Nonheme-iron histochemistry for light and electron microscopy: a historical, theoretical and technical review*

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Summary. We reviewed the methods of nonheme-iron histochemistry with special focus on the underlying chemical principles. The term nonheme-iron includes heterogeneous species of iron complexes where iron is more loosely bound to low-molecular weight organic bases and proteins than that of heme (iron-protoporphyrin complex). Nonheme-iron is liberated in dilute acid solutions and available for conventional histochemistry by the Perls and Turnbull and other methods using iron chelators, which depend on the production of insoluble iron compounds. Treatment with strong oxidative agents is required for the liberation of heme-iron, which therefore is not stained by conventional histochemistry. The Perls method most commonly used in laboratory investigations largely stains ferric iron, but stains some ferrous iron as well, while the Turnbull method is specific for the latter. Although the Turnbull method performed on sections fails in staining ferrous iron or stains only such parts of the tissue where iron is heavily accumulated, an in vivo perfusion- Turnbull method demonstrated the ubiquitous distribution of ferrous iron, particularly in lysosomes. The Perls or Turnbull reaction is enhanced by DAB/silver/gold methods for electron microscopy. The iron sulfide method and the staining of redox-active iron with H2O2 and DAB are also applicable for electron microscopy. Although the above histochemical methods have advantages for visualizing iron by conventional light and electron microscopy, the quantitative estimation of iron is not easy. Recent methods depending on the quenching of fluorescent divalent metal indicators by Fe2+ and dequenching by divalent metal chelators have enabled the quantitative estimation of chelatable Fe2+ in isolated viable cells.

Received December 7, 2006

* This work was supported by a grant from the Karuji Memorial Foundation for Medical Research (Grant B to R. Meguro, 2005) and a Grant-in-Aid (18700336 to R. Meguro and 14657123 to K. Shoumura) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Introduction

Iron is one of the essential trace metals in living organisms. Iron is present at the active sites of those molecules that are responsible for essential biological functions such as oxygen transport, electron transfer, and DNA synthesis. These functions depend on the chemical properties of iron that can change valence (redox capability) to catalyze oxidation-reduction reactions. However, this property of iron causes a paradoxical generation of highly reactive and, as a result, cytotoxic hydroxyl radicals (OH·) through the Fenton reaction or iron-catalyzed Haber-Weiss reaction in the presence of H2O2 or O2·. The Fenton reaction was reported in 1876, and the Haber-Weiss reaction in 1934 (reviewed in Halliwell and Gutteridge, 1999; Crickton et al., 2002).

Fe2+ + H2O2 → OH· + OH− + Fe3+
(Fenton reaction)
and

Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2
Fe^{2+} + H_2O_2 \rightarrow OH^- + OH^- + Fe^{3+}

(Iron-catalyzed Haber-Weiss reaction)

OH^- is highly reactive and oxidizes biologically important macromolecules including membrane lipids, DNA, proteins, and carbohydrates, causing necrotic or apoptotic cell death, or the initiation of carcinogenesis (Halliwell and Gutteridge, 1999). Because of the high reactivity of OH^- affecting macromolecules close to its site of formation (site specificity), the localization of catalytic iron is critical in the development of tissue injury. The substrate of the Fenton reaction, H_2O_2, is rapidly permeable across biological membranes (Halliwell and Gutteridge, 1999).

To prevent the hazardous spreading of catalytic active iron, organisms have developed two systems to treat iron strictly in nontoxic forms: the iron-transport (transferrins) and iron-storage/sequestration (ferritin and hemosiderin) systems (reviewed in Aisen et al., 2001; Crichton et al., 2002; Ponka 2003; Taketani 2005). Furthermore, cellular iron content is tightly regulated by post-transcriptionally controlling the expression of the transferrin receptor and ferritin (Crichton et al., 2002; Ponka, 2003). Despite this strictly controlled iron-metabolism, there exists considerable evidence suggesting that both the cell death caused by iron-catalyzed OH^- generation and the peroxidation of essential cellular molecules are the central pathogenesis of various degenerative diseases: haemochromatosis, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, Friedrich’s ataxia, Huntington’s chorea, Hallervorden-Spatz syndrome, ischemia-reperfusion injury of various organs, and degeneration of the retina. Furthermore, in view of the importance of DNA damage in carcinogenesis, iron-catalyzed OH^- generation can be a risk factor for cancer (reviewed in Halliwell and Gutteridge, 1999; Gordon, 2000; Pinero and Connor, 2000; Berg et al., 2001; Chiuch, 2001; Pandolfo, 2002; Crichton et al., 2002; Götz et al., 2004).

In experimental animal studies and pathological studies of the diseases for which iron-mediated cell degeneration is suspected, the histochemical visualization of tissue iron has proved a useful approach in assessing the contribution of iron in the generation of tissue damages. An early important contribution of nonheme-iron histochemistry (Tiermann-Schmertz method, see Section 4.2) was the characterization of Hallervorden-Spatz disease (Hallervorden and Spatz 1922), which was clinically represented by gradually increasing stiffness of all four limbs, clubfoot deformity, severe dysarthria, emotional disorders, and severe dementia. Brains affected by this disease demonstrated massive depositions of iron in the basal ganglia, nerve cell degeneration, and an increase in the number of glial cells. More recently, mitochondrial damage caused by nonheme-iron accumulation and the increased production of highly toxic free radicals have been implicated in Friedreich’s ataxia (Gordon, 2000; Pandolfo, 2002). Iron deposits in degenerated cardiac muscle cells were demonstrated by the Perls method (see Section 4.1) in the heart of Friedreich’s ataxia (Sanchez-Casis et al., 1976; Larnarch et al., 1980). Recently, abnormal iron accumulation was also demonstrated by MRI in the dentate nucleus of the cerebellum (Waldvogel et al., 1999). Most patients affected with this disease have a point mutation and trinucleotide repeat expansion in both alleles of this ataxia in addition to an abnormality of the nuclear gene encoding the nuclear-encoded mitochondrial (ataxin (the protein regulating mitochondrial iron metabolism).

The history of nonheme-iron histochemistry is lengthy. Perls (1867) performed pioneer work in this field. He used potassium ferrocyanide and HCl (acid-ferrocyanide) to visualize tissue iron. The next year, Quincke demonstrated tissue iron with ammonium sulfide (see Section 6; Quincke 1896). Thereafter, various modifications of these methods and novel ones were proposed and tested. There are several early reviews focusing on iron-histochemistry by Gomori (1936), Okamoto (1937), Lillie (1948), and Bunting (1949). More recently, Pearse (1985) critically reviewed various methods of iron-histochemistry. Since then, new combinations of old techniques and innovative techniques have been published.

The demand for the visualization of tissue iron, particularly the cellular and subcellular localization and the redox-state, is increasing with the recent surge in our understanding of the iron metabolism in physiological and pathological conditions as well as developments in MRI techniques, which make iron stores visible in the normal and diseased human body (Schenck and Zimmerman, 2004, Haake et al., 2005).

This review seeks to present historical, theoretical, and technical aspects of iron-histochemistry, particularly for a better understanding of the chemical principles of the reactions that will enable the appropriate use of the methods and interpretation of the results in laboratory investigations. Furthermore, a detailed knowledge of how the techniques of iron-histochemistry were developed, modified, and improved should provide a sound basis for further improvement of the techniques and the development of new ones. Also discussed in Section 12 are recommended protocols of nonheme-iron histochemistry for light and electron microscopy.
1. Biological iron is categorized into heme- and nonheme-iron

The iron of organisms is categorized into heme- and nonheme-iron according to its chemical structure and metabolism (Fontecave, 1998; Cotton et al., 1999; Tsiftsgolou et al., 2006). Heme-iron is an iron-protoporphyrin complex and a prosthetic group of hemoglobin, myoglobin, cytochromes, and oxidation/reduction enzymes (Table 1). Heme is synthesized in mitochondria from protoporphyrin and Fe\(^{2+}\) by ferrochelatase. For the degradation of heme-proteins, the protein moiety is digested by lysosomal enzymes, while iron release from heme requires the oxidative cleavage of the protoporphyrin ring and is carried out by hemeoxygenases (HO-1 and HO-2) using molecular oxygen in the mitochondria or microsomes (reviewed in Kikuchi et al., 2005; Tsiftsgolou et al., 2006).

