J. Pharm. Pharmacol. 1994, 46: 1000–1003
Received February 24, 1994
Accepted April 18, 1994

Iron-binding Affinity of Bacterial Vaccine Polysaccharides which Contain Phosphodiester Linkages as Part of the Polymer Chain and of Other Polyphosphates, Including DNA

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Abstract—The interaction of iron (II) with bacterial polysaccharides, possessing phosphodiester bonds as part of their polymer chain, has been studied by equilibrium binding dialysis using atomic absorption spectrophotometry. Ferrous ions were found to bind with a stoichiometry of one per two phosphates and with a binding constant of about $2{\cdot}5 \times 10^3\ M^{-1}$. Similar results, but with larger (ca $1 \times 10^4\ M^{-1}$) binding constants were observed with DNA. This interaction helps explain the depolymerization of polyphosphates which has been observed in the presence of iron salts, and highlights the need to avoid iron contamination of vaccines (and other substances) which contain phosphodiester bonds. The interaction may also be a means of iron sequestration in bacteria which possess these cell-surface polyphosphates.

Natural polymers which contain phosphodiester bonds are widespread. The phosphodiester bond is a structural feature of nucleic acids, phospholipids and of bacterial capsular and cell-wall polysaccharides. These polysaccharides are often major metabolic products and may comprise up to 50% of the dry weight of Gram-positive bacterial cell-walls (Baddiley 1972; Ward 1981). In recent years capsular materials have achieved importance through their use as vaccines against diseases caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. The efficacy of the vaccines is highly dependent upon the molecular size of the polysaccharide and specifications for this property are stated in Pharmacopoeial (British Pharmacopoeia 1993) recommendations and must be met in order for these vaccines to be used in the UK (and elsewhere). A number of factors have been shown to effect this property including the presence of iron. For example, mannitol (55 mM) plus ferrous iron (1 mM), caused the mol. wt of the extracellular polysaccharide from *Neisseria meningitidis* type A (which possesses a repeat unit of *N*-acetyl-α-D-mannosamine linked 1,6 through phosphodiester bonds) to be reduced from 200 kDa to less than 10 kDa, whereas in a similar (except for iron content which was $< 1\ \mu M$) preparation of the polysaccharide and mannitol, the mol. wt remained high at about 180 kDa (Newland et al 1989).

It is known that iron, when present in biological systems containing oxygen, can cause substantial structural damage because of the formulation of reactive oxygen species (Halliwell & Gutteridge 1989) and furthermore that this damage is most pronounced in the vicinity of sites which bind the ions (site specific damage). The binding of metal ions to macromolecules is nevertheless of considerable biological importance and a convenient method for investigating the phenomenon is by equilibrium dialysis. The experimental data can be presented in terms of the Scatchard model which treats the binding in terms of multiple equilibria and is particularly applicable to macromolecules which contain only one class of binding site.

Using this approach, Lambert et al (1975) studied the interaction of magnesium ions with the teichoic acids of *Lactobacillus buchneri* and *Staphylococcus aureus* H and concluded that the phosphodiesters bound Mg^{2+}, and suggested that this interaction was an important part of the mechanism by which magnesium was assimilated by these bacteria. More recent reports have noted that a high mol. wt inorganic polyphosphate is produced (Noegel & Gotschlich 1983) by *Neisseria gonorrhoeae* and that it appears to be a means by which iron is sequestered from the environment (West et al 1988) into this organism which apparently lacks a siderophore.

We have, therefore, used equilibrium dialysis to study the interaction of Fe^{2+} with a number of polysaccharides and related compounds which contain phosphodiester bonds under controlled pH and temperature to determine the nature of such interactions, and to better understand the causes of depolymerization of biomolecules containing these bonds.

Materials and Methods

Materials

Teichoic acid (sodium salt) from *Staphylococcus aureus* H cell wall was prepared as described previously (Tarelli & Coley 1979). Extracellular polysaccharides from *Streptococcus pneumoniae* type 3, 6B, and 19F were the bulk materials used for formulation of vaccines and were provided by Dr M. Corbel, Divison of Bacteriology, National Institute for Biological Standards and Control, UK. DNA (sodium salts) from *E. coli* and from herring sperm, 2,2′-dimethylglutaric acid, sodium polyphosphate (sodium phosphate glass type 75+) and amylose were obtained from

