Hypothesis

FREE MANGANESE(II) AND IRON(II) CATIONS CAN ACT AS INTRACELLULAR CELL CONTROLS

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1. Introduction

It is accepted that while free Mg(II) and Ca(II) ions act as controls of both intra- and extra-cellular activities, copper(II) and zinc(II) ions perform as bound ions only and the very low level of the free ions have no known function. The intermediate strength of binding of metal ions such as Fe(II) and Mn(II) and the consequent intermediate concentrations of the free ions suggests that these two metals could act simultaneously both in catalytic and control roles. This article is a detailed enquiry into this possibility.

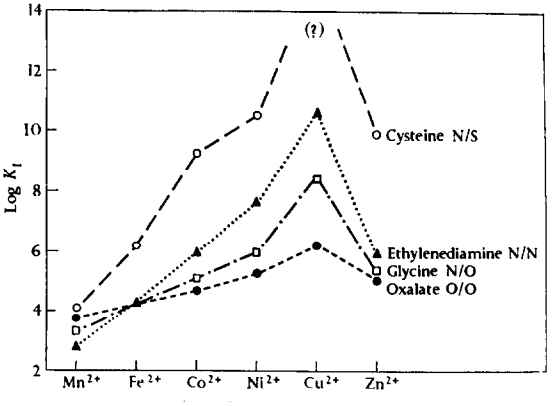

Fig.1. The stability constants of complexes of the first transition series of divalent cations. Note the slopes of the lines. Effective constants at pH 7 are reduced by competition from H^+. Mg(II) and Ca(II) complexes are all weaker than Mn(II) complexes.

2. Metallo-enzymes and metal-activated enzymes

2.1. *Very slow and fast exchanging metal/protein complexes*

The conventional classification of metal requiring enzymes is into metal-activated enzymes and metallo-enzymes [1,2]. The classification is based on the observation that most magnesium- and calcium-requiring enzymes need the addition of free aquo cations to the proteins (apo-enzymes) after their isolation in order to show full activity. Examples are to be found amongst the many nucleases, ATP-requiring enzymes, phosphoglucomutases and phospholipases A2. On the other hand, there is a large number of zinc, copper and some nickel enzymes which have been isolated as intact, i.e., stoicheiometric, metallo-enzymes from which the metal exchanges extremely slowly, e.g., carbonic anhydrase, laccase, urease. The explanation for these observations is simple. To a first approximation the side-chains of proteins bind metal ions, just as model ligands do, with affinities which follow the Irving–Williams series (fig.1), so that metal ions such as Mg^{2+} and Ca^{2+} can only bind to their special proteins with binding constants of $\sim 10^3$–10^7 whereas Zn^{2+}, Cu^{2+} and Ni^{2+} bind with constants $>10^{12}$. Low binding strength permits exchange, high binding strength does not.

Now due to the nature of the strength of metal-ion binding, the availability of metal ions and proteins, and the biological pH it has been shown that these 2 extreme groups of metals would be expected [2] to bind to very different protein side-chains. Mg^{2+} and Ca^{2+} were predicted to bind to only O-donor ligands rarely assisted by perhaps N-donor imidazole (Mg^{2+}). Cu^{2+}, Zn^{2+} and Ni^{2+} were predicted to bind to 2 or more N- or S-donors with perhaps 1 (or 2) carboxylate donors. These almost necessary conclusions from model stability constant data have been fully confirmed by detailed X-ray structural studies (table 1).

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Table 1
Coordination sites for some divalent metal ions in proteins

Metal ion	Ligands (coord. no.)	Protein (example)
Zn(II)	4 S; (4)	Alcohol dehydrogenase
	3 N; (4,5)	Carbonic anhydrase
	10, 2 N; (4,5)	Carboxypeptidase
Cu(II)	2 N, 2 S; (4)	Azurin
	4 N; (4,5)	Superoxide dismutase
Ca(II)	6 O; (6–8)	Parvalbumin
Mg(II)	2 O; (6)	ATP in proteins

Table 2
Trivalent metallo-proteins

Protein	Metal	Position in cell
Transferrin	Fe(Mn)	Plasma protein
Ferritin	Fe	Intracellular store
Superoxide dismutase	Fe, Mn	Mitochondria
Acid phosphatase	Mn, Fe	Extracellular[a]
Some oxygenases	Fe	Intracellular
Rubredoxin	Fe	Intracellular

