Inorganic-Biochemical Perspectives of Sporadic Prion Diseases

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Preface

Between 1980 and roughly 1996, about 750,000 cattle infected with *BSE* (bovine spongiform encephalopathy) were slaughtered for human consumption in Great Britain, and it is now clear that BSE, also known as "*mad cow disease*" is not merely a UK phenomenon, nor is it merely an economic nuisance. The sudden and explosive increase of BSE in recent Europe (1990-2000) may have been spread among cattle by the feeding of infected offal, but the majority of cases of naturally occurring prion diseases arise *sporadically with no known cause*. Thus, *the most important problem to be solved is to elucidate the intrinsic chemical mechanism of the prion diseases which arise sporadically,* i.e., we must answer the questions:

What induces the conversion of normal prion protein into an abnormal isoform, and how the abnormal isofom forms without the infected offals ?

Many years ago ALS (amyotrophic lateral sclerosis) patients were collectively found in the New Guinea and Papua islands, and its origin has been attributed to the subterranean water, which contains much Al^{3+} *and* Mn^{2+} *ions*. In Alzheimer's disease specific region such as the hippocampus and the motor cortex contain elevated iron levels relative to normal, and abnormalities in brain iron metabolism have been described for several neurodegenerative disorders, including Alzheimer's diseases, Parkinson's disease, Huntington's, and prion diseases. Investigations of scrapie, CJD, and chronic wasting disease clusters in Iceland, Slovakia and Colorado, respectively have indicated that the soil in these regions is *low in copper* and *higher in manganese.*

Above facts suggest that the sporadic *prion* and other *neurodegenerative diseases* are closely related with the function of several transition-metal ions, and *thus* ***inorganic-biochemical perspectives are necessary in order to elucidate the chemical mechanisms of pathogenesis of these diseases.***

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Chapter I. Introduction

Between 1980 and roughly 1996, about 750,000 cattle infected with *BSE* (bovine spongiform encephalopathy) were slaughtered for human consumption in Great Britain, and it is now clear that BSE, also known as "*mad cow disease*" is not merely a UK phenomenon, nor is it merely an economic nuisance. In fact, it may be an impending world-wide health crisis, and in recent months several other European countries have found BSE in their cattle herds, and over the past few years about 100 mostly young individuals have fallen victim to a fatal condition known as new *variant Creutzfeld-Jacob disease* (vJCD). [1-3] BSE and vJCD are one of the transmissible spongiform encephalopathies (TSEs, or *prion disease*) which are group of fatal neurodegenerative disorders that include BSE, vJCD, *scrapie* of sheep, chronic wasting diseases (CWD) of mule deer and elk, as well as Gestmann-Straussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) of humans. [4,5] At present it is generally recognized that BSE may have originated from a *scrapie agent* infecting small ruminants, which have been recycled through cattle and disseminated through the use of contaminated meat and bonemeal. Compelling evidence links vCJD to exposure to beef infected with BSE prions, and recent studies have suggested that blood-borne transmission of CJD is *highly* possible.

Some 250 years ago, a sheep disease that presented with excitability, itching, ataxia and finally paralysis and death was recognized and this is known today as *scrapie* in English-speaking countries, "the trembles" in France, "trotting disease" in Germany and "itching disease" in Japan, reflecting the gamut of its symptoms. The first major advance in scrapie research took place in 1936 when Cuille and Chelle succeeded in transmitting the disease to sheep and goats by inoculating them with lumbar cord of diseased animals. Subsequently, transmission to mice and hamsters provided more-convenient experimental models. It was soon recognized that the transmissible agent had quite extraordinary properties, such as unusually long incubation periods, measured in months to years, and uncommon resistance to high temperature, formaldehyde treatment and UV irradiation. Enriching fractions from Syrian hamster (SHa) brain for scrapie infectivity led to the discovery of the *prion protein* (PrP), and at present it is generally accepted that the central event in TSEs is the

post-translational conversion of the normal cellular prion protein (PrP^{C}) into an abnormal isoform of called *scrapie PrP* (PrP^{Sc}) that *has a high-β-sheet content* and *is associated with transmissible disease.* (see Figure 1) [6] These misfodled prions (PrP^{Sc}) ultimately kills neurons and leaves the brain riddled with holes, like a sponge, and the 1997 Nobel Prize in Physiology and Medicine was awarded to Professor *S. Prusiner* of the University of California, San Francisco, for his contributions towards the identification of the infectious agent that causes TSEs.

PrP^{C} is a glycoprotein expressed on the surface of many cell types (see Figure 2) and the fact that the protein is *expressed in neurons at higher levels* than in any other cell types suggests that PrP^{C} has special importance for neurons. Additionally, PrP^{C} is highly concentrated at the *synapse* and there is evidence for intense localization not only as central nerves synapse but also at endplates. PrP^{C} is linked to the cell membrane by glycosylphosphatidylinositol (GPI) anchor. (see Fig. 2) [7] It has one or two sugar chains that are closely linked to the C-terminus and also exists in a non-glycosylated form. PrP^{Sc} is extracted from affected brains as highly aggregated, detergent-insoluble materials that is not amenable to high-resolution structural technique. PrP^{Sc} is covalently indistinguishable from PrP^{C}. During infection, theunderlying molecular events that lead to the conversion of PrP^{C} to the scrapie agent remain ill defined.

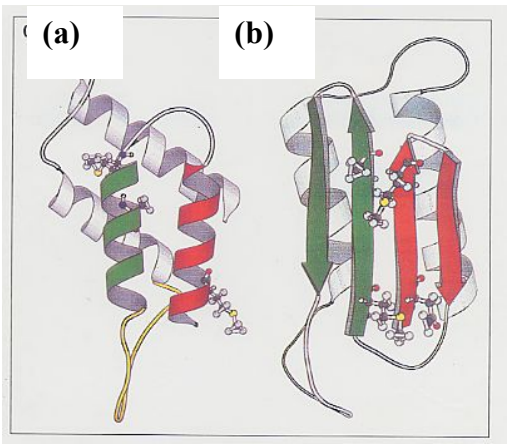

Figure 1. Plausible models for the tertiary structures of PrP^{Sc} and PrP^{C}.

(a) The proposed three-dimensional structure of $PrP^{C.}$ It has been believed that helices 1 and 2 are converted into a β-sheet structure during the formation of PrP^{Sc}.

(b) The proposed three-dimensionalstructure of PrP^{Sc}.

(S. B. Prusiner, *Trends Biochemical Sciences*, 1996, **21**, 482)

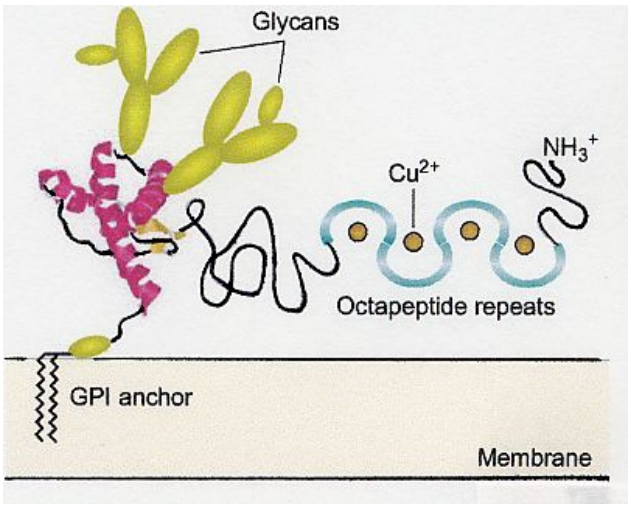

Figure 2. Model of PrP^C structural domains.

The folded C-terminal portion of PrP^C that contains the short β-sheet strands and the α-helix is based on a model derived from NMR-based coordinates of residues of hamster PrP. (B. Caughey, *Trends Biochemical Sciences*, 2001, **25**, 235)

Recent studies have showed that PrP^C not only binds *copper* (Cu) within the octarepeat region located in the unstructured N-terminus, but under certain specific circumstances may bind along the C-terminal structured domain of protein fragments. Furthermore, recombinant PrP^C can also bind other metals such as manganese at both the octarepeats and the C-terminal sites. [8] Indeed, accumulating evidence suggests that metallochemical alterations may play a role in the pathogenesis of prion diseases and other neurodegenerative diseases. It has been demonstrated that both recombinant and brain-derived PrP have *superoxide dismutase* (SOD)-like activity when Cu is bound to the octarepeat region resulting in conformational changes to the protein. [8]

The sudden and explosive increase of BSE in recent Europe may have been spread among cattle by the feeding of infected offal [9-12] but the majority of cases of naturally occurring prion diseases arise *sporadically with no known*

cause. Thus, ***the most important problem to be solved is to elucidate the intrinsic chemical mechanism of the prion diseases which arise sporadically,*** i.e., we must answer the questions:

What induces the conversion of PrP^{C} to PrP^{Sc} and how the PrP^{Sc} forms in the life process without the infected offals ?

The sporadic neurodegenerative diseases are in general *endemic*; many years ago ALS (amyotrophic lateral sclerosis) patients were collectively found in the New Guinea and Papua islands, and its origin has been attributed to the drinking subterranean water, which contains much *Al^{3+} and Mn^{2+} ions*, and in these regions many patients of Alzheimer's and Parkinson's diseases were found, [13] and increased aluminum levels were reported in the hippocampus of patients with Alzheimer's disease. [14] In Alzheimer's disease specific region such as the hippocampus and the motor cortex contain elevated iron levels relative to normal, whereas the occipital cortex contains decreased levels, and abnormalities in brain iron metabolism have been described for several neurodegenerative disorders, including Alzheimer's diseases, Parkinson's disease, Huntington's, and prion diseases. [14-17] Investigations of scrapie, CJD, and chronic wasting disease clusters in Iceland, Slovakia and Colorado, respectively have indicated that the soil in these regions is low in copper and *higher in manganese*, and Brown et al. observed striking elevation of manganese ion accompanied by significant reduction of copper ion bound to purified PrP in all sCJD (sCJD = *sporadic CJD*) variants. [18] Brown et al. have reported that it loses the SOD-like activity when Cu is replaced with Mn in recombinant PrP, and also that Cu binding to PrP purified from sporadic CJD was significantly decreased while the binding of Mn and Zn was markedly increased. [18] These results suggest that altered metal-ion occupancy of PrP plays a pivotal role in the pathogenesis of prion diseases.

In this book we will show the new concept on the "oxidative stress" induced by the metal ions such as copper, manganese, and iron, etc, [19] *to lead to the sporadic prion diseases and other neurodegenerative diseases which include ALS, Alzheimer's and Parkinson's,* ***and will postulate the comprehensive***

chemical mechanism of the sporadic prion diseases..

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Chapter II Parkinson's Disease: Deficiency of Neurotransmitters and Neural Cell Death due to Oxidative stress

In October 2000, the Nobel Assembly awarded the Nobel Prize in Physiology and Medicine to *Carlsson* of the University of Gothernburg in Sweden and to two pioneers in the study of nerve cell communications; Paul *Greengar*d of Rockefeller University in New York City, who figured out how dopamine and other neurotransmitters trigger target neurons when they bind at the *synapse*, the junction between two nerve cells; and Eric *Kandel* of New York's Columbia University, who built on these insights to demystify some aspects of learning and memory. Carlsson overturned conventional wisdom by proving that dopamine, once thought to be merely a precursor in the synthesis of the neurotransmitter norepinephrine (see Figure 3), is an important nervous system messenger in its own right. They gave rabbits a drug that depletes norepinephrine in the brain, putting the animals into a temporary stupor. Carlsson found that the rabbits could be roused with injections of L-dopa, which the brain converts to dopamine. Later they discovered that Parkinson's diseases results from degeneration of dopamine-producing neurons in the brain involved movement control. That finding led to the use of L-dopa as a therapy for Parkinson's patients.

Greengard et al. have found that in most neurons the neurotransmitters exert their effects by triggering a so-called second messenger inside the target cells. This in turn activates an enzyme that adds phosphate groups to cellular proteins, setting off a chain of events that alter nerve cell properties. To date, they have identified more than 100 brain proteins phosphorated as a result of neurotransmitter activity, including that serves as a kind of master control switch for dopamine. The link between phosphorylation and nerve cell signaling inspired the research of Kandel into how the brain learns and remembers. They demonstrated that the responses of *Aplysia'*s nerve cells to various stimuli were amplified according to the strength and duration of the stimuli.