On the other hand, nonheme-iron contains heterogeneous species of the iron complex (Table 1): 1) nonheme-iron loosely bound to various organic bases of low molecular weight (nonprotein-bound iron); 2) nonheme-iron tightly bound to proteins for transport of iron; 3) nonheme-iron proteins where iron contributes to the protein structure such as iron-sulfur proteins and nonheme-iron enzymes; and 4) the iron of storage or sequestration (ferritin and hemosiderin).

2. Cellular processing of nonheme-iron differs according to the solubility and reactivity of iron ions, Fe\(^{2+}\) and Fe\(^{3+}\)

Nonheme-iron in biological tissues exists in a ferrous (Fe\(^{2+}/\text{Fe(II)}\)) or ferric (Fe\(^{3+}/\text{Fe(III)}\)) state. Here, we describe some important chemical properties of Fe\(^{2+}\) and Fe\(^{3+}\) that determine the cellular processing and subcellular localization of these ions. In aqueous solutions, Fe\(^{3+}\) and Fe\(^{2+}\) are present as hexaaqua complexes, [Fe(H\(_2\)O\(_6\))]\(^{3+}\) and [Fe(H\(_2\)O\(_6\))]\(^{2+}\), respectively. The solubility of Fe\(^{2+}\) and Fe\(^{3+}\) in aqueous solutions depends on the pH of the solvents. Fe\(^{3+}\) is almost completely precipitated as Fe(OH)\(_3\) at the physiological pH and the maximal concentration of Fe\(^{3+}\) is only about 10\(^{-22}\) M, whereas the solubility of Fe(OH)\(_3\) is much greater than Fe(OH)\(_2\); and the maximal concentration of Fe\(^{2+}\) reaches ca. 10\(^{-1}\) M (Arredondo and Núñez, 2005). This indicates that Fe\(^{2+}\)

<table>
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<tr>
<th>Table 1. Examples of heme- and nonheme-iron</th>
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<td><strong>Heme-iron</strong></td>
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<td>Heme-iron of oxygen-transport</td>
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<td>Heme-iron of electron-transport proteins</td>
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<td>Heme-iron of oxidation/reduction enzymes</td>
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<tr>
<td><strong>Nonheme-iron</strong></td>
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<td>Iron loosely bound to low molecular weight organic bases</td>
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<td>Iron tightly bound to transport proteins</td>
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<td>Iron-sulfur proteins</td>
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<td>Nonheme-iron enzymes</td>
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<td>Nonheme-iron enzymes</td>
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<td>Iron of storage</td>
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can exist as the hexaaqua complex at the physiological pH, while Fe\(^{3+}\) cannot. This fact is highly significant because soluble Fe\(^{3+}\) can catalyze the generation of highly reactive, toxic OH\(^-\) in the presence of H\(_2\)O\(_2\) (Fenton reaction) at the physiological range of pH. Furthermore, in the reductive environment of the cell, reducing agents such as ascorbic acid, thiols, and superoxide (O\(^-\)) reduce loosely bound Fe (III) to Fe (II).

To avoid the risk of the generation of OH\(^-\), Fe\(^{3+}\) is strongly bonded to transporter proteins (transferrins) for the intercellular transport of iron. Furthermore, iron is sequestered and stored in the ferritin, which can accommodate up to 4,500 Fe atoms in the form of ferritidyde (5Fe\(^{3+}\)[Fe\(^{3+}\)O\(_4\)] - 9H\(_2\)O) with minor forms similar to magnetite (Fe\(^{3+}\)Fe\(^{3+}\)O\(_4\)) or hematite (ferric oxide, Fe\(^{3+}\)O\(_2\)) (Crichton and Ward, 1992; Harrison and Arosio, 1996; Chasteen and Harrison, 1999). Iron is also sequestered in hemosiderin masses of phagolysosomes, where iron is present in a form similar to the ferritiodyde core of ferritin (reviewed in Crichton and Ward, 1992; Harrison and Arosio, 1996). It should be stressed that the iron of intercellular transport and intracellular sequestration are strictly in an insoluble ferric form except for the Fe (II) of magnetite.

Iron release from nonheme-iron complexes occurs in endosomes (transferrin) and phagolysosomes (hemosiderin and other iron-protein complexes) wherein pH is respectively maintained at 5.5-6.0 (Mellman et al., 1986) and 5.2-5.6 (Geisow et al., 1981) by an ATP-dependent proton pump. The released Fe\(^{3+}\) is reduced to Fe\(^{2+}\) by endosomal (and lysosomal) ferric reductase that uses ascorbate as the reducing agent (Núñez et al., 1990). The presence of a small pool of Fe\(^{2+}\) was histochemically demonstrated in endosome/lysosome by the perfusion-Turnbull method, suggesting the reduction of Fe\(^{3+}\) in those organelles (Meguro et al., 2005). Fe\(^{2+}\) is then exported to cytosol by the Fe\(^{2+}\) transporter (Crichton et al., 2002; Ponka, 2003), where Fe\(^{2+}\) is considered to loosely complex with low-molecular weight organic bases forming the so-called low-molecular weight iron pool (LIP), which may contain some Fe (III) salts as well due to cytosolic oxidation. LIP is the transient intermediate between the iron released from the endosome/lysosome and transient storage in ferritin or for final use in mitochondria and other organelles. Because of its reactivity and cytotoxicity, the LIP size is maintained in very small amounts.

3. Nonheme-iron is liberated in acid solutions

Classically, biological iron has been categorized into ‘masked’ and ‘not masked’ iron according to the availability of free iron for histochemical demonstration (Zaleski, 1887; Macallum, 1895; Meyer, 1906; Okamoto, 1937). The ‘masked’ iron includes heme-iron and iron tightly bound to unknown compounds. Tissue treatment with strong oxidants is required for the release of iron from these compounds (see Section 11), while ‘not masked’ iron includes more loosely bound nonheme-iron that is liberated in acid solutions. Therefore, nonheme-iron histochemistry is usually carried out at a low pH using dilute HCl, acetic acid, or trichloroacetic acid. We consider below the chemical mechanism of iron release from each nonheme-iron species.

3.1. Iron loosely bound to low molecular weight organic bases

This species of iron constitutes the LIP, where iron is considered to be ionically bonded to low molecular weight organic bases such as phosphate (e.g. ATP, ADP, nucleotide, and polar heads of membrane phospholipids and other phosphate esters), carboxylate (organic acids such as citrate and lactate), carbonate, and ascorbate. This species of iron is also called labile iron or chelatable iron.

Iron can exist in both ferrous and ferric forms in LIP. The presence of ferric iron in the cytosol was suggested by the demonstration of an ATP-dependent uptake of iron from Fe (III)-citrate and Fe (III)-ATP in rat liver nuclei (Gurguera and Meneghini, 1996). Studies using fluorescent transition-metal indicators demonstrated chelatable ferrous iron in the nucleus and mitochondria of viable hepatocytes at the pH range of 6.2–8.5 (Petrat et al., 2001, 2002). The estimated amount of low-molecular weight, chelatable iron was very small, some 0.5–1.0 \(\mu\)M (determined by bleomycin-DNA damage method, Crichton and Ward, 1992) or 5.8 +/- 2.6 \(\mu\)M (determined by fluorescent histochemistry, Petrat et al., 2001).

