqueous phase was estimated from the total activity added to the system found by counting a sample of the original aqueous phase.

Results

A number of substances known to induce ionic permeability in model and biological membranes were tested for their ability to transfer Fe(II) across the phospholipid bilayers of multi-lamellar liposomes. The results of some typical experiments are shown in Fig. 1. Of all the compounds that were available to us, A23187 at 25 μM was the most effective in inducing a flux of Fe(II). The efflux of Fe(II) was detected by the increase in absorbance at 562 nm of the Fe(II)-chelate dye ferrozine which also served as a sink maintaining the activity of the Fe(II) in the external aqueous phase at a very low level. Other carboxylate ionophores definitely capable of inducing a leak of Fe(II) include (in order of effectiveness) lysocellin > X537A > Br-X537A > A28695A (Fig.

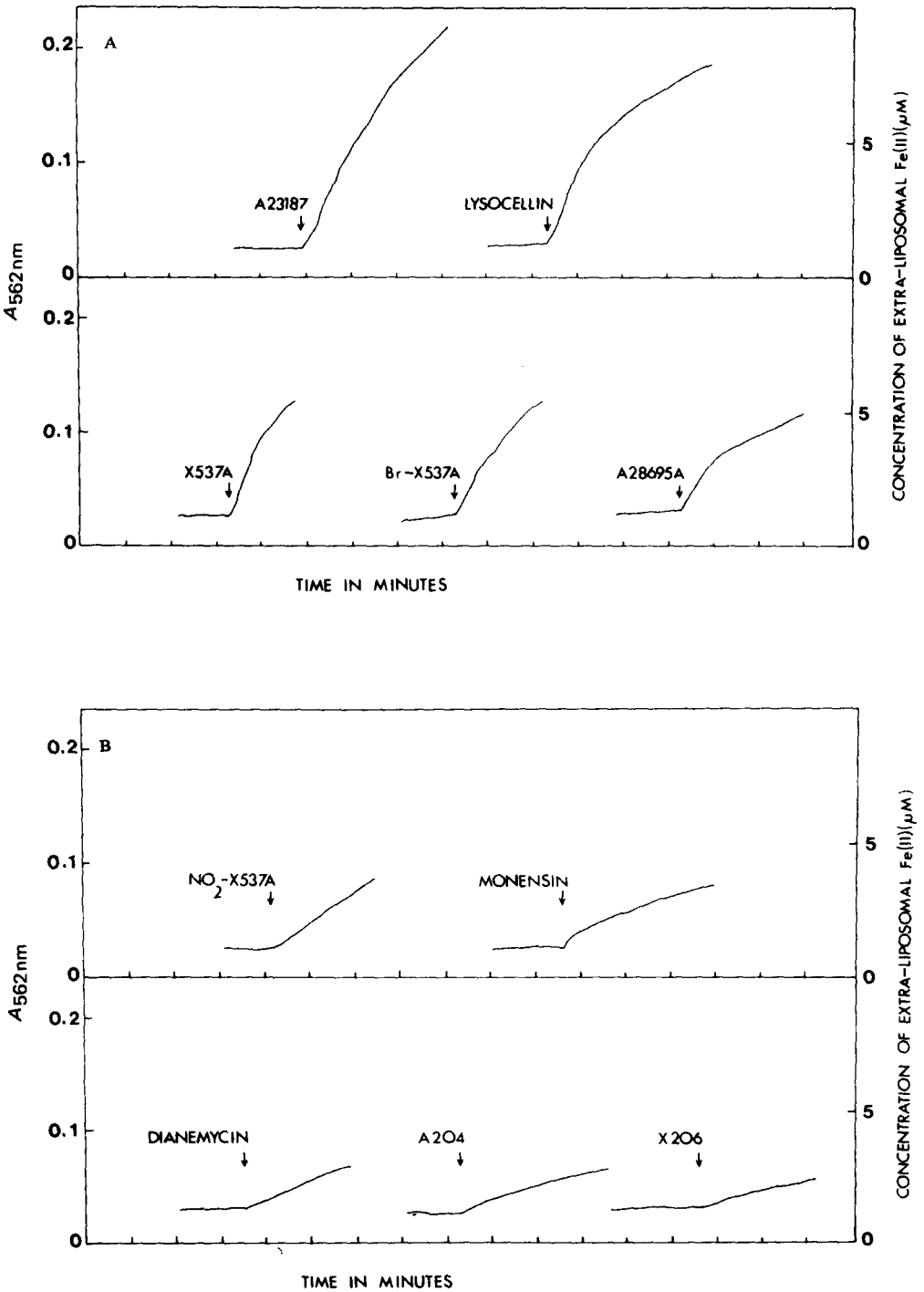

Fig. 1. Release of Fe(II) from liposomes induced by various carboxylate ionophores. Liposomes (1 mM total lipid) were suspended in a solution containing Bis-Tris, ascorbic acid and ferrozine and the pH adjusted to 6.5. Ionophores dissolved in ethanol were added to give a final concentration of 25 μM. Maximum absorbance change equivalent to total release of liposomal Fe(II) = 0.865 ≡ 37 μM Fe(II) in the extraliposomal solution.

1A). With all these ionophores total loss of the included Fe(II) (equivalent to that released by addition of the non-ionic detergent Triton X100 0.5%) could be achieved by allowing the reaction to continue for about 30—60 min. We could also detect an efflux of Fe(II) from