[a] Extracellular refers to those enzymes which are placed in vesicles such as lysosomes as well as to those which are outside the confines of the cytoplasmic membrane

Looking at fig.1 and table 1 we are left with a group of metal ions, manganese(II), iron(II) and cobalt(II) of intermediate character. How are they bound in biological systems? One answer is given by the nature of many iron and cobalt enzymes – they are based on the binding of small organic ring chelates and not on the binding of free metal ions, i.e., in haem and corrin, which are made under kinetic control (see [3]). These small chelates do not exchange metal ions. They provide the great advantage of the maintenance of the integrity of many iron- and cobalt-dependent proteins. Hence corrin- and haem-dependent enzymes are true metallo-enzymes. Chlorin even permits magnesium to be retained permanently in a protein and there appears to be a nickel cofactor of a similar kind.

However, it remains true that there are a number of manganese- and iron- but probably no cobalt-requiring enzymes which have no such small chelating ligand. Cobalt bound directly to proteins is very unlikely to be generally biologically useful since cobalt is a relatively very rare metal. Before we turn to the remaining iron and manganese enzymes we should observe one other facet of manganese and iron chemistry. Manganese and iron have two possible oxidation states open to them, M(II) and M(III). We have been concerned so far (see fig.1) with M(II), but we know that such ions are not available equally in all parts of biological space. Fe(III) is the overwhelming form of iron in the oxidising atmosphere *outside cells* at pH 7, bound or not bound, whereas Mn(II) is plentiful outside cells in an unbound form. In the presence of particular anionic ligands, for example of many carrier proteins, Mn(III) predominates however. Table 2 lists the manganese(III) and iron(III) proteins known, and there is included in it 1 or 2 extracellular enzymes. They are all true metallo-proteins and show very little dissociation since the binding of these M(III) ions like that of Cu(II), is very strong and kinetically stable, though the ligands are known to include more anionic, oxygen-donor groups, e.g., carboxylate and phenolate together with 1 or 2 imidazoles (table 2). One of these proteins is particularly interesting since it supplies iron to cells, i.e., transferrin, which could transport both Fe(III) and Mn(III) from the storage systems, e.g., ferritin, present perhaps in all cells, to all regions of an organism. Since transferrin is a specific protein and probably has a different surface geometry when bound to Mn(III) as opposed to Fe(III) and is recognised by receptors on cell surfaces it follows that the input of Fe or Mn to different types of cell could be very different. Once inside the cell these 2 metal ions will be reduced to a much higher degree.

Inside cells the redox potential is much lower, let us say ~0.0 V and free M(II), both Fe(II) and Mn(II), but rather little M(III), must be present. As far as M(III) is concerned the binding to proteins now includes that by thiolate, which was oxidised and not available outside cells. There is therefore an extra class of metallo-proteins of low redox potentials which involves Fe(III) but probably not Mn(III) thiolate complexes. The preferential binding of Fe(III) may well be due to its greater stability in a site of tetrahedral geometry (table 2). Thiolates together with S^{2-} (in cells only) do not only form strong complexes with Fe(III) but also form polynuclear complexes of Fe(III) and Fe(II) which give rise to the metallo-enzymes generally classed as iron–sulphur proteins. These proteins often have high binding constants and usually do not lose metal on isolation. The classification of metallo-proteins and metallo-enzymes and its

separation from metal-activated proteins and enzymes is now apparently complete. The observations allowed the suggestion that truly catalytic functions resided in metallo-proteins, using transition metal ions, where the metal ion does not dissociate and has no direct control function, and that truly control functions resided in metal-activated proteins, group IIA cations, where the metal has a poor catalytic role but a ready dissociation [4]. Parallels could be drawn between 2 organic small molecules, cAMP which is a control compound with no catalytic role and exchanges rapidly from proteins (cf. Mg^{2+} and Ca^{2+}) and flavin which is a catalyst and which does not exchange (cf. haem, Cu^{2+} or Zn^{2+}). Further examination of organic cofactors leads one to ask if there is a parallel metal ion to NAD which has a catalytic and a control function and dissociates readily but slowly from enzymes.