The solubility of the salt of a weak acid depends on the pH of the solution. Fe(H\(_2\)O)\(_6\)\(^{3+}\) (Ka = 6 \times 10\(^{-9}\)) and Fe (H\(_2\)O)\(_6\)\(^{2+}\) (Ka < 6 \times 10\(^{-9}\)) are weak acids, and the bases of their salts in LIP, e.g. citrate, carbonate, phosphate, ascorbate, or ATP, are protonized by a strong acid (e.g. HCl) that is used for iron-histochemistry — for example,

\[
\text{Fe(III)-citrate} + 3\text{H}^+ \rightarrow \\
\text{Fe}^{3+} + \text{citric acid} (K_a = 7.4 \times 10^{-4})
\]

This causes more Fe (III)-citrate salt to dissociate to maintain a solubility equilibrium. Accordingly, the higher
the hydrogen ion concentration is, i.e. the lower the pH, and the greater the solubility of such salts. However, the estimated amounts of nonheme-iron in LIP are too small to be distinctly visualized by histochemical methods which depend on the production of insoluble iron precipitate, probably only yielding a pale staining of the cytoplasm.

3.2. Transferrins

Transferrins are intercellular iron-transporter proteins that carry two Fe\(^{3+}\) ions per molecule. Iron-binding sites consist of six ligands — four from the protein and two from carbonate (Crichton et al., 2002). Fe\(^{3+}\) is strongly bonded to transferrin; the iron-binding constant of transferrin-Fe(II) is 10\(^{23}\) while that of citrate-Fe(III) is 10\(^{10}\) (Halliwell and Gutteridge, 1999). In the cell, Fe\(^{3+}\) is liberated from transferrin in endosomes, where the pH is maintained at 4.5 +/- 0.2 by an ATP-dependent proton pump. In this acidic environment, the protonation of a carbonate anion initiates iron release from transferrin (El Hage Chahine and Pakdaman, 1995). In vitro, Fe\(^{3+}\) was liberated from transferrin in 2% acetic acid (pH 2.75), and free iron was visualized with Ferene-S, an indicator of Fe\(^{2+}\), after the reduction of ferric iron with thioglycolic acid (Chung, 1985).

3.3. Ferritin and hemosiderin

In the cytosol, the mobilization of iron from ferritin requires a reduction to the ferrous state by some reducing agents. Ferritin molecules taken into lysosomes are degraded by protease, and Fe\(^{3+}\) is liberated in the lysosomal acidic environment. Hemosiderin is a proteolytic degradation product of ferritin in phagolysosome, and its nonheme-iron compositions are comparable to those of ferritin (Crickson and Ward, 1992; Harrison and Arosio, 1996). Although the mechanism of iron release from hemosiderin has not been determined, a reductive iron release comparable to that for ferritin has been suggested (Meguro et al., 2005).

In vitro, Fe\(^{3+}\) can be directly liberated from ferritin and hemosiderin in an aqueous buffer at pH 4.5 without any reducing agent (O’Connell et al., 1985; Ozaki et al., 1988), probably by the acid-induced denaturation of ferritin protein and replacement of iron by protons:

\[
\begin{align*}
5 \text{Fe}^{III} \cdot \text{O}_2 \cdot 9 \text{H}_2 \text{O} \text{ (ferrihydrite)} & + 30 \text{H}^{+} \rightarrow \\
10 \text{Fe}^{3+} & + 24 \text{H}_2 \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{Fe}^{III} \cdot \text{O}_2 \text{ (hematite)} & + 6 \text{H}^{+} \rightarrow 2 \text{Fe}^{3+} + 3 \text{H}_2 \text{O} \\
\text{Fe}^{III}, \text{Fe}^{II} \cdot \text{O}_2 \text{ (magnetite)} & + 8 \text{H}^{+} \rightarrow 2\text{Fe}^{3+} + \text{Fe}^{2+} + 4 \text{H}_2 \text{O}
\end{align*}
\]

3.4. Iron-sulfur proteins

The iron of the iron-sulfur protein is bonded to sulfur atoms by coordinate covalent bonds making iron-sulfur clusters — [2Fe-2S], [3Fe-3S] or [4Fe-4S] — which contain iron in oxidized or reduced forms (Cotton et al., 1999). Iron atoms are bridged by sulfur atoms and coordinated by cysteine residues of protein. To release iron from iron-sulfur proteins, protein unfolding is necessary, by which iron-sulfur centers are exposed (Leal et al., 2004). Iron is easily liberated from iron-sulfur proteins after denaturation of the proteins. Kuo and Fridovich (1988) observed iron release from ferredoxin in a sodium acetate buffer at pH 5.0 by visualizing free iron with diaminobenzoic acid-2HCl and H\(_2\)O\(_2\). Leong et al. (1992) also observed iron release from ferredoxin in a the solution (pH<1.0) consisting of 10% methanol and 10% trichloroacetic acid.

4. Nonheme-iron histochemistry: Perls and Turnbull methods

The above findings together indicate that iron is liberated from non-heme iron species at a low pH, which enables the visualization of free Fe\(^{3+}\) or Fe\(^{2+}\) by the Perls or Turnbull method described below.

4.1. Perls method

The Perls method is very old but has continued to be most commonly used for its visualization of nonheme iron in laboratory work because of its specificity for iron, simplicity of procedure, and low cost. The Perls method depends on the formation of insoluble Prussian blue by treating tissue sections with acid-ferrocyanide.

Perls (1867) introduced Berlin blue (Prussian blue) to demonstrate iron in various tissues and organs of autopsy material. This method was originally described by Grohe (1861), who observed the precipitation of Berlin blue when he gave a small amount of potassium ferrocyanide solution and HCl to the decomposed tissue of cadavers exposed to air or water for a long time. These authors gave no precise information of the concentrations of potassium ferrocyanide and HCl they used. Perls only indicated that he used dilute potassium ferrocyanide. Both Grohe and Perls treated tissues first with potassium ferrocyanide and then added a small amount of HCl. This procedure is proper because iron is released from the tissue in acid solutions and lost in the absence of ferrocyanide ion. It is noteworthy that these works were carried out before the paper of Svante Arrhenius who first described in detail the
ionic dissociation of salts in 1887 (Pauling, 1988).

Previous investigators used various preparations of the Perls mixture. Gömörí (1936) used a high concentration of Perls solution consisting of a mixture of equal volumes of 10% ferrocyanide and 20% HCl. He claimed that excess potassium ferrocyanide reduces the solubility of Prussian blue. This notion, however, was mistaken because excess potassium ion causes the generation of so-called ‘soluble’ Prussian blue, delaying the precipitation of the reaction product (see below). Furthermore, high concentrations of HCl can dissolve Prussian blue. Lison (1936) used a mixture (pH 0.6–0.75) of equal volumes of 2% HCl and 2% potassium ferrocyanide; this was shown to provide the best results by a study by Bunting (1949). Lilie (1948) used equal parts of 5% acetic acid and 2% potassium ferrocyanide at 80 °C. However, heating of the solutions is absolutely unnecessary. Among these modifications, Lison’s mixture has been most popular in recent studies (e.g., Hill and Switzer, 1984; Morris et al., 1992; Moos and Mollgard, 1993; Meguro et al., 2003, 2005; Asano et al., 2005; Moos et al., 2006 and others).

Nonheme-Fe (III) bonded to organic bases, proteins, the polar head of membrane lipid, and nucleotide is liberated in acid solutions to react with ferrocyanide ion, [Fe(III) (CN)₆]³⁻, to form Prussian blue. Though insoluble or soluble Prussian blue has been described, Prussian blue is all insoluble.

\[
\begin{align*}
4 \text{Fe}^{3+} + 3[\text{Fe}^{II} (\text{CN})_6]^{4-} & \rightarrow \\
\text{Fe}^{II}_x[\text{Fe}^{II}(\text{CN})_6]_y \cdot x\text{H}_2\text{O} & \quad (x = 14-16) \\
\text{(Prussian blue)}
\end{align*}
\]

or

\[
\begin{align*}
\text{Fe}^{3+} + [\text{Fe}^{II} (\text{CN})_6]^{4-} + K^+ & \rightarrow \\
\text{KFe}^{III}[\text{Fe}^{II} (\text{CN})_6] & \\
\text{(So-called ‘soluble’ Prussian blue)}
\end{align*}
\]

It should be noted that the reactions occur simply between the anion and cation, which implies that the reactions occur only when free $\text{Fe}^{3+}$ is available to acid-ferrocyanide. In other words, the Perls method stains nonheme-iron only in an ionic form. The Turnbull method stains nonheme-Fe (II) following the same principle (see Section 4.4).

