Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: a possible physiological function for *myo*-inositol hexakisphosphate

Phillip T. HAWKINS, David R. POYNER,* Trevor R. JACKSON, Andrew J. LETCHER, David A. LANDER and Robin F. IRVINE†
Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K.

1. The ability of *myo*-inositol polyphosphates to inhibit iron-catalysed hydroxyl radical formation was studied in a hypoxanthine/xanthine oxidase system [Graf, Empson and Eaton (1987) J. Biol. Chem. **262**, 11647–11650]. Fe^{3+} present in the assay reagents supported some radical formation, and a standard assay, with 5 μM Fe^{3+} added, was used to investigate the specificity of compounds which could inhibit radical generation. 2. InsP_6 (phytic acid) was able to inhibit radical formation in this assay completely. In this respect it was similar to the effects of the high affinity Fe^{3+} chelator Desferral, and dissimilar to the effects of EDTA which, even at high concentrations, still allowed detectable radical formation to take place. 3. The six isomers of InsP_5 were purified from an alkaline hydrolysate of InsP_6 (four of them as two enantiomeric mixtures), and they were compared with InsP_6 in this assay. Ins(1,2,3,4,6)P_5 and D/L-Ins(1,2,3,4,5)P_5 were similar to InsP_6 in that they caused a complete inhibition of iron-catalysed radical formation at > 30 μM. Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,4,5,6)P_5, however, were markedly less potent than InsP_6, and did not inhibit radical formation completely; even when Ins(1,3,4,5,6)P_5 was added up to 600 μM, significant radical formation was still detected. Thus InsP_5s lacking 2 or 1/3 phosphates are in this respect qualitatively different from InsP_6 and the other InsP_5s. 4. *scyllo*-Inositol hexakisphosphate was also tested, and although it caused a greater inhibition than Ins(1,3,4,5,6)P_5, it too still allowed detectable free radical formation even at 600 μM. 5. We conclude that the 1,2,3 (equatorial–axial–equatorial) phosphate grouping in InsP_6 has a conformation that uniquely provides a specific interaction with iron to inhibit totally its ability to catalyse hydroxyl radical formation; we suggest that a physiological function of InsP_6 might be to act as a 'safe' binding site for iron during its transport through the cytosol or cellular organelles.

INTRODUCTION

myo-Inositol hexakisphosphate (phytic acid, InsP_6) is a major component of plant storage tissues (Cosgrove, 1980), where it is generally believed to be a phosphate store, and Ins(1,3,4,5,6)P_5 is found in large quantities in erythrocytes of avian, reptilian and amphibian species (Johnson and Tate, 1969), where it modulates haemoglobin oxygen binding (see Bartlett, 1982). Until the fairly recent past, these two higher inositol polyphosphates, with these specific functions, were believed to be confined to these particular tissues. However, investigations of inositol phosphates in animal cells revealed that InsP_6 is found probably in all animal cells [see Stephens et al. (1991) for references], and presently there is no known exception to suggest other than that InsP_6 is universal in eukaryotic tissues. InsP_5s are also found in various extents in animal tissues, the predominant isomer being Ins(1,3,4,5,6)P_5 (Stephens et al., 1991).

The levels of Ins(1,3,4,5,6)P_5 can be modulated after cell stimulation, probably because there are complex routes of synthesis to it from receptor-generated Ins(1,4,5)P_3 [e.g. Stephens and Downes (1990) and see Shears (1989) for a review]. However, at least in the slime mould *Dictyostelium discoideum*, presently the only organism in which the route of synthesis of InsP_6 has been fully elucidated, InsP_6 can be synthesized [with Ins(1,3,4,5,6)P_5 as an obligate intermediate] by sequential phosphorylations of inositol and with no lipid intermediate (Stephens and Irvine, 1990). The breakdown route of InsP_6 is not documented yet, but in slime moulds it is predominantly dephosphorylated in the 3 and 5 positions (Stephens and Irvine, 1990; Stephens et al., 1991), and in animals there is a 3-phosphatase with a high affinity for InsP_6 (Nogimori et al., 1991).

The reasons for the apparently universal occurrence of InsP_6 in eukaryotic cells are not known. It is found at estimated levels up to 600 μM (Martin et al., 1987), and clearly represents a considerable investment in cell energy. Various functions have been suggested, and these have been summarized elsewhere (Stephens and Irvine, 1990; Carpenter et al., 1989). In 1984, Graf et al. showed that InsP_6 was a particularly effective inhibitor of iron-catalysed hydroxyl radical ($OH^{\cdot}$) formation, and suggested that it might make a useful food additive (Graf et al., 1984, 1987; Graf and Eaton, 1990). They also suggested that, given its high level in dry storage tissues of higher plants, evolution may already have put it to that use, i.e. that it is there as a natural antioxidant (Graf et al., 1987; Berridge and Irvine, 1989). A natural extension of this line of reasoning is that animal cells may also use InsP_6 as an endogenous iron chelator. It is clear that animal cells possess iron in a low-molecular-mass pool which may be important for the transport of iron between transferrin (the major route of iron entry into cells), ferritin (the major iron depot in cells), and various cell destinations (e.g. haem-containing enzymes); this pool may also be important in various disease states (e.g. haemochromatosis: Jacobs, 1977; Grohlich et al., 1979; Crichton, 1979; Morgan, 1977; Fontecave and Pierre, 1991; Britton et al., 1990; Nielson et al., 1993). The nature of the low-molecular-mass iron pool is presently unclear, but it has in the past been postulated to represent iron chelated with citrate (Morley and Bezhorovainy, 1983), phosphate (Pollack et al., 1985), amino acids (Bakkeren et al., 1985; Deighton and Hider,