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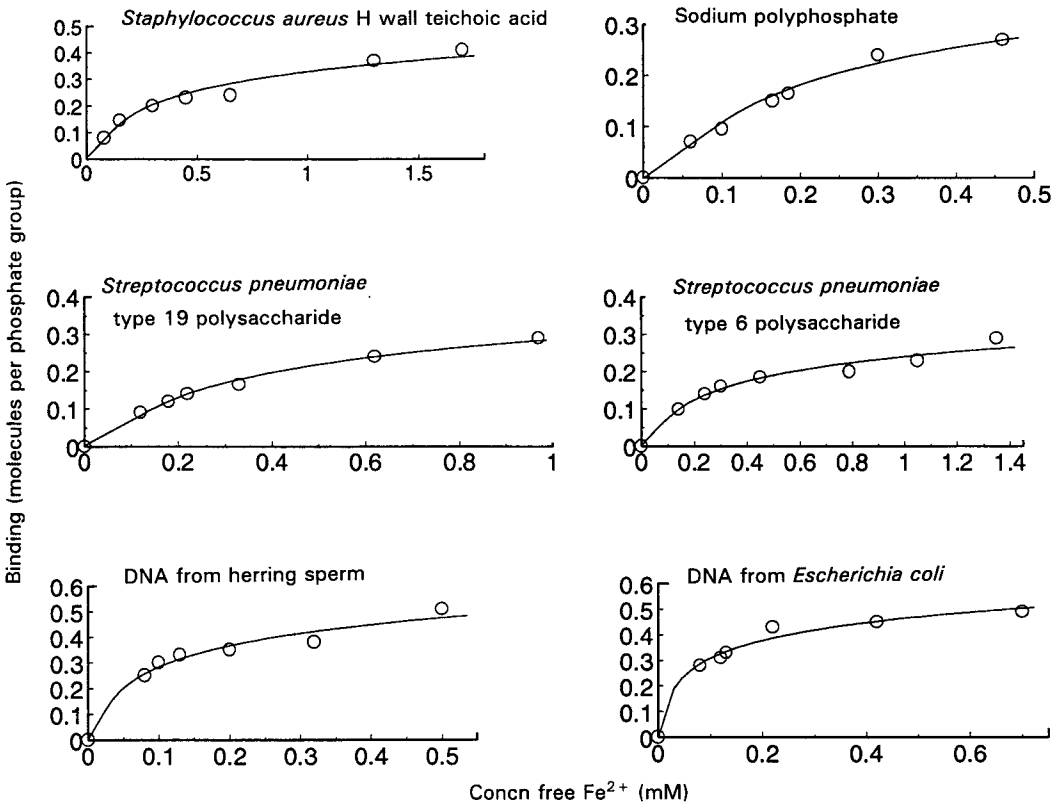

FIG.1 The binding of Fe^{2+} to polyphosphates expressed as isotherms.

Sigma Chemical Co., Dorset, UK. Ferrous ammonium sulphate and sodium hydroxide were AnalaR grade from BDH, Poole, Dorset, UK. Double-glass distilled water with an iron content (determined by atomic absorption spectrophotometry) $< 10\,\mu g\,L^{-1}$ was used throughout. Neutral borosilicate glass ampoules (acid washed) as used (Campbell 1974) for the preparation of WHO International Biological Standards were used for the dialysis experiments. Before sealing they were fitted with a polythene capillary leak plug to obtain a nitrogen (oxygen-free) atmosphere within the ampoule (Campbell 1974; Tarelli 1987). Seamless viscose cellulose dialysis tubing (Visking Co., Chicago, IL, USA) was used to carry out the equilibrium studies. Samples for analysis were dissolved in 0·2% nitric acid and the iron content was then determined using a Perkin-Elmer atomic absorption spectrophotometer model 1100B fitted with a graphite furnace; duplicate analyses were performed. Phosphorus was determined by the method of Chen et al (1956).

Measurement of Fe^{2+} binding

Solutions of the polymers at concentrations of approximately 1 mg mL^{-1} (corresponding to about 1 μmol phosphorus mL^{-1}) were prepared in 2,2′-dimethylglutaric acid buffer (0·1 mM, 0·01% sodium azide, pH 5·0) which had been degassed under vacuum and then saturated with oxygen-free nitrogen. Portions (1·0 mL) of these solutions were dispensed into bags made of 20/32 Visking dialysis tubing, the bags sealed and placed in ampoules containing 5 mL of the buffer described above. An appropriate amount (to give a range 0·1–2 mM Fe^{2+}) of freshly prepared ferrous ammonium sulphate in the same buffer was then added. Each ampoule was fitted with a polythene capillary leak plug, the ampoules back filled with nitrogen, to prevent oxidation of Fe^{2+}, and then sealed. The sealed ampoules were then continuously tumbled for 18 h at 37°C after which the concentrations of iron both inside and outside the dialysis bags were measured by atomic absorption spectrophotometry.