Parkinson's disease (PD) is a common neurodegenerative disorder that is

clinically characterized by tremor, bradykinesia, rigidity, and loss of postural reflexes. It is generally believed that the major symptoms of PD are caused by a striatal *dopamine (DA) deficiency*, secondary to degeneration of nigrostriatal dopaminergic neurons and possibly a decreased DA-biosynthetic capacity in the surviving cells. [1,2] Although the DA loss is most pronounced, norepinephrine, serotonin, and melanin pigments are also decreased, whereas cholinergic activity seems to be increased. The selective loss of specific neurons in the central nervous system (CNS) is a characteristic feature for PD and other common neurodegenerative disorders, such as Alzheimer's disease, Huntington's chorea, amyotrophic lateral sclerosis (ALS), and also "mad cow disease". Although originally discounted, hereditary factors have emerged as the focus of research in PD; recent studies suggest that hereditary factors play an important role in sporadic PD, and two genes are clearly associated with the diseases; α-synuclein and parkin, and as a third, gene ubiquitin C-terminal hydroxylase L1. [3]

Let us at first consider the *biochemical synthesis of dopamine*. The chemical mechanism of dopamine synthesis has been elucidated by the biochemists, and the result is illustrated in Figure 3. Dopamine is synthesized from phenylalanine and tyrosine, one of the 20 essential amino-acids through the oxygenation reaction at the benzene ring by the enzymes, phenylalanine hydroxylase (PAH) or tyrosine hydroxylase (TH) (see Figure 4). It should be noted here that the oxygenation at the benzene ring does not occur in the air without the catalyst, *and thus it is necessary for us to know the detail chemical mechanism of the enzymes, TH or PAH, and or tryptophan hydroxylase, which catalyzes the formation of* ***serotonin*** *from* ***tryptophan****;* the deficiency of serotonin has been proposed to induce several metal diseases, such as depression etc.

TH is a *non-heme iron protein* that uses one molecule of dioxygen to hydroxylate its amino acid and tetrahydropterin substrates to hydroxy-amino acids and 4a-hydroxytetrahydropterins, respectively. (see Figure 4) [4,5] As the 4a-hydroxytetrahydropterins subsequently dehydrates and is regenerated by the NADH-dependent enzyme dihydropteridine reductase, it is frequently termed a cofactor for the peteridine-dependent hydroxylases. The cofactor (BH_4) is the most abundant of the unconjugated tetrahydropterins in mammalian tissues and is considered to be the naturally tetrahydropterin substrate for these enzymes. The active site structure and catalytic mechanism of the aromatic amino acid

hydroxylases have been investigated by kinetic and spectroscopic techniques, as well as by site-directed mutagenesis. *The iron is necessary for catalytic turnover*, (see chapter V) and the tetrahydropterin and amino acid substrates bind close to the iron(II) center, but probably without a direct coordination to the metal center. Thus, ***it is clear that iron-deficiency should lead to deficiency of neurotransmitters, such as dopamine, serotonin, etc.***

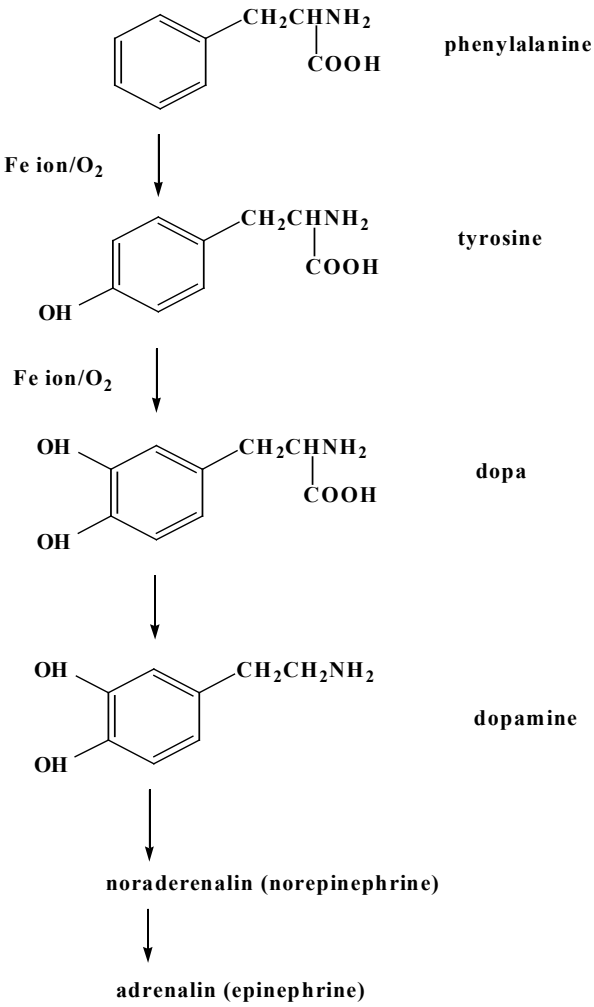

Figure 3. Synthesis scheme of neurotransmitters

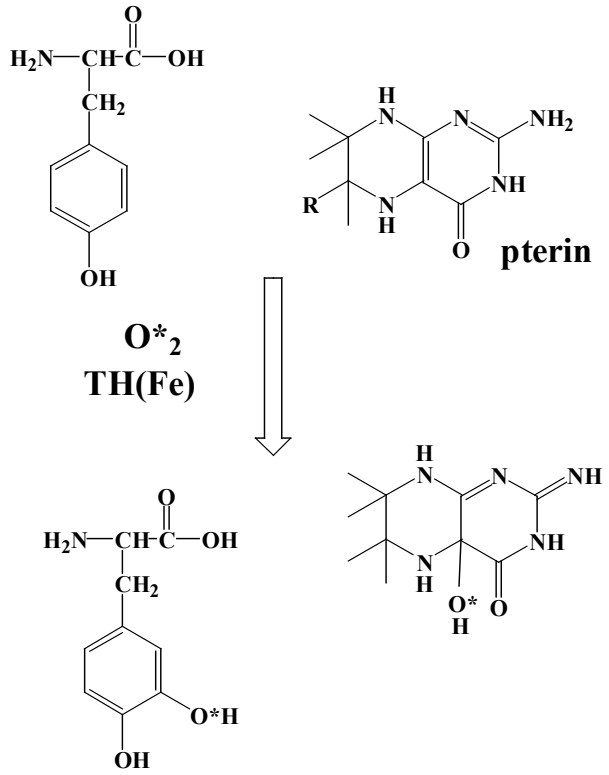

Figure 4. Formation of dopa from tyrosine catalyzed by TH

As indicated in Chapter I, increased brain iron concentrations at some special regions have been described in Parkinson's disease, [1] *but these iron ions do not contribute to the formation of dopamin*e; the reason will be developed in Chapter VI. The cause of nigral cell death in the Parkinson's disease remains unsolved, but many authors have pointed out the hypothesis that the cellular degeneration observed results from ***oxidative stress***. [2,6-8] *Oxidative stress manifests itself as an increased oxidation of cellular constituents (lipids and proteins) and DNA damage*. *Lipid peroxidation and protein damage* have been observed in the SN of PD patients, which *suggests* that oxidative stress is involved in the pathogenesis of this disease.

The increased iron ions observed in these Parkinson's and Alzheimer's disorders has been *frequently* suggested to play a role in catalyzing the production of the *so-called* oxygen free radicals via the metal dependent reduction of hydrogen peroxide. This reaction, sometimes referred to as the Fenton reaction, *may* involve the reaction of hydrogen peroxide with ferrous ion to produce the potentially damaging hydroxyl radical (OH•) (*see below*), and many authors have insisted that this OH• should be a main active oxygen species in the oxidative stress,

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\bullet$$

but it should be noted that free intracellular ferrous iron concentration have been calculated to be very low, below 10^{-8} *M, [9] and nobody has succeeded in confirming the* OH• *formation by the reliable chemical methods.*

I would like to show that* OH• *does not exert the oxidative stress in the living cell, and to propose the new concept for oxidative stress in this monograph (see chapter III) [10] and also show that deficiency of neurotransmitters due to the abnormal iron metabolism in brain leads to the neural cell death.

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Chapter III Nishida's Concept on Activation of Oxygen Molecule

The electronic configuration of the oxygen atom is $1s^2 2s^2 2p^4$. When two O atoms combine to form O_2, the same orbital types combine if they are of equal or approximately equal energy. Thus, the 1s, 2s, $2p_x$, $2p_y$, and $2p_z$ on one oxygen combine with the similar orbitals on the other oxygen to give, in each case, two MO's. The five AO's on each atom give rise to ten MO's in the molecule. [1]

Since the electrons will occupy the orbitals in the order of increasing energy, we must arrange our MO's in an energy sequence so that we can place our sixteen electrons properly. One of the most instructive ways to do this is by means of the molecular orbital energy diagram method. In the ***oxygen molecule*** we have the combination of both 1s and 2s orbitals to give four σ type orbitals, [1] two bonding and two antibonding, each of them
occupied by two electrons. Because the 1s electrons are not valence electrons, we usually pay little heed to them. The combination of the 2s orbitals does not result in any net bonding. (see Figure 5)

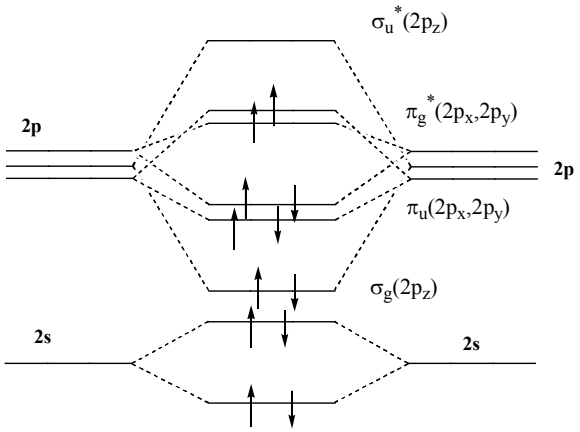


Figure 5. MO scheme for O_2.

The three atomic p orbital levels in the isolated atom are of equal energy (degenerate); but when we bring one atom into the field of the other, the p_z orbitals pointing toward the other atom start to interact to form a σ bond between the two atoms. the corresponding σ^* orbital are generated. These orbitals $\sigma_g(2p_z)$ and $\sigma_u^*(2p_z)$, are in fact σ molecular orbitals because they are symmetric with respect to rotation around the internuclear axis (in this case, z-axis). The bonding interaction is quite large, and hence the splitting is also relatively large. (see Figure 5)

The p_x and p_y orbitals on each oxygen combine to each from a π set: $\pi_u(2\pi_u(2p_x)$, $\pi_g^*(2p_x)$, $\pi_u(2p_y)$, $\pi_g^*(2p_y)$. The p_x and p_y orbitals are perpendicular to each other. Now, if we refer to our MO energy diagram, we see that six electrons of 2p orbitals are referred to as valence electrons, that is these electrons occupy $\sigma_g(2p)$ and $\pi_u(2p)$. If we follow the principles used for the periodic classification, the two electrons must go separately into the $\pi_g^*(2p_x)$ and $\pi_g^*(2p_y)$ orbitals, *with spin parallel* (Hund's rule: see Figure 5 and Table 2). The two unpaired electrons in the π^* orbitals give rise to the *paramagnetic properties of molecular oxygen*. The diradical character and accompanying paramagnetism of oxygen constitute its outstanding property.

The occupation of antibonding orbitals by one or more electrons cancels some of the bonding attraction between the atoms. In the O_2 example, we have two π bonding orbitals, each doubly occupied, and a σ bonding orbital, doubly occupied, or a total of three bonding orbitals. However, each of the two electrons in an antibonding orbital cancel the bonding effect of an electron in a bonding orbital, and so the net bonding in oxygen can be considered to result from a double bond. Evidence for the effect of occupation of the antibonding orbitals comes from bond distances. In the ground state, the bond distances between oxygen atoms is 1.207 A. (see Table 1) However, when O_2 is ionized by loss of an electron from one of the π^* antibonding orbitals, the resulting O_2^+ is 1.123 A, a considerable decrease, indicative of stronger bonding in the ion. The bond lengths of O_2^- (a radical anion) and O_2^{2-} are 1.28 and 1.49 A, respectively, confirming the fact that electrons have been added to antibonding orbitals.