liposomes treated with NO_2-X537A > monensin > dianemycin > A204 > X206 (Fig. 1B). As far as we are aware, this is the first report of divalent ionophoric activity for many of these compounds. The following carboxylate ionophores were without effect: nigericin, A28695B, acetyl-X537A. Also the cyclic depsipeptide valinomycin, the polypeptide gramicidin D and the cyclic polyenes nystatin and amphotericin B were ineffective.

The most active substances were also capable of inducing an inward movement of Fe(II) into red cells (Fig. 2). In this experiment there was no means of maintaining the activity of Fe(II) on the distal (or trans) side of the membrane at a continuously low level and it is probably for this reason that we were only able to detect significant inward movements of Fe(II) with those ionophores which were the most effective in the liposome efflux experiments. With the cyclic polyenes amphotericin B and nystatin, which proved inactive as permeability inducing agents for Fe(II), we were able to demonstrate that ion

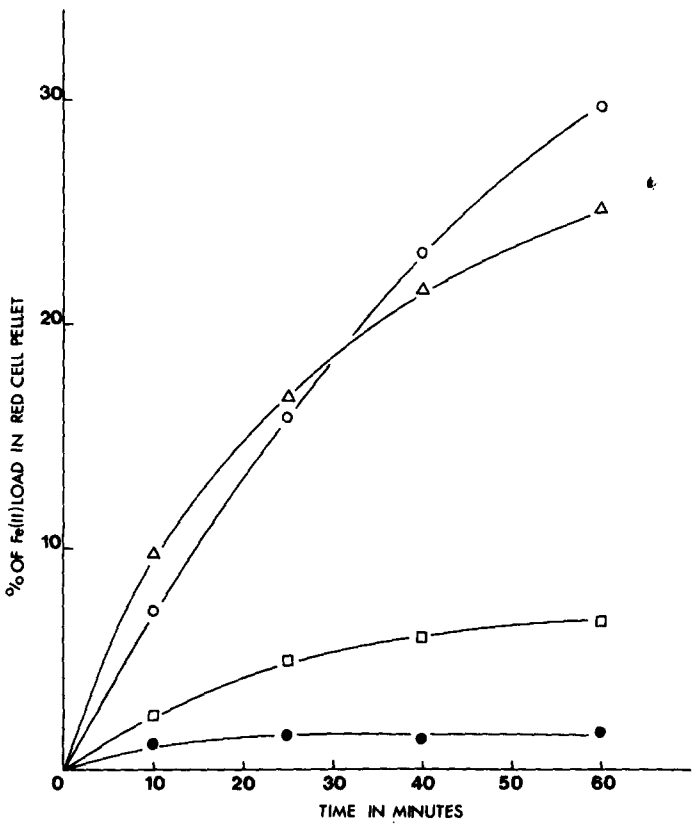

Fig. 2. Time course of uptake of ^{59}Fe(II) by human red cells treated with carboxylate ionophores at 50 μM. ○, A23187; △, Br-X537A; □, X537A; ●, control (no ionophore). After exposure to ^{59}Fe and ionophores, the red cells were separated by centrifugation through silicone oil.

pathways for monovalent ions had indeed been generated, since extensive lysis occurred when Na^+ was substituted for Et_4Am^+ as the main extracellular cation. Na^+ readily penetrates amphotericin and nystatin channels but Et_4Am^+ is impermeant on account of its larger size [12].