4.2. Chemistry of Prussian blue and related compounds

Prussian blue or iron (III) hexacyanoferrate (II) was the first artificially manufactured color, which was, accidentally discovered by the color maker Diesbach of Berlin in 1704 (Coleby, 1939; Boddy-Evans, 2006). Soon after its discovery, Prussian blue became widely used across Europe for painting because of its low cost. Many famous artists used Prussian blue, e.g., Monet, Van Gogh, and Picasso (in his ‘Blue Period’, Boddy-Evans, 2006).

Many chemists investigated the molecular structure of Prussian blue, and at the beginning of 20th century the formula of $\text{KFe}^{III}[\text{Fe}^{II}(\text{CN})_6]\cdot\text{H}_2\text{O}$ was obtained (Müller, 1914 in Coleby, 1939), and finally, $\text{Fe}^{III}[\text{Fe}^{II}(\text{CN})_6] \cdot x\text{H}_2\text{O} (x = 14-16)$ has been given (Buser et al., 1977; Herren et al., 1980).

Prussian blue is a deep blue, crystal precipitate. The crystal consists of a lattice-like network of Fe (III) bonded to the nitrogen of cyanide and Fe (II) bonded to the carbon of cyanide, $\text{Fe}^{III}-\text{N} = \text{C}-\text{Fe}^{II}$ (Fig. 1A). The cubic unit cell dimension is 1.16 or 1.02 nm (Fig. 1A, Robin, 1962; Buser et al., 1977). Each unit cell consists of fourteen Fe (II) and twelve Fe (III) atoms. The interior of the unit cell accommodates six molecules of $\text{H}_2\text{O}$ coordinated to Fe (II) and eight molecules of $\text{H}_2\text{O}$ which are assumed to be linked by hydrogen bonds to coordinated $\text{H}_2\text{O}$ (Herren et al., 1980). Prussian blue is a type of mixed valence compound. Intense charge transfer transition occurs along the cyanide bridges between Fe (III) and Fe (II). This causes an absorption band in the red region of the visible spectrum around 700 nm and the deep blue color (Robin, 1962; Robin and Day, 1968).

Electron microscopy of Prussian blue and Turnbull blue deposits shows a single or aggregate of cuboid or angular shape crystals of 20–200 nm in diameter (Fig. 1B; Parmley et al., 1978; Meguro et al., 2005), particularly in the cells, e.g. splenic macrophages, where nonheme-iron is heavily accumulated. The protein shell of ferritin can house up to 4,500 iron atoms (Chasteen and Harrison, 1999), but usually contains only 2,000–2,500 Fe atoms in most tissues (Aisen et al., 2001). When all these iron atoms are released and trapped by ferrocyanide ions, the crystal size may be enough to be visualized by the Perls method and electron microscopy.

The so-called ‘soluble’ Prussian blue is not really soluble but consists of small particles of ferric ferrocyanide dispersed in water like a colloidal solution, in which potassium ion enters into the hole of the unit cell of Prussian blue. Therefore, blocking the hole with some other cations can inhibit the formation of ‘soluble’ Prussian blue and enhance the precipitation of Prussian blue. Such blocking cations are Li⁺, Na⁺, K⁺, Mg²⁺, and Ca²⁺ (Itaya and Uchida, 1986).

As already noted by Perls (1867), Prussian blue is not stable at a higher pH (> 7.0). Hydroxide ions decompose Prussian blue to hydrated ferric oxide, ferric hydroxide, and ferrocyanide ions (Holtzman, 1945; Scholz et al., 2001).

\[
\text{Fe}^{III}[\text{Fe}^{II}(\text{CN})_6] + 8\text{OH}^- \rightarrow \\
\text{(Prussian blue)}
\]
Fig. 1. A: The unit cell of Prussian blue crystal according to Buser et al. (1977) and Herren et al. (1980). Dark blue and light blue circles indicate Fe (II) and Fe (III), respectively. Adjoining Fe (II) and Fe (III) are bridged by $\equiv \text{C} \equiv \text{N}$. Water molecules accommodated within the cell are not shown to avoid complexity. B: A splenic macrophage of the rat treated by the perfusion-Perls method (pH 1.0) and OsO₄ postfixation but without DAB intensification or counterstaining with lead or uranium salt. Numerous cuboid crystals of various sizes accumulate in two large phagolysosomes. A small number of small crystals are scattered in the cytoplasmic matrix and nucleus (n) but not in mitochondria (m). Bar = 200 nm

$$4\text{Fe}^{III} \text{O} \text{(OH)} + 3[\text{Fe}^{II} \text{(CN)}_6]^{3-} + 4\text{H}^+ \quad \text{(Ferric oxide)}$$

and

$$\text{KFe}^{III} \text{[Fe}^{II} \text{(CN)}_6] + 3\text{OH}^- \rightarrow \quad \text{(So called 'soluble' Prussian blue)}$$

$$\text{Fe}^{III} \text{(OH)}_3 + \text{K} \text{[Fe}^{II} \text{(CN)}_6]^{3-} \quad \text{(Ferric hydroxide)}$$

A brown precipitate, FeO(OH) or Fe(OH)₃, is formed by these reactions. Therefore, treatment of Perls-stained sections with a higher pH medium for counter-staining or cover slipping should be avoided. Holtzman (1945) suggested that the incorporation of Ni²⁺ into Prussian blue improves resistance to alkaline attack by immersion in a solution of nickel (II) salt. By this treatment, some Fe of the Prussian blue is replaced by Ni²⁺, forming a precipitate whose hue shifts towards a more greenish-blue.

Ferrocyanide ion, [Fe⁷⁺CN]⁶⁻⁻, and ferrous iron produce a white precipitate (Everitt's salt or a reduced form of Prussian blue or Prussian white). The reaction is

$$\text{Fe}^{II} + \text{K} \text{[Fe}^{II} \text{(CN)}_6] \rightarrow \text{K} \text{[Fe}^{II} \text{(CN)}_6] + 2\text{K}^+ \quad \text{(Everitt's salt)}$$

Everitt's salt is slowly oxidized in the air and aqueous solution to produce Prussian blue. This indicates that the Perls method can stain Fe (II) as well as Fe (III), as emphasized by Meguro et al. (2003), in contrast to the general belief that the Perls method is specific for Fe (III). Indeed, Nishimura (1910) used the oxidative production of Prussian blue to stain nonheme Fe (III). His method consists of the following procedure: 1) the tissue sections are treated with concentrated ammonium sulfide, (NH₃)₂S, for one hour; 2) after washing in d. w., the sections are treated in a mixture of equal volumes of 2% potassium ferricyanide and 1% HCl for 1 to 1.5 hours; 3) the sections are thoroughly washed in d. w., 0.5% HCl for 1 min, and d. w.. In this method, tissue nonheme Fe-(III) is first reduced to ferrous form by a strong reducing agent, (NH₃)₂S:
\[
\text{Pr-Fe}^{3+} + (\text{NH}_3)_2 \text{S} \rightarrow \text{Pr-Fe}^{3+} \text{S} + 2\text{NH}_3\text{OH} \quad (\text{Pr} = \text{proteins})
\]

Then the Fe\(^{2+}\) released from Pr-Fe\(^{3+}\) S in the acid solution reacts with ferricyanide ion to produce a white precipitate, Evertis’s salt, which then is oxidized to Prussian blue during thorough washing in d. w.

Ferrocyanide ion makes insoluble precipitates with various metal ions other than iron in a wide range of pH. These include Cu\(^{2+}\) and Zn\(^{2+}\), giving dark brown and white precipitates, respectively. These precipitates can be differentiated from those of Prussian blue by the color difference. Furthermore, these precipitates do not catalyze the oxidative polymerization of DAB by H\(_2\)O\(_2\) (Meguro et al., 2003).

