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Iron and transition metal transport into erythrocytes mediated by nifedipine degradation products and related compounds

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Abstract

The aim of this investigation was to determine the mechanism of action of the nitrosophenylpyridine derivative of nifedipine (''nitrosonifedipine'', NN) on Fe(II) transport into erythrocytes. Nifedipine is rapidly degraded to NN by daylight. We used rabbit erythrocytes, NN, and several chemically related substances, and examined their effects on the transfer of iron and other transition metals (cadmium, cobalt, manganese, nickel, zinc) into and out of the cells. NN mediated the transfer of iron and zinc but not the other metals into the cell cytosol. The transfer of Fe(II) was not affected by changes in cell membrane potential and could not be ascribed to free radical production. Two hydroxamic acid compounds chemically related to NN also stimulated iron and zinc uptake, but no evidence was obtained for cell-induced transformation of NN to them. *In vivo*, NN is probably converted to a lactam derivative. This compound was found to have no effect on iron uptake by the cells. It is concluded that NN has a relatively high specificity for the transport of iron compared with other transition metals, and small changes in its structure markedly affect this action. Also, because the lactam to which NN is converted *in vivo* is inactive, it is unlikely that nifedipine will affect iron metabolism after therapeutic administration.

Keywords: Iron transport; Erythrocytes; Nitrosophenylpyridine derivative of nifedipine; Transition metals

1. Introduction

Nifedipine (**1**) is a Ca^{2+} channel blocking drug that is widely used in clinical medicine. It is light-sensitive and in the presence of daylight is readily converted into a series of products that are no longer active against Ca^{2+} channels. In a previous study, we investigated whether iron can enter erythroid cells via Ca^{2+} channels by the use of channel antagonists, including nifedipine [1]. They had little effect on iron transport. However, after exposure to light, the photodegradation products of nifedipine were found to strongly stimulate ferrous iron (Fe(II)) transfer into and out of the cells. The active constituent of these products was isolated by chromatographic techniques and shown to be the nitrosophenylpyridine derivative of nifedipine (''nitrosonifedipine''). It was suggested that this substance is able to act as an iron ionophore [1].

The high capacity of nitrosonifedipine (**2**) to mediate Fe(II) transport across the cell membrane of erythroid cells is quite unusual and in magnitude greatly exceeds that of known iron transport processes [1]. Under normal circumstances, mature erythroid cells (erythrocytes) have no capacity to transport iron, and their cell membrane is virtually impermeable to the metal. It is important to determine the mechanism of action of nitrosonifedipine since this information will help in the elucidation of membrane permeability and the properties of substances that can mediate iron transport across cell membranes. It may also help (a) to explain the side-effects of nifedipine that are unrelated to its action as a Ca^{2+} channel antagonist, and (b) in the design of substances that can be used to release iron from cells in clinical conditions associated with iron overload, such as thalassaemia and genetic hemochromatosis.

The present investigation was undertaken to investigate the mechanism of action of nitrosonifedipine on iron

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Abbreviations: BHA, 2[3]-*t*-butyl-4-hydroxyanisole; CP20, 1,2-dimethyl-3-hydroxypyridin-4-one; DFO, desferrioxamine; PCV, packed cell volume; PIH, pyridoxal isonicotinoyl hydrazone.

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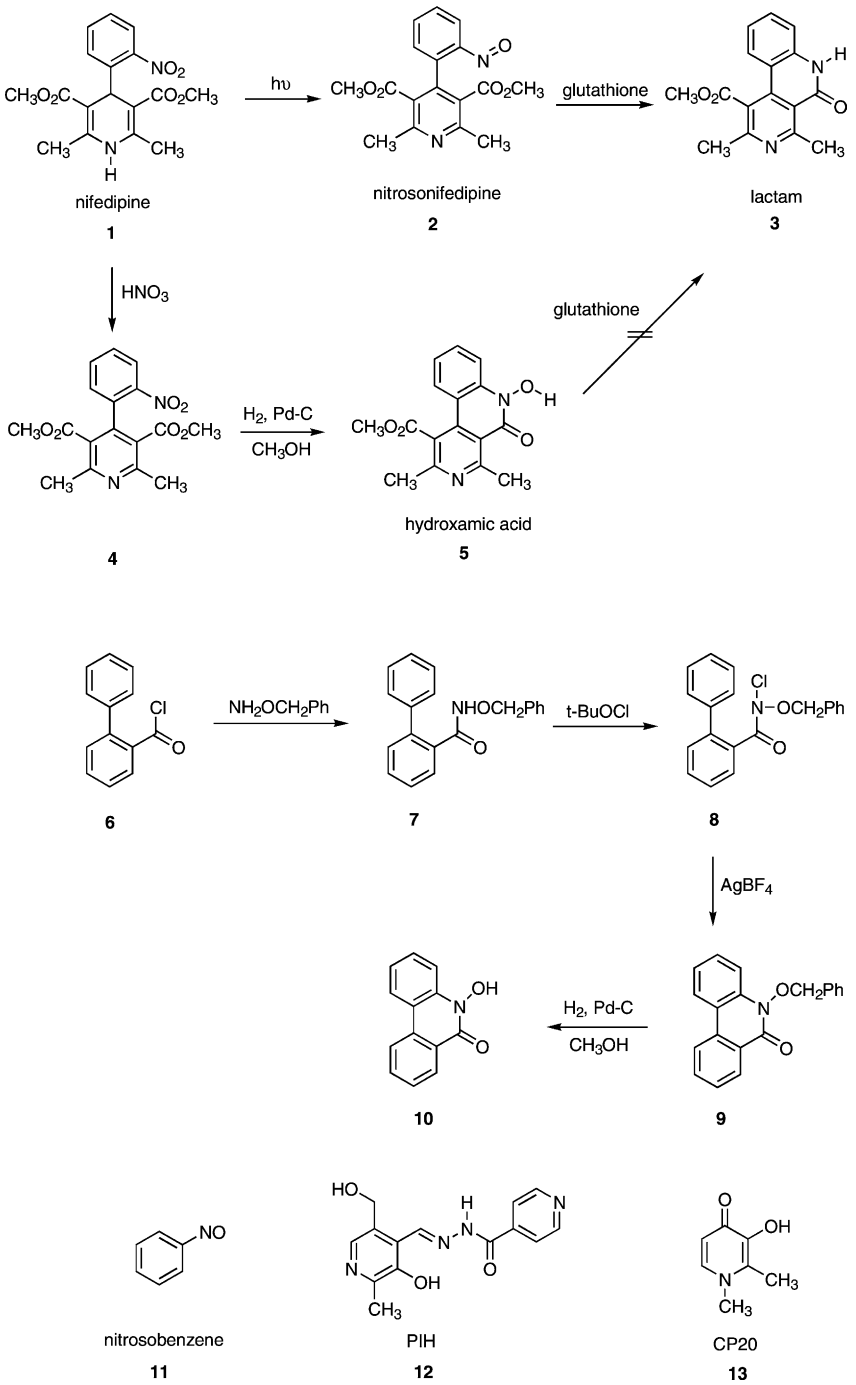

Fig. 1. Structures of nifedipine and other substances used in the investigation. The figure also shows the reactions used to synthesize some of the compounds and the numbers used to designate them in the text.

transport into and out of erythrocytes. We used rabbit cells and several substances chemically related to nitrosonifedipine as well as nitrosonifedipine, and examined the action of these substances on the transfer of Fe(II), Fe(III), and other transition metals into and out of the cells. In addition, some comparisons were made with two iron chelators, PIH and CP20, which have been studied widely and have been used in the treatment of iron overload disorders. The structures of the compounds used in this investigation and their numerical designations (compounds **1** to **13**) are given in Fig. 1.