Addition of A23187 to Fe(III)-containing liposomes suspended in ascorbate (0.1 M) caused no efflux of Fe, which would have been detectable as Fe(II)-ferrozine upon reduction in the extraliposomal solution. Subsequent addition of PMS (10 μM) produced an efflux of Fe (Fig. 3A). PMS alone was without effect but Fe flux could be obtained upon subsequent addition of A23187 (Fig. 3B).

In view of our stated aims, it was of importance to test whether the ionophores which were capable of transporting Fe(II) could be used to deplete liposomes which had been loaded with the iron storage protein ferritin. In ferritin the iron is present as the stable Fe(III) oxy-hydroxide polymer [9], but upon reduction to Fe(II) it can be released [10]. When liposomes loaded with ferritin were suspended in a reducing environment (ascorbic acid, 0.1 M) together with the Fe(II) chelate dye ferrozine (0.5 mM) no change in absorbance due to the release of Fe(II) could be detected over a period of several hours. As shown in Fig. 4 the addition of A23187 (25 μM) alone has no effect. Only when the ionophore was added together with PMS (10 μM) or TMPD (10 mM) did a pronounced efflux of Fe(II) occur. We understand the activity of PMS and TMPD in this system (and in the Fe(III) experiment related above) to be due to their well known ability to act as carriers of reducing electrons in lipid phases [11]. As with Fe(III) liposomes, the addition of PMS alone had no effect: subsequent addition of A23187 to PMS-treated ferritin-containing liposomes produces an efflux of Fe(II). The effects were much more pronounced at pH 5.5 than at pH 6.5 and this is probably related to the greater ease with which the Fe(III) of ferritin is reduced to Fe(II) at low pH [10].

The possibility exists that the reduction of ferritin and the subsequent liberation of Fe(II) could arise from non-specific damage to the phospholipid membranes, particularly in the case of experiments in which the membranes are treated with several compounds. In order to test for this β-methyl umbelliferyl phosphate was included within the ferritin liposomes to act as a marker of non-

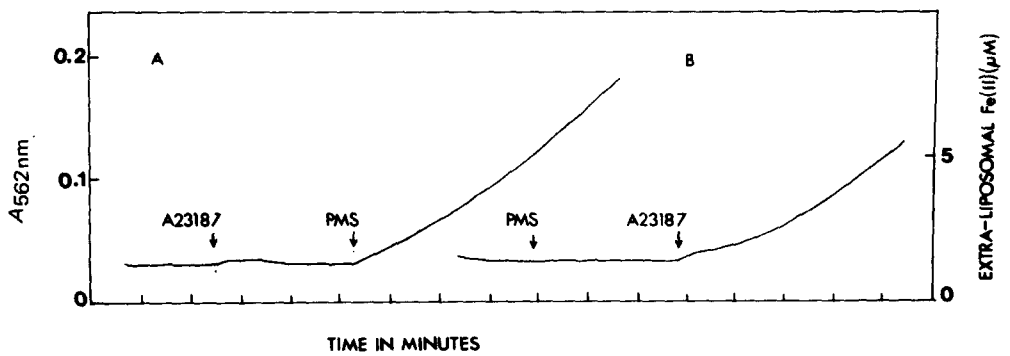

Fig. 3. Release of Fe(II) from Fe(III)-loaded liposomes treated with A23187 and PMS. See Fig. 1. and Methods for details of experiment.