4.3. Turnbull method

The Turnbull method uses acid-ferricyanide instead of the acid-ferricyanide in the Perls method. The Turnbull method depends on the generation of insoluble blue precipitate (Turnbull blue) formed by the reaction between ferricyanide ion and Fe\(^{3+}\).

To our knowledge, the first study in which acid-ferricyanide was used was that of Zaleski (1887). He treated tissue sections from idiopathic thrombocytopenic purpura with 1% ferro- or ferricyanide and then with 2–3% HCl and observed an extensive positive reaction for acid-ferricyanide in various organs. He also noted a small region positively stained by acid-ferricyanide (Turnbull method) in the gray matter of the brain. However, there was no such reaction throughout other organs.

The Turnbull blue method was usually not used in its simple form but was usually performed after pretreatment of the tissue sections with (NH\(_3\))\(_2\) S (Tirmann-Schmeltzer method, Gömöri, 1936), which reduces nonheme Pr-Fe\(^{3+}\) to Pr-Fe\(^{3+}\) S. This was because iron in the tissue was believed to be present invariably or mostly in a ferric form. This belief came from the failure to demonstrate nonheme Fe (II) by simple use of the Turnbull method on sections (Gömöri, 1936), very probably because tissue nonheme Fe (II) was readily oxidized in the air and aqueous solutions. Important neuropathological works using the Turnbull blue reaction (Tirmann-Schmeltzer method) appeared in the early 1920’s (Spatz, 1922; Hallervorden and Spatz, 1922). However, the Tirmann-Schmeltzer method soon dropped out of use because the reduction of ferric iron by (NH\(_3\))\(_2\) S proved to be incomplete; tissues were still positively stained by the Perls method after treatment with (NH\(_3\))\(_2\) S. An exceptional Turnbull-positive reaction without pretreatment with (NH\(_3\))\(_2\) S was reported in chronic hemorrhagic lesions (Mori et al., 2001), the kainic acid-injured hippocampus of the rat (Wang et al., 2002), and some parts of aged human brains (Morris et al., 1992). These tissues showed increased accumulations of iron as demonstrated by the Perls method, strongly suggesting that the reductive generation of toxic ferrous iron is inevitable in the tissue where nonheme-iron is densely accumulated. A new application of the Turnbull blue, the perfusion-Turnbull method, enabled the visualization of catalytic Fe\(^{2+}\) in normal and pathological conditions of experimental animals (Meguro et al., 2003, see Section 4.5).

4.4. Chemistry of Turnbull blue and related compounds

The origin of Turnbull blue is found in the Nobel lecture of Sir William Ramsay (1904), who discovered rare gases. He stated in the lecture that his grandfather on his father’s side, William Ramsay, a chemical manufacturer in Glasgow, made Turnbull’s blue with his partners, the Messrs Turnbull. This suggests that Turnbull blue was discovered in the early 19th century.

Turnbull blue deposits are larger than those of Prussian blue, while deposits of Prussian blue are denser, more homogeneous and more sharply outlined than those of Turnbull blue, as already noted by Gömöri (1936). The hue of Turnbull blue also differs somewhat from that of Prussian blue. However, magnet chemistry, Möebius spectroscopy, and X-ray studies concluded that the final product of Prussian blue and Turnbull blue is always Fe (III) hexacyanoferrates (II) or Fe\(^{III}\)\([\text{Fe}^{II} (\text{CN})_6]^3^-\) \cdot xH\(_2\)O (x = 14-16) (Cotton et al., 1999). The difference in the tones for Prussian blue and Turnbull blue are thought to reflect the difference in the particle size; the average particle size is larger in Turnbull blue than in Prussian blue.

Turnbull blue is produced by mixing Fe\(^{2+}\) with [Fe\(^{III}\) (CN)\(_6\)]\(^{3-}\):

\[3\text{Fe}^{2+} + 2[\text{Fe}^{III} (\text{CN})_6]^3^- \rightarrow \text{Fe}^{III} [\text{Fe}^{III} (\text{CN})_6]; \quad \text{(Turnbull blue)}\]

On the other hand, when solutions of Fe\(^{3+}\) and [Fe\(^{III}\) (CN)\(_6\)]\(^{3-}\) are mixed, a brown solution (ferric ferricyanide) forms; ferric ferricyanide does not dissociate but does not precipitate.

\[\text{Fe}^{3+} + [\text{Fe}^{III} (\text{CN})_6]^3^- \rightarrow \text{Fe}^{III} [\text{Fe}^{III} (\text{CN})_6] \quad \text{(Ferric ferricyanide)}\]

Ferric ferricyanide is a strong oxidant and reacts with tissue reducing agents such as ascorbic acid, melanin, lipofuscin, chromaffin granules, and proteins with thiols (SH) (Adams, 1956; Lillie and Donaldson, 1974, Chinoy and Sanjeevan, 1978). Because the standard reduction potential (E\(^\circ\)) of Fe\(^{3+}\) (E\(^\circ\) = +0.771 V) is larger than that of [Fe\(^{III}\) (CN)\(_6\)]\(^{3-}\) (E\(^\circ\) = +0.36 V), Fe\(^{III}\) is better reduced to Fe\(^{II}\) (Karyakin, 2001):
Fe^{III}[Fe^{III}(CN)_{6}] + K^+ + Pr-SH →
KFe^{II}[Fe^{III}(CN)_{6}] + 1/2 Pr-S-Pr+ + H^+

The intermediate reduction product is KFe^{II}[Fe^{III}(CN)_{6}]_{1/2} Fe^{III}[Fe^{III}(CN)_{6}]_{1/2} (Crumbliss et al., 1984). This compound is greenish blue (Berlin green) and finally oxidized to Prussian blue (Ware, 2003). This suggests that, in the presence of some reducing agents in the tissue, the ferricyanide reaction (Turnbull method) can stain Fe^{3+} released from the tissue. However, ferric ferricyanide is soluble, and the small amount of ferric ferricyanide generated in the tissue is readily diluted and lost during tissue treatments. Furthermore, staining by the Turnbull method can be inhibited by pretreatment with a divalent metal chelator, 2, 2’-dipyridyl (Meguro et al., 2005). These findings strongly support that the Turnbull method is specific for nonheme-Fe (II) and does not stain Fe (III).

4.5. In vivo perfusion-Perls and -Turnbull methods

We have developed highly sensitive methods of nonheme-iron histochemistry for animal experiments, the perfusion-Perls and -Turnbull methods (Meguro et al., 2003).
Compare Figure 2, A and B which show a rat kidney stained by the conventional Perls method performed on tissue sections and by the perfusion-Perls method, respectively. The higher sensitivity of the perfusion-Perls method is obvious. There was no staining in the sections treated by the conventional Turnbull method, whereas the perfusion-Turnbull method lightly stained the kidney.

Our methods depend on the production of insoluble and stable Fe(III)- or Fe(II)-compounds (Prussian blue and Turnbull blue) in vivo, preventing the postmortem redox change of nonheme-iron, inevitable loss of loosely bound iron, and oxidation of ferrous iron during tissue treatments. Particularly, the perfusion-Turnbull method demonstrated the presence of Fe(II) in lysosomes of various kinds of cells (Meguro et al., 2005) and a reduction of nonheme-Fe (III) to Fe (II) in the ischemic brains (Yu et al., 2001) and stress-induced gastric lesions (Asano et al., 2005). Furthermore, the method proved to be instrumental in the study of iron transport across the brain capillary endothelium (Moos et al., 2006).

Another important advantage of the perfusion-Perls and -Turnbull methods is that it allows visualization of the catalytic iron available at a different pH, such as a physiological (pH 7.4), acidic (pH 6.5 +/-0.3), endosomal (pH 4.5 +/-0.2) and phagolysosomal (pH 5.2–5.6) pH, by choosing the pH of Perls and Turnbull solutions. These perfusion methods have an additional benefit of easy identification of iron-containing organs and tissues, which are stained blue. For electron microscopy, because of the low pH of the solutions used in these methods, tissue stabilization by perfusion with 4% paraformaldehyde and 1.5% glutaraldehyde in PBS (pH 7.4) is necessary prior to the perfusion with the solution for perfusion-Perls or -Turnbull histochemistry (Meguro et al., 2005, see also Section 12.2).

