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Effect of Metals on Mutagenesis and DNA Repair

by Toby G. Rossman*

Unlike the situation with organic compounds, metals do not show a high correlation between carcinogenicity and mutagenicity. An agent may be mutagenic by causing misreplication of DNA due to alterations of the DNA template, decreased fidelity of DNA polymerase, or inhibition of the proofreading of DNA replication. In addition, bacteria have an inducible, error-prone DNA repair system (SOS repair) whose activity results in mutagenesis. In the best studied example of metal mutagenesis, chromate, there is little evidence for the involvement of the SOS system. Metals may act as comutagens by inhibiting the repair of damage to DNA caused by another agent. This has been demonstrated for arsenite. Comutagens would not be detected by standard screening methods.

Introduction

Most organic carcinogens or their metabolites have been shown to bind to DNA (*1*). In many cases, specific adducts have been identified. Although DNA repair mechanisms can remove much of this damage, some adducts have been shown to persist for many generations (*2*). That persistent damage to DNA leads to carcinogenesis is suggested by the human genetic disease Xeroderma pigmentosum. Patients with this disease have defects in the repair of UV-induced pyrimidine dimers, and multiple skin cancers arise on parts of the body exposed to sunlight. A recent review of this disease has been published by Setlow (*3*).

The realization that many carcinogens derive their activities from their abilities to react with DNA has led to the development of a number of short-term tests based on mutagenicity. Because of their simplicity, sensitivity, economy, and short time scale, bacterial systems have been useful for studies on the mutagenicity of carcinogens. With some exceptions, agents which are mutagenic to bacteria are also mutagenic to animal cells. Metal mutagenicity studies have been carried out almost exclusively in bacterial systems, although some studies on the effects of metals on animal cell chromosomes have been carried out. Chromosomal abnormalities are discussed in a separate paper on that subject (*4*), and only gene mutations will be discussed in this paper.

Metal mutagenesis has been reviewed by Flessel (*5*). In 1951, manganese was shown to be a bacterial mutagen (*6*). Since that time chromate (Cr VI) has been established as a mutagen in a variety of bacterial systems. Other metal compounds reported to mutagenize *S. typhimurium* include ferrous sulfate, *cis*-diamminoplatinum tetrachloride, and selenate but not selenite. Negative results were reported for arsenite and arsenate (*5*). In *E. coli*, molybdenate and arsenite have been reported as mutagens (*7*). However, attempts by this author to demonstrate mutagenesis by arsenite, using a variety of protocols, yielded only negative results (*8*). Negative results in the *E. coli* system were reported for compounds of tungsten, molybdenum, zinc, cadmium and mercury (*5*).

In general, the strains of bacteria used for mutagenesis testing of metals have given either inconsistent results, or results which do not correlate well with the carcinogenicity of the metals. It has been known for some time that the Ames test does not predict well for metals suspected or known

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to be carcinogenic (*9*). The Ames tester strains have been developed for increased sensitivity toward mutagens which form bulky lesions on DNA and work via an error-prone DNA repair pathway (*10*). As will become clear in this review, a number of other mechanisms by which metals could be mutagenic exist. In addition, some metals might act as comutagens rather than as primary mutagens, and a different test procedure is needed to demonstrate comutagenesis.

Mechanisms of Mutagenesis

Drake and Baltz (*11*), in a review of biochemical mechanisms of mutagenesis, have divided mutagenic mechanisms into two major classes. Class I involves directly induced base mispairing, whereas Class II applies to agents which interrupt normal DNA replication by preventing base-pairing of any kind at the damaged site, and mutations result as errors in DNA repair. Since there are many mechanisms by which direct base mispairing can occur, and since error-prone DNA repair has not been proven to occur in animal cells, I shall use a different method of classification based on possible sites at which metals might act in causing mutations.

Misreplication Due to Altered DNA

Some of the earliest examples of analysis of mutagenic mechanisms were performed on agents which chemically alter the DNA (*11*). For example, nitrous acid, which deaminates cytosine to uracil and adenine to hypoxanthine, generates point mutations. More recently, some alkylating agents,such as ethyl methane sulfonate, have been shown to cause direct base mispairing due to the formation of alkylation products on the purine and pyrimidine oxygens (*12*). The mutagenicity of bisulfite (SO_2 in solution) is due to the deamination of cytosine to uracil (*13*).

In order for metals to cause mutations by this mechanism, the metal must either bind to DNA in such a way as to cause base mispairing during DNA replication, or it must cause a chemical alteration of the DNA by another mechanism. The binding of metals to nucleic acids has been reviewed by Sundaralingam (*14*) and Eichhorn (*15*). In general, metal complexes affect neither the nucleotide geometry nor their conformations. Exceptions are the alterations in bondlengths and angles in cadmium-GMP complex and in a few other cases where N(7) is the sole site of a transition metal binding. Also, some platinum complexes contain the rare trans C(4')-C(5') conformation in the sugar.

Metals can bind to bases, phosphate groups, or sugars in nucleotides. Phosphate is the strongest coordinating group for most metals. In general, the stability of metal compounds to nucleosides reflects the stability of binding to phosphate (*15*). Lesions on the phosphate groups or sugars of DNA are assumed to be of little biological consequence unless gross distortions of the DNA helix result. Metals which bind strongly to phosphates tend to stabilize the DNA helix (increase in T_m). The order of binding to phosphates in preference to bases is Mg (II) > Co (II) > Ni (II) > Mn (II) > Zn (II) > Cd (II) > Cu (II).

The purine bases exhibit higher reactivity towards metal ions than do the pyrimidines. The ring nitrogen of purines is favored over the amino nitrogens or keto oxygen. The N(7) position of purines is the favored binding site for Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}, while Cu^{2+} and Cd^{2+} tend to bind to N(3) of cytosine and N(1) of adenine. It should be noted that the N(7) position of purines is not involved in base pairing and its alteration would not be expected to have mutagenic consequences. No information was available on the binding sites of compounds of arsenic, chromium, selenium or beryllium to nucleotides or polynucleotides.

Metal compounds might also form cross-links in DNA. The preferred binding of Hg^{2+} to alternating poly d(A-T) involves cross linking to two thymine residues. A model for the binding of *cis*-diammino platinum suggests binding to the N(7) atoms of two adjacent purines on the same strand.

A great deal of work needs to be done in the area of metal DNA complexes, with particular attention to carcinogenic metals. The biological consequences of metal binding to DNA needs to be examined as well. Many carcinogenic metals have been shown to cause infidelity in DNA replication (*16*). The mechanism of this effect could be via metal interactions with DNA polymerase, or via metal interactions with DNA itself. If the binding of the metal to DNA or a synthetic polynucliotide is tight enough, these mechanisms could be distinguished by binding of the metal to the template and washing away of free metal prior to the misincorporation assay.

Indirect evidence for damage to DNA can be obtained by comparing the toxicities of metal compounds in strains of bacteria which are proficient in DNA repair and in strains defective in some DNA repair pathway. This is the basis for the Pol test and the rec assay (*17*, *18*). A number of metal compounds have been tested in the latter, which compares toxicities in rec^+ and rec^- strains of *B. subtilis*. Positive results (greater toxicity in rec^-) were reported for: $AsCl_3$, $NaAsO_2$, Na_3AsO_4, K_2CrO_4, $K_2Cr_2O_7$, CH_3HgCl, $CH_3COOHgC_6H_5$, $MnCl_2$,

$Mn(NH_3)_2$, $MnSO_4$, $Mn(CH_3COO)_2$ and $(NH_4)_6Mo_7O_{24}$. More work should be done to correlate biochemical studies on alterations to DNA by metal compounds with studies on enhanced toxicity of metals to DNA repair-deficient bacteria.

In animal cells, indirect evidence for damage to DNA by metal compounds might be obtained by studying the recovery of DNA synthesis after removal of an inhibitory dose of the metal. As pointed out by Painter (*19*), DNA-damaging agents can be distinguished from other agents which inhibit DNA replication by the continued inhibition of DNA replication after the removal of the DNA damaging agent. In contrast, agents which inhibit DNA replication by another mechanism usually show an immediate recovery of DNA replication upon their removal. In this system, 5*mM* $NiCl_2$ was inhibitory to DNA replication, but since recovery upon its removal was rapid, it did not behave like a DNA damaging agent. It would be of interest to see how other metal compounds behave in this system.

To summarize, a number of metal compounds have been shown to bind to purines and pyrimidines, and a number of compounds have been shown to have greater toxicity in DNA repair-deficient mutants of bacteria. Thus far, there is no clear correlation between binding to DNA and mutagenicity. No particular DNA-metal complex has been demonstrated as a premutational lesion, and there is no information as to other types of DNA damage, such as deamination, which might be caused by metals.

Misreplication Due to Decreased Fidelity of DNA Polymerase

Errors in replication may be caused by agents which decrease the fidelity of DNA replication by affecting the DNA polymerase directly, rather than by damaging the DNA template. The effects of metals on the fidelity of DNA replication will be covered in the paper on infidelity of DNA synthesis.

Inhibition of Proofreading

Prokaryotic DNA polymerases contain a 3′-5′ exonuclease function which acts to excise newly incorporated (3′-terminal) mis-matched nucleotides. This is known as the proofreading function of the polymerase. A model for mutagenesis via alterations in proofreading comes from studies on phage T_4 mutators (mutants which exhibit a higher than normal frequency of spontaneous mutations). Mutators of T_4 sometimes have a DNA polymerase with low 3′-5′ exonuclease to polymerase ratios, which results in leaving too many mismatched bases in DNA (*11*). There is evidence that the carcinogenic metal beryllium can specifically inhibit the 3′-5′ exonuclease function (*20*). The carcinogen azathioprine has also been reported to act in this manner (*21*).

However, in one study of the effects of metals on *E. coli* DNA polymerase I, the 3′-5′ exonuclease function of the polymerase was not inhibited by metal salts at concentrations which caused a loss of fidelity of the polymerase (*22*).

Eukaryotic DNA polymerases do not contain a 3′-5′ exonuclease function. It is possible that proofreading is carried out by a separate enzyme. Evidence for such an activity has been reported (*23*). This question must be resolved before the effects of metal compounds on proofreading can be determined in eukaryotic cells.

Mutagenesis via Error-Prone DNA Repair

In bacteria, some agents have been shown to cause mutations only when an error-prone DNA repair system (SOS system) is induced. Agents which are mutagenic by this mechanism are those which cause lesions on DNA which interrupt normal DNA replication by preventing base-pairing of any kind at the damaged site. Strictly speaking, this system is not really a repair system since lesions on DNA are not removed.

The best studied example of an agent which is mutagenic via the SOS system is ultraviolet light (*24*). Mutagenesis after UV-irradiation in *E. coli* requires the $recA^+$ and $lexA^+$ gene products and protein synthesis. It has been suggested that one of the induced proteins might alter DNA polymerase activity, allowing DNA replication past a lesion which previously had constituted a block to replication (e.g. a pyrimidine dimer). Since lesions of this sort are noncoding, nucleotides inserted opposite them must be random, and therefore a high probability for mutagenesis exists (*25*).

In bacteria with an SOS system, an agent which inhibits either the induction or action of this system will behave as an antimutagen. A few years ago, my coworkers and I reported such an effect for arsenite (*26, 27*). If *E. coli* is exposed to 1*mM* sodium arsenite after UV irradiation, both survival and mutagenesis are decreased. The most likely explanation for this effect is the inhibition of induction of the SOS system.

If an agent causes mutations solely via the SOS system, the agent will be unable to mutate strains of bacteria which have genetic defects in the SOS

system (i.e., *recA*⁻ or *lexA*⁻). Based on studies in strains of *E. coli,* such a mechanism of action has been proposed for $NaAsO_2$, $K_2Cr_2O_7$ and $(NH_4)_6Mo_7O_{24}$ (*7*). However, others were unable to demonstrate mutagenesis by arsenite in *E. coli* (*8*) or in Salmonella (*28*) or by salts of molybdenum in *E. coli* (*29*). In the case of chromate, Venit and Levy found mutagenesis in a *lexA*⁻ (*exrA*⁻) strain (*29*). Thus, chromate mutagenicity probably does not occur via the SOS system in bacteria.

The existence of an SOS-like system in eukaryotic cells is controversial. The best evidence comes from studies on the enhanced survival of irradiated viruses when grown on cells which have previously been UV- or x-irradiated (*30*), or treated with low doses of carcinogens (*31*). There is also evidence that this repair is error-prone (*32*). However, another interpretation of this phenomenon has been presented (*33*). It has been suggested that the mechanisms by which mutations occur in eukaryotic cells are constitutive, in contrast to the inducible systems in prokaryotic cells (*34, 35*). Thus, it is premature to speculate about the effects of metals on a system which may not exist in eukaryotic cells.

Effects on DNA Repair Leading to Comutagenesis

The function of DNA repair systems is to restore the informational content of DNA which has been damaged. With the exception of the SOS system described above, DNA repair processes generally suppress mutagenesis, i.e., most DNA repair is relatively error-free. The high incidence of skin cancers in patients with xeroderma pigmentosum suggests that unrepaired damage to DNA can also lead to carcinogenesis. Thus, any agent which interferes with (error-free) DNA repair is likely to act as a comutagen and a cocarcinogen.

Most of the discussion which follows will concern excision repair pathways. In bacteria, a number of post-replication repair (or recovery) systems exist whose function is to fill in daughter strand gaps opposite lesions, which are thought to arise due to blockage of replication at the lesion and resumption further on. These gaps may be filled in by a recombinational process or via the SOS system described above (*11, 24, 25, 33, 36, 37*). In eukaryotic cells (as in prokaryotes), DNA made immediately after UV irradiation has a smaller molecular weight than normal. With time, the molecular weight enlarges until a normal size is seen, suggesting the existence of daughter strand gaps and subsequent filling of the gaps. There is no evidence for recombinational repair in eukaryotic cells. A model for post-replication repair in eukaryotic cells, in which gaps are filled by *de novo* DNA synthesis, has been presented. More recently, a number of other models and re-interpretations of data have been suggested (*33, 36-39*). Because of the confusion in the field of eukaryotic post-replication repair, in which its very existence is in doubt (*33*), it would be premature to discuss the effects of metal compounds on this system.