2. Materials and methods

2.1. Materials

The radioisotopes $^{57}CoCl_2$, $^{63}NiCl_2$, $^{65}ZnCl_2$, and $^{109}CdCl_2$ were purchased from Amersham Australia, and $^{59}FeCl_3$ and $^{54}MnCl_2$ were purchased from Dupont. PIH was synthesised as described by Ponka *et al*. [2], nitrosobenzene was synthesised as described by Coleman *et al.* [3], and CP20 was provided by Dr. E. Baker. The other

biochemical reagents were purchased from the Sigma Chemical Co.

2.2. Synthesis and characterisation of nifedipine (**1**), "nitrosonifedipine" (**2**), and compounds **3**, **4**, **5**, **9**, and **10**

2.2.1. Dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (nifedipine) (**1**)

The following procedure is based on that described by Phillips [4]. A solution of 2-nitrobenzaldehyde (10.2 g, 68 mmol), methyl acetoacetate (21.1 g, 181 mmol), and concentrated ammonium hydroxide solution (10 mL of 25%, w/w) in ethanol (40 mL) was heated under reflux in a steam bath for 2 hr. The solution was diluted with water (50 mL) and extracted with dichloromethane (2 × 50 mL). The extract was washed with water, dried ($MgSO_4$), and evaporated, and the residue was recrystallized from dichloromethane/petrol to give the title compound as yellow crystals (18.5 g, 79%), m.p. 173–174° (lit. [5] 172–173°). ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.28, s, 6H, 2 × CH_3; 3.54, s, 6H, 2 × OCH_3; 5.68, s, 1H, H4; 6.17, s, 1H, NH; 7.21, ddd, $J_{4'5'}$ 7.1, $J_{4'3'}$ 8.2, $J_{4'6'}$ 1.9 Hz, 1H, H4′; 7.44, m, 2H, H5′, H6′; 7.64, dd, $J_{3'4'}$ 8.2, $J_{3'5'}$ 1.2 Hz, 1H, H3′.

2.2.2. Dimethyl 2,6-dimethyl-4-(2-nitrosophenyl)-pyridine-3,5-dicarboxylate ("nitrosonifedipine") (**2**)

A solution of nifedipine (**1**) (4.40 g) in dichloromethane (800 mL) was purged with argon and then irradiated for 4 hr in an Oliphant photochemical chamber reactor with a bank of 16 fluorescent tubes emitting at 350 nm. The solvent was evaporated and the residual solid was recrystallized from dichloromethane/petrol to give the title nitroso compound as green needles (2.61 g, 63%), m.p. 94° (lit. [6] 93°). Mass spectrum *m/z* 328 (M^+, 18%), 284 (22), 269 (100), 267 (33), 253 (40), 252 (28), 237 (24), 193 (28), 152 (28). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.60, s, 6H, 2 × CH_3; 3.32, s, 6H, 2 × OCH_3; 6.47, ddd, $J_{6'5'}$ 8.0, $J_{6'4'}$ 1.1, $J_{6'3'}$ 0.4 Hz, 1H, H6′; 7.37, ddd, $J_{5'6'}$ 8.0, $J_{5'4'}$ 7.3, $J_{5'3'}$ 1.3 Hz, 1H, H5′; 7.46, ddd, $J_{4'3'}$ 7.6, $J_{4'5'}$ 7.3, $J_{4'6'}$ 1.1 Hz, 1H, H4′; 7.67, ddd, $J_{3'4'}$ 7.6, $J_{3'5'}$ 1.3, $J_{3'6'}$ 0.4 Hz, 1H, H3′. ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 23.3, 2 × CH_3; 51.9, 2 × OCH_3; 107.6, CH; 127.1, C; 128.8, CH; 130.5, CH; 135.0, CH; 139.9, C; 144.3, C; 155.3, C; 161.5, C; 167.5, 2 × CO. Electronic spectrum (dichloromethane) λ_{max} (log ε) 230 (4.20), 283 (3.95), 312 nm (3.82).

2.2.3. Methyl 2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylate (**3**)

Solutions of nitrosonifedipine (**2**) (77 mg, 0.24 mmol) in ethanol (70 mL) and glutathione (700 mg, 2.4 mmol) in water (100 mL) were mixed, and the resulting solution was heated at 60° for 30 min. The solution was concentrated on a rotary evaporator to about half its original volume and cooled in ice. The resulting crystals were collected by filtration and dried in a vacuum desiccator over phosphoric oxide to give the title lactam **3** as colourless needles (16 mg, 24%), m.p. 275–276° (lit. [6] 257–263° (dec), lit. [7] 270–272°). ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.70, s, 3H, CH_3; 3.25, s, 3H, CH_3; 4.02, s, 3H, OCH_3; 7.16–7.38, m, 2H, aromatic; 7.58, dd, *J* 8.2, 8.2 Hz, 1H, aromatic; 7.85, d, *J* 8.4 Hz, 1H, aromatic. These data are very similar to those reported [6,7] for **3** prepared by other methods.

2.2.4. Dimethyl 2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (**4**)

This was prepared by the general procedure of Phillips [4]. A mixture of nifedipine (21.3 g) and 4 M nitric acid (150 mL) was heated on a steam bath for 2 hr. The cooled mixture was made alkaline with aqueous sodium hydroxide, extracted with ether (3 × 150 mL), and the extract washed with water, dried ($MgSO_4$), and evaporated. The solid residue (17.8 g) was recrystallized from dichloromethane/petrol to give the title compound as very pale yellow rhombs (9.97 g, 47%), m.p. 103–104° (lit. [8] 103–105°). ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.63, s, 6H, 2 × CH_3; 3.48, s, 6H, 2 × OCH_3; 7.17, ddd, $J_{6'5'}$ 6.6, $J_{6'4'}$ 2.3, $J_{6'3'}$ 0.1 Hz, 1H, H6′; 7.53, ddd, $J_{4'5'}$ 9.4, $J_{4'3'}$ 7.5, $J_{4'6'}$ 2.3 Hz, 1H, H4′; 7.60, ddd, $J_{5'4'}$ 9.4, $J_{5'3'}$ 1.5, $J_{5'6'}$ 6.6 Hz, 1H, H5′; 8.18, ddd, $J_{3'4'}$ 7.5, $J_{3'5'}$ 1.5, $J_{3'6'}$ 2.3 Hz, 1H, H3′.

2.2.5. Methyl 6-hydroxy-2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylate (**5**)

The following procedure is based on the general method of Kim [9]. A solution of dimethyl 2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (**4**) (222 mg, 0.67 mmol) in ethyl acetate (15 mL) was stirred in an atmosphere of hydrogen over 5% palladium on carbon catalyst (50 mg) until 26 mL (1.2 mmol) of hydrogen had been absorbed. The precipitated product was dissolved by the addition of ethyl acetate, and the catalyst was removed by filtration. The filtrate was evaporated, and the residue was subjected to vacuum liquid chromatography on silica gel. Elution with 40% ethyl acetate in light petroleum followed by recrystallization from dichloromethane/petrol gave the title compound as colourless needles (116 mg, 60%), m.p. 190–195° (lit. [7] double m.p. 210°, 215°). Mass spectrum *m/z* 298 (M^+, 64%), 281 (41), 256 (100), 213 (40), 185 (33), 137 (33), 129 (76), 111 (37), 110 (42). ^{1}H NMR (300 MHz, DMSO-D_6) δ 2.60, s, 3H, CH_3; 3.04, s, 3H, CH_3; 3.96, s, 3H, OCH_3; 7.31–7.80, m, 4H, aromatic; 11.42, s, 1H, OH. This spectrum is similar to that reported previously [6]. ^{13}C NMR (75.5 MHz, DMSO-D_6) δ 22.6, CH_3; 27.2, CH_3; 53.1, OCH_3; 113.2, CH; 113.6, C; 117.0, C; 120.0, C; 122.5, CH; 125.2, CH; 136.9, C; 138.4, C; 155.4, C; 155.3, C; 161.8, CO; 169.9, CO.