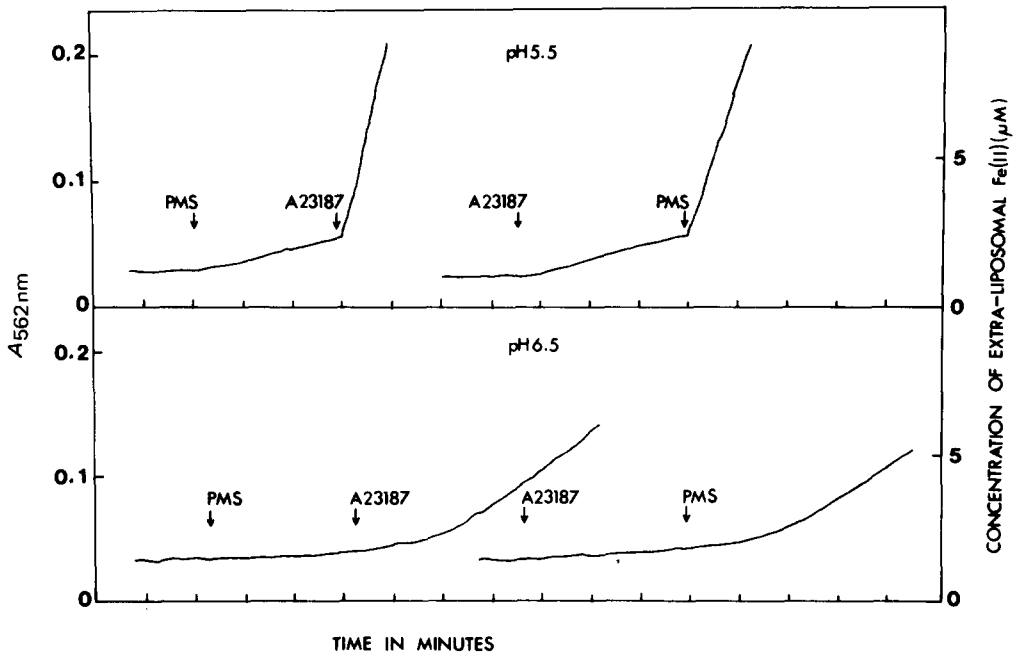

Fig. 4. Release of Fe(II) from liposomes loaded with ferritin treated with A23187 and PMS at pH 5.5 and pH 6.5. Maximum absorbance change in this experiment resulting from release of total ferritin Fe(III) = 2.42 ≡ 104 μM Fe(II).

specific leakage. Addition of alkaline phosphatase to the external medium resulted in the conversion of any extraliposomal substrate to the fluorescent product β-methyl umbelliferone. We never detected any increment in the fluorescence although the method was sufficiently sensitive to detect as little as 0.1% of the internal marker substrate.

The carboxylate ionophores A23187, X537A, Br-X537A and lysocellin are capable of transferring Fe(II) into bulk organic phases. We titrated Fe(II) at pH 4 and pH 5 in a two phase system containing A23187 (0.1 mM with respect to the organic phase). In this system, by re-extracting the aqueous phase with toluene/butanol (70/30, saturated with water) and measuring fluorescence (365—455 nm) we were unable to detect the presence of A23187 in the aqueous phase (limit of detection, 1 μM) and so over 99% of the ionophore is retained by the organic phase. Under these conditions, and by approaching the limit of solubility of Fe(II) in the aqueous phase (see Fig. 5) we found that complexation of Fe(II) by A23187 in the organic phase approached the stoichiometry represented by $Fe(A23187)_2$. In the presence of citrate (added to stabilise Fe(III)) we were unable to detect the formation of an organic soluble Fe complex.This could be either due to the failure of complexation by A23187 of Fe(III) or to the high stability of the Fe(III) citrate complex in aqueous solution.

Transfer of Fe(II) into the organic phase mediated by these ionophores occurs without the additional requirement of another lipid soluble anion (cf. valinomycin [13]) and so it would appear most likely that they form electro-neutral complexes with Fe^{2+} as has been reported for Ca^{2+} and other divalent