* Present address: Department of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET, U.K.
† To whom correspondence should be sent.

1989) and ATP (Weaver and Pollack, 1989). However, given the apparently high affinity and kinetic lability of iron–InsP_6 complexes (e.g. Cosgrove, 1980; Poyner et al., 1993) and the documented ability of phosphates to exchange iron between iron-binding proteins (e.g. Cowart et al., 1986; Morgan, 1977), it seemed an attractive possibility that InsP_6 represents a component of this low-molecular-mass iron-pool.

Direct evidence for such a function for InsP_6 will be difficult to obtain (it may have to come from eliminating InsP_6 from a cell by genetic means), but we have here sought some indirect evidence by investigating the specificity of the interaction of inositol polyphosphates with iron. We find that although all the inositol polyphosphates tested will interact with iron and inhibit $OH^{\cdot}$ formation (generated from superoxide anion and H_2O_2), only those with a 1,2,3 (equatorial–axial–equatorial) phosphate grouping cause a complete inhibition. This precise and specific effect lends some credibility to the possibility that this is one reason why eukaryotic cells maintain fairly high levels of cytosolic InsP_6.

MATERIALS AND METHODS

In all experiments involving ferric iron, the desired concentrations were obtained by diluting (at least 20-fold) concentrated stock solutions immediately before use; this was done to avoid the formation of insoluble iron hydroxide precipitates.

InsP_6 and InsP_5 isomers

These were prepared from InsP_6 (purchased from Sigma) by alkaline hydrolysis and ion-exchange chromatography as described in Stephens (1990) and Stephens et al. (1991). They were checked for purity by chromatography on polyethyleneimine–cellulose (Spencer et al., 1990), and, as shown in Figure 1, each InsP_5 fraction is distinct and apparently largely free of contamination with other inositol phosphates. The InsP_6 was also purified by h.p.l.c., and in some experiments, InsP_6 from mung beans (*Phaseolus aureus*) (a gift from L. Stephens) was used. *scyllo*-Inositol hexakisphosphate was generously given by Dr. M. F. Tate (Waite Institute, Adelaide, South Australia, Australia).

Catechol-decolouring assay

Solutions were made containing 0.25 mM catechol and the appropriate concentration of chelator, and then made up to 0.25 mM Fe^{3+} with $FeCl_3$. The pH was adjusted to 7.0 with dilute HCl or NaOH and after 15 min the absorbance was read at 575 nm (at 25 °C). The chelator–catechol complexes had no significant absorbance at this wavelength in the absence of added iron.

Hydroxyl radical generation assay

This was essentially performed as described by Graf et al. (1984, 1987) with some modifications. The principle of the assay is the iron-catalysed formation of $OH^{\cdot}$ from superoxide anion radical ($O_2^{-\cdot}$) generated by xanthine oxidase/hypoxanthine) and H_2O_2 (the reactions involved are thought to be:

$$Fe^{3+} + O_2^{-\cdot} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-})$$

This assay is based on the original work of Beauchamp and Fridovich (1970). The formation of $OH^{\cdot}$ is monitored by reaction with dimethyl sulphoxide and measurement of resulting formaldehyde by the Hantz reaction (Nash, 1953). We tried to make