2.2. *Metal/protein complexes showing intermediate exchange rates*

There is a class of intracellular Mn(II) and Fe(II) enzymes which on isolation requires a catalytic metal ion to be added (table 3). It is to these enzymes which we turn next. They use either Mn(II) or Fe(II) but not Ni(II), Zn(II) or Cu(II). With Mn(II) enzymes it is sometimes the case that Mg(II) will substitute with varying degrees of success from organism to organism. The binding constants of these enzymes are known to be $\sim 10^8$. They therefore need a standing concentration of Mn(II) or Fe(II) of $\sim 10^{-8}$ M since the metal ion dissociates from the enzyme quite rapidly. They fall exactly between metallo-enzymes and metal enzyme complexes as would be expected from thermodynamic studies of model ligands (fig.1). Moreover in the 2 Mn(II) and Fe(II) proteins for which structural data are available we know that the liganding groups to the metals are intermediate in character between those which bind Ca(II) and those which bind Zn(II). Concanavalin A binds to Mn(II) through one imidazole group and several oxygen donors [5]. Such a site is insufficiently electronegative to retain ions such as Cu(II), Zn(II) and Ni(II) but the presence of 1 nitrogen donor discriminates heavily in favour of Mn(II) against Mg(II) and Ca(II). A second potential way of discriminating against the heavier transition metal ions and yet maintaining selectivity versus the ions Mg(II) and Ca(II) is to increase the bond lengths to the nitrogen (especially) and oxygen donor ligands or by increasing the coordination number, i.e., the size of the metal binding cavity. Binding between cations and neutral nitrogen bases is very sensitive to the distance, M–N, since the linkage depends for its strength on overlap of orbitals and not electrostatic interaction.

Table 3
Readily dissociable Fe(II) and Mn(II) proteins

Protein	Location
(a) Mn(II) Proteins	
Superoxide dismutase	Mitochondria, prokaryotes
Glycosyl transferase	Membranes of vesicles
Malate and isocitrate dehydrogenases (?)	Mitochondria
Oxygen-generating enzyme	Thylakoids
Concanavalin A	Plant sap
(b) Fe(II) Proteins	
Aconitase	Mitochondria
Superoxide dismutase	Prokaryote cytoplasm
Ferredoxin II	Sulphur bacteria cytoplasm
Many oxygenases	Prokaryote cytoplasm
Lysine/proline oxidases	Cytoplasm
Chelatase for porphyrin	Mitochondria

In fig.1 we have compared ligands which are good donors (binding through strong orbital overlap to a large degree) with poor donors (binding through electrostatic terms largely) to show that the complexes of the second group of ligands but not of the first group of ligands was of comparable strength to Fe(II) and Mn(II) as to the later transition metals, say Zn(II). The alternative possibility for making the binding of the 2 groups of metal ions more equal, to increase bond lengths, can be managed in 2 ways:

(1) At low coordination number, say 4-coordinate, the ligand atoms can be constrained by the ligand (protein) framework so that the donor atoms cannot collapse to their optimal bond distances with the metal ion;

(2) At high coordination number mutual repulsion between the donor ligand atoms will limit their ability to make close contact with the smaller cations at the end of the transition series.

Both these circumstances restrict the overlap of ligand and metal orbitals more critically for Zn(II), radius 0.6 Å, than for Mn(II), radius 0.75 Å. In fact the demand for shorter bond lengths later in a series of complexes of the divalent transition metal ions with a

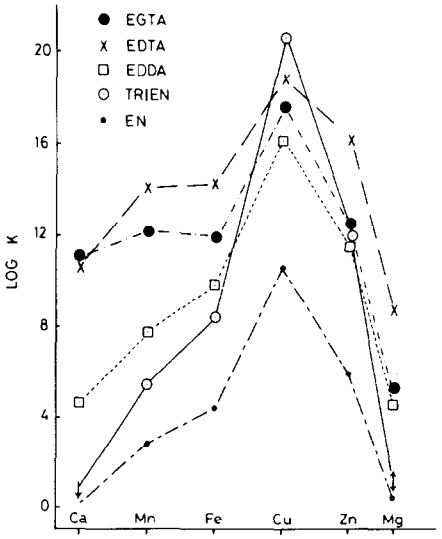

Fig.2. The stability constants of complexes of divalent cations with the ligands: EN, ethylenediamine; TRIEN, triethylenetriamine; EDDA, ethylenediaminediacetate; EDTA, ethylenediamine tetra-acetate; EGTA, 2,2'-ethylenedioxy bis ethylimidotetra-acetate. Note that the slope of the plots increases with increasing numbers of N-donors but finally decreases with increasing numbers of O-donors.