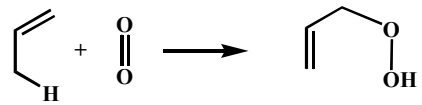
Table 1. Electronic and structural properties of oxygen and its derivatives

	Bond order	compounds	O-O Distance(A)	binding energy kcal/mol	ν(O-O) cm^{-1}
O_2^+	2.5	O_2AsF_6	1.123[a]	149.4	1,858[c]
O_2	2	O_2	1.207[a]	117.2	1,554.7[d]
$O_2(^1\Delta_g)$	2	O_2	1.216[e]	94.7	1,483.5[g]
O_2^-	1.5	KO_2	1.28		1,145[h]
O_2^{2-}	1	Na_2O_2	1.49	48.8	842[j]

[a]J. C. Abraham: *Quart. Rev. Chem. Soc*., 10, 407(1956); [b]G. Herzberg, "Molecular Spectra and Molecular Structure", 2nd edition. [C] J. Shamir, J. Beneboym and H. H. Classen, *J. Am. Chem. Soc*., 90, 6223 (1968). [d]Ref. b. [e]M. Kasha and A. U. Khan, *Ann. N. Y. Acad. Sci*., 171, 5 (1970). [f]Calculated from the data in footnote d. [g]L. Herzberg and G. Herzberg, *Astrophys. J*., 105, 353(1947). [h]J. A. Creighton and E. R. Lippencott, *J. Chem. Phys*., 40, 1779(1964). [i] S. N. Foner and R. L. Hudson, *J. Chem. Phys*., 36, 2676 (1962). [j]J. C. Evans, *J. Chem. Soc*., D, 682 (1969).

It is known from chemical studies that O_2 can be converted from its ground triplet state to a singlet state if energy is supplied, usually in the form of light in the presence of a photosensitizer. Two types of *singlet oxygen* are known (see Table 2) and of these *$^1O_2(^1\Delta_g)$ is more interesting*. Singlet state $^1O_2$ ($^1\Delta_g$) has a reactivity which is quite different from that of triplet O_2; for example, $^1O_2$ ($^1\Delta_g$) reacts very rapidly with alkenes at room temperature to give allylic peroxides or conjugated dienes to give cyclic peroxides. (***see below***)

1,3-Addition(ene-reaction)



1,4-Addition(Endperoxide formation)

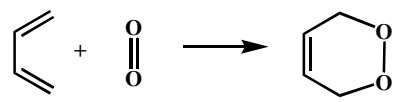


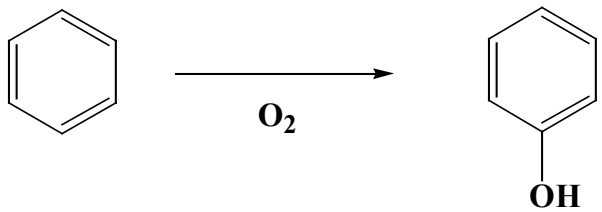
Table 2 Electronic configurations of singlet oxygen

State	$\pi^*(2p_x)$	$\pi^*(2p_y)$	Energy
$^1\Sigma_g^+$	↑	↓	155 kJ(~13,000 cm^{-1})
$^1\Delta_g$	↑↓		92 kJ(~8,000 cm^{-1})
$^3\Sigma_g^-$	↑	↑	0 (ground state)

Oxygen activation in the Oxygenases

Despite its greater reactivity, it is unlikely that singlet O_2 is involved in very many biological oxygenase reactions. For one thing, most of the reactions catalyzed by oxygenases bear little overall resemblance to known reactions of singlet O_2; for example most reactions of singlet O_2 with organic compounds give peroxide products, whereas peroxides are the ultimate products of few oxygenase reactions. Also, singlet O_2 does not react with alkanes or unactivated aromatic compounds, both of which are frequently substrates for oxygenase reactions. However, the most persuasive argument against the involvement of singlet O_2 in biological reactions is that the lowest energy singlet state ($^1\Delta_g$) is 22 kcal/mole higher in energy than the ground state triplet, and it is not apparent how an enzyme could supply electronic energy of that magnitude. [4]

In the previous chapter, we have demonstrated that the oxygenation at the benzene ring (of phenylalanine or tyrosine) does not occur in the air without the catalyst.



Probably the single most important reason for the low kinetic reactivity of O_2 is that O_2 has a triplet ground state---it is a diradical. On the other hand, the stable reduction products (H_2O_2 an H_2O) of O_2, and essentially all stable organic compounds, including the reactants and products of oxygenase reactions, are singlets. The direct reaction of a triplet molecule with a singlet to give singlet products is a spin-forbidden process, i.e., it will not occur readily. In addition to this, the reaction of O_2 with an organic compound to give a triplet product is usually considerably endothermic, and thus cannot occur with most biological molecules at physiological temperature.

How do oxygenases cope with the fact that O_2 is a triplet and still get it to react with organic compounds? ***There appear to be two general methods*** which biological systems have evolved to circumvent this problem. The ***one method*** by which biological systems circumvent the problem that O_2 is a triplet is to have the initial reaction of O_2 occur by a free radical mechanism. An example of particular relevance to biological chemistry is the reaction fully reduced flavin (FH_2) with O_2. This reaction has been shown to proceed by a radical mechanism, but it only proceeds readily because the intermediate flavin seminquinone radical is stabilized by extensive delocalization in the isoalloxazine ring system.

$$FH_2 + O_2 \quad \rightarrow \quad FH\cdot + HO_2\cdot \quad \rightarrow \quad F + H_2O_2$$

The other method involves complexing the triplet O_2 to a transition metal ion which itself has unpaired electrons are more popular in the biological systems. The enzymes which catalyze the insertion of oxygen atom(s) of O_2 molecule into the organic substrates are called “oxygenases”, and many reports have been published on the chemical mechanisms of the enzymes (see later chapter). However, several important problems remain unsolved on the mechanism of oxygenases at present. [10]

Nishida’s Concept on the “Active oxygen species”

Until now, much efforts have been devoted to detection and identification

of the so-called "active-oxygen species", for example a Fe(V)=oxo species, as proposed for cytochrome P-450, one of the monooyxgenases (see Figure 6), but the Fe(V)=O has not been detected in the reaction cycle of the cytochrome P-450 as yet. [10] Many biochemists have considered that "active oxygen species" generates in the reaction course with the enzyme and oxygen, and then it reacts with substrate. But it should be noted here that there are many reports to indicate that enzyme binds oxygen only in the presence of substrate to form the ternary complex, ESO_2, in which oxygen and substrate interact to give a product. However the detail on the interaction between oxygen and the substrate has never been discussed.

I have pointed out the importance of the electronic interaction between oxygen and the substrate in the ternary complex ESO_2, and **proposed the new concept on the oxygen activation,** [5-7,9] *i.e.*, the substrate and peripheral organic moieties around the metal ion plays an important role in activating oxygen, and determining the reaction pathway and the products in the oxygenases, and postulated the new mechanism for P-450 as illustrated in Figure 7. [6] In the case of cytochrome $P\text{-}450_{CAM}$, the presence of threonine-252 is very important as a peripheral group, as shown in Figure 8. I also reported that the reactivity of the Fe(III)-OOH as **an electrophile** is similar to that of the Fe(V)=O species in terms of the EHMO calculation (see Figure 9: Polyhedron, 13 (1994), 2473), and pointed out that the intrinsic active species in the cytochrome P-450 should be a Fe(III)-OOH species as shown in Figure 7.

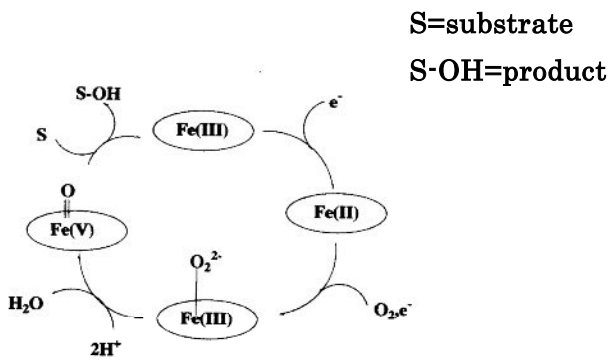


Figure 6. Reaction cycle in P-450 proposed by Groves.

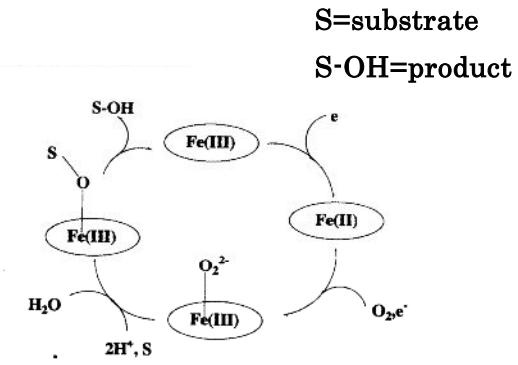


Figure 7. Reaction cycle in P-450 postulated by Nishida.

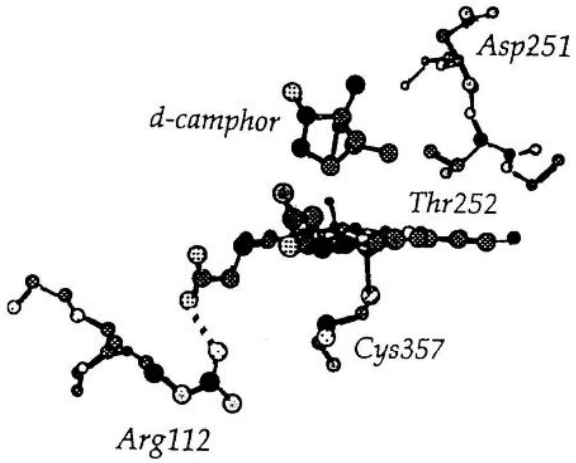


Figure 8. Structure of P-450 in the presence of substrate

In addition to the above, I have observed ***that in many cases oxygen (O_2) and hydrogen peroxide ion (O_2^{2-}) exhibits chemical reactivity similar to that of singlet oxygen ($^1\Delta_g$) in the presence of several metal ions such as iron(II), iron(III) or copper(II);*** [5-7] **in these cases, the electronic structures of triplet oxygen or peroxide molecule is changed through the coordination to a metal**

ion, and this is greatly promoted by the presence of peripheral organic group or substrate. [5-7, 9]

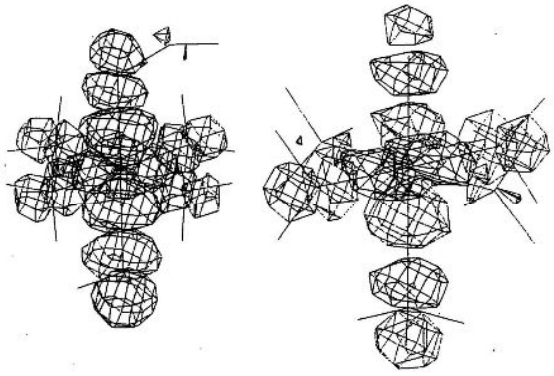


Figure 9. LUMO of Fe(V)=O (right) and Fe(III)-OOH (left)
(Polyhedron, 13 (1994), 2473)

These findings are *especially important* to elucidate the "gain-of-function" observed for ALS patients, and also to investigate the chemical mechanism in several oxygenases, such as Lipoxygenase, TH, PAH, and tryptophan hydroxylase, which will be developed in the subsequent chapters (Chapter V); in the latter three cases the participation of pterin to promote the interaction between oxygen and Fe(II) ion should be stressed. [9] My original idea on the mechanism of oxygenases [5-7, 9] is quite consistent with the recent publications.[11]

Thus I think that the studies to detect, isolate, and identify the so-called "active oxygen species" such as Fe(V)=O species proposed in P-450 [10] may be interesting **on the synthetic chemical point of view, but it is nonsense from the biological point of view.**

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Chapter IV. Copper(II)-hydroperoxide Adduct in Amyotrophic Lateral Sclerosis (ALS) and Prion Diseases

"Gain-of Function" of Copper(II)-hydroperoxide Adduct

Amyotrophic lateral sclerosis (ALS) is a progressive, devastating syndrome that affects both upper and lower motorneurons and results in limb and facial motor weakness, atrophy, and death. [1,2] The age-adjusted world-wide incidence of ALS is 0.5-3 per 100,000 person years (without obvious race-related differences) Older males and postmenopausal females are most typically related. Familial ALS (fALS) accounts for less than 10 % of diagnosed cases, with sporadic ALS (sALS) comprising the remainder of diagnoses. Although the pathogenesis of ALS remains unknown, notable progress has been made in identifying molecular processes potentially involved in ALS-mediated motor neuron injury.