2.2.6. Stability of the hydroxamic acid **5** towards glutathione

Solutions of hydroxamic acid **5** (30 mg, 0.100 mmol) in ethanol (30 mL) and glutathione (316 mg, 1.03 mmol) in water (40 mL) were mixed, and the resulting solution was

heated at 60° for 30 min. TLC analysis showed the presence of starting material; the characteristic blue fluorescence observed for the lactam **3** under 254 nm radiation was not detected. The solution was concentrated on a rotary evaporator to about half its original volume and cooled in ice. The resulting crystals were collected by filtration and dried in a vacuum desiccator over phosphoric oxide to give unchanged **5** as colourless needles (27 mg), m.p. 192–194°.

2.2.7. 5-Benzyloxy-6(5H)-phenanthridone (*9*)

The following is based on the general procedure of Glover *et al.* [10]. A solution of biphenylene-2-carboxylic acid (3.00 g) in anhydrous benzene (5 mL) was added dropwise to gently refluxing freshly distilled thionyl chloride (5.00 g). After the addition was complete, the mixture was heated under reflux for 30 min before the benzene and excess thionyl chloride were evaporated. The residue was distilled (Kugelrohr) at ~150°/0.5 Torr to give biphenylene-2-carbonyl chloride as a colourless oil (2.80 g, 79%). The foregoing acid chloride (2.33 g, 10 mmol) was added dropwise to an ice-cold stirred suspension of *O*-benzylhydroxylamine hydrochloride [11] (1.60 g, 10 mmol) in a mixture of anhydrous dichloromethane (25 mL) and triethylamine (2.00 g, 20 mmol). The mixture was stirred at room temperature overnight, diluted with dichloromethane (75 mL), and then washed successively with dilute hydrochloric acid, 10% sodium carbonate solution, and brine. The dichloromethane layer was dried ($MgSO_4$) and evaporated. The residue was subjected to radial chromatography eluting with petrol containing increasing amounts of ethyl acetate. Evaporation of the main band afforded *N*-benzyloxybiphenyl-2-carboxamide as a solid (1.60 g, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.72, br s, 2H, OCH_2; 7.10–7.26, br m, 2H, aromatic; 7.25–7.32, m, 3H, aromatic; 7.34–7.45, m, 7H, aromatic; 7.50, ddd, *J* 7.5, 7.5, 1.4 Hz, 1H, aromatic; 7.60, br d, *J* 7.5 Hz, 1H, aromatic. ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 77.9, OCH_2; 127.5, CH; 128.0, CH; 128.5, CH; 128.6, CH; 128.7, CH; 129.0, CH; 130.1, CH; 130.6, CH; 132.3, C; 135.0, C; 139.6, C; 139.9, C; 167.3, CO.

A solution of *N*-benzyloxybiphenyl-2-carboxamide (1.0 g, 3.0 mmol) and *t*-butyl hypochlorite (1.0 g, 10 mmol) in anhydrous benzene (15 mL) was stirred overnight in the dark. The benzene and excess *t*-butyl hypochlorite were evaporated under vacuum to give *N*-chloro-*N*-benzyloxybiphenyl-2-carboxamide as a colourless gum (0.91 g). A portion of the foregoing compound (0.35 g, 1.0 mmol) in anhydrous benzene (25 mL) was treated with silver tetrafluoroborate (0.25 g, 1.3 mmol), and the mixture was stirred in the dark at room temperature overnight. The precipitated silver chloride was removed by filtration and was washed with chloroform. The combined organic filtrate was washed with water, dried ($MgSO_4$), evaporated, and the residue submitted to radial chromatography. Elution with 10% ethyl acetate in petroleum gave 5-benzyloxy-6(5*H*)-phenanthridone (**9**) as a crystalline solid (0.094 g, 30%), which crystallized from dichloromethane/petrol as colorless needles, m.p. 145–146°. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.30, s, 2H, OCH_2; 7.29–7.81, m, 10H, aromatic; 8.25, d, *J* 8.0 Hz, 1H, aromatic; 8.27, d, *J* 8.0 Hz, 1H, aromatic; 8.60, dd, *J* 8.0, 1.2 Hz, 1H, aromatic. ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 77.0, OCH_2; 112.9, CH; 118.3, C; 121.9, CH; 123.0, CH; 123.1, CH; 126.3, C; 128.0, CH; 128.6, CH; 129.1, CH; 129.8, CH; 132.5, CH; 133.0, C; 133.9, C; 136.3, C; 157.5, CO.

2.2.8. 5-Hydroxy-6(5H)-phenanthridone (*10*)

A solution of 5-benzyloxy-6(5*H*)-phenanthridone (54 mg) in methanol (10 mL) was stirred in an atmosphere of hydrogen over 10% palladium on carbon (25 mg) until hydrogen uptake ceased. The methanol was evaporated, the residue was extracted with hot chloroform and filtered, and the filtrate was concentrated until crystallization began. 5-Hydroxy-6(5*H*)-phenanthridone was obtained as colorless rhombs (35 mg, 92%), m.p. 256–257° (lit. [12] 257–258°). ^{1}H NMR (500 MHz, DMSO-D_6) δ 7.36, ddd, *J* 8.0, 8.0, 0.9 Hz, 1H, aromatic; 7.63, ddd, *J* 7.8, 7.8, 1.0 Hz, 1H, aromatic; 7.67, dd, *J* 7.5, 7.5 Hz, 1H, aromatic; 7.77, d, *J* 8.0 Hz, 1H, aromatic; 7.85, ddd, *J* 7.7, 7.7, 1.2 Hz, 1H, aromatic; 8.38, dd, *J* 8.0, 1.3 Hz, 1H, aromatic; 8.49, d, *J* 7.3 Hz; 8.55, d, *J* 8.2 Hz, 1H, aromatic; 11.37, br s, 1H, OH. ^{13}C NMR (125.75 MHz, DMSO-D_6) δ 113.1, CH; 117.3, C; 122.7, CH; 122.8, CH; 123.4, CH; 125.4, C; 127.5, CH; 128.2, CH; 130.0, CH; 132.3, C; 132.5, CH; 136.9, C; 156.7, CO.

2.3. Cells

Blood was obtained from adult rabbits by bleeding from a marginal ear vein, using heparin as an anticoagulant. The blood cells were washed four times with 10–15 vol. of ice-cold 0.15 M NaCl and centrifuged at 2000 *g* for 10 min at 4°. The supernatant and buffy coat (leucocytes and platelets) were removed after each centrifugation. For metal uptake experiments the cells were then suspended in 0.15 M NaCl at a PCV of 25–30%. For metal efflux experiments, the cells were washed one more time in 0.15 M KCl and then suspended in 0.15 M KCl.

2.4. Metal solutions

The solutions of the metal salts were prepared immediately before use by mixing samples of the radioactive isotopes with divalent non-radioactive chloride salts of Fe, Cd, Co, Mn, or Zn, or with $FeCl_3$, and diluting with 0.27 M sucrose to give solutions with 20 times the desired final concentrations of the metals.

2.5. Incubation procedures

2.5.1. Metal uptake

Samples of the cell suspensions (usually 60 μL) were incubated at 37° in an oscillating, temperature-controlled

water bath in 2 mL solution containing the radioactive metals. Most incubations were performed for 10 min in 0.15 M NaCl–0.01 M Tris–HEPES, pH 7.4. Except where indicated to the contrary, the metal concentration was 20 μM. Solutions of the nifedipine derivatives and the other compounds tested were prepared at 200 times the desired final concentrations in DMSO. Aliquots of these solutions (10 μL) or DMSO for controls were added to the cell suspension and incubated with them for 10 min at room temperature (except where indicated below) before addition of the radioactive metals and incubation at 37°. After incubation the cells were washed three times with ice-cold 0.15 M NaCl, hemolysed with 1.5 mL of 0.01 M Tris–HEPES, pH 7.4, centrifuged to separate cytosolic and membrane fractions, and counted for radioactivity as described previously [13]. These experiments were performed with mature erythrocytes, which do not contain nuclei or other intracellular organelles. Hence, the centrifugation step separates the cells into two fractions that contain only cytosol and cell plasma membranes (referred to as membranes).