5. Intensification of Prussian blue and Turnbull blue reactions

Prussian blue and Turnbull blue are redox-active (i.e. able to receive or give off e-) and able to catalyze the oxidation/reduction of H₂O₂ which is decomposed to H₂O and O₂:

Fe⁶⁺[Fe⁷⁺(CN)₆]³⁻ + 1/2 H₂O₂ →
Fe⁷⁺ + [Fe⁷⁺(CN)₆]⁴⁻ + H⁺ + O₂ (oxidation of H₂O₂)
Fe⁷⁺ + [Fe⁷⁺(CN)₆]⁴⁻ + 1/2 H₂O₂ →
Fe⁶⁺[Fe⁷⁺(CN)₆]³⁻ + OH⁻ (reduction of H₂O₂)

Then the reaction is

Fe⁶⁺[Fe⁷⁺(CN)₆]³⁻ + H₂O₂ → H₂O + O₂

By giving some oxygen recipient (chromogens), it is possible to enhance Prussian blue and Turnbull blue reactions. These include 3', 3'-diaminobenzidine (DAB, Nguyen-Legros et al., 1980), phenylhydrazine HCl (Sumi et al., 1985) and others. These chromogens are also used to detect redox-active iron (see Section 7).

5.1. DAB intensification for the Perls and Turnbull methods

The Prussian blue method is greatly improved by the use of DAB (Nguyen-Legros et al., 1980) as shown in Figure 2C. The method depends on the oxidative/reductive degradation of H₂O₂ by Prussian blue coupled with the oxidative polymerization of DAB. The visibility of the DAB reaction (dark brown) is improved by adding CoCl₂ to the DAB/H₂O₂ solution (Adams, 1981). CoCl₂ turns the DAB reaction product from brown to black (Fig. 2D). Although the chemical mechanism of this effect has not been described, the molecular structures of DAB and polymerized DAB (pDAB) strongly suggest that aromatic amine is the most likely binding site for Co⁺. To accelerate DAB penetration into 80 µm thick vibratome sections of the human brain, LeVine (1997) added 1% Triton X-100 to DAB/H₂O₂ solution.

The quantitative estimation of the amount of iron is difficult from the intensity of the DAB reaction, but the lowest concentration of iron visible by the reaction can be determined by spot tests. Calculating from the data (Fig. 3A, B) of Moos and Mølgaard (1993), the lowest concentration of iron detectable by Prussian blue-DAB and Prussian blue-DAB plus silver-gold-uranyl nitrate method was 375 and 25 µM, respectively. By the Perls-DAB/CoCl₂ intensification, we observed a positive reaction for FeCl₂ with the smallest amount of 100 µM (Iwatsuki et al., unpublished data). Masini et al. (1985) found a consistent production of malondialdehyde (one of the endproducts of the peroxidation of unsaturated fatty acid) in isolated rat liver mitochondria at the presence of 100 µM of nonheme-iron. This implies that the Perls-DAB/CoCl₂ and Perls-DAB-silver-gold-uranyl nitrate method clearly visualize the location of catalytic iron, which can participate in the peroxidation of cellular macromolecules.

5.2. Post-DAB intensification for the Perls and Turnbull methods

The visibility of pDAB deposits on Prussian blue can be further improved by various methods (post-DAB intensification), particularly for electron microscopy. pDAB deposits behave as a weak reducing agent, as demonstrated by its reactivity to ferric ferrocyanide, Fe₃[Fe⁶⁺(CN)₆] (Nemes, 1987). In the presence of pDAB,
Fig. 3. Electron microscopic nonheme-iron histochemistry in the rat kidney. The kidney was treated by the perfusion-Perls method (pH 1.0), DAB intensification and post-DAB intensification with methenamine-silver/gold substitution. A–C: An epithelial cell of the proximal convoluted tubule (PCT). Electron-dense grains accumulate on the dark DAB deposits in the lysosome (l) and are scattered in the cytoplasmic matrix, particularly at the apical (A and B) and basal parts (C) of the PCT. Asterisks in A and B indicate the same endosome. Note that a small number of cuboid crystals (Prussian blue) in B and C are surrounded by dark osmified pDAB, on which fine gold grains accumulate. Comparing the size of the cuboid crystals and that of the surrounding pDAB, DAB treatment enhances Prussian blue 3–5 times. D: The basal part of distal convoluted tubule (DCT). A small number of fine grains are observed along the membranes of some mitochondria in DCT. bl: basal lamina, m: mitochondria, mv: microvilli. Bars = 500 nm (A), 200 nm (B–D)
Fe$^{II}$[Fe$^{III}$(CN)$_6$] is reduced to Fe$^{II}$[Fe$^{III}$(CN)$_6$] (Turnbull blue). Such metal ions as Au$^{III}$ (E$\text{r}^\circ$ = +1.50 V) and Ag$^+$ (E$\text{r}^\circ$ = +0.8 V) can be used instead of Fe$^{II}$[Fe$^{III}$(CN)$_6$] for metal-mediated post-DAB intensification—note that the reduction potential of Fe$^{II}$ is +0.771 V. Of these metal ions, Au$^{III}$ is best incorporated in pDAB (Siegesmund et al., 1979; Newman et al., 1983). OsO$_4$ (E$\text{r}^\circ$ = +0.64 V) also effectively intensifies pDAB deposits, but a small amount of pDAB is difficult to differentiate from the background staining by OsO$_4$. Among these metals, Ag salts are most commonly used because of their prominent chemical property which once silver metal is laid down on tissue reducing agents, rapidly catalyzes the further reduction of silver ions and greatly accelerates the reaction (Newman and Jasani, 1998).

The gold-substituted silver-intensification of pDAB has been described (Fig. 3, Görcs et al., 1986; Goto et al., 1992; Moos and Malligárd, 1993; Teclemarian-Mesbah et al., 1997; Schroeter et al., 2004). In this method, Ag$^+$ given in the form of silver-methenamine (a complex ion) is reduced by pDAB and precipitates on its surface, in which sodium tetraborate accelerates the reduction of Ag$^+$, and then Ag is substituted by Au$^{III}$. Tissue reducing groups can reduce Ag$^+$ to generate the background staining; therefore, the tissues are treated with a reducing agent, 8% thioglycolic acid (Görcs et al., 1986) or 0.5% sodium borohydrate (Teclemarian-Mesbah et al., 1997) prior to the silver-intensification.

5.3. Silver intensification of Prussian blue

Parmley et al. (1988) described a method to intensify Prussian blue reaction by treatment with silver salt (1% silver protein for 15-30 min in the dark). By this method, a small electron dense silver deposition is evident on Prussian blue deposits, making small ferrocyanide-reactive sites clearly visible. This method depends on the formation of silver-ferrocyanide by the substitution of the Fe$^{II}$ of Prussian blue with Ag$^+$ but not by the reduction of Ag$^+$ by Prussian blue.

Fe$^{II}$[Fe$^{II}$(CN)$_6$] + 12Ag$^+$ →
(Prussian blue) Ag$_{12}$[Fe$^{II}$(CN)$_6$] + 4Fe$^{III}$
(Silver ferrocyanide)

This mechanism was determined by the finding that silver deposits (silver-ferrocyanide) were removed with NH$_2$OH, which, however, could not make reduced silver (elementary silver) soluble:

Ag$_{12}$[Fe$^{II}$(CN)$_6$] + 12 OH$^-$ →
(Silver ferrocyanide) 12Ag(OH) + 3 [Fe$^{II}$(CN)$_6$]$^{4-}$

and

Ag(OH) + OH$^-$ → [Ag(OH)$_2$]

6. Nonheme-iron histochemistry: iron sulfide methods

This method depends on the sulfidation of tissue iron using (NH$_4$)$_2$S, H$_2$S or Na$_2$S.