A significant discovery in ALS research was the identification of a genetic defect associated with 10-15 % of fALS kindreds. *The involved gene, SOD1, encodes a cytosolic from of superoxide dismutase (SOD)*, and identified mutations in exons, 1,2,4, and 5 of the SOD1 gene all appear to reduce Cu/Zn-SOD stability.

Differences in the location of three missence mutations apparently affect both clinical fALS severity and measured SOD activity levels. For example, a very short progressive form of fALS in two Japanese kindreds is associated with a His46Arg point mutation on the SOD molecule. This mutation may alter Cu^{2+}-binding to the enzymatic catalytic site and reduces erythrocyte lysate SOD activity by approximately 20 %. In Caucasian fALS kindreds with rapid rates of clinical disease progression, many SOD1 mutations instead code for the dimer contact region and appear to destabilize SOD dimer formation. Two such point mutations (Ala4Val and Gly41Ser) produce an enzyme with less than 5-% of normal Cu/Zn SOD activity, and death of affected patients occurs after less than one year of symptom onset.

To understand ALS pathogenesis, we must understand how altering SOD

activity can induce cell injury. The SOD's are well-known enzymes that catalyze the disproportionation reaction of superoxide ion, [3] which is considered to be one of the reaction oxygen species (ROS), into oxygen and hydrogen peroxide.

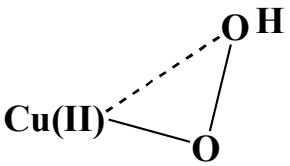
$$O_2^- + Cu(II) \rightarrow O_2 + Cu(I) \quad (1)$$

$$Cu(I) + O_2^- \xrightarrow[H^+]{} O_2H_2 + Cu(II) \quad (2)$$

The crystal structure of the SOD(Cu/Zn) was already determined, [4] which is illustrated below (Figure 10). The copper and zinc ions are bridged by anionic form of imidazole ring of His.

The reaction mechanism of this enzyme has been investigated by many authors. Very recently Nishida et al. have postulated new mechanism for this enzyme based on the results used by the model compounds. We have pointed out the importance of formation of a *copper(II)-OOH species* as an intermediate (see **Scheme-I**) in the second step (2) above, and this hydrogen peroxide produced is *immediately* moved from the *wild-type enzyme* because of the *negligible* interaction between hydrogen peroxide and the copper(II) ion and the surrounding organic groups; the former is due to a distorted square pyramidal structure of the copper(II) ion in the enzyme, and *the hydrogen peroxide* is destroyed into water and oxygen.

Scheme-I



In 1997, Yim et al. have reported that a fALS mutant (Gly93Ala = G93A) exhibits an enhanced free radical-generating activity, while its dismutation activity is identical to that of the wild-type enzyme. [6] In Figure 11, ESR spectra of DMPO-OH radical adducts formed in solution containing H_2O_2 and the human SOD enzymes. These are indicating that fALS symptoms are not associated with the reduction in the dismutation activity of the enzyme. They

reported that the mutant and wild-type enzymes contain one copper ion per subunit *have identical dismutation activities*, however, the free-radical generating activity of the mutant, as measured by spin-trapping method at low H_2O_2 concentration, is enhanced relative to that of the wild-type and G93A, wild-type < G93A < A4V.

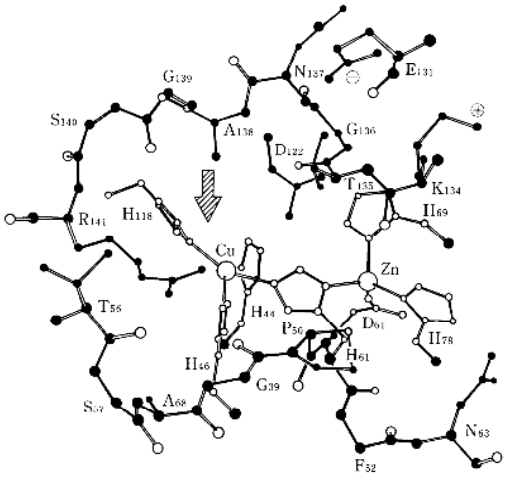


Figure 10. Crystal structure of SOD (Cu/Zn)

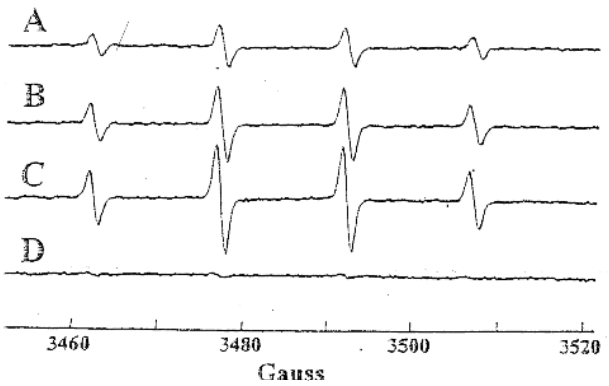
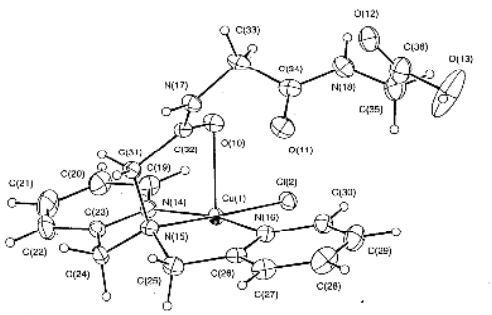


Figure 11. ESR spectra of DMPO-OH radical adducts formed in solutions containing H_2O_2 and the human SOD(Cu/Zn). A; wild-type SOD(Cu/Zn), B; G93A mutant SOD, C; A4V mutant SOD, D; heat-inactivated SOD (Yim *et al.*, J. Biol. Chem., 1997, 272, 8861).

Siddique et al., have determined the crystal structures of human SOD, along with two other SOD structures, and have established that the fALS mutations do not change any active-site residues involved in the electrostatic recognition of the substrate, the ligation of the metal ions or the formation of the active-site channel, but *only the slight change in the neighborhood around the copper(II) ion is detected*. On the basis of rigorous studies defining the structural and energetic effects of conserved hydrophobic packing interactions in proteins, six of the fALS mutations would be expected to destabilize the subunit fold or *the dimer contact*. The most frequent fALS mutations would disrupt both, the subunit fold and the dimer interface. [7,8]



Structure of $[Cu(dpgt)]^+$

In order to obtain the comprehensive solution for the *correlation between the structural change in mutations and pathogenesis of ALS*, we have studied the reactivity of a *copper(II)-OOH*, proposed as an important intermediate in the SOD reaction. For this purpose, we have synthesized many copper(II) compounds with the ligands which contain N,N-*bis*(2-picolylmethyl)amine moiety, as illustrated in Figure 12. [9] The crystal structure of $[Cu(dpgt)Cl]^+$ is illustrated below; the structural features of all other compounds are essentially similar to each other, but *the slight change was introduced around the copper(II) ion*.

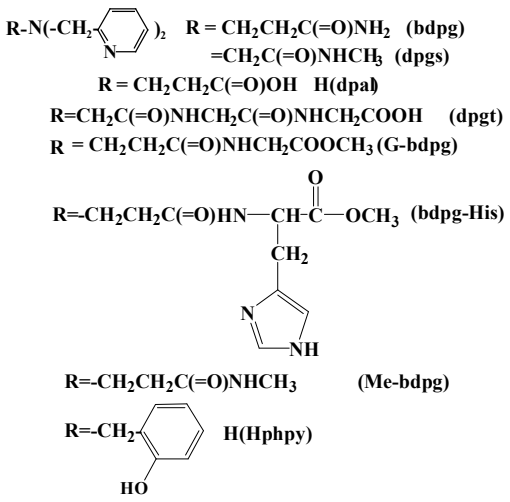
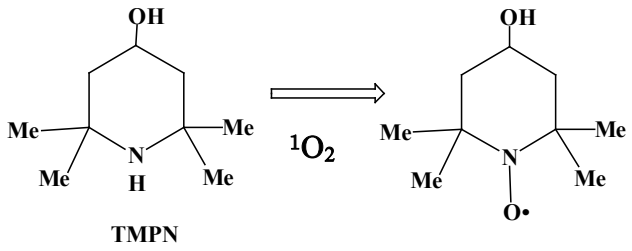


Figure 12. Chemical structures of the ligands used in our study

We have measured the ESR spectra of the solution containing a copper(II) complex and spin-trapping reagent, such as PBN (α-phenyl-N-*t*-butylnitrone) and TMPN (N,N,N',N'- tetramethyl-4-piperidinol), specific reagents for OH• radical and singlet oxygen ($^1\Delta_g$) (**Scheme-II**), respectively. [10]

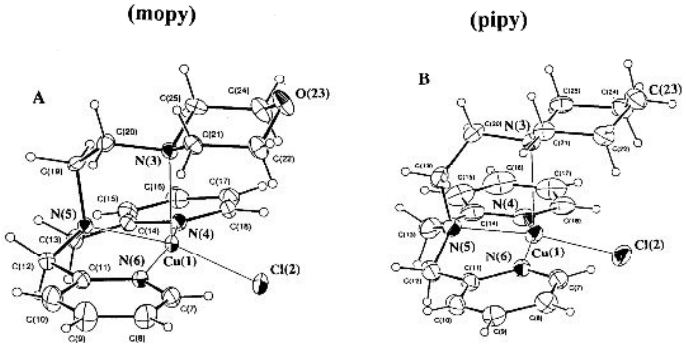


TMPN

Scheme-II

No ESR signal due to the formation of radical of PBN was detected when the copper (II) complex was mixed with H_2O_2 and PBN. However, strong peaks due to *nitron radical formation* of the corresponding TMPN **(Scheme-II)** was detected in some cases; especially comparison between the $Cu(pipy)Cl^+$ and

the Cu(mopy)Cl^+ is interesting. Structural features of the two compounds are essentially the same, instead the difference of the oxygen atom on the morphorin ring of Cu(mopy)Cl^+ complex is replaced by the $-CH_2$ in the Cu(pipy)Cl^+ complex. (see the figure below)

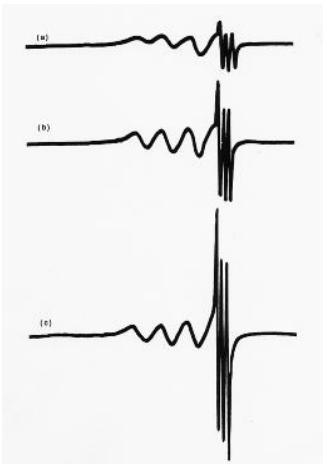


In the case of Cu(pipy)Cl^+, no formation of the *nitron radical* was observed; on contrast to this, high activity for the radical formation by the Cu(mopy)Cl^+ complex was detected as illustrated in Figure 13. The similar high activity for radical formation of TMPN was also observed for the copper(II) complex with H(phpy), $[Cu(Hphpy)Cl]^+$. In this case, similar to the Cu(mopy)Cl^+ complex, the addition of the H_2O_2 to the copper(II) solution does not induce the change in ESR spectrum due to the copper(II) ion; but the addition of TMPN leads to the dramatic change in the ESR signals attributed to the copper(II) species (i.e., the change of hyperfine structure values due to copper atom). These are all comprehensively elucidated on the assumption that the complex formation of copper(II), hydrogen peroxide, and TMPN occurs only when three reagents are present in the solution, (see the Figure 14), and unique reactivity of the hydrogen peroxide observed is detected only when the intermediate is formed in the solution.