2.5.2. Metal efflux

Samples of the cell suspensions were first labelled with the radioactive metals by incubation for 30 min at 37° with 20 μM solutions of the metals in 0.15 M KCl–0.01 M Tris–HEPES, pH 7.0. (This leads to efficient uptake of all of the metals tested [14].) The cells were then washed four times with ice-cold 0.15 M NaCl, suspended in 2 mL of 0.15 M NaCl–0.01 M Tris–HEPES, pH 7.4, containing 100 μM DFO, and 10 μL of DMSO or solutions of the nifedipine derivatives were added. Incubation was then performed for periods of up to 30 min at 37°, followed by centrifugation to obtain the efflux solution, and hemolysis of the cells and recentrifugation as for the uptake experiments. Radioactivity was counted in the efflux solution and in the two fractions of the cells.

2.6. Analytical methods

The PCV was determined by the microhematocrit method. Partition of ^{59}Fe from an aqueous solution to an organic solvent was performed by shaking 1.0 mL of the ^{59}Fe solution in 0.15 M NaCl, 0.01 M Tris–HEPES, pH 7.4, with 1.0 mL of organic solvent followed by centrifugation and sampling the organic solvent for measurement of radioactivity. The nifedipine derivatives were added to the aqueous phase before the addition of the solvent. The radioactivity of ^{63}Ni was measured in a Beckman LS6500 liquid scintillation counter (Beckman Instruments Inc.) and the other radioisotopes in a three-channel γ-scintillation counter (1282 Compugamma; LKB Wallac). Transmembrane potential differences were measured by the method of Macey *et al.* [15].

2.7. Statistics

The experiments were performed three or four times. Results are presented in the figures as means ± SEM. Significance of differences between the means ($P < 0.05$) was assessed using Student's *t*-test.

3. Results

3.1. Fe(II) uptake

As found earlier using nitrosonifedipine (**2**) obtained by photodegradation of nifedipine [1], *de novo* synthesized **2** greatly enhanced Fe(II) uptake by erythrocytes. The majority of the Fe(II) taken up by the cells was transferred through the membrane to the cytosolic fraction of the cells and accumulated there in a linear manner with respect to time of incubation for at least 20 min (results not shown). Hence, in the subsequent experiments, a 10-min uptake time was used so that the cells would be sampled during the linear phase of uptake and the results would represent a rate of uptake.

The amount of Fe(II) taken up by the cells was dependent upon both the concentration of iron and of **2** (Fig. 2). As little as 0.5 μM **2** led to markedly increased Fe(II) uptake, while the effect on uptake to the cytosol was maximal at approximately 10 μM, as was confirmed in later experiments (Fig. 3). However, uptake to the membrane increased further as the concentration of **2** was raised above 10 μM (Fig. 3). In most of these experiments, the total uptake of Fe(II) by the cells (cytosol plus membrane) considerably exceeded the amount of **2** present in the incubation solution when compared on a molar basis. For instance, from the data in Fig. 2 it was calculated that the ratio of Fe(II) uptake to **2** present varied from 1 to 5 and 2 to 10 when the Fe(II) concentration was 10 or 40 μM, respectively, as the **2** concentration was changed from 0.2 to 10 μM.

The mechanism by which **2** stimulates Fe(II) uptake was investigated by examining the possible effects of transmembrane potential difference and free radical-induced changes in the cell membrane on the uptake process. We also compared the effects of **2** on Fe(II) uptake with those of certain chemically related compounds (**3**, **4**, **5**, **10**, **11**) and of two iron chelators that have been studied in some detail previously, PIH (**12**) and CP20 (**13**).

Membrane potential was altered by the addition of valinomycin (0.5 μM) to the incubation medium. This increased membrane permeability for K^+ and changed the potential from -9.0 ± 3.0 to -71 ± 8.9 mV (mean ± SEM, N = 3). However, this was not accompanied by a significant change in iron uptake to the cytosolic or membrane fractions of the cells incubated with 0.2, 2, or 20 μM Fe(II) in the presence of 10 μM **2** (results not shown).

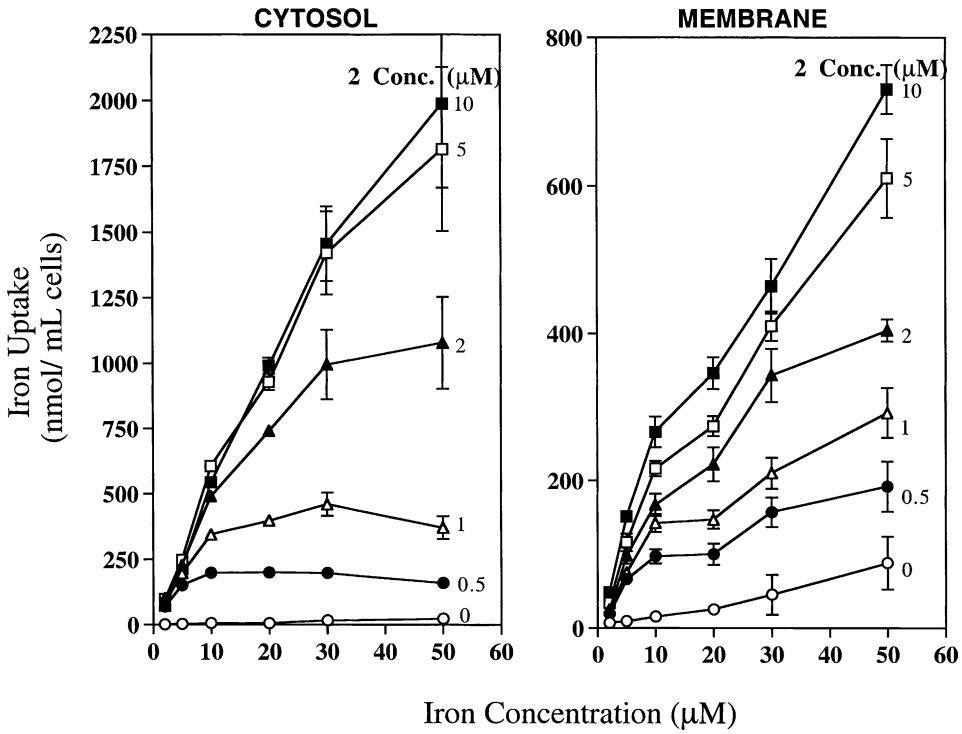

Fig. 2. Effect of the concentrations of Fe(II) and **2** on iron uptake by rabbit erythrocytes. The cells were incubated for 10 min at 37° with 2–50 μM Fe(II) in the presence of 0–10 μM **2**; then they were washed and separated into cytosolic and membrane fractions. The results are the means ± SEM of four experiments.

An increase in membrane permeability to iron due to free radical production and lipid peroxidation in the cell membrane has been reported for iron uptake by other cell types [16,17]. The possibility that a similar effect was responsible for the results of the present investigation was therefore investigated by the addition of reagents that act as free radical producers or scavengers to the incubation medium. The free radical producers tested were *t*-butyl hydroperoxide (20 μM), cumene hydroperoxide (10 μM), H_2O_2 (100 μM), and 3-methylindole (100 μM). The scavengers were 2[3]-*t*-butyl-4-hydroxyanisole (50 μM), *N*,*N*′-diphenyl-1,4-phenylenediamine (20 μM), melatonin (1 μM), α-tocopherol (100 μM), and superoxide dismutase (200 IU/mL). None of these reagents, with the exception of superoxide dismutase, had a significant effect on Fe(II) uptake in the presence of 10 μM **2**. Superoxide dismutase produced a 12% reduction in Fe(II) uptake (results not shown).