Excision Repair

Excision repair of damaged DNA involves removal of a piece of DNA containing the damage and resynthesis (repair replication), using the complementary strand as template. The two major pathways of excision repair (Fig. 1) differ in the initial steps prior to repair replication. UV-induced pyrimidine dimers and large carcinogen-DNA adducts are repaired by a pathway known as nucleotide excision repair, in which the first step is incision of the damaged DNA by an endonuclease which recognizes the damage and cleaves the phosphodiester bond near the damage. The other major pathway is base excision repair. Here, such damage as uracil in DNA (which can result from deamination of cytosine), hydrated and ring-saturated bases, and small adducts are recognized by specific *N*-glycosylases, which cleave the *N*-glycosyl bond between the base and the sugar, leaving an apurinic or apyrimidinic (AP) site. An endonuclease which recognizes AP sites then performs an endonucleolytic cleavage. In both major pathways, polymerase, exonuclease and ligase action forms a patch of new DNA (repair patch). In animal cells, nucleotide excision repair is thought to result in larger patches than does base excision repair, suggesting that perhaps the exonuclease steps of these two repair systems are not identical. A more detailed discussion of excision repair is given elsewhere (*33, 36, 37*).

Evidence suggests that excision repair in prokaryotes and eukaryotes is an error-free process. Since the template strand is undamaged, repair replication should be as faithful as DNA replication itself. Mutants of bacteria which are defective in excision repair tend to be more readily killed and mutated by agents whose damage is not repaired (*24, 36*). Damaged DNA is more likely to have lethal and mutagenic consequences if the damage persists to replication than if it undergoes excision repair. The demonstration that the mutation frequency in UV-irradiated human fibroblasts is decreased when cells are kept in a confluent state after irradiation (where excision repair can occur but DNA replication cannot) is taken as evidence that excision repair in human cells is also an

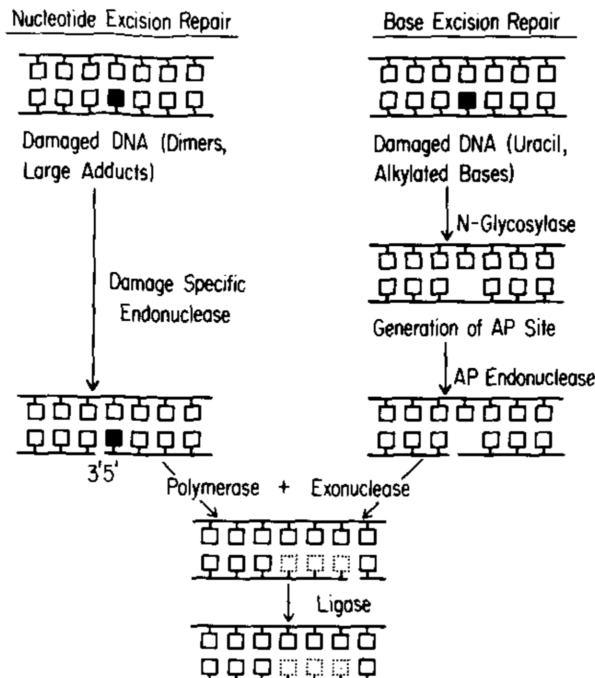

FIGURE 1. Pathways for excision repair: (□) normal bases; (■) damaged bases; (⬚) repaired bases.

error-free process (*40*).

The effects of metal compounds on excision repair systems can be assayed by growing cells in the presence and absence of a nontoxic concentration of the metal compound after exposure of the cells to a DNA-damaging agent, and scoring for survival and mutagenesis. Since there are a variety of enzymes which recognize damage to DNA, agents which cause different types of damage should be tested. If the metal compound inhibits excision repair, an enhancement of the mutation frequency (comutagenesis) and decrease in survival should be seen. In bacterial systems, strains deficient in excision repair will not show this effect. Biochemical assays can be used to pinpoint the steps in repair which are affected. If the repair enzymes have been identified, direct assays of the effects on these enzymes by metal compounds can be carried out.

My laboratory has recently found that low concentrations of arsenite (but not arsenate) act as a comutagen with ultraviolet light in *E. coli* (*41*). When a *uvrA* mutant (which cannot excise pyrimidine dimers) is used, no comutagenesis is seen. This suggests that arsenite can inhibit excision repair in *E. coli*. Studies on other metal compounds are being planned.

Mismatch Repair

Mismatch repair is a form of excision repair which operates preferentially on the newly synthesized daughter strand of DNA to remove replication errors. It differs from the previously described proofreading, which corrects replication errors only at the 3′ terminus. Mismatch repair can be assayed in *E. coli* by the ability to convert λ heteroduplex DNA to the homoduplex (*42*). Genetic evidence suggests that the parental and daughter strand DNA can be distinguished by the presence of methyl groups on parental DNA and their absence in the newly synthesized daughter strands (*43*).

In mammalian cells, newly synthesized DNA is also undermethylated (*44*). Since mammalian cells have been shown to convert SV_{40} heteroduplex DNA (*45*), mutation suppression by a methylation-instructed mismatch repair system is likely to exist in eukaryotic cells.

Agents which inhibit mismatch repair might act as mutagens by preventing correction of spontaneous replication errors, or as comutagens by preventing correction of mismatches due to DNA which has been altered by a mutagen. It would be of interest to determine the effects of metals on this system, perhaps by assaying the conversion of heteroduplex DNA. The enzymology of mismatch repair is not well understood (*45*). If a mammalian nuclease which specifically recognizes mismatches in DNA were identified, the effects of metals on this enzyme might be of interest.

Other Pathways of Damage Correction

Recently, two alternative modes by which damaged DNA can be repaired have come to light. Neither of these modes involve cleavage of the phosphodiester bond and the subsequent repair replication characteristic of excision repair. Both involve only correction of the damaged base, and no studies on the effects of metals on these systems have been carried out.

When DNA is damaged by carcinogenic alkylating agents, a number of alkylation products are formed. One which is now thought to be of critical importance in carcinogenesis is O^6-alkylguanine. In animal tissues, there is evidence that removal of O^6-methylguanine from DNA can occur (*46*). This activity may be induced by prolonged exposure to alkylating agents. If there are enzymes which can remove a methyl group from O^6-methylguanine, it is possible that other enzymes might exist which could remove other types of damage directly, without requiring the excision repair pathways.

A second recent finding involves an enzyme (sometimes called "insertase") which is able to insert a purine into apurinic sites in DNA (*47*). Apurinic sites can arise spontaneously, can be generated by chemical action, or can result from the action of an *N*-glycosylase. Base excision repair may be avoided by the reinsertion of a base at the AP site. The purine which is inserted also appears to be the correct one. However, the authors speculate that direct purine insertion might be more error-prone than an excision repair pathway (*47*). So far, there have been no reports of a pyrimidine insertase.

Why Carcinogenic Metals Are Not Mutagenic in Microbial Systems

Unlike organic carcinogens, carcinogenic metals cannot be predicted with high accuracy in bacterial mutagenesis tests. Of the metals suspected or known to be carcinogenic, only chromate has given consistently positive results. Even in this case, chromate is a very weak mutagen which can best be detected in a fluctuation test rather than in standard agar plate assays (*48*). Reasons for the failure of bacterial mutagenesis tests to detect the mutagenicity of carcinogenic metals may be due to technical problems, such as precipitation of the metal in the medium commonly used. There are a number of other possibilities concerning the mutagenicity of carcinogenic metal compounds.

(1) As pointed out by Rosenkranz et al. (*17*), strongly bacteriocidal agents can obscure mutagenicity if the results are expressed as mutants/plate, without taking into account the survival level.

(2) Bacteria and mammalian cells may differ in their mutagenic response to metals. So far, few metal compounds have been tested in mammalian systems for mutagenicity, a subject which should have high priority.

(3) Carcinogenic metals may be comutagens rather than mutagens. Comutagenesis might occur by inhibition of (error-free) DNA repair pathways or by the formation of additional lesions on DNA by the combined action of metal plus mutagen.

(4) Bacterial strains in current use may be genetically incapable of giving a positive mutagenic response to metals. If, as suggested by Sirover and Loeb (*16*), carcinogenic metals cause infidelity in DNA synthesis, bacteria might be able to correct the errors by mismatch repair. Strains of bacteria lacking mismatch repair might be more suitable for studies on metal mutagenesis.

(5) Finally, it is altogether possible that there is no correlation between carcinogenicity and mutagenicity (or comutagenicity) of metals.

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Metal-Induced Infidelity of DNA Synthesis

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In this paper, we consider the effects of metal ions on the accuracy of catalysis by DNA polymerases. Certain activating and nonactivating metal ions have been shown to diminish the fidelity of DNA synthesis *in vitro* with a variety of DNA polymerases. There is a significant correlation between the metals that decrease fidelity and those that have been reported to be mutagenic and carcinogenic. Thus, metal carcinogens are no exception to the general postulate that carcinogens can be identified by their interactions with DNA.

The Present State of Knowledge

A number of metals have been shown to be mutagens and carcinogens (*1*) and to affect the accuracy of DNA replication (*2*). *In vivo* systems are too complicated to begin to unravel the mechanisms by which metals induce mutations and the effects of metal ions on the fidelity of DNA replication. Our approach to this problem has been to examine DNA synthesis *in vitro*, to determine the effects of different metal ions on the fidelity of this process, and then to ask whether alterations in the fidelity of DNA synthesis are related to the mutagenic and carcinogenic properties of these metals. Prior to considering these studies in detail, it is instructive to consider the mechanism of DNA synthesis *in vitro* and methods for measuring fidelity of DNA synthesis.

Mechanism of DNA Polymerization

The requirements for catalysis by various DNA polymerases appear to be similar. Synthesis proceeds by a sequential addition of nucleotide monomers (deoxynucleoside monophosphates) with the concomitant release of pyrophosphates (*3*). DNA polymerases are part of a unique class of enzymes in that they primarily take direction from another molecule, a template. In cells, the template is DNA. Synthetic polydeoxynucleotides and polyribonucleotides can also serve as templates for most DNA polymerases *in vitro*. Synthesis is started on the 3′-hydroxy terminus of a primer-strand hybridized onto a template strand. The primer can be an oligonucleotide, one strand of double-stranded DNA, a hairpin loop of single-stranded DNA, or a fragment of RNA hybridized onto DNA. Thus, DNA polymerases only elongate already existing polynucleotide chains; they fail to initiate chains *de novo* as do RNA polymerases. The substrates of all known DNA polymerases are deoxynucleoside triphosphates that are complementary to the template. Based on the similar requirements for activity and a spectrum of similar kinetic parameters, it is a reasonable expectation that there is a common mechanism for catalysis by DNA polymerases from different sources (*4*).

DNA polymerases can be classified as zinc metalloenzymes (*5*) in that they contain tightly bound zinc, which is required for activity (*6*). In addition, these enzymes require an added metal for catalysis, which in cells is presumably Mg^{2+}. *In vitro*, Mg^{2+} has been shown to coordinate the enzyme with the substrate in the form of an enzyme-metal-substrate complex. Mn^{2+}, Co^{2+}, Ni^{2+}, and in certain cases, Zn^{2+}, have been shown to substitute for Mg^{2+} as activators (*7*). Analysis of *E. coli* DNA polymerase I-Mn^{2+}-substrate complexes indicates that in the absence of template, the

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enzyme alters the conformation of the deoxynucleoside triphosphate substrate to that which it would occupy in double helical DNA (*8*). The function of the metal activator in the DNA polymerase reaction *per se* has recently been extensively reviewed (*9, 10*).

Fidelity of DNA Synthesis

On the basis of spontaneous mutation rates in procaryotic and eucaryotic cells, stable misincorporation of a base during DNA replication is estimated to occur with a frequency of 10^{-8} to 10^{-11} per base-pair synthesized (*11*). This accuracy appears to be achieved by a multistep process (*12*). The differences between correct and incorrect Watson-Crick base-pairings involve only one or two hydrogen bonds. This difference in free energy, ΔG has been estimated to account for an error rate of approximately 10^{-2} (*13*). DNA polymerases also participate in base-selection, reducing the error rate to values approaching 10^{-5} (*4*). Presumably, other cellular mechanisms increase accuracy during or immediately after DNA replication. At present, studies on the effects of metals on fidelity *in vitro* are limited to analysis with purified DNA polymerases.

Assays of Fidelity with Polynucleotide Templates

Until very recently, all assays of the fidelity of DNA synthesis *in vitro* measured the ability of DNA polymerases to copy homopolymer or alternating copolymer templates. These templates contained only one or two nucleotides and the mismatched nucleotide was identified simply as one not complementary to the template nucleotides. Using this assay, one can observe the effects of both activating and non-activating metals on the fidelity of DNA synthesis. The template that we have chosen for critical measurements of fidelity is poly[d(A-T)], a synthetic polynucleotide consisting of deoxythymidine and deoxyadenosine monophosphates. Poly[d(A-T)] can be synthesized to contain less than 1 in 2×10^6 mistakes by using a *de novo* reaction with *E. coli* DNA polymerase I (*14*). Copied correctly, only dAMP and dTMP should be incorporated into the newly synthesized product. By using [α-^{32}P]-dTTP, unlabeled dATP, and [^{3}H]-dGTP or [^{3}H]-dCTP, one can simultaneously measure the incorporation of complementary and non-complementary nucleotides (*15*). The incorporation of either dCTP or dGTP would represent errors. The frequency of misincorporation is obtained from the ratio of [^{3}H] to [^{32}P] in the acid-insoluble product. Control experiments are required to show that the [^{3}H] label in the reaction product is in the noncomplementary nucleotides and not in any radioactive contaminants. Also, it must be demonstrated that the noncomplementary nucleotides are covalently incorporated in phosphodiester linkage. Using nearest-neighbor analysis, one can determine the distribution of the noncomplementary nucleotides. Measurements of the frequency of misincorporation by DNA when copying polynucleotide templates have been summarized in a recent review (*12*). In general, they vary from 10^{-3} for DNA polymerases from RNA tumor viruses to 10^{-5} for procaryotic DNA polymerases. It should be noted that the error rates of the procaryotic DNA polymerases, those with a $3' \rightarrow 5'$ exonuclease, are similar to those of the eucaryotic DNA polymerases, enzymes that do not have an accompanying exonuclease (*16*). Thus, the exonuclease in procaryotic DNA polymerases is not necessarily the major determinant of fidelity.