The Fe^{2+} binding was calculated (Morawetz 1965) in terms of mols of iron bound per mol of phosphorus and the results expressed as binding isotherms, i.e. r vs A, where r = number of Fe^{2+} bound per phosphate group and A = equilibrium concentration of Fe^{2+} in solution (mM), and as Scatchard (Scatchard 1949) plots, i.e. r/A vs r.

Results and Discussion

All the polyphosphates listed in Table 1 bound Fe^{2+} at pH 5·0 (which corresponds to the pK_a of the phosphodiester group) as can be seen from similar isotherm plots (Fig. 1.)

The Scatchard plots computed from these data are shown in Fig. 2 and appear linear indicating that the Fe^{2+}-binding sites on the individual molecules are equivalent and that there are no interactions between adjacent sites. From Fig. 2 the association constants may be obtained (from the slope) and the stoichiometry (from the r intercept), and these are listed in Table 1.

The association constants found for the bacterial polysaccharides were approximately $2{\cdot}5 \times 10^3\ M^{-1}$ and are of similar magnitude to the association constants reported

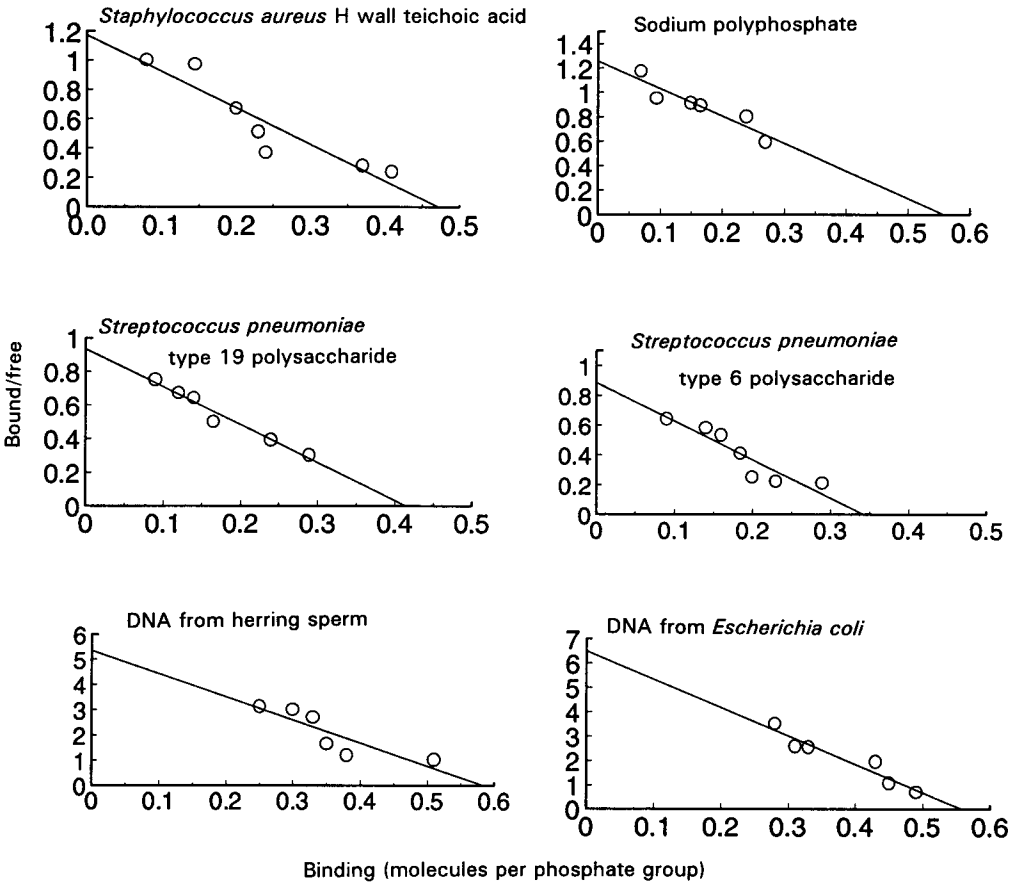

FIG 2. The binding of Fe^{2+} to polyphosphates expressed as Scatchard plots.