Two compounds chemically related to **2**, compounds **5** and **10**, stimulated Fe(II) uptake to the cytosol while others, **3**, **4** and **11**, as well as **12** and **13**, had no effect (Fig. 3). Iron uptake to the membrane was also enhanced by **5** and **10**

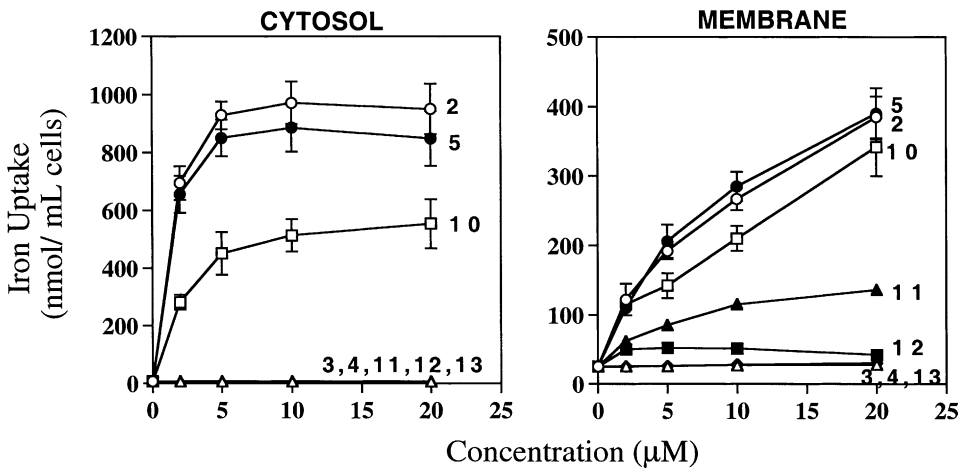

Fig. 3. Effect of various concentrations (0–20 μM) of **2**, **5**, **10**, **11**, **12**, **3**, **4**, and **13** on Fe(II) uptake by rabbit erythrocytes. The cells were incubated for 10 min at 37° with 20 μM Fe(II) in the presence of the reagents. Values are the means ± SEM of three experiments.

and, to a lesser extent, by **11** and **12**. The effects of **5** were very similar to those of **2**, while **10** was only about half as effective in stimulating Fe(II) uptake to the cytosol, although it was nearly as effective with respect to iron uptake to the membrane.

The similarity of the effects of **2** and the hydroxamic acid **5** also raised the possibility that the action of **2** is the result of its cell-induced transformation to **5** and that this is the active substance. To test this, we measured the time course of Fe(II) uptake mediated by the two substances when they were added to the cells either 10 min before or simultaneously with the radiolabelled Fe(II). The rate of iron uptake was almost the same in the presence of each of the two substances and was not influenced by the time of their addition to the cells (results not shown).

It has been demonstrated that, *in vivo*, **2** is converted rapidly to the lactam, **3** [18,19]. It was considered possible that this conversion could occur during incubation of **2** with erythrocytes and that the observed effects could be attributed to the action of **3**. However, as demonstrated above, **3** lacks the capacity of **2** to mediate Fe(II) uptake by erythrocytes. The ability of the cells to convert **2** to **3** under the incubation conditions used in these experiments was examined as follows. Erythrocytes were incubated with 10 μM **2** for various times up to 60 min at 37°, centrifuged, and the supernatant used in a second incubation with ^{59}Fe using fresh samples of cells. Compared with **2** which had been incubated in the absence of cells, the cell supernatants produced approximately 80% as much Fe(II) uptake even after a 60-min preincubation with 5 times as many cells as used during iron uptake measurements (Fig. 4).

3.2. Uptake of other divalent metals and Fe(III)

The specificity of the action of **2** on other divalent metals as well as iron was assessed by performing incubations with Cd^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Fe(III) under the same conditions as were used to increase Fe(II) uptake. Only Zn^{2+} and Fe(III) were transported into the cytosol but at a much lower rate than Fe(II). Co^{2+} and Ni^{2+} as well as Zn^{2+} and Fe(III) were taken up by the membrane fraction of the cells (Fig. 5). However, the uptake of the other metals was lower than that of Fe(II) or Fe(III), especially at lower concentrations of **2**. Although the membrane uptake of Fe(III) was greater than that of Fe(II) at all concentrations of **2** except 20 μM, this could be attributed to a much larger uptake of Fe(III) than Fe(II) in the absence of **2**. If these values are subtracted from the uptake found in the presence of **2,** the uptake of Fe(II) mediated by **2** is found to be greater than that of Fe(III).

Of the substances chemically related to **2** that were tested, only the hydroxamic acids **5** and **10** had any effect on divalent metal uptake, and their actions were confined to Co^{2+}, Zn^{2+}, and Ni^{2+} (Fig. 6). None of the substances influenced Mn^{2+} or Cd^{2+} uptake by the cells (results not shown). Compound **10** strongly stimulated Co^{2+}, Zn^{2+}, and Ni^{2+} uptake to the cytosol and, to a lesser degree, to the membrane. Compound **5** mimicked the action of **2**, stimulating Zn^{2+} uptake into the cytosol and Co^{2+}, Zn^{2+}, and Ni^{2+} uptake to the membrane. Compounds **5** and **10** at 10 μM also stimulated Fe(III) uptake to the cytosol, to levels approximately 30% as great as Fe(II) uptake (results not shown).

3.3. Metal efflux from erythrocytes

To study the effects of **2** and related compounds on metal efflux from erythrocytes, the cells were first incubated with the radiolabelled metals under conditions that allow efficient uptake of all of the metals under investigation. They were then washed and reincubated in the presence of DFO plus the compounds, since it was shown previously that **2**

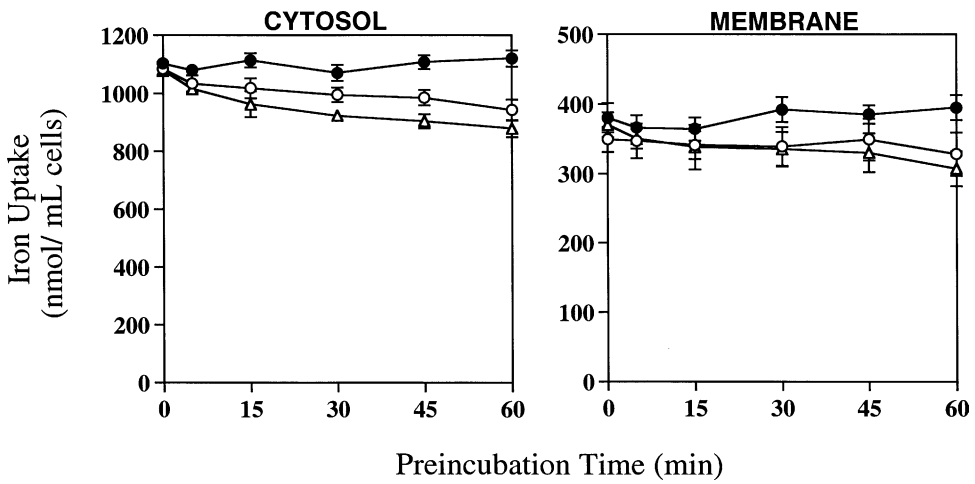

Fig. 4. Effect of preincubation of **2** with erythrocytes on its subsequent ability to mediate Fe(II) uptake by fresh samples of cells. Compound **2** (10 μM) was incubated at 37° with no cells (●), 60 μL cells (○), or 300 μL cells (Δ) of PCV of 25% in 2 mL NaCl, pH 7.4, for 0–60 min. The cell suspension was then centrifuged at 2000 *g* for 10 min at 4°, and the supernatant was used to measure Fe(II) uptake by a fresh sample of cells in the standard manner. The figure shows the means ± SEM of three experiments.