Fe (III) + S$^{2-}$ + e$^-$ → Fe (II) S
Fe$^{II}$ + S$^{2-}$ → Fe (II) S

This method was originally described by Mayer as early as 1850 (in Quincke, 1896). He used (NH$_4$)$_2$S for the gross observation of iron-containing organs after an intravenous injection of Fe-lactate. Quincke (1986) introduced this method for the microscopic histological study of tissue iron; tissue sections were treated with (NH$_4$)$_2$S to form black iron sulfide (Fe$_3$S$_4$). The combination of Turnbull and Quincke's methods produced the Tielmann-Schmerzer method (see Section 4.2).

Later, Timm (1958) found the precipitation of physically reduced silver ion on the metal sulfide core, and this lead to the development of more sensitive metal histochemistry (Timm's method). Timm's method was modified and improved by later authors (e.g., Danscher and Zimmer, 1978; Danscher 1981; Zdolsek et al., 1993). By the modified Timm's method, Zdolsek et al. (1993) observed Ag deposits within the endosomes/lysosomes of cultured macrophages in addition to a pronounced reduction in staining following deferoxamine pretreatment. Comparable results were described by Yu et al. (2003).

The sulfide method is not specific for iron, but produces various divalent metal sulfides including ZnS (white to yellow precipitate) and CuS (black precipitate). Furthermore, the strongly alkaline solution of (NH$_4$)$_2$S would expose the heme of heme proteins by denaturation of the protein moiety, making heme-iron reactable with S$^{2-}$.

7. Histochemical demonstration of 'reductively' iron

'Reductively' iron is a species of nonheme-iron that can catalyze oxidation-reduction reactions, including loosely bound Fe$^{III}$ or Fe$^{II}$ in LIP and endosomes/lysosomes. H$_2$O$_2$ given to this system is oxidized or reduced by 'reductively' iron:

Fe$^{II}$ + 1/2 H$_2$O$_2$ → Fe$^{III}$ + O + H$^+$ (oxidation of H$_2$O$_2$)
Fe$^{III}$ + H$_2$O$_2$ → Fe$^{II}$ + OH$^-$ + H$^+$ (reduction of H$_2$O$_2$)

By giving some chromogens oxidatively polymerized by these oxidants, the reaction site can be visualized. Sumi et al. (1985) used phenylhydrzone-HCl as a chromogen to visualize ferric iron that was nonspecifically endocytosed.
by the liver sinusoid cells after an injection of ferric nitrate via the portal vein in the rat. This method was claimed to have a sensitivity superior to the Perl's method in light-microscopic observations, but it has not been used for electron microscopy.

Smith et al. (1997), Sayre et al. (2000), and Fauchoux et al. (2003) used DAB as a chromogen for the visualization of redox-active iron in the tissue. They treated methacarn (methanol:chloroform:acetic acid, 60:30:10)-fixed paraffin sections or fresh frozen sections with modified Karnovsky medium (0.45% H2O2 and 0.05% DAB, or 3% H2O2 and 0.075% DAB in a 50 mM Tris-HCl buffer at pH 6.5 or 7.6). DAB is oxidatively polymerized to form a brown precipitate. By this method, they observed increased staining for redox-active iron in the substantia nigra of Parkinson patients and the brain lesions of Alzheimer's disease together with an increased accumulation of Fe (III) demonstrated by the Perl's method. These results again suggest that an increased accumulation of nonheme-Fe (III) is indeed the site for the generation of catalytic iron. This method is applicable by perfusion in experimental animal studies and for electron microscopic observations.

8. Fluorescence histochemistry of nonheme-iron

The methods of nonheme-iron histochemistry described above possess great advantage for visualizing nonheme-iron in tissues and organs by conventional light and electron microscopy. However, these methods have difficulty in quantifying the amount of nonheme-iron.

Epsztejn et al. (1997) and Petrat et al. (1999, 2000, 2001, 2002) developed a method which enables the quantitative determination of the chelatable iron pool in isolated, viable mammalian cells. The method depends on the quenching of fluorescent metal indicators (calcine, phen green PG, SK, or rhodamine B-[(1,10-phenanthroline-5-yl)aminocarbonyl] benzyl ester) by chelatable ferrous iron, and quenching by administration of a known amount of various divalent metal chelators (salicylaldoxime isonicotinoyl hydrazone, 2,2'-dipyridyl or 1,10-phenanthroline). Fluorescence measurements were performed using digital fluorescence or a laser scanning microscope. They estimated amounts of 0.2–1.5 μM of chelatable iron in rat erythroid and myeloid cells, and 9.8 +/- 2.9 or 2.5 +/- 2.2 μM in rat hepatocytes. Petrat et al. (2001, 2002) further demonstrated the presence of chelatable iron in the nuclei of hepatocytes (6.6 +/- 2.9 μM) and endothelial cells (11.8 +/- 3.9 μM) and in mitochondria (12.2 +/- 4.9 μM).

9. Other staining methods for nonheme-iron

Schmelzer (1933) described a method in which tissue iron was visualized as dark red Rhodainestinal (ferric thiocyanate). This method was used in some earlier studies but soon fell out of use because the method requires tissue treatment with very toxic volatile thiocyanic acid.

For the light microscopic study of nonheme-iron, hematoxylin (Mallory's method) and ferrous iron chelators (e.g., bathophenanthroline or 2, 4, 6-tri-[2'-pyridyl]-1, 3, 5 triazin) which form an insoluble Fe2+-chelator complex are used (Hukill and Putt, 1962; Pearce, 1985). However, these methods have rarely been used, probably because hematoxylin also stains other tissue substances and a reduction of Fe(III) is required before treatment with divalent chelators.

10. Tissue fixation for nonheme-iron histochemistry

The tissue fixatives for nonheme-iron histochemistry should be buffered to obtain a pH value around 7 to minimize the possibility of missing this form of iron. These include 10% formalin, 4% paraformaldehyde (for light microscopy) or 4% paraformaldehyde + 0.1–1.5% glutaraldehyde (for electron microscopy) in a 0.01 M phosphate or cacodylate buffer (pH 7.4). Smith et al. (1997) treated hippocampal tissue from autopsy cases with methacarn (methanol:chloroform:acetic acid, 60:30:10) and embedded the tissues in paraffin. Acetic acid favors the tissue penetration of the fixative. However, the pH of this solution is low (2.75) and can cause some iron loss before treatment with ferrocyanide. For paraffin embedding, we recommend tissue dehydration with graded ethanol after buffered-aldehyde fixation.

Fauchoux et al. (2003) used blocks of human brains frozen in dry ice reduced to powder and stored at -80 °C without prior tissue fixation. The blocks were cut at 20 μm on a cryostat, and the sections were treated for the visualization of redox-active iron (Section 6). The tissues can be fixed after mounting on slides. This method preserves the tissue iron well.

11. Strong oxidizing agents are required to liberate heme-iron

Finally, we consider the chemical conditions required for iron release from heme-proteins. The iron of heme proteins is bonded to four nitrogen atoms of
protoporphyrin and to the protein through the imidazol of histidine (hemoglobin and myoglobin) or a thiol of cysteine (cytochrome P-450) by coordinate covalent bonds. Heme-iron is one of the ‘masked’ iron species because it is not stained by the Perls method in its simple form, i.e., heme-iron is not liberated by tissue treatment with dilute HCl, acetic acid, or trichloroacetic acid. It should be stressed that these acids do not behave as an oxidant like sulfuric or nitric acid.

Iron was released from transferrin and ferredoxin by treatment with 10% trichloroacetic acid, but there was no iron release from myoglobin (Leong et al., 1992). More recently, Rebouche et al. (2004) described a method for the microanalysis of iron in animal tissues. They treated a tissue homogenate with 1M HCl and 10% trichloroacetic acid at 95 °C for 1 h. In spite of such strong chemical and physical treatment, less than 2% of the iron was released from hemin or myoglobin, while 100% of the iron from ferritin was released under the same condition. Furthermore, desferrioxamine does not withdraw iron from heme, while it is capable of removing iron from ferritin and hemosiderin (Keberle, 1964).

