Iron is a transition metal that forms chelates and complexes with various organic compounds, also with phenolic plant secondary metabolites. The ligands of iron affect the redox potential of iron. Electrons may be transferred either to hydroxyl radicals, hydrogen peroxide or molecular oxygen. In the first case, oxidative stress is decreased, in the latter two cases, oxidative stress is increased. This milieu-dependent mode of action may explain the non-linear mode of action of juglone and other secondary metabolites. Attention to this phenomenon may help to explain idiosyncratic and often nonlinear effects that result in biological assays. Current chemical assays are discussed that help to explore these aspects of redox chemistry.

Introduction

Iron is a transition metal. Transition metals are elements whose atoms have incomplete d sub-shells. They usually occur in two or more oxidation states and easily form complexes and chelates. Complexes are designated as chelates if bonds to more than one binding site of the ligand are formed. Many organic molecules that contain unsaturated bonds, oxygen, nitrogen or sulphur functions, qualify as ligands of transition metals. For biological chemistry, the transition metals of the fourth period, from Mn to Zn, are especially important; the dominant oxidation state is $\text{M}^{2+}$ as found in oxides and sulphides. The relative affinity of the metal ion to non-metal atoms or anions, which can donate or share electrons, increases as follows: $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} (\text{Ni}^{2+}) < \text{Cu}^{2+} > \text{Zn}^{2+}$

Atmospheric oxygen is relatively unreactive in the triplet ground state ($\text{O}_3$). It has to be activated energetically to singlet oxygen ($\text{O}_2$) or by reduction to other reactive oxygen species, the superoxide anion radical ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) or the hydroxyl radical ($\cdot\text{OH}$). Reduced transition metals, especially $\text{Fe}^{2+}$ and $\text{Cu}^{+}$, represent important electron donors for one electron transfer reaction that may reduce molecular oxygen (1) to $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ to $\cdot\text{OH}$ (2). The latter reaction is known as the Fenton reaction.$^{2,3}$

\[ \text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^- \] (1)

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \] (2)

This mini review focuses on iron as an exemplary transition metal and its effects on the redox chemistry of complexes with the phenolic secondary metabolites. (±)-Catechin, a flavan-3-ol, and juglone, a naphthoquinone, represent the discussed examples. The applied methods and conclusions from two recent papers from our lab$^{4,5}$ are discussed in relation to other papers that focus on similar questions.$^{6-8}$

Redox- and Iron Homeostasis

In living tissues, iron is complexed by various organic ligands because of the high reactivity of transition metals to form these complexes. In plants, a common ligand is nicotinamide (vitamin B$_3$) (3)$^9$

\[ \begin{align*}
\text{Nicotinamide} & \rightarrow \text{NH}_2 \text{O} \\
\text{O} & \text{N}
\end{align*} \] (3)

Nicotinamide shows chemical functions that are important for the complexation of iron: unsaturated bonds, nitrogen and oxygen. These functions provide electrons for the complex. Many plant metabolites, both primary and the so-called secondary, are characterized by the same functional groups. The catalytic efficacy of iron for the reactions outlined above, however, is affected decisively by the identity of the organic ligand in the complex.$^{10}$ Recently, in a comprehensive review, the role of iron in complex with variable ligands in combination with $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ was pointed out to merit more attention.$^{11}$ Iron and, to a lesser degree, other transitory metals are involved in many degradation processes in nature that include, at the first glance, unrelated areas such as disease development in vertebrates$^2$ and litter decomposition in...
soil. The most reactive and destructive ROS is ‘OH; it only needs to acquire one electron to form water and thus hydroxyl radicals react very fast with any organic molecule that happens to be close enough. Amongst others, it oxidizes fatty acids (lipid peroxidation), DNA and proteins. Consequently, cells have evolved various protective mechanisms that include strongly reducing low-molecular metabolites, such as ascorbic acid and glutathione, carotenoids and tocopherols in the chloroplasts of plants, as well as enzymes, superoxide dismutase, catalase and ascorbate peroxidase. Their concerted actions aim to maintain the redox homeostasis in the cell. Iron homeostasis is closely linked to redox homeostasis. Iron ions participate in electron transfers, not only to reduce H₂O₂ to ‘OH as in the Fenton reaction but also in the respiratory electron transport chains and, in plants, in photosynthesis and the biosynthesis of chlorophyll.