It should be remembered here that in the previous section, we stated that --however, the most persuasive argument against the involvement of singlet O_2 in biological reactions is that the lowest energy singlet state ($^1\Delta_g$) is 22 kcal/mole higher in energy than the ground state triplet, and it is not apparent how

an enzyme could supply electronic energy of that magnitude--. *Our present results clearly show that some copper (II) chelates can activate the hydrogen peroxide to exhibit high reactivity similar to that of the singlet oxygen ($^1\Delta_g$). [10]*

Figure 13. ESR spectra of the solution containing $[Cu(mopy)Cl]^+$, H_2O_2, and TMPN. (a) measured immediately at the addition of hydrogen peroxide, (b) after 5 minutes addition of hydrogen peroxide, (c) after 15 minutes addition of hydrogen peroxide



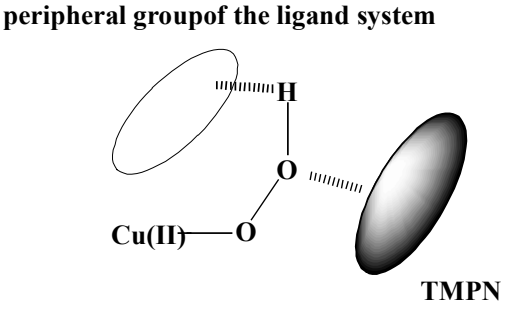
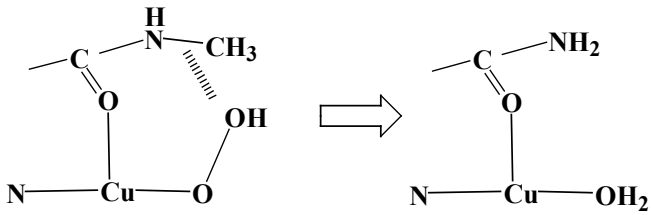


Figure 14. Assumed intermediate among copper(II) chelate, H_2O_2 and TMPN.

In order to get further information on the reactivity of a copper (II)-OOH species, we have measured the ESI-Mass spectra of the solutions of copper (II) compounds and hydrogen peroxide. When hydrogen peroxide was added to the Cu(Me-bdpg)Cl solution (see Figure 12), the formation of [Cu(bdpg)Cl], not [Cu(dpal)], was detected by ESI-Mass spectra. [11] These are clearly indicate that Cu(II)-OOH species can cleave the peptide at the C-N bond *oxidatively, not hydrorytically*, because the hydrolytic cleavage may give Cu(dpal) species from the Cu(Me-bdpg) compound.



We also reported that some copper(II) complexes exhibit high activity to oxygenate the methionine residue of amyloid beta-peptide(1-40) at sulfur atom [12], and decompose the several proteins [13] in the presence of hydrogen peroxide. All these facts may indicate that the "*gain-of-function*" of the mutant SOD is due to formation of a *long-lived highly reactive copper(II)-OOH* as an intermediate in the process of mutant SOD reaction; the chemical structures around the copper(II) in the mutant SOD is slightly changed, and this gives an unexpected effect on the reactivity of a copper(II)-OOH as observed in our papers. *In the mutant SOD* the C-N bond cleavage by the Cu(II)-OOH may give great changes in the surface of SOD, leading to destabilizing of the dimer contact of the SOD enzyme. [14] Recent studies have shown that this destabilizing of the dimer contact of the SOD enzyme should be a most serious risk factor to induce ALS. (O.-Matsumoto and Fridovich, Proc. Natl Acad. Sci. USA., 99(2002), 9010; Yamanaka and Cleveland, Neurology, 65(2005), 1859).

It has been generally believed that hydrogen peroxide is relatively inert and not toxic to cells, but ***our experimental facts clearly show that formation and***

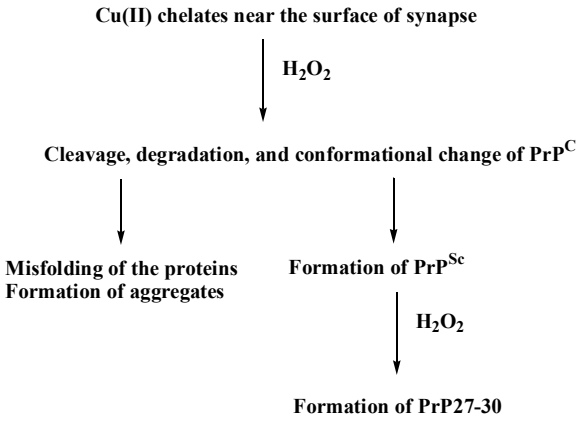
existence of a highly reactive Cu(II)-OOH species is an intrinsic origin for oxidative stress in the pathogenesis of ALS, [15] and that the reactivity of the Cu(II)-OOH is determined by the structural properties of the intermediate, chemical interactions of copper(II)-OOH species with peripheral groups and substrate (see Figure 14), [16] and clearly show that OH· radical does not play a role in this process.

Copper(II)-OOH in Sporadic Prion Diseases

PrP^C is a glycoprotein expressed on the surface of many cell types and its genetic code was identified only after the isolation of an abnormal isoform, PrP^{Sc} from brains of mice that were infected with the disease scrapie. [17-19] It is generally recognized that PrP^C is a copper-containing protein (at most 4 copper ions are present within the octarepeat region located in the unstructured N-terminus (Figure 2 in page 5)). Since 1996 there has been increasing evidence that PrP^C increases cellular resistance to oxidative stress. Cerebelle neurons and astrocytes from PrP^C knockout mice are more sensitive to superoxide toxicity, whereas cells with higher levels of PrP^C expression are more resistant to oxidative stress. [17] Analysis of recombinant mouse and chicken PrP^C has lead to the *discovery of an important "gain-of-function"* following the formation of the PrP^C copper complex; PrP^C has been shown to contribute directly to cellular SOD activity. Recombinant PrP^C that has as least two atoms of copper bound specifically has an activity similar to that of superoxide dismutase.

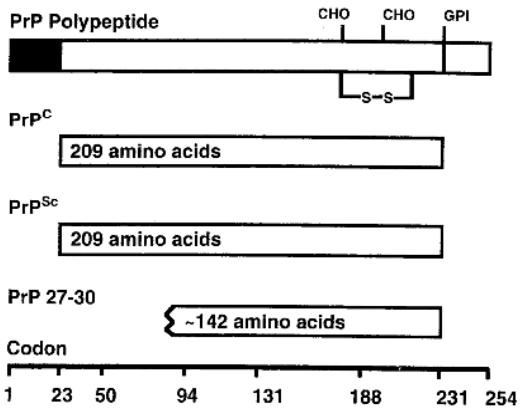
The copper at the synapse is released in vesicles and studies of copper concentration have suggested that the level can reach 250 mM locally. The copper released in this way appears to be taken up rapidly by the neurons, and deployed within 30 minutes of this process. It is unknown in what from this copper is bound, however it is probable that the copper is chelated to some peptides or amino acids because there is little free copper found in the body. [20] It has been pointed out that the copper(II) chelate compounds which across the membrane may originate from the cleavage of the PrP^C [17]. Based on the results described in the previous section it seems quite likely that *these copper(II) chelates react with hydrogen peroxide to yield a Cu(II)-OOH species, giving serious effects toward the PrP^C such as oxygenation at methionine residue,*

conformational change(i.e., formation of PrP^{Sc}), and degradation of protein, if hydrogen peroxide is present in the vicinity of synapse (see **Scheme-III**).



Scheme-III

As described in Chapter I, the misfolded prions (PrP^{Sc}) ultimately kills neurons and leaves the brain riddled with holes, like a sponge. In addition to PrP^{Sc}, another protease-resistant PrP of 27-30 kDa, which is called as PrP27-30 was extracted from affected brains. (see the Figure below)



It should be noted here that PrP27-30 is derived from only PrP^{Sc} (not from PrP^{C}), and no difference in amino acid sequence between PrP^{C} and PrP^{Sc} have been identified. Based on these facts we may assume that the chemical environment around the copper ion in the PrP^{Sc} should be different from those in the PrP^{C}; this situation is similar to the difference observed between the those around copper(II) ions in the wild-type and mutant SOD enzyme. Thus, it is most likely that the "gain-of-function" in the PrP^{Sc} due to a "*highly reactive*" *Cu(II)-OOH formation may occur* as described for the mutant SOD molecule, which leads to the cleavage of the peptide bonds around the copper ion (near at about 90 site), giving PrP27-30 in the presence of hydrogen peroxide. (see **Scheme III**)

We reported that some copper(II) complexes exhibit high catalytic activity to oxygenate the sulfur atom of methionine of amyloid beta-peptide in the presence of hydrogen peroxide.[12] Oxidation of methionine residues in the prion protein by the hydrogen peroxide attracts recent interests; [24] and it has become apparent that Met 129, a residue located in a polymorphic position in human PrP and modulating risk of prion diseases, was also easily oxidized as was Met 134. The structural effect of H_2O_2-induced methionine oxidation leads only to a modest increase in β-sheet structure. *Several experimental facts observed for the native prion proteins* [21,22,23] *seem to be consistent with our results as described in the previous section,* and *the presence, formation, and the serious roles of hydrogen peroxide in the biological oxidative stress have been confirmed by the present authors.* [15,25] All these findings support our proposal [15] that ***hydrogen peroxide, which derives from the abnormal iron ion metabolism,*** *(see Chapter VI)* ***should be the serious origin for the oxidative stress in sporadic prion diseases (***see **Scheme III**)**.**

PrP^{Sc} formation from PrP^{C} during Infection

PrP^{Sc} is extracted from affected brains as highly aggregated, detergent-insoluble materials that is not amenable to high-resolution structural technique. PrP^{Sc} is covalently indistinguishable from PrP^{C}. During infection, the

underlying molecular events that lead to the conversion of PrP^{C} to the scrapie agent remain ill defined. *In vitro experiments* have shown that when PrP^{C} was taken out of the context of the membranes, it can binds selectively to PrP^{Sc} and be converted to a protease-resistant state that is indistinguishable from that of PrP^{Sc} itself. A schematic model of this two-step process is suggested. [18] Although this PrP^{Sc}-induced conversion reaction was originally demonstrated under the cell-free conditions containing non-physiological denaturants, it has been adapted to much more physiologically compatible conditions. In fact, *in situ* conversion reactions have been demonstrated using intact, TSE-infected brain slices, revealing that both amyloid plaque and diffuse deposits of PrP^{Sc} have the ability to induce conversion. The PrP^{Sc}-associated converting activity correlates with scrapie infectivity in guanidine hydrochloride denaturation studies, and further studies based on the protein-protein interaction should be necessary for the purposes.

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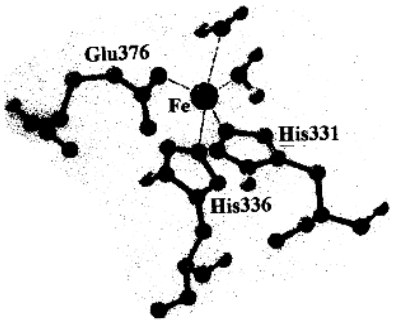
Chapter V. Oxygen Activation in Tyrosine Hydroxylase and its Derivatives:Factors to Prevent the Formation of Neurotransmitters

As shown in Chapter II, the mammalian aromatic amino acid hydroxylases (phenylalanine, tyrosine, and tryptophan hydroxylases; PAH, TH, and TPH, respectively) are a unique class of monooxygenases in their use of tetrahydropterins as obligatory cofactors. [1,2] These enzymes play important roles in mammalian metabolism: PAH initiates the detoxifications of high level of phenylalanine. Phenylalanine hydroxylase catalyzes the formation of tyrosine*; mutations in the enzyme are the most common cause of phenylketonuria.* Tyrosine hydroxylase catalyzes the formation of dihydroxylphenylalanine, the first step in the biosynthesis of the *catecholamine neurotransmitters, including*



dopamine. Tryptophan hydroxylase catalyzes the formation of 5'-hydroxytryptophan, the first step in the biosynthesis of the neurotransmitter *serotonin*. These enzymes are monooxygenases, incorporating one atom of oxygen from molecular oxygen into the substrate and reducing the other atom to water. The two electrons required for the reduction of the second atom to water are supplied by the tetrahydrobiopterin (BH_4) substrate. Phenyalanine hydroxylase is present at relatively abundant levels in liver. *Both tyrosine and tryptophan hydroxylases are found in the central nervous system*. TH is also present in the aderenal gland, a common source of the naturally occurring enzyme.

In the absence of bound substrates or inhibitors, the active site can be identified from the location of the iron atom. This assignment is supported by

the results of Maitines, who used the paramagnetic effects of the metals in TH and PAH to show that both the aromatic ring of the amino acid substrates ate the tetrahydropterin bind closely to the metal. In both structures the iron is located in a hydrophobic cleft 10 A from the surface. The structure of the iron site in TH is shown below. [1,2] There are thee amino acid ligands to the metal, histidine 331 and 336 and glutamate 376. In addition, there are two solvent molecules 2.0 A from the iron, resulting in square pyramidal geometry with histidine 331 as the axial ligand.

A direct relationship between the iron content and the activity was subsequently established for both PAH and TH enzymes. The iron is in the ferric form when the enzymes are purified. Reduction of the iron to the ferrous state is required for PAH to be active, and metal reconstitution studies have shown that TH requires ferrous iron for activity. Tetrahydroptrins can reduce the iron atom in either enzyme, suggesting that BH_4 is the physiological reductant.