Table 1. Association constant (slope) and stoichiometry (intercept on r axis) as determined from the Scatchard plots (Fig. 2).

Polyphosphate from (repeat unit of polysaccharide)	Association constant (M^{-1})	Stoichiometry
Staphylococcus aureus H [→ 1(-4-*O*-α-D-GlcNAc)-ribitol-5-OPO_3^-] (Tarelli & Coley 1979)	$2{\cdot}5 \times 10^3$	0·46
Streptococcus pneumoniae 6B [→ 2)-α-D-Galp-(1 → 3)-α-D-Glcp-(1 → 3)-α-L-Rhap-(1 → 3)-Ribitol-(1-OPO_3^-] (Rebers & Heidelberger 1961)	$2{\cdot}6 \times 10^3$	0·34
Streptococcus pneumoniae 19F (1 → 4)-β-D-ManNAc-(1 → 4)-α-D-Glcp-(1 → 2)-α-L-Rha-(1-OPO_3^-] (Jennings et al 1980)	$2{\cdot}3 \times 10^3$	0·41
Sodium polyphosphate	$2{\cdot}3 \times 10^3$	0·55
DNA (herring sperm)	$9{\cdot}3 \times 10^3$	0·58
DNA (*E. coli*)	$11{\cdot}6 \times 10^3$	0·56

(Lambert et al 1975) for magnesium ion binding to teichoic acids. This suggests that Fe^{2+} interacts with phosphodiesters in a similar manner (probably at the same site) and may in fact be a means by which the bacterium is able to sequester environmental iron as well as magnesium for intracellular incorporation which is vital for cell growth and survival.

Sodium polyphosphate (similar to the polyphosphate found in *Neissieria gonorrhoeae*) bound Fe^{2+} with an association constant based on a pyrophosphate repeat unit of $2{\cdot}3 \times 10^3\ M^{-1}$, the same as those observed for the polysaccharides. It is noteworthy that a neutral polysaccharide amylose showed no binding of Fe^{2+} (data not shown). Significant binding was, however, found for *Streptococcus pneumoniae* type 3 extracellular polysaccharide (data not shown) which contains (Reeves & Goebel 1941) equimolar amounts of D-glucose and D-glucuronic acid, indicating that Fe^{2+} binding may be an important property of other types of polyanions located at or near to a bacterial cell surface especially for those organisms lacking phosphodiester polymers.

Two samples of nucleic acids (from bacterial and from animal origin) were also shown to bind Fe^{2+}. A higher apparent association constant ($\sim 1 \times 10^4\ M^{-1}$, Table 1) was found in these cases suggesting that sites other than the phosphodiesters or tertiary structures are influencing the strength of binding. Scatchard analyses are strictly applicable if only one type of binding site exists within a molecule

and consequently, the interpretation of these data should be treated with some caution, although our results unequivocally show that, under the conditions employed, Fe^{2+} binds to DNA.

The stoichiometry of the interactions can be determined from the Scatchard plots (as the intercept on the r axis) and are listed in Table 1. For the polysaccharides these were reasonably close to 0·5, which would indicate that two phosphate groups are involved in the binding of each Fe^{2+}. The experimentally observed values (< 0·5) may be the result of chain termini effects, inhomogeneity of the polymer (e.g. partial *O*-acetylation) or to depolymerization. The value (> 0·6) observed for DNA suggests that other structural features may be influencing the binding.

As might be expected some changes to the polymers were observed under the conditions employed. Phosphorus determination was routinely performed on the equilibrium solutions from each side of the dialysis membranes. For the smaller macromolecules studied, increasing amounts of phosphorus were found outside the dialysis bags when higher concentrations of Fe^{2+} were used. In the case of the teichoic acid from *Staphylococcus aureus* H (mol. wt approx. 10 kDa) about 10% of the phosphorus was present outside the dialysis bag when the concentration of Fe^{2+} was 1 mM; less than 1% was observed under similar conditions but in the absence of Fe^{2+}. Similarly the sodium polyphosphate (mol. wt ~ 6 kDa) showed a steady increase of dialysable phosphorus with increasing Fe^{2+} concentration and at 0·5 mM Fe^{2+} about 10% was detected outside the dialysis bag. For the larger molecules (mol. wt > 300 kDa) this phenomenon was not observed, probably because the small extent of bond fission would, on the whole, give rise to fragments large enough to be retained by the dialysis membrane under the experimental conditions used. These results accord with our unpublished observations that polysaccharides which contain phosphodiester linkages, depolymerize in the presence of iron salts very much more readily than those in which this linkage is absent.