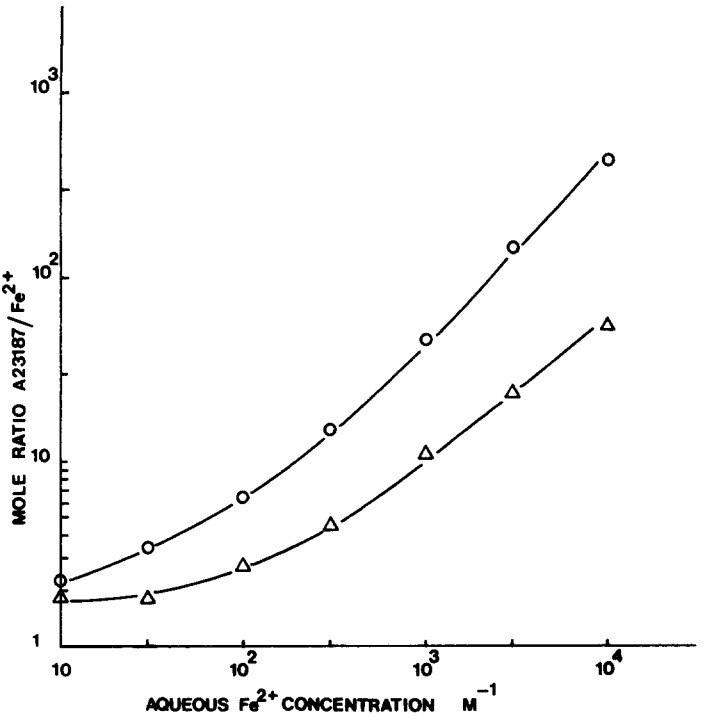

Fig. 5. Titration curves of Fe(II) with A23187 in the two phase system at pH 4 (○) and pH 5 (△). The organic phase was toluene/*n*-butanol (70/30) containing A23187 (0.1 mM). Aqueous phase was buffered with Bis-Tris (50 mm) and Fe(II) was added together with ascorbate (in 5 × molar excess) and a trace of ^{59}Fe. The data are presented in the double reciprocal format on logarithmic sclaes in order to accomodate the wide range of Fe concentrations.

cations [4,14]. Further support for this notion comes from the observation (Fig. 6) that the extent of the extraction is dependent on the pH of the bulk aqueous phase at equilibrium. The affinity of A23187 for Fe^{2+} in the two phase system is much greater than for Ca^{2+} as shown by the ability of the ionophore to transfer Fe^{2+} into the organic phase from aqueous phases of much lower pH. Pfeiffer and Lardy [4] have shown how data of this type may be used to derive an overall equilibrium constant for the reaction

$$2[AH]_{org} + [M^{2+}]_{aq} = [A_2M^{2+}]_{org} + 2[H^+]_{aq} \quad (1)$$

where the chelate ($AH = A^- + H^+$) binds to the divalent cation M^{2+} with 2 : 1 stoichiometry. Here the overall equilibrium constant k' is given by

$$k' = \frac{[A_2M^{2+}]_{org}[H^+]^2_{aq}}{[M^{2+}]_{aq}[AH]^2_{org}} \quad (2)$$

We performed extractions over a range of pH values at different concentrations of Ca^{2+} and Fe^{2+}. Substitution of the mid-point values in Eqn. 2 gives the following values of k' for A23187: Ca^{2+} (at 5 and 50 mM), 1.9 and $6.0 \cdot 10^{-8}$; Fe(II) (at 5 and 50 mM), 2.4 and $12.0 \cdot 10^{-3}$. k' values for the ionophores X537A, Br-X537A and lysocellin (all measured at 500 mM Fe(II)) were 3.4, 8.4 and $16 \cdot 10^{-9}$, respectively.