**Assays for the Exploration of Iron Redox Chemistry**

Ligands compete for transition metals and this complicates experimental setups. Consequently, designing and performing assays that explore the chemistry of variably liganded iron require care. It is important to decrease undesired complexation of iron by reactants used in the assay. Here, chelates with EDTA facilitate the exploration of the chemistry of the test compound in the non-liganded state provided that the chelate is formed before the test compound and other reagents are added to the solution. One assay that allows exploring the redox chemistry of test compounds is the deoxyribose degradation assay, which is added last, reduces Fe²⁺ (added as FeCl₃) to Fe²⁺.

The reduced iron enters the Fenton reaction. H₂O₂ and hydroxyl radicals are produced that specifically attack 2-D-deoxyribose. Malonyldialdehyde, a decomposition product of 2-D-deoxyribose is quantified colourimetrically. The Fenton reaction also runs if iron is chelated by EDTA (4).

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \cdot \text{OH} \]  \hspace{2cm} (4)

Similarly, phenolic secondary metabolites, such as (±)-catechin and juglone, chelate iron ions if EDTA was not added to the solution previous to the addition of the test compound (5).

Our results suggest that, if iron is chelated by juglone and not by EDTA, its redox potential is probably shifted to the anodic direction. As a result, iron is more easily reduced and more difficultly oxidized. Conversely, (±)-catechin shifted the redox potential of iron in the complex to the cathodic direction. As a result, iron is more difficultly reduced and more easily oxidized. Nevertheless, the redox potentials generally depend on the concentrations of the reactants and reaction products (Nernst equation).

The variant of the deoxyribose assay that facilitated the detection of this effect only contained Fe³⁺ but no H₂O₂ and ascorbic acid. The H₂O₂ that was required by the Fenton reaction was formed by the reduction of molecular oxygen to O₂⁻ by iron that was chelated either by EDTA or by (±)-catechin and juglone, which also may reduce it. The O₂⁻ dismutates then to molecular oxygen and H₂O₂. The results obtained by our two studies strongly support the view presented by Kell: inappropriate iron chelation may contribute to disease development due to the fact that different ligands may either increase or decrease the redox potential of iron that catalyzes the generation of ROS, especially that of ‘OH. In the assessment of those reactions, the quality of the milieu has to be taken into consideration. If hydroxyl radicals are formed close enough to the liganded reduced iron, then Fe²⁺ may reduce OH⁻ to water. From this fact it becomes apparent that liganded iron whose redox potential is shifted to the anodic direction may affect both pro- and antioxidative effects that are ascribed to the ligand.

Chvátalová and colleagues provide a possible explanation for the behavior of (±)-catechin as a ligand. They show that catechols as ligands facilitate the autoxidation of Fe²⁺ to Fe³⁺ more compared to phenolic compounds lacking ortho-dihydroxy groups. The experiments were carried out at a pH of 7.4, the same as found in the cytosol. Unfortunately, many studies carried out by chemists are performed at a higher pH due to the higher redox reactivity facilitated by this milieu. Chvátalová and colleagues used ferrozine that selectively chelates Fe²⁺ ions to determine the autoxidation of Fe³⁺ in the complex. They provided evidence that supports our observations by an independent method that was applied to phenolic acids with similar catechol functions. The ferrozine assay, however, has a distinct disadvantage: at higher concentrations, the catechol competes with ferrozine for the iron. The modified versions of the deoxyribose assay, however, are not affected by comparable competitive reactions between ligands.