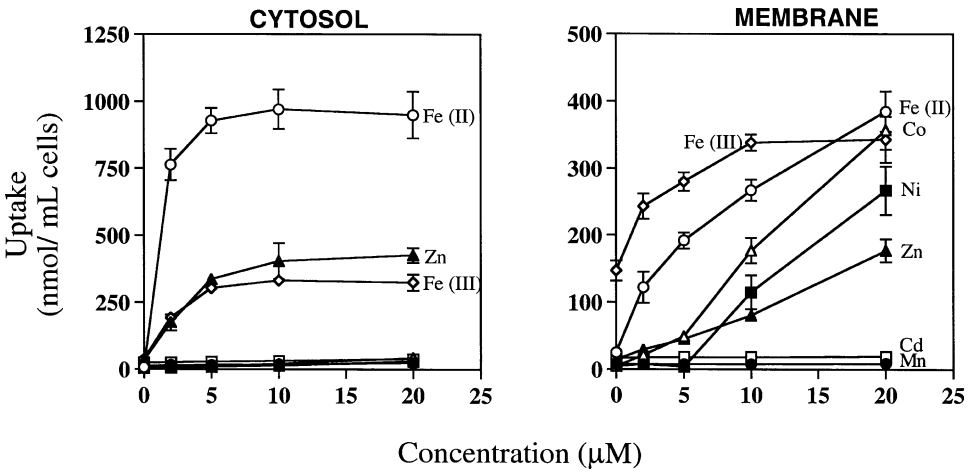

Fig. 5. Concentration-dependent effects of **2** on the uptake of divalent metals (Fe, Co, Zn, Ni, Cd, and Mn) and Fe(III) by rabbit erythrocytes. The incubations were performed for 10 min at 37° in the presence of 0–20 μM **2** and a 20 μM concentration of the metals. There was virtually no uptake of Co, Cd, Mn, or Ni in the cytosolic fraction of the cells. The results are the means ± SEM of three experiments.

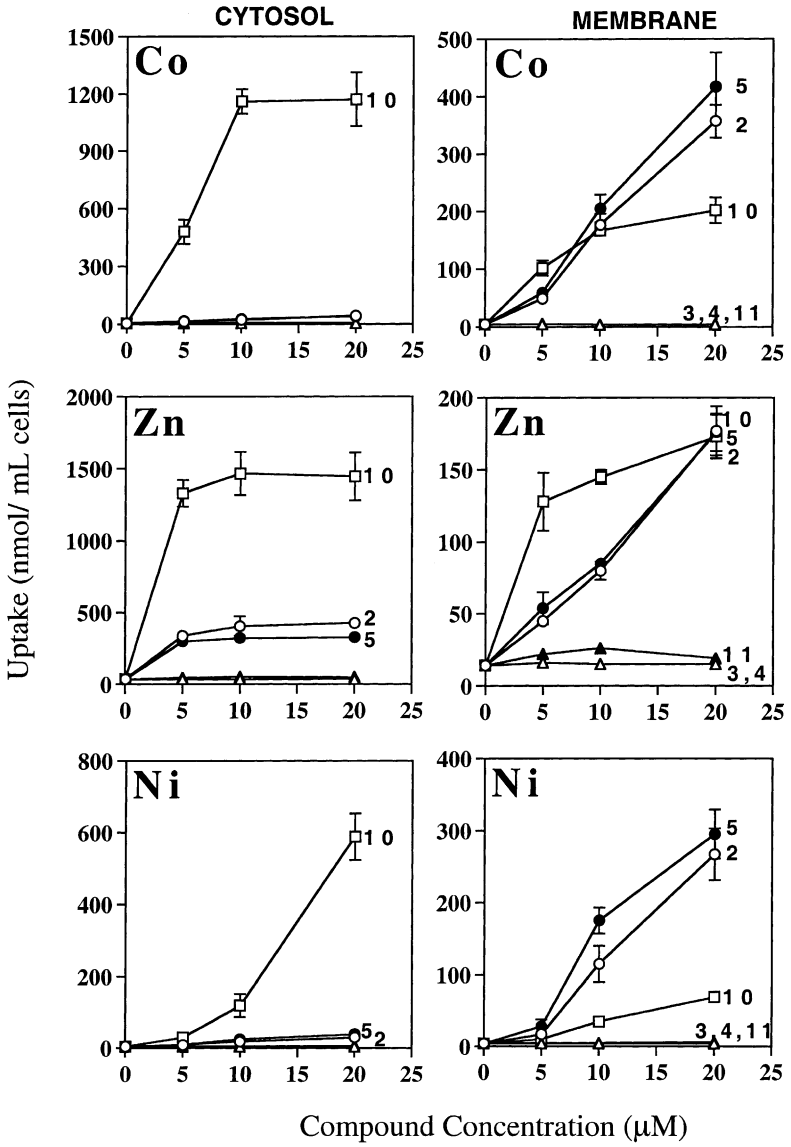

Fig. 6. Effects of **2** and related compounds on Co, Zn, and Ni uptake by rabbit erythrocytes. The cells were incubated for 10 min with 20 μM concentrations of the metals in the presence of 0–20 μM **2** (○), **5** (●), **10** (□), **3**, **4** (Δ), or **11** (▲). Values are the means ± SEM of 3–4 measurements.

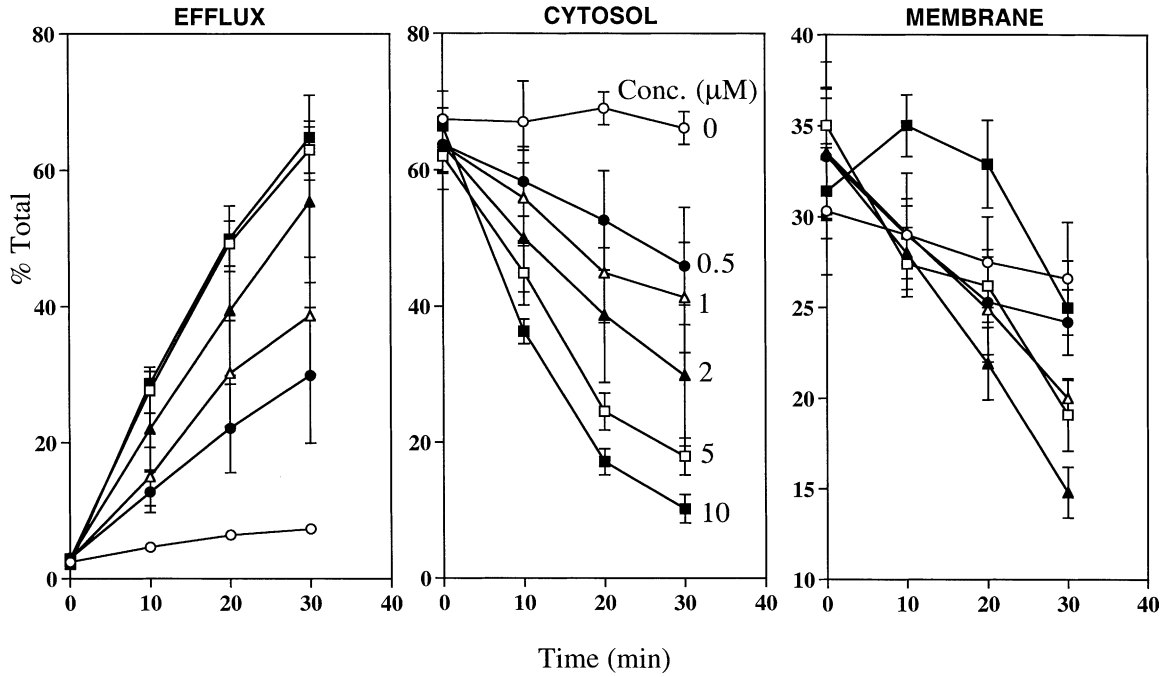

Fig. 7. Efflux of iron from rabbit erythrocytes in the presence of various concentrations of **2**. The cells were prelabelled with ^{59}Fe as described in the text, washed, and reincubated at 37° in the presence of 100 μM DFO and 0 (○), 0.5 (●), 1 (Δ), 2 (▲), 5 (□), or 10 (■) μM **2**. The figure shows the percentage of the ^{59}Fe present in the efflux solution, cytosol, and membrane of the cells after 0-, 10-, 20-, and 30-min reincubation at 37°. Each value is the mean ± SEM of four experiments.

stimulates iron efflux only if DFO, an iron chelator that does not enter erythrocytes, is present in the extracellular fluid [1]. Under these conditions, **2** was found to mediate iron efflux from the cells in a concentration-dependent manner (Fig. 7). Most of the iron lost from the cells was derived from the cytosol, although there was some decrease in membrane ^{59}Fe (Fig. 7).