given set of ligand atoms is often reflected in a change of structure from 6-coordinate Mn(II) to 4-coordinate Zn(II). Clearly a constrained large cavity rather than a flexible one will favour Mn(II) rather than Zn(II) to some degree. These expectations are illustrated in fig.2 which shows that the discrimination between Zn(II) and Fe(II) associated with the 2 nitrogen donors of ethylene diamine (or 2 imidazoles of carboxypeptidase) is progressively lost in EDTA and EGTA complexes. The stability data for EGTA show that discrimination for Zn(II) over Mn(II) effectively disappears. The probable explanation of the construction of proteins which are able to retain Mn(II) or Fe(II) in the presence of some free Zn(II) using protein side-chain donors including nitrogen donors is then the relatively large loss at long bond lengths or at high coordination number of stability of the Zn(II) complexes. Sites such as that provided by EGTA with the nitrogen of ethylenediamine replaced by imidazole and the oxygen donors replaced partly by water are known in hemerythrin (fig.3) [6]. Note that both it and concanavalin A provide 6-coordinate sites. Discrimination in favour of Fe(II) over Mn(II) is based here on larger numbers of nitrogen bases within complexes of high coordination number, (fig.1,2).

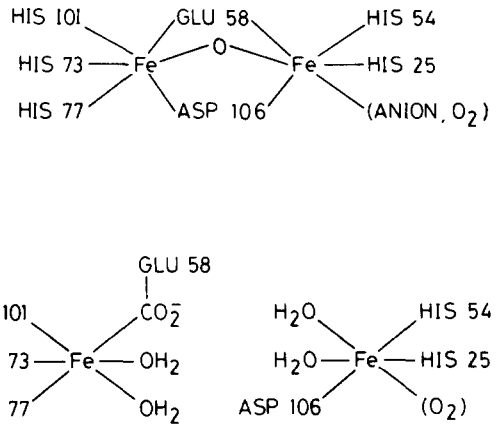

Fig.3. The known structure of hemerythrin in the Fe(III) state and the possible structure of the same protein in the Fe(II) state.

3. Cell compartments

Whereas iron is required to be pumped into all cells, and is so pumped by special carrier/receptor systems this is not known to be the case for manganese. It is extremely likely in fact that Mn(II) is pumped out of cells in the same way as Ca(II) since it is close to Ca(II) in radius. Further understanding of the pumping of Mn(II) must await experiment, e.g., pumping into chromaffin granules [7] and mitochondria [8]; there may be separate pumps for Mn(II) which do not pump Ca(II). They could be based on the differences in coordination shown in concanavalin A [5] and the differences in stability constant shown in fig.2.

All cells are not alike and statements about the free ion concentration require us to distinguish the transport systems of prokaryotic and eukaryotic systems. The single cell membrane of the prokaryotes is known to pump in iron chelates [9] and to pump out calcium ions. Generally we know that calcium pumps also pump manganese but not smaller ions such as magnesium which is close in radius to Fe(II). It is very likely then that both due to inward and outward pumps [Fe(II)] > [Mn(II)] in the cytoplasm of prokaryotes. Fe(II) may be quite high in prokaryote cells since it is essential for haem synthesis (see below, [9,10]).

Haem is made by the insertion of Fe(II) into porphyrin by a chelatase. This insertion enzyme requires free Fe(II) which is obtained from the iron

stored in the cell in the form of ferritin. The ferritin is made directly from the iron uptake probably via Fe(II). Now ferritin is effectively a precipitate of $Fe(OH)_3$ and we can calculate the free Fe(II) in equilibrium with this precipitate knowing the redox potential of the inside of the cell, (~0.0 V), the pH (~7.0) and the solubility product of $Fe(OH)_3$ (10^{-38}), [Fe(II)] must be $\sim 10^{-6}$ M. Although this estimate could be wrong by as much as 10^3 it indicates that the free [Fe(II)] in prokaryote cells is probably close to the free [Ca(II)].