As shown in Figure 4, one of the products of the reactions catalyzed by all three hydroxylases is a 4a-hydroxypterin. The oxygen atoms in both the amino acid and the pterin products have been shown to come from molecular oxygen. The formation of this compound establishes that there must be a reaction between molecular oxygen and tetrahydropterin during catalysis. It has been accepted that 4a-peroxytetahydropterin is the intermediate results of the reaction of the tetrahydropterin and molecular oxygen; Based on the model studies with both flavins and pterin, this reaction was assumed to occur in two steps; initial slow, single-electron transfer to form superoxide and the pterin cation radical followed by rapid combination of the two radicals. The 4a-peroxypterin may be considered as a candidate for the hydroxylating intermediate, since the analogous 4a-peroxyflavin is thought to be the hydroxylating intermediate in the flavin phenol hydroyxlases. Fitzpatrick et al. have proposed [2] that the range of the reaction resembles the reaction catalyzed by cytochrome P-450-dependent hydroxylases [3], in which the hydroxylating intermediate has been believed to be a high-valence iron-oxo species, and thus it seems likely that a more reactive species containing the iron, an iron-oxygen species is involved.

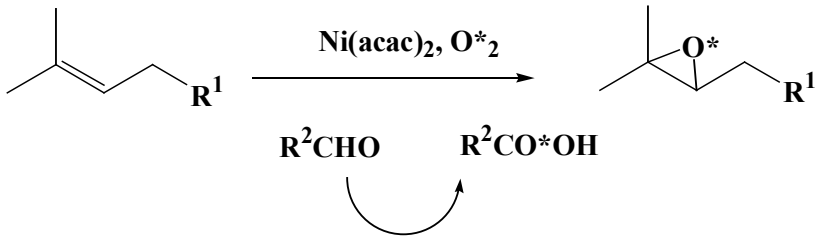
Dix and Benkovic reported [4] that the amount of hydroxylated amino acid

formed agreed well with the amount of 4-hydroxypterin, suggesting that the oxygen-oxygen bond is not cleaved unless hydroxylation occurs, and no hydrogen peroxide was detected. Iron is clearly required for activity, and the mutation of the ligands to the iron results in the loss of detectable activity. Electron paramagnetic resonance spectrum of enzyme frozen at 7 K during turnover does not show any ESR-detectable species, and this can be rationalized by formation of the hydroxylating intermediate being much slower than its substitution reaction. An additional potential role for the iron atom would be to assist in the reaction of tetrahydropterin and molecular oxygen to form the peroxypterin. The ^{18}O kinetic isotope effects determined by Francisco et al. are consistent with either a single-electron transfer from tetrahydropterin to oxygen, or with equilibrium binding of O_2 to the iron atom followed by single-electron transfer from the tetrahydropterin to the iron-bound oxygen. Dix et al have suggested the peroxypterin-iron complex as a reasonable intermediate, and pointed out that heterolytic cleave of the oxygen-oxygen bond of the species would generate the peroxypterin and the iron-oxo hydroxylating species directly.

I cannot agree with the opinion that high-valent iron-oxo species is an active species in the cytochrome P-450, [5] and since there is no reasonable chemical evidences to support the peroxopterin formation proposed by Dix et al., we have started the study on the mechanism of these hydroyxlases, and proposed quite different mechanism from those published before, and we would like to point out that only our mechanism can elucidate the mechanism of the hydroxylses reasonably, and give a reasonable explanation for the correlation between the neurodegenerative diseases and metal ions.

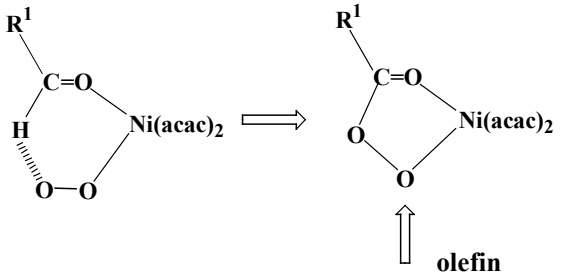
Nishida's mechanism for the Hydroxyalses

In 1991, Mukaiyama et al. have reported that in the presence of a catalytic amount of bis(1,3-diketonato)nickel(II) complex, trisubstituted and exo-tetrminal olefins or norbornene are smoothly monooxygenated into the corresponding epoxides in high to quantitative yields on treatment with aldehyde under an atmosphere pressure of oxygen at room temperature.[6]



This report have attracted much attention for the chemists, because it is generally recognized that the nickel(II) complexes are in generally in the oxidation state II, and redox reactions concerning the nickel(II) ion are almost rare. In the previous papers, we already postulated that in some metal compounds a transition metal ion such iron(III) or copper(II) can activate oxygen without change the oxidation state of the metal ions, [7] ***which is quite against the common sense of general chemists***.

According to the Nishida's mechanism, the epoxidation by the Mukaiyama reaction can be explained as follows: in the reaction mixture, six-coordinate Ni(II) species of acetylacetone, oxygen, and aldehyde, is formed as illustrated in (A) below, to give a nickel(II) complex with *acylhydroperoxide through the* ***insertion of O_2*** *into C-H bond of the aldehyde*, and this reacts with olefin to give the *epoxide and the corresponding acid through the heterolytic cleavage of the O-O bond of the acylhydroperoxide* (B). [8,9]



(A) (B)

In this scheme, the oxidation state of the nickel(II) is unchanged throughout the reaction. The formation of the intermediate **(A)** is promoted *through the interaction of unpaired electrons in the d-orbital of the nickel (II) ion and that of the oxygen molecule* (see the Figure 15), and is also p*romoted through electronic*

interaction with aldehyde molecule. In this process, it is anticipated that the ***oxygen coordinated to the nickel(II) is activated to behave as singlet oxygen*** ***($^1\Delta_g$),*** which was confirmed by us experimentally, [8,9] and this will give a reasonable explanation for the facile formation of the acylhydroperoxide as illustrated in (B) above.

It should be noted here that *the Mukaiyama reaction is very similar to those of the hydroxylases*, such as TH, i.e., two oxygen atoms of O_2 are transferred to the substrate and pterin, respectively in TH; the aldehyde used in the Mukaiyama reaction is replaced by pterin in TH. Thus, we may elucidate the reaction mechanism of the iron-containing hydroxylases by the use of scheme described for the Mukaiyama reaction as shown in **Scheme-IV**, that is the oxygen in the intermediate is activated through interaction with ***pterin, substrate, and d-electron of the Fe(II) ion***, to react with the substrate, giving the hydroxylated product, which is quite consistent with the observed facts. (Fitzpatrick, J. Mol. Biol., **359** (2006), 299.) *In the latter process, it is likely that the substrate promotes the O-O cleavage, as proposed for cytochrome P-450, etc*. [5]

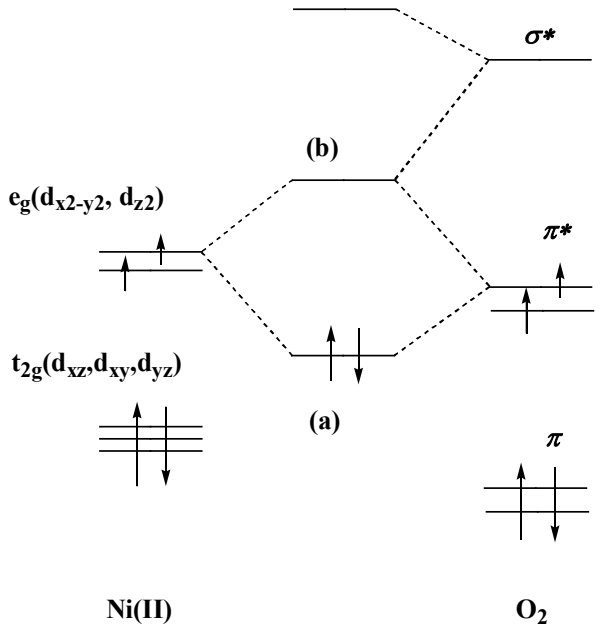
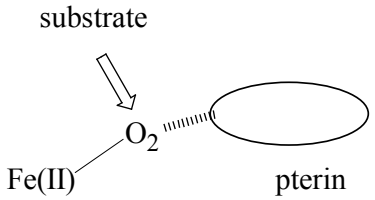


Figure 15. MO scheme for Ni(II)-O_2 system. The formation of orbital **(b)** may be responsible for the appearance of reactivity similar to singlet O_2 ($^1\Delta_g$).

Scheme-IV



substrate:phenylalanine,
tyrosine

In our mechanism the weak interaction between the *unpaired electrons of d-electron and oxygen* is very important, and this interaction was already confirmed experimentally [10-12]. Thus, when iron ion in the hydroxylase is replaced by Al^{3+}, the enzyme cannot give the desired product, because Al^{3+} ion has no unpaired electron. Generally the interaction between unpaired electrons of d-electron and oxygen should be originally weak, but under the special conditions, i.e., in the presence of coenzyme such as pterin, etc., this interaction occurs, and *electronic property of the oxygen in the complex is quite changed from that of the stable triplet oxygen in the air*.

In the Mukaiyama reaction, when the $Co(acac)_2$ was used instead of nickel(II)-acetylacetonato complex, the Co(II) ion is oxidized to a corresponding Co(III) species. [9] This is because that the activated oxygen in the intermediate can oxidize the Co(II) ion, and the resulted Co(III) is greatly stabilized; in the case of nickel(II) compound the oxidation to a Ni(III) is impossible because the Ni(III) state is very unstable in the air, but the best yields of the products are obtained in the case of Ni(II) complex used. The change of the oxidation state of the metal ion is not suitable for this oxygenation reaction

Difference between Fe(II) and Mn(II) ions in Oxidation Reaction

The α-keto acid-dependent enzymes are distinguished from other non-heme iron enzymes by requirement of an α-keto acid cofactor as well as Fe(II) and O_2 for reactivity. (see Figure 16) [13] They now constitute a large class of enzymes which are essential in the biosynthesis of many biological compounds.

Examples of these enzymes are illustrated below: In general, the reactions

catalyzed by these enzymes involve the oxidation of an unactivated C-H bond to give either hydroxylated products. In these systems, α-keto acid may behave as pterin in TH, and the aldehyde in Mukaiyama reaction, respectively.

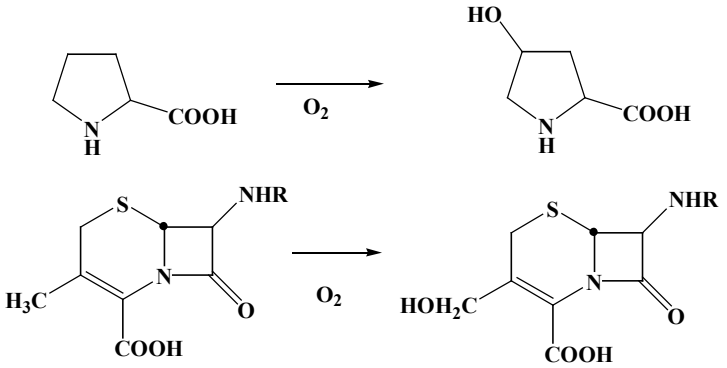
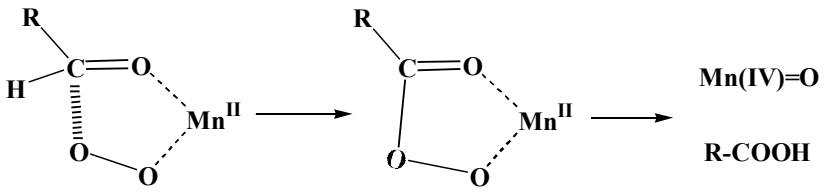


Figure 16. Examples of α-keto acid-dependent dioxygenases. [13]

In general, these enzymes require 1 equiv of Fe(II), and α-keto acid (usually α-ketoglutarate), and ascorbate for full activity. Substitution with other divalent metal ions (Zn(II), Mn(II), Co(II), Mg(II), and Ni(II)) results in the complete loss of enzymatic activity [13] These facts are clearly show that the oxidation activity by the Fe(II) ion is completely different from those of other metal ions, *especially Mn(II)*. This should be due to that *Mn(II) compounds are in favour of the "two-electron oxidation" reaction and readily oxidized to an Mn(IV) ion, whereas "one-electron reaction", for the Fe(II) species*. This is clearly exemplified in our recent results., *i.e.,* Mn(II) ion of the complex $Mn(ntb)ClClO_4$, which is very stable in air, is readily oxidized to a Mn(IV) ion in the presence of reducing agent, cycloyhexane-carboxyaldehyde in air; the mechanism of the Mn(IV) formation is written below. [14]



These are indicating that in the biological iron-containing hydroxylases, the replacement of the iron ion by a Mn(II) ion will give rise to great loss of the enzymatic activity. It should be remembered here that the recent investigations of scrapie, CJD, and CWD clusters in Iceland, Slovakia, and Colorado have indicated the soil in these regions is low in copper and higher in manganese as described in Chapter I. Brown et al. have reported that prion protein loses the SOD-like activity when Cu is replaced with Mn in recombinant PrP, and also that Cu binding to PrP purified from sporadic CJD was significantly decreased while the binding of Mn and Zn was markedly increased. It should be quite likely that the Mn(II) ions in the prion protein may degrade the surrounding peptide bonds in the presence of aliphatic aldehyde, such as 4-hydroxy-2-nonenal, frequently observed for the iron-overload patients, (Zainal et al., Free Radical Biology Medicine, 26(1999), 1181) and in the presence of hydrogen peroxide, because under these conditions formation of a Mn(IV) species is facile and these Mn(IV) species is *highly reactive* towards proteins and DNA, etc.