Compound **5** stimulated iron efflux to a degree similar to that of **2** (Fig. 8), while **3**, **4**, **10**, **11**, **12**, and **13** had no significant effect (results not shown). Compound **10** caused a shift of iron from the cytosolic to the membrane fraction of the cells but had little effect on efflux to the incubation solution. DFO by itself produced no significant efflux of iron (or other metals) or redistribution between cytosol and membrane (Fig. 8).

When the other divalent metals were examined, an effect was found only with Co^{2+} (Fig. 8). None of the reagents affected efflux of Mn^{2+}, Cd^{2+}, Zn^{2+}, or Ni^{2+} (results not shown). With Co^{2+}, **10** was found to stimulate efflux. This was associated with a drop in cytosolic Co^{2+} but no change in membrane Co^{2+} (Fig. 8).

3.4. Two-phase partition of iron

The effect of **2** and related compounds on the partition of iron from an aqueous to an organic phase was tested initially using **2** and a variety of organic solvents. The highest level of partitioning was found with 1-octanol, which was used in subsequent studies, while lower levels of partitioning were found with cyclohexane, petroleum

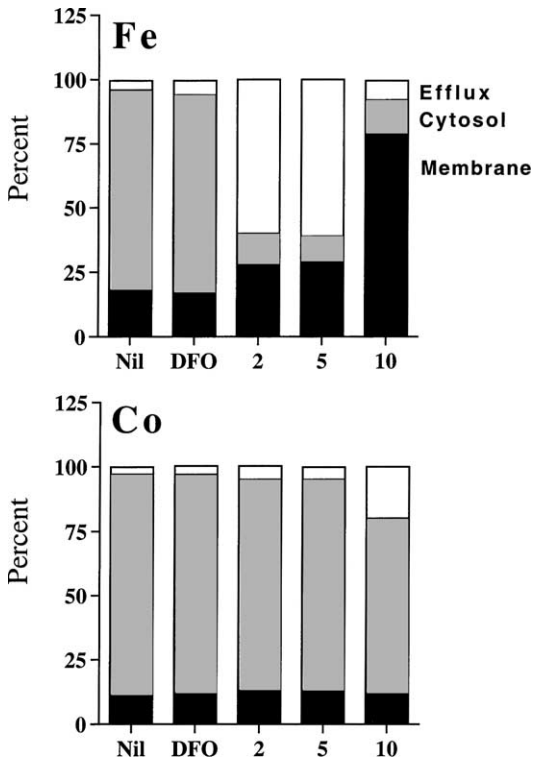

Fig. 8. Iron and cobalt efflux from erythrocytes mediated by DFO, **2**, **5**, and **10**. The cells were prelabelled with ^{59}Fe or ^{57}Co, washed, and reincubated at 37° for 30 min in the presence of no reagents (Nil), 100 μM DFO, or 100 μM DFO plus 10 μM **2**, **5**, or **10**. The figure shows the percentage of the ^{59}Fe or ^{57}Co that was present in the efflux solution, cytosol, and membrane at the end of the reincubation period. Each value is the mean of four experiments.

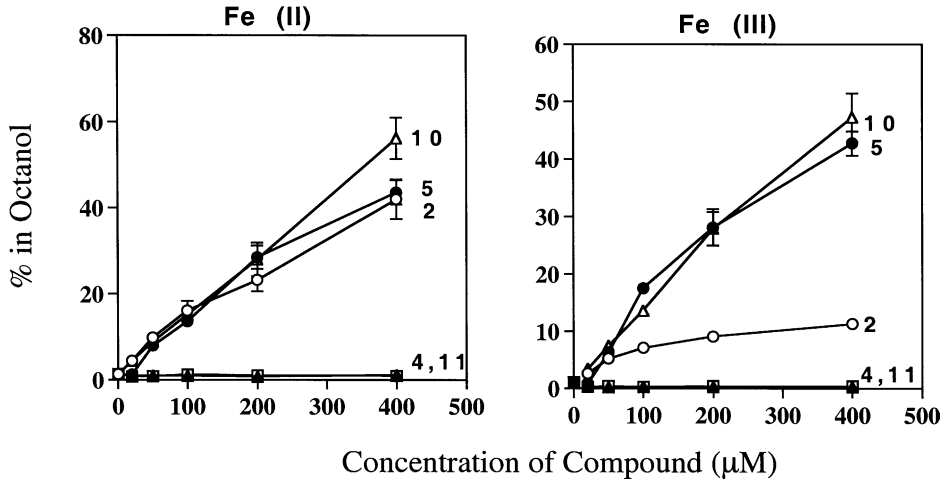

Fig. 9. Partitioning of Fe(II) and Fe(III) from the aqueous phase to 1-octanol in the presence of various concentrations of **2**, **5**, and **10**. Compounds **4** and **11** produced no measurable partitioning of iron. Values are the means ± SEM of three experiments.

ether, *n*-hexane, and carbon tetrachloride. Compounds **2**, **5**, and **10** were approximately equally effective in mediating iron partitioning Fe(II) into 1-octanol, while the nitro compound **4** and nitrosobenzene (**11**) were without effect (Fig. 9). Compounds **5** and **10** also partitioned Fe(III) into the octanol phase to a degree similar to that for Fe(II), but **2** was less effective with Fe(III) than with Fe(II) (Fig. 9).

4. Discussion

These experiments show that synthetic **2** has similar effects on iron transport into erythrocytes as reported previously for the photodegradation product of nifedipine [1]. This confirms that the active substance is the nitroso derivative of nifedipine. Using the pure compound, we have obtained results quantitatively similar to those of the earlier work, although the pure substance was found to be slightly more active per unit weight than the substance derived from photodegradation of nifedipine. Thus, at a 20 μM iron concentration, maximal Fe(II) uptake was found to occur at a concentration of **2** of 10 μM, whereas in the earlier work uptake was slightly higher at 20 μM than at 10 μM. These differences may be due to the presence of impurities in the earlier preparations.

A striking feature of the results is the very high rate of Fe(II) transport into the cells in the presence of **2** and **5**. This is illustrated by the following calculation. At 5–10 μM concentrations of these compounds and 20 μM Fe(II), the rate of Fe(II) uptake was equivalent to about 5 mmol/L cells/hr. The iron concentration in mature erythrocytes is approximately 20 mmol/L. Hence, if the rate of Fe(II) uptake in the presence of **2** or **5** continued in a linear manner, it would take only 4 hr to acquire this amount of iron, whereas the developing red cell *in vivo* takes 4–5 days to do this.