Metals Activators and Fidelity

The activating metal for DNA polymerase *in vivo* is Mg^{2+}. *In vitro*, DNA polymerases from animal (*16*), viral (*17*), and bacterial sources can also use Mn^{2+}, Co^{2+}, or Ni^{2+} as the activating metal. With AMV DNA polymerase and an activated DNA template, the maximal rates of nucleotide incorporation with Mn^{2+}, Co^{2+}, and Ni^{2+} were 65%, 25%, and 7%, respectively, of that achieved with Mg^{2+} (*18*). Minimal activity has also been reported with Zn^{2+}. The effects of Mg^{2+} and Mn^{2+} concentrations on the incorporation of complementary and noncomplementary nucleotides with poly [d(A-T)] as a template are illustrated in Fig. 1. At activating concentrations of Mg^{2+} (*2mM*), human placenta DNA polymerase-β incorporates one molecule of dGTP for every 40,000 molecules of dTTP and dATP polymerized (*17*). This error rate is invariant with respect to Mg^{2+} concentration. At the optimal activating concentration of Mn^{2+} (*0.1mM*), the error rate was 1 in 15,000. At greater than activating concentrations of Mn^{2+}, there was a progressive decrease in the incorporation of the complementary nucleotide but not of the noncomplementary nucleotide, thus yielding a further increase in the frequency of misincorporation. At concentrations as great as *2mM*, the error rate approached 1 in 3600, and nearest-neighbor analysis indicated that each misincorporation occurred as a single-base substitution. A similar enhancement in the error rate is observed with both activating and inhibiting concentrations of Co^{2+}. The decreased fidelity with increased Mn^{2+} concentration has been observed

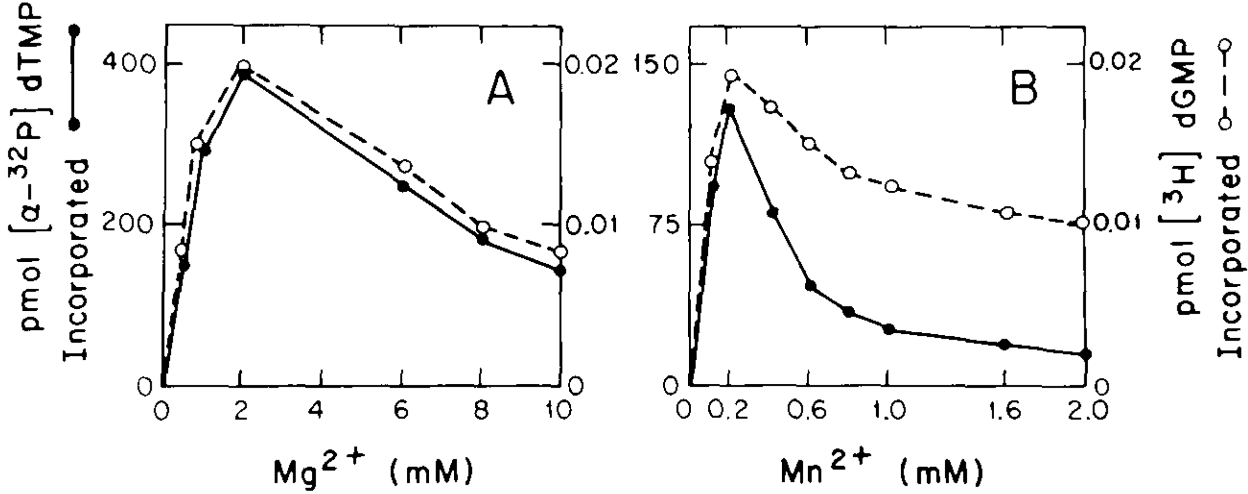


FIGURE 1. Effect of metal activators on the fidelity of human placenta DNA polymerase-β with poly [d(A-T)] as a template (*17*).

with all templates and noncomplementary nucleotides tested. An absolute increase in the rate of incorporation of the noncomplementary nucleotide at high Mn^{2+} concentration can be demonstrated by simply using more DNA polymerase in the assay or prolonging the time of incubation.

The enhancement of misincorporation with an alternate metal activator appears to be a characteristic finding with DNA polymerases (Table 1). Substitution of Mn^{2+} for Mg^{2+} results in an increase in misincorporation by *E. coli* DNA polymerase I (*7*), T_4 DNA polymerase (*19*), DNA polymerase-α (*17, 20*) DNA polymerase-β (*17*), and avian myeloblastosis virus (AMV) DNA polymerase (*15, 21*). The fact that Mn^{2+} and Co^{2+} alter the fidelity of the DNA polymerases that do not have an associated exonuclease (AMV, DNA polymerases-α and -β) indicates that for these DNA polymerases the metal ions do not promote misincorporation by inhibiting an error correcting exonucleolytic activity. Ni^{2+} can also substitute for Mg^{2+} as a metal activator. However, the amount of synthesis achieved with Ni^{2+} as the metal activator has not been sufficient to accurately measure the changes in the fidelity of DNA synthesis with any DNA polymerase except AMV DNA polymerase, in which case Ni^{2+} promotes misincorporation (*18*).

In order to relate the measurements with alternate metal activators to a situation that would be expected to occur in cells, the effects of these activators on Mg^{2+}-activated DNA synthesis have been investigated. Co^{2+}, Mn^{2+}, and Ni^{2+} have been shown to enhance misincorporation by DNA polymerases in the presence of activating amounts of Mg^{2+} (*17, 21, 22*). Thus, these metal activators could alter the fidelity of DNA polymerases in cells.

Nonactivating Metal Ions and Fidelity

Beryllium, a known carcinogen, has been shown to decrease the fidelity of catalysis with *M. luteus* DNA polymerase (*23*) and AMV DNA polymerase (*24*). Be^{2+} is unable to substitute for Mg^{2+} as a metal activator. However, as a nonactivating cation,

Table 1. Error rates with different metal activators.[a]

DNA polymerase	Mg^{2+} (5*mM*)	Mn^{2+} (0.1 *mM*)	Mn^{2+} (2 *mM*)	Co^{2+} (0.4 *mM*)	Co^{2+} (5 *mM*)
AMV	1/1,680	1/760	1/500	1/1,100	1/200
E. coli I	1/20,000	1/10,000	1/1,000	1/7,500	1/7,000
Human placenta-α	1/6,000	1/1,900	1/300	1/1,300	1/450
Human placenta-β	1/20,000	1/9,000	1/2,000	1/5,000	1/1,300

[a]All assays were carried out with poly [d(A-T)] as a template and dATP, [α^{32}P]dTTP and [^{3}H]dGTP at 25 μM (*12*).

Be^{2+} alters the fidelity of DNA synthesis in the presence of Mg^{2+}. Preincubation of the enzyme, but not the template, primer or substrates with high concentrations of Be^{2+} resulted in an increased error rate (*24*). This finding suggests that Be^{2+} can interact with some noncatalytic site on DNA polymerase and thereby alter the fidelity of DNA synthesis. Be^{2+} has also been shown to alter the fidelity of DNA polymerase-α from human fibroblasts (*25*), DNA polymerases-α and -β from human placenta and *E. coli* DNA polymerase I (*17*). Since the eucaryotic and viral DNA polymerases lack an exonuclease, these results mitigate against the hypothesis of Luke et al. (*23*) that Be^{2+} interacts with the exonucleolytic site on procaryotic DNA polymerases.

Screening for Metals that Alter Fidelity

To date, over 40 metal compounds have been tested in graded concentrations for their effects on the fidelity of DNA synthesis. The method of analysis and the results are summarized in Figure 2. In the initial study, 22 of these metal salts were tested by using a triple-blind protocol in which the assays, computations, and designation of each unknown compound with respect to fidelity were carried out independently (*2*). Compounds which increased infidelity by greater than 30% at two or more concentrations were scored as positive. Metals were designated as carcinogens or mutagens by an evaluation of the literature prior to assessment of their effects on fidelity. An enhancement in the infidelity of DNA synthesis was observed with all of the known mutagens and/or carcinogens tested at that time (Ag, Be, Cd, Co, Cr, Mn, Ni, Pb). The evidence in the literature on the mutagenicity or carcinogenicity of three of the metal ions was considered equivocal. Of these, Cu^{2+} increased misincorporation; Fe^{2+} and Zn^{2+} did not alter fidelity. All other metal salts that were tested were considered to be neither carcinogenic nor mutagenic, and they did not increase misincorporation. Only a few of the metal salts that did not alter fidelity are listed in Figure 2.

With only a few exceptions, these results have been confirmed by Miyaki et al. (*22*) and Sirover et al. (*7*), using *E. coli* DNA polymerase I, and by Seal et al., using DNA polymerases-α and -β from human placenta (*17*). Most recently, we have observed that neither arsenic (AsO_4, As_2O) nor selenium (SeO_2) diminish fidelity with *E. coli* DNA polymerase I (*26*). Furthermore, Se, which has been reported to have an anticarcinogenic effect (*27*), does not reduce the mutagenic effect of Mn in titration experiments containing varying amounts

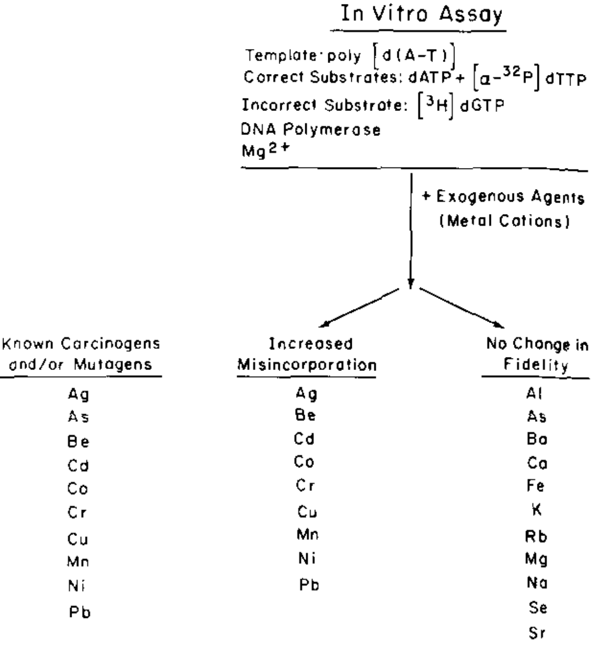


FIGURE 2. Screening for metal ions that alter fidelity. The data are compiled from the literature (*2, 26, 32*).

of these two metal ions. We have also examined the effect of different ionic species of chromium and have found that both Cr (III) and Cr (VI) alter the fidelity of *E. coli* DNA polymerase I (*26*). Chain initiation by RNA polymerases can be stimulated to Pb^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+}, whereas Zn^{2+}, Mg^{2+}, Li^{+}, Na^{+} and K^{+} are inhibitory (*28*). The similarity between the effects caused by particular metal ions on fidelity with DNA polymerases and on chain initiation with RNA polymerase could point to metal interactions with the DNA template as the common underlying mechanism for these two phenomena.

Fidelity of DNA Synthesis with Natural DNA Templates

All of the aforementioned studies on the effects of metal ions on the fidelity of DNA synthesis depended on measuring the incorporation of noncomplementary nucleotides by using synthetic polynucleotide templates of limited composition. It has been assumed that the results with such a model system are similar to those that would be obtained copying natural DNA containing all four bases. It is known, however, that slippage of the primer relative to the template can occur when primed templates of a repeating nucleotide se-

quence are copied. Thus, metal-mediated changes in the fidelity of DNA synthesis could result from such slippage of the primer on the template, an event that presumably does not occur during copying of natural DNA templates. Also unique to homopolymers or repeating heteropolymers is the fact that a single noncomplementary nucleotide can occupy a looped-out structure without changing the reading frame of subsequent codons. Thus, metals could enhance misincorporation by increasing the frequency of such looped-out structures. To circumvent these limitations, a system has been recently developed (*29*) to monitor the fidelity of *in vitro* DNA synthesis using a natural DNA template, DNA from the bacteriophage φX174 carrying a suppressible nonsense mutation, amber 3 (*am3*) (Fig. 3). Certain nucleotide substitutions within the *am3* locus that occur during *in vitro* replications of this DNA will cause a reversion to the wild type phenotype. Such reversions are detected by the bioassay method diagrammed in Figure 3. Thus, measurement of the reversion frequency of the progeny phage indicates the accuracy with which the DNA in the region of this mutation was copied.

This assay system has been used with AMV DNA polymerase (*30*) and *E. coli* DNA polymerase I (*31*). With homogeneous AMV DNA polymerase Mg^{2+}, and equal concentration of nucleotides, the *in vitro* mutation rate is approximately 1 in 1000. With *E. coli* DNA polymerase I, variations in the divalent metal ion activator used in the copying reaction markedly affects the reversion frequency of copied φX174 *am3* DNA. Thus, the calculated error rate observed with 5*mM* Mg^{2+} can be increased severalfold by the substitution of Mn^{2+} or Co^{2+} for Mg^{2+}. The error rate can also be increased by copying in the presence of inhibiting concentrations of Mg^{2+} or by the presence of Cr^{3+} or Cr^{6+} in Mg^{2+}-activated reactions (*32*). These limited studies suggest the metal mutagens and carcinogens also diminish accuracy with natural DNA templates. By determining the sequence of the DNA synthesized in the presence of mutagenic metals using the φX174 DNA template, it should be possible to define the specificity of interactions of these metals with the template nucleotides.

Mechanism of Genetic Miscoding by Metals

The exact mechanism by which certain divalent metal ions decrease the fidelity of DNA synthesis *in vitro* is not known. On the basis of the available data, three alternatives can be unambiguously eliminated, while three others may still be considered viable mechanisms and will require further investigation.

The following three possibilities by which metal

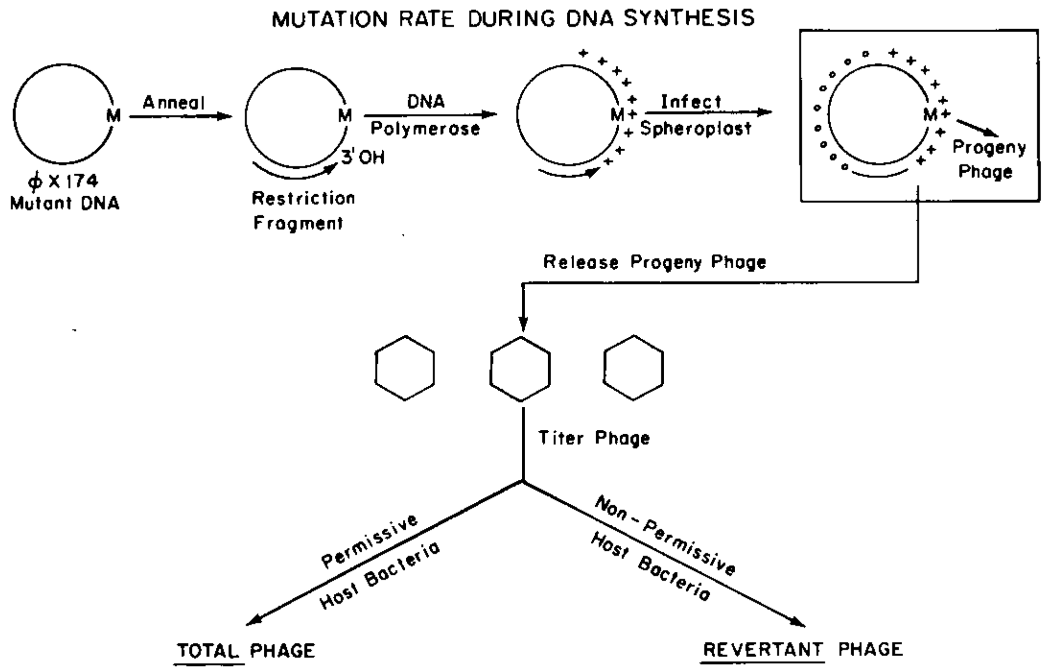


FIGURE 3. φX174 fidelity assay (*29*, *31*).

ions decrease the fidelity of *in vitro* DNA synthesis are no longer tenable mechanisms.