When we turn to eukaryote cells we must consider its various different compartments. Calcium, and we may suppose Mn(II), is removed from the cytoplasm by all membranes so that it is concentrated in vesicles and organelles as well as outside the cell [8]. The cytoplasm must be low in Mn(II). It is impossible to make a direct estimate of [Mn(II)] but for reasons given below it is likely to be around 10^{-6} M in many vesicular spaces and considerably less than this in the cytoplasm. It is known to be concentrated by mitochondria and chloroplasts. Iron is known to be high in mitochondria especially [10]. In fact it is likely that Fe(II) is pumped into mitochondria (and perhaps chloroplasts) as it is into the cytoplasm of prokaryotes since all haem is synthesised there. Chelatase is a mitochondrial enzyme. It seems probable that the distributions of iron and manganese are those shown in fig.4 with little free Fe(II) present anywhere in eukaryotes except in mitochondria (chloroplasts) but free [Mn(II)] quite high everywhere except in the cytoplasm. The cytoplasms of eukaryotic and prokaryotic cells are then quite different.

Although it is not yet possible to prove the assertion that free Fe(II) is distributed mainly in mitochondria of eukaryotes while free Mn(II) is distributed into many vesicles and into the environment outside cells it is a striking observation that many of the enzymes which in vitro require the addition of free Fe(II) or free Mn(II) are in exactly these compartments in vivo (table 3). There are very few cytoplasmic Fe and Mn dissociable enzymes in eukaryotes. Support for this deduced distribution comes from the nature of different superoxide dismutases.

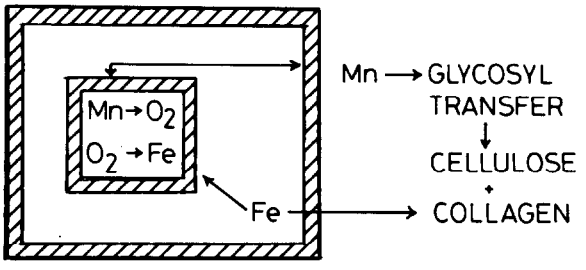

Fig.4. The distributions of Mn(II) and Fe(II) showing Mn(II) inside vesicles and organelles and outside the cell while Fe(II) is largely concentrated in mitochondria. The figure also shows the connection of both iron and manganese with oxygen metabolism and extracellular structures, see text.

4. The superoxide dismutases

It is a curious observation that prokaryotes and mitochondria have manganese or iron superoxide dismutases while the cytoplasm of eukaryotes contains the copper enzyme. All 3 enzymes are efficient and it is very likely that all act by cycling between different redox states. In fact all 3 have about the same redox potential (+0.3 V) [11]. The difference between the enzymes is in their stability. While both Cu(II) and Cu(I) are bound with high and equal stability, the binding of Mn(II) and Fe(II) in their respective enzymes is not so strong and many orders of magnitude ($>10^5$-fold) weaker than the M(III) states. On isolation both the manganese and iron enzymes are known to lose metal. Thus although copper superoxide dismutase is a true metallo-enzyme the other 2 are only metallo-enzymes in the M(III) state. Given that the redox potential of the cytoplasm of eukaryotes is below +0.3 V and that both Fe(II) and Mn(II) are pumped out of this space these enzymes are likely to be unstable in the cytoplasm. Once eukaryotic cells had evolved, a copper dismutase became an extremely useful advantage. There is of course the added advantage that the removal of the required free (mutagenic) Fe(II) and Mn(II) from the compartment which contains DNA is of great importance in long-lived multicellular organisms while it matters little to short-lived prokaryotes and could help their adaptive mutation.

The instability and distribution of superoxide dismutases lends strong support to a main thesis of this article that mononuclear Mn(II) and Fe(II) proteins are only stable in environments where there is a reasonably high concentration of free metal ions.