**Iron Chelators as Siderophores**

Today, invasive plant species represent a major challenge for conservation and considerable research efforts are directed at obtaining improved insights into the underlying processes. This research field represents a good example to discuss the ecological interpretation of iron complexation. Secondary metabolites have obtained some attention as they were proposed to act as chemical weapons for invasive plants. They may also function as siderophores that facilitate the uptake of iron and other transition metals. The improved micronutrient uptake then contributes to improved performance of the plant. A recent study focussed
on 8-hydroxyquinoline. This secondary metabolite is exuded by the roots of *Centaurea diffusa*, an invasive knapweed species in North America. Originally, attention was directed to this compound in attempts to elucidate its role as a chemical weapon because it was shown to possess pronounced phytotoxic activities. Tharayil and colleagues, by contrast, pursued the hypothesis that 8-hydroxyquinoline complexes iron and thus improves the uptake of this transition metal by roots in a nonspecific fashion. Fe$^{3+}$ forms a rather lipophilic complex (6) that, however, is the uptake of this transition metal by roots in a nonspecific fashion that 8-hydroxyquinoline complexes iron and thus improves.

The notable lipophilicity of the complex supports the hypothesis of improved iron uptake efficacy of Tharayil and colleagues. The observation that complexes with iron and copper reduce the phytotoxic effects of 8-hydroxyquinoline contradicts the documented pro-oxidative activity of the complex by other authors. Reduced iron and copper ions in the complex with 8-hydroxyquinoline easily reduce hydrogen peroxide and oxygen. But, depending on its presence, it also can reduce hydroxyl radicals. Manganese—here the authors unfortunately did not specify the oxidation state but we assume it was II—and zinc (II) did not decrease the toxicity of 8-hydroxyquinoline in the experiments carried out by Tharayil and colleagues. Manganese (II) is good in reducing but less competitive in forming complexes; zinc (II) does not reduce and has lower affinity to complexes. The alleviating effects of the copper and iron complexes that were observed in those experiments may have been caused by a non-linear hormetic mode of action. Scavenging of ROS by lower concentrations of the test compound, if they are present, and generation of ROS by higher, if they are absent, may contribute to these hormetic effects. Unfortunately, the classical statistics used to estimate EC$_{90}$ assumes a sigmoid dose-response relation that neglects non-linear effects.

In the previously focused study, the iron uptake mechanism of *Centaurea maculosa*, another invasive knapweed species, was also investigated to compare it with *C. diffusa*. This *Centaurea* sp., which is more or less conspecific with *C. stoebe*, a species that occurs in dry wastelands in central and eastern parts of Europe, was shown to exude (±)-catechin, a flavan-3-ol. Tharayil and colleagues observed that *C. maculosa* was also able to take up iron more efficiently than *C. solstitialis*, a further invasive knapweed species that lacks the exudation of either 8-hydroxyquinoline or (±)-catechin. The authors suggest that this process may utilize a chelate complex similar to that of 8-hydroxyquinoline with (±)-catechin (7). The ligands of the thus acquired iron and of course the amount of the iron itself then determine if the uptake is beneficial to the plant or not. The border between improved micronutrient acquisition and heavy metal stress is narrow. At least our results suggest that iron-(±)-catechin complexes shifts the redox potential of iron to a more negative value. If mutants that possess deficiencies in iron uptake are assayed, as it is the case with the 8-hydroxyquinoline study, the reductions initiated by Fe$^{3+}$ contribute more to maintenance than to disturbance of the redox homeostasis.