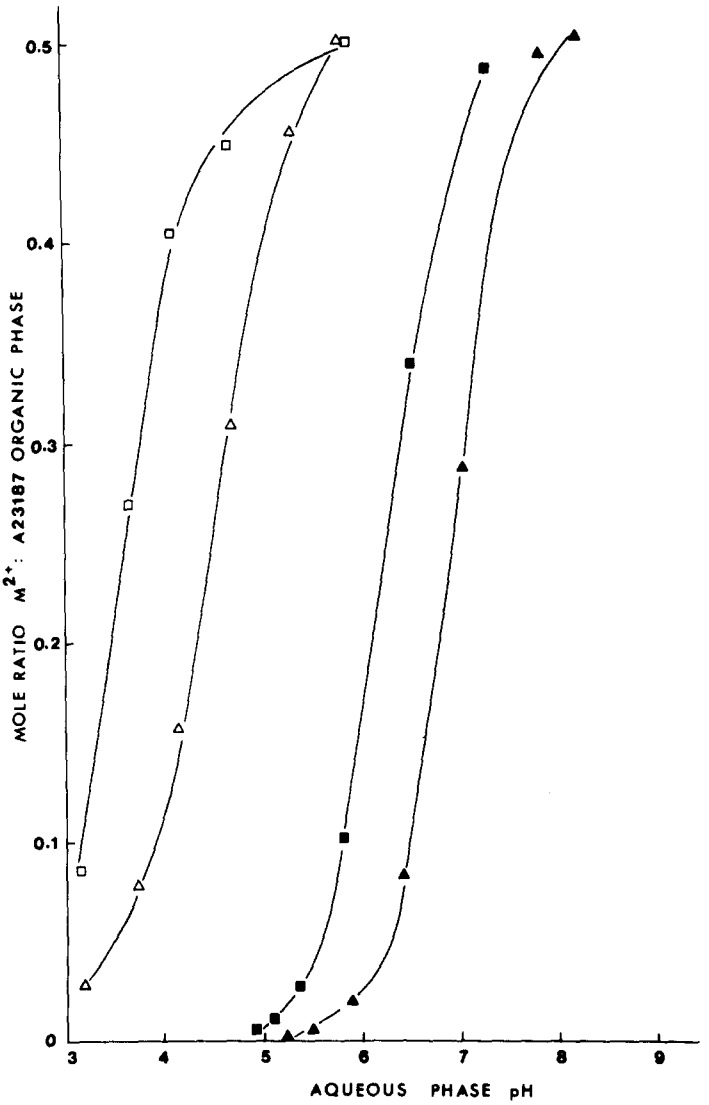

Fig. 6. The dependence on metal ion concentration and pH of the aqueous phase on extraction of Fe^{2+} and Ca^{2+} into toluene : butanol (3 : 1) by A23187. □, Fe, 50 mM; △, Fe, 5 mM; ■, Ca, 50 mM; ▲, Ca, 5 mM. The extraction (ordinate) is expressed as the degree of saturation of the ionophore with the assumption of 2 : 1 stoichiometry.

The data are inevitably complicated by the particular buffers used and the presence of ascorbic acid, and this possibly explains our inability (in the case of A23187: the other ionophores were only tested at a single concentration of Fe since their affinity was so low) to assign unique constants for each of the 2 ions. Another way of expressing this dilemma is that we observed an excessive shift in the pH for mid-point saturation: instead of the predicted 0.5 pH units, we observed shifts of 0.65 and 0.85 for Ca^{2+} and Fe(II) respectively for a 10-fold change in the metal ion concentration on the assumption of 2 : 1 stoichiometry. Even allowing for this discrepancy, the apparent overall equilib-

rium constant for complexation of Fe^{2+} by A23187 is approximately 5 orders of magnitude greater than for Ca^{2+} and fits into the range of values reported for other transition metal cations [4].

Discussion

Low molecular weight iron transport compounds have been widely reported in microbial systems [15]. All of these show high specificity for Fe(III) and so there is a tendency for these compounds to promote the oxidation of Fe(II). In spite of its instability Fe(II) is found in many animal systems as a product of the reduction of Fe(III) and its subsequent release from Fe(III) storage and transport proteins, especially ferritin and transferrin [16]. The presence of a pool of Fe(II) in reticulo-endothelial cells resulting from the catabolism of haemoglobin has been postulated [17]. Consequently, a lipid soluble Fe(II) transport compound which does not bind Fe(III) and chelates Fe(II) reversibly would be of interest in the study of the role of Fe(II) in iron metabolism.

Of all the ionophores tested, A23187 produced the greatest efflux of Fe(II) from liposomes. Using the model system described, even ionophores having comparatively low affinities for Fe(II) could be induced to show ionophoric activity due to the presence of ferrozine having high affinity for Fe(II) (log $k = 15.56$ [18]). The Fe(II) efflux curves do not have the linear form of zero order kinetics and frequently an abrupt reduction in rate was observed shortly after addition of ionophore (see for example the curves for lysocellin and A28695A in Fig. 1A). We believe that this may be due to the multicompartmental nature of the liposomes. For this reason, in comparing the efficacy of the different ionophores, only the inital rates are of interest.

In red cells, where no substantial sink capable of binding Fe(II) is present, only those ionophores shown to be most effective in promoting the efflux of Fe(II) from liposomes (see Fig. 1A) caused Fe(II) influx.

Our results show that A23187 is unable to transport Fe(III) across liposomal membranes. Only when PMS is added to the system to reduce Fe(III) to Fe(II) is A23187 able to release Fe from Fe(III) loaded liposomes.