5. The iron and manganese dissociable enzymes

We have now shown that a certain class of iron and manganese enzymes dissociate. Their existence is completely in accord with the chemical nature of

Table 4
Iron – uptake components

Precursor	Iron uptake: chelating agent	Fe(II) requiring related enzyme
Citrate	Schizokinen	Aconitase (Krebs cycle enzymes)
Citrate	Citrate	Aconitase
2,3-Dihydroxybenzoic acid	Enterobactin	Oxygenases
Ornithine (proline)	Siderochromes	Hydroxylating enzymes
Lysine	Mycobactin P	Hydroxylases
Glycine succinate	Porphyrin	Chelatase
		α-Ketoglutarate-dependent hydroxylase

metal binding sites and stability constant data. These enzymes are located in particular parts of space in different types of cells. We turn next to their functional significance. We must remember that a growing cell needs a relatively large amount of iron and has devised special mechanisms for its uptake [9,10,13,14]. Consideration of those proteins which are classed as dissociable iron enzymes shows that the substrates which they handle are related to the very molecules which are required in the synthesis of chelating agents for this iron uptake (table 4). In the middle column of table 4 are the major known small chelating agents which are used by a variety of cells and probably mitochondria too in the capture of iron. The precursors of these chelating agents are given in the first column. Related enzymes which handle the substrate, or a very closely linked molecule in a metabolic path, are given in the third column. Consider the first two entries where citrate is either involved directly or is a precursor of an iron chelating agent. A major pathway of citrate metabolism is to isocitrate via aconitase. Aconitase is an enzyme which requires free Fe(II) ions. Under circumstances in which Fe(II) is very low citrate will accumulate increasing the concentration of iron scavenging molecules. This is also true of dihydroxybenzoic acids since their oxidative breakdown depends on a dissociable Fe(II) oxygenase but they are also incorporated into iron scavenger chelates. The siderochromes and mycobactin P are prepared by prior oxidation of proline (then ornithine) and lysine. In many cells these 2 amino acids can be oxidised on an alternative pathway leading to crosslinking agents for celluloses and collagen. The latter enzymes, prolyl and lysyl monooxygenases, both require Fe(II) so that when the metal level is low alternative oxidation to give the scavenging chelating agents can occur. Finally at low levels of iron, porphyrin synthesis is restricted and not just haem production since the direct production of succinate from α-ketoglutarate by dioxygenases, or prior to that from citrate, requires iron. The link between scavenging for iron and metabolism is shown in fig.5. The iron acts as a control as well as in catalysis, the control being exerted over the synthesis of its scavenging chelates both in mitochondria and in the cytoplasm of prokaryotes. Fig.6 illustrates the relationship of the iron control to the Krebs cycle.

Aconitase, in table 4, needs special mention. It is

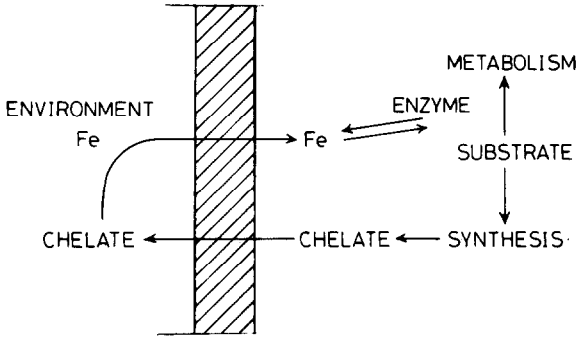

Fig.5. The link between mitochondrial or prokaryote cytoplasmic free iron, Fe(II) and free Fe(III) in the environment is controlled by the synthesis of scavenging chelates. This synthesis is regulated by the free Fe(II) itself by the switching on or off of dissociable enzymes which are dependent upon Fe(II).