Under most concentrations of Fe(II) and **2** used in these experiments, the quantity of Fe(II) in molar terms that was taken up by the cells exceeded the amount of **2** initially present in the incubation solution. To achieve this, **2** could be acting as a stimulator of an Fe(II) transport process, a channel former, or an ionophore. An effect on known Fe(II) transport processes was ruled out by earlier experiments [1]. Moreover, such processes either disappear or diminish considerably when immature erythroid cells such as reticulocytes mature into mature erythrocytes [14], yet Fe(II) uptake mediated by **2** tends to be greater in mature cells than in reticulocytes [1]. Channel formation as a means of action of **2** is unlikely since Fe(II) uptake mediated by **2** was not affected by a change of membrane potential. Hence, it is probable that **2**, **5**, and **10** act as lipophilic ionophores, having the ability to transport the Fe(II) into and across the cell membrane, deposit it at these sites, and return to the incubation solution. Presumably there are binding sites in the cytosol and cell membrane that can compete with these compounds for the Fe(II). Only when there is a membrane impermeable chelator such as desferrioxamine outside the cell is the direction of iron exchange reversed.

Compounds **2** and **5** produced similar results with respect to all aspects of Fe(II) transport that were studied in the present work. They also had a similar capacity to partition Fe(II) into 1-octanol. Hence, their mode of action is probably the same, acting as Fe(II) ionophores. Compound **10** was less effective in mediating Fe(II) uptake to the cytosol than were compounds **2** or **5** even though it partitioned Fe(II) to 1-octanol as well as they did. Thus, it also probably acts as a lipophilic ionophore but with quantitatively different properties from **2** and **5**. The ability of metal complexing agents to mediate metal exchange across cell membranes depends largely on their binding affinity for the metals and their lipophilicity [20,21]. In addition, the presence of binding sites and their affinities for the metals in the cell membrane and cytosol will influence the distribution of the metals in the cell. All of these factors probably came into play in the present studies

to produce the observed results. As judged from the partition of Fe(II) into 1-octanol, **2**, **5**, and **10** have similar lipophilicity, but this does not rule out the possibility that **10** is more soluble in the cell membranes than are the other two compounds. Alternatively, or additionally, its affinity for Fe(II) may be higher so that it exchanges the Fe(II) with binding sites in the cell less effectively than **2** or **5**. Either property could lead to a relatively greater accumulation of Fe(II) in the membranes compared with cytosol than with **2** or **5**, and movement of iron from the cytosol to membranes during the efflux experiments.

A remarkable property of **2** and **5** was their high degree of selectivity for the transport of Fe(II) into the cytosol when compared with the other metals examined in this study. Only Zn^{2+} was transported to the cytosol by these compounds, but to a lesser extent than Fe(II). However, Co^{2+} and Ni^{2+} as well as Zn^{2+} were taken up by the membrane fraction of the cells. By contrast, **10** carried all of these metals including Fe(II) into the cytosol. As discussed above, these results are probably dependent upon the solubility of the metal complexes in the cell membrane, the affinity of the compounds for the metals, and the number and affinity of binding sites for the metals within the cells.

Compounds **5** and **10** are hydroxamic acids, which are known to be strong chelators of Fe(III) [21]. However, the partition studies show that they can complex Fe(II), although probably much less strongly than Fe(III). The lower affinity for Fe(II) would aid dissociation of the iron within the cell and its accumulation there, thus accounting for the greater cellular uptake of Fe(II) than Fe(III) produced by **5** and **10**. Probably **2** has an affinity for Fe(II) similar to that of **5**, as well as lipophilicity, resulting in the observed similarity in iron transporting properties. Compound **2** also had some affinity for Fe(III) and some ability to mediate Fe(III) uptake by the cells, although lower than for Fe(II). Compounds **12** and **13** are lipophilic chelators of Fe(III), which is probably why they showed little activity in this study which used Fe(II). Overall, the results discussed above are compatible with the conclusion that **2**, **5**, and **10** can function as lipophilic ionophores for iron, cobalt, zinc, and nickel.

Fe(II) uptake by the cells was not affected by the addition of valinomycin. This K^+ ionophore increases the membrane conductance of K^+ and changes the membrane potential of erythrocytes from one dependent upon the $[Cl_i/Cl_o]$ distribution ratio to one dependent upon K^+. In the present study this was accompanied by a change in membrane potential from −9.0 to −71 mV (inside relative to outside). The fact that such a large change in potential difference did not affect Fe(II) uptake indicates that the iron enters the cells in the form of an electrically neutral complex with **2**.

Iron-induced free radical production can lead to lipid peroxidation and damage to cell membranes. This may result in increased permeability to Fe(II) [16,17] and could provide an explanation for nitrosonifedipine-stimulated Fe(II) uptake by erythrocytes. However, several scavengers of free radicals were found to have little effect on Fe(II) uptake in the presence or absence of **2**, while free radical producers that may be expected to lead to increased uptake were also without effect. Hence, there is no evidence that cell membrane changes due to iron-catalysed lipid peroxidation are involved. More likely, as concluded above, **2** forms a neutral complex with Fe(II) and acts as an ionophore which transfers the iron across the cell membrane.

Compound **2** is rapidly reduced to the lactam **3** when incubated with glutathione [18,19], and this procedure was used to produce **3** in the present study. The same change was observed when **2** was incubated in plasma or blood, and after intravenous injection into rats [18,19]. Hence, it appears that **2** is rapidly reduced to a stable lactam **3** *in vivo*, and any **2** produced by photodegradation of nifedipine *in vivo* would be converted to the lactam and have no effect on cellular iron metabolism. However, related compounds that are stable *in vivo* may have some effects and would be worthy of investigation. Compounds **5** and **10** are possible examples of such compounds.

Erythrocytes are known to possess cell membrane reductase activity [22], and this may contribute to the production of the lactam that occurs in blood and *in vivo*. Indeed, some decrease in Fe(II) transporting activity was found after incubation of **2** with erythrocytes. However, this effect was small, probably due to the small quantities of erythrocytes used in the present investigation, so that Fe(II) uptake in the presence of **2** continued in a linear manner during a 20-min incubation and was not affected significantly by the addition of **2** to the cells 10 min prior to adding the iron.

Compound **2** has been shown to promote iron uptake by human epidermal keratinocytes [23] and gallium uptake by cultured tumour cells [24]. On the basis of these investigations it was proposed that the nifedipine derivative may be of value clinically, by mediating iron excretion via the skin in patients with iron overload [23] and to improve the imaging of tumours by the use of ^{67}Ga [24]. However, the observations that **2** is rapidly converted to a stable lactam compound **3** *in vivo* [19] and that this product does not mediate Fe(II) transport in erythrocytes (present study) suggest that it does not have the potential for use *in vivo*. Moreover, this conversion is probably the reason why the effects of alterations of iron metabolism have not been reported for patients undergoing treatment with nifedipine.

In terms of three-dimensional structure and polarity, the nitro compound **4** and nitrosonifedipine **2** are very similar, yet only the latter is active in Fe(II) transport. The nitroso group of **2** is unlikely to be solely responsible for this property as the parent nitrosobenzene (**11**) is inactive. Nitrosobenzene (PhNO) is reduced rapidly to phenylhydroxylamine (PhNOH) in a number of cell systems, [25,26], and it is possible that in the current system an analogous reduction of the nitroso group of **2** could generate a hydroxylamine group that cyclises onto the neighbouring ester group to deliver the hydroxamic acid

5. Indeed, a synthetic sample of **5** was found to be almost as active as **2**. In addition, the simpler hydroxamic acid **10** was also observed to possess activity. However, the effect of **2** on Fe(II) uptake was observed immediately after its addition to the cells, and with very short incubation times it was just as great as that of **5**. Hence, it is unlikely that the action of **2** is due to conversion to **5**. Probably the comparable effects of these two substances result from their similar lipid solubility and ability to complex the divalent metals. Indeed, their abilities to mediate the partitioning of Fe(II) into 1-octanol were almost identical.

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