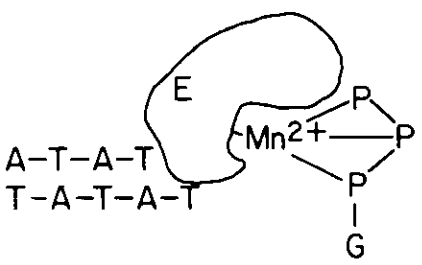
Precipitation of Noncomplementary Nucleotides. It can be argued that the observed increase in error frequency at high metal concentration represents the selective acid precipitation of metal ion complexes containing unincorporated noncomplementary nucleotides. However, physical and enzymatic studies of the products synthesized with AMV DNA polymerase (*33*), *E. coli* DNA polymerase (*7*), and DNA polymerases-α and -β (*17*), indicate that the noncomplementary nucleotides are incorporated into a polynucleotide chain, predominantly as single-base substitutions.

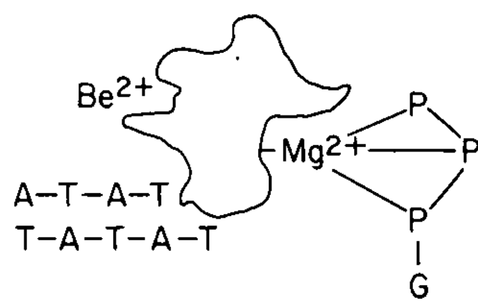
Metal-Substrate Interactions. Metal-induced infidelity does not appear to result from selective interactions between particular metals and particular nucleotides. For example, it could be argued that Co^{2+} selectively interacts with the complementary nucleotide and reduces its effective concentration in the reaction mixture. However, at a high concentration of Co^{2+} (5*mM*), the incorporation of dGTP as the complementary nucleotide with a poly (C) template is markedly inhibited, whereas the incorporation of dGTP as the noncomplementary nucleotide with poly [d(A-T)] as the template is undiminished (*18*). Similar results have been obtained with Mn^{2+} using various DNA polymerases with different template combinations.

Inhibition of "Proof-reading" Exonuclease by Metal Ions. The possibility that decreases in fidelity with divalent metal ions are mediated by inhibition of 3′ → 5′ exonucleolytic activity is also unlikely. Eucaryotic DNA polymerases and DNA polymerases from RNA tumor viruses are devoid of such an activity (*16*), yet mutagenic metal ions decrease the fidelity of these enzymes. Detailed studies on the effect of Mn^{2+} on fidelity, exonucleolytic activity, and monophosphate generation have been carried out with *E. coli* DNA polymerase I. Under conditions in which Mn^{2+} diminishes fidelity, there is no diminution of the 3′ → 5′ exonucleolytic activity (*7*).

The decrease in fidelity of metal ions during *in vitro* DNA synthesis can be explained most directly by any one or more of the following types of interactions (Fig. 4).

Altered Substrate Conformation. The ability of Mn^{2+}, Co^{2+}, Ni^{2+}, and possibly Zn^{2+} to substitute for Mg^{2+} as a metal activator focuses on the possibility that the mechanism of change in fidelity by these metals occurs by a substitution at the substrate binding site on the polymerase. Using a variety of DNA polymerases, the frequency of misincorporation at activating concentrations of Mn^{2+} and Co^{2+} is 2- or 3-fold that observed with Mg^{2+}. Magnetic resonance studies indicate that the interaction of the metal activator involves an enzyme-metal-substrate bridge complex involving the γ-phosphoryl group of the substrate (*35*). Studies with *E. coli* DNA polymerase I in the absence of template indicate that the bound metal changes the conformation of the substrate to that of the nucleotidyl





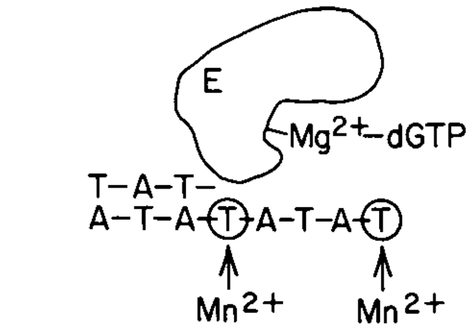


FIGURE 4. Possible mechanisms for metal-induced infidelity during DNA synthesis (see text).

unit in double-helical DNA. Sloan et al. (*8*) noted that this conformation could reduce the frequency of misincorporation. Thus, it could be argued that differences in conformation of the bound substrate with different metal activators might account for differences in the fidelity of DNA synthesis, particularly at activating concentrations of these cations. However, the current data are not sufficient to eliminate the possibility that differences in fidelity reflect interactions of metal ions with the template and not the enzyme even when the metal serves as an activator. Thus, the parallel incorporation of complementary and noncomplementary nucleotides at activating metal concentrations could simply indicate that polymerization is a rate-limiting event and the metal-mediated change in fidelity could be at a site other than the substrate site on the enzyme.

Altered Enzyme Conformation. The decrease in fidelity observed at inhibiting concentrations of metal activators suggests binding of metals at sites in addition to the catalytically active site. Ancillary binding sites for Mn^{2+} were detected on *E. coli* DNA polymerase I by nuclear magnetic resonance studies (*35*). The demonstrations that nonactivating metal ions alter the fidelity of other DNA polymerases is compatible with this concept. Also, evidence has been presented that Be^{2+}, a nonactivating cation, binds to AMV DNA polymerase directly and diminishes the fidelity of DNA synthesis *in vitro* (*24*). Thus, interactions of metals or metal-nucleotide complexes at distant sites could change the conformation of the polymerase so as to promote misincorporation. To date, attempts in our laboratory to generate an altered DNA polymerase with diminished fidelity by treatment with denaturing agents and heat have not been successful.

Altered Template-Base Specificity. The direct interaction of metal ions with phosphates and bases on polynucleotides have been measured by a number of physical techniques (*36*). Studies on the interaction of Mn^{2+} with activated DNA template by paramagnetic resonance (*35*) indicate 5 ± 2 very tight sites and 52 weaker sites having an invariant association constant of 68 μM. The largest decreases in fidelity with Mn^{2+} were observed at much higher concentrations (2-5mM). Weak Mn^{2+} binding sites on *E. coli* DNA polymerase I have been reported (*35*). However, it is also possible that very weak binding sites on polynucleotides are responsible for diminished fidelity, and these would not be observed in the magnetic resonance experiments. Eichhorn and collaborators initially observed that metal ions can cause enhanced mispairing upon renaturation of polynucleotides (*36*). Conceivably, the metal ions can directly interfere with complementary base-pairing or cause a shift in the keto-enol equilibria of the nucleotide. Recent studies by Murray and Flessel (*37*) indicate that Mn^{2+} and Cd^{2+} promote mispairing during hybridization of the synthetic templates. Moreover, the mispairing with Mn^{2+} can be demonstrated to occur at millimolar concentrations.

Public Health Implications

The results in this working paper suggest that mutagenic metal ions alter the fidelity of DNA synthesis. This has been demonstrated with purified DNA polymerases using both synthetic and natural DNA templates. We argue that in studying fidelity of DNA synthesis by DNA polymerases, one is studying mutagenesis *in vitro*. Correlations observed between alterations in fidelity *in vitro* and mutagenicity or carcinogenicity *in vivo* are in accord with the hypothesis that infidelity during DNA synthesis may cause mutations. However, we recognize that metal ions have many other effects *in vivo*. Considerable evidence will be required to document whether or not alterations in the fidelity of DNA synthesis are causally associated with mutations and malignancy. Irrespective of a defined mechanism, the correlation between alterations in fidelity and mutagenicity and/or carcinogenicity indicates the practicality of using fidelity assays as a screen for evaluating possible mutagens and carcinogens. Since these assays are carried out *in vitro* in defined homogeneous systems, it is possible to design experiments to understand how metals alter the fidelity of DNA synthesis.

With respect to metals, diminished fidelity and somatic mutations, the following hypothesis can be generated. Metal-induced mutations may occur by the interaction of metal ions with the DNA template or with the DNA polymerase. In the latter case, a normal polymerase could be exposed to an abnormal concentration of physiologically required metals, or to exogenous metals that are usually not present during cellular metabolism. Alternatively, metal ions that are normally not used for DNA replication could serve as activators for DNA polymerases that have been previously altered. In either case, an abnormal polymerase-metal combination might decrease the fidelity with which the DNA is replicated, and thus lead to the synthesis of DNA containing mutations. This newly synthesized DNA may contain certain critical errors (e.g., genes which code for altered polymerases). Furthermore, continued replication of the DNA by an altered polymerase or in the presence of mutagenic metals could also lead to an accumulation of additional errors during subsequent rounds of replica-

tion. Such critical errors and/or cascading errors caused by an accumulation of mutations may account for the progressive change in cellular properties during tumor progression.

Required Research

To date, only the initial studies have been conducted on genetic miscoding by metals. The main gap in our knowledge concerns the relationship between the studies with metals and purified polynucleotides *in vitro*, as well as those involving infidelity by DNA polymerases, to the realities of chromosomal replication in cells. The central question is whether or not the infidelity of DNA synthesis by metals is causally associated with mutagenesis and perhaps carcinogenesis. The approach to the problem requires an analysis of the effects of metals on the accuracy of DNA synthesis at three different levels of cellular organization.

At the Level of DNA Polymerases

Current evidence suggests that metal ions can alter the accuracy of catalysis by DNA polymerases from a variety of cells. The fact that infidelity of DNA synthesis correlates with mutagenic or carcinogenic properties of these metals may be only circumstantial when considering the multiple effects of metals in cells. The mechanism by which metals alter the fidelity of DNA synthesis has not been detailed. Careful physical and biochemical measurements are required on the binding of metals to DNA templates and polymerases before mechanistic details can evolve. Of interest is the possibility that particular metals interact preferentially with different nucleotides on DNA templates. An analysis of changes in sequence using natural DNA templates, such as ϕX174 DNA copied in the presence of different metal ions, should provide more definitive results concerning specificity of metal-nucleotide interactions.

At the Level of DNA Replicating Complexes

The most faithful DNA polymerase copies polynucleotide templates *in vitro* with an error rate approximating 10^{-5}. This rate is considerably greater than the spontaneous mutation rates per nucleotide synthesized observed in cells (10^{-8}-10^{-11}). Clearly, other proteins in cells function in accuracy of DNA synthesis. From studies with *E. coli* DNA polymerase I andT_4 DNA polymerase, it is apparent that DNA replication proceeds by the concerted action of multiple proteins (*3*). Further studies are required to determine the effect of each of these proteins on the replication process. With the establishment of enzyme/protein complexes capable of replicating DNA faithfully *in vitro*, it will be necessary to study the effects of metals on these complexes. An analysis of the effects of metals on the accuracy of catalysis by DNA replicating proteins is perhaps somewhat closer to the complexity involved in chromosomal replication in human cells.

At the Cellular Level

The main problem in studying detrimental effects of metals at the cellular level is that metals are required for many cellular processes. Nevertheless, a number of studies on cells in tissue culture demonstrate the mutagenic potential of metals. Thus, a study of the effects of metals on the fidelity of DNA synthesis *in vivo* is important since replication of DNA is required for the establishment of mutational alterations. It would be desirable to extend these studies using various eukaryotic cells that are defective in different pathways of DNA repair, since these cells would be more likely to exhibit metal-induced mutational alterations. There is also a large gap in knowledge of metal content and localization in cells. An analysis of metal content of eukaryotic cells, particularly in the nucleus and chromosomes, is required to better define the relationship between studies on fidelity *in vitro* and mutation rates in cells.

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Chemistry of Carcinogenic Metals

by Arthur E. Martell*

The periodic distribution of known and suspected carcinogenic metal ions is described, and the chemical behavior of various types of metal ions is explained in terms of the general theory of hard and soft acids and bases. The chelate effect is elucidated, and the relatively high stability of metal chelates in very dilute solutions is discussed. The concepts employed for the chelate effect are extended to explain the high stabilities of macrocyclic and cryptate complexes.

Procedures for the use of equilibrium data to determine the speciation of metal ions and complexes under varying solution conditions are described. Methods for assessing the interferences by hydrogen ion, competing metal ions, hydrolysis, and precipitation are explained, and are applied to systems containing iron(III) chelates of fourteen chelating agents designed for effective binding of the ferric ion. The donor groups available for the building up of multidentate ligands are presented, and the ways in which they may be combined to achieve high affinity and selectivity for certain types of metal ions are explained.

Carcinogenic Metals

A large number of metal ions and their complexes are now considered to be primary carcinogens. The evidence for carcinogenicity of metal ions has been reviewed recently (*1-6*), and no attempt will be made in this paper to go into this subject in detail. The purpose of this review is to consider the properties and coordination chemistry of carcinogenic metal ions, the reactions that they probably undergo in physiological systems, and to consider how the speciation of metal ions in the form of their complexes and chelates may be related to carcinogenic effects. It is first necessary, therefore, to define the scope of this paper in terms of the metals to be discussed.

Carcinogenic metals may be classified in three general categories based on the nature and mechanism of carcinogenesis: radioactive metals, chemical carcinogens, and surface oncogens. This paper will be limited to consideration of metals that function as carcinogens through chemical interaction with biological systems. Metals that achieve carcinogenic effects solely as the result of the production of high-energy particles and/or electromagnetic radiation will not be discussed. Also beyond the scope of this paper are the carcinogenic effects of solid materials, which seem to be more closely related to physical properties and surface characteristics rather than to the chemical nature of the solids.

The periodic distribution of metals that have been recognized as chemical carcinogens is presented in Figure 1. This distribution is interesting in that the metals involved fall into several groups. The more basic metal ions that generally form labile complexes are for the most part not carcinogenic. On the other hand, a large fraction of the fourth period elements excepting groups 1A, 2A, and 7B, (but including many first row transition metals) have been found to have carcinogenic effects. The lanthanides and actinides, that form relatively basic metal ions of +3 and +4 charge, also seem to be generally noncarcinogenic. Details of the nature of the evidence for carcinogenicity of the metals circled in Figure 1 have been reviewed recently (*5, 6*) and the considerations involved will not be repeated here.