A further point of note is that under aerobic conditions oxidation of bound Fe(II) to Fe(III) would probably occur within minutes. This is expected by analogy with the reported (Harris & Aisen 1973) facilitation of autoxidation of Fe(II) by low mol. wt Fe(III) complexing agents, including phosphate. Oxidation should then result in a higher affinity between iron and the polyphosphates.

In conclusion, the results described here show that polyphosphates bind Fe^{2+} and consequently highlight the importance of avoiding their contamination with iron salts, for example, during production of polyphosphate-type vaccines, standards and reference reagents or other substances containing this functionality. The interaction may contribute, through cell surface polymers, to a mechanism by which a bacterium is able to sequester iron from its environment. The binding of iron to polyphosphates will, however, induce depolymerization and for such polysaccharides used as vaccines this will result in a loss of efficacy, with obvious serious consequences.

Acknowledgements

Dr J. M. C. Gutteridge (NIBSC) is thanked for helpful discussions.

References

Baddiley, J. (1972) Teichoic acids in cell walls and membranes of bacteria. Essays Biochem. 8: 35–77

British Pharmacopoeia (1993) Her Majestys Stationery Office, London, p. 1200

Campbell, P. J. (1974) International biological standards and reference preparations II. Procedures used for the production of biological standards and reference preparations. J. Biol. Standard. 2: 259–267

Chen, P. S., Toribara, T. Y., Warner, H. (1956) Microdetermination of phosphorus. Anal. Chem. 28: 1756–1758

Halliwell, B., Gutteridge, J. M. C. (1989) The chemistry of oxygen radicals and other oxygen derived species. In: Halliwell, B., Gutteridge, J.M.C. (eds) Free Radicals in Biology and Medicine. Clarendon Press, Oxford, pp 22–81

Harris, D. C., Aisen, P. (1973) Facilitation of Fe(II) autoxidation by Fe(III) complexing agents. Biochim. Biophys. Acta 329: 156–158

Jennings, H. J., Rosell, H. G., Carlo, D. J. (1980) Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type-19 (19F). Can. J. Chem. 58: 1069–1074

Lambert, P. A., Hancock, I. C., Baddiley, J. (1975) The interaction of magnesium ions with teichoic acid. Biochem. J. 149: 519–524

Morawetz, H. (1965) Polyelectrolytes. In: Mark, H., Flory, P.J., Marvel, C.S., Melville, H.W. (eds). Macromolecules in Solution. Interscience, New York, pp 340–342

Newland, P., Bingham, B., Tarelli, E., Thomas, A. H. (1989) High performance liquid chromatography of meningococcal polysaccharides. J. Chromatogr. 483: 406–412

Noegel, A., Gotschlich, E. C. (1983) Isolation of a high molecular weight polyphosphate from *Neisseria gonorrhoeae*. J. Exp. Med. 157: 2049–2060

Rebers, P. A., Heidelberger, M. (1961) The specific polysaccharide of type VI pneumococcus. II. The repeating unit. J. Am. Chem. Soc. 83: 3056–3059

Reeves, R. E., Goebel, W. F. (1941) Chemoimmunological studies on the soluble specific substance of pneumococcus. V. The structure of the type III polysaccharide. J. Biol. Chem. 139: 511–519

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. Ann. NY Acad. Sci. 51: 660–672

Tarelli, E. (1987) A hydrolytic procedure giving reproducible analytical conditions. Anal. Biochem. 167: 145–147

Tarelli, E., Coley, J. (1979) ^{13}C NMR spectra of some ribitol teichoic acids. Carbohydr. Res. 75: 31–37

Ward, J. B. (1981) Teichoic and teichuronic acids: biosynthesis, assembly and location. Microbiol. Rev. 45: 211–243

West, S. E. H., Penniall, R., Sparling, P. F. (1988) Polyphosphate-mediated release of iron from transferrin. A possible mechanism for aquisition of iron from transferrin by *Neisseria gonorrhoeae*. In: Poolman, J. T. (ed.) Gonococci and Meningococci. Kluwer Academic, Dordrecht, Netherlands, pp 837–841