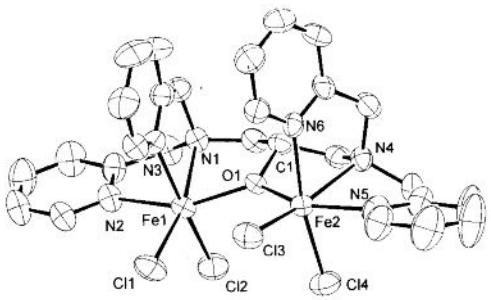
"Manganism" and its origin

As a nutrient, manganese is an essential component of several enzymes; a deficiency can lead to heart and bone problems and in children, stunted growth. However, it has been known since 1837 that workers in manganese mines can develop ***manganism***, [17] a dreaded illness marked by Parkinson's-like tremors, violent outbursts, and hallucinations. Victims have lesions in the globus pallidus and striatum of the basal ganglia, a part of the brain involved in fine muscle control. When manganese (this should be manganese oxide) is inhaled, blood ferries it from the lungs to the brain, where it can readily cross the blood-brain barrier. As stated before, ***excess manganese ions in the brain should lead to the deficiency of neurotransmitters*****,** and this should give the most reasonable explanation for the "manganism" observed. Several studies have demonstrated that iron deficiency increases transport of orally administered manganese into the body as well as delivery to the brain. (Aschner *et al*. Ann. NY Acad Sci. 1012(2004), 115; Gunter et al., NeuroToxicology, 27(2006), 765; Erickson and Aschner, NeuroToxicology, 27(2006), 125; Heilig et al., Am. J. Physiol. Cell Mol Physiol., 290(2006), L1247)

Many countries in the world completed phasing out leaded gasoline (gasoline containing tetraethyl-lead), paving the way for widespread use of a manganese-based compound, MMT, in gasoline. (MMT=methyl cyclopentadienyl manganese tricarbonyl) The additive increases octane level, which boosts engine performance and enables fuel to be burned more evenly. ***This means that manganese oxide, one of the famous chemical carcinogens, is widely spread in the sky,*** *and the human inhale the manganese oxide every day, which is stored in the lung.* ***The use of MMT should be stopped as soon as possible!***

Al^{3+} ion in Neurodegenerative Diseases

As described above, it is clear that an unpaired d-electron plays an important role in activating O_2 in the iron-containing enzymes. If the Fe(II) is replaced by an another metal with no unpaired d-electrons such as Al^{3+} ion, it is quite likely that such enzymes cannot activate O_2, thus giving rise the deficiency of the neurotransmitters. In order to get more information on this problem, we have compared the reactivity of the Al(III) compounds with those of the corresponding Fe(III) compounds. We have prepared several binuclear iron(III) complexes with H(HPTB) and H(HPTP) shown below, and determined the crystal structure of $Fe_2(HPTP)Cl_4ClO_4$, which is illustrated below. (H(HPTP) =N,N,N',N'-tetra(2-pyridylmethyl)- 1,3-diaminopropanol)).



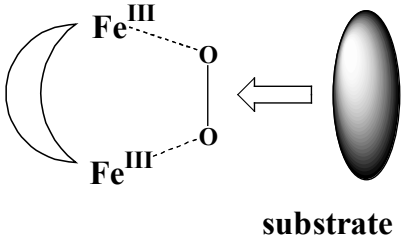
Structure of $Fe_2(HPTP)Cl_4$ cation.

We have found these two binuclear iron(III) complexes give high positive *TBARS* in the reaction with linolenic acid in the air [18,19]; here TBARS are

malondialdehyde (which gives pink products with TBA; λmax=532 nm) or monoaldehyde derivatives (gives orange products), which are formed from the peroxidation of linolenic acid. (TBA= 2-thiobarbituric acid; see Figure 17). [20]

Above results have been elucidated on the assumption that oxygenation of O_2 into the linoleic acid proceeds without the change of oxidation state of Fe(III) through forming an intermediate containing two iron(III) ions, O_2, and linoleic acid, as illustrated in **Scheme-V**; this is very similar to that proposed for the elucidation of Mukaiyama reaction and lipoxygenase. In this case, *the interaction between the two unpaired electrons of two iron(III) atoms and O_2 (* ····· in Scheme-V, see below) *is necessary to activate the O_2*, which is also promoted via interaction with the substrate, linolenic acid.

Scheme-V



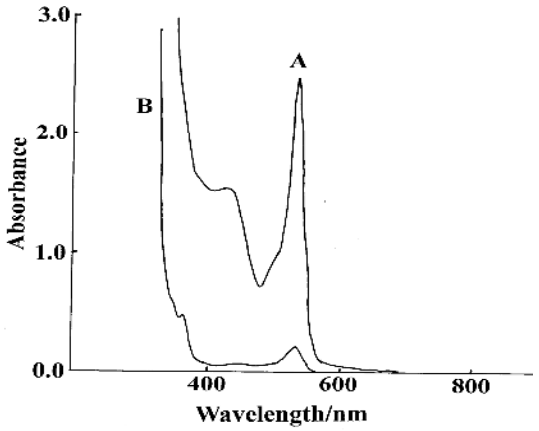


Figure 17. Absorption spectra of the reaction mixtures treated with TBA according to the usual procedures. [18,19]
A: $Fe_2(HPTP)Cl_4ClO_4$ and linolenic acid,
B: $Al_2(HPTP)(OH)Cl_2(ClO_4)_2$ and linolenic acid

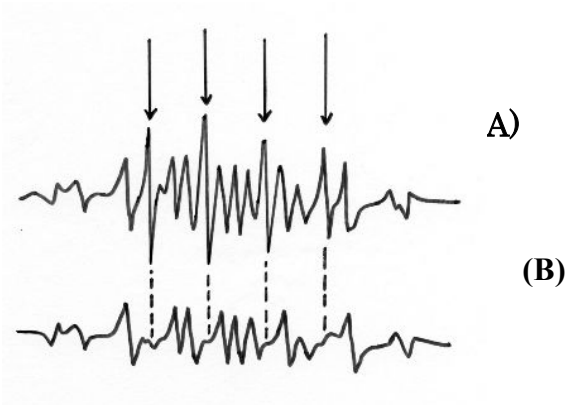
The binuclear Al(III) complex, $Al_2(HPTP)(OH)Cl_2(ClO_4)_2$ was also isolated. The activities for the oxygenation of linolenic acid by these two binuclear Fe(III) and Al(III) compounds were compared by measuring the quantities of the ***TBARS compounds***, and the results are shown in Figure 17. This clearly shows that

Al(III) complex exhibit no activity for the oxygenation of linoleic acid (the absorbance at 532 nm is much lower than that due to the corresponding iron(III) complex), and this can be attributed to the absence of unpaired d-electron in Al(III) complex, and this also supports the importance of role of unpaired d-electron in the activation of O_2.

The similar fact was also observed when the DMPO, which is one of the famous spin-trapping agent for OH• radical, was added to the solution containing binuclear iron(III) complex, $Fe_2(HPTP)(OH)\ (NO_3)_2^{2+}$;strong four signals which correspond to the formation of DMPO-OH have appeared as illustrated in Fig. 18, [21] whereas no such signal was detected by the addition of DMPO to the solutions of $Fe(edta)^-$ and $Al_2(HPTP)(OH)Cl_2(ClO_4)_2$. This clearly indicates that the formation of DMPO-OH is not due to the presence of OH• in the solution. Above mysterious fact was elucidated by the similar way as described for the oxygenation of linolenic acid through the formation of the intermediate similar to that assumed for the linolenic acid (**Scheme-V**), O_2 is activated to interact with the DMPO, leading to DMPO-OH formation. These demonstrate that the results obtained by the use of spin-trapping reagents, ***especially for the formation of OH• by DMPO,*** are doubtful, and the results reported hitherto *should be re-investigated, and thus I cannot support the discussion on the presence of so-called Fenton reaction in the biological systems.*

Figure 18. ESR spectra of the solutions added by DMPO. [21]

A: $Fe_2(HPTP)(OH)(NO_3)_2^{2+}$ (upper) B: Fe(edta)Na (lower)



As shown in the previous Chapter, ALS and Parkinson's and Alzheimer's patients were collectively found in the New Gunia and Papua island, and its origin has been postulated to be the drinking subterranean water, which contains much concentrations of Al^{3+} and Mn^{2+}, and increased aluminum and manganese levels were reported in the hippocampus and the motor cortex patients with Alzheimer's disease [15] and in the brain of CJD patients [16], respectively Thus, it seems likely that the elevated concentration of these Al^{3+} and Mn^{2+} is a one of the serious origins of these e***ndemic sporadic*** neurodegerative diseases, and this should be closely related with the fact the replacement of the iron ion by *another metal ion leads to the loss of activity of the enzymes which play a central role in the synthesis of neurotransmitters*.

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Chapter VI Non-specific Iron ion and Abnormalities in Brain Iron Metabolism

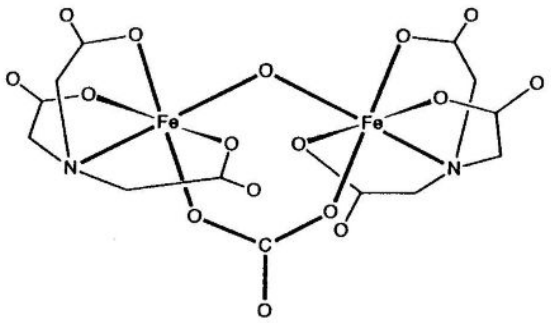
Human iron metabolism and absorption has been the subject of a recent review. Normal human males contain 3-5 g of iron (often less in females) and of this, two-thirds is in circulating red cells as hemoglobin and 15-25 % in storage in ferritin and hemosiderin. [1] The remaining iron is in muscle myoglobin (about 8 %) and in cytochromes and iron-containing enzymes. Plasma transferrin accounts for only 3 mg of Fe, but the daily exchange if iron through plasma transferrin is ten times this account. Transferrin therefore plays a central role in

iron distribution. Iron delivery by transferrin to etythroid and many non-erythroid cells involves interaction of transferrin with specific receptors followed by endocyosis and recycling of apotransferrin and receptor. These receptors are present in low amounts on phagocytosis cells which receive their iron from degraded red cell hemoglobin. In the case of hepatocytes it has been proposed that iron is released by reduction at the plasma membrane rather than receptor-mediated endocytosis, but there is no evidence for a diferric reductase in rat liver *plasma membrane.* ***Under pathological conditions (e.g., iron* overload syndrome**) the amount of iron not bound to transferrin may become important (these are called ***non-specific iron ions)***. This is distributed to organs in an inappropriate manner ***leading to iron overload in parenchymal organs such as liver and pancreas***.

Genetic (idiopathic or hereditary) ***haematochromatosis*** is one of the most common genetic disorder in western populations, particularly among Celtic peoples. This disease is associated with greatly increased (sometimes 50-fold) deposits of storage iron, predominantly as haemosiderin, in the liver and other tissues due to abnormally high absorption from the gut, although there seems to be little or no ferritin in the duodenal absorption cells. The excess iron cannot be eliminated and must be stored. Elevated body iron leads to increased iron in storage (and not to increases in hemoglobin, myoglobin, etc. except in the treatment of iron deficiency anaemia) Under normal iron loading, ferritinis the major storage form, but in diseases of iron overload, the capacity to synthesize ferritin levels off and haemosiderin becomes predominant.