That the formation of the Fe(II) complex with some of the carboxylate ionophores was dependent on the displacement of protons was demonstrated by extraction into organic phases from aqueous solutions over a range of pH. Of all the compounds tested A23187 was found to have the highest affinity for Fe(II), about 5 orders of magnitude greater than for Ca^{2+}. The values for the overall equilibrium constant k' that we obtained are in the same range, but not identical with those previously published for certain other divalent transition metals [4]. The differences are probably due to the special conditions of our experiments. Buffer dependence has been reported previously when it was shown that extraction of Ca^{2+} was inhibited in the absence of buffer [19]. Thus, strict comparisons are not possible unless conditions are the same. In order to insure that at the alkaline end of the pH range studied, the iron remained in the Fe(II) oxidation state, a 10 molar excess of ascorbate was present in the aqueous phase. It was also present in the aqueous phase used for the Ca^{2+} extractions to enable a valid comparison of data obtained for Fe and Ca to be made. This excess of ascorbate may have caused the disparity in

estimates of k' obtained at 5 mM and 50 mM metal ion concentration. Another limit on the system was the low solubility of Fe(II) at high pH [20]. Hence extractions of Fe(II) using X537A, Br-X537A and lysocellin were constrained by a number of factors: (1) oxidation of Fe(II); (2) limited solubility of Fe(II); (3) low affinities of the ionophores for Fe(II). Nevertheless, our estimates of the k' values for Fe(II) complexes of these 3 ionophores fit reasonably well with the predicted values based on previously published data for X537A with other cations [21].

Finally, the demonstration of Fe(II) release from ferritin loaded liposomes indicates a new possibility for the therapeutic application of ionophores. Iron intoxication of the tissues has been described in a number of conditions, and some of these are accompanied by an iron deficiency anaemia due to the inability to mobilise stored iron [5,6]. It would be necessary however to design an "ironophore" of high specificity. A23187 is highly toxic (LD_{50} (mouse, intravenously) ≈ 4.5 mg kg^{-1} (Hamill, R.L., personal communication)) and this probably arises from its activity as a calcium carrier, which would result in uncontrolled and widespread activation of calcium-dependent tissue functions in vivo [1,2].

References

1 Gomperts, B.D. (1977) The Plasma Membrane: Models for Structure and Function, Academic Press, London
2 Gomperts, B.D. (1976) in Receptors and Recognition, Vol. 2A (Cuatrecasas, P. and Greaves, M.F., eds.), Chapman and Hall, London
3 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501—530
4 Pfeiffer, D.R. and Lardy, H.A. (1976) Biochemistry 15, 935—943
5 Cartwright, G.E. and Lee, G.R. (1971) Brit. J. Haematol. 21, 147—152
6 Jacobs, A. (1976) Brit. J. Haematol. 34, 1—4
7 Young, S., Baker, E., Gomperts, B.D. and Huehns, E.R. (1975) in Proteins of Iron Storage and Transport In Biochemistry and Medicine, (Crichton, R.R., ed.), pp. 417—425, North-Holland/American Elsevier.
8 Six, H.R., Young, W.W., Kei-ichi, U. and Kinsky, S.C. (1974) Biochemistry 13, 4050—4058
9 Michaelis, L., Coryell, C.D. and Granick, S. (1943) J. Biol. Chem. 148, 463—480
10 Granick, S. and Michaelis, L. (1943) J. Biol. Chem. 147, 91—97
11 Kimelberg, H.K. and Lee, C.P. (1970) J. Memb. Biol. 2, 252—262
12 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30—43
13 Haynes, D.H. and Pressman, B.C. (1974) J. Memb. Biol. 18, 1—21
14 Pressman, B.C. (1973) Fed. Proc. 32 1698—1703
15 Neilands, J.B. (1973) in Inorganic Biochemistry. Chapter 5, vol. 1 (Eichhorn, G.L., ed.), Elsevier
16 Frieden, E. (1973) Nutrition Revs. 31, 41—44
17 Lipshitz, D.A., Simon, M.O., Lynch, S.R., Dugard, J., Bothwell, T.H. and Charlton, R.W. (1971) Brit. J. Haematol. 21, 289—303
18 Gibbs, C.R. (1976) Anal. Chem. 48, 1197—1201
19 Pfeiffer, D.R., Reed, P.W. and Lardy, H.A. (1974) Biochemistry 13, 4007—4017
20 Sillen, L.G. and Martell, A.E. (1964) Stability Constants of Metal Ion Complexes. Special Publication No. 17 of the Chemical Society, London
21 Degani, H. and Frieden, H.L. (1974) Biochemistry 13, 5022—5032