It is thus clear that heme-iron is much more strongly bonded than nonheme-iron. Heme-iron was stained by the Perls method after treating tissues with ammonium peroxydisulfate gas (NH₄)₂S₂O₈, Okamoto, 1937) at 30 °C for 12 h, a strong alkaline ammonium sulfide, (NH₄)₂S or NH₄HS (Macallum, 1895) for several days or 30% H₂O₂ for a short period (Pearse, 1985). These findings indicate that tissue treatment with strong oxidizing agents is required to liberate heme-iron.

12. Recommended protocols for histochemical demonstration of tissue nonheme-iron

Here we describe the recommended protocols of the histochemical demonstration of nonheme-iron by the Perls or Turnbull methods performed on tissue sections and supplemented by intensification with DAB, DAB/CoCl₂ or DAB + methenamine-silver/gold-substitution. The tissue sections treated by in vivo perfusion-Perls and Turnbull methods can be intensified in the same way. For simplicity of description, the protocols are presented separately for light or electron microscopy. Throughout the procedure, deionized d. w. used, and every section treatment is performed in a water bath at 25 °C, except for methenamine-silver intensification performed at 60 °C.

12.1 Light microscopy

1. Tissue fixation
For animal experiments, transcardially perfuse with 4% paraformaldehyde (PA) in PBS (pH 7.4) after a flush with heparinized saline, and for autopsy material, immerse small pieces of tissue in 4% PA in PBS (pH 7.4). Frozen tissues can be fixed after cutting sections on a cryostat at 30 μm and mounting on glass slides.

2. Preparation of tissue sections
Cut paraffin embedded sections at a 5-7 μm thickness. Mount sections on silan-coated slides. Sections treated by the perfusion-Perls or -Turnbull method (Section 4.5) are processed according to the procedure 4.

3. Staining by the Perls or Turnbull method
After dewaxing/rehydration (paraffin sections), incubate sections for 30 min in ‘freshly’ prepared 1% potassium ferrocyanide (Perls method) or 1% potassium ferricyanide (Turnbull method) in d. w. Adjust the pH of the solutions to below endosomal pH 5.5–6.0, but usually around 1.0.

4. DAB intensification

1) After washing in d. w. (3 volumes), treat sections in methanol containing 0.01 M NaNO₃ and 0.3% H₂O₂ for 60 min.
2) Wash sections in 0.1 M phosphate buffer (pH 7.4, 3 volumes).
3) Treat sections in the solution containing 0.025% 3, 3’-diaminobenzidine-4HCl (DAB, Sigma, St Louis, Mo, USA) and 0.005% H₂O₂ in a 0.1M phosphate buffer (pH 7.4) for 30 min. Add 0.5 ml of 1% CoCl₂ to 100 ml of the above solution.
4) Wash sections in d. w. (3 changes) to stop the DAB/H₂O₂ reaction.
5) If required, sections can be lightly counter-stained with neutral red or eosin.

5. Control sections
To prepare control sections, treat the sections as described above but omit the staining by the Perls or Turnbull method or treat sections with high pH (6.0–7.0) Perls or Turnbull solution.

6. Results

1) The Perls or Turnbull method stains nonheme-iron pale blue to deep blue, according to the amount of iron (Fig. 2A, B). Nonheme-iron is more intensely stained by the perfusion-Perls method (Fig. 2B) than the Perls method performed on sections (Fig. 2A). CoCl₂ improves the visibility of the fine DAB/H₂O₂
reaction product by turning it from brown to black (Fig. 2, C, D), particularly when sections are counterstained with neutral red or eosin.

2) Control sections show no iron deposits.

12.2 Electron microscopy

1. Tissue fixation
For animal experiments, transcardially perfuse with 4% paraformaldehyde and 0.5–1.0% glutaraldehyde in PBS (pH 7.4) after a flush with heparinized saline. For autopsy material, immerse the tissue in 4% paraformaldehyde and 1.5% glutaraldehyde in PBS (pH 7.4). Deep-frozen materials can be fixed after cutting sections on a cryostat.

2. Preparation of tissue sections
Cut sections at 40–50 μm on a vibratome, collect sections in 0.005 M PBS (pH 6.0–7.0), and treat sections with freshly made 0.5% sodium borohydride in Tris-buffered saline for 10 min.

3. Staining by the Perls or Turnbull method
Incubate free float sections for 30 min in ‘freshly’ prepared 1% potassium ferrocyanide (Perls method) or 1% potassium ferricyanide (Turnbull method). Adjust the pH of the solution to below endosomal pH 5.5–6.0, but usually around 1.0 by adding HCl. The tissues treated by the perfusion-Perls or Turnbull method are cut at 40–50 μm on a macroslicer, and processed for DAB intensification.

4. DAB intensification
1) After washing in PBS, incubate sections in PBS (pH 7.4) containing 0.01 M NaNO₃ and 0.3% H₂O₂ for 30 min.
2) Wash sections in PBS (pH 7.4) and treat sections in the solution containing 0.025% DAB and 0.005% H₂O₂ in a 0.1M phosphate buffer (pH 7.4) for 30 min.

5. Methenamine-silver/gold-substitution
After washing in d. w., treat sections according to the protocol by of Teclemariam-Mesbah et al., (1997).

6. Postfixation, dehydration, and embedding
1) Treat sections in 1% OsO₄ for 1 h.
2) After washing in d. w., dehydrate sections in graded ethanol, treat with propylene oxide, and embed in an Epon mixture.
3) Cut plastic sections at 90–100 nm and observe without staining with lead and uranium salts.

7. Control sections
To prepare control sections, treat sections as described above but omit the staining by the Perls or Turnbull method or treat sections with high pH (6.0–7.0) Perls or Turnbull solution.

8. Results
1) DAB intensification/OsO₄ postfixation produces a homogeneously electron-dense pDAB-OsO₄ complex (Fig. 3).
2) Methenamine-silver intensification/gold-substitution produces electron-dense fine grains (Au deposits) on a dark pDAB-OsO₄ complex (Fig. 3).
3) Control sections show no electron-dense deposits.

13. Concluding remarks
We carefully examined the chemical principles underlying the heretofore reported methods of nonheme-iron histochemistry. Most of the chemical principles are simple and describable by general chemistry. Nonheme-iron histochemistry has been an informative tool for experimental and pathological investigations, particularly for the estimation of the involvement of nonheme-iron in degenerative changes of the tissues. For example, nonheme-iron histochemistry played a crucial role in characterizing Hallervorden-Spatz disease. The history of nonheme-iron histochemistry is very old. Early authors introduced various unique methods to visualize tissue nonheme-iron. These methods have been theoretically and practically tested and improved, and several selected methods continue to be the main tools for the histopathological examination of tissue nonheme-iron. More recently, new modifications and new applications of old methods for light and electron microscopy have enabled the visualization of tissue nonheme-iron in much greater detail and in a form more comparable to the vital state.

Iron has a central primary role in oxygen transport, electron transfer, and DNA synthesis. However, iron is not properly regulated; it can catalyze the generation of highly reactive, and therefore toxic, free radicals. Abnormal iron accumulation is associated with a wide spectrum of degenerative diseases of organs, particularly those of the central nervous system. The subcellular localization and redox-state of nonheme-iron and the expression of iron-regulating molecules in different types of cells remains largely unsettled although there has been a considerable accumulation of knowledge about the cellular localization of nonheme-iron in both normal and diseased organs. Electron microscopic histochemistry for nonheme-iron
and iron-regulating molecules should provide the answers

to these queries.

Acknowledgements

The authors thank Professor Kunio Ohzeki (Inorganic
Chemistry) at Hirosaki University, Faculty of Science and
Technology, for his critical reading of the manuscript and
useful suggestions.

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