**Iron Chelators as Chemical Weapons**

At present, a vivid debate is under progress if (±)-catechin is toxic and stable enough in soil to act as a chemical weapon, the controversial positions are summarized elsewhere.7,8 Very recently, Pollock and colleagues published a study that pointed out the conditionality of the phytotoxic effects of (±)-catechin that is caused by complexation of various metals in the soil.7 The rationale behind the presented research is conclusive as (±)-catechin as ligand determines the redox chemistry of the metal in the complex or chelate. Pollock and colleagues observe that iron and copper as chelators contribute to the instability of (±)-catechin. This is not astonishing as iron and copper are involved in enzymatic and non-enzymatic reactions resulting in the generation of ‘OH that ultimately attack biopolymers such as cellulose and lignin in litter decomposition.12 Most probably, the (±)-catechin in iron and copper complexes that was added to the pot cultures in the experiments carried out by Pollock and colleagues may have been decomposed by this chemistry. The addition of iron and copper to the soil may have accelerated the process. Our experiments with iron-catechin complexes suggest that the iron in the complex is less oxidative. In soils, however, iron may be complexed by other ligands, e.g., humic acids. In this study, the most toxic metal in the complex with (±)-catechin is calcium, actually classified as soft and not a transition metal. Calcium is well known to decrease the availability of phosphorus for root uptake; it might have been replaced by more competitive metals in the complex and then reacted with phosphate or the phosphate became a part of the calcium-(±)-catechin complex. In both cases, this might have led to a dramatic decrease in the bioavailability of phosphorus. Unfortunately, the authors did not consider this aspect but the complexity of the research problem usually limits the manageable experimental approaches.
There is, however, one issue that the development of more informative assays cannot address: Many of the existing studies, including the study by Pollock and colleagues,\(^7\) that try and have tried to provide evidence that (±)-catechin is a chemical weapon for invasive plants purchased the compound from a source that has recently been implicated as unreliable. Contaminations probably affected the results. Thus, one of the key publications that originally documented the phytotoxicity of (±)-catechin in vitro was retracted recently.\(^27\) The studies that we carried out that originally documented the phytotoxicity of (±)-catechin in probably affected the results. Thus, one of the key publications has recently been implicated as unreliable. Contaminations for invasive plants purchased the compound from a source that tried to provide evidence that (±)-catechin is a chemical weapon with juglone 4 and (±)-catechin 5 show that, in terms of toxicity, (±)-catechin does not compare to juglone but is much less efficient in causing pro-oxidative effects. In terms of radical scavenging, antioxidative activity, both compounds are more similar. We interpret the redox cycling properties of both compounds as beneficial for the producing plants in terms of surviving biotic and abiotic stress.\(^3\) Juglone is usually compartmented as glycoside in the plant, with good reasons; the free species would be too reactive. Small amounts of deglycosylated juglone, however, may serve as a redox signal in the tissue.

**Conclusions and Perspective**

The chemistry of transitions metal complexes represent an enigma even to researchers that are well versed in chemistry. Nevertheless, this chemistry seems to play a pivotal role in biological chemistry; its dimension has been reviewed recently\(^8\) and the more than 2,400 references listed in this review serve well to characterize the scope of the dimension. Most probably, the main interest of the scientific community will be directed to the role of complexes of iron in disease development. For ecologists and biologists, however, it may prove equally beneficial to pay more attention to redox chemistry because it may provide valuable hints to obtain improved insights into a wide range of phenomena in various scales, from single cells to ecosystems. Especially if we deal with biologically active metabolites, we have to be aware that not only their functional groups and the applied dosage but also the milieu defines the effect. Many biological metabolites reduce. The acquisition of electrons, oxidation, can be a destructive process. The reduction of strong oxidants, e.g., \(\cdot OH\), helps a cell to survive. Conversely, the reduction of molecular oxygen may lead to the formation of \(\cdot OH\). Transition metals are involved in this chemistry. We have good reasons to assume that the quality of the ligand decides the rate of \(\cdot OH\) formation from molecular oxygen. Cells use the formed ROS as signals and are thus able to react to a wide range of biotic and abiotic stress. Too strong disturbances of the redox homeostasis, however, induce apoptosis, programmed cell death.\(^28,29\)

The variants of the deoxyribose assay that were developed by us\(^5\)—the main merit goes to the first author—are simple to perform and also allow non-chemists to explore the transition metal mediated redox chemistry of plant metabolites. Several of these variants were designed with attention to decrease ligand concentration between reagents added to the solution and the test compound. They also comply with recommendations for further research directions voiced by authoritative reviewers of polyphenol redox chemistry.\(^1\) The results that we can expect to obtain from these assays may help to understand various idiosyncratic effects observed in physiological and ecological studies; we exemplified this for the results from two recently published papers.\(^20\)

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