Normal cells store iron mainly in ferritin molecules, but under conditions of iron overload some of it is shunted into another source of storage form known as haemosiderin. Several data support the hypothesis that haemosiderin is a degradation product of ferritin, and this has been confirmed for phytosiderin, an insoluble iron-containing product from pea seed, which was found to contain a peptide derived from the ferritin subunit. These findings indicated that ferritin is taken up by lysosomes, and that subsequent processing involved a partial dissolution of the core, and degradation to siderosomal ferritin and to the insoluble haemosiderin. Thus the haemosiderin from various iron-loaded animals were consistently found to *have ferrihydrite-like iron cores* similar to ferritin, and haemosiderin is typically insoluble, as isolated, in contrast to the

soluble ferritin, but it can be solubilized by treatment with alkali and detergents, and also with the *several amino acids or small peptides,* ***so-called non-specific iron, which exist in brain as soluble dimeric (or polymeric) compounds,*** similar to $Fe_2(HPTP)Cl_4^+$, or with (nta); the latter complex is of a μ-oxo bridged dimeric structure, $[Fe_2O(nta)_2(CO_3)]^{2-}$, as illustrated below. [2,3] *It should be noted here that these binuclear iron(III) compounds give rise to increased oxidation of lipids, proteins, DNA damages, and also cell death in the presence of hydrogen peroxide. [3-6]*



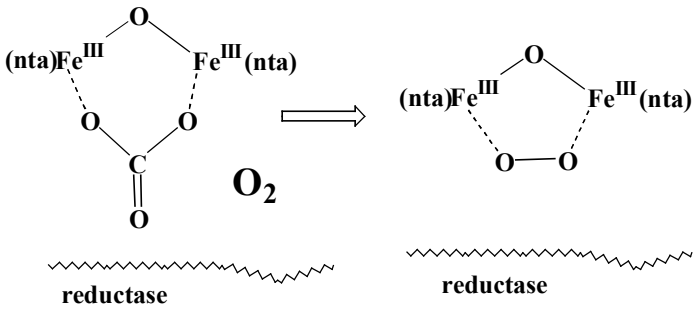
Hydrogen Peroxide Formation by binuclear iron(III) Species:
A renal carcinogen, Fe(III)-(nta) Complex

It is noteworthy that the oxidation of the TMPD, one of the famous one-electron donor, is greatly accelerated in the presence of the binuclear iron(III) complex, $Fe_2(HPTP)Cl_4^+$ or binuclear copper(II) complex, [7,8] to *give much quantity of hydrogen peroxide (see the following equation)***;** this indicates that the *soluble dimeric* iron(III) species can produce hydrogen peroxide in the presence of reducing agent and O_2.

$$Fe^{III}{}_2(HPTP)Cl_4^+ + 2TMPD + O_2 \rightarrow Fe^{III}{}_2(HPTP)Cl_4^+ + 2TMPD^+ + O_2^{2-}$$

In the biological systems, there are many enzymes that operate as reductase, such as glutathione reductase, etc. If the dimeric iron(III) chelates approaches to this enzyme under an aerobic condition, the reductase may give hydrogen peroxide, and thus it is quite reasonable to assume that oxidative degradation of the proteins and sugars may occur in the vicinity of this enzyme in the .presence

of the iron carcinogen, Fe-nta via formation of binuclear iron(III)-peroxide adduct (see below), and this should be due to fact that the carbonato ion in the original binuclear iron(III)-(nta) complex is easily released from the compound. ([4] and Nishida et al., Chem. Lett., 1994, 641; Synth. Reac. Inorg. Metal-org. Nano-Metal Chem., 36(2006), 373).



This may give a reasonable explanation to the facts observed by Okada et al., they reported that redox-active iron caused free radical injuries in the proximal tubes of mice kidneys after injection of a renal carcinogen Fe(III)-(nta) and induced the apoptosis of the proximal tubular epithelial cells. But, this injury was not observed in the distal portion, and at this proximal portion glutathione reductases are operating. (Okada et al., Arch. Biochem. Biophys., 301(1993), 138; Kawabata et al., Carcinogenesis, 18(1997), 1389; Kafel et al., Chemosphere, 65(2006), 963).

In the previous chapter, we have shown that hydrogen peroxide should be an intrinsic factor to induce the pathogenesis of ***sporadic scrapie and BSE*** as illustrated in **Scheme-III** in Chapter IV. Very recently we have reported that the capillary electrophoresis method (CE) is very suitable to investigate the conformation change of the proteins and aggregation states of the proteins in solution. [Nishida, Annu. Report CIN, 2004, 1; Nishida et al., Synth. Reac. Inorg. Metal-org. NanoMetal Chem., 35(2005), 379; Nishida et al., Synth. Reac. Inorg. Metal-org. NanoMetal Chem., 35(2005), 677; Chem. Lett., 34(2005), 141; Nishida et al., Z. Naturforsch., 62c(2006), 273] As an example, two CE profiles of SOD and transferrin are illustrated in Figure 19. Although the concentrations of the two solutions are the same, the peak intensities are quite different from

each other, and this has been rationalized on the fact that SOD has a rigid dimeric structure in solution, but that of apo-transferrin is more flexible. In fact, at least three components are observed in the CE of holo-transferrin. We also have found that the presence of excess hydrogen peroxide (and also some non-specific iron observed that species such as $[Fe_2(HPTP)Cl_4]^+$, *etc*) induces the loosening or dissociation of dimeric structure of SOD molecule (see Figure 20), [9] which has been considered to be one of the main origins of ALS (see Chapter IV). Thus, it seems quite likely that ***sporadic ALS*** is related with the excess hydrogen peroxide (and also some non-specific iron species and copper(II) chelates [9,10]) in brain, and the similar discussion may be applied to the elucidation of ***sporadic prion diseases*** as described before. (see Chapter IV).

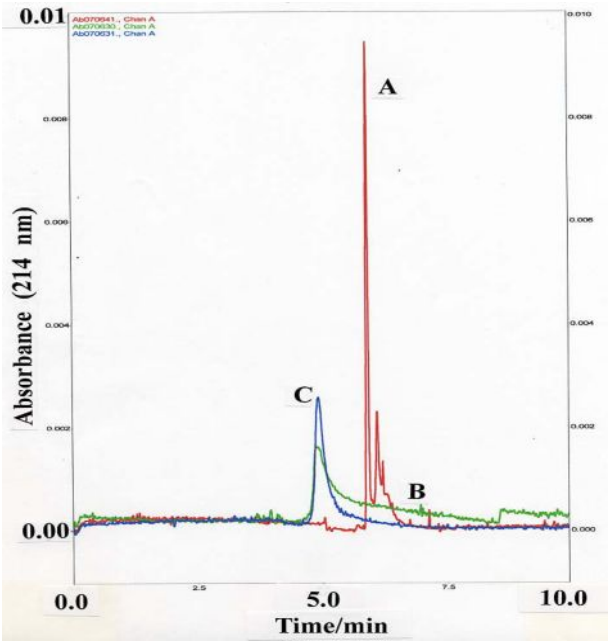


Figure 19. CE profiles of the solutions (protein 0.5mg/1ml). A: SOD B and C (after 60 min.), Apo-transferrin. (Chem. Lett., 34(2005), 141)

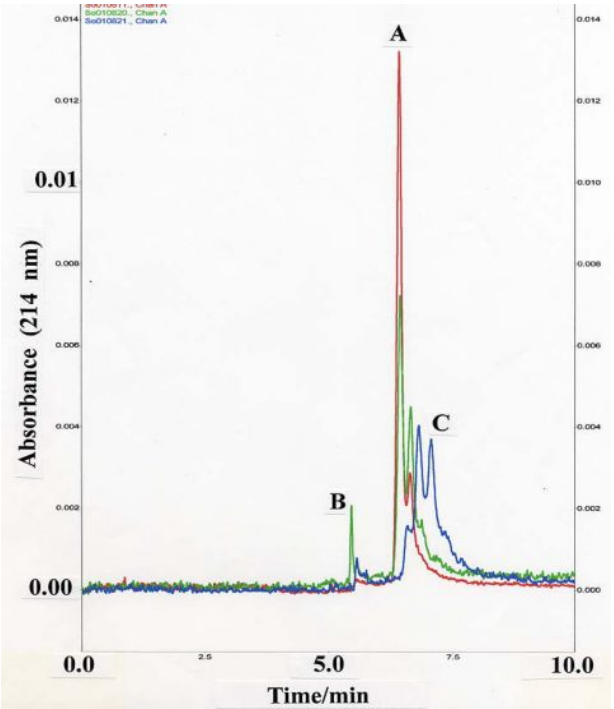


Figure 20. CE profiles of the solutions (protein 0.5 mg/1ml) containing SOD.A: SOD only. B hydrogen peroxide solution was added to Solution A, (0 min.) and C, after 60 min**.** [9]

Abnormal Iron Metabolism due to Increased Al^{3+} and Mn^{2+} Ions in brain

As shown in Chapter I, increased aluminum levels were reported in the hippocampus of patients with Alzheimer's disease and specific regions such as the hippocampus and the motor cortex contain elevated iron levels. In contrast to iron levels, ferritin levels were differentially altered in the brain of patients with Alzheimer's disease. *This suggests that iron overload syndrome may be induced through the accumulation of exogenous metal ions such as* Al^{3+} *and*

Mn^{2+}; as stated above, the accumulation of Al(III) and Mn(II) ions in brain will lead to the deficiency of dopamine and other neurotransmitters, and under this circumstances the brain will order the transferrin to transport the iron ion to the enzyme, but the transported metal ions including Fe(III) and Al(III) should be discarded to the tissue, because the enzyme is loaded with the metal ion, Al(III), etc.; this is consistent with *the many facts observed*. [11,12]

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Chapter VII Summary

We have investigated the chemical mechanism of pathogenesis of *sporadic prion diseases* based **on the *new concept* on "oxidative stress"** induced by the metal ions; ***our conclusion is summarized as follows:***

1) In iron-containing enzymes, tyrosine-, phenylalanine-, and tryptophan-hydroxylase, Fe(II) ion activates O_2 through weak interaction between the unpaired electrons of Fe(II) ion and O_2, which is promoted by pterin, *giving rise to hydroxylated benzene ring. Other metal ions such as Al(III) or Mn(II) cannot activate O_2 by the similar ways*, leading to the deficiency of neurotransmitters, and to the elevated iron levels in brains. Elevated iron levels in brains and abnormalities in brain iron metabolism have been detected for Alzheimer's and Parkinson's diseases, and these iron ions serve to the formation of the *so-called non-specific iron ions*, and
2) The *so-called non-specific iron ion**s***, generally soluble polynuclear Fe(III) species, exhibit unique reactivity toward O_2, and gives hydrogen peroxide in the presence of reducing agents. The hydrogen peroxide thus produced readily forms a metal-OOH, hydroperoxo-metal chelates of Fe(III) or Cu(II) chelates. These metal-OOH species are highly reactive to oxidation of cellular constituents, such as proteins, and DNA, which lead to *DNA damages*, *mutant proteins, misfolded proteins, and protein aggregations*. Thus the formation of "highly reactive" copper(II)-OOH species *in vivo* is closely related with the initial step of pathogenesis of sporadic ALS and prion diseases.

Based on these results, we would like to propose that the most important risk factor to induce sporadic prion diseases is the abnormal accumulation of Al(III) or Mn(II) ions in brain, which leads to the abnormalities in brain iron metabolism (formation of non-specific iron ions), and in fact the cell-death due to "iron-overload syndrome" was already reported by several authors. Thus, the

sudden and explosive increase of scrapie and BSE in the last decade in Europe may be partially due to "acid rain", because the acid rain makes Al(III) and Mn(II) ions soluble in the subterranean aquifers.

In the 21st century we must do many works to prevent the increases of Al^{3+} and Mn^{2+} ions in brain in our dairy life, and make adequate countermeasures for "acid rain". Strong acid rain dissolves out the iron ions from the surface of the mountains and hills, leading to iron-deficiency in many trees, which should be a critical origin for the abnormal autumn tints frequently observed in recent Japan. I already proposed new methods to maintain the healthy forests and woods in Japan, which should be the best way towards the acid rain, **and to maintain the healthy brain in human lives, leading to decrease or prevention of metal diseases such as depression, and violent or rough children, *etc*.**