The metals that are recognized as the most potent carcinogens are limited to a relatively small number: beryllium, cadmium, nickel, and chromium. Beryllium is the only exception to the generalization mentioned above that the more basic metal ions are not carcinogenic. Evidence for beryllium

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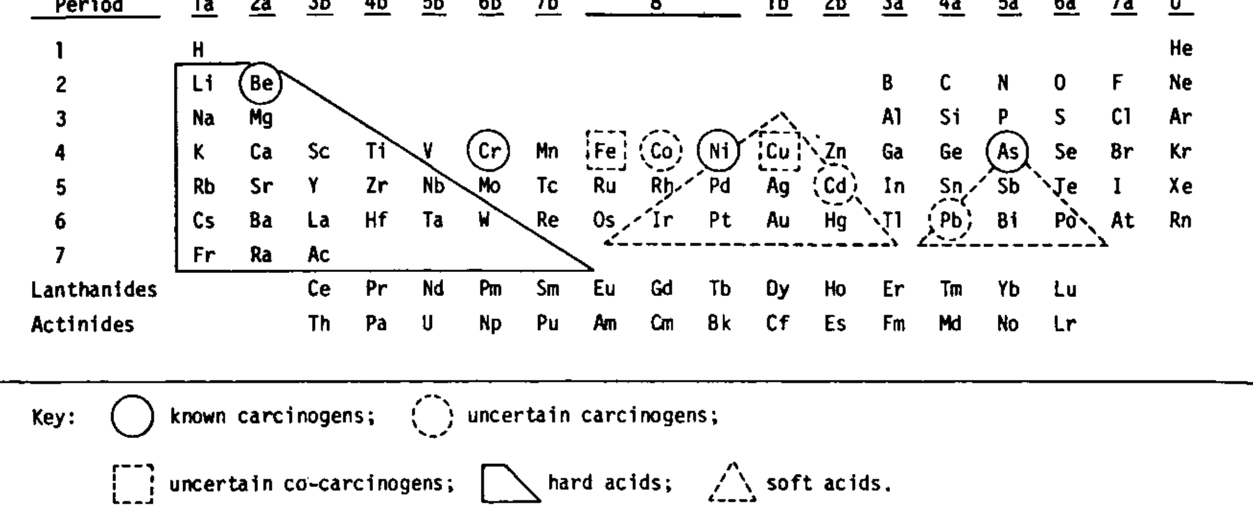


FIGURE 1. Periodic classification of elements forming compounds having definite carcinogenic effects in man or suggestive of carcinogenicity.

carcinogenicity is well established (*5, 7, 8*). Because of its small ionic radius, it would be expected to displace magnesium(II) from enzymes such as RNA polymerase and deoxythimidine kinase. Accordingly, it is not surprising that the carcinogenic effects of beryllium seem to be associated with the high affinity of beryllium compounds for the cell nucleus.

It should be pointed out that the philosophy behind the periodic classification of carcinogenic metals that has been generally employed in the past seems to have involved an inherent assumption that a positive carcinogenic effect of any compound or complex of a metal is sufficient to label the metal as carcinogenic. It is becoming obvious, however, that the interaction of a metal ion with a biological system depends on the nature of the compounds or complexes that are formed, and that changes in the complexing or chelating agent may greatly change its properties, including its carcinogenic effects. A widely recognized example of this principle is the case of chromium, indicated in Figure 1 as a carcinogen. The most frequently observed route involves lung cancer resulting from the inhalation of chromium(VI) by workers in chromium metallurgy and dichromate manufacture. Long induction periods are frequently observed and the actual carcinogenic chromium compound or compounds have not been identified. The nature of the chromium exposure leading to cancer is still controversial.

The conflicting results obtained with various types of metal compounds, and the dependence of the carcinogenic effects observed for a particular metal on the nature of the metal compound involved indicates that clinical results on metal carcinogenicity should be related to the particular metal compound or complex, rather than to the metal in general. Thus it appears that a simple periodic classification illustrated by Figure 1 can be very misleading, and that many of the compounds of the metals indicated as carcinogenic may be quite harmless.

The importance of metal speciation in carcinogenicity is inferred in a recent correlation with metal electronegativities (*9*). It appears that the positively demonstrated and suspected carcinogenic metals are grouped within the electronegativity range 1.1-1.9, with very few exceptions. The ions of the more electropositive metals form very labile complexes and generally have low ligand affinity. The metal ions in the higher electronegativity range form highly covalent bonds with soft donor groups (e.g., mercaptides) and undergo very sluggish exchange reactions with ligands generally found in biological systems. Metal ions in the intermediate electronegativity range have considerable affinity for the nitrogen and oxygen donor groups in many biomolecules, and interact with them with measurable reaction rates. Thus it seems that complex and chelate formation is a common characteristic of metal ions that are found to have carcinogenic properties.

Finally, it should be pointed out that a large number of the carcinogenic metals indicated in Figure 1 are essential to life in trace or moderate concentrations. Metal ions that are essential for certain physiological functions and for the activa-

tion of certain enzymes, include nearly all of the first transition series (V, Cr, Mn, Fe, Co, Ni, Cu) as well as Mo, Zn, Cd, and Sn. This interesting dichotomy of essentiality and carcinogenicity and its implications with respect to the Mantel-Bryan (*10*) nonthreshold model of carcinogenesis, and the application of the Delaney clause to food additive legislation has recently been pointed out (*11*).

Formation of Metal Complexes and Chelates

The large amount of experimental data now available on the reactions of electron donors (complexing or chelating ligands) with electron acceptors (hydrogen ion, metal ions) has been correlated in a qualitative manner with the nature and properties of the donors and acceptors through the use of empirical classification involving type (a) ionic, and type (b) covalent, chemical bonding (*12*). Type (a) acceptors consist of the more basic metal ions that tend to form complexes having ionic bonds with little covalent character. The term "hard" was later introduced for the ionic type (a) acceptors and donors, which have low polarizability, and the term "soft" was suggested for type (b) acceptors and donors, which generally have relatively high polarizability (*13*). Thus "hard" and "soft" acceptors were designated as "hard" and "soft" acids, while "hard" and "soft" donors were given the term "hard" and "soft" bases. As would be expected when the principal attractive forces are coulombic, the stabilities of the complexes formed from hard acids and bases increase with increasing ionic charge and decreasing ionic radius. Hard metal ions are generally strongly hydrated in aqueous solution, and form their most stable complexes with negative flouride, oxygen, and nitrogen donors, and to a somewhat lesser extent with negative chloride and sulfur donors. The softest metals on the other hand, such as Ag(I), Hg(I), and Hg(II) form complexes with donor atoms having stability order S > O, P > N, and $I^- > Br^- > Cl^- >> F^-$.

On the basis of these criteria, all the soft acids are situated in two roughly triangular areas of the periodic system (Fig. 1), while the hard acids are generally found in a triangular lefthand area of the periodic system, as indicated. The properties of these acceptors will also vary greatly with charge, as indicated above. Also, it is obvious that the softness of a donor atom will increase with an increase of negative charge. The effect of charge on donors is not as important as it is for acceptors, since there are only two well known binegative donor atoms, the oxide and sulfide anions, O^{2-} and S^{2-}. Details of the effect of ionic charge and other properties of metal ions and ligand donor atoms on the stabilities and covalencies of metal complexes have been described and analyzed by Ahrland (*14*). Various numerical parameters directed toward treating the concept of hard and soft acids and bases in a semiquantitative manner have also be discussed (*15, 16*).

Another semiempirical correlation of the stabilities of complexes with variation in the electropositive (vs. electronegative) character of metal ions (i.e., hard and soft acids) was pointed out some time ago by Martell (*16*). Such a correlation, indicated in Figure 2, shows increasing stability with increasing electronegativity and increasing charge of the metal ion. Increased electronegativity would be expected to increase covalency of the coordinate bonds formed, since it would result in closer matching of the electronegativies of donor and acceptor. The effect of charge may be in part coulombic (in view of the fact that ligand is negative) and in part due to greater polarization of the negative charge of the ligand toward the metal ion. The use of electronegativity as a parameter is similar in principle to a parameter based on electron affinities used by Ahrland (*14*) to explain relative degrees of hardness and softness of metal ions.

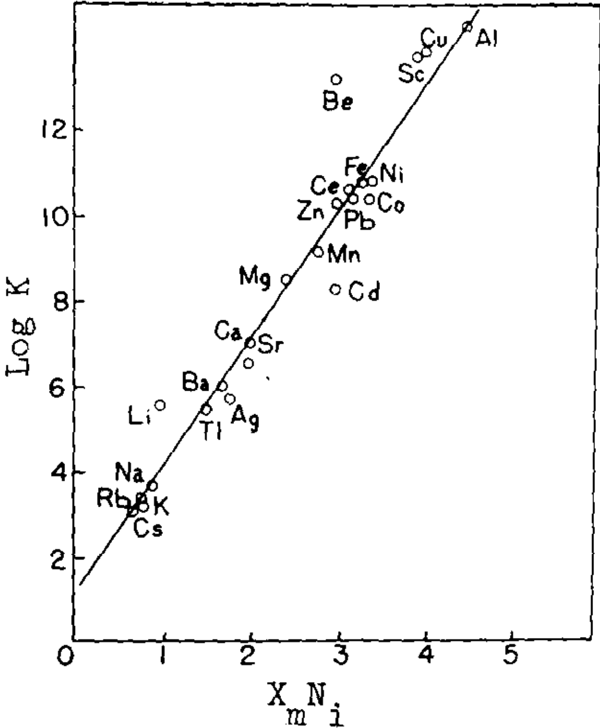


FIGURE 2. Correlation of stability constants, K, of dibenzoylmethane chelates with product of electronegativity of the metal, X_m, and ionic charge, N_i

Metal Chelates and the Chelate Effect

It is widely recognized that metal chelates are much more stable in aqueous solutions than simple complexes of the same metal ions with monodentate ligands containing similar donor atoms. Thus any increment of stability due to the "chelate effect" is superimposed on the factors cited above, such as electronegativity and hard and soft character of donors and acceptors. Since nearly all of the natural and artificially introduced coordination compounds that are found in biological systems are metal chelates, it is appropriate at this point to consider the factors that control stability and other properties of metal chelate compounds.

The coordination tendencies of metal ions in aqueous solution result in the formation of aquo complexes containing several coordinated water molecules, and which are designated as "aquo" metal ions. The majority of the frequently encountered metal ions have coordination numbers of six, and are bound to six water molecules usually arranged in a manner more or less approximating a regular octahedron. Relatively large, highly-charged metal ions, such as those of the actinides and lanthanides, and also Zr(IV), and Hf(IV) have coordination numbers of eight, while other metal ions of lower charge, such as those of Zn(II), Be(II), Al(III), Ga(III), and Mg(II) seem to have coordination numbers of four or six, depending on the ligands and the reaction conditions. For metal ions of intermediate and low basicity the covalent character of the coordinate bonds is sufficient to maintain a fixed number of donor groups in a specific geometric arrangement about the metal center. For the more basic metal ions of intermediate or low ionic charge, the number and geometrical arrangement of coordinated donor groups may vary considerably, and a dynamic equilibrium involving a distribution of structures may be assumed to exist.

A metal chelate compound is merely a metal complex or coordination compound in which two or more of the donor atoms coordinated to the metal ion are bound together by some kind of chemical linkage. According to this general description, metal chelate compounds would not have special properties that distinguished them from simple complexes. On the other hand when a chelate ring meets certain structural and constitutional specifications, its stability is increased and it may also have additional properties that cannot be achieved in simple complexes with independent donor groups. The principal properties of metal chelate compounds are described below.

The formation of highly stable metal chelates in aqueous solution have important applications in biological systems. The use of chelating ligands with a sufficient number of donor groups to match the coordination requirement of a metal ion makes it possible to achieve 1:1 stoichiometry in the formation of a chelate compound—an important property if stability of the chelate compound is to be maintained in extremely dilute solutions. The conceptual and thermodynamic basis for the special properties of metal chelate compounds has been described in a number of reviews (*17-19*).

For a metal ion of coordination number six, for example, the reactions involving monodentate and sexadentate ligands may be compared, as follows:

$$M + 6A \rightleftarrows MA_6 \qquad \beta_{MA_6} = [MA_6]/[M][A]^6 \quad (1)$$

$$M + L \rightleftarrows ML \qquad \beta_{ML} = [ML]/[M][L] \quad (2)$$

For the complex MA_6, the units of β_{MA_6} are molarity to the negative sixth power, and the degree of formation of the complex is proportional to the sixth power of the free ligand concentration, which in very dilute solution can become a vanishingly small number. The degree of formation of the chelate compound ML of the sexadentate ligand, however, is much less sensitive to concentration, and decreases linearly with the first power of the free ligand concentration.

The high stability of metal chelates relative to metal complexes in dilute solution is clearly related to the values of the entropies of dilution of the complexes and chelates relative to the entropies of dilution of the dissociated species with which they are in equilibrium. This experimental fact (that metal chelates are much less dissociated in dilute solution) is illustrated in Table 1, which compares the degrees of dissociation of coordination compounds containing zero, three, and five chelate rings. An average chelate effect of 10^2 per chelate ring is assumed as the basis of the arbitrary stability constants employed—a result that would be achieved if the donor groups of the ligands have approximately equivalent metal ion affinity. The superior stability of the metal chelate in dilute solution, and the striking effect of increasing the number of metal chelate rings, is dramatically illustrated by a comparison of the percent dissociation of the metal chelates and complexes indicated for unit and thousandth molar solutions.

As pointed out by Adamson (*20*), the entropy-related chelate effect, which was assigned a value of 10^2 in log β per chelate ring, is a result of the use of unit molality (≅ unit molarity) as the standard

Table 1. Comparison of dissociation of metal complexes and chelates in dilute solution.

Donor groups per ligand	No. of chelate rings	Equilibrium quotient	β_x	1.0 *M* Complexes		1.0×10^{-3} *M* Complexes	
				Free [M]	%Dissociation	Free [M]	% Dissociation
1	0	$\frac{[MA_6]}{[M][A]^6}$	$10^{18}\ M^{-6}$	6×10^{-4}	6×10^{-2}	2×10^{-4}	20
2	3	$\frac{[MB_3]}{[M][B]^3}$	$10^{24}\ M^{-3}$	5×10^{-7}	5×10^{-5}	1×10^{-7}	1×10^{-2}
6	5	$\frac{[ML]}{[M][L]}$	$10^{28}\ M^{-1}$	1×10^{-14}	1×10^{-12}	3×10^{-16}	3×10^{-11}

reference state for solutes in aqueous solutions. The value of the chelate effect would vary considerably if some other concentration were selected as the reference state. It would increase considerably for 10^{-3} M and would vanish for unit mole fraction standard state. Regardless of these considerations, the relative degrees of dissociation of the model compounds in Table 1 remain as an experimental fact.