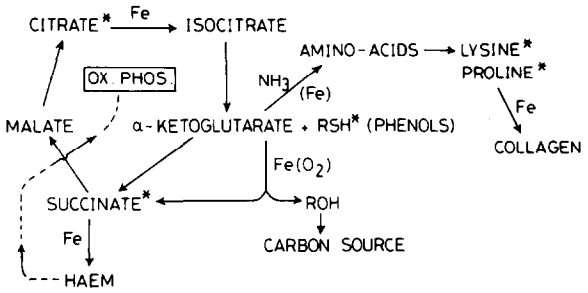

Fig.6. Some of the enzymes controlled by free Fe(II) in cells (mitochondria). The link between citric acid cycle intermediates and the scavenging chelates for Fe(II) is indicated by an asterisk on the substrates which can be used to synthesise chelating agents (table 4).

known now that aconitase is an iron–sulphur protein and has a dissociable Fe_4S_4 unit. Therefore even what was thought to be a robust unit of metallo-enzymes is not tightly bound in aconitase. A similar dissociable Fe_4S_4 unit is found in a control electron-transfer protein of sulphur bacteria. The functional value of the dissociation $Fe_4S_4 \rightleftarrows Fe_3S_3 + Fe^{2+} + S^{2-}$ in these bacteria has been discussed in [12]. Dissociable iron enzymes may have a greater importance in control than we know at present.

Finally, we turn to the manganese enzymes. Apart from their involvement in *organelle enzymes* especially in superoxide dismutase and oxygen generation of eukaryotes, the function of other Mn(II) proteins is associated with saccharides. There are on the one hand the Mn(II) lectins which bind to cell surfaces and appear to stimulate cell division and the Mn(II) enzymes involved in glycosyl transfer. There is a strong indication here that external Mn(II) is an external control over connective material, at least in plants, and cell surfaces in other organisms (fig.4). In plants an insufficiency of Mn(II) means that photosynthesis is much reduced and it would then seem to be wise to adjust the external development of external saccharides by simultaneously stopping glycosyl transfer and, using lectins, reducing cell division. (There is a parallel in animal cells in that low iron not only restricts the development of mitochondria and therefore restricts aerobic cells in the same way as low manganese restricts photo-cellular growth but low iron reduces the synthesis of hydroxylysine and hydroxyproline both of which are required for collagen, a major constituent of the connective tissue of animal cells.)

6. Redox potential control of Fe(II) concentration

There is another possible limitation on the availability of iron in a cell: the redox potential of the medium. In the above discussion the cellular redox potential was set somewhat arbitrarily at 0.0 V. It may well be that some cells have a much lower redox potential when Fe(II) is more available from Fe_2O_3 (ferritin) but there is an increase in another restriction if H_2S is present. The solubility product of FeS is $\sim 10^{-17}$ when at 10^{-3} M H_2S, i.e., 10^{-10} M S^{2-} at pH 7, the concentration of Fe(II) is limited to 10^{-7} M. In more oxidising solutions than 0.0 V, [Fe(II)] is reduced due to its oxidation and removal in Fe(III) complexes. Under any conditions which reduced Fe(II) increased iron scavenging occurs but the use to which the iron is put may be different under oxidising and reducing circumstances. From the above it can be seen that the Fe(II) level could be fixed quite independently from the rate of iron entering a compartment but many constraints exist to prevent it rising above $\sim 10^{-6}$ M. It may be that in oxidising conditions the input of iron to the cell will be greatest as [Fe(II)] will then be lowest.

7. Conclusion

This article is intended to stimulate an experimental investigation of the significance of free Mn(II) and Fe(II) ion concentrations inside and outside cells. Stability constant data for both model and protein complexes indicate that these free concentrations may well lie within 10^{-6}–10^{-10} M. Examination of pumping mechanisms for these ions show that these concentrations could be different in different cell compartments and from one cell type to another. Iron and manganese are deeply involved in major reaction pathways. Control over the concentration of the free cations could then lead to metabolic controls of the conventional kind normally associated with calcium activation. Unlike calcium however iron and manganese may work on the integration of pathways: manganese on the control over glycosyl transfer linked to mitochondrial or chloroplast activity; and iron on the link between the citric acid cycle and collagen production. While the last remarks are speculative they are based on knowledge of the enzymes which require dissociable manganese or iron.

Finally, cellular differentiation and even division is

dependent on Mn(II) lectins and on Fe(II) uptake, either through increase in scavengers for iron or increase in receptors for transferrins in multicellular organisms. Free Fe(II) and Mn(II) concentrations may be used not as rapid triggers of cell function, as Ca(II) is used, but as more slowly acting regulators integrating many pathways. Much work on the role of these metal ions is needed and the author hopes that this article will stimulate research into the roles of these 2 metal ions as regulators.

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