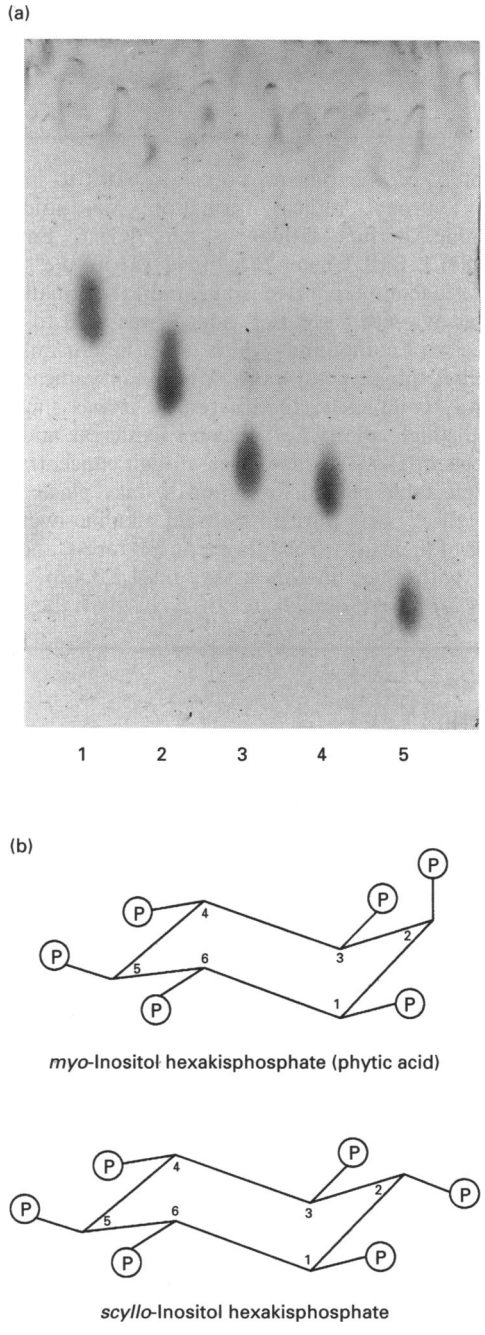

Figure 1 Isomers of InsP_5

(**a**) The six isomers of InsP_5 (four of them as two enantiomeric mixtures) were prepared as described in the Materials and methods section, and this Figure illustrates their apparent purity when analysed by polyethyleneimine–cellulose t.l.c. (see the Materials and methods section for details). Lanes are as follows: 1, Ins(1,2,3,4,6)P_5; 2, D/L-Ins(1,2,3,4,5)P_5; 3, D/L-Ins(1,2,4,5,6)P_5; 4, Ins(1,3,4,5,6)P_5; 5, InsP_6. (**b**) We illustrate, in its predominant chair conformation (Emsley and Niazi, 1981), InsP_6 (left) with its carbon atoms numbered. Note that the 2-phosphate is axial, whereas the others are all equatorial; thus the 1,2,3 phosphates form a unique equatorial–axial–equatorial grouping. Below, for comparison, is *scyllo*-inositol hexakisphosphate, which has all six of its phosphates equatorial.

this assay linear with respect to enzyme, substrates, time and added Fe^{3+}. Under the conditions originally described by Graf et al. (1984), the reaction saturated with enzyme concentration and over time, even under conditions where the original substrate (hypoxanthine) was consumed to $< 10\%$ as calculated by formaldehyde formation and the enzyme was still active (as judged by further substrate addition). Eventually, however, we arrived at assay conditions essentially linear with everything but added Fe^{3+} concentration (see the Results section); these conditions included the addition of a small quantity of H_2O_2 to encourage a 'pseudo-first-order' generation of $OH^{\cdot}$ radicals with respect to added iron. The standard assay (e.g. as used for Figures 5 and 6 below) mixture contained, in a final volume of 1 ml, 20 mM Trizma (Sigma) buffered to pH 7.5 with HCl, 50 mM dimethyl sulphoxide, 0.3 mM hypoxanthine, 5 μM $FeCl_3$ (added from 50 μM stock freshly prepared), 0.5 μM H_2O_2, 18 m-units of xanthine oxidase (Grade III; Sigma; from buttermilk). After 15 min (at 25 °C) the fractions were quenched by 2 min immersion in a boiling-water bath, and 250 μl formaldehyde reagent was added. This consists of 15 g of ammonium acetate, 0.3 ml of acetic acid and 0.2 ml of acetylacetone in a final volume of 100 ml of water. The tubes were incubated at 60 °C for 15 min to develop the colour, and the absorbance was measured at 410 nm.

RESULTS

Interaction of Fe^{3+} and higher inositol polyphosphates

Although this investigation is concerned primarily with the relative efficacy of some naturally occurring inositol polyphosphates to inhibit iron-catalysed $OH^{\cdot}$ formation, we began by investigating the overall ability of InsP_6, and the InsP_5 isomers used here, to bind Fe^{3+}. This was done indirectly, and in two ways. First, increasing concentrations of InsP_6 were mixed with 1 mM $Fe_2(SO_4)_3$ (unbuffered, pH 6.5, or buffered to pH 7.0) and their ability to prevent precipitation of $Fe(OH)_3$ was assessed by the formation of stable clear solutions. InsP_6 at 100 μM had a significant inhibitory effect on precipitation, and at 1 mM inhibited precipitation completely (results not shown). Given the extremely low solubility of $Fe(OH)_3$ these data imply a very high affinity of Fe^{3+} for InsP_6 (i.e. an overall affinity constant for Fe^{3+} binding to InsP_6 $> 10^{18}$; Biederman and Schindler, 1957). Because of the semi-quantitative nature of this assay we did not explore extensively the potency of the four InsP_5 isomers (see below), but, when equimolar with Fe^{3+}, all four also completely inhibited precipitation, implying that they too have a very high affinity for Fe^{3+}. The important point is that in the experiments on iron-catalysed $OH^{\cdot}$ formation described below, when any InsP_5 or InsP_6 is added in excess of Fe^{3+}, the Fe^{3+} will be very largely bound to the inositol phosphate.

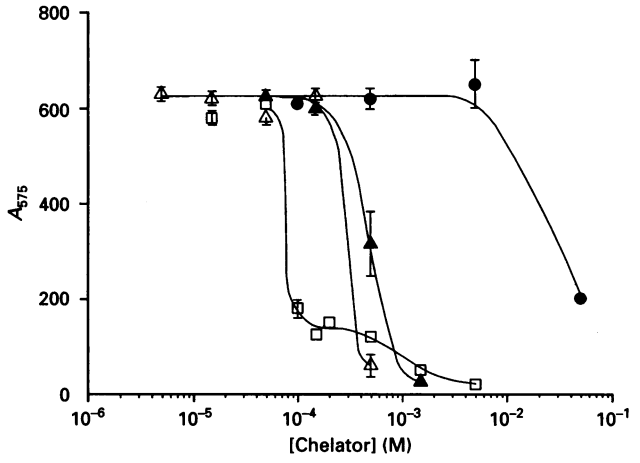

Figure 2 Interaction of Fe^{3+} with chelators assayed by decolorization of catechol/Fe^{3+}

Assays were carried out as described in the Materials and methods section. Fe^{3+} and catechol are at 0.25 mM. □, InsP_6; ▲, Na_2EDTA; ●, Na_2ATP; △, Desferral. The data are means ± S.E.M. ($n = 3$) of independent samples; where no error bars are shown they lie within the sizes of symbols.

Some idea of the relative affinity of InsP_6 for Fe^{3+} was deduced by competition experiments measuring the decolorization of $FeCl_3$/catechol complexes (see the Materials and methods section). Any compound that is able to compete with catechol for Fe^{3+} in the same concentration range as the Fe^{3+}–catechol complex (0.25 mM in this case) must have an affinity for Fe^{3+} that is of a similar order to, or greater than, that of catechol (the K_1 for which is approx. 10^{-20}; Martell and Smith, 1982). The data (Figure 2) show that InsP_6, EDTA and Desferral all fall into this category; the greater potency of InsP_6 compared with the other two chelators is presumably because InsP_6 has multiple phosphates which are capable of chelating Fe^{3+} with high affinity (i.e. more than one Fe^{3+} can be bound per InsP_6; Graf et al., 1987). The discontinuity in the InsP_6 inhibition curve (Figure 2) may imply differing affinities between the phosphates, or interactions between the phosphates as they progressively bind Fe^{3+}, but we have not explored this further. Another point that emerges from Figure 2 is that InsP_6 is considerably more potent in this assay than ATP. We do not know exactly the intracellular concentrations of InsP_6 (see the Introduction), but the data imply that, even in the presence of physiological levels of ATP, InsP_6 is likely to compete very well with ATP for this cation. Finally, we investigated the relative affinities of other cations by using the radical formation assays described below and exploring the ability of the cations to reverse the inhibitory effect of InsP_6 on radical formation catalysed by 5 μM Fe^{3+}. Of these, only Al^{3+} was effective at releasing Fe^{3+} from InsP_6 (as judged indirectly by radical formation), whereas Zn^{2+}, Ni^{2+}, Mn^{2+}, Pb^{2+} and Ca^{2+} were without discernible effect (results not shown). Taken all together, these data suggest a very high affinity interaction between inositol polyphosphates and Fe^{3+}, and our aim was then to investigate to what extent this interaction inhibits the ability of Fe^{3+} to generate $OH^{\cdot}$ radicals.

Linearity of radical forming assay

As we were interested in quantitative differences between the ability of inositol polyphosphates to interact with iron, we have tried to make the hypoxanthine/xanthine oxidase system linear with respect to added Fe^{3+}. This has provided some problems (see below and the Materials and methods section). The formation of the $OH^{\cdot}$ radical is a multi-stage process involving more than one oxidation state of iron [see the Materials and methods section and Graf et al. (1984, 1987)] and the reaction between $OH^{\cdot}$ and dimethyl sulphoxide causes a variety of products to be formed (see Halliwell and Gutteridge, 1985), only one of which (formaldehyde) is being measured in the assays, and these factors may explain some of the complexities that we have encountered.

$OH^{\cdot}$ formation proceeded in the absence of added Fe^{3+}, and this was inhibited by low concentrations of the Fe^{3+} chelator Desferral (Halliwell and Gutteridge, 1985; Graf et al., 1987). From this we deduced that it is due to Fe^{3+} in the reagents, and

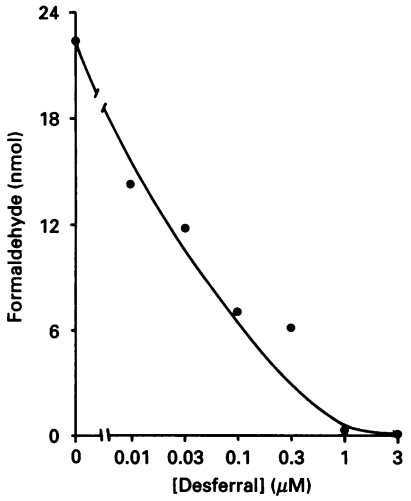

Figure 3 Effect of Desferral on OH• radical generation with no added Fe^{3+}

The assay was as described in the Materials and methods section, but with no exogenous Fe^{3+} added (i.e. only Trizma, hypoxanthine, H_2O_2 and xanthine oxidase at the concentrations given in the Materials and methods section, with increasing concentrations of Desferral). Typical of three independent experiments.

we traced the Tris buffer as the principal source; the reaction was to some degree proportional to Tris concentration, and changing to a purer form of Tris (Trizma, Sigma) decreased the reaction rate. Nevertheless, we could not eliminate this entirely, and titration with Desferral (Figure 3) suggested that less than 1 μM Fe^{3+} was present.

Addition of more Fe^{3+} (Figure 4a) gives an erratic but highly reproducible curve, with a high level of activity at 1 μM added Fe^{3+} which then decreased and levelled off. We did not see this erratic behaviour with 40 μM EDTA present (Figure 4b), and so we believe this is to do with the inherent difficulties of (a) multiple reactions and products, of which we are only measuring one [see above and Halliwell and Gutteridge (1985)] and (b) the inevitable difficulty of working with very low levels of unbuffered multivalent cations.

As a final compromise we added 5 μM Fe^{3+}, and we have had to accept that quantitative conclusions must be limited. For blanks we used heat-killed enzyme, though there was a very slight colour development (< 5% of that with enzyme) in its absence. Extensive exploration of blanks has convinced us that a high concentration of InsP_6 or Desferral (in excess of Fe^{3+}) does truly reduce OH• generation to zero within the limits of detection of this method.

Inhibition of radical formation by *myo*-inositol polyphosphates

The effect of all the InsP_5 isomers [four of them as two enantiomeric pairs; see Stephens et al. (1991)] on OH• generation in our standard assay is shown in Figure 5. If 50 μM Fe^{3+} was used instead of 5 μM, similar results were obtained for InsP_6 and Ins(1,3,4,5,6)P_5 (the only two Ins$P$s of which we had sufficient for high concentrations), but, as is to be expected, more of each inositol phosphate was needed to achieve the same percentage inhibition (results not shown). It is clear that the compounds fall into two distinct groups (Figure 5). InsP_6 at about 10–30 μM causes a complete inhibition (see above), and we found that InsP_6 preparations from several sources (prepared from mung beans;

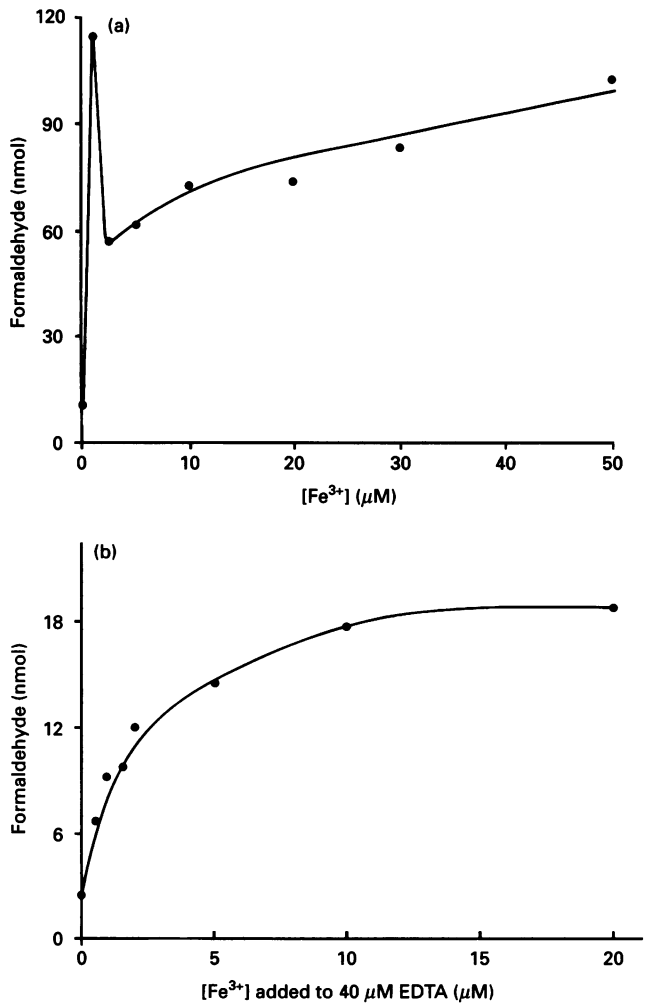

Figure 4 Effect of Fe^{3+} concentration of OH• radical generation

(**a**) In the absence of any chelator, Fe^{3+} was added in increasing amounts from a freshly prepared 100 μM $FeCl_3$ stock (the pH of the assay was not altered significantly by addition up to 50 μM). For assay conditions, see the Materials and methods section. This result is typical of four independent experiments. (**b**) In the presence of 40 μM EDTA (i.e. assay conditions exactly as standard, but with 40 μM EDTA present in all tubes, and $FeCl_3$ added in increasing concentrations). Results are typical of two independent experiments.

h.p.l.c.-purified from Sigma InsP_6) were similar in their potency and ability to inhibit completely the radical generation (results not shown). Ins(1,2,3,4,6)P_5 and D/L-Ins(1,2,3,4,5)P_5 are similar to InsP_6. They appear slightly less potent than InsP_6, but it is clear that they reach the same end point, i.e. complete inhibition under our assay conditions. Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,4,5,6)P_5 are profoundly different. They are less potent and, most importantly, they do not cause a complete inhibition under our assay conditions. We did not have sufficient D/L-Ins(1,2,4,5,6)P_5 to increase it to a very high concentration, but we increased Ins(1,3,4,5,6)P_5 to 600 μM, and still it had not inhibited radical formation completely (Figure 5). Neither Ins(1,4,5)P_3 nor Ins(1,3,4,5)P_4 caused any inhibitory effect at all up to 500 μM, and both in fact caused a slight (approx. 20%) stimulation, and ATP at 1 mM caused only a small inhibition (results not shown).

The implication from these data overall is that the 1,2,3 phosphate grouping in InsP_6 is essential for the complete inhibition of free-radical formation. In this context, it is noteworthy

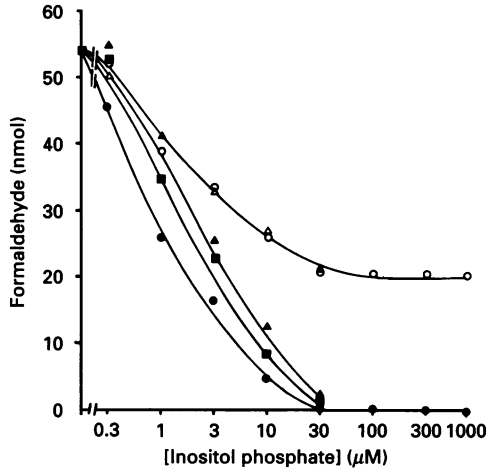

Figure 5 Effects of *myo*-inositol polyphosphates on OH• generation

The assay was as described in the Materials and methods section (standard assay), with 5 μM added Fe^{3+}. Inositol phosphates were added from 1 mM stock solutions. This is a result pooled from two experiments, and is typical of six independent experiments. ●, InsP_6; ○, Ins(1,3,4,5,6)P_5; △, D/L-Ins(1,2,4,5,6)P_5; ▲, Ins(1,2,3,4,6)P_5; ■, D/L-Ins(1,2,3,4,5)P_5.

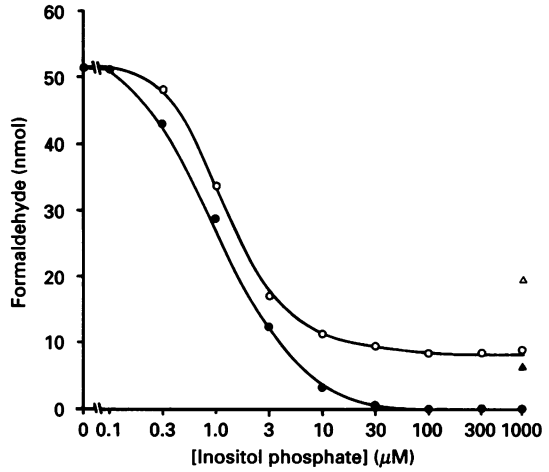

Figure 6 Effect of *scyllo*-inositol hexakisphosphate on OH• generation

Conditions were as described for Figure 5. ●, InsP_6 (*myo*-inositol hexakisphosphate); ○, *scyllo*-inositol hexakisphosphate. Also shown are data points in the same experiments for: ▲, EDTA; △, Ins(1,3,4,5,6)P_5. Similar results were obtained in an identical experiment.

that, notwithstanding the semi-quantitative assay, we can see that a considerable excess of InsP_6 over Fe^{3+} is required (Figure 5), which contrasts with the data of Figure 2, from which we deduced that multiple phosphate moieties on InsP_6 are binding Fe^{3+} tightly; this apparent contradiction could be because to cause complete inhibition of radical formation the 1,2,3 grouping must bind all the iron (and moreover it must do so in competition with the other phosphate moieties on the InsP_6). We should note, however, that Graf et al. (1984) found complete inhibition of radical formation at iron/InsP_6 ratios of less than 1, and we have no explanation for this other than differences in the assay (see above).

Inhibition by other potential chelators

In agreement with Graf et al. (1984, 1987) and Graf and Eaton (1990) we found that whereas Desferral caused a complete inhibition (Figure 3), EDTA did not (see also Beauchamp and Fridovich, 1970). EDTA was more potent than InsP_6 at low concentrations, but even at millimolar levels there was still some reaction occurring, whereas, in parallel incubations, InsP_6 at 30 μM caused complete inhibition (results not shown).

The 2-phosphate is the only axial one in InsP_6 in the predominant chair conformation (Emsley and Niazi, 1981; Figure 1b), and it is therefore an intriguing possibility that an equatorial–axial–equatorial conformation has the precise geometry for this complete inhibition of radical generation. We were fortunate to gather some more direct information on this by the gift of some synthetic *scyllo*-inositol hexakisphosphate from Dr. M. F. Tate. *scyllo*-Inositol hexakisphosphate is identical with InsP_6, except that all its phosphate groups are equatorial (Figure 1b). When we tested this in the assay we found that it is clearly more effective at reducing OH• formation than Ins(1,3,4,5,6)P_5, especially at high concentrations, but it is also clearly and reproducibly unable to block this completely (Figure 6). This goes some way to confirming the implication above, that it is the equatorial–axial–equatorial grouping of InsP_6 (*myo*-inositol hexakisphosphate) which is uniquely (in the present inositol phosphate context) able to prevent iron entirely from catalysing OH• formation.

Conclusions and physiological significance

The data presented above enable us to group the ion chelators studied into two divisions, based on whether, given sufficient concentrations, inhibition of the iron-catalysed OH• radical formation is complete. Graf et al. (1984) drew a similar line: only Desferral and InsP_6 in their experiments would stop this completely, whereas other chelators allowed some activity to proceed. The reasons for this are not clear, though Graf et al. (1984, 1987) suggested that the exclusion of water from the co-ordination complex or iron and an inhibition of Fe^{3+} reduction (which forms Fe^{2+}, an essential catalyst in the generation of OH• radicals by the mechanisms studied here; see the Materials and methods section) might both contribute to this phenomenon. The precise mechanisms are not our concern here. What is striking is that our results suggest that a unique conformation of the phosphate grouping in InsP_6 is probably responsible. The binding of iron to InsP_6 is likely to be very complex, with a number of different iron/InsP_6 stoichiometries possible, each possessing individual and possibly quite different stability constants [compare, for example, the large polynuclear complexes described containing Fe^{3+} and ATP (Mansour et al., 1985)], and thus detailed physical studies are likely to be required before this postulated interaction can be interpreted at the molecular level. It is interesting, however, that the distinctive behaviour of the InsP_5 isomers in the radical generation assay [i.e. the qualitative difference between D/L-Ins(1,2,4,5,6)P_5/Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,3,5,6)P_5/Ins-(1,2,3,4,6)P_5] is closely mirrored by their relative abilities to inhibit InsP_6 binding to cerebellar membranes (Poyner et al., 1993) and their grouping by chromatography in the presence of HCl on polyethyleneimine–cellulose (Figure 1) and Dowex-1 resin (Cosgrove, 1980), indicative perhaps of some common physical basis for these observations.

As discussed in the Introduction, our current knowledge of InsP_6 metabolism in animal cells implies that the 2-phosphate may be the last to be added, and the 3-phosphate the first to be removed; also, it is apparent that Ins(1,3,4,5,6)P_5 is probably the

only InsP_5 which is found at cellular concentrations close to (sometimes greater than) InsP_6 (Stephens et al., 1991; McConnell et al., 1991). So, one could argue that InsP_6 is unique among inositol phosphates in its high cellular levels, and its ability to bind iron entirely 'safely', i.e., in a way that completely prevents iron-catalysed formation of OH$^\bullet$ radicals. Since iron-catalysed OH$^\bullet$ radical formation is an event potentially lethal to living cells (Halliwell and Gutteridge, 1985), this property further serves to suggest that InsP_6 may be a physiologically relevant ligand for iron in cells (see the Introduction).

In conclusion, as Graf et al. (1987) have emphasized, the ability of InsP_6 to prevent OH$^\bullet$ radical formation entirely is unusual among chemical compounds, and on present knowledge it is unique amongst endogenous chemicals. Our observations here point to this property being due to the 1,2,3 (equatorial–axial–equatorial) phosphate grouping, a conformation probably only found in InsP_6 out of the inositol phosphates which exist in cells at high levels. We believe that it is therefore a reasonable suggestion that this may be one reason for InsP_6's universal occurrence in cells, and that it provides a possible physiological function of this molecule.

We are most grateful to Dr. Max Tate for generously providing *scyllo*-inositol hexakisphosphate. We would like to thank Frank Cooke and Kit Erleback for some technical assistance. We also thank Dr. Tate, Dr. Len Stephens, Dr. Ed Constable, Dr. Anne Theibert and Professor Barry Halliwell for helpful discussions. P.T.H. is a Lister Fellow, T.R.J. is a Royal Society Jaffé Fellow. Some of this work was performed while D.P. was a Medical Research Council Fellow, funded by CellTech.

REFERENCES

Bakkeren, D. L., Jeu-Jaspars, C. M. H., Van der Heul, C. and Van Eijk, H. G. (1985) Int. J. Biochem. **17**, 925–930
Bartlett, G. R. (1982) Anal. Biochem. **124**, 425–431
Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. **245**, 4641–4646
Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–205
Biederman, G. and Schindler, P. (1957) Acta Chem. Scand. **11**, 731–740
Britton, R. S., Ferrali, M., Magiera, C. J., Recknagel, R. O. and Bacon, B. R. (1990) Hepatology **11**, 1038–1043
Carpenter, D., Hanley, M. R., Hawkins, P. T., Jackson, T. R., Stephens, L. R. and Valleo, M. (1989) Biochem. Soc. Trans. **17**, 3–5
Cosgrove, D. J. (1980) Inositol Phosphates, Elsevier, Amsterdam
Cowart, R. E., Swope, S., Loh, T. T., Chasteen, N. D. and Bates, G. W. (1986) J. Biol. Chem. **261**, 4607–4614
Crichton, R. R. (1979) CIBA Found. Symp. **65**, 57–75
Deighton, N. and Hider, R. C. (1989) Biochem. Soc. Trans. **17**, 490
Emsley, J. and Niazi, S. (1981) Phosphorus Sulfur Relat. Elem. **10**, 401–407
Fontecave, M. and Pierre, J. L. (1991) Biol. Met. **4**, 133–135
Graf, E. and Eaton, J. W. (1990) Free Radical Biol. Med. **8**, 61–69
Graf, E., Mahoney, J. R., Bryant, R. G. and Eaton, J. W. (1984) J. Biol. Chem. **259**, 3620–3624
Graf, E., Empson, K. C. and Eaton, J. W. (1987) J. Biol. Chem. **262**, 11647–11650
Grohlich, D., Morley, G. D. and Bezkorovainy, A. (1979) Int. J. Biochem. **10**, 803–806
Halliwell, B. and Gutteridge, J. M. C. (1985) Free Radicals in Biology and Medicine, Oxford University Press
Jacobs, A. (1977) CIBA Found. Symp. **51**, 91–106
Johnson, L. F. and Tate, M. E. (1969) Can. J. Chem. **47**, 63–73
Mansour, A. N., Thompson, C., Theil, E. C., Chasteen, N. D. and Sayers, D. E. (1985) J. Biol. Chem. **260**, 7975–7979
Martell, A. E. and Smith, R. M. (1982) (eds.) Critical Stability Constructs, vol. 5, p. 340, Plenum Press, New York
Martin, J., Foray, M., Keen, G. and Satre, M. (1987) Biochim. Biophys. Acta **931**, 16–25
McConnell, F. M., Stephens, L. R. and Shears, S. B. (1991) Biochem. J. **280**, 323–329
Morgan, E. H. (1977) Biochim. Biophys. Acta **499**, 169–177
Morley, C. G. D. and Bezkorovainy, A. (1983) IRCS Med. Sci. **11**, 1106–1107
Nash, T. (1953) Biochem. J. **55**, 416–421
Nielson, P., Düllermann, J., Wulfhekel, U. and Heinrich, H. C. (1993) Int. J. Biochem. **25**, 223–232
Nogimori, K., Hughes, P. J., Glennum, M. C., Hodgson, M. E., Putney, J. W., Jr. and Shears, S. B. (1991) J. Biol. Chem. **266**, 16499–16506
Pollack, S., Campana, T. and Weaver, J. (1985) Am. J. Hematol. **19**, 75–84
Poyner, D. R., Cooke, F., Hanley, M. R., Reynolds, J. M. and Hawkins, P. T. (1993) J. Biol. Chem. **268**, 1032–1038
Shears, S. B. (1989) Biochem. J. **260**, 313–324
Spencer, C. E. L., Stephens, L. R. and Irvine, R. F. (1990) in Methods in Inositide Research (Irvine, R. F., ed.), pp. 39–43, Raven Press, New York
Stephens, L. R. (1990) in Methods in Inositide Research (Irvine, R. F., ed.), pp. 7–30, Raven Press, New York
Stephens, L. R. and Downes, C. P. (1990) Biochem. J. **265**, 435–452
Stephens, L. R. and Irvine, R. F. (1990) Nature (London) **346**, 580–583
Stephens, L. R., Hawkins, P. T., Stanely, A. F., Moore, T., Poyner, D. R., Morris, P. J., Hanley, M. R., Kay, R. R. and Irvine, R. F. (1991) Biochem. J. **275**, 485–499
Weaver, J. and Pollack, S. (1989) Biochem. J. **261**, 787–792

Received 15 February 1993/7 May 1993; accepted 21 May 1993