Other Factors Influencing Stabilities of Metal Complexes and Chelates

Table 2 indicates that there are many factors in addition to the entropy-based chelate effect that must be taken into account in order to fully understand metal-ion affinites of multidentate ligands. Mutual coulombic repulsions between donor groups in metal chelates are important, and the extent to which these repulsions are overcome in the free chelating ligand relative to the coulombic repulsions that the corresponding unidentate ligands must undergo in complex formation is a manifestation of the enthalpy-based chelate effect. This property, which greatly increases stability constants of chelates, is inherent in the enthalpies of formation of the chelating agents in solution. This enthalpy effect is developed to the highest possible degree in macrocyclic and cryptand ligands in which the donor groups are held at geometric positions that are relatively close to the positions that they would have when combined with metal ions. Thus stability and specificity of both natural and synthetic multidentate ligands are achieved by the arrangement of donor groups in positions favorable for satisfying the coordination requirements of the appropriate metal ions. In biological macromolecules this objective may be achieved by the positioning of donor groups in favorable geometric arrangements through, for example, the folding of a polypeptide chain. Specificity of synthetic ligands depends on the development of a molecular framework that will achieve similar results, either through ring formation or the utilization of rigid aromatic structures.

Donor Groups

Examples of donor groups that may be built into synthetic and natural ligands are shown in Figure 3. These constitute a partial list involving only the more common donor groups. For the donors involving oxygen atoms, for example, analogous ligands in which sulfur atoms replace one or more oxygens are also possible, and many such ligands are avail-

Table 2. Factors influencing solution stabilities of complexes and chelates.

Enthalpy effects	Entropy effects
Variation of bond strength with electronegativities of metal ions and donor atoms of ligands	Number of chelate rings
Ligand field effects	Size of the chelate ring
Enthalpy effects related to the conformation of the uncoordinated ligand	Arrangement of chelate rings
Steric and electrostatic repulsions between ligand donor groups	Changes of solvation on complex formation
Heats of desolvation of metal ion and ligand	Steric interferences with chelates ring formation
Other coulombic forces involved in chelate ring formation	Entropy variations in uncoordinated ligands
	Effects resulting from differences in configurational entropies of the free and coordinated ligands

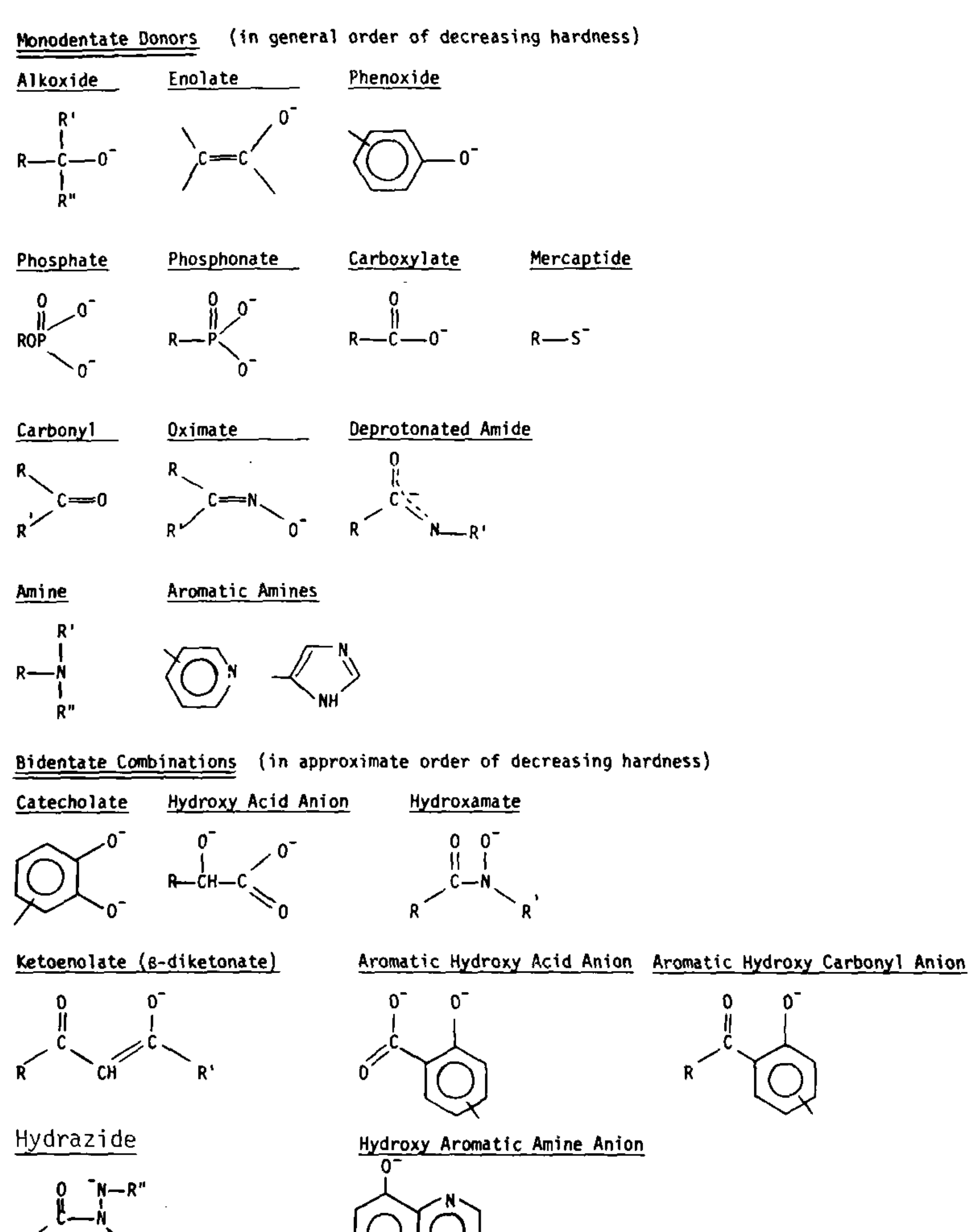


FIGURE 3. Types of donor groups in natural and synthetic ligands.

able. The bidentate donor groups are those in which the donor atoms function in a cooperative fashion through resonance interactions. Thus for bidentate functional groups containing an unsymmetrical formal charge, the resonance effects distribute the charge partially or equally between the donor atoms, depending on the molecular structure, thus making both donor atoms effective in metal ion coordination. Several of these bidentate donor groups are synthesized in microbial systems for metal ion transport. Examples are the microbial iron(III) carriers that contain one or more catechol or hydroxamic acid groups (*21-26*). These functional groups have also been incorporated into synthetic analogs of the microbial iron carriers for the treatment of iron overload disease (*27*, *28*).

Examples of Chelating Ligands

The polyaminopolycarboxylates indicated in Table 3 constitute a familiar series of synthetic ligands that have been widely used in biological systems as well as for analytical and commercial purposes. The first three members of the series, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), and diethylenetriaminepentaacetic acid (DTPA) are well known. The two higher members of the series are available only in small quantities for experimental purposes.

For metal chelates in which all of the amino and carboxylate donor groups of the ligands in Table 3 are coordinated to the metal ion, there are n-1 five-membered chelate rings, where n is the total number of nitrogen and oxygen donors (one oxygen per carboxylate group). This extent of chelate ring formation is generally achieved for most of the well-known di- and trivalent metal ions, provided that the coordination number of the metal ion does not exceed n. Typical examples of metal chelates of NTA, EDTA and DTPA are illustrated by I-IV.

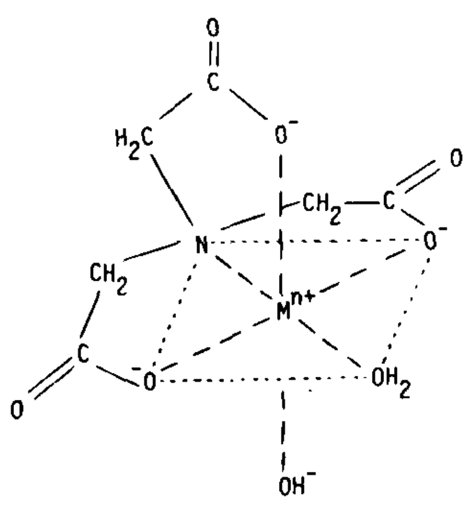
The data in Table 3 indicate that continued increase in the number of donor atoms in ligands of this type does not produce a parallel increase in the stability constants of chelates formed with divalent metal ions. A decrease eventually occurs, with the maximum value for Ca(II) ion obtained with DTPA. For Cu(II) the maximum stability occurs with triethylenetetraminehexaacetic acid (TTHA). For metals of higher ionic charge, the maximum stabilities are not known because of the lack of stability constant data for the higher ligands, but probably occur with tetraethylenepentamineheptaacetic acid (TPHA) or the next higher member of the series. This type of behavior is rationalized by the view that for basic metal ions such as Ca^{2+}, La^{3+}, and Th^{4+}, the stabilities of the corresponding chelates of these ligands depends on the ability of the ligands to form an "ionic cage" about the metal ion. For moderate to low coordination number, this objective is best achieved with EDTA or DTPA. For TTHA some of the carboxylate donor groups will not be coordinated, probably leaving an unbound pair at one end of the ligand. On that basis TTHA would present only four negative charges to the calcium(II) ion while DTPA would provide five. Similar considerations would be expected for the higher members of this series of ligands in the coordination of basic metal ions of higher charge and coordination number, such as the tripositive lanthanides and tetrapositive actinides.

It is apparent from the above remarks that the ligands in Table 3 form stable chelates with a wide variety of metal ions but are not very specific: i.e., they do not effectively distinguish between various metal ions. For metal ions of increasingly higher charge (increasing "hard acid" character), higher stability can be obtained by simply increasing the number of carboxylic acids, that are only moderately hard basic groups. In the design of new chelating ligands, higher selectivity may be achieved by replacing one or more carboxylate groups by one or more coordinating groups having more selective coordinating properties. Greater stability and selectivity have been achieved by the introduction of functional groups having high affinity for the metal ion under consideration. An example of how ligands may be varied in this manner follows.

It has been known for a long time that phenolate oxygen donors have very high affinity for the ferric ion. Highly specific sexadentate ligands for the Fe(III) ions have been synthesized by modifying the EDTA structure so as to provide two carboxylate and two phenolate donor groups attached to the basic ethylenediamine framework. The ligands ethylene-*N*,*N'*-bis-2-(*o*-hydroxphenyl)glycine (EHPG) (*29*) and *N*,*N'*-bis(o-hydroxybenzyl)ethylenediamine-*N*,*N'*-diacetic acid (HBED) (*30*), illustrated in Table 4, have affinities for Fe(III) from 9 to 14 orders of magnitude over those of EDTA. On the other hand the phenolate ligand has little selectivity over EDTA for other metal ions such as those of Zn(II) and Ni(II). In the case of Ca(II), the phenolate analogs are poorer ligands than EDTA. The much higher effectiveness of *N*,*N'*-(bis)(2-hydroxybenzyl)-ethylenediamine-*N*,*N'*-diacetic acid (HBED) over EHPG for iron(III) is considered to be due to the much more favorable steric orientation in HBED of the carboxylate groups, which bind the ferric ion much more strongly than is possible with EHPG.

The high affinity of HBED and EHPG for iron(III) is due to the presence of two phenoxide groups in

I NTA Chelate



II Hydroxo NTA Chelate

III EDTA Chelate

IV DTPA Chelate

each ligand. These donor groups are "hard bases" and match the "hard acid" character of the iron(III) ion. It was pointed out above that oxygen atoms of catechols and hydroxamic acids are also hard bases and are highly effective for iron(III). Two examples of microbial ligands containing hydroxamate (*24*) and catecholate (*25*) donor groups are illustrated by formulas V and VI, respectively. The six oxygen donors in each ligand are arranged octahedrally around the iron(III) ion. The higher stability of the catecholate-type chelate [enterobactin-iron(III)] is due to the higher basicity and higher ionic charge of the six phenolate donor groups in dissociated enterobactin. The relative stabilities in biological systems, however, are not as different as is indicated by the relative values of the stability con-

Table 3. Stabilites of 1:1 chelates of NTA and EDTA and Their analogs (25°C, 0.10*M* ionic strength).

Ligand	Log formation constants Ca^{2+}	Cu^{2+}	La^{3+}	Th^{4+}
NTA	6.57	12.96	10.47	12.4
EDTA	10.4	18.7	15.2	23.2
DTPA	10.7	21.1	19.5	28.78
TTHA	9.9	20.3	23.1	31.9
TPHA	9.0	~20	>27	>>32

Table 4. Stabilities of chelates of EDTA analogs containing phenolic groups (25°C; 0.10*M* ionic strength)

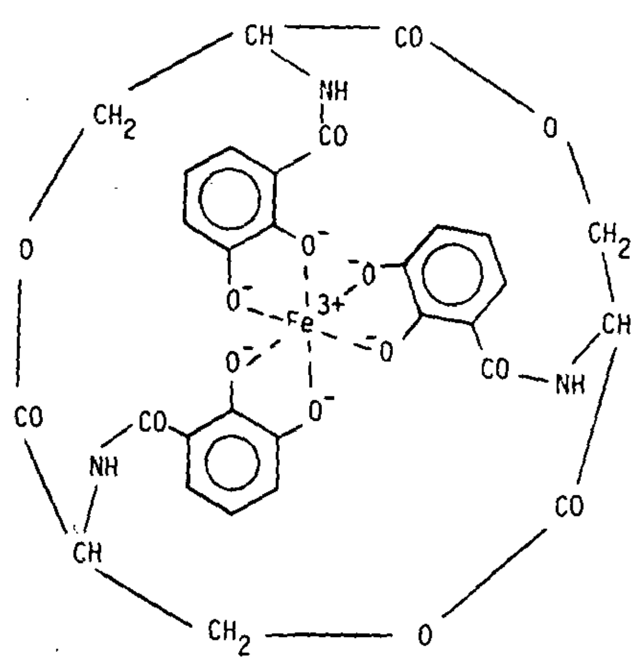
Ethylenebis-*o*-hydroxyphenylglycine (EHPG)

N,N′-bis (*o*-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid (HBED)

	EHPG log K_{ML}	EHPG Δ log K^{a}	HBED log K_{ML}	HBED Δ log K^{a}
Cu^{2+}	23.90	5.20	21.38	2.68
Ni^{2+}	19.60	1.14	19.31	0.79
Zn^{2+}	16.80	0.36	18.38	1.93
Ca^{2+}	7.20	-3.41	9.29	-1.32
Fe^{3+}	33.91	8.91	39.68	14.68

[a] $\Delta \log K = \log K_{ML} - \log K_{ML}^{EDTA}$.

V Ferrichrome A [R $= CH = C(CH_3)CH_2COOH$]



VI Iron (III)-enterobactin Chelate

stants given. This moderating effect which is due to proton competition for the ligand donor groups, is explained below.

Selectivity through Design of Molecular Framework of Coordinating Ligand

The chelating ligands described above are generally flexible molecules that have quite different structures in solution in the absence and in the presence of metal ions. Thus the process of metal chelate formation requires reorientation of the ligand structure and close approach of polar and charged donor atoms. These requirements result in adverse enthalpy (coulombic repulsion) and entropy effects (loss of rotational and vibrational freedom). Both affinity and specificity for metal ions may be greatly increased by the use of more rigid molecular frameworks so that the donor groups are positioned very close to the orientations that they would have in the metal chelate. Well-known examples of this type of ligand are provided by the

porphyrins and the phthalocyanines, which coordinate metal ions with four completely resonance-linked aromatic nitrogen donor atoms arranged in a square plane around the central metal ion. In many cases the coordination requirements of the coordinated metals are satisfied by two additional monodentate ligands in the axial positions. The literature on the coordination chemistry of these complexes is very extensive and the chemistry of these systems will not be described in this paper. Interested readers are referred to a recent review (*31*) and references cited therein.

The "crown ethers", other macrocyclic ligands, and the cryptate ligands are examples of cyclic chelating agents that are more flexible than the porhpyrins and phthalocyanines. However, the ring systems in these ligands may be varied so as to produce cavities surrounded by donor atoms in sufficiently restricted positions to discriminate between metal ions differing in ionic radius. The following is a brief discussion of these ligands and their chelates because of their close relationship to the ionophores.

The so-called crown ethers are macrocyclic ligands containing ether oxygens as coordinating donor groups surrounding more or less well-defined cavities. The special property of these ligands is their ability to form stable coordination complexes of alkali metal and alkaline earth ions. The crown ethers, and other macrocyclic ligands containing other donor atoms such as nitrogen and sulfur, consist of single rings with varying numbers of coordinating donor atoms. While these ligands may form two-dimensional rings, the aliphatic bridges between the donor atoms are flexible and three-dimensional complexes are readily formed. Ligands containing three polyether (or polyamine or polythioether) strands joined by two bridgehead nitrogens provide three-dimensional cavities that are more well-defined than is the case for the simple macrocycles, and have higher specificities for metal ions of varying ionic radius. The inclusion complexes formed are called cryptates, and the metal-free ligands are designated as cryptands.

Representative topologies of crown and cryptand ligands are given in Figure 4. The simple macrocycle A may have a wide variety of coordinating heteroatoms. For the polyethers, rings varying from 9 to 60 atoms, with from 3 to 20 oxygen atoms have been synthesized. With two bridgehead nitrogens cryptands represented by B may be synthesized so as to provide one or more coordinating atoms in each bridge. With four bridgehead nitrogens, the cryptand molecule may have a cylindrical or spherical shape, as indicated by C and D, respectively. It is seen that these structures can be expanded with additional bridges and bridgehead atoms to form a wide variety of conformations. With variations in the number of kind of donor atoms in each bridge, it is seen that the possible number of cryptand ligands is almost limitless.

The Macrocyclic Effect

The stability constants for the formation of the Cu(II) and Ni(II) chelates of typical four-nitrogen macrocycles and their open-chain tetramine analogs are presented in Table 5. The remarkably higher stability of 5-7 orders of magnitude for the macrocyclic complex is typical for comparisons of this nature. The term "macrocyclic effect", which may be up to ten times larger than the chelate effect for analogous mono- and polyamine complexes, was ascribed by Margerum et al. (*32*, *33*) to differences in the degrees of hydration of the open-chain and cyclic ligands, the latter being much less solvated. Thus formation of the macrocyclic complex takes place with much less expenditure of desolvation energy. These arguments are based on measured thermodynamic parameters of formation of chelates of the type illustrated in Table 5, and the assumption that the thermodynamic properties of the final metal chelates are very much alike, with or without the additional chelate ring. This effect may account for a major part of the macrocyclic effect. It is suggested here, however, that entropy considerations are also important factors contributing to higher stability of the macrocyclic chelate. The

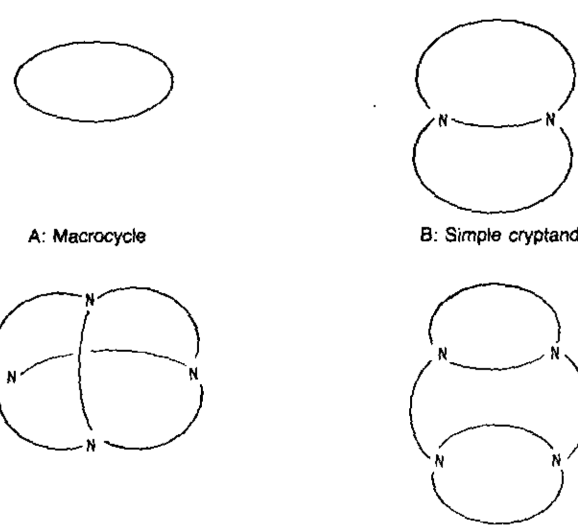


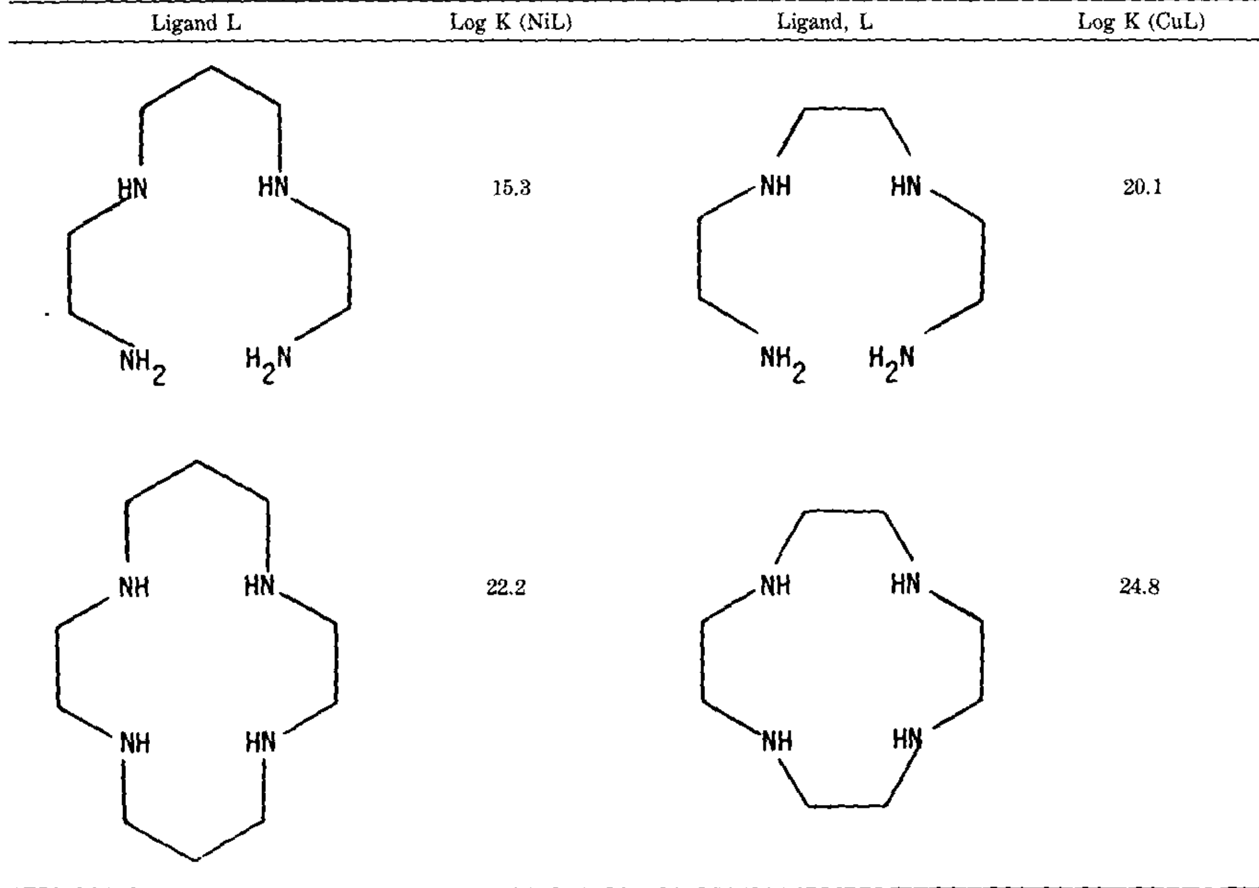
FIGURE 4. Representative topologies of crown and cryptand ligands.

open chain ligand has an extended configuration, which is not indicated in Table 5, and formation of the chelate results in significant negative entropy effects because of the restriction of rotational and vibrational motion of the ligand. Such restrictions do not occur in the formation of the macrocyclic chelates, since the free ligands exist initially in conformations which are close to those that are required for coordination of the metal ion.

The Cryptate Effect

The "cryptate effect" has been described by Lehn and co-workers (*34*, *35*) as the increase in the stability of a macrocyclic chelate resulting from the formation of an additional connecting bridge to form macrobicyclic ligands, or cryptands. This effect, which is illustrated in Table 6, is even larger than the macrocyclic effect for the same metal ions (Table 7). The factors described above that produce the macrocyclic effect may be further extrapolated to explain the even higher stabilities of cryptates. This increase is at a maximum when the three-dimensional cavity between the donor atoms closely fits the dimensions of the metal ion under consideration. From a thermodynamic point of view the cryptate effect is due to both enthalpic and entropic factors favoring formation of the metal cryptate. The lower hydration of the cryptand (relative to open chain and macrocyclic ligands) is an important factor. It has been pointed out by Lamb et al. (*36*), however, that other enthalpic factors must be involved since the cryptate effect has been shown to increase with a decrease of dielectric constant. It is now suggested that this "other enthalpy factor" is the overcoming of the coulombic repulsions between the polar or charged donor groups in the cryptand relative to macrocyclic and open chain ligands, in which the polar groups are free to move farther apart to a much greater extent than is possible for the analogous metal cryptates. It is also suggested that there is an inverse relationship between the hydration energy and coulombic repulsion enthalpic terms. In solvents of high dielectric

Table 5. Formation constants of macrocyclic chelates and of analogous chelates of open-chain polyamines.



Ligand L	Log K (NiL)	Ligand, L	Log K (CuL)
	15.3		20.1
	22.2		24.8

constant the polar groups will be highly solvated and the coulombic repulsions between the donor groups will be mitigated by the solvent molecules associated with the ligand donor atoms. It is these solvent molecules that must be displaced in the formation of a metal complex or chelate. On the other hand, in solvents of low dielectric constant there is less solvation, so that this effect must be less important. Because of the lower solvation however, the coulombic repulsions between the negatively charged donor groups that must be overcome in formation of a metal complex or chelate are relatively much larger. Both of these enthalpic factors work against metal ion coordination, and both are at a minimum in the formation of metal cryptates.

Entropy factors also provide important contributions toward the higher stabilities of cryptates, since the formation of a cage of donor atoms prior to coordination of the metal ion results in less loss of vibrational and rotational entropy in the process of metal ion coordination. Further rigidity may be built into the framework of both macrocyclic ligands and cryptands by incorporating aromatic rings or other groups that restrict rotation of various parts of the ligand molecule, thus further decreasing the entropy loss on coordination, and favoring higher stabilities of the macrocyclic and cryptand chelates.

While cryptates represent the extreme in the achievement of molecular structures that favor metal complex formation, there are kinetic disadvantages to their application in chemical and biological systems. The closer the fit of the wrap-around ligand to the metal ion, the slower will be the rate of formation and dissociation of its chelates, and the slower will be the rate at which one metal ion displaces another from such complexes. This problem may not be a very serious one for many of the labile, highly ionic chelates formed by the alkali and alkaline earth metal ions. For transition metal ions having an appreciable covalent component in their coordination compounds, the protective ligand shell around cryptate and even some macrocyclic chelates may render the metal ion quite inert to dissociation and exchange reactions.

Ionophores

Ionophores are natural and synthetic ligands that can transport metal ions across low-dielectric constant barriers such as lipids, organic solvents, and biological membranes. This functional definition encompasses a wide variety of possible chemical

Table 6. Examples of the cryptate effect.[a]

Ligand	log K				
	Na^+	K^+	Ca^{2+}	Sr^{2+}	Ba^{2+}
	3.26	4.38	4.4	6.1	6.7
	7.21	9.75	7.60	11.5	12.0

[a]95 vol % methanol.

Table 7. Macrocyclic effect for analogous metal Ions as in Table 6.[a]

	log K	
	K^+	Ba^{2+}
	1.96	2.34
	5.35	6.56

[a]90 vol % methanol.

structures. The best known ionophores at present typically have ether or carbonyl oxygens arranged in the form of a cage or ring around a cavity sufficiently large to hold and coordinate various metal ions. In addition to the neutral but polar ether and carbonyl oxygens, certain ionophores contain one (or more) carboxylate groups, that assist in charge neutralization as well as coordination of the metal ion. Others may have aliphatic hydroxyls that seem to assist with maintaining the desired conformation through hydrogen bonding, but coordination of the metal ion by hydroxyl oxygen is not precluded. The ionophores studied thus far are either macrocyclic ligands or open chain compounds that can assume a macrocyclic conformation through head-to-tail hydrogen bonding. Although synthetic cryptates have not generally been considered as ionophores, there is no reason why they should not function as well or better than the macrocyclic ionophores, although for certain metal ions there may be kinetic problems, as mentioned above. Work on the use of cryptand ligands for metal ion transport in low-polarity media is no doubt in progress at the present time in many laboratories.

The properties of ionophores and their metal chelates have been described in detail in several recent reviews (*38-41*). For the purposes of this paper only a few typical examples will be discussed: valinomycin (VII), a macrolide actin (VIII), dicyclohexyl-18-crown-6 (IX), and monensin (X).

Valinomycin is a cyclic dodecadepsipeptide consisting of alternating amino acid and hydroxy acid residues condensed through the carboxylic acid, amino, and hydroxyl groups. The peptide carbonyls form a three-dimensional cage that accommodates K^+ more efficiently than Na^+, with a displacement constant $K_d = [Na^+][KCr^+]/[K^+][NaCr^+]$ of about 10^4.

The macrolide actins (VIII) are cyclic tetraesters that provide eight oxygens arranged approximately at the apices of a cube for coordination of a central metal ion. The coordinating oxygens are derived from alternating ester carbonyls and heterocyclic ether oxygens. The synthetic crown polyethers, represented by dicyclohexyl-18-crown-6 (IX), are somewhat less efficient as chromophores than some of the natural compounds, but have the advantage of higher chemical stability since they are not subject to hydrolytic attack. It seem likely that the efficiency of the crown ethers may be improved by the introduction of alkyl groups and the careful building of suitable chirality into the parent polyethers.

Monensin (X) is an example of the naturally occurring carboxylic acid ionophores. It consists of a linear arrangement of ether-containing heterocyclic rings. The chirality of these ring systems and adjacent asymmetric carbons favors a cyclic arrangement of the molecule, which is further stabilized by head-to-tail hydrogen bonding. There are also two hydroxyl oxygens that seem to be involved in metal coordination.

Because of the membrane permeability of their metal complexes, ionophores have unique biological activity in altering transmembrane metal ion gradients and electrical potentals. Various ionophores differ in ion selectivity as the result of different geometries of the donor atoms surrounding the metal ion-accepting cavity. The metal ion complexing and transport functions of ionophores has led to an increasing list of applications as novel drugs for many purposes, including cardiovascular applications. They are also of interest from a purely chemical and bioinorganic point of view for studies of metal chelation processes *in vivo* and *in vitro*, as well as in chemical and biological media of varying ionic strength. In addition to providing new tools for biological research, ionophors now have practical applications as additives to poultry and livestock feed for increasing the efficiency of meat production, and for the development of potentially useful drugs in man. In view of the rapid large-scale increases in commercial ionophore usage, it seems urgent that our knowledge of their chemical and biochemical reactions be increased so as to improve our understanding of the nature of their pharmacological and toxicological effects.

Effective Stabilities of Metal Chelates in Solution

As indicated above, the formation of metal complexes and chelates in biological systems is controlled by the stability (equilibrium) constants (K_n) for combination of metal ions with each ligand present. It is a common fallacy to consider that the ligands with the highest stability constants for a given metal ion will result in the highest degree of formation of the corresponding metal complexes. There are many competing reactions that prevent the existence of such a simple parallel relationship between published stability constants and degree of formation of the chelate compounds. Stability constants are generally directly applicable only under special conditions which are most favorable for chelate formation—in the absence of competing ions and in the optimum pH region in which the ligand is fully dissociated. Generally, the effectiveness of a chelating agent *in vivo* is reduced substantially by competing ions that may be present in the

VII Valinomycin

VIII Macrolide Actins

IX Metal Complex of Dicyclohexyl-18-crown 6

X Monensin

biological system under consideration. Neglecting other competing ligands, calcium ions, about $10^{-3}M$ in physiological systems, hydrogen ions ($10^{-7}M$) and hydroxide ions ($10^{-7}M$) constitute the most serious interferences which compete with either the ligand for the metal ion or with the metal ions for the ligand. Thus the greater the basicity of the chelating agent, the greater is its affinity for the metal ion, but this effect is paralleled by a higher affinity for protons, so that hydrogen ions may provide very strong competition for the metal ion, except for relatively high pH conditions under which the ligand is fully dissociated (deprotonated). This competition is due to the formation of acid forms of the ligand, thus lowering the concentration of the free basic form of the ligand available for metal binding. It should also be noted that these competing reactions may vary considerably from ligand to ligand. The relative effectiveness of various ligands in coordinating metal ions will differ considerably from the relative stability constants if they exhibit considerable differences in proton affinity.

In the design of ligands to achieve selectivity of metal binding in biological systems, it is helpful to calculate interferences by the use of the appropriate ligand protonation constants, the binding constants of competing metal ions for the ligand(s) in question and the hydrolysis constants of the metal ions.

In the case of proton interference, the term α_L, which represents the fraction of ligand in its completely deprotonated form, is frequently employed

$$[L^{m-}] = T_L \alpha_L \quad (3)$$

where T_L is the sum of the concentrations of free ligand and its protonated species (i.e., the sum of the concentrations of all non metal-coordinated forms of the ligand). The formation of the protonated species is governed by the corresponding equilibrium constants.

$$\beta_i^H = [H_iL]/[H^+]^i[L] \quad (4)$$

$$\alpha_L = (1 + [H^+]\beta_1^H + [H^+]^2\beta_2^H + \ldots [H^+]^n\beta_n^H)^{-1} \quad (5)$$

The corresponding expressions for calcium ion and hydroxide ion interference are given by Eqs. (6) and (7), respectively, and the effective binding constant, K_{eff}, of the metal chelate is given by Eq. (8).

$$\alpha_{CaL} = ([Ca]\beta_{CaL})^{-1} \quad (6)$$

$$\alpha_M = (1 + [OH^-]\beta^{OH}_1 + [OH^-]^2\beta^{OH}_2 + \ldots [OH^-]^m\beta^{OH}_m)^{-1} \quad (7)$$

$$\log K_{eff} = \log \beta_{ML} - n \log(\alpha^{-1}_{CaL} + \alpha^{-1}_L) - \log \alpha^{-1}_M \quad (8)$$

Equation (8) takes into consideration only the soluble mononuclear hydroxy complexes of the metal. In concentrated solutions, and in the presence of a precipitate of the insoluble hydroxide of the metal ion, the situation is considerably more complex. Generally, however, the conditions are such that only soluble mononuclear hydroxy metal species need be considered, and interference by hydroxide ion, as well as hydrogen ion, is dependent only on pH. If one uses 7.4 as the pH value of greatest interest for physiological systems, the effective metal binding constants of natural and synthetic ligands may be compared. Some representative values are given in Table 8, in which Fe^{3+} is selected as an important biological metal ion which as a hard acid requires hard bases for effective binding in aqueous systems. The ligands containing phenolic groups (1-5 and 11-13) have very high proton affinities and the value of K_{eff} is considerably lower than the stability constants β_n. For the aminopolycarboxylic acid ligands (7-10) there is less proton competition than for the phenols, but the K_{eff} values are reduced by strong Ca^{2+} competition.

Reaction Kinetics

The equilibrium principles described above cannot be applied to the determination of speciation, or changes of speciation, of metal complexes and chelates in biological systems without considering reaction kinetics. Most of the metal ions of interest because of carcinogenic effects establish equilibrium in aqueous solutions with simple chelating and complexing ligands at moderate or rapid rates. Thus most aquo ions would be converted rapidly to complexes or chelates such as acetates, citrates, glycinates, etc., and equilibria would be established in fractions of a minute or less. The main exceptions to this generalization are the trivalent ions, Cr^{3+}, Co^{3+}, and Rh^{3+}. Simple complexes of these ions may require a few days to reach equilibrium. The theoretical basis for this behavior was first described by Taube (*42*).

For the more labile metal ions, combination with multidentate ligands to form complexes containing relatively large numbers of chelate rings may occur very slowly. Rates of substitution may be greatly decreased by steric effects, as in macrocyclic, cryptate, and ionophore complexes of the transition metal ions. In addition, for metal chelates having large numbers of chelate rings, the ligands must "unwrap" from around the metal ion before the latter may be transferred to another chelating ligand. The kinetics of such processes, and the way ligand-ligand metal ion transfer may be catalyzed by additional weak ligands, have been described by Margerum (*43*). An interesting example of this type of catalysis is the acceleration by nitrilotriacetic acid (NTA) of the transfer of iron(III) from transferrin to desferriferrioxamine. Metal chelates of highly multidentate ligands that are rigid and cannot unfold represent the extreme in slow substitution rates. Thus the porphyrin chelates of otherwise reasonably labile transition metal ions are kinetically extremely stable and their rates of

Table 8. Iron (III) affinities of chelating agents in terms of standard and effective stability constants.[a]

No.	Name	Structure	Formula of chelate	n	log β_n	Log K_{eff}
1	2,3-Dihydroxynaphthalene-6-sulfonic acid, H_3L		FeL_3^{6-}	3	44.2	18.20
2	Salicylic acid, H_2L		FeL_3^{3-}	3	35.3	8.01
3	8-Hydroxyquinoline, HL		FeL_3	3	36.9	20.9
4	1,8-Dihydroxynaphthalene-3,6-sulfonic acid, H_4L		FeL_2^{5-}	2	37.0	20.81
5	3-Isopropyltropolone, HL		FeL_3	3	32.0	21.77
6	Acetoxyhydroxamic acid, HL	CH_3—C(=O)—NH—OH	FeL_3	3	28.3	13.10
7	Nitrilotriacetic acid, H_3L	N(CH_2COOH)$_3$	FeL_2^{3-}	2	24.3	8.17
8	Ethylenediaminetetraacetic acid, H_4L	($HOOCCH_2$)$_2$N—CH_2CH_2—N(CH_2COOH)$_2$	FeL^-	1	25.0	8.10
9	Diethylenetriaminepentaacetic acid, H_5L	($HOOCCH_2$)$_2$NCH_2CH_2N(CH_2COOH)CH_2CH_2N(CH_2COOH)$_2$	FeL^{2-}	1	28.0	10.96

[a]Standard data used in these calculations are: $\log \beta_1^{FeOH} = 11.09$; $\log \beta_2^{FeOH} = 21.96$; $-\log K_{sp}\,Fe(OH)_3 = 10^{41}$; $\log \beta_4^{FeOH} = 24.4$; $-\log K_w = 13.795$; pH = 7.40.

Table 8. (continued)

No.	Name	Structure	Formula of chelate	n	log β_n	Log K_{eff}
10	Triethylenetetraminehexa-acetic acid, H_6L	$(HOOCCH_2)_2NCH_2CH_2(N(CH_2COOH)CH_2CH_2)_2N(CH_2COOH)_2$	FeL^{3-}	1	26.8	10.61
11	N,N′-Bis(*o*-hydroxybenzyl)-ethylenediamine-N,N′-diacetic acid, H_4L		FeL^{-}	1	39.7	20.78
12	Ethylenebis-N,N′-(2-*o*-hydroxy-phenyl)glycine, H_4L		FeL^{-}	1	33.9	16.20
13	Enterobactin, H_6L		FeL^{3-}	1	52	25
14	Desferrioxamine B, H_3L	$CH_3-C(=O)-N(OH)-(CH_2)_5NHCO(CH_2)_2-C(=O)-N(OH)-(CH_2)_5NHCO(CH_2)_2-C(=O)-N(OH)-(CH_2)_5NH_2$	FeL	1	30.6	16.34

metal-ion displacement or transfer are generally immeasurably low.

Future Research Needs

It was suggested above that information on the speciation of metal chelates in biological systems is essential for an understanding of their functions and reaction mechanisms. Metal complexes introduced into the body may undergo exchange with one or more of the large number of natural ligands present in physiological systems, so that the reactions observed may not be characteristic of the original species.

An interesting example of this type of chemical behavior of metal chelates in physiological systems is provided by recent experience with the use of gallium(III) citrate and a number of other gallium(III) complexes and chelates as radiopharmaceuticals. It seems that, regardless of the nature of the gallium compound employed, the patterns of adsorption of radioactive gallium on tumors remained very similar. Eventually it was realized that all moderately stable and weak gallium(III) complexes were converted to the more stable transferrin chelate. Thus the patterns observed turned out to be characteristic of the gallium(III) chelate of transferrin, which has hard phenolate donor groups that form very stable chelates of other trivalent ions as well as of iron(III). For a gallium(III) chelate to retain its integrity in physiological systems, it must be administered in the form a chelate which is significantly more stable than the gallium(III) chelate of transferrin or any other trivalent metal ion receptors that may be present. It seems, therefore, that the study of metal ion reactions in biological systems requires prior information on the stabilities of chelates formed with any synthetic and naturally-occurring ligands that may be present, and that such information is essential to the understanding of the mechanisms of action of metal compounds in the body.

As recently pointed out by Furst (*2*), the carcinogenicity of metal compounds seems to be related to metal-nucleic acid interactions, which may profoundly influence the replication process. Certainly more information is needed concerning the selectivity of binding of metal ions to the bases and phosphate oxygens of DNA.

In nucleic acids the donor groups available for metal ion coordination are the phosphate oxygens of the ribose phosphate backbone, the oxygen and nitrogen atoms of the bases, and to a considerably lesser extent the ribose hydroxyl groups. The donor groups of the bases have affinity for the more electronegative transition metal ions, while more basic metal ions coordinate with the phosphate oxygens. The latter are normally neutralized and coordinated by magnesium ions. The binding of toxic metal ions by nucleic acids either through displacement of Mg^{2+} ions, or by combination with the heterocyclic nitrogen bases, may alter their conformation and structure and lead to impaired function.

DNA polymerase involved in replication requires the interaction of proteins with the DNA template, and Mg^{2+} ions are essential in this process. The substitution of unnatural metal ions such as Be^{2+} for Mg^{2+} results in serious errors in replication. DNA polymerase is bound to DNA by Zn^{2+} ions, while Mg^{2+} binds deoxynucleotide triphosphates to the enzyme. The activating metal ions for RNA polymerase systems are activated by Mg^{2+} and Mn^{2+}, and inhibited by a considerable number of other metal ions. Both replication and transcription require Mn^{2+} and Mg^{2+} for activation, which involves the unwinding of double-stranded DNA and subsequent rewinding. The displacement of these activating metal ions by other metal ions would be expected to strongly interfere with the replication process. The much higher affinity of Be^{2+} over Mg^{2+} for the phosphate oxygens, for example, could lead to irreversible changes in structure and function.

Since most essential trace metals are carcinogenic, it seem probable that the observed carcinogenic properties occur when there is an imbalance, or a large excess, of a metal ion over the concentration required for normal function, or over the amounts that can be handled by the protective mechanisms available in the body. On the basis of these tentative concepts it becomes obvious that the testing of these theories and the development of an understanding of metal carcinogenesis will require a much higher level of knowledge than now exists on the speciation of metals in the various compartments of the body and the changes of speciation that occur when metal ions migrate from one compartment to another, as the result of changes of conditions and of changes in the natural ligands available. Only with such information will it be possible to carry out controlled experiments on carcinogenic metal compounds under conditions such that the nature of the activating